**Universität für Bodenkultur Wien**
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**DISSERTATION**

# Enzymatic synthesis and functionalization of bio-based polyesters

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# Preamble

This thesis is divided into five sections.

Section 1 (Chapters 1 and 2) present to the reader the aim of the work and an introduction to the biocatalyzed synthesis and functionalization of polyesters. This section describes the importance of bio-based polyesters in the Bioeconomy context and underlines the important role of enzymes as green catalysts for several polyesters synthetic and hydrolysis reactions.

Section 2 (Chapters 3 and 4) includes two publications on enzymatic synthesis of polyesters. The first one describes the use of a novel enzyme for the synthesis of bio-based aliphatic polyesters. The second one describes the use of a well-known commercial enzyme preparation for the synthesis of aliphatic aromatic polyesters.

Section 3 (Chapters 5 and 6) lists two publications on enzymatic hydrolysis of bio-based polyesters and functionalization of enzymatically activated poly(L-lactic acid) (PLA). A biocatalyzed coupling reaction on the surface of PLA films and the biocatalyzed hydrolysis of poly(ethylene furanoate) (PEF) with relative characterization of the reaction products are described in this section.

Section 4 (Chapters 7 and 8) concludes the results of the present thesis with a general conclusion and an appendix. Scientific publications, oral presentations, secondments and awards are listed in this section.

# Abstract

The work described in this thesis was carried out in the context of the EU-7$^{th}$ Framework Programme for Research and Technological Development within the REFINE project. The aim was to develop new skills and expertise in sustainable green materials manufacturing and technologies and applications for the development of a greener and more sustainable society. This project was based on the unique combination of knowledge along the production chain in polymer and materials science, biotechnology and applications, ecological impact and Life Cycle Analysis.

Following the given tasks, the individual focus of this thesis was on hydrolytic enzymes as green catalysts with an enormous unexploited potential for the synthesis, hydrolysis and functionalization of polyesters.

In a first step, the biocatalyzed synthesis of polyesters was studied. In this context we investigated the synthetic potential of *Thermobifida cellulosilytica* cutinase 1 (Thc_cut1), an hydrolytic enzyme previously reported for the hydrolysis of poly(ethylene terephthalate). Production, purification and immobilization of the biocatalyst together with the synthesis of aliphatic bio-based polyesters with $M_w$ of around 1900 and $M_n$ of around 1000 Da was investigated and compared to lipase B from *Candida antarctica* (CaLB) or cutinase from *Humicola insolens* (HiC). Computational studies were also carried out for a better comparison of these biocatalysts. Additionally, the use of a commercial preparation of CaLB for the synthesis of aliphatic-aromatic oligoesters in a simple one-step, one-pot reaction system was described. A detailed analysis of the reaction products showed that the best combination of diester and polyol was consisting of dimethyl isophthalate with 1,10-decanediol that led to a $M_w$ as high as 1512 Da and a conversion of 87% after 96 h of reaction.

In a second instance, biocatalysts were investigated for their ability to hydrolyze and functionalize chemically-produced bio-based polyesters. HiC was explored for the surface hydrolysis of poly(L-lactic acid) (PLA) films. A change of water contact angle from 74.6 to 33.1° was measured while the roughness and waviness were an order of magnitude higher in comparison to the blank. Surface functionalization was demonstrated using two different techniques, 14C-radiochemical analysis and X-ray

photoelectron spectroscopy using $^{14}$C-butyric acid sodium salt and 4,4,4-trifluorobutyric acid as model molecules, respectively. Moreover, the enzymatic hydrolysis of poly(ethylene furanoate) (PEF) was also successfully carried out. PEF powders of various molecular weights (6, 10 and 40 kDa) were synthetized and their susceptibility to enzymatic hydrolysis was investigated for the first time. According to LC/TOF-MS analysis, Thc_cut1 liberated both 2,5-furandicarboxylic acid and oligomers of up to DP4 for all the tested samples. Hence, in summary in this work the high potential of environmentally friendly biocatalysts both for production and processing of polymers was demonstrated.

# Kurzfassung

Diese Dissertation wurde im Rahmen des EU-REFINE Projektes im EU-7th Framework Programme for Research and Technological Development durchgeführt. Das generelle Ziel des EU-Projektes war die Entwicklung neuer Technologien und Fähigkeiten bei der umweltfreundlichen und nachhaltigen Produktion von Materialien. Das Projekt vereinte in einzigartiger Art und Weise Expertise in den Bereichen Materialwissenschaften, Biotechnologie sowie ökologischer Bewertung entlang der gesamten Produktionskette von Polymeren.

Basieren auf diesen generellen Zielen war der Schwerpunkt dieser Dissertation die Erforschung des Potentials hydrolytischer Enzyme zur Entwicklung umweltfreundlicher Verfahren für die Synthese, Hydrolyse und Funktionalisierung von Polyestern.

In einer ersten Phase wurde die biokatlytische Synthese von Polyestern untersucht. In diesem Zusammenhang wurde das Potential einer Hydrolase, nämlich der Cutinase 1 von *Thermobifida cellulosilytica* bekannt für die Hydrolyse von Poly(ethylene terephthalate) untersucht. Verfahren für die Produktion, Reinigung und Immobilisierung dieses Enzyms wurde entwickelt und aliphatische biobasierten Polyester mit $M_w$ und $M_n$ von ca. 1900 bzw. 1000 Da konnten erfolgreich synthetisiert werden. Diese Ergebnisse wurden zu Synthesen mittels der Lipase B aus *Candida antarctica* (CaLB) sowie der Cutinase von *Humicola insolens* (HiC) verglichen und bioinformatische Studien durchgeführt. Mittels CaLB wurde in weiterer Folge die Synthese von aliphatisch-aromatischen Oligoestern in einfachen einstufigen “one-pot” Systemen studiert. Dabei kristallisierte sich eine Kombination aus Dimethylisophthalat und 1,10-Decanediol als beste Ausgangsprodukte heraus was zu Oligoestern mit einem $M_w$ von 1512 Da führte mit einem Umsatz 87% nach 96 Stunden.

In der zweiten Phase der Arbeit wurde das Potential von Enzymen zur Hydrolyse und Funktionalisierung von Polyestern untersucht. Dabei wurde HiC erfolgreich für die Oberflächenhydrolyse von Poly(L-Milchsäure) (PLA) eingesetzt und resultierte in einer Reduktion des Wasser-Kontaktwinkels von PLA von 74.6 zu 33.1° einhergehend mit einer Erhöhung der Rauheit der Oberfläche. Das Potential von Enzymen zur gezielten Funktionalisierung von PLA Oberflächen wurde durch Einsatz

spezieller Methoden wie der $^{14}C$- Markierung der zu koppelnden Moleküle oder mittel XPS gezeigt. Schließlich konnte erstmals eine enzymatische Hydrolyse des biobasierten Polyesters Poly(ethylene furanoat) (PEF) an selbst synthetisierten Filmen mit Molekulargewichten von 6 bis 40 kDa nachgewiesen werden. Dabei konnte mittels LC/TOF-MS Analyse gezeigt werden, dass von der Cutinase Oligomere bis zu einem Polymerisationsgrad von DP4 freigesetzt wurden. Insgesamt konnte also dieser Arbeit das Potential umweltfreundlicher Biokatalysatoren sowohl zur Produktion wie auch zur Verarbeitung von bio-basierten Polymeren dargestellt werden.

# 1

## Aim of the thesis

The work described in this thesis was carried out in the context of the REFINE project, funded by the 7th Framework Programme for Research and Technological Development of the European Union. The aim of the project was to develop new skills and expertise in sustainable green materials manufacturing, technologies and applications for the development of a greener and more sustainable society. In order to achieve these goals several approaches were pursued by the partners, including - but not limited- to enzymatic processing, ionic liquids chemistry, synthesis in supercritical carbon dioxide, use of terpenes, etc. This project was based on the unique combination of knowledge along the production chain in polymer and materials science, biotechnology and applications, ecological impact and Life Cycle Analysis.

The aim of this thesis was to explore the potential of enzymes for the synthesis, hydrolysis and functionalization of polyesters, one of the most important classes of polymers actually present on the market. The use of hydrolytic enzymes as interesting green biocatalysts for several applications was investigated in the context of:

1. Enlarging and improving the biocatalyst selection for polycondensation reactions.
2. Exploring the synthesis of aromatic-aliphatic oligoesters *via* mild and sustainable routes.

3. Investigating several biocatalysts in the hydrolysis of poly(L-lactic acid) polyesters for a subsequent
4. Enzymatic functionalization of the activated polymer surfaces.
5. Enzymatic hydrolysis of the novel bio-based and industrially interesting poly(ethylene furanoate) (PEF).

# 2

## Introduction

In the chemical industry, together with petrochemicals and fine chemicals, polymers are one of the major products accounting, only in Europe, for more than 60,000 companies employing over 1.45 million people [1]. The production and commercialization of renewable biobased polymers is expected to continuously grow by 2020, thus representing a real alternative to fossil carbon sources derived polymeric products. Biodegradable plastics will be widely used in disposable products whereas non-biodegradable bioplastics will be aimed at durable applications and recycling. The term “bio-based plastics” generally refers to all those polymers obtainable by processing synthetic polymeric materials based on building blocks obtained after fermentation of natural feedstock [2].

In Nature, enzymes catalyze the breakdown of naturally occurring polymers such as lignocellulose and also polyesters such as cutin. Scientific advances in the field of biocatalysis have led to develop suitable enzymes and reaction conditions for the synthesis and functionalization of polymers and polyesters in particular [3-5]. Experts and stakeholders generally agree that one of the crucial factors that will affect the success of renewable polyester is the productivity and robustness of bioconversions, which should be greatly improved to become cost-effective. This comprehensive introduction will focus the attention on the chemical building blocks already available for the production of the next generation polyesters that aims at the massive reduction on the carbon footprint by addressing the biotechnological production of the

most relevant bio-based monomers but also the recent advances aiming at the sustainable production and processing of renewable and biodegradable polyesters.

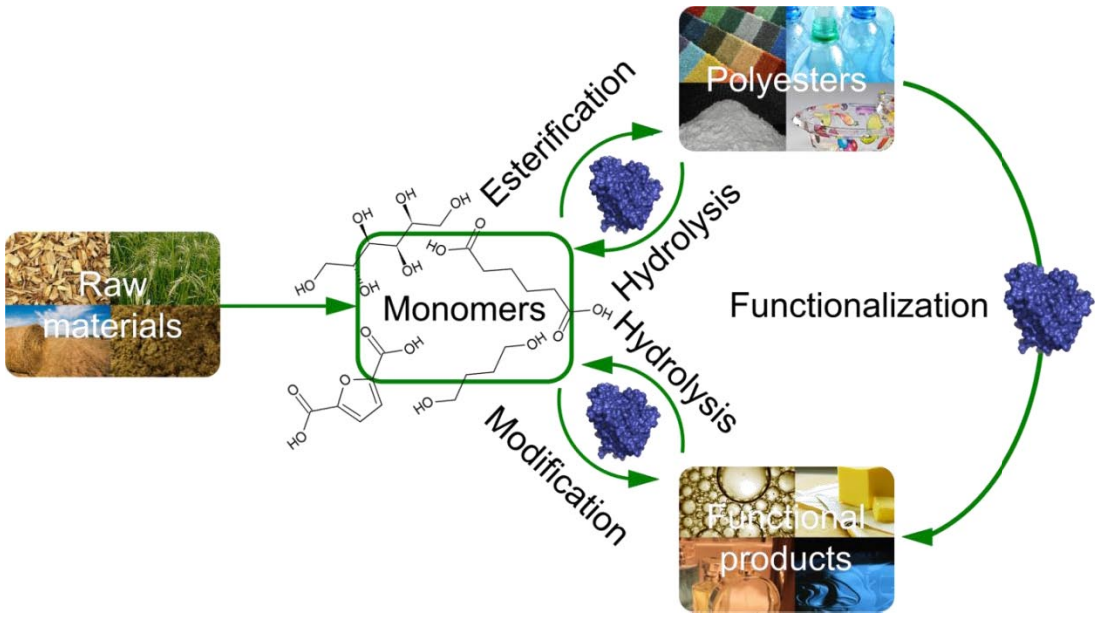



**Figure 1.** Enzymatic circle for the synthesis, functionalization, modification, and hydrolysis of bio-based polyesters. (From Pellis et al. **2016**, *Trends Biotechnol.*, DOI: http://dx.doi.org/10.1016/j.tibtech.2015.12.009).

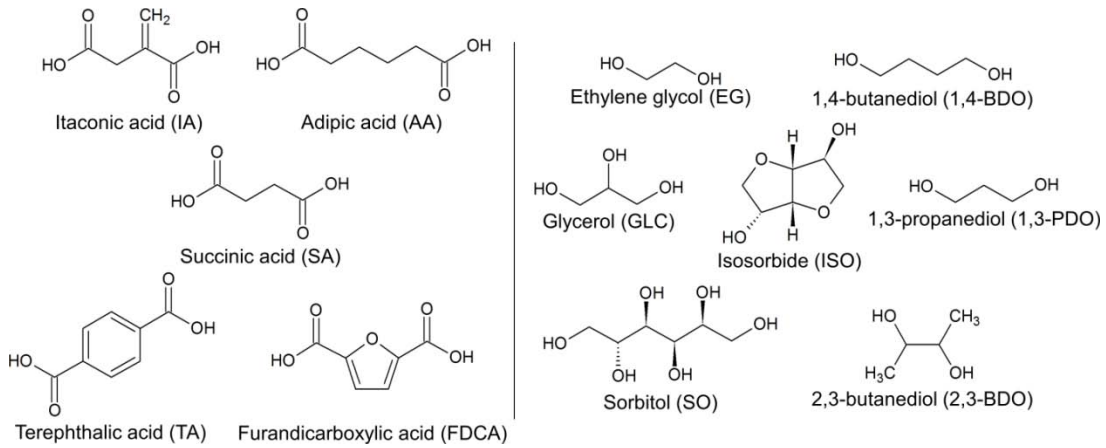
## 2.1 Bio-based monomers and polyesters

The possibility to synthetize polyesters from bio-based monomers has attracted considerable industrial interest in the last decade [6]. This first introductory section will briefly analyze and discuss the most relevant industrial-interesting biotechnological strategies for the production of the bio-based monomers that could further be processed into commercial products. In fact, among polymers, polyesters are a widely used class with applications ranging from clothing to food packaging and from the car industry to biomedical applications.

**Historical successful examples: PLA and PHAs**

The most famous example of polyester synthetized from bio-based monomers is indeed poly(lactic acid) (PLA), currently the most important bio-based polyester in terms of volume, with a capacity of approximately 180,000 tons/y. [7] PLA derives from **lactic acid**, an alpha hydroxy acid usually produced *via* bacterial fermentation. Several processes were invented and patented over the years [8, 9] and commercial bio-based PLA is available on the market since many years already.

Another product of industrial success are **poly(hydroxyalkanoate)s** (PHAs), biodegradable microbial polyesters commercially produced *via* bacterial fermentation of a large variety of carbon sources and agricultural wastes like sweet potatoes or soy starch or whey [10]. Commercial availability of PHAs is reported since the '80s when polyhydroxybutyrate (PHB), commercialized under the name of Biopol, was made available in the US by Monsanto [11]. PHAs in general are known to be environmentally friendly, biodegradable, compostable plastics with the additional advantage to degrade harmlessly in soil after a few months [12].




**Scheme 1.** The most important bio-based dicarboxylic acids and polyols currently available for polyesters synthesis. (Adapted from Pellis et al. **2016**, *Polym. Int.*, DOI: 10.1002/pi.5087)

**Latest developments**

After giving a brief overview of the successful stories of bio-based products, in this section a survey of the most recent advances for the production of bio-based monomers (overview in Scheme 1) is presented.

**Adipic acid.** The global demand of adipic acid (AA) was estimated to be 2.6 Mtons year$^{-1}$ with a yearly growing rate of 3-5%. High-purity fiber-grade AA is used to produce nylon 66, while low-purity AA is used to produce polyurethanes (Fig. 2).

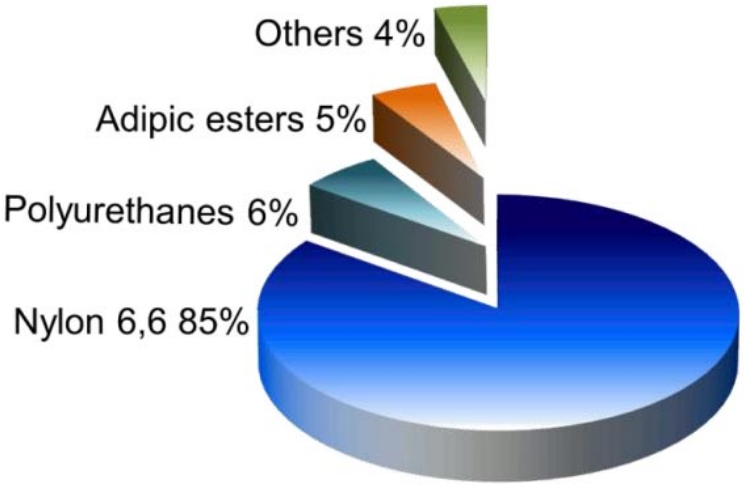



**Figure 2.** The most common commercial polymeric products derived from the bio-based monomer adipic acid (AA). (From Pellis et al. **2016**, *Polym. Int.*, DOI: 10.1002/pi.5087)

The synthesis of AA from renewable carbon sources such as glucose derived from starch or cellulose is a promising alternative route to this important commodity chemical [13]. Several start-up companies such as Rennovia, and Verdezyne have developed biobased routes to produce AA with the final aim to create 100% biobased nylon. Such approaches demonstrated are of extreme interest since demonstrated to be cost competitive with the conventional cyclohexane oxidation process. [14]

**Succinic acid.** Like lactic acid and many other bio-based monomers, also for succinic acid (SA) the most important production process from renewable feedstock is microbial fermentation of different glucose sources by various microorganisms [15]. Such processes actually are in use by two companies: the Myriant's biorefinery in Lake Providence (Louisiana, USA) that employs grain sorghum grits as its saccharificable starting material [16] and the Reverdia process (used by DSM and Roquette) where ethanol and SA are co-produced through glucose fermentation. Both processes run with genetically modified anaerobic bacteria, in such a way that alcoholic fermentation sustains the SA production [17].

The high interest towards SA is given by the fact that this dicarboxylic acid is a key component/intermediate in the production of several solvents, adhesives, printing inks, magnetic tapes, coating resins, plasticizers, emulsifiers, deicing compounds, chemical and pharmaceutical intermediates [18]. In addition, SA can be hydrogenated to obtain **1,4-butanediol** (BDO) (that can be in turn carbonylated to AA) (Figure 3). The successful production of biobased BDO on commercial scale was achieved from the partnership between Genomatica and DuPont Tate & Lyle. Five million pounds were produced by direct fermentation using conventional sugars as feedstock.

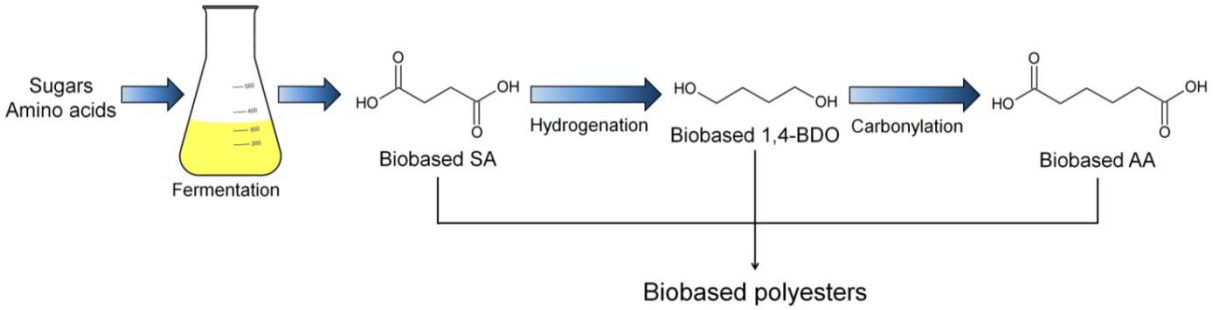



**Figure 3.** Biotechnological process for the production of biobased succinic acid (SA) and its derivatives 1,4-butanediol (1,4-BDO) and adipic acid (AA). (From Pellis et al. **2016**, *Polym. Int.*, DOI: 10.1002/pi.5087)

**Terephthalic acid.** Terephthalic acid (TA) is one of the most important monomers for polyester synthesis since it is one of the starting monomer (together with **ethylene glycol** (EG) for the production of poly(ethylene terephthalate) (PET). TA is currently produced via catalytic oxidation of petrochemical p-xylene and has an estimated global market of 50 Mton/year [19]. Nevertheless, several routes already exist enabling the synthesis of for biobased p-xylene: i) pyrolysis of biomass; ii) yeast fermentation of sugars into iso-butanol and iii) chemical conversion of carbohydrates; iv) cycloaddition of acrylic acid and isoprene (both biobased); v) use of biomass derived furfural as starting monomer [20-22]. Notably, the announced production of biobased 'plant bottle' by Coca Cola company starting from bio sourced PET is expected to have a major impact on the PET industry. At the moment, the Coca Cola company in partnership with Gevo uses biobased TA derived from iso-butanol. The bottles on the marked are nowadays composed of up to 30% of biobased monomers, in particular of EG derived from sugar cane [23]. The huge increase of biodiesel production over the last few years where **glycerol** (GLC) represents a byproduct, have led to a dramatic price reduction, making this monomer a very attractive starting material for the production of EG and 1,2-propylene glycol *via* catalytic

hydrogenolysis [24]. Even if such bio-based pathway is already technically feasible, the process resulted to be not efficient in terms of carbon and oxygen yields. Processes for EG production via xylitol, sorbitol and GLC are more sustainable, although still require further development. Nowadays the ethylene oxidation process is still the most widely used for EG production

**Furandicarboxylic acid.** Dehydration of sugars available within biorefineries can lead to a family of products, including dehydrosugars, furans, and levulinic acid. Furandicarboxylic acid (FDCA) is a member of the furan family, and is usually synthesized by oxidative dehydration of glucose using oxygen, or electrochemistry [19]. Moreover the conversion can also be carried out by oxidation of 5-hydroxymethylfurfural. FDCA has been suggested as an important renewable building block because it can substitute TA in the production of various polyesters due to comparable properties of the final material (eg. PET and PBT) [25]. The versatility of this compound is also evident when considering various FDCA based derivatives accessible via relatively simple chemical reactions [26].

The primary technical barriers for the production of FDCA from renewable materials include the development of effective and selective sugar dehydration, which is currently an uncontrolled process. In addition, the control of FDCA reactivity make difficult to develop efficient esterification reaction for FDCA-based polyesters synthesis [27]. Recently, Avantium (Geleen, The Netherlands) has announced a new technology that involves a highly reactive catalyst together with an efficient separation technology, which would result in economic feasible production of FDCA starting from 2016. The company is currently running a pilot plant with a 40 tons/year capacity. The planned industrial production capacity is estimated to be between 30 and 50 ktons/year [28].

The use of FDCA in the production of bio-based alternatives to PET is expected to capture over 60% of global FDCA production by 2020. Since PET is widely used in food and packaging industry, there is a strong interest in developing bio-based alternative polymers. In particular, a combination of FDCA with EG will lead to 2,5-furandicarboxylate (PEF); such polymer is expected to be commercialized in 2018 with a production range of about 300 ktons/year [29].

Table 1 summarizes the actual status of the biotechnological production of bio-based monomers and their corresponding industrial applications.

**Table 1.** Overview of the biotechnological routes and status of the industrially most relevant bio-based monomers that can be employed in the synthesis of bio-based polyesters. (Adapted from Pellis et al. **2016**, *Trends Biotechnol*., DOI: http://dx.doi.org/10.1016/j.tibtech.2015.12.009)

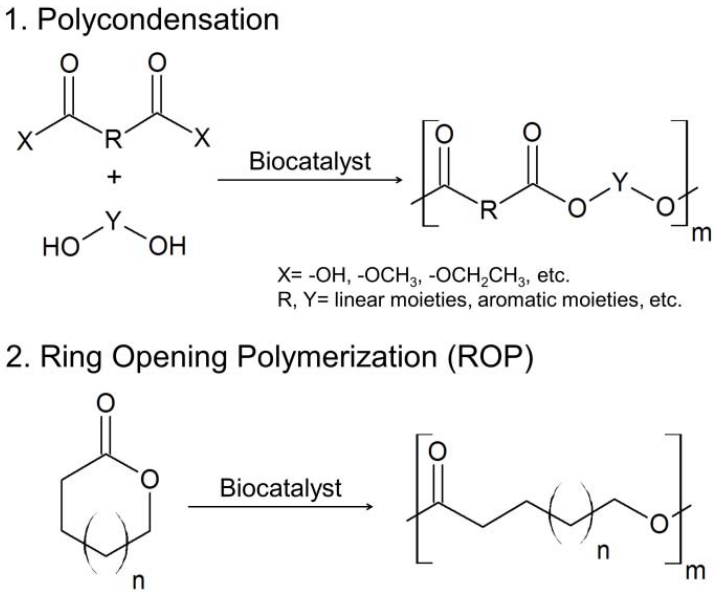
| Monomer | Biotechnological route | Company | Status | Application of the corresponding bio-based polyesters |
|---|---|---|---|---|
| Sorbitol | Fermentation + hydrogenation | Roquette, ADM | Market | Functional polyesters; coatings |
| Isosorbide | Sorbitol dehydration | Roquette | Market | Thermosetting resins |
| Ethylene glycol | Ethanol dehydration | India Glycols Ltd, Greencol Taiwan | Market | PET; PEF |
| 1,3-propanediol | Fermentation | Du Pont, Tate & Lyle, Metabolic Explorer | Market | PTT; fibers; elastomers; polyester-urethanes |
| 1,4-butanediol | Fermentation, succinic acid hydrogenation | BioAmber, Genomatica, Mitsubishi | Market | PBAT; PBS; PBT |
| Adipic acid | Fermentation + hydrogenation | Celexion LLC, BioAmber, Rennovia, Verdezyme | Market | Resins; polyester-amines; polyester-urethanes |
| Itaconic acid | Fermentation | Qingdao Kehai Biochemistry, Itaconix | Market | Photocurable precursors; plasticizers |
| Lactic acid | Fermentation | Nature Works, BASF, Purac, Cargill, BBCA, Galactic | Market | PLA |
| Succinic acid | Fermentation | BioAmber, Myriant, Reverdia, BASF, Purac, Succinity | Market | Textiles; coatings; PBS; PBT |
| Terephthalic acid | Isobutylene oxidation, fermentation | Virient, Annellotech, Genomatica | Pilot plant | PET; coatings |
| Levulinic acid | Fermentation, acid treatment of C6 sugars | GFBiochemicals, Bio-on, Biofine Renewables | Market | Coatings, hyperbranched dendrimeric polyesters |
| Malic acid | Fermentation | Novozymes | Pilot plant | Functionalized chiral polyesters |
| 2,5-furandicarboxylic acid | Fermentation + dehydration + oxidation | Avantium | Pilot plant | PEF; polyester-urethanes |

## 2.2 Biocatalyzed synthesis of bio-based polyesters

After development of the inorganic and the Ziegler-Natta catalysts, during the 1980s, an alternative enzyme-based polymerization strategy arose as alternative for polymer synthesis. Esterases, and in particular lipases, catalyze the hydrolysis of fatty acid esters in aqueous environments. [4] Some of these hydrolases were found to be stable in organic solvents where they are able to catalyze reverse reactions namely esterification and transesterification. [30] Hence, these enzymes were studied regarding synthesis of aliphatic - and to a lesser extend of aromatic - polyesters. [31] Hydrolases for polyester production are relatively stable, commercially available and easily produced. Among lipases, undoubtedly the most widely used biocatalyst for polyester synthesis is the lipase B from *Candida antarctica* (CaLB), due to its commercial availability as a free and immobilized catalyst.

**Polycondensation vs Ring Opening Polymerization (ROP)**

Polyester synthesis can be accomplished *via* polycondensation of dicarboxylic acids with polyols (Scheme 2, top).




**Scheme 2.** Routes for the enzymatic synthesis of biobased polyesters. 1) Polycondensation reaction of diacids (or their diesters) with polyols. 2) Ring opening polymerization (ROP) of lactones. (From Pellis et al. **2016**, *Polym. Int.*, DOI: 10.1002/pi.5087)

Early reports on enzymatic polycondensation of dicarboxylic acids and polyols indicate the formation of only low molecular weight products. In order to produce polyesters of high molecular weight, it is necessary to remove the byproducts (water or alcohol in the case of diesters) formed during the reaction in order to shift the

equilibrium to the polymerization reaction [32]. Equilibrium can be shifted towards polyesterification by using diesters instead of dicarboxylic acids, since the volatility of the byproduct (alcohol) is higher than water (byproduct from dicarboxylic acids) [33]. Likewise, vinyl esters of dicarboxylic acids have been reported to be very effective monomers for enzyme-catalyzed polymerizations since the vinyl alcohol (polycondensation leaving group) is irreversibly tautomerized to acetaldehyde leading to the desired polyester in high yields; even if high acetaldehyde concentration might determine a decrease of enzyme activity, clearly affecting biocatalyst reuse. An effective and commonly method to increase the polymer molecular weight and the reaction yield is to perform the reaction under vacuum conditions to remove by products and boost thermodynamic equilibrium towards the synthesis [34].

In the 90s the first enzymatic ring opening polymerizations (ROP) of lactones to give the corresponding polyesters was described (Scheme 2, bottom). ROP of lactones and carbonates does not produce a leaving group during the reaction course [35]. Unsubstituted lactones with a 4-17 carbon atoms ring size were polymerized using various lipases both in bulk and in various solvents [36]. A representative example of ROP is given by the synthesis of poly(caprolactone) (PCL) starting from ε-caprolactone (ε-CL). Toluene has been selected as solvent for the CaLB-catalyzed ROP, since it is able to solubilize substrates and products while retaining the activity of the biocatalyst [37]. Water plays a major role in ROP reactions and it is important to ensure its removal from the reaction system to achieve good conversions and high molecular weight products. A recent work from Gross and co-workers reported the synthesis of polyesters with $M_w$ of 163 kDa after only 15 min of reaction, starting from ω-pentadecalactone and using a reactive extrusion technique [38].

**Biocatalysts availability**

In 1984, Okumura and co-workers published the first study where a lipase from *Aspergillus niger* was demonstrated to catalyze the polycondensation of several dicarboxylic acids and polyols to obtain short oligomers [39]. Following this study, lipases gained interest as catalyst for polyester synthesis and several members of this family were investigated to explore their potentialities in this field [31]. In the 90s, Linko and co-workers conducted a wide range of studies where they compared different lipases. From these experimental evidences, the most promising biocatalysts for the polyester synthesis resulted to be the lipase from *Mucor miehei*

[31]. Using the above mentioned enzyme, polymers of AA and 1,4-BDO were obtained with a molecular weight of over 40 kg $mol^{-1}$ [32].

After these studies carried out between 1980 and the '90s, CaLB arose as the biocatalyst of choice and it still remains to be the most commonly used enzyme for synthetic applications [40, 41].

**Table 2.** Most recent enzymatic processes for synthesis and functionalization/hydrolysis of bio-based polyesters. (Adapted from Pellis et al. **2016**, *Trends Biotechnol.*, DOI: http://dx.doi.org/10.1016/j.tibtech.2015.12.009)

| Biocatalyst | Material class | Goal |
|---|---|---|
| *Candida antarctica* lipase B | Lactic-acid based | ROP of D,D-lactide |
| | Terephthalic acid-based<br>Ethylene glycol-based<br>2,5-furandicarboxylic acid-based | Synthesis of aromatic/aliphatic polyesters |
| | Sorbitol-based | Synthesis of hydroxy-functional polyesters |
| | Adipic acid-based<br>1,4-butanediol-based<br>Isosorbide-based | Synthesis of aliphatic polyesters |
| | Itaconic acid-based<br>1,4-butanediol-based | Synthesis of vinyl-functional polyesters |
| | Glycerol-based<br>Itaconic acid-based | Branched-controlled polyesters, epoxide-containing polyesters |
| | Malic acid-based<br>Adipic acid-based | Copolymers of L-malic acid, adipic acid and 1,8-octanediol |
| *Humicola insolens* cutinase | Adipic acid-based<br>1,4-butanediol-based | Synthesis of aliphatic polyesters |
| | Terephthalic acid-based<br>Ethylene glycol-based | PET hydrolysis |
| | Lactic-acid based | Surface functionalization of PLA films |
| *Thermobifida cellulosilytica* cutinase 1 | Terephthalic acid-based<br>Ethylene glycol-based | Surface functionalization of PET films |
| *Thermobifida fusca* cutinase | Terephthalic acid-based<br>Ethylene glycol-based | Degradation of PET nanoparticles |
| *Thermobifida halotolerans* esterase | Terephthalic acid-based<br>Ethylene glycol-based | PET hydrolysis |
| | Lactic-acid based | PLA hydrolysis |
| *Thielavia terrestris* cutinase | Succinic acid-based<br>1,4-butanediol-based | PBS hydrolysis |
| | Terephthalic acid-based<br>Ethylene glycol-based | PET hydrolysis |

The most used preparation of CaLB is the commercially available Novozym® 435, consisting of the lipase adsorbed on a macroporous acrylic resin. [4] The biocatalyst displays different activity and selectivity according to the medium used for the reaction, so that supercritical $CO_2$ was also tested as an alternative to organic

solvents is used as reaction solvent instead of the most commonly used organic solvents or bulk reactions [42, 43]. The need of enlarging substrate specificity for the synthesis of new polyesters has boosted the study on different hydrolases, especially belonging to cutinase family. Cutinase from *Humicola insolens* (HiC) was studied by Gross and co-workers for both ROP and polycondensation reactions (Figure 4) [44, 45]. The improvement of the catalytic activity of CaLB towards ROP of D-lactic acid has been also achieved through protein engineering approaches [46]. Recently also the cutinase 1 from *Thermobifida cellulosilytica* was investigated for the synthesis of biobased polyesters with a combined computational-experimental approach [47]. Table 2 summarizes the most recent enzymatic polyesters processes.

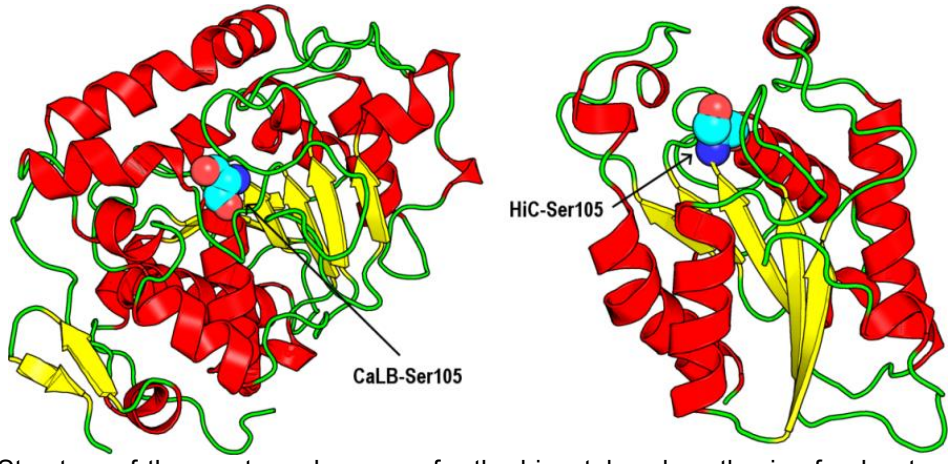



**Figure 4.** Structure of the most used enzyme for the biocatalyzed synthesis of polyesters nowadays *Candida antarctica* (CaLB) on the left, compared with the emerging cutinase from *Humicola insolens* (HiC) on the right (Structures retrieved from Protein Data Bank, codes 1TCA and 4OYY respectively). Catalytic serine for each enzyme is highlighted in sphere representation. (From Pellis et al. **2016**, *Polym. Int*., DOI: 10.1002/pi.5087)

## Advantages and challenges of biocatalyzed polymerizations

Suitable activity and specificity of enzymes are not sufficient to guarantee its efficient application to polyester synthesis. The biocatalysts must be immobilized [48-50] in order to allow its recovery and reuse. That implies the necessity of tailored immobilization protocols preventing the detachment of the protein from the support and, therefore, the contamination of the product. Moreover, the reaction configuration must preserve the integrity of the support, which easily undergoes fragmentation under mechanical and magnetic stirring conditions [51, 52]. This is even more relevant in the case of solvent-free polymerizations, where the viscosity of the reaction system requires vigorous mixing [53]. New covalent immobilization methods and reactor configurations exploiting thin-film systems have been recently developed to overcome these technological limitations [4, 5]. Finally, downstream processing

still represents a major challenge in enzymatic synthesis of polyesters. Supercritical carbon dioxide ($scCO_2$) [54] and ionic liquids [55] (Figure 4) were explored as possible greener solutions to limit the use of conventional toxic organic solvents.

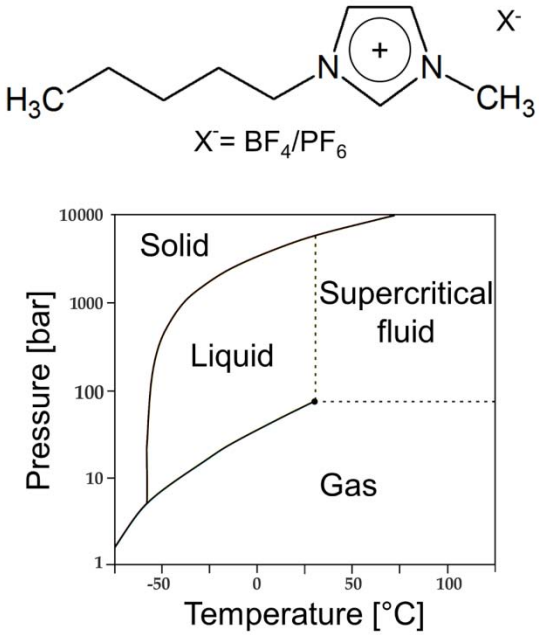



**Figure 4.** Ionic liquids used for the biocatalyzed synthesis of polyesters (top) and phase diagram of $scCO_2$ (bottom).

These scientific and technological challenges still prevent the biocatalyzed production of polyester at an industrial scale. Nevertheless, the research in this field is motivated by the necessity of replacing classical chemical routes employing toxic catalysts [56]. Moreover, temperatures required by chemical polycondensation (generally >200 °C) [57, 58] cause undesired side reactions (i.e. dehydration of the polyols or β-scissions of polyesters to acid and alkene terminal groups) and degradation of chemical unstable monomers. For example, siloxane, epoxy and vinyl moieties represent unsuitable functional groups which react in an uncontrolled way [59]. On the other hand, such functional polyesters are of extreme interest in the biomedical field due to their biodegradability and lower toxicity. In this case enzymatic catalysis represents an appropriate way to obtain functional polyesters containing sensitive groups. For example, itaconic acid (or its derivatives) was polymerized with several polyols to give side-chain functionalized polyesters where the vinyl moiety was preserved after the reaction and could be therefore used for further functionalizations/cross-linking of the polymer [4, 5]. Similar polyesters containing a lateral epoxy moiety were also reported after a polycondensation carried out using CaLB as catalyst [61].

A future challenge for enzymatic polycondensations certainly comprises the incorporation of aromatic dicarboxylic esters as substrates in order to produce PET-like aromatic-aliphatic polyesters. Several attempts to polymerize such compounds were reported [62, 63] and they lead to products with modest molecular weight.

## 2.3 Enzymatic hydrolysis/functionalization of bio-based polyesters

Ever since plastics were introduced into human daily life, a significant improvement of stability and durability was achieved, producing materials strongly resistant to environmental conditions (eg. PE bags). With the increase of the worldwide demand over the years, the amount of plastic materials started to be an environmental problem due to the durability in the environment after disposal, leading to ecological problems like the formation of the plastic ocean patches or its presence in rivers [64]. Nevertheless, polymers with a heterogeneous atom backbone composition such as polyesters, polyamides and polyurethanes can be degraded by microorganisms and isolated enzymes [65, 66]. Most importantly, mild enzymatic reactions allow to finely tune and control the degree of hydrolysis and functionalization of polymeric surface, which can be exploited for further functionalization and advanced applications (Figure 5) [3, 67].

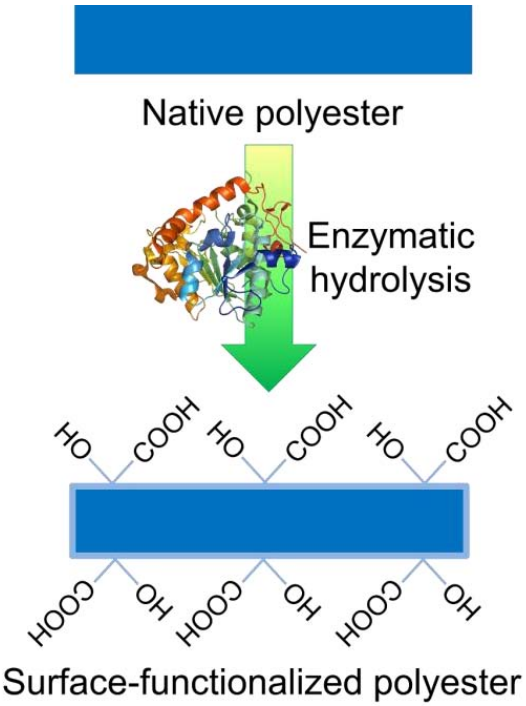



**Figure 5.** Enzymatic functionalization of polyesters. The biocatalyst hydrolyzes only the surface chains of the polymer leaving the bulk properties unaltered. (From Pellis et al. **2016**, *Polym. Int.*, DOI: 10.1002/pi.5087)

### Enzymatic functionalization of PLA

PLA is a fascinating polymer: it can be obtained from renewable agricultural sources and its production consumes $CO_2$ while providing consistent energy savings. Due to its biocompatibility and processability properties, PLA is used for biomedical applications exploiting the fact that it can be absorbed by the human body. It is also

widely used as a packaging material, taking advantage of its ability to biodegrade [68] Partial hydrolysis of the outer layer of the polymer for the generation of either carboxyl or hydroxyl groups cannot only enhance its biocompatibility [69], but also promote the coupling of molecules carrying various functionalities. Compared with PET, PLA is more sensitive to alkaline hydrolysis and nonwoven PLA undergoes a 100% weight loss after only a 30-min incubation with 10-20% NaOH. Controlled enzymatic hydrolysis was shown to be an alternative to alkaline treatments to avoid material damage. Different lipases from *Aspergillus niger*, *Candida cylindracea*, and *Candida rugosa* were tested on nonwoven PLA and optimum surface hydrolysis conditions were reported, enabling the preservation of their mechanical or structural properties [70]. One of the most-studied enzymes for the hydrolysis of PLA is proteinase K, which catalyzes the hydrolysis of PLA by acting on amorphous regions between the crystalline regions, rather than on the folding chains.

Cutinases have also been investigated for their ability to hydrolyze PLA films and/or fibers and are gaining increasing attention for applications in polymer surface functionalization [70, 71]. Ribitsch et al. reported that cutinase from *Thermobifida halotolerans* hydrolyzes PLA films, leading to the release of lactic acid monomers. The resulting polar groups on the PLA surface cause a decrease in the water contact angle (from approximately 76° to the complete spread of the water drop on the polymer surface) [71].

The enzymatic functionalization of PLA has biomedical application relevance due to the possibility of increasing its biocompatibility while leaving its bulk properties unaltered. Nyanhongo et al. reported the activation of the surface of PLA membranes via hydrolysis catalyzed by HiC, followed by the coupling of unfolded human serum albumin to the newly generated superficial carboxylic groups. The hydrophilic groups of the grafted human serum albumin were important to promote the interaction between the PLA membrane and cells, resulting in up to 2.4 times higher cytocompatibility [69].

Enzymes were also used for the selective excavation of the PLA-based core of block copolymer micelle assemblies and their shell cross-linked nanoparticle analogs. The hydrolysis rate of PLA from the cores of the block copolymer micelles was significantly higher than for bulk PLA [72].

**Enzymatic modification of PET**

PET is currently the most produced polyester worldwide, with applications as a synthetic textile fiber, film, medical device, and packaging material.

Despite the optimal mechanical properties, the main drawback of PET is its highly hydrophobic nature, showing a poorly wettable surface that is difficult to functionalize. Industrial applications frequently require an activated or reactive surface to graft molecules, such as flame retardants or water-soluble dyes. Several methods, including plasma or chemical treatments, caused aging effects (which decrease the resulting surface hydrophilicity) or a consistent reduction of the polymer weight and strength [73]. Enzymatic hydrolysis showed the potential to overcome such limitations and provide surface functionalization without affecting the bulk properties of the polymer [3]. Several polyesterases were reported to be active in the hydrolysis of PET [71, 74]; in particular, cutinases from *Thermobifida* species were demonstrated to be active either on PET or on small PET model substrates [71, 75-78]. A correlation was found between the kinetic parameters of soluble substrates, the release of hydrolysis products, and the final degree of PET hydrophilization. Interestingly, the hydrolysis efficiency of two highly homologous cutinases from *Thermobifida cellulosilytica* was found to be influenced by the electrostatic and hydrophobic properties of the enzyme surface in proximity to the active site [73]. Several PET-based substrates ranging from films [73] and fibers [76] to nanoparticles [76] were enzymatically hydrolyzed using cutinases as biocatalysts and, in the case of cutinase from *Thermobifida fusca*, the hydrolysis products were observed to cause inhibition of the enzyme [79].

Cutinases from *Fusarium solani*, *Pseudomonas mendocina*, and *Humicola insolens* were also reported to be active on PET [80]. As already mentioned, PET is largely used for the manufacturing of plastic bottles, which uses a low crystallinity polymer to achieve high bottle transparency. This particular form of PET is an optimal substrate for enzymatic hydrolysis. Therefore, cutinases were suggested for PET recycling [81] in a process that could overcome the quality limitations of current recycling strategies based on blending. The enzyme hydrolyzed the polymer to its constituent monomers (TA and EG) so that the freshly produced monomers could then be promptly repolymerized after separation of dyes and contaminants. The hydrolytic properties of cutinases are also exploited in detergent formulations, where enzymes act as effective antipilling agents by partially hydrolyzing fuzz from polyester fibers [82, 83]

Recent developments in the field of biocatalyzed hydrolysis of PET are focused mainly on improvement of the activity of enzymes via approaches of enzyme engineering, targeting both the active site and sorption properties (Figure 6).

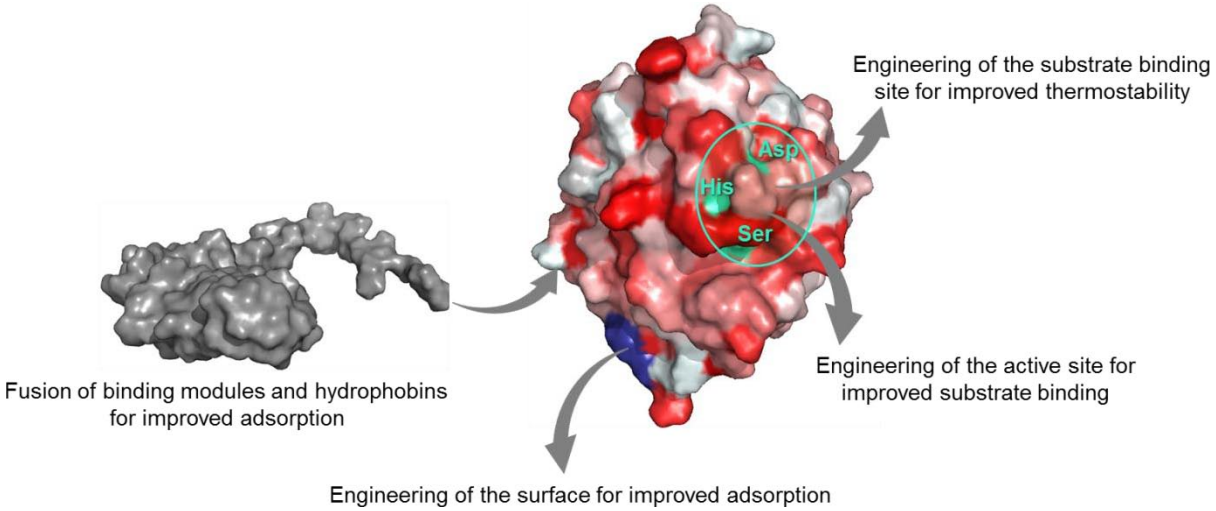



**Figure 6.** Biocatalyst engineering strategies for enhanced hydrolysis of aromatic-aliphatic polyesters. (From Pellis et al. **2016**, *Trends Biotechnol.*, DOI: http://dx.doi.org/10.1016/j.tibtech.2015.12.009)

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# The Closure of the Cycle: Enzymatic Synthesis and Functionalization of Bio-Based Polyesters


## Abstract

The polymer industry is under pressure to mitigate the environmental cost of petrol-based plastics. Biotechnologies contribute to the gradual replacement of petrol-based chemistry and the development of new renewable products, leading to the closure of carbon circle. An array of bio-based building blocks is already available on an industrial scale and is boosting the development of new generations of sustainable and functionally competitive polymers, such as polylactic acid (PLA). Biocatalysts add higher value to bio-based polymers by catalyzing not only their selective modification, but also their synthesis under mild and controlled conditions. The ultimate aim is the introduction of chemical functionalities on the surface of the polymer while retaining its bulk properties, thus enlarging the spectrum of advanced applications.


## Trends

- Different integrated biotechnological advances are gradually replacing petrol-based chemistry and contribute to the development of new chemicals and plastics. Some bio-based polymers, such as PLA, are chemically synthesized and are already available on an industrial scale.
- A long-term contribution to the production of renewable building-blocks and monomers is expected from biotechnology research on the bioconversion of $CO_2$ and microbial electrocatalysis.
- Advanced applications of polymers are obtainable by introducing chemical functionalities on the surface of the polymer while retaining its bulk properties. Such modifications can change the superficial hydrophobicity as well as introduce a ‘pendant’ as anchoring point or for successively chemical modifications. These possibilities are of key importance, especially for bio-medical applications.
- Biocatalyzed polymerization is not yet economically competitive. The conventional process configurations and reactors used in chemical synthesis do not respond to the complexity of the bio-catalytic systems. Thus, the need to improve mass transfer while preserving the integrity of the biocatalyst still requires a specific tailored solution.
- Robust enzyme immobilization, as well as thin film conditions or ionic liquids, are some of the solutions proposed for overcoming such limitations.

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## Introduction

For several decades, the demand for polymers and plastics derived from fossil fuels has grown at a faster rate than for any other group of bulk materials, and expectations are that this trend will continue until 2020 [1]. With the worldwide increase in demand, the amount of plastic material released to the environment has become a significant problem because such material does not biodegrade easily or quickly, if at all, leading to ecological problems such as the formation of plastic patches in the ocean or in rivers [2]. The UN Environmental Programme estimated that the overall natural capital cost of plastic use in the consumer goods sector each year is US$75 billion, representing financial impacts resulting from issues such as pollution of the marine environment or air pollution caused by incinerating plastic. Of the natural capital costs of plastic, 30% are due to greenhouse gas emissions from raw material extraction and processing [3].

Biotechnology research has responded to the need to mitigate the environmental impact of plastics with research and technological innovations that now enable the biotechnological production of bio-based monomers from renewable carbon (see Glossary) on an industrial scale [4].

Among polymers, polyesters are a widely used class with applications ranging from clothing to food packaging and from the car industry to biomedical applications. The possibility to synthesize polyesters from bio-based monomers is demonstrated by PLA, currently the most important bio-based polyester in terms of volume, with a capacity of approximately 180,000 tons/y. Renewable polyesters can be also biosynthesized by microorganisms through complex regulatory pathways responding to external stimuli, including poly(hydroxyalkanoate)s (PHAs), which are biodegradable microbial polyesters commercially produced *via* fermentation, although we do not discuss them here [5].

Research aiming at developing the next generation of bio-based polyesters must not only address their sustainability, but also pursue their competitiveness in terms of their superior technological and functional properties. Biocatalysis goes a step further by enabling the synthesis of structured, functionalized, and biodegradable polyesters through highly selective and benign synthetic processes. Moreover, biocatalysts enable targeted hydrolyses and modifications of polyesters that are not possible with conventional chemical strategies [6, 7]. Here, we illustrate how polymer chemistry is already benefitting from a range of biotechnological advances that enable the

environmentally sustainable production of high-quality polyesters with new functional properties. Innovations in the field of bio-based polyesters are a paradigm of the increasingly intimate integration between biotechnologies and sustainable chemistry, which responds to the pressing challenges of a circular economy [8].

## Bio-based monomers and polyesters

The percentage of chemical production based on biotechnology is estimated to increase from less than 2% in 2005 to approximately a quarter of all chemical production by 2025. [9, 10]. The largest contribution will come from the conversion of renewable carbon into chemicals *via* biotechnological routes. Factors boosting the integration between biotechnology and chemistry include the expected decrease in petroleum production and concerns about $CO_2$ emissions; however, there is also the need to improve public confidence in the chemical industry. Although only 7% of worldwide petroleum consumption is currently used for chemical production, bio-based processes leading to platforms of chemical building blocks will create higher added-value compared with current biofuel production processes [4].

By 2030, the market value of bio-based building blocks is expected to reach €3.2 billion, whereas the demand for fermentation-based chemical building blocks was less than €700 million in 2013[i].

Such building blocks could either be produced from renewable carbon through green chemical conversion routes or *via* microbial conversions. The incorporation of fermentative production of basic building blocks as unit operations in integrated **biorefineries** [4] is dependent on intense research activities ranging from microbial strain development and engineering to fermentation and down-stream processing optimization. Different critical analyses of research advances for enhancing the commercial potential of specific building blocks have been extensively reviewed elsewhere [11, 12].

Strong research efforts are also currently directed towards the bioconversion of $CO_2$ into chemical building blocks by designing artificial metabolic routes or through microbial electrocatalysis. However, intense fundamental research is still needed before consumers will benefit from the practical applications of such technologies [12]. One of the key end uses for bio-based building blocks is expected to be in the production of bio-based polymers, with a projected market value for renewable plastic of €5.2 billion by 2030 [10]. Recent industrial analysis (November 2015) suggested that worldwide production capacity will triple from 5.7 million tons in 2014 to nearly 17 million tons in 2020[ii]. The most relevant monomers already available for use in fermentation technologies or in chemical processes and that are already tested in the synthesis of polyesters are detailed in Table 1.

**Table 1.** Overview of the biotechnological routes and status of the industrially most relevant bio-based monomers that can be employed in the synthesis of bio-based polyesters.

| Monomer | Biotechnological route | Ref. | Company | Status | Application of the corresponding bio-based polyesters | Ref. |
|---|---|---|---|---|---|---|
| Sorbitol | Fermentation + hydrogenation | [14] | Roquette, ADM | Market | Functional polyesters; coatings | [10, 11, 15, 16] |
| Isosorbide | Sorbitol dehydration | [17] | Roquette | Market | Thermosetting resins | |
| Ethylene glycol | Ethanol dehydration | [15] | India Glycols Ltd, Greencol Taiwan | Market | PET; PEF | |
| 1,3-propanediol | Fermentation | [18] | Du Pont, Tate & Lyle, Metabolic Explorer | Market | PTT; fibers; elastomers; polyester-urethanes | |
| 1,4-butanediol | Fermentation, succinic acid hydrogenation | [19] | BioAmber, Genomatica, Mitsubishi | Market | PBAT; PBS; PBT | |
| Adipic acid | Fermentation + hydrogenation | [20] | Celexion LLC, BioAmber, Rennovia, Verdezyme | Market | Resins; polyester-amines; polyester-urethanes | |
| Itaconic acid | Fermentation | [21] | Qingdao Kehai Biochemistry, Itaconix | Market | Photocurable precursors; plasticizers | |
| Lactic acid | Fermentation | [22] | Nature Works, BASF, Purac, Cargill, BBCA, Galactic | Market | PLA | |
| Succinic acid | Fermentation | [23] | BioAmber, Myriant, Reverdia, BASF, Purac, Succinity | Market | Textiles; coatings; PBS; PBT | |
| Terephthalic acid | Isobutylene oxidation, fermentation | [24] | Virient, Annellotech, Genomatica | Pilot plant | PET; coatings | |
| Levulinic acid | Fermentation, acid treatment of C6 sugars | [25] | GFBiochemicals, Bio-on, Biofine Renewables | Market | Coatings, hyperbranched dendrimeric polyesters | [26] |
| Malic acid | Fermentation | [27] | Novozymes | Pilot plant | Functionalized chiral polyesters | [28] |
| 2,5-furandicarboxylic acid | Fermentation + dehydration + oxidation | [29] | Avantium | Pilot plant | PEF; polyester-urethanes | [30] |

Polyesters are generally produced through chemical processes such as ring opening polymerization (ROP) or *via* polycondensation of di-and trifunctional polyols with dicarboxylic acids or their diesters and/or anhydrides. The resulting bio-based polyesters can be endowed with various properties, and are classified as a function of their sustainable qualities as renewable, biocompatible, and/or biodegradable. Therefore, they are not biologically degradable per se since their monomers might be linked together *via* chemical bonds recalcitrant to enzymatic hydrolysis [13].

Polyesters synthetized via enzymatic catalysis are also biodegradable because the enzymes used for catalyzing the synthesis of ester bonds are also able to catalyze their hydrolysis. Nevertheless, some chemically synthesized ester bonds can be also hydrolyzed enzymatically: although PLA is produced *via* conventional chemical ROP starting from bio-based lactic acid, it is formed by enzymatically hydrolysable ester linkages. However, it is classified as bio-based biodegradable polyester [14]. Further information on the environmental classification and certification of plastics is available from European Bioplastics.[iii]

Bio-based chemicals and polymers suffer severe economic competition from cheaper products synthesized by conventional routes from hydrocarbons that were optimized more than 100 years ago. PLA-based products had already been developed by the 1940s and 1950s, but their production became economically viable only 70 years later. This demonstrates the importance of optimizing the productivity and robustness of bioconversions to achieve cost-effective production.

The success of bio-based polyesters does not rely solely on their capacity to replace fossil-based polymers while being economically competitive. Rather, the next generation of bio-based polyesters should bring entirely new advanced chemical and functional properties to the polymer scenario. This challenge has been addressed over the past decade by exploiting the unique selectivity and efficiency of biocatalysts (Table 2); here, we analyze the potential and limitations of the enzymatic approach for the hydrolysis, modification, and functionalization of bio-based polyesters [15-17].

Glossary

- **Biodegradable**: property of a material that enables it to be chemically dissolved by bacteria, fungi, or enzymes.
- **Biorefinery**: the process that entails refining of biomass in a commercial context for the production of fuels, chemicals, polymers, materials, food, feed, and value-added ingredients.
- **Cutinases**: (E.C. 3.1.1.74) hydrolytic enzymes that catalyze the hydrolysis of the ester bonds of cutin, a natural polyester of the plant cuticle.
- **Immobilization of the biocatalyst**: enzymes are formulated to become insoluble and enabling recycling. The enzymatic proteins can be either anchored on solid matrixes, cross-linked, or entrapped in porous materials.
- **Lipases**: (E.C. 3.1.1.3) hydrolytic enzymes that catalyze the hydrolysis of triglyceride ester bonds.

- **Prodrug**: an inactive compound that is metabolized (i.e., converted within the body) into a pharmacologically active drug.
- **Renewable resource**: organic natural resource that can replenish to overcome usage and consumption, either through biological reproduction or other naturally recurring processes.
- **Stirred tank reactor**: a particular type of chemical reactor, usually considered the simplest type of reactor, where mixing is provided by a mechanical stirrer system, such as a turbine wing or a propeller.

## Enzymatic functionalization and modification of polyesters

Polymers with a heterogeneous atom backbone composition, such as polyesters, polyamides, and polyurethanes, can be degraded by microorganisms [18]. Since 1997, several isolated lipases have been reported to catalyze the hydrolysis of aliphatic polyesters, while only a few hydrolases are active on aromatic-aliphatic polyesters [18]. These polyester-degrading enzymes represent a milder and selective alternative to chemical or physical treatments aiming at introducing chemical functionalities to the surface of polymers while retaining their bulk properties. One of the major advantages of enzymatic polyester modification is the possibility of fine tuning and controlling the degree of functionalization by operating under mild conditions [17, 19]. Here, we describe the major advances in enzyme-catalyzed hydrolysis of bio-based polyesters achieved over the past few years, focusing on the two most industrially relevant polymers, PLA and PET.

### Enzymatic functionalization of PLA

PLA is a fascinating polymer: it can be obtained from renewable agricultural sources and its production consumes $CO_2$ while providing consistent energy savings. Due to its biocompatibility and processability properties, PLA is used for biomedical applications exploiting the fact that it can be absorbed by humans. It is also widely used as a packaging material, taking advantage of its ability to biodegrade [20].

Partial hydrolysis of the outer layer of the polymer for the generation of either carboxyl or hydroxyl groups cannot only enhance its biocompatibility [21], but also promote the coupling of molecules carrying various functionalities. Compared with poly(ethylene terephthalate) (PET), PLA is more sensitive to alkaline hydrolysis and nonwoven PLA undergoes a 100% weight loss after only a 30-min incubation with 10-20% NaOH. Controlled enzymatic hydrolysis was shown to be an alternative to alkaline treatments to avoid material damage. Different lipases from *Aspergillus niger*, *Candida cylindracea*, and *Candida rugosa* were tested on nonwoven PLA and optimum surface hydrolysis conditions were reported, enabling the preservation of their mechanical or structural properties [22]. One of the most-studied enzymes for the hydrolysis of PLA is proteinase K, which catalyzes the hydrolysis of PLA by acting on amorphous regions between the crystalline regions, rather than on the folding chains. Tsuji and co-authors reported how proteinase K hydrolyzes PLA via both endo- and exo- chain scission.

**Table 2.** Most recent enzymatic processes for synthesis and functionalization/hydrolysis of bio-based polyesters.

| Biocatalyst | Material class | Goal | Ref. |
|---|---|---|---|
| CaLB | Lactic-acid based | ROP of D,D-lactide | [36,37] |
| | Terephthalic acid-based<br>Ethylene glycol-based<br>2,5-furandicarboxylic acid-based | Synthesis of aromatic/aliphatic polyesters | [38, 39] |
| | Sorbitol-based | Synthesis of hydroxy-functional polyesters | [40] |
| | Adipic acid-based<br>1,4-butanediol-based<br>Isosorbide-based | Synthesis of aliphatic polyesters | [33, 41] |
| | Itaconic acid-based<br>1,4-butanediol-based | Synthesis of vinyl-functional polyesters | [34] |
| | Glycerol-based<br>Itaconic acid-based | Branched-controlled polyesters, epoxide-containing polyesters | [34, 42] |
| | Malic acid-based<br>Adipic acid-based | Copolymers of L-malic acid, adipic acid and 1,8-octanediol | [28] |
| HiC | Adipic acid-based<br>1,4-butanediol-based | Synthesis of aliphatic polyesters | [43] |
| | Terephthalic acid-based<br>Ethylene glycol-based | PET hydrolysis | [44] |
| | Lactic-acid based | Surface functionalization of PLA films | [35, 45] |
| *Thermobifida cellulosilytica* cutinase 1 | Terephthalic acid-based<br>Ethylene glycol-based | Surface functionalization of PET films | [46, 47] |
| *Thermobifida fusca* cutinase | Terephthalic acid-based<br>Ethylene glycol-based | Degradation of PET nanoparticles | [48, 49] |
| *Thermobifida halotolerans* esterase | Terephthalic acid-based<br>Ethylene glycol-based | PET hydrolysis | [50] |
| | Lactic-acid based | PLA hydrolysis | |
| *Thielavia terrestris* cutinase | Succinic acid-based<br>1,4-butanediol-based | PBS hydrolysis | [51] |
| | Terephthalic acid-based<br>Ethylene glycol-based | PET hydrolysis | |

**Cutinases** have also been investigated for their ability to hydrolyze PLA films and/or fibers and are gaining increasing attention for applications in polymer surface functionalization [22, 23]. Ribitsch et al. reported that cutinase from *Thermobifida halotolerans* hydrolyzes PLA films, leading to the release of lactic acid monomers. The resulting polar groups on the PLA surface cause a decrease in the water contact angle (from approximately 76° to the complete spread of the water drop on the polymer surface) [23].

The enzymatic functionalization of PLA has biomedical application relevance due to the possibility of increasing its biocompatibility while leaving its bulk properties unaltered. Nyanhongo et al. reported the activation of the surface of PLA membranes

*via* hydrolysis catalyzed by cutinase from *Humicola insolens* (HiC), followed by the coupling of unfolded human serum albumin to the newly generated superficial carboxylic groups. The hydrophilic groups of the grafted human serum albumin were important to promote the interaction between the PLA membrane and cells, resulting in up to 2.4 times higher cytocompatibility [21].

Enzymes were also used for the selective excavation of the PLA-based core of block copolymer micelle assemblies and their shell cross-linked nanoparticle analogs. The hydrolysis rate of PLA from the cores of the block copolymer micelles was significantly higher than for bulk PLA [24].

**Enzymatic modification of PET**

PET is currently the most produced polyester worldwide, with applications as a synthetic textile fiber, film, medical device, and packaging material. It is synthesized from ethylene glycol (EG) and terephthalic acid (TA). The latter is currently produced *via* catalytic oxidation of petrochemical p-xylene and has an estimated global market of 50,000 kton/y.

The most recent advances towards bio-based PET rely on the strong integration of chemistry and biotechnology. Bio-based p-xylene can be produced directly from isobutanol obtained by fermentation of $C_5$/$C_6$ sugars, which is then chemically converted to p-xylene [25]. Over the past few years, the lab-scale production of bio-based TA has been reported via the cycloaddition of acrylic acid and isoprene (both bio-based) to obtain fully bio-based TA with a 94% yield [26]. Moreover, bio-based TA was obtained from furfural, which is chemically produced from inedible cellulosic biomass. In 2009, the Coca-Cola Company announced the production of a bio-based PET bottle as a result of the use of 30% bio-based EG and, more recently, a 100% bio-based PET bottle was developed in partnership with the US company Virent. The latter produces bio-based p-xylene via aqueous phase chemical reforming of $C_5$/$C_6$ sugars [25].

Despite the optimal mechanical properties, the main drawback of PET is its highly hydrophobic nature, showing a poorly wettable surface that is difficult to functionalize. Industrial applications frequently require an activated or reactive surface to graft molecules, such as flame retardants or water-soluble dyes. Several methods, including plasma or chemical treatments, caused aging effects (which decrease the resulting surface hydrophilicity) or a consistent reduction of the polymer weight and strength [27]. Enzymatic hydrolysis showed the potential to overcome such limitations

and provide surface functionalization without affecting the bulk properties of the polymer [19]. Several polyesterases were reported to be active in the hydrolysis of PET [23, 28]; in particular, cutinases from *Thermobifida* species were demonstrated to be active either on PET or on small PET model substrates [23, 29-32]. A correlation was found between the kinetic parameters of soluble substrates, the release of hydrolysis products, and the final degree of PET hydrophilization. Interestingly, the hydrolysis efficiency of two highly homologous cutinases from *Thermobifida cellulosilytica* was found to be influenced by the electrostatic and hydrophobic properties of the enzyme surface in proximity to the active site [27]. Several PET-based substrates ranging from films [19, 27] and fibers [30] to nanoparticles [33] were enzymatically hydrolyzed using cutinases as biocatalysts and, in the case of cutinase from *Thermobifida fusca*, the hydrolysis products were observed to cause inhibition of the enzyme [33].

Cutinases from *Fusarium solani*, *Pseudomonas mendocina*, and *H. insolens* were also reported to be active on PET. [34] Of these, HiC was the most active due to its stability after prolonged incubation at 70 °C, which corresponds to an optimal hydrolysis temperature, being very close to the Tg of PET. At this temperature, the enzyme benefits from higher mobility of the polyester chains in the amorphous phase, thus increasing the accessibility of HiC to the ester bonds [34].

As already mentioned, PET is largely used for the manufacturing of plastic bottles, which uses a low crystallinity polymer to achieve high bottle transparency. This particular form of PET is an optimal substrate for enzymatic hydrolysis. Therefore, cutinases were suggested for PET recycling [35] in a process that could overcome the quality limitations of current recycling strategies based on blending. The enzyme hydrolyzed the polymer to its constituent monomers (terephthalic acid and ethylene glycol) so that the freshly produced monomers could then be repolymerized after separation of dyes and contaminants. The hydrolytic properties of cutinases are also exploited in detergent formulations, where enzymes act as effective antipilling agents by partially hydrolyzing fuzz from polyester fibers [36, 37] (Box 1).

Recent developments in the field of biocatalyzed hydrolysis of PET are focused mainly on improvement of the activity of enzymes via approaches of enzyme engineering, targeting both the active site and sorption properties (Box 1 and Figure 1).

Box 1. Engineering of cutinases for PET hydrolysis

Although cutinases are the most active enzyme class in terms of PET hydrolysis, the need for more efficient hydrolytic processes has pushed the development of rationale enzyme-engineering strategies to enhance the hydrolytic activity of cutinases. The rationale behind most engineering strategies applied so far was to enlarge the active site space and increase its hydrophobicity, resulting in the better binding of bulky substrates [84]. Introduction of mutations Leu81Ala and Leu182Ala in the cutinase from Fusarium solani pisi increased four- and fivefold, respectively, the activity towards PET fibers. Not only the active site architecture is important; although less understood, the polarity of the enzyme surface is also a key factor. In the case of *Thermobifida fusca* cutinase, mutation outside of the active site, such as the introduction of a nonpolar alanine in the mutant Ile218Ala and double mutant Gln132Ala/Thr101Ala, improves significantly the hydrolysis of PET [85]. In the case of *Thermobifida cellulosilytica* cutinase 2, exchange of Arg29 and Ala30, located outside the active site, by Asp and Val, respectively, increased the hydrolysis activity up to threefold [86].

Fusion of different binding modules to catalytic domains and, thus, increasing the surface catalytic activity at the interface, has been an evolutionary strategy developed by nature to improve degradation of complex polymers [87]. This approach inspired the fusion of binding modules to the C-terminal of a PET-hydrolyzing enzyme to drive enzyme adsorption on the polymer surface and increase its hydrolytic activity [88]. The hydrophobicity of the C-terminal-fused binding domain affects enzyme absorption on PET: cutinases equipped with hydrophobic binding modules adsorb to a higher extent compared with those carrying hydrophilic binding modules, leading to almost four times higher activity compared with the native biocatalysts. Recently, Hydrophobins were successfully fused to the same cutinase, increasing its PET hydrolytic activity [81]. Engineering the hydrophobicity of the binding domain also led to a better polymer-enzyme interaction. In the case of a *T. fusca* cutinase binding module construct, mutations Trp68Leu or Trp68Tyr on the binding module resulted in a 1.5-fold improvement towards PET [89].

Increasing thermostability also enhances polymer hydrolysis. Since temperature increases the mobility of the polymer chains and eases the enzymatic action [90], the improvement of enzyme thermal stability represents the aim of another rational engineering strategy. In cutinase from *Saccharomonospora viridis* AHK190 [91],

mutations Ser226Pro and Arg228Ser led to a 6 °C increase in the enzyme melting temperature and a 1.5 increase in PET hydrolytic activity.

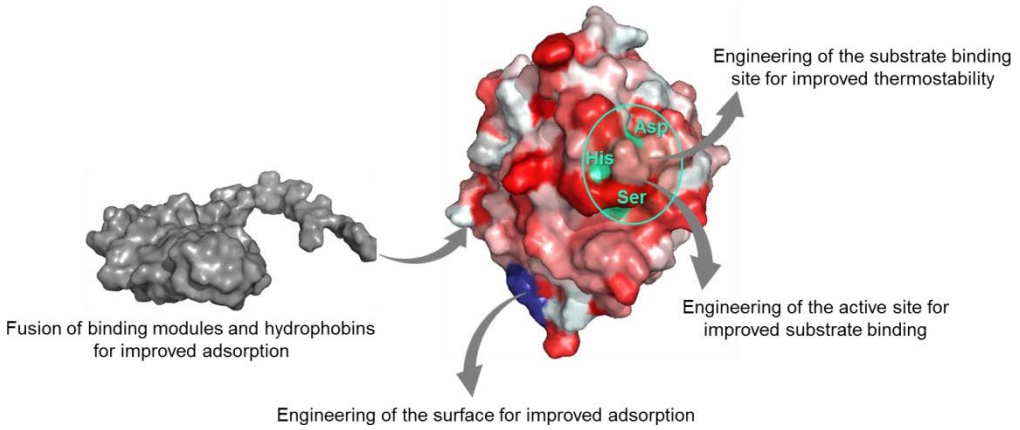



**Figure 1.** Biocatalyst engineering strategies for enhanced hydrolysis of aromatic-aliphatic polyesters.

## Biocatalyzed synthesis of functionalized polyesters

### Greener synthesis under challenging reaction conditions

Polycondensation of dicarboxylic acids with polyols is the primary route by which commercial polyesters, such as PET, PPT, and poly(butylene adipate-co-terephthalate) (PBAT) are currently synthetized [11]. Classically, the synthesis is conducted using a wide array of toxic metal catalysts at temperatures that may exceed 150-250 °C [38, 39]. These conditions are optimal for polymerization because they reduce the system viscosity, although they also cause undesired side reactions of chemical unstable substrates, such as epoxy and vinyl moieties [6]. In these cases, the selectivity and extraordinary activity of enzymes under mild conditions represent a solution for circumventing the limitations of conventional chemical polycondensations. Moreover, enzymes make the use of toxic and unselective metal catalysts unnecessary.

Enzymatic polycondensation is made possible by esterases, in particular lipases, which are active in low-water media and can catalyze esterification and transesterification in organic solvents or in solvent-free bulk systems. Enzymatic processes can synthesize aliphatic and, to a lesser extent, also aromatic polyesters [40, 41].

On the way towards greener processes, Takamoto et al. investigated the enzymatic synthesis of polyesters in $scCO_2$ *via* both polycondensation (divinyl adipate and 1,4-BDO) and ROP (of ε-CL) [42]; however, in both cases, work up of the reaction involving dissolution of the product in organic solvent and filtration of the biocatalyst was necessary. An alternative solution was the introduction of ionic liquids as green reaction solvents, which are able to solubilize arrange of monomers and are characterized by high thermal stability. This approach is particularly interesting in the case of high melting point substrates, which cannot be polymerized in bulk [43].

**Immobilization of the biocatalyst** is mandatory in these synthetic processes, first to avoid the aggregation of the hydrophilic enzyme molecules, second, to recycle the expensive enzyme and, finally, to prevent the contamination of product by the enzyme protein [44]. Currently, more than 90% of the academic work on enzymatic polyesters synthesis uses Novozym® 435, a formulation of lipase B from *Candida antarctica* (CaLB), immobilized *via* adsorption on methacrylic resins that works efficiently also in solvent-free systems at temperatures of 70-90 °C [6].

However, the pioneering work of Binns [45] demonstrated that, during one single cycle of polycondensation between adipic acid (AA) and 1,4-butanediol (BDO), 10% of the protein detaches from the Novozym® 435 carrier and contaminates the product. This is mainly the consequence of the weak anchoring of the lipase on the carrier through physical adsorption on the methacrylic resin [46]. Recently, the problem was overcome by applying covalently immobilized CaLB [15, 16] (Box 2).

Temperatures ranging from 60 to 90 °C are used in solvent-free systems to reduce viscosity and vigorous mixing is applied to improve mass transfer [47], but this combination causes considerable stress on the biocatalyst, affecting severely the economic viability of the process. In these reaction conditions, the integrity of the biocatalyst is a major problem. This fact was underlined by Korupp et al., who described the scale-up of a lipase-catalyzed polyester synthesis using the commercially available Novozym® 435 in bulk systems. The authors managed to obtain bio-based polyesters from glycerol (GLC) and AA with a molecular weight of 2-3 kDa, but noticed that a protein content of 0.48 g $kg^{-1}$ was present in the final product. This implies a 45% destruction of the immobilized preparation during the reaction [47]. The most recent studies indicate that classical **stirred tank reactors** are inappropriate for efficient enzymatic polycondensation, whereas processes on thin films [15] and in turbo reactors [48] assure good mass transfer while causing low stress to the enzymatic preparations (Box 2).

Box 2. New perspectives for making enzymatic polycondensation scalable

In 2010, Gross reviewed the wide array of different polyesters synthesized via enzymatic catalysis on a laboratory scale and identified the main factors hampering the exploitation of this wealth of knowledge on an industrial scale [6]. Since then, research advances have resulted from Gross' call for improved activity and robustness of biocatalysts under manufacturing systems (e.g., stir tank reactors). Recent studies proposed alternative integrated solutions for enzymatic polycondensation since they pointed out the inadequacy of batch and stirred tank reactors for these highly viscous systems [15, 16]. Processes were configured by combining robust covalently immobilized lipases with thin-film conditions. The advantages of working under thin-film conditions are several, including: (i) the integrity of the biocatalyst is preserved because no mechanical stirring is required; (ii) mass and heat transfer are highly efficient; and (iii) thin-film processes provide an

easy route for fast removal of volatile components and water under reduced pressure.

The concept was experimentally validated first on the laboratory scale (10-g monomers) in the synthesis of BDO with adipic acid (AA) and dimethyl itaconate (DMI) and ensured the integrity of the biocatalyst over eight reuse cycles. On a pilot scale (10-kg monomers) the thin-film system was operated continuously with in turbo reactors [48]. Interestingly, it was demonstrated that the polycondensation of AA with different polyols can be conveniently carried out in two steps: after an initial biocatalyzed oligomerization, the enzyme can be removed and the reaction can be thermodynamically driven thanks to the removal of co-product (i.e., water). This solution allows reduction of the exposure of the biocatalyst to stressing factors.

Regarding the improvement of enzyme activity, monomolecular dispersion of the native enzyme would lead to the highest reaction rate, but contamination of the product with the enzyme must be avoided. As an alternative solution, it was demonstrated that distributing the catalyst on the widest carrier surface facilitated the enzyme-substrate approach and promoted polycondensation [16]. CaLB covalently immobilized on carriers with low protein loading was shown to be a practical and economical solution to the problem, although further improvement might come from the development of novel cheaper, renewable, and efficient carriers. This conclusion is applicable more generally not only to solvent-free enzymatic polycondensations, but also to any biocatalyzed process hampered by mass transfer limitations and viscosity.

**Functionalized polyesters from biocatalysis**

The use of enzymes in polymer synthesis is primarily directed to the preparation of highly structured polyesters characterized by a regular presence of functional groups, which are of great interest especially in drug formulations. Hydroxyl-, thiol-, or carboxyl-functional pendant groups along the macromolecular chains facilitate covalent anchorage of **prodrugs** [49].

By changing the monomer ratios of copolymers or the chemical structures of the pendant groups, the hydrophobic-hydrophilic balance and the degradation rate of polymers can be adjusted to the needs of different applications. Furthermore, by post modification of pendant functional groups, novel comb, graft, or network polymers can be prepared. In this case, enzymatic catalysis represents an appropriate way to obtain functional polyesters containing sensitive groups. The bio-based monomer

itaconic acid, for example, was polymerized with several polyols to give side-chain functionalized polyesters where the vinyl moiety was preserved. Molecular weights obtainable through lipase catalysis are relatively modest (1-2.5 kDa) due to the scarce inherent reactivity of itaconic acid. However, the vinyl group is prone to further functionalizations and/or cross-linking of the polymer [15, 16, 50]. Computational studies guided 'substrate engineering' approaches for selecting diol structures favorable to chain elongation in the polycondensation of dimethyl itaconate [16]. Similar polyesters containing a lateral epoxy moiety have also been reported [51]. Malic acid represents another interesting bio-based dicarboxylic acid because it carries a hydroxy group and it is also chiral. Li et al. used (L)-malic acid and adipic acid in different ratios together with1,8-octanediol in reactions catalyzed by CaLB that led to polyesters with a molecular weight of up to 10 kDa [52]. The products demonstrated that CaLB accepts only the primary hydroxyl group of the diol for the polycondensation, whereas the secondary hydroxyl group of malic acid remains available for further modifications.

In addition to the monomers reported in Table 1, glycerol has a major role as renewable monomer since it is largely available on the market as an inexpensive co-product of the transesterification of triglycerides in biodiesel synthesis or from bioethanol production [4]. The chemical synthesis of polyesters with multifunctional monomers, such as glycerol, needs strict synthetic control to prevent branching and formation of gels [53]. For example, CaLB demonstrated regioselectivitytowards1,3-OH groups in the polycondensation of glycerol with esters of itaconic acid [16] and epoxide monomers [6, 50, 51].

A future challenge for enzymatic polycondensations lies in the incorporation of aromatic dicarboxylic esters to produce PET-like aromatic-aliphatic polyesters. The attempts to polymerize such compounds reported so far [54, 55] demonstrated a modest monomer conversion rate. A recent study reported the synthesis of furan-based polyesters starting from the bio-based 2,5-bis(hydroxy methyl)furan, but the obtained molecular weights were below 3.0 kDa [56]. Comparable results ($M_n$ of 23.7 kDa) were obtained by combining the dimethyl ester of the furan dicarboxylic acidwith1,10-decanediol [57].

The difficulty of obtaining high-molecular-weight polyesters by enzyme catalysis has been recognized as one of the major limitations towards the industrial exploitation of this environmentally benign methodology. However, this problem can be

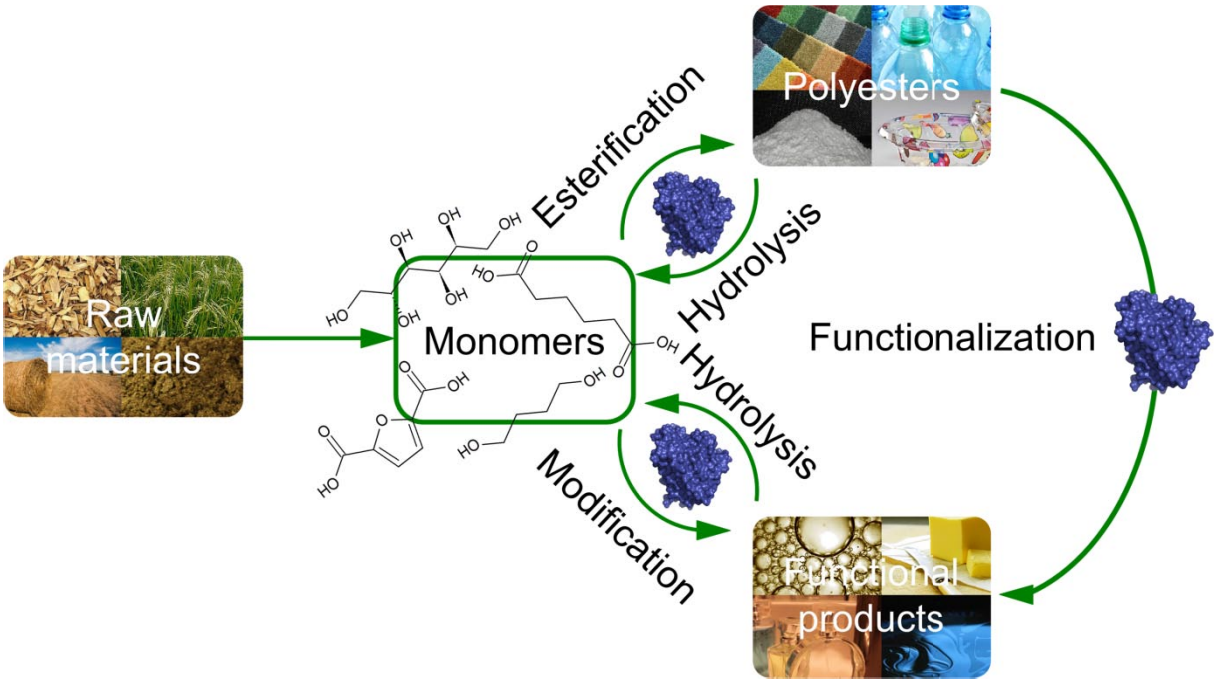
circumvented by the synthesis of oligomers, or telechelic pre-polymers, with functional ends. The synthesis of oligomers also prevents the precipitation of polymers during the synthesis [58, 59]. The functional ends of the telechelics canbeusedinasecondchemicalstepaftertheremovalofthebiocatalystand films with different properties are obtained. Currently there is a strong need for enzymes with novel selectivities due to an increasing range of bio-based building blocks with potential for the synthesis of structurally different specialty polymers and products for the pharmaceutical or food industry [6]. Although this problem might be approached by enzyme engineering, the only study addressing the mutation of enzymes for polyester synthetic purposes is related to a CaLB mutant that showed 90-fold increased activity for the ROP of D-lactic acid [60].

Cutinase enzymes have emerged as interesting alternative to lipase biocatalysts because of their wide substrate specificity. HiC was found to have high activity at 70 °C and to catalyze polycondensation and ROP reactions of an array of substrates [61, 62], with a selectivity preference towards long dicarboxylic acids ($C_{10}$-$C_{13}$) [63].

## Concluding remarks

Biotechnological conversion of renewable carbon into chemical building blocks for polymers and other chemicals is paving the way for the sustainable innovation of chemistry (Figure 2). Although industrial projections indicate that, by 2025, sustainable biotechnologies will contribute to the production of a quarter of chemicals and polymers, their success will depend not only on technological factors. Availability of renewable feedstock not competing with food is one of the issues, together with the capacity of new products to penetrate the market. Concerning the first issues, there is much excitement around the bioconversion of $CO_2$ by chemo-enzymatic routes or through microbial electrocatalysis, but strong research efforts are still required in this field. In the future, algal technologies and other non-conventional sustainable sources of biomass (e.g., insects) may also contribute to relieve the pressure on soil exploitation.

In the meantime, research must be directed not only towards the replacement of existing petrol-based polymers, but also for conferring higher value and competitive functions to bio-based polymers (see outstanding questions). Biocatalysis already contributes not only to the selective modification and degradation of bio-based polymers, enabling the closure of the carbon circle, but also to the *in vitro* synthesis of advanced polymers, which are not accessible by chemical routes. Despite major advances achieved using modern genetic tools, efforts are still needed to not only enlarge the portfolio of enzymes endowed with necessary selectivities, but also for the optimization of biocatalyzed processes moving away from conventional chemical-engineering paradigms. These integrated efforts are necessary for the success of the new generation of bio-based polymers, which must compete with well-established products, optimized through decades of chemistry research and innovation practices.




**Key Figure.** Enzymatic circle for the synthesis, functionalization, modification, and hydrolysis of bio-based polyesters.

## Outstanding questions

- How long will it take to develop a range of different bio-based monomers available on an industrial scale?
- How long will it take to integrate bio-based building block production together with biocatalyzed polymerization in integrated biorefineries?
- What engineering or screening approach would be most appropriate for obtaining enzymes able to convert efficiently aromatic monomers in polycondensation processes?
- How can we design enzymes and processes that enable the biocatalyzed synthesis of polymers with higher molecular weight?
- How ‘sustainable’ and ‘renewable’ are biocatalyzed processes making use of petrol-based resins for enzyme immobilization? In addition, what would be the most promising renewable biomass or material for replacing organic resins as an immobilization matrix?
- Will biotechnology succeed in the development of appropriate $CO_2$ conversion routes to obtain high-value products and chemicals?

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# 3

## Enlarging the tools for efficient enzymatic polycondensation: structural and catalytic features of cutinase 1 from *Thermobifida cellulosilytica*

**Abstract**

Cutinase 1 from *Thermobifida cellulosilytica* is reported for the first time as an efficient biocatalyst in polycondensation reactions. Under thin film conditions the covalently immobilized enzyme catalyzes the synthesis of oligoesters of dimethyl adipate with different polyols leading to higher $M_w$ (~1900) and $M_n$ (~1000) if compared to lipase B from *Candida antarctica* or cutinase from *Humicola insolens*. Computational analysis discloses the structural features that make this enzyme readily accessible to substrates and optimally suited for covalent immobilization. As lipases and other cutinase enzymes, it presents hydrophobic superficial regions around the active site. However, molecular dynamics simulations indicate the absence of interfacial activation, similarly to what already documented for lipase B from *Candida antarctica*. Notably, cutinase from *Humicola insolens* displays a "breathing like" conformational movement, which modifies the accessibility of the active site. These observations stimulate wider experimental and bioinformatics studies aiming at a systematic comparison of functional differences between cutinases and lipases.

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## 3.1 Introduction

The rising demand for advanced polyesters, displaying new functional properties, has boosted the development of new biocatalyzed routes for polymer synthesis, where enzymes concretely respond to the challenge of combining benign conditions with high selectivity and efficient catalysis. Enzymes are attractive sustainable alternatives to toxic catalysts used in polycondensation, such as metal catalysts and tin in particular [1]. Moreover, they enable the synthesis of functional polyesters that are otherwise not easily accessible by using traditional chemical routes because of the instability of some monomers under the elevated temperatures used in traditional approaches [2]. For example, it has been reported that itaconic acid (and its esters) were polymerized in the presence of different polyols leading to side-chain functionalized oligoesters, where the preserved vinyl moiety is exploitable for further functionalization [3-5]. Polymeric products containing epoxy moieties have been also synthesized enzymatically [6]. Although the size of polymers obtainable through biocatalysis might be modest [4], the molecular weight of oligomers can be enhanced by combining chemical or thermal methods. Hydrolases, and more specifically *Candida antarctica* lipase B (CaLB), are the most widely investigated enzymes [7-9] in ring opening polymerization (ROP) reactions and in the polycondensation and of a wide array of monomers [2, 10].

While various immobilized-CaLB preparations have been studied and applied in polyesters synthesis, the potential of other esterases remain unexplored [8]. Besides CaLB, Gross and co-workers reported also the activity of cutinase from *Humicola insolens* (HiC) in the polycondensation of linear dicarboxylic acids and their esters (eg. adipic acid, diethyl sebacate) [11, 12] and its application in the ring opening polymerizations of lactones (eg. ε-caprolactone, ω-pentadecalactone) [13, 14]. More recently, the same cutinase showed an extraordinary hydrolytic activity towards aliphatic/aromatic polyesters [15].

Concerning the catalytic properties of cutinase enzymes, various fungal cutinases have been isolated and characterized16 since these enzymes are involved in plant pathologies caused by the depolymerization of cutin, a three-dimensional polymer of inter-esterified hydroxyl and epoxy-hydroxy fatty acids with chain lengths mostly between 16 and 18 carbon atoms [17]. Interestingly, also pancreatic lipase has been reported to hydrolyze cutin, thereby releasing oligomers and monomers [18].

The interest of cutinases as biocatalysts arises from different studies addressing their applications on unnatural substrates. Because of that, cutinases are potentially applicable to industrial processes including hydrolysis of milk fats, petrol manufactory, as well as production of detergents, structured triglycerides, surfactants, flavor esters, chiral pharmaceuticals and agrochemicals [19-22]. Recently, cutinase from Fusarium solani pisi showed a consistent synthetic activity for the production of polyamides [23, 24]. Fungal cutinases from *Penicillum citrinum*, [25] *Thielavia terrestris* [26] or *Thermobifida* species [27, 28] have been also applied in the hydrolysis of commercial aliphatic/aromatic polyesters such as poly(lactic acid) (PLA), poly(1,4-butylene adipate-co-1,4-butylene terephthalate) (PBAT), poly(butylene succinate) (PBS) and poly(ethylene terephthalate) (PET) without affecting the bulk properties of the polymers [15].

Although there are some indications of potential applications of cutinases in polymer chemistry, scientific literature is lacking from a systematic analysis of structural and functional properties of cutinases and a rationalization of differences between lipase and cutinase enzymes, on the light of the fact that they share the specificity towards highly hydrophobic substrates. The most detailed studies involve the crystal structure of cutinase from *Fusarium solani* pisi (Fsp) [29], which shows an easily accessible, by solvents and substrates, active site where the catalytic serine is not buried into the protein core. In analogy with lipases, Fsp has mobile α-helices domains defining the active site entrance, but the enzyme does not undergo conformational changes preventing the active site accessibility [16, 30]. Notably, while the activity of most lipases is greatly improved at water-lipid interfaces, it is known that CaLB does not exhibit significant conformational modifications ascribable to interfacial activation [31] and recent bioinformatics analysis support the idea that CaLB is functionally and structurally assimilable to esterases [32].

In the present work, we introduce the cutinase 1 from *Thermobifida cellulosilytica* (Thc_cut1) as a biocatalyst able to catalyze the synthesis of linear polyesters with a higher efficiency as compared to lipase B from *Candida antarctica* or cutinase from *Humicola insolens*. The latters are among the few enzymes reported so far for the synthesis of polyesters. Although the potential of Thc_cut1 and of some engineered mutants in the hydrolysis of PET was recently documented [33, 34] its synthetic activity has been never explored before. The data here presented indicate that the covalently immobilized Thc_cut1 catalyzes, under solvent-less and thin film

conditions [4, 5], the synthesis of an array of linear biobased oligoesters both in solvent and bulk systems, leading to improved conversions and number average molecular weight ($M_n$) when compared to CaLB and HiC employed under the same conditions. A further advantage of this cutinase is represented by its structural features that enable a much higher recovery of enzymatic activity upon covalent immobilization, which is of crucial importance for practical industrial applications [35]. A preliminary computational study provides the first structural analysis of Thc_cut1 and tries to shed light on the different behavior of this enzyme as compared to CaLB and HiC.

## 3.2 Results and discussion

**Homology model of cutinase 1 from *Thermobifida cellulosilytica* (Thc_cut1): preliminary structural analysis and comparison with *Humicola insolens* cutinase (HiC)**

The crystal structure of *Thermobifida cellulosilytica* cutinase (Thc_Cut1) has not been solved yet. Therefore, a model was constructed by homology modelling using the protein sequence from the NCBI GenBank nucleotide sequence HQ147785 [33]. We use as a template the structure of *Thermobifida fusca* (PDB36 code 4CG137), which shares 99.23% of sequence identity with Thc_Cut1 and differs only for residues 19 and 137 (Arg and Ser in *Thermobifida fusca* cutinase are replaced by Ser and Thr in Thc_Cut1) (Fig. 1). The final 3D model of Thc_cut1 was highly reliable as indicated by a GMQE value of 0.99. The Thc_cut1 catalytic triad is constituted by Ser131, His209 and Asp177 whereas Tyr61 and Met132 form the oxyanion hole. Interestingly, although Thc_cut1 and HiC belong to the same cutinase family (E.C. 3.1.1.74), they are quite different as indicated by superimposition of the crystal structure of HiC (PDB code 4OYY) [38] with the 3-D model of Thc_cut1. HiC consists of a polypeptide chain of only 193 amino acids (19.89 kDa), whereas Thc_cut1 is composed by 262 amino acids (28.18 kDa).

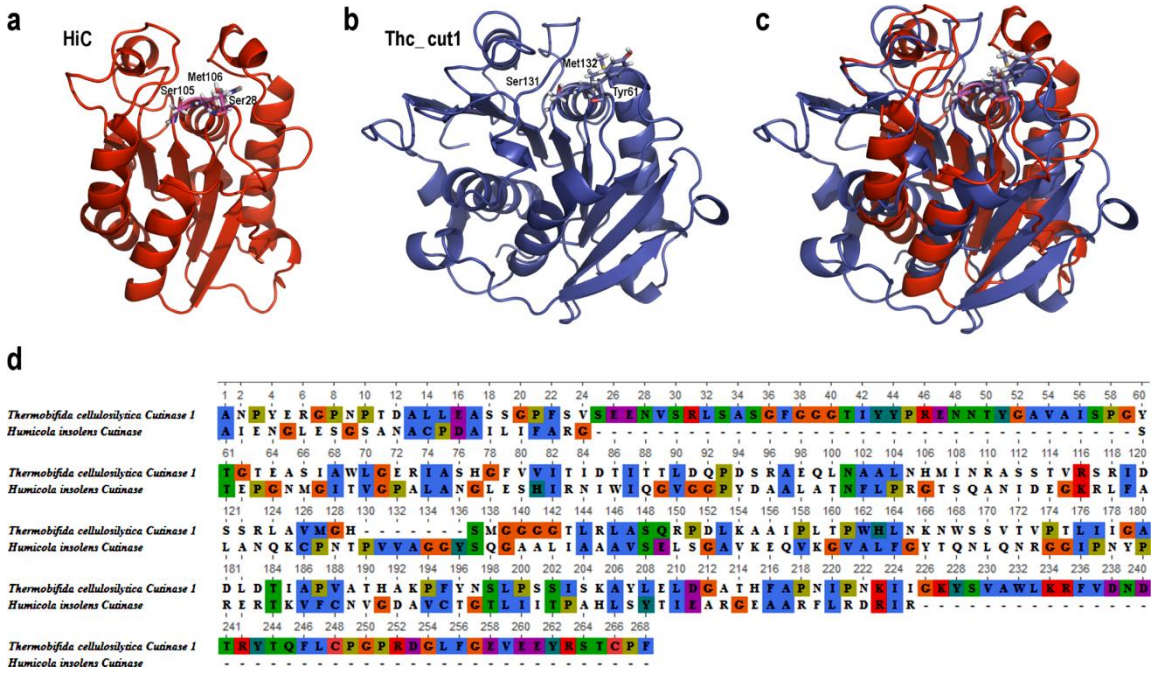


**Figure 1.** Representation of the three-dimensional structures of HiC (a), Thc_cut1 (b) and the two cutinase structures superimposed on the bases of their catalytic residues (c). Catalytic serines and the residues forming the oxyanion hole of each enzyme are represented in sticks mode and are labeled. Sequence alignment (d) was guided by the superimposition of residues forming the catalytic machinery. Residues are colored according to Clustal W color scheme.

Moreover, the sequence alignment (Figure 1) shows only 9% of sequence identity. The catalytic serine (Ser105 and Ser131 for HiC and Thc_cut1 respectively) and the residues forming the oxyanion hole (Ser28, Met106 and Tyr61, Met132 for HiC and Thc_cut1 respectively) were taken as a reference for performing the structure superimposition Figure 1c). The comparison of the two enzyme structures (Figure 1a and b) reveals that the two enzymes share a α/β hydrolase fold but the main difference is related to the location and accessibility of their active sites. While Thc_cut1 has a catalytic triad placed in a superficial and accessible groove, the active site of HiC (Ser105, His188, and Asp175) is placed in a deeper cavity.

**Application of CaLB, HiC and Thc_cut1 for polycondensation reactions**

As previously reported by our group and others, covalent immobilization is an important pre-requisite in enzymatic polycondensation [2, 4]. Obviously, a mono-molecular dispersion of the native enzyme would lead to the highest reaction rate and higher molecular weights, as largely documented in the literature [2]. However, contamination of reaction product with free enzyme must be avoided and recovery of the biocatalyst is mandatory for an economic process.

In the present study, the two cutinases and CaLB were immobilized on epoxy activated organic polymeric resins (EC-EP/M from Resindion S.r.l.) [4]. As reported in Table 1, more than 99% of the two cutinases and 87% of CaLB was bound onto EC-EP within the first two hours. The prompt adsorption and binding of Thc_cut1 (Fig. S1 of SI) is most probably ascribable to the occurrence of hydrophobic interactions between the hydrophobic areas of the proteins and the resin, as previously reported for lipases but never documented for cutinases [5]. Notably, less hydrophobic resins led to poorer results (Fig. S1 of SI).

**Table 1.** Immobilization yields and recovered activities of different hydrolases immobilized on EC-EP epoxy-carrier using 10 mg of protein per g of dry resin in 10 mL buffer. Immobilization was performed in 0.1 M Tris-HCl buffer pH 7 at 21 °C for 24 h.

| Enzyme | Bound enzyme (%)* | Recovered activity (%)^ |
|---|---|---|
| Thc_cut1 | >99 | 37 |
| CaLB | 87 | 8 |
| HiC | >99 | 23 |

* Calculated by evaluating the residual activity and protein concentration in the supernatant.
^ Percentage of enzyme activity exhibited by the immobilized preparation when compared to the soluble form.
All results are the average of two independent immobilization procedures.

The covalently immobilized enzymes were termed iThc_cut1, iHiC and iCaLB and their hydrolytic activities were respectively of 13 ± 2, 8 ± 1 and 17± 2 U $g^{-1}$. The two immobilized cutinases retain a much higher percentage of the original activity, especially in the case of Thc_cut1 (37%). Indeed, the poor immobilization yield observed with CaLB (8%) is in line with different studies that have already reported and commented the difficulties encountered in the efficient immobilization not only of CaLB39 but also lipases from *Pseudomonas sp*. [40] and from *Candida rugosa* [41].

A possible rational explanation of the higher immobilization yields of the two cutinases comes from the analysis of distribution of Lys residues on the surface of the three enzymes (Figure 2).

The primary amino group of Lys is the main candidate for the formation of covalent bonds via nucleophilic attack of epoxy functionalities. [31, 42] The Lys residues are located far from the active sites of Thc_cut1 and HiC, and that is expected to favour the correct orientation of the enzyme upon binding as well as the accessibility of the active site (Figure 2). In contrast, two out of the nine Lys residues of CaLB are situated close to the active site.

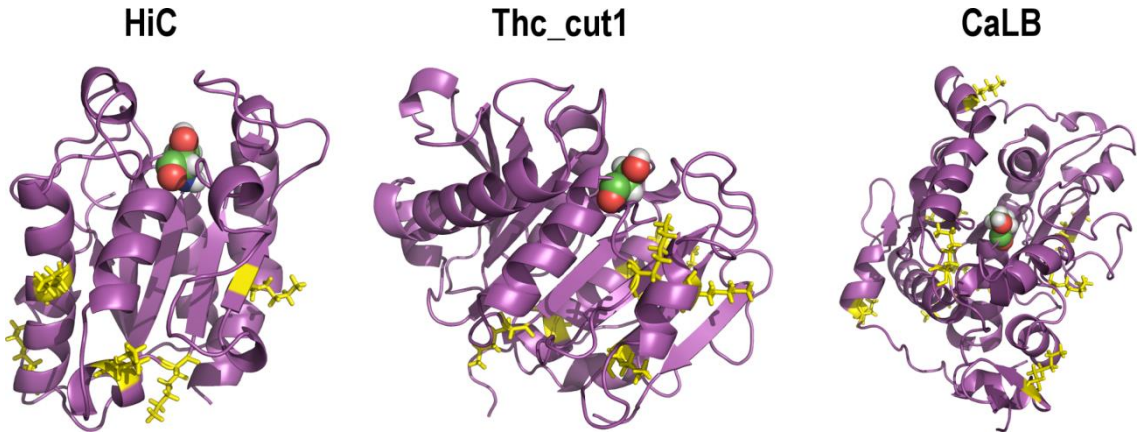


**Figure 2.** Lysine residues on the surface of the three hydrolases, highlighted in yellow sticks mode. The catalytic Ser of each enzyme is represented in sphere mode.

The three hydrolases were also compared in terms of hydrophilic-hydrophobic balance of their surface. It is largely recognized that the enzyme surface properties affect not only enzyme stability [43] but also the efficacy of different protocols for enzyme immobilization [31]. Lipases generally display a polarization of the hydrophilic and hydrophobic areas, in agreement with the natural evolution of these enzymes which are able to act on hydrophobic substrates. The hydrophobic side of the enzyme corresponds to the active site, which is normally positioned at the water-lipid interface. On that respect, Figure 3 shows that more than 50% of the surface of

the two cutinases is hydrophobic, which is purposeful to the approaching and recognition of the hydrophobic cutin, their natural substrate. HiC is considerably smaller (193 aa) when compared to CaLB and Thc_cut1 (317 and 262 residues respectively) and it is also evident how the active site of Thc_cut1 is the most superficial and accessible.

It is important to point out that CaLB is expressed in Aspergillus sp. and the analysis of the primary sequence of CaLB indicates the presence of a N-glycosylation site at Asn 74. As previously reported, the glycan masks a hydrophobic spot on CaLB surface [39]. Consequently, the overall hydrophobicity of glycosylated CaLB is comparable or even lower as compared to Thc_cut1, which is expressed in E. coli and is not glycosylated. This observation is also in agreement with the high affinity of Thc_cut1 for the hydrophobic EC-EP carrier (Table 1) [39].

All polycondensation reactions were carried out using enzymatic preparations with a water content below 0.1% w $w^{-1}$ in order to avoid competing hydrolytic reactions. The stability of the immobilized enzymes was investigated in terms of protein detachment from the support and resulted to be less than 2%. Indeed, it is known that magnetic and mechanical mixing are responsible for damage of carriers and thin-film reactors have already demonstrated to preserve the integrity of EC-EP resins while overcoming the viscosity of solvent-less polycondensation [4, 42, 44]. The observed stability of the enzyme preparations can be of industrial interest in the view of an upscaling of the process [44].

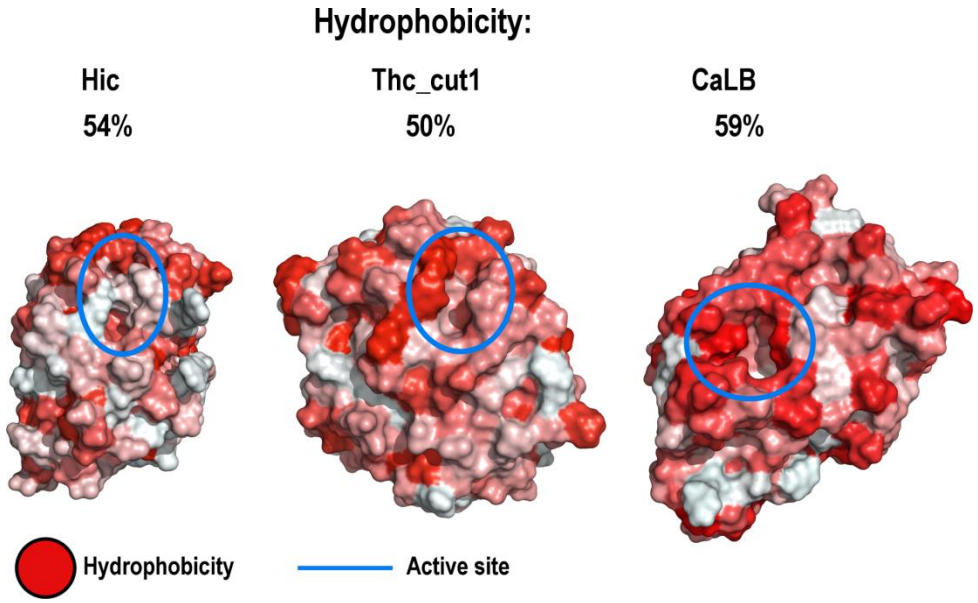


**Figure 3.** Comparison of the hydrophobicity of the surface of the three hydrolases. The openings of the active sites are highlighted within cyan circles. The extent of the surface hydrophobicity of the three enzymes was calculated and represented by using the color_h script of the PyMOL software.

**Comparison of Thc_cut1, HiC and CaLB in the polycondensation of DMA with BDO**

In order to compare the behaviour of the three enzymes, a model reaction between dimethyl adipate (DMA) and 1,4-butanediol (BDO) was investigated. These monomers are widely used in polymer synthesis and their biobased production gained further interest in the recent years. Polycondensations were conducted using a thin-film solvent-free system [5] at 70 °C and 100 kPa (Table 2) [11]. The investigation included also the biocatalyst Novozym® 435, because it is the enzymatic preparation most widely used in polycondensation, although it has been demonstrated that it causes protein contamination and it does not allow an efficient recycling [4].

**Table 2** Polycondensation of DMA with BDO by different hydrolases at 24 h at 70 °C and 100 kPa , using 10% w $w^{-1}$ of biocatalyst.

| Enzymatic preparation | Conversion (%)[Λ] | $M_w$[*] | $M_n$[*] | PD[*] |
|---|---|---|---|---|
| Novozym® 435 | 78 | 1040 | 561 | 1.85 |
| iCaLB | 76 | 888 | 528 | 1.68 |
| iThc_cut1 | 86 | 1923 | 985 | 1.95 |
| iHiC | - | - | - | - |

[Λ] Calculated via $^1$H-NMR by comparing the ratio between the polyol methylene groups adjacent to -OH area ($B_1$) and the internal methylene groups area of DMA ($A_1$, assumed as constant). All reactions were performed in duplicates.

[*] Calculated via GPC calibrated with low molecular weight polystyrene standards ranging from 250-70,000 Da.

Interestingly, the polycondensation catalyzed by iThc_cut1 led to the highest monomer conversion (86% calculated by $^1$H-NMR analysis) with reaction products reaching a $M_w$ of 1923 Da (Figure S2-S4 in SI). The data appears quite promising when considered that the commercial Novozym® 435 gave 78% monomer conversion with $M_w$ of 1040 Da (Figure S5-S7 in SI) notwithstanding previous studies documented the tendency of such formulation to release part of the free enzyme in the reaction mixture [4].

Regarding the use of HiC, Hunsen et al. claimed that the covalently immobilized enzyme was able to catalyze the polycondensation of adipic acid with $C_4$, $C_6$ and $C_8$ linear polyols in solvent-less condition at 70 °C and 10 mm Hg (about 1.3 kPa) [12]. Our attempts to synthetize similar polyesters starting from dimethyl ester, although at 100 kPa, gave no observable product even when the free HiC enzyme was employed. Monomer conversions of around 10% were obtained only using adipic acid as monomer.

It must be underlined that polyesters of much higher Mw were reported in studies employing adsorbed CaLB (e.g. Novozym® 435) in polycondensation of other different monomers but in the present study, our interest was mainly focused on esters of adipic acid and BDO as they are bio-based monomers available at industrial scale. Previous studies [45, 46] indicated that these short chain monomers led to polyesters with $M_w$ in the range of 600-2,200 Da. However, in such cases the detachment of the native enzyme was observed and its dispersion in the reaction mixture. As recently demonstrated, the fine and homogeneous distribution of the biocatalyst affects the elongation of polymers much more than the specific activity of the biocatalyst.5 Therefore, in the case of Novozym® 435 polycondensation is catalyzed both by the immobilized biocatalyst and by the fraction of native CaLB dispersed in the reaction mixture [4].

Taken these factors into account, the size of oligoesters here reported is of the same order of what previously obtained using covalently immobilized CaLB for the polycondensation of similar monomers [4, 5]. Experimental data, combined with computational information, indicate that Thc_cut1 is a promising biocatalyst for applications in polycondensation reactions and it is particularly suitable for being covalently immobilized on EC-EP.

**Polycondensation of dimethyl adipate catalyzed by iThc_cut1 using different diols**

In order to assess the substrate specificities of iThc_cut1 towards different monomers, a set of qualitative screening reactions was carried out using DMA and diols with different chain-lengths ($C_2$-$C_{12}$). These preliminary reactions were carried out in bulk and monitored by means of $^1$H-NMR and indicated that iThc_cut1 was able to catalyze the polycondensation of DMA with BDO, HDO, ODO and DDO (Table 3). The production of short chain oligoesters was demonstrated by ESI-MS. The longest reaction product was an 8 units oligomer obtained in the reaction between DMA and BDO while the most abundant products were trimers, tetramers and pentamers in all the performed reactions (Figure S8 in SI).

Further quantitative information on the efficiency of iThc_cut1 was obtained by studying the time-course of the polycondensation of DMA with $C_4$, $C_6$ and $C_8$ linear diols using a thin-film reaction system at environmental pressure and in solvent-free conditions (Figure 4) [47].

**Table 3.** Polyesterification of DMA with EG, PDO, BDO, HDO, ODO and DDO catalyzed by 10% w $w^{-1}$ iThc_cut1 with a hydrolytic activity of 13 U $g^{-1}$ for 24 h.

| Dicarboxylic acid (A) | Polyol (B) | Area -$CH_2$-OCO- ($B_1$)* | Area -$CH_2$-$CH_2$-OCO- ($A_1$)* | Monomer conversion (%)* |
|---|---|---|---|---|
| DMA | EG | X | 4.0 | X |
| | PDO | X | 4.0 | X |
| | BDO | 1.79 | 4.0 | 45 |
| | HDO | 1.49 | 4.0 | 37 |
| | ODO | 1.36 | 4.0 | 34 |
| | DDO | 1.04 | 4.0 | 26 |

* Calculated via $^1$H-NMR by comparing the ratio between the polyol methylene groups adjacent to -OH area ($B_1$) and the internal methylene groups area of DMA ($A_1$, assumed as constant). All reactions were performed in duplicates.

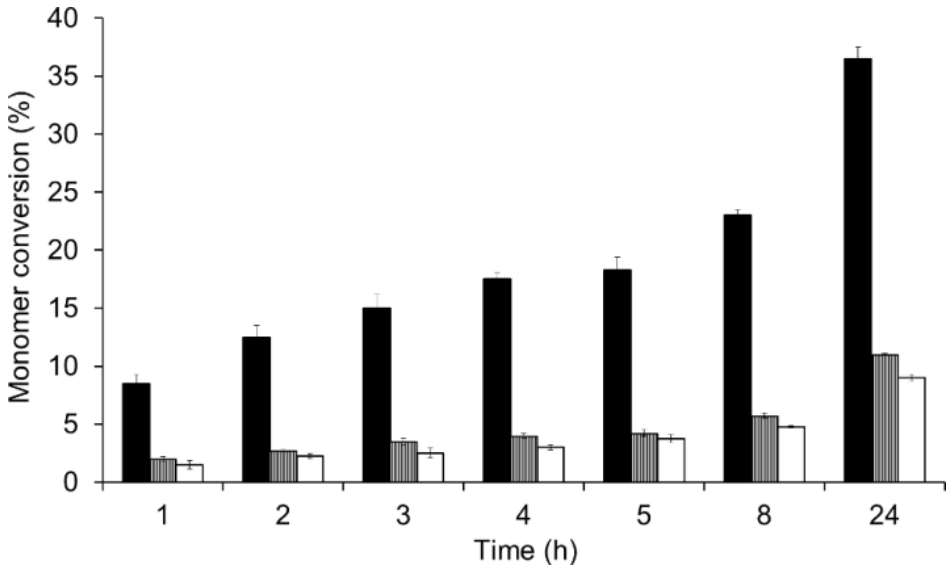


**Figure 4.** Time-course of the solvent-free polycondensation of DMA with BDO (black bars), HDO (stripe bars) and ODO (white bars) catalyzed by immobilized Thc_cut1 having a hydrolytic activity of 7 U $g^{-1}$. Monomer conversion was calculated via $^1$H-NMR. All reactions were performed in duplicates. It must be noted that these reactions were catalyzed by an enzyme preparation displaying a much lower activity (7 ± 2 U $g^{-1}$) in order to allow suitable monitoring of the polycondensation reaction time course while maintaining the same monomer-biocatalyst ratio (10% w $w^{-1}$).

From Figure 4 it appears that Thc_cut1 is more efficient in the polycondensation of $C_4$ diol leading to 37% of monomer conversion in 24 h while the $C_6$ and the $C_8$ dialcohols were converted only by 11% and 9% respectively. However, the observed rate of conversion may be ascribed not only to different enzyme specificity but also to different viscosity of the reaction systems under solvent-less conditions. Indeed, while BDO is a liquid, the other polyols are solid at 25 °C and they are simply dispersed in DMA before heating at 70 °C to obtain a homogeneous phase. The possible effect of viscosity and mass transfer on data in Figure 4 was confirmed by carrying out the polycondensation in two different organic solvents, namely toluene and tetrahydrofuran (THF). It has been already reported that HiC is active in several organic solvents while there are no respective data on Thc_cut1. The reactions were

carried out by solubilizing the monomers in organic solvent at a concentration of 0.2 M and Table 4 reports the results obtained in toluene, since no polymerization product was observed using THF. The polycondensation of DMA with $C_4$, $C_6$ and $C_8$ linear polyols led to monomers conversions ranging from 50 to 55% after 24 h of reaction with $M_w$ distributions of 400-450 Da.

**Table 4.** Polycondensation of DMA with $C_4$, $C_6$ and $C_8$ linear polyols at 24 h catalyzed at 70 °C and 100 mkPa in toluene using 10% w $w^{-1}$ iThc_cut1 with a hydrolytic activity of 13 U $g^{-1}$.

| Linear Polyol | Conversion (%)[Λ] | $M_w$[*] | $M_n$[*] | PD[*] |
|---|---|---|---|---|
| BDO | 50 | 435 | 400 | 1.09 |
| HDO | 52 | 440 | 453 | 1.17 |
| ODO | 55 | 551 | 465 | 1.19 |

[Λ] Calculated via $^1$H-NMR by comparing the ratio between the polyol methylene groups adjacent to -OH area ($B_1$) and the internal methylene groups area of DMA ($A_1$, assumed as constant). All reactions were performed in duplicates.
[*] Calculated via GPC calibrated with low molecular weight polystyrene standards 250-70,000 Da.

It must be underlined that the activity of Thc_cut1 in the polycondensation of BDO and dimethyl adipate opens interesting perspectives for the enzymatic synthesis of polyesters. Gross and co-workers reported that cutinase from *Humicola insolens* accepts preferably $C_6$ and $C_8$ diols for the polymerization with adipic acid while the $C_4$ diol was scarcely converted [12]. Moreover, previous studies reported also that HiC accepts preferably $C_{10}$ and $C_{13}$ diacids, while only little activity was detected for substrates with a $<C_{10}$ carbon chain [11]. The same work also documented that CaLB catalyzes the polycondensation of $C_3$-$C_8$ linear polyols with sebacic acid at 70 °C in bulk, although the study did not report information on the rate of conversion of monomers but only the increase of the $M_n$ over time [11].

The time course of Figure 5 shows how Thc_cut1 converts the linear diols in toluene with similar efficiency, thus confirming a possible effect of viscosity and mass transfer in conversions reported in Table 3 and Figure 4. However, some solvent effect on the conformation and accessibility of the enzyme cannot be excluded.

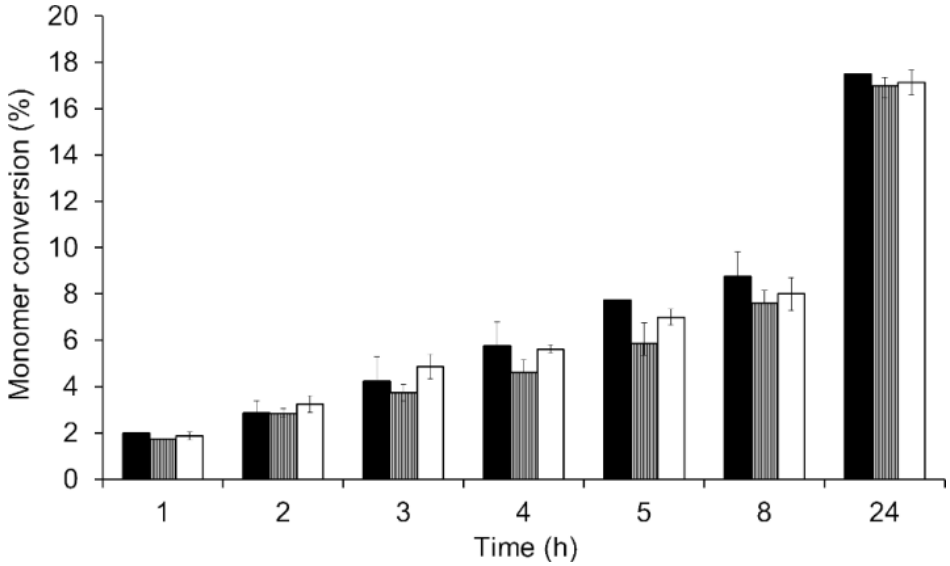


**Figure 5.** Time-course of the polycondensation of DMA with BDO (black bars), HDO (stripe bars) and ODO (white bars) in toluene. The reactions were catalyzed by immobilized Thc_cut1 displaying lower activity (7 ± 2 U $g^{-1}$) in order to facilitate the monitoring of the polycondensation while maintaining the same monomer-biocatalyst ratio (10% w $w^{-1}$).Monomer conversion was calculated via $^{1}$H-NMR. All reactions were performed in duplicates.

The results obtained in the study of the biocatalyst recyclability (Figure 6) demonstrate that by using a thin-film reaction system the covalently immobilized Thc_cut1 retains most of its activity after 10 synthetic cycles. Details of time course are available in ESI, Figure S9.

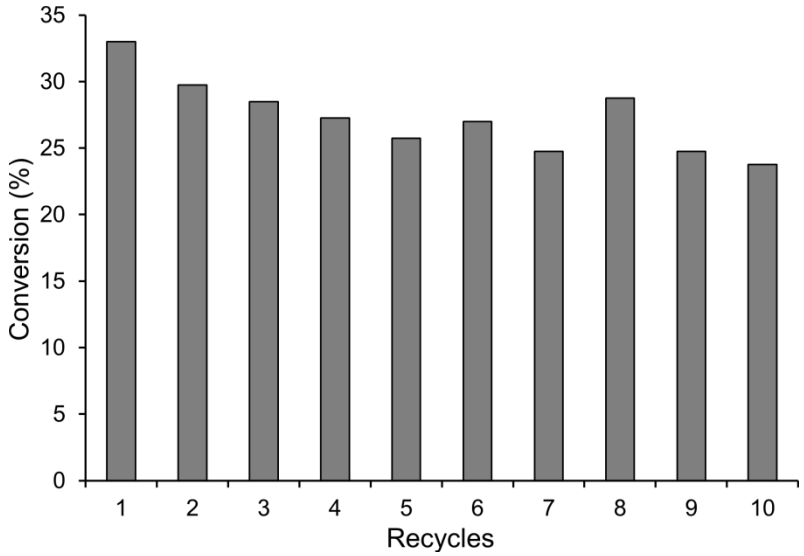


**Figure 6.** Evaluation of the recyclability of the Thc_cut1 covalent preparation over 10 cycles expressed as a percentage of the BDO monomer reacted after 4 h of reaction.

It must be underlined that our previous studies demonstrated that also the covalently immobilized CaLB displays good recyclability and no protein leaching using the same reaction conditions, whereas the adsorbed CaLB preparations Novozym® 435

undergoes a progressive detachment of the enzyme and decrease of enzymatic activity [4].

**Comparison of dynamic behavior of Thc_cut1, HiC, and CaLB in different media**

In order to investigate possible solvent effects on the conformation and accessibility of Thc_cut1, a conformational analysis was carried out by running MD simulations for 10 ns at 343 K in explicit toluene. HiC and CaLB were also included in the study for comparison and Figure S10 in SI reports a comparison of the starting structure (crystals for HiC and CaLB, homology model for Thc_cut1) with the conformation obtained after 10 ns of MD simulation in toluene.

Root Mean Square Fluctuation (RMSF) was also calculated for each simulated protein [31], in order to identify the most flexible domains (Figure 7). Interestingly, the analysis pointed out two very flexible and mobile domains overlooking the catalytic Ser105 of HiC. After 10 ns simulations in toluene the HiC active site increases its accessibility remarkably and assumes the shape of an open “chasm”. This observation might suggest that HiC has a behaviour similar to lipases, which are members of the same serine-hydrolase superfamily. Lipases are known for undergoing dynamic opening and activation when exposed to hydrophobic phases, as a result of the movements of some flexible domains, also referred as “lid” [31].

Because CaLB is missing the typical “lid”, it is not characterized by the interfacial activation phenomena. This is confirmed by the RMSF analysis of Figure 7, which indicates the presence of a domain endowed with modest flexibility in the proximity of the opening of the active site corresponding to a small putative lid unable to close the active site [31].

In Thc_cut1 the regions surrounding the opening of the active sites appear of scarce mobility, while there are terminal loops undergoing wider fluctuation. Conversely, the superficial and groove shaped active site of Thc_cut1 undergoes very limited conformational modifications in toluene (Figure S10 in ESI).

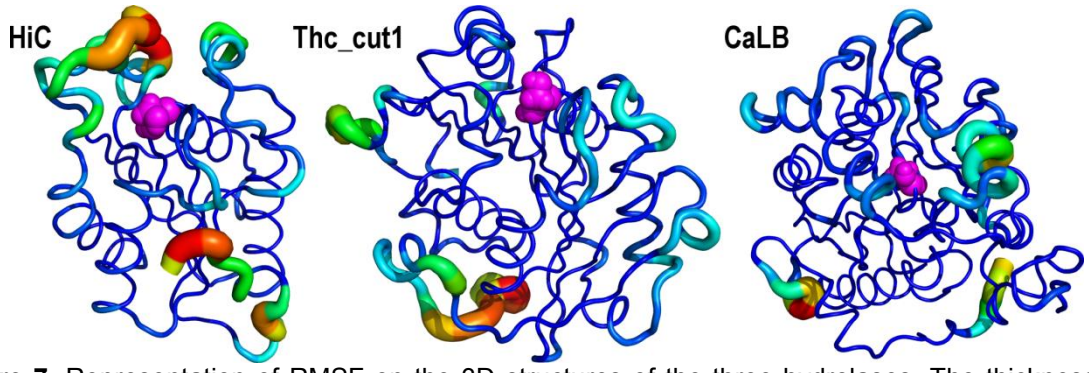


**Figure 7.** Representation of RMSF on the 3D structures of the three hydrolases. The thickness and "color temperature" (from blue to red) are correlated with the fluctuation observed during the 10 ns MD simulations in explicit toluene at 343 K. The thicker and red regions correspond to the highest RMSF values. The catalytic serine of each enzyme is highlighted in pink sphere mode.

In order to shed light to this lipase-like conformational behaviour of HiC, further dynamic simulations were run in explicit water. Our previous studies on a number of lipase enzymes illustrated how in explicit water they undergo a kind of "closure" of the active sites with a restriction of the accessibility. On the contrary, the conformation of HiC after 10 ns simulation in explicit water at 343 K becomes open and the active site is fully accessible. Quantitative details of the amplitude of the movements of domains overlooking the catalytic serine can be observed in Figure 8b, where it appears clear how the crystal structure used as a starting point for the MD computations is the less accessible. Interestingly, this structure corresponds to the crystal obtained in the presence of a hydrophobic inhibitor (diethyl-p-nitrophenyl-phosphate). No significant conformational variations were observed for CaLB and Thc_cut1.

Overall, computational studies here presented document how HiC and Thc_cut1 are considerably different in terms of structure and conformational behaviour. Indeed, HiC presents highly mobile domains, a kind of "lid" overlooking the active site. Although this feature is shared by most lipase enzymes, the MD simulations indicate that the active site of HiC remains open and accessible both in water and in hydrophobic environment. Nevertheless, the crystal structure shows how the putative "lid" is indeed able to assume conformations that reduce the active site accessibility in the presence of a hydrophobic inhibitor. Of course, this behaviour deserves further investigations, since it would be interesting to perform bioinformatics analysis to understand more about structural and functional differences between cutinases and lipases.

The hydrophobic surface appears to be a common feature of lipases and cutinases but it must be noted that very few studies address the differences between lipases and cutinases. Pioneering studies indicated how cutinase enzymes are able to

hydrolyze fatty acid esters and emulsified triacylglycerol as efficiently as lipases, but without any interfacial activation [48, 49]. Structural and computational investigations of cutinase from *Fusarium solani* pisi documented how the loops surrounding the catalytic site are flexible domains [30]. The same studies also indicated that the absence of any significant structural rearrangements upon binding to non-hydrolyzable substrates represents an important feature of cutinase, which is shared by *Candida antarctica* lipase B [50]. Other investigations reported that the atoms involved in cutinase oxyanion hole formation do not move upon inhibitor binding whereas significant displacements occur in *Rhizomucor miehei* lipase and human pancreatic lipase upon inhibition [51].

The present study indicates that there is no unified picture for illustrating structural and conformational properties of all cutinases. The negligible conformational mobility of Thc_cut1 is indeed comparable with the CaLB behaviour whereas the conformational modifications occurring in HiC are compatible with a “brief-like” motion able to modulate the access to the hydrophobic active site.

On the light of these preliminary evidences, a comprehensive future computational and bioinformatic comparison could elucidate the structure function relationships of these interesting enzymes in more detail.

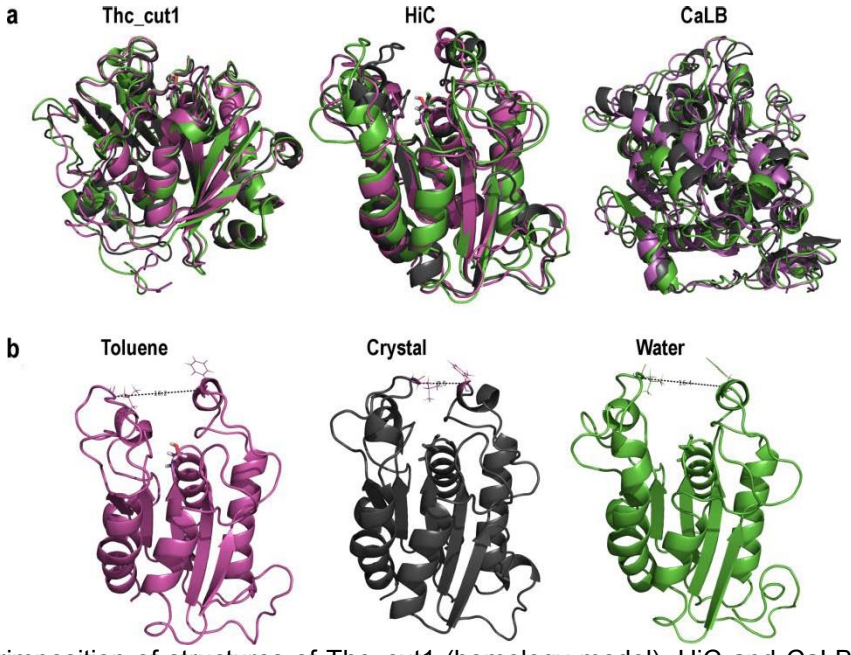


**Fig 8.** A: Superimposition of structures of Thc_cut1 (homology model), HiC and CaLB (crystals) with conformations obtained after MD simulations at 343 K. Legend: gray = starting 3D structure; pink = after 10 ns MD in toluene; green = after 10 ns MD in water. B: Comparison of the accessibility of HiC active site expressed as the distance between $C_\alpha$ of Phe 70 and Lys 167. Pink (toluene) = 16.2 Å; grey (crystal structure after removal of inhibitor) = 8.5 Å; green (water) = 16.4 Å.

## 3.3 Conclusions

The urgency of more sustainable, selective and efficient routes for the synthesis of new generation polyesters was addressed by introducing cutinase 1 from *Thermobifida cellulosilytica* (Thc_cut1) as new enzyme suitable for polycondensation reactions.

The disclosure of some methodological problems hampering the polycondensation procedures used so far (unsuitability of adsorbed immobilized biocatalysts as well as of batch reactors) motivated this study and led to new steps towards rational optimization of the in vitro enzymatic synthesis of polyesters. Covalently immobilized Thc_cut1 catalyzes, under thin film conditions [4, 5], the synthesis of an array of linear biobased oligoesters both in solvent and bulk systems, leading to improved conversions and $M_n$ when compared to lipase B from *Candida antarctica* (CaLB) and cutinase from *Humicola insolens* (HiC) employed under the same conditions. A further advantage of this cutinase is represented by its structural features enabling a much higher recovery of enzymatic activity upon covalent immobilization, which is of crucial importance for practical industrial applications. [35] Preliminary computational studies provide the first structural analysis of Thc_cut1 and shed light on the different conformational behaviour of this enzyme as compared to CaLB and HiC. Structural analyses indicate that Thc_cut1 has a very superficial and fully accessible active site both in aqueous and hydrophobic media. Interestingly, Thc_cut1 shares some structural and conformational properties with lipase B from *Candida antarctica*, whereas cutinase from *Humicola insolens* has highly mobile domains able to modify the accessibility of its active site. Such remarkably different behaviour of these two cutinases should lead to further comprehensive bioinformatics analysis able to elucidate structural and functional differences among cutinases and lipases, two enzyme classes sharing highly hydrophobic surfaces and the ability to hydrolyze insoluble substrates.

## 3.4 Experimental section

**Chemicals and reagents.**

EC-EP/M and EC-HFA/M Sepabeads were kindly donated by Resindion S.r.l., (Mitsubishi Chemical Corporation, Milan, Italy). EC-EP/M beads have average pore diameter of 10–20 nm, particle size in the range of 200-500 µm and water retention around 55-65%. Dimethyl adipate (DMA), ethylene glycol (EG) and 1,2-propanediol (PDO) were purchased from Sigma-Aldrich. 1,4-butanediol (BDO), 1,6-hexanediol (HDO), 1,8-octanediol (ODO) and 1,12-dodecanediol (DDO) were purchased from Merck. All other chemicals and solvents were also purchased from Sigma-Aldrich at reagent grade, and used without further purification if not otherwise specified.

**Enzymes.**

The recombinant *Thermobifida cellulosilytica* cutinase 1 (Thc_cut1) was produced and purified as previously described. The organism used for the expression was *E. Coli*. [33] Novozym® 435 was purchased from Sigma-Aldrich (product code: L4777) containing *Candida antarctica* lipase B immobilized on macroporous acrylic resin with a specific activity of >5000 U $g^{-1}$ (PLU Units, determined by producer). Lypozyme CaLB (protein concentration of 8 mg $mL^{-1}$) was a kind gift from Novozymes (DK). The cutinase from *Humicola insolens* (HiC) (protein concentration of 11.2 mg $mL^{-1}$) was a gift from Novozymes (Beijing, China) and was purified as previously described [15] prior to use.

**Activity assay for native lipase and cutinases.**

Activity was measured at 21 °C using p-nitrophenyl butyrate (PNPB) as a substrate as previously reported by Ribitsch et al. with some modification. [27] PNPB was selected because lipases and cutinases display different substrate specificity and a general test for esterase activity was preferred rather than the typical tributyrin hydrolysis assay. In any case, no direct comparison between lipase and cutinase activity was reported. The final assay mixture was made up of 200 µL of solution B and 20 µL of enzyme solution (solution A: 86 µL of PNPB and 1000 µL of 2-methyl-2-butanol; solution B: 40 µL of solution A and 1 mL of 100 mM Tris-HCl buffer at pH 7). The increase of the absorbance at 405 nm due to the hydrolytic release of p-nitrophenol (ε 405 nm= 9.36 mL (µmol cm)$^{-1}$) was measured over time using a Tecan plate reader using plastic 96 well plates. A blank was included using 20 µL of buffer instead of enzymatic solution. The activity was calculated in units (U), where 1 unit is

defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute under the given assay conditions.

**Activity assay for immobilized enzymes.**

Activity was measured at 21 ˚C using PNPB as substrate. The final assay mixture was made up of 0.1 mL of the substrate solution (86 µL of PNPB and 1000 µL of 2-methyl-2-butanol), 11 mL of 100 mM Tris-HCl buffer at pH 7 and 20 mg of immobilized enzyme preparation. The increase of the absorbance at 405 nm due to the hydrolytic release of p-nitrophenol (ε 405 nm= 9.36 mL (µmol cm)$^{-1}$) was measured over time with a HACH Lange benchtop spectrophotometer using plastic cuvettes. A blank was included using beads where glycine was used instead of enzyme as blocker for the epoxy-activated beads. The activity was calculated in units (U), where 1 unit is defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute under the given assay conditions.

**Evaluation of enzyme leaching.**

Immobilized enzyme preparations were incubated as described above without adding the PNPB solution. Samples were taken after 5, 10, 15 and 30 min and the biocatalyst was removed via filtration. The substrate solution was added to the supernatant and the residual esterase activity was assessed as described above.

**Protein quantification.**

Protein concentrations were determined by using the BioRad protein assay (Bio-Rad Laboratories GmbH, Vienna, Cat.No: 500-0006). Briefly, 10 µL of the sample was added into the wells of a 96-well micro-titer plate (Greiner 96 Flat Bottom Transparent Polystyrene). As soon as all the samples were placed into the wells, 200 µL of the prepared BioRad reaction solution were added to the wells (BioRad Reagent diluted 1:5 with m$QH_2O$). The plate was incubated for 5 min at 21 ˚C and 400 rpm. The buffer for protein dilution (0.1 M Tris-HCl pH 7) was used as blank and BSA (bovine serum albumin) as standard. The absorption after 5 min was measured at λ=595 nm and the concentration calculated from the average of triplicate samples and blanks.

**Immobilization of Thc_cut1, HiC and CaLB on epoxy-activated beads.**

The epoxy-activated beads were washed with ethanol (2 times) and double distilled $H_2O$ (2 times) prior to use. A total of 1.0 g of dry epoxy-activated beads were suspended in 10 mL of 1 mg mL$^{-1}$ enzyme solution in 0.1 M Tris-HCl buffer pH 7 at

21 °C for 24 h on a blood rotator. Samples were withdrawn over time. The progress of the immobilization was monitored by evaluating the residual activity and protein concentration in the supernatant and data are reported in Figure S1 of ESI. It must be noted that Tris-HCl buffer was selected as immobilization medium because native Thc_cut1 was produced in this same buffer and the exchange of buffer would cause a loss of enzymatic activity (data not shown). After the immobilization, the enzyme preparations were extensively washed with 0.1 M Tris-HCl buffer pH 7 in order to remove all the non-covalently bound protein adsorbed on the support. Finally, in order to block the unreacted epoxy groups, the enzymatic preparations were incubated in 45 mL of 3 M glycine for 24 h at 21 °C as previously reported [52]. The enzyme preparations were extensively washed with 0.1 M Tris-HCl buffer pH 7 and dried for 48 h at 30 °C under reduced pressure (13.3 kPa) in a desiccator containing silica gel prior to use (if not otherwise specified). The immobilized preparations are termed iThc_cut1, iCaLB and iHiC, respectively.

**Moisture determination.**

0.2 g of immobilized enzymatic preparation was weighted in a tarred weighting bottle (A), dried for 6 h at 120 ± 5 °C, cooled down in a dessicator until constant weight was reached and weighted again (B). The moisture content was calculated as follows:

$$\text{Moisture content (\%)} = [(A-B)/A] \times 100$$

A table with the calculated water content of the immobilized preparations can be found in ESI (Table S1). All determinations were conducted in duplicates.

**Enzymatic polycondensation of DMA e BDO using a thin-film reaction system under solventless conditions.**

6.0 mmol A and 6.0 mmol B and the biocatalysts iThc_cut1, iCaLB, iHiC or Novozym® 435 (10% w $w^{-1}$ respect to the total amount of monomers) were incubated in a 50 mL round bottom flask connected to a rotary evaporator at 70 °C and 100 kPa for 24 h. The molar ratio of A and B was 1.0:1.0. During the polymerization process the biphasic system became a monophasic homogeneous transparent solution. The final product was a viscous sticky colorless liquid which was solubilized in DCM. After solvent evaporation, the crude product was analyzed by GPC, ESI-MS and $^{1}$H-NMR without any further purification. All reactions were performed in duplicates and compared to a control without enzyme.

**Screening of activity of iThc_cut1 in the polycondensation of dimethyl adipate (A) and diols with different chain length (B).**

A fast preliminary screening of the substrate specificity of Thc_cut1 towards different diols was performed by incubating 5.0 mmol of A, 5.0 mmol of B and iThc_cut1 (10% w $w^{-1}$ respect to the total amount of monomers). These semi-quantitative preliminary tests were carried out using common 4-mL reaction vials at atmospheric pressure and 70 °C and applying magnetic stirring for 24 h. The molar ratio of A and B used was 1.0:1.0. During the polymerization process the initial biphasic system became a monophasic homogeneous transparent solution. The final products were solubilized in tetrahydrofuran (THF) and filtered in order to remove the biocatalyst. After solvent evaporation, the crude products were analyzed by gel permeation chromatography (GPC), Electrospray Ionization-Mass analysis (ESI-MS) and proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) without any further purification. All reactions were performed in duplicates and compared to a control without enzyme. The same protocol was applied for the reactions conducted in organic solvent using 12-mL reaction vials and a concentration of monomers of 0.2 M.

**GPC.**

Samples were dissolved in THF (250 ppm BHT as inhibitor) and filtered through filter paper (595 ½, Whatman GmbH, Dassel, Germany). In case of liquid samples, the starting solvent was removed under reduced pressure. Gel permeation chromatography was carried out at 30 °C on an Agilent Technologies HPLC System (Agilent Technologies 1260 Infinity) connected to a 17369 6.0 mm ID x 40 mm L HHR-H, 5 µm Guard column and a 18055 7.8 mm ID x 300 mm L GMHHR-N, 5 µm TSKgel liquid chromatography column (Tosoh Bioscience, Tessenderlo, Belgium) using THF (250 ppm BHT as inhibitor) as eluent (at a flow rate of 1 mL $min^{-1}$). An Agilent Technologies G1362A refractive index detector was employed for detection. The molecular weights of the polymers were calculated using linear polystyrene calibration standards (250-70000 Da).

**$^1$H- NMR.**

Nuclear magnetic resonance $^1$H and $^{13}$C measurements were performed on a Bruker Avance II 400 spectrometer (resonance frequencies 400.13 MHz for $^1$H) equipped with a 5 mm observe broadband probe head (BBFO) with z-gradients. $CDCl_3$ was used as NMR solvent if not otherwise specified.

**Electrospray Ionization Mass Spectrometry (ESI-MS).**

The crude reaction mixtures were analyzed on Esquire 4000 (Bruker) electrospray positive ionization by generating the ions in an acidic environment. Around 10 mg of sample was dissolved in 2 mL of methanol containing 0.1% v $v^{-1}$ formic acid. The generated ions were positively charged with m $z^{-1}$ ratio falls in the range of 200-1000. The subsequent process of deconvolution allows the reconstruction of the mass peaks of the chemical species derived from the analysis of the peaks generated.

**Recyclability of Thc_cut1: polycondensation between DMA and BDO.**

The recyclability study was carried out on a scale of 1.5 mL (1.6 g of monomers) according to the following procedure: DMA (1.0451 g, 6.0 mmol) and BDO (0.5407 g, 6.0 mmol, molar ratio 1.0:1.0) were mixed in a 50-mL round-bottom flask. The two monomers are liquid and completely miscible. The addition of the biocatalyst (0.1586 g of Thc_cut1, 10% w $w^{-1}$ respect to the total amount of monomers) started the reaction, which run for 4 h at 50 °C under atmospheric pressure (100 kPa) in the flask connected to a rotary evaporator. The conversion of DMA was monitored at 1, 2, 3 and 4 h by withdrawing volumes (about 50 µL) of the fluid crude reaction mixture that were dissolved in $CDCl_3$ and analyzed by $^1$H-NMR.

The products and the unreacted monomers were sufficiently fluid to be filtered under reduced pressure without any addition of solvent. The immobilized biocatalyst (beads diameter 200-500 µm) was fully recovered at the end of the reaction by means of a sintered glass filter, equipped with cellulose filters. The biocatalyst was not rinsed in order to prevent any detrimental effects of solvent treatments. The recovered biocatalyst was employed for the following synthetic cycles under the conditions above described by adding the same amount of fresh monomers. It was also verified that no reaction occurred in the absence of enzyme.

**Construction and analysis of the homology model of Thc_cut1.**

The *Thermobifida cellulosilytica* cutinase 1 (Thc_cut1) protein sequence was taken from the NCBI GenBank nucleotide sequence HQ147785 [33]. The translated protein sequence was used as input for building a homology model of the Thc_cut1 3D structure using the SWISS-MODEL server [53]. As a template the structure of cutinase from *Thermobifida fusca* was used (PDB36 code 4CG1): [37] the two enzymes share high homology and differ in just two amino acids. The catalytic triad

and the oxyanion hole were individuated by visual inspection taking as a reference the organization of other serine-hydrolases [32].

The final 3D structure of Thc_cut1 was obtained by SWISS-MODEL server [53] and evaluated by means of GMQE with a value of 0.99 (GMQE is a scoring function for homology model quality evaluation; it assumes values between 0 and 1 where higher numbers indicate higher model reliability). The final 3D structure is available in ESI (Structure Thc_cut1).

**Structural and sequence comparisons.**

Structure comparisons of cutinase from *Humicola insolens* (HiC) and cutinase 1 from *Thermobifida cellulosilytica* (Thc_cut1) were performed by the software PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC). 3D-structure of Thc_cut1 was generated by homology model as previously indicated; HiC crystal structure 4OYY (crystal obtained in 0.1 M Tris-HCl pH 8.5, 50 mM lysine, PEG MME 2K 11% v/v of 50% w/v stock solutions in the presence of diethyl-p-nitrophenyl-phosphate as inhibitor) [38] was taken from Protein Data Bank (PDB). [36] Structural superimposition was performed by considering catalytic residues as a reference: the catalytic serine (Ser105 and Ser131 for HiC and Thc_cut1 respectively) and the residues forming the oxyanion hole (Ser28, Met106 and Tyr61, Met132 for HiC and Thc_cut1 respectively). Subsequently, the structural superimposition was used as a reference for the sequence alignment of the two cutinases. Sequence alignment was visualized by the software UGENE [54]; aligned residues are coloured according to Clustal W colour scheme [55].

**Surface analysis.**

The representation and the calculation of the hydrophobic enzyme surfaces were performed by the color_h python script [56] for the software PyMOL. Protein structures were visualized and recorded using the PyMOL software. The 3D-structures used for the hydrophobicity comparisons were retrieved from the PDB with the code 4OYY [38] for HiC and 1TCA [57] for CaLB, whereas the homology model was used for Thc_cut1.

**Molecular dynamics simulations.**

The structure of HiC 4OYY [38] was taken from PDB and used as starting point for the MD simulation after removal of the inhibitor diethyl-p-nitrophenyl-phosphate. The

1TCA [55] crystallographic structure was used for computing for CaLB (crystal obtained in acetate buffer 20 mM pH 3.6, 20% polyethylene glycol 4000, 10% isopropanol). The Thc_Cut1 structure was obtained by homology modelling as described above. Both HiC and CaLB starting structure contains just one protein molecule and the crystal water, whereas concerning the Thc_Cut1 structure, crystal water was retrieved from the 4CG1 template structure. The protonation state was calculated at pH 7.0 using the PDB2PQR server [56] based on the software PROPKA [57]. Subsequently, each protonated enzyme structure, together with its crystal water, was defined according to OPLS force field [58], inserted in a cubic box of 216 $nm^3$ and solvated with explicit solvent (either TIP4 water or toluene as defined by literature) [59]. Thus, each enzyme system was minimized using the software GROMACS version 4 [60] using a steepest descendent algorithm for 10,000 steps. Afterwards, equilibration MD simulations were performed with the software GROMACS version 4 for 5 ns at 343 K in an NVT environment keeping enzymes position restrained, thus allowing the equilibration of the solvent particles (toluene and crystal water); Particle Mesh Ewald (PME) algorithm [61] for the calculation of electrostatic interactions was employed, v-rescale algorithm [62] for temperature and Berendsen algorithm [63] for pressure were also employed. Finally, after the removal of the every restraint on protein position, each enzyme was simulated for 10 ns at 343 K in NVT environment using the same parameters as before.

**Acknowledgements**

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# 4

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


## Abstract

The application of *Candida antarctica* lipase B in enzyme-catalyzed synthesis of aromatic-aliphatic oligoesters is reported here. The obtained oligomers 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. Reaction conditions and enzyme selectivity for polymerization 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.


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## 4.1 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 applications had an exponential growth. Nowadays, enzymes are widely used in multi-ton-scale processes as detergent and feed additives, for starch conversion, in textile applications, and in many other applications related to polymer degradation, notably polysaccharide degradation [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 society 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 polymers, 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 aromatic-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, polycondensation reactions using halogen-activated diesters produce reaction side products which need special handling for discarding. Five years later, Uyama et al. reported that also divinyl esters can be used at this purpose [17] but, unfortunately,

the produced 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 simple 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 polycondensation of the renewable furandicarboxylic acid dimethyl 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 favourable conditions for enzyme catalyzed synthesis 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 selectivity 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 conditions [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].

## 4.2 Materials and methods

### 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 reagents and solvents were reagent grade and used without further purification if not otherwise specified.

### Enzymatic preparations

Novozym® 435 is a commercial formulation (Sigma Aldrich) of lipase B from *Candida 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 preparation water content was determined to be below 0.3% w $w^{-1}$. 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.

### Hydrolytic activity assay

The activity of enzyme preparations was assayed as previously reported [15] by following the tributyrin hydrolysis and by titrating, with 0.1 M sodium hydroxide, the butyric acid that is released during the hydrolysis. An emulsion composed of 1.5 mL of tributyrin, 5.1 mL of arabic gum emulsifier (0.6% w $v^{-1}$) and 23.4 mL of m$QH_2O$ was prepared in order to obtain a final molarity of tributyrin of 0.17 M. Successively, 2 mL of 0.1 M potassium-phosphate buffer pH 7.0 were added to 30 mL of the tributyrin emulsion. The mixture was incubated in a thermostated vessel equipped with a mechanical stirrer at 30 °C. After pH stabilization, 50 mg of the biocatalyst were added. The consumption of 0.1 M NaOH was monitored for 20 min. One unit of activity (U) was defined as the amount of immobilized enzyme required to produce 1 μmol of butyric acid per min at 30 °C.

### Synthetic activity assay

Quantification of the synthetic enzyme activity was performed based on the enzymatic synthesis of propyl laurate as previously described [6]. Briefly, 7.2 g

(0.12 mol) of 1-propanol and 24 g (0.12 mol) of lauric acid were weighted into a 100 mL glass bottle. The solution was heated up to 60 °C until the lauric acid was completely dissolved. A sample at time= 0 min was taken and the biocatalyst was added, while maintaining the solution under continuous stirring. Samples were taken at different reaction times. The assay is based on the determination of acid values of the reaction mixture by titration with 0.1 M KOH in ethanol using phenolphthalein as indicator. The acid value indicates the residual unreacted lauric acid. One unit of enzyme activity is defined as the amount of enzyme protein required to produce 1 µmol of propyl laurate per minute under standard conditions (T=60 °C).

**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 *Candida antarctica* (CaLB) (10% w $w^{-1}$ with respect to the total amount of monomers) was then added to the reaction mixture and the reaction proceeded for 96 h in a Carousel 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.

**$^1$H-NMR**

Nuclear magnetic resonance $^1$H-NMR measurements were performed on a Bruker Avance II 400 spectrometer (resonance 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.

**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.

## 4.3 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), dimethyl phthalate (1,2e) were reacted with the linear polyols ethylene glycol (2p), 1,4-butanediol (4p), 1,6-hexanediol (6p), 1,8-octanediol (8p), 1,10-decandediol (10p) and 1,12-dodecanediol (12p) were investigated. All the reactions were catalyzed by lipase B from *Candida antarctica* (CaLB) (see Figure 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 polymerization of dicarboxylic acids divinyl esters with polyols [17] and of various aliphatic substrates [13, 16]. The used temperature (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].

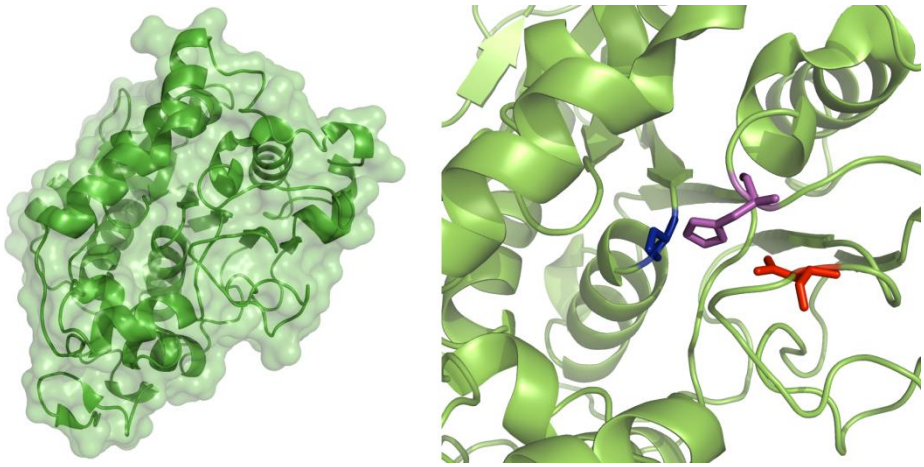

**Figure 1.** Structure of the biocatalyst *Candida antarctica* lipase B (CaLB) (left) and zooming of the catalytic triad (right). Red: Aspartic acid (Asp187); Blue: Serine (Ser105); Magenta: Histidine (His224).

**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 polycondensation 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 (see Electronic Supplementary Information Figure 1s) 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 (filtration 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 polymerizations 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.

**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 (%)* |
|---|---|---|
| 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 |

* 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

This first screening phase revealed that aromatic dicarboxylic acids were not suitable for enzymatic polycondensation 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 serious 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.

**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 favouring the nucleophilic attack of the acyl carbon by the deprotonated alcohol. The enzyme catalyzes the reaction by stabilizing the negatively charged oxyanion by means of electrostatic interactions between Thr40 and Gln106 inside the so-called oxyanion 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-furandicarboxylic acid dimethyl ester with linear

polyols. In this study, enzymatic polymerization was also conducted using tetrahydrofuran (THF) as more polar solvent when compared to toluene. In this case, no polycondensation products were observed for 1,2e, 1,4e and the reaction conducted without biocatalyst while a limited conversion ranging from 11 to 20% was found for all the linear polyols (see Electronic Supplementary Information, Table 1s). 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).

**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 (%)* |
|---|---|---|
| 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% |

* 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

The molecular weights and the polydispersity index of the reaction products (Figure 2) indicates 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 isophthalic 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.

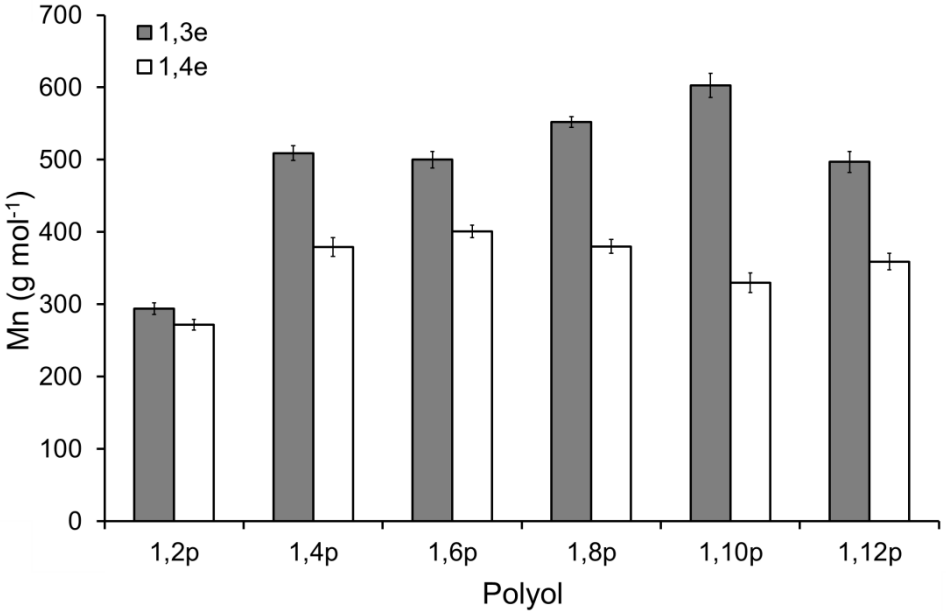


**Figure 2.** Number average molecular weight ($M_n$) distribution for the reactions between dimethyl isophthalate (1,3e), dimethyl terephthalate (1,4e) and the $C_2$-$C1_2$ linear polyols. Reaction conducted in toluene for 96 h using Novozym® 435 as biocatalyst. 1,2p: ethylene glycol; 1,4p: 1,4-butanediol; 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.

For all the used polyols, the obtained $M_n$ was higher for the reactions where 1,3e is used as aromatic moiety (Figure 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).

## 4.4 Concluding remarks

In this work, immobilized lipase B from *Candida 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 dimethyl phthalate. The most suitable diols for the enzymatic synthesis were the $C_4$-$C_{10}$ 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 modelling studies are needed in order to understand the poor reactivity of dimethyl terephthalate and the effect of the solvent on the reaction progression.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author Contributions**

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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# 5

# Biocatalyzed approach for the surface functionalization of poly(L-lactic acid) films using hydrolytic enzymes


## Abstract

Poly(lactic acid) as a biodegradable thermoplastic polyester has received increasing attention. This renewable polyester has found applications in a wide range of products such as food packaging, textiles and biomedical devices. Its major drawbacks are poor toughness, slow degradation rate and lack of reactive side-chain groups. An enzymatic process for the grafting of carboxylic acids onto the surface of poly(L-lactic acid) (PLLA) films was developed using *Candida antarctica* lipase B as a catalyst. Enzymatic hydrolysis of the PLLA film using *Humicola insolens* cutinase in order to increase the number of hydroxyl and carboxylic groups on the outer polymer chains for grafting was also assessed and showed a change of water contact angle from 74.6 to 33.1° while the roughness and waviness were an order of magnitude higher in comparison to the blank. Surface functionalization was demonstrated using two different techniques, $^{14}$C-radiochemical analysis and X-ray photoelectron spectroscopy (XPS) using $^{14}$C-butyric acid sodium salt and 4,4,4-trifluorobutyric acid as model molecules, respectively. XPS analysis showed that 4,4,4-trifluorobutyric acid was enzymatically coupled based on an increase of the fluor content from 0.19 to 0.40%. The presented $^{14}$C-radiochemical analyses are consistent with the XPS data indicating the potential of enzymatic functionalization in different reaction conditions.


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## 5.1 Introduction

Together with poly(caprolactone) (PCL), poly(lactic acid) (PLA) is one of the most widely studied polyesters due to its wide field of application and biodegradability. [1-3] PLA has excellent biocompatibility, biodegradability and processability properties [4-6] which makes it an attractive material for biomedical purposes [7, 8]. The most common strategies to modify the bulk properties of PLA like mechanical characteristics include copolymerization with other monomers [9, 10] and blending. [11-13] There is an increasing interest in modifying polymer surfaces in order to improve properties like hydrophilicity and/or creating reactive anchor groups for further functionalization. The latter includes for example covalent immobilization of bioactive compounds or decoration of nanoparticles for targeted drug delivery [14-16] while retaining the bulk properties.

Surface functionalization of polyesters is usually achieved via wet chemistry, [17-19] photografting [20-22] or plasma treatment. [23, 24] In order to avoid the use of harsh chemicals and reduce the energy consumption different enzymes have been investigated to improve the surface hydrophilicity of renewable polyesters such as PLA [25-27] or even recalcitrant synthetic polyesters like poly(ethylene terephthalate) (PET). [28-30] Enzymes do not only work at mild process conditions and are not restricted to planar surfaces like plasma treatment but can specifically introduce modifications on polymer surface while leaving the bulk properties unchanged. Improved biocompatibility of PLA, was achieved by coupling human serum albumin on cutinase activated membranes [31]. The functionalized material showed an increased antioxidant capacity as well as a higher osteoblast cell proliferation. In a similar approach, polyamide materials were functionalized in a two-step enzymatic process starting with limited enzymatic surface hydrolysis [32].

The aim of the present work was to functionalize the surface of PLA films using hydrolases, both for surface “activation” and for the subsequent coupling of a model molecule. To monitor the surface functionalization process and avoid interferences due to adsorbed enzyme protein, a new method using $^{14}$C-radiolabeled molecules was used for higher sensitivity. This enzymatic coupling approach is a promising environmentally friendly way to create PLA based materials where the surface is functionalized while leaving the bulk properties unchanged.

## 5.2 Materials and methods

### Chemicals and reagents

L(-)-lactide was a gift from PURAC (Gorinchem, Netherlands). n-Butyric acid [1-14C] sodium salt with a concentration of 1 mCi $mL^{-1}$ in EtOH and a specific radioactivity of 50-60 mCi $mmol^{-1}$ was purchased from American Radiolabeled Chemicals Inc. (St. Louis, USA). Poly(L-lactic acid) PLLA films, thickness 0.05 mm, were from Goodfellow (Cambridge, UK). All the other chemicals and solvents were purchased from Sigma-Aldrich at reagent grade, and used without further purification if not otherwise specified.

### Enzymes

Recombinant *Candida antarctica* lipase B (fCaLB) (62288 SIGMA), specific activity 9 U $mg^{-1}$, protein content 90%, water content 0.01% and CaLB immobilized on macroporous acrylic resin (L4777 SIGMA), specific activity >5000 U $g^{-1}$, water content 0.01% (aCaLB) were from Sigma-Aldrich. *Humicola insolens* cutinase (HiC) was a gift from Novozymes (Beijing, China), 11.2 mg $mL^{-1}$ was purified as previously described [33] prior to use. Covalently immobilized CaLB (cCaLB) on epoxy-activated beads, particle size 100-300 µm, specific activity 2237 U $g^{-1}$, water content 0.03% was kindly provided by SPRIN S.p.a. (Trieste, Italy).

### Esterase activity assay

Esterase activity was measured at 21 °C using p-nitrophenyl butyrate (PNPB) as a substrate as previously reported by Ribitsch et al. with some modification. [26] The final assay mixture was made up of 10 µL of the substrate solution (86 µL of PNPB in 1000 µL of DMSO), 1000 µL of 100 mM Tris-HCl buffer at pH 7 and 100 µL of enzyme solution. The increase of the absorbance at 405 nm due to the hydrolytic release of p-nitrophenol (ε 405 nm = 9.36 mL (µmol cm)$^{-1}$) was measured over time using a Jasco V-630 spectrophotometer using plastic cuvettes. A blank was included using 100 µL of buffer instead of enzyme. The activity was calculated in units (U), where 1 U is defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute under the given assay conditions.

### Synthetic activity assay

Quantification of the synthetic enzyme activity was performed based on the enzymatic synthesis of propyl laurate. Briefly, 7.2 g of 1-propanol and 24 g of lauric

acid were weighted into a 100 mL glass bottle. The solution was heated up to 60 °C until the lauric acid was completely dissolved. A sample at time = 0 min was taken and the biocatalyst was added, while maintaining the solution under continuous stirring. Samples were withdrawn at different reaction times. The assay is based on the determination of acid values of the reaction mixture by titration with 0.1 M KOH in ethanol using phenolphthalein as indicator. The acid value indicates the residual unreacted lauric acid. One unit of enzyme activity is defined as the amount of enzyme protein required to produce 1 µmol of propyl laurate per minute under standard conditions (T = 60 °C).

**Protein quantification**

Protein concentration was determined with the BioRad protein assay (Bio-Rad Laboratories GmbH, Vienna, Cat.No: 500-0006). Briefly, 10 µL of the sample was added into the wells of a 96 well micro-titer plate. As soon as all the samples were in the wells, 200 µL of the prepared Bio-Rad reaction solution were added (BioRad Reagent diluted 1:5 with Fresenius water). The plate was incubated for 5 min at 21 °C and 400 rpm. Buffer (0.1 M Tris-HCl pH 7) was used as blank and BSA as standard. The absorption after 5 min was measured at λ = 595 nm and the concentration calculated from the average of triplicates.

**PLLA Hydrolysis**

Prior to the treatment, PLLA films were cut into pieces (20 × 20 mm) and washed in three consecutive steps for 30 min at 37 °C and 130 rpm. In a first step, a solution of 5 g $L^{-1}$ Triton-X100 was used, in the second step a 100 mM $Na_2CO_3$ solution was used, followed by double distilled water (ddH2O). The *H. insolens* cutinase was diluted in 100 mM Tris-HCl buffer pH 7 and used in different concentrations. Chemical hydrolysis was performed using NaOH in concentrations from 0.05 to 0.1 M. Incubations were performed at 130 rpm and 37 °C for the enzymatic treatment and at 21 °C for the alkaline treatment. Hydrolysis products were quantified by using high performance liquid chromatography. Controls were performed by incubating the PLLA with buffer solution.

**PLLA functionalization**

After the hydrolytic treatment, PLLA films were incubated in a 0.2 M butyric acid solution in anhydrous n-heptane containing different concentrations of n-butyric acid [1-14C] sodium salt. Different preparations and amounts (calculated in U $g^{-1}$) of

*Candida antarctica* lipase B as biocatalyst were used. For the X-ray photoelectron spectroscopy (XPS) measurements the functionalization was carried out using a 0.2 M 4,4,4-trifluorobutyric acid solution in n-heptane as above.

**Synthesis of PLLA oligomers**

The preparation of the PLLA oligomers was carried out as previously described [34] with some modification. Preparation of (S)-2-([S]-2-hydroxypropanoyloxy) propanoic acid (oLACT): 25.003 g of (3S,6S)-3,6-dimethyl-1,4-dioxane-2,5-dione in water (150 mL) were stirred at 45 °C for 4 h. The reaction mixture was then freeze dried to obtain the product as a clear colorless oil.

Synthesis of benzyl 2-([S]-2-hydroxypropanoyloxy) propanoate (pLACT): 24.008 g of oLACT, 43.350 g of benzyl bromide, 50.608 g of triethylamine and 250 mL of $CH_2Cl_2$ were put in a 500 mL glass bottle under ice. Triethylamine and the $CH_2Cl_2$ were later removed under reduced pressure. The subsequent workup of the reaction was performed according to the method cited above.

Enzymatic synthesis of 1-([1-(benzyloxy)-1-oxopropan-2-yl]oxy)-1-oxopropan-2-yl butyrate (pLACT-BA): for the synthesis of pLACT-BA 0.2049 g (0.81 mmol) of pLACT and 0.0718 g (80.81 mmol) of butyric acid (BA) were put in a 4 mL glass vial with 10% w/w immobilized CaLB as catalyst under nitrogen atmosphere. The reaction mixture was then stirred for 96 h at 100 °C. A control reaction was carried out at the same conditions without catalyst. Detailed spectral information of the model substrates can be found in Supporting information.

**HPLC-RI**

Hydrolysis samples were precipitated following the Carrez method and filtered through 0.20 µm Nylon filters (GVS, Indianapolis, USA). The analytes were separated by high performance liquid chromatography (HPLC) using refractive index detection (1100 series, Agilent Technologies, Palo Alto, CA) equipped with an ICSep-ION-300 column (Transgenomic Organic, San Jose, CA) of 300 mm by 7.8 mm and 7 µm particle diameter. Column temperature was maintained at 45 °C. Samples (40 µL) were injected and separated by isocratic elution for 40 min at 0.325 mL $min^{-1}$ in 0.005 M $H_2SO_4$ as the mobile phase.

**GPC**

Samples were dissolved in THF (250 ppm BHT as inhibitor) and filtered through filter paper (595 1/2, Whatman GmbH, Dassel, Germany). In case of liquid samples, the

starting solvent was removed under reduced pressure. Gel permeation chromatography was carried out at 30 °C on an Agilent Technologies HPLC System (Agilent Technologies 1260 Infinity) connected to a 17369 6.0 mm ID × 40 mm L HHR-H, 5 µm Guard column and a 18055 7.8 mm ID × 300 mm L GMHHR-N, 5 µm TSKgel liquid chromatography column (Tosoh Bioscience, Tessenderlo, Belgium) using THF (250 ppm BHT as inhibitor) as eluent (at a flow rate of 1 mL $min^{-1}$). An Agilent Technologies G1362A refractive index detector was employed for detection. The molecular weights of the polymers were calculated using linear polystyrene calibration standards (400-2,000,000 Da).

**Hydrophobicity measurements**

Hydrophobicity of the sample was measured via Water Contact Angle (WCA). WCA of the PLLA films were measured before and after exposure to the enzymatic treatments. The protein was washed away from the surface using three consecutive washing steps [26, 28] as described in the PLLA hydrolysis section. Polymer films were analyzed with a Drop Shape Analysis System DSA 100 (Kruss GmbH, Hamburg, Germany) using ddH2O as test liquid with a drop size of 2 µL, deposition speed 100 µL $min^{-1}$. Water contact angles were measured via the static sessile drop method after 5 s. Data are obtained from the averages of the measurements taken from at least eight different points of three sample surfaces. In this way the measured angle was constant and reproducible though all the samples and remarkable differences before and after the enzymatic treatment were evidenced [28, 35].

**Atomic force microscopy (AFM)**

All AFM-microscopy was performed on a Bruker Dimension Icon AFM (Bruker, Karlsruhe, Germany) in tapping mode. The probes used were OTESPA (AFM Bruker, Karlsruhe, Germany) and had nominal values of k = 42 N $m^{-1}$, f0 = 300 kHz and rtip<10 nm. Scan speed was 1 Hz, with gain factors and set point kept as constant as possible with only small adjustments to get good feedback. Scan size for all images was 1 $\mu m^2$, with a resolution of 512 pixels $line^{-1}$ for the reference PLLA and the non-pre-treated samples. A resolution of 256 pixels $line^{-1}$ was used for the pre-hydrolyzed samples. This change was performed to lower the effect of uncontrolled oscillations of the cantilever on these samples. Evaluation of AFM images was performed using the free Gwyddion 2.28 image analysis software. For all images a tilt correction on the topography image was performed and the minimal value set to zero before data

analysis. Roughness analysis evaluating $r_a$, $r_q$, $w_a$ and $w_q$, was performed by evaluating the length of the image diagonals. The width of the analysis strip was 5 pixels (256 pixels line$^{-1}$ resolution images) and 10 pixels (512 pixels line$^{-1}$ image resolution) respectively, with a roughness cut-off of 0.1 for all images.

**$^{14}$C-radiochemical analysis**

The functionalized films were put into plastic vials containing 10 mL scintillation cocktail (Optiphase Hisafe 3, Perkin-Elmer) and assayed for $^{14}$C on a Perkin-Elmer Tri-Carb 3110 liquid scintillation counter (dpm mode, 20 min counting time). Liquid samples (100 µL) were mixed with scintillation cocktail (2 mL) and assayed for $^{14}$C as described above.

**X-ray photoelectron spectroscopy (XPS)**

XPS was performed on an Multiprobe UHV-surface-analysis system (Omicron Nanotechnology) equipped with a DAR400 x-ray source with Al anode and a quartz-crystal monochromator XM 500 using an x-ray excitation energy of 1486.70 eV (Al Ka1-line). The monochromated x-ray line width was of 0.3 eV. The analyzed surface area was of approximately 1 mm$^2$. The reaction mixture was then stirred for 25 h at 21 °C and monitored via TLC.

**Lyophilization**

Freeze drying was conducted using a Christ Freeze dryer Beta 1-16, 220 V, 50 Hz, 1.2 kW.1-dimensional roughness and waviness of the graphs was evaluated from line spectra of the image diagonals as rq and wq, that is the root mean square height deviation of the mean line. The cutoff frequency was chosen as 0.1.

**Flash column chromatography**

For the purification of pLACT a Büchi flash column chromatography system equipped with a C-620 Control Unit, a C-660 Fraction Collector and a C-640 UV spectrophotometer (Büchi, Switzerland) was used. Sepacore® HP 120 g Büchi Silica cartridges were used as stationary phase and a mixture of 1:1 hexane: ethyl acetate was used as mobile phase for separations.

**TLC**

Analytical thin layer chromatography (TLC) was performed on commercial Macherey-Nagel plates coated with silica gel Alugram® SIL G/UV254 (0.2 mm thick using

hexane/ethyl acetate in a ratio of 1:1 as the mobile phase. The analytes were detected using a UV lamp or via staining with a $KMnO_4$/KOH solution.

**LC/TOF-MS**

Samples were dissolved in acetonitrile (AcN) with 0.1% formic acid at a concentration of 1 mg $mL^{-1}$ and filtered through 0.20 µm Nylon filters. Liquid chromatography/electrospray/time-of-flight mass spectrometry (LC/ESI/TOF/MS), in positive ionization was used to separate and identify oLACT and pLACT. The analytes were separated using an HPLC (1260 series, Agilent Technologies, Palo Alto, CA) equipped with a reversed-phase C18 rapid resolution column (Zorbax Eclipse XDB, Agilent Technologies) of 50 mm by 2.1 mm and 1.8 µm particle diameter. Column temperature was 25 °C. Mobile phase A was water with 0.1% formic acid, and mobile phase B consisted of can with 0.1% formic acid. The mobile phase composition was maintained at the initial conditions (90% A) for 0.5 min, followed by a linear gradient progressed to 100% B in 35 min, after which the mobile phase composition for 5 min. The next 10 min mobile phase was set back to the initial conditions. The flow rate was 0.4 mL $min^{-1}$ and the injection volume was 1 µL. This HPLC system was connected to a time-of-flight mass spectrometer (6230 TOF LC/MS, Agilent Technologies) equipped with an electrospray interface under the following operating parameters: capillary 3500 V, nebulizer 20 psig, drying gas 10 L $min^{-1}$, gas temperature 325 °C, fragmentator 200 V, skimmer 65 V, OCT 1 RF Vpp 750 V. The mass axis was calibrated using the mixture provided by the manufacturer over the m/z 50-3200 range. A second orthogonal sprayer with a reference solution was used as a continuous calibration using the following reference masses: 121.050873 and 922.009798 m/z. Spectra were acquired over the m/z 100-3000 range at a scan rate of two spectra per second.

**NMR**

Nuclear magnetic resonance $^{1}H$ and $^{13}C$ measurements were performed on a Bruker Avance II 400 spectrometer (resonance frequencies 400.13 MHz for $^{1}H$ and 100.63 MHz for $^{13}C$) equipped with a 5 mm observe broadband probe head with z-gradients. $CDCl_3$ was used as NMR solvent if not otherwise specified.

## 5.3 Results and discussion

The biodegradable and biobased polyester PLLA is increasingly being used in many fields from textiles to biomedical usually involving chemical functionalization. Current covalent PLLA surface-modification methods usually involve the use of UV irradiation for the grafting of molecules onto the PLLA surface. However, this technique leads to monomer migration into the film bulk [5] and a noticeable decrease of its molecular weight after irradiation [36]. In this work we describe a novel nondestructive enzymatic method for surface functionalization of PLLA films that prevents bulk modification of the polyester and at the same time allows a controlled grafting onto the polymer surface. In a first step new reactive groups are created onto the polymer surface by limited enzymatic hydrolysis of the outer PLLA polyester layers, followed by a subsequent lipase-catalyzed coupling of a molecule containing carboxylic functionalities onto the PLLA surface.

**Enzymatic hydrolysis of PLLA films**

In a first step, enzymatic surface hydrolysis of PLLA was studied. Therefore, the release of lactic acid as quantified with HPLC-RI (Figure 1) was compared for changes in bulk properties as measured by using GPC. After 24 h of hydrolysis, a maximum of 42 mg $L^{-1}$ of lactic acid was released. Longer incubation times of 72 h were also assessed leading to a release of 113 mg $L^{-1}$ of lactic acid. No release of lactic acid was detected in control conditions. The results from GPC measurements showed that there were only slightly changes in Mw, Mn and polydispersity indices of the PLLA after 72 h of treatment (Table 1). This indicates that the hydrolysis preferentially occurred on the polymer surface while the bulk properties were not significantly altered in contrast to chemical alkaline hydrolysis. Chemical PLLA hydrolysis such as alkaline hydrolysis, can lead to unexpected results on the degradation rate depending on the polymer sources [37]. As alkali-based treatments strongly affect the bulk properties of the PLLA films, this technique is not suitable if the final goal is to achieve only a surface-functionalization of the polymer. In addition, the residual alkali is not easily removed after the treatment [9].

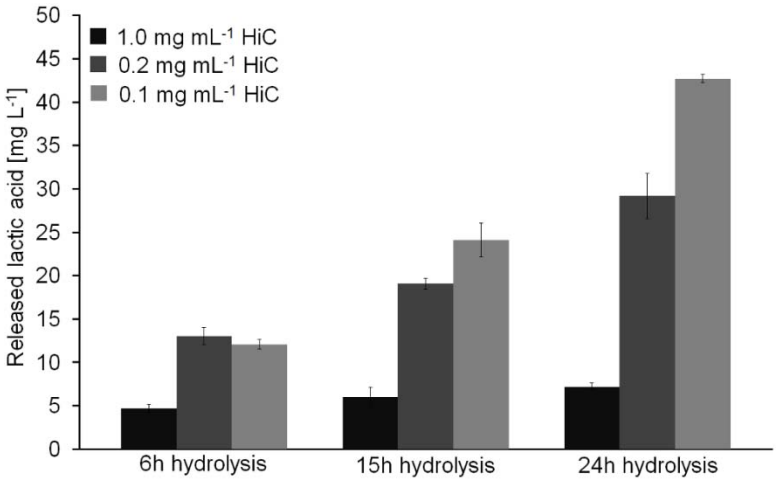


**Figure 1.** HPLC-RI analysis of the released lactic acid after poly(L-lactic acid) films hydrolysis using *Humicola insolens* cutinase (HiC) as catalyst in 0.1 M TRIS buffer at pH 7. Control reactions performed using buffer solution led to no detectable hydrolysis products. All reactions were performed in triplicates. Error bars show the standard deviation.

Limited partial hydrolysis of the PLLA as performed here generates hydroxyl and carboxylic groups leading to hydrophilization of the surface. Therefore, water contact angle (WCA) measurements after complete enzyme removal were used to quantify the hydrophobicity decrease upon enzymatic surface hydrolysis. Compared to the blank (74.6 ± 0.7°), the maximum hydrophilicity of the WCA was reached already after 24 h of hydrolysis (33.1 ± 1.7°) with a marginal further decrease after longer incubation for 48 h (33.7 ± 2.1°) and 72 h (36.5 ± 0.9°). The WCA value of the starting PLLA film was 73.5 ± 1.2°. Previously, slightly higher values were obtained for enzymatic hydrolysis of PLLA membranes which are most likely due to a larger surface area [31]. Nevertheless, these results indicated that 24 h surface hydrolysis should be enough to activate the PLLA for further functionalization. At those particular conditions described by Nyanhongo et al. the hydrophilicity increase achieved with the enzymatic treatment, 58 ± 3°, was comparable to the one achieved with a chemical treatment 52 ± 5° (hydrophobicity of the starting material: 78 ± 2°) [31].

**Table 1.** Number-average molecular weights and polydispersity indices of the PLLA films before and after the enzymatic surface hydrolysis.

| Treatment | $M_n$ (g $mol^{-1}$)[a)] | $M_w$ $M_n^{-1}$[a] |
|---|---|---|
| Starting PLLA | $34.6*10^3$ | 4.8 |
| Blank 24 h | $34.3*10^3$ | 4.8 |
| Blank 48 h | $35.3*10^3$ | 4.7 |
| Blank 72 h | $34.0*10^3$ | 4.8 |
| Hydrolysis 24 h | $34.6*10^3$ | 5.0 |
| Hydrolysis 48 h | $33.3*10^3$ | 5.0 |
| Hydrolysis 72 h | $34.3*10^3$ | 5.0 |

a) Determined using GPC calibrated with narrow molar mass polystyrene standards.

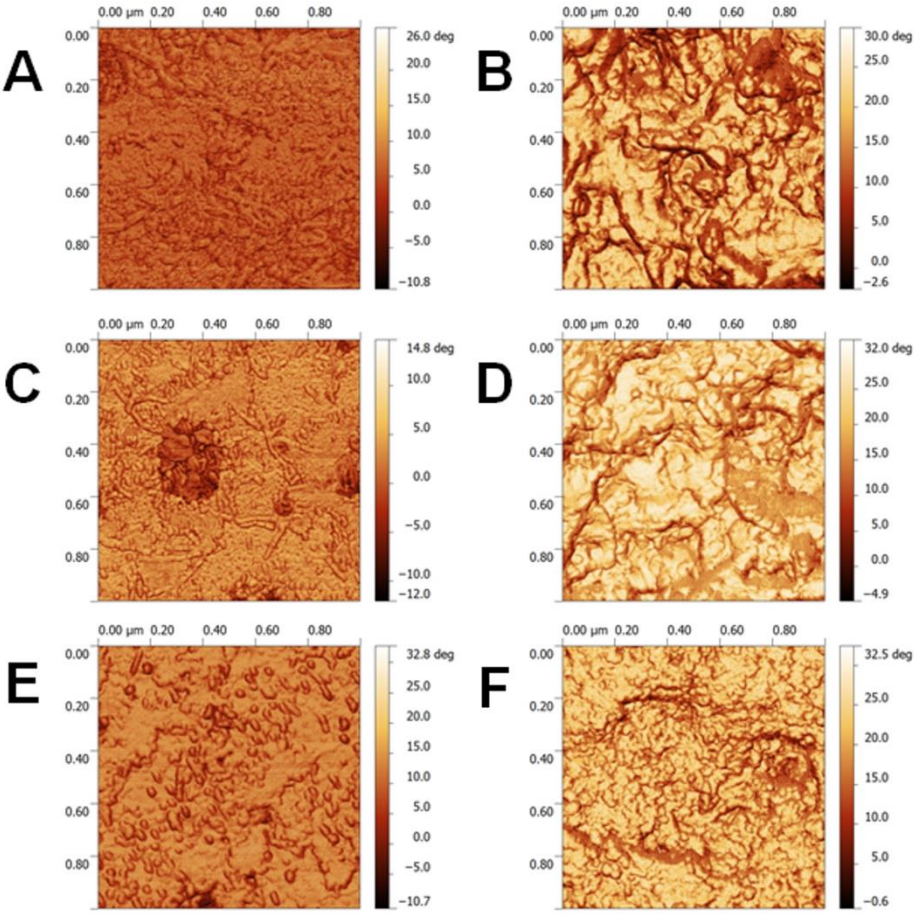


Figure 2. Atomic force microscopy phase images of the surfaces of PLLA films after enzymatic functionalization with and without enzymatic prehydrolysis. (A) PLLA blank (24 h in buffer), (B) 24 h hydrolyzed with cutinase, (C) Nonhydrolyzed/enzymatic coupling blank, (D) Hydrolyzed/enzymatic coupling blank, (E) Nonhydrolyzed/enzymatic coupling, (F) Hydrolyzed/enzymatic coupling. All AFM pictures were scanned over an area of 1 µm × 1 µm. For the enzymatic coupling 880 U of aCaLB were used. All reactions were performed in triplicates.

AFM analysis of the enzymatically hydrolyzed samples revealed distinct morphology changes created on the surface (Figure 2). Compared with the untreated PLLA (Figure 2A), which shows a nearly ideally smooth surface, with small evenly scattered structures, the enzymatically hydrolyzed samples (Figures 2B, 2D and 2F) showed a rougher surface. Roughness and waviness (Supporting information, Table S1) were an order of magnitude higher compared to the blank (Figures 2C and 2E), with valley-to-peak distances of about 500 nm on a scan length of only 1000 nm.

Collectively, these results strongly indicate that carboxylic and hydroxyl groups can be generated enzymatically on PLLA films using the cutinase from *H. insolens* with low extent of damage to the material. WCA measurements showed that the treatment leads to a strong decrease of the surface hydrophobicity (more than 40° after 24 h of treatment in comparison with the blank) due to the new carboxyl and hydroxyl generated groups without affecting the bulk structure of the polymeric material. The same degree of modification (as measured with WCA) is not possible by using alkali

treatment without a concomitant strong decrease of the PLLA molecular weight [38]. Controlled surface hydrolysis of PLLA for activation of the material for further functionalization is investigated in this study. Moreover, hydrophilization per se is required for certain applications such as to improve cell affinity [39].

**Enzymatic synthesis of PLLA model oligomers**

In a first step, enzymatic esterification of PLLA was studied using oligomers as PLLA models. Therefore, a -COOH benzyl protected lactide dimer (pLACT) was successfully synthesized in order to obtain a PLLA dimer with only one reactive end (-OH group). The esterification of pLACT with butyric acid was then carried out using *Candida antarctica* lipase B (CaLB) adsorbed onto macroporous acrylic resin as catalyst. CaLB is able to catalyze the esterification reaction of various dicarboxylic acids with diols [40, 41] in different solvents [42, 43] and is also capable to transesterify aromatic dicarboxylic acid esters with diols [44]. The synthetic route that leads to pLACT coupled with butyric acid (pLACT-BA) is shown in Figure 4. The compounds were used in a 1:1 ratio in bulk.

In order to demonstrate that CaLB is able to catalyze the esterification of the PLLA model pLACT as shown in Figure 4, reaction mixtures were analyzed by LC/TOF-MS. Despite the occurrence of some uncatalyzed background conversion [45], the difference between blank and enzymatic reaction appeared to be highly significant. Since an absolute quantification of the reaction product with LC/MS-TOF analysis is not possible due to the presence of $Na^+$ adducts, we calculated the difference between blank and enzymatic treatment according from the UV spectra of the HPLC peaks identified as pLACT and pLACT-BA by TOF-MS. A pLACT-BA/pLACT ratio of 0.02 at $\lambda$ = 260 nm (absorption maximum of the benzyl group) was found for the blank while a pLACT-BA/pLACT ratio of 0.46 was found for the CaLB catalyzed reaction. The model reaction thus demonstrated that CaLB is a suitable catalyst for the esterification reaction between the protected dimer pLACT with a carboxyl containing molecule (butyric acid). The reaction was also performed using n-heptane as solvent and showed similar results (pLACT-BA/pLACT ratio for the enzymatic reaction of 0.34, pLACT-BA/pLACT ratio for the blank reaction of 0.01).

**CaLB-catalyzed functionalization of PLLA films**

After proven that CaLB is able to catalyze the esterification of PLLA oligomers, in a next step the lipase enzymatic esterification of PLLA films making use of the

carboxylic and hydroxyl groups enzymatically generated on the film surface was investigated. Since enzymatically activated PLLA films were not soluble in butyric acid the reaction was carried out in an organic solvent as previously reported for other polyesters [17, 46]. Since PLLA is known to be soluble in most of the chlorinated and aromatic organic solvents [47], the first step involved identification of an appropriate solvent system not dissolving the PLLA film while being suitable for the enzymatic synthesis. n-heptane was found to fit both requirements: non solubility of the PLLA film and possibility of conducting an esterification reaction using CaLB as catalyst. Incubation of n-heptane with the polymer film revealed no dissolution or degradation of the polymer after up to 72 h of incubation both with and without prior enzymatic activation.

After solvent selection, the next challenge was to develop a sensitive method to detect the binding of the molecule on the polymer surface during optimization of reaction conditions. Commonly techniques like ATR-FTIR, SEM and hydrophobicity measurements were found to be unable detect the coupled molecule. ATR-FTIR is usually a very powerful technique to detect surface functionalization of polymers [17, 46] but IR-light is penetrating into the polymer bulk, making the analysis of the outer layers of the polymer involved in the above described functionalization unfeasible. [36] SEM was used for the study of several polylactides [39, 48] but for our system it turned out to be a destructive technique. Hydrophobicity measurements, really helpful in the study of the hydrolysis of several poly esters, [26, 28] were not suitable to detect the newly bound molecules on the polymer surface because of the limited amount of molecule that was coupled via the enzymatic treatment. Therefore, $^{14}$C-radiolabeled butyric acid was used for esterification together with $^{14}$C-radiochemical analysis of the treated film.

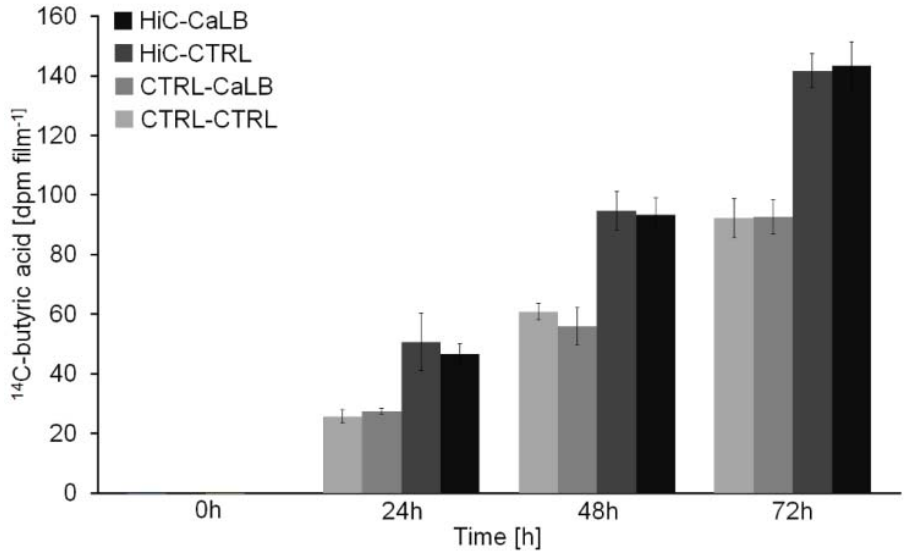


**Figure 3.** *Candida antarctica* lipase B (CaLB) catalyzed functionalization of PLLA films with butyric acid with and without prior enzymatic surface hydrolysis by *Humicola insolens* cutinase (HiC). The experiments were conducted using a starting concentration of $^{14}$C-radiolabeled butyric acid of 58 kdpm mL$^{-1}$ using n-heptane as solvent. For the enzymatic coupling 880 U of aCaLB were used. All reactions were performed in triplicates. Error bars show the standard deviation.

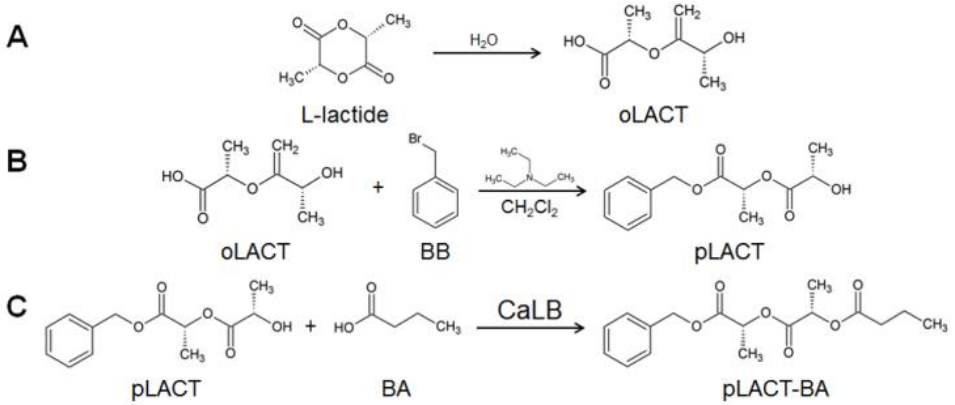


**Figure 4.** Reaction scheme of the pLACT-BA synthesis. (A) Ring opening of L-lactide in order to obtain oLACT. (B) Protection of the -COOH end group of the oLACT using benzyl bromide (BB) in order to obtain the protected dimer with only one functional end (pLACT). (C) Enzymatic esterification of pLACT with butyric acid (BA) using CaLB as biocatalyst (reaction performed both in bulk and in n-heptane as reaction solvent).

Both the pre-hydrolyzed and the non-pretreated PLLA films, showed consistently higher scintillation counts in presence of CaLB compared to the PLLA samples that had not undergone the enzymatic treatment. In all cases some background sorption of the radiolabeled molecule was detected. Sorption studies indicated that the amount of butyric acid in the initial reaction solution determines the base level of sorption of butyric acid on the film and a direct correlation between the amount adsorbed and the initial radiolabeled butyric acid concentration was clearly seen. In the presence of the enzyme, a clear increase on the amount of butyric acid over time on the PLLA was observed (Figure 3).

Interestingly, both the pre-hydrolyzed and the non-pretreated samples show similar levels of functionalization. This may be due to the fact that in an apolar reaction environment such as n-heptane, the newly generated polar surface groups (-OH and -COOH), undergo hydrophobicity recovery. This recovery in air has already been widely described for polymers modified via corona discharges or plasma. [49] Previous studies demonstrated that the hydrophilic surface is unstable and that there is a rearrangement of the polymer chains to a more thermodynamically stable state. Based on the above mentioned findings, we hypothesize a polymer reorientation also for our PLLA/n-heptane reaction system. The cleaved surface chains are turning inside the polymer matrix, with the uncleaved chains (such as those found in the non-hydrolyzed sample) becoming exposed to the enzyme for the coupling reaction. This effect was investigated by conducting hydrophobicity measurements after the partial hydrolytic treatment and incubation of the samples for 24 h in n-heptane. The solvent was removed incubating the samples at 30 °C for 24 h under a continuous air flow. Water contact angle measurements (WCA) revealed indeed a hydrophobicity recovery during the coupling reaction, in which the WCA after enzymatic hydrolysis increased from 33.1 ± 1.7° to 53.3 ± 2.82° after incubation for 24 h in n-heptane. These data clearly confirm that the PLLA films undergo a significant surface hydrophobicity recovery when incubated in apolar solvents explaining the similar relativities of pre-hydrolyzed and non-pretreated samples. It is important to note the effect of the amount of dosed enzyme on the degree of esterification achieved. Using 880 U of aCaLB, the amount of dpm per film detected was 15.5 ± 1.3 counts for the non-pretreated sample and 13.8 ± 1.9 counts for the pre-hydrolyzed sample; while using 88 U of enzyme, the amounts of dpm per film detected were 9.0 ± 0.8 and 7.3 ± 0.5 counts, respectively. Both experiments were conducted using the same concentration of butyric acid (0.2 M) with a $^{14}$C-radiolabeled substrate of 35 kdpm mL$^{-1}$, which lead to a background butyric acid sorption of 6.5 ± 2.4 counts for the non-pretreated sample and 7.5 ± 2.6 for the pre-hydrolyzed sample (Supporting information, Table S2).

The clear influence of the enzyme dosage on the second step in the polymer functionalization, led us to investigate whether the type of CaLB preparation would affect the esterification yield. Therefore we performed experiments comparing lyophilized CaLB (fCaLB) with adsorbed CaLB (aCaLB) and covalently immobilized CaLB (cCaLB). In the first case, the enzymatic treatment (400 U) showed a

consistent effect (22.7 ± 2.1 dpm per film for non-pretreated PLLA, 23.3 ± 1.0 dpm per film for pre-hydrolyzed PLLA) in comparison with the blank (11.0 ± 0.8 dpm per film for non-pretreated PLLA, 13.3 ± 0.5 dpm per film for pre-hydrolyzed PLLA). The functionalization was only seen when using at least 400 U of fCaLB. These experiments were conducted using a starting concentration of $^{14}$C-radiolabeled substrate of 42 kdpm mL$^{-1}$. In the case of cCaLB (860 U, starting concentration of $^{14}$C-radiolabeled substrate of 58 kdpm mL$^{-1}$), a comparable esterification after 24 h as previously described for the adsorbed enzymatic preparation (aCaLB) was observed (Supporting information, Table S3).

Interestingly, there were no significant differences in terms of final butyric acid content regardless what the first step was. Especially remarkable is the fact that the enzymatic generation of hydroxyl groups did not increase the amount of butyric acid bound on the polymer film after incubation with neither immobilized nor free CaLB. However, independently from the first step a significant increase of the amount of butyric acid grafted was detected with any of the used CaLB preparations.

The surface composition of the enzymatically functionalized films was further characterized by XPS [50] using fluorine “labeled” butyric acid. The XPS spectra indicate that both the CaLB treated and untreated samples contain fluorine atoms and consequently fluorobutyric acid on the surface while the starting PLLA film and the same samples after 24 h of hydrolysis only contain carbon and oxygen (H excluded). Nevertheless, despite some adsorption, there was a clear increase in the amount of fluorobutyric acid from 0.19% to 0.40% upon enzymatic esterification. These data are therefore consistent with $^{14}$C-radiochemical analysis and provide an additional means of quantification of the amount of substrate coupled to the surface of the PLLA film.

Overall the $^{14}$C-radiochemical method proved to be very sensitive and allowed the detection of even small amounts of butyric acid enzymatically grafted onto the PLLA surface. This technique is not influenced by enzyme potentially adsorbed and not-completely removed from the film prior to analysis. This has been reported to produce artifacts in XPS analysis for monitoring enzymatic surface hydrolysis of PET [51, 52]. However, in this study we have circumvented this problem by using a substrate “labeled” with fluorine which is neither present neither in the polymer nor in the enzyme preparation [53, 54].

## 5.4 Concluding remarks

An innovative enzymatic method for the surface functionalization of poly(L-lactic acid) films is presented. PLLA films were functionalized with $^{14}$C-radio labeled butyric acid or 4,4,4-trifluorobutyric acid using *Candida antarctica* lipase B as catalyst in n-heptane at a reaction temperature below the glass transition temperature (T < Tg) of the biopolymer. The feasibility of the functionalization was investigated via $^{14}$C-radiochemical analysis while the surface composition was investigated via XPS analysis. No significant difference in the yield was observed while functionalizing pre-hydrolyzed and non-pre-hydrolyzed polymer. This is most probably due to a rearrangement of the outer polymer chains in the hydrophobic reaction environment. It is indeed remarkable how PLLA films maintained their bulk properties despite the enzymatic functionalization of the surface in contrast to other commonly used surface modification methods previously reported so far. The results presented here form the basis for further developments on enzymatic functionalization to tune PLLA properties using more complex substrates like those of biomedical interest (e.g. antimicrobial peptides for smart packaging). Moreover protease-catalyzed peptide synthesis on solid supports in aqueous environments was already reported. [55, 56] Consequently, enzymatic polymer surface functionalization e.g. with antimicrobial peptides could be an interesting approach.

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The authors declare no financial or commercial conflict of interest.

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# 6

# Enzymatic hydrolysis of poly(ethylene furanoate)


## Abstract

The urgency of producing new environmentally-friendly polyesters strongly enhanced the development of bio-based poly(ethylene furanoate) (PEF) as an alternative to plastics like poly(ethylene terephthalate) (PET) for applications that include food packaging, personal and home care containers and thermoforming equipment. In this study, PEF powders of various molecular weights (6, 10 and 40 kDa) were synthetized and their susceptibility to enzymatic hydrolysis was investigated for the first time. According to LC/TOF-MS analysis, cutinase 1 from *Thermobifida cellulosilytica* liberated both 2,5-furandicarboxylic acid and oligomers of up to DP4. The enzyme preferentially hydrolyzed PEF with higher molecular weights but was active on all tested substrates. Mild enzymatic hydrolysis of PEF has a potential both for surface functionalization and monomers recycling.


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## 6.1 Introduction

Enzymatic hydrolysis of aromatic/aliphatic polyesters has first been demonstrated in the 1990ies for poly(ethylene terephthalate) (PET) with several lipases. [1] Ever since, an increasing number of scientific publications and patents focused on enzymatic hydrolysis of the most important polyester PET can be found. [2-6] Enzymes hydrolyzing PET are already used for finishing of PET based textiles or in detergents. There is also a large potential for enzymatic processing of PET based biomedical devices, automotive parts or packaging materials, and additionally for enzymatic recycling [5]. However, since aromatic/aliphatic polyesters are not synthetized in nature, their hydrolysis by enzymes specific to natural polyesters (e.g. cutin) is relatively slow. Therefore, modern tools of genetic engineering have recently been exploited to enhance the efficiency of PET hydrolyzing enzymes. These included adaptation of the active site [7], enzyme surface engineering of the sorption properties to hydrophobic PET [8], attachment of binding modules or hydrophobins [9-11] or enhancing enzyme stability at temperatures close to the glass transition [12, 13].

Despite environmentally friendly processing of polymers, a recent challenge is the replacement of petroleum-based monomers by renewable building blocks [14, 15]. One of the most promising monomers as bio-based substitute for terephthalic acid (TA) is 2,5-furandicarboxylic acid (FDCA), which could be easily produced via fermentative routes [16]. Consequently, in the near future PET is expected to be partially substituted by poly(ethylene furanoate) (PEF) in several applications [17]. Recently, Avantium (Geleen, The Netherlands) has announced a new technology involving a highly efficient separation technology and catalyst which would result in economically feasible production of FDCA starting from 2016. The planned industrial production capacity is estimated to exceed 300,000 t/y in 2018 while the company has established collaborations with major end-users from the food and beverage industry (http://avantium.com/yxy/YXY-technology.html, 2015).

Polyesters containing furan moieties have already been investigated for decades, starting with Moore and Kelly's pioneering research and continuing with more recent studies mostly based on the use of di-functional monomers derived from furfural. However, the synthesis of PEF, the furan homologue of PET, has only been reported more recently [18].

Several research groups have recently investigated PEF synthesis and its mechanical properties [19] as well as the thermal behaviour compared to PET and poly(ethylene naphthalene) (PEN) [20]. Vannini et al. also reported the possibility to substitute ethylene glycol with 1,3-propanediol in order to obtain a material with excellent barrier properties as function of the crystallinity [21]. Other 2,5-furandicarboxylic acid (FDCA) copolymers were investigated by Gubbels et al. and Papageorgiu et al. who produced some fully bio-based polyesters using 2,3-butanediol and 1,4-butanediol respectively as polyols [22, 23]. Recently, the enzymatic synthesis of FDCA-based polyesters was studied by the groups of Loos and Boeriu [24, 25] with *Candida antarctica* lipase B previously reported for the synthesis of aliphatic [26, 27] and aromatic [28] polyesters. However, apart from numerous reports around the emerging topic of PEF production and application, there is no information on enzymatic hydrolysis of PEF. Nevertheless, especially for bio-based PEF, biotechnological processing for functionalization and recycling of monomers could have a large potential to replace harsh chemicals (eg. alkali) as demonstrated for PET [2, 29]. Consequently, in this study, enzymatic hydrolysis of PEF with different molecular weights was investigated.

## 6.2 Materials and Methods

### Enzymes, chemicals and reagents

The recombinant *Thermobifida cellulosilytica* cutinase 1 (Thc_Cut1) was produced and purified as previously described. [2] Dimethyl furan dicarboxylate was purchased from Fluorochem (Hadfield, UK). All other chemicals and solvents were purchased from Sigma-Aldrich (Vienna, Austria) at reagent grade, and used without further purification if not otherwise specified.

### Enzyme activity assay

Esterase activity was measured at 21 ˚C using p-nitrophenyl butyrate (PNPB) as a substrate as previously reported by Ribitsch et al. [29] with some modification. The final assay mixture was made up of 200 µL of solution B and 20 µL of enzyme solution (Solution A: 86 µL of PNPB and 1000 µL of 2-methyl-2-butanol; Solution B: 40 µL of Solution A and 1 mL of 0.1 M Tris-HCl buffer, pH 7). The increase of the absorbance at 405 nm due to the hydrolytic release of p-nitrophenol (ε 405 nm= 9.36 mL (µmol cm)$^{-1}$) was measured over time using a Tecan infinite M200pro plate reader (Maennedorf, Switzerland) using plastic 96-well micro-titer plates (Greiner 96 Flat Bottom Transparent Polystyrene). A blank was included using 20 µL of buffer instead of enzyme solution. The activity was calculated in units (U), where 1 unit is defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute under the given assay conditions.

### Protein quantification

Protein concentration was determined as previously reported [30] using the BioRad protein assay (Bio-Rad Laboratories GmbH, Vienna, Cat.No: 500-0006). Briefly, 10 µL of the protein solution was added into the wells of a 96-well micro-titer plate (Greiner 96 Flat Bottom Transparent Polystyrene). As soon as all the protein solutions were placed into the wells, 200 µL of the prepared BioRad reaction solution were added to the wells (BioRad Reagent diluted 1:5 with mQ water). The plate was incubated for 5 min at 21 °C and 400 rpm. The buffer for protein dilution (0.1 M Tris-HCl pH 7) was used as blank and BSA (bovine serum albumin) as standard. The absorption after 5 min was measured at λ=595 nm and the concentration calculated from the average of triplicate samples and blanks.

**PEF synthesis**

The synthesis of PEF was performed by using a two stage melt-polycondensation method, as previously described by Papageorgiu et al. 2014 [20] with some modifications. A two necked round bottom flask equipped with a distillation bridge was used as reaction vessel. The reaction was performed under oxygen and water free conditions. The apparatus was dried in oil pump vacuum (0.001-0.1 mbar) and heating from the outside with heat gun to 180 °C. After cooling to room temperature the apparatus was flushed with $N_2$. This procedure was repeated three times before the apparatus was charged with the chemicals in a counter flow of $N_2$. Dimethylfuran-2,5-dicarboxylate (DMDC) and 1,2-ethylene glycol (EG) were added in a 1:3 (DMDC:EG) molar ratio. Then 450 ppm (with respect to mass of DMDC) of tetrabutyl titanate were added. The mixture was heated to 160 °C in an oil bath, and stirred with a magnetic stirring bar, while the whole apparatus was kept under nitrogen atmosphere. The temperature was kept for 2 h. The temperature was then increased to 170 °C and kept constant for 2 h. Afterwards, the temperature was increased to 190 °C for another 2 h. During this reaction phase the methanol generated in the transesterification reaction of DMDC and EG was collected in the receiving flask. In the second step of polycondensation, the receiving flask was emptied to avoid exceeding foaming, whereupon the pressure in the apparatus was reduced to 0.05-0.1 mbar in a time span of 30 min. In the meantime, the temperature was increased to 220 °C and kept for 2 h, followed by a last temperature increase to 235 °C which was kept for 4 h. This protocol was meant to remove the excess of EG, which was collected in the receiving flask. Finally, the apparatus was cooled down to room temperature and the product, a white solid, was recovered from the reaction vessel. The solid reaction product was then grounded in a mortar and extensively washed with methanol prior to drying.

In order to obtain PEF with higher molar mass the following adaptations were made: the magnetic stirring was replaced by a sealed precision glass (KPG) stirrer to ensure better mixing of the highly viscous reaction mixture. In addition, the heating protocol in the second polycondensation step was changed as follows: After heating to 220 °C this temperature was kept for 1 h, followed by a temperature increase to 235 °C and keeping this temperature for 2 h. Finally, the temperature was increased to 250 °C and kept for 2.5 h. The PEF samples differed in their appearance: the lower $M_n$ samples (6 and 10 kDa) being white powders and the higher $M_n$ polymer (40 kDa)

(see ESI, Figure S1) being a brownish brittle powder with glassy appearance. The brownish brittle material was dissolved in concentrated trifluoroacetic acid and precipitated therefrom by addition of water, a white viscous but not sticky solid was obtained. When this solid brown material was analyzed by GPC, IR and NMR the analysis results showed no noteworthy deviation from the material before this precipitation treatment. For the subsequent steps of enzymatic hydrolysis only purified PEF was used as substrate.

**NMR**

Nuclear magnetic resonance $^{1}H$ measurements were performed on a Varian Unity Inova NB high resolution spectrometer (resonance frequencies 499.90 MHz for $^{1}H$-NMR). Tetramethylsilane or the residual solvent peak was set as internal standard. Chemical shifts (δ) are reported in parts per million (ppm). Deuterated trifluoroacetic acid ($CF_3COOD$) was used as NMR solvent if not otherwise specified.

PEF Spectral information: $^{1}H$-NMR ($CF_3COOD$): δ 7.41 (s, Ar, 2H), 4.83 (s, -Ar-COO-$CH_2$-$CH_2$-OH, 4H). 13C-NMR ($CF_3COOD$) the signals can be attributed to the corresponding atoms: 64.2 ppm ($CH_2$), 120.5 ppm (CH), 146.6 ppm ($C_q$, furan) and 160.6 ppm ($C_q$, ester).

**FTIR spectroscopy**

Infrared spectra of the PEF powder were collected using a Bruker Tensor 37 FT-IR spectrophotometer (4000-600 $cm^{-1}$) with integrated ATR module. The background signal is air, which was subtracted automatically from each spectrum. The wavenumbers are reported in $cm^{-1}$.

PEF Spectral information: ATR-FTIR (solid, cm-1): 3116, furan v=CH; 1733, ester vC=O; 1575, furan vC=C; 1266, 1227 and 1150, ester vC-O; 1044, furan ring breathing; 987, 859 and 831, furan deformation vibrations.

**GPC**

Gel permeation chromatography (GPC) in hexafluoroisoproanol (HFIP) was performed on a system equipped with a Waters 1515 Isocratic HPLC pump, a Waters 2414 refractive index detector (35 °C), a Waters 2707 auto sampler, and a PSS PFG guard column followed by two PFG-linear-XL (7 µm, 8 × 300 mm) columns in series at 40 °C. HFIP with potassium trifluoroacetate (3 g $L^{-1}$) was used as eluent at a flow rate of 0.8 mL $min^{-1}$. The molecular weights were calculated against poly(methyl methacrylate) standards (Polymer Laboratories, $M_p$= 580 Da up to $M_p$= 7.1×106 Da).

**Differential Scanning Calorimetry (DSC)**

Thermal analysis was conducted on a DSC 822e equipment (Mettler Toledo, Switzerland), which was previously calibrated using indium standards according to the method provided by Mettler Toledo. All measurements were performed in the presence of dry nitrogen purge (20 mL $min^{-1}$), at heating and cooling rates of 10 K $min^{-1}$. Samples, with a weight spanning between 5-10 mg, were heated in aluminum plates. Recorded data were evaluated using STARe SW 9.10 software. The degree of crystallinity (χc) was calculated from the first heating ramp according to the equation below:

$$\chi_c = \frac{\Delta H_m - \Delta H_{cc}}{\Delta H_m^0}$$

where ΔHm is the enthalpy of melting, ΔHcc is the enthalpy of cold crystallization and ΔHm0 is the enthalpy of fusion per gram of a perfect crystal of infinite size. The latter is 187 J $g^{-1}$ for 100 % crystalline PEF. [31]

**PEF hydrolysis**

The hydrolysis of PEF was performed according to a procedure previously described for PET [2]. The PEF powder was milled in order to homogenize the size of the particles. 10.0 mg of PEF powders of various $M_n$ (6, 10 and 40 kDa) were incubated with 1.0 mL of Thc_Cut1 solution (dosage was 5 μM in 0.1 M $K_2HPO_4/KH_2PO_4$ buffer, pH 7) for different time intervals (8, 24, 48 and 72 h). Incubation was conducted at pH 7 on an orbital shaker set at 100 rpm at 50 °C since good stability and activity over time was previously reported for these conditions [9, 10, 32].

**HPLC-DAD analysis**

After enzymatic hydrolysis, proteins were precipitated using 1:1 (v $v^{-1}$) ice-cold methanol. Samples were centrifuged (Hettich MIKRO 200 R, Tuttlingen, Germany) at 14,000 g at 0 °C for 15 min. The supernatant was than collected and filtered through a 0.2 μm PTFE filter into an HPLC vial. The used HPLC was a Hewlett Packard 1050 Series equipped with a Hewlett Packard 1050 Series automated sample injector and a Hewlett Packard 1100 Series photodiode array detector. For the analysis of 2,5-furandicarboxylic acid (FDCA), a reversed phase column RP-C18 (Poroshell 120 EC-C18, 2.7 μm, 3.0x5.0 mm with pre-column) was used. Analyses were carried out using a gradient of 0.1% formic acid, mQ water and methanol as specified in Table T1 (see ESI). The flow rate was set to 0.75 mL $min^{-1}$ and the column was maintained at a temperature of 40 °C. The injection volume was 10 μL. Detection of the analytes

was performed with a photodiode array detector at the wavelength of 254 nm. A post time run of 5 min to equilibrate the column at the initial gradient conditions was also considered.

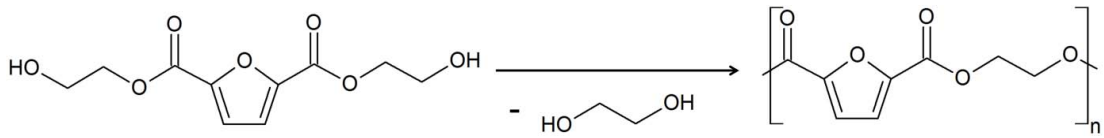
**LC/TOF-MS**

Samples were diluted in methanol to a concentration of 1 mg $mL^{-1}$ and filtered through 0.20 µm PTFE filters. Liquid chromatography/electrospray/time-of-flight mass spectrometry (LC/TOF-MS), in positive ionization was used to separate and identify the release products after enzymatic PEF hydrolysis. The analytes were separated using an HPLC (1260 series, Agilent Technologies, Palo Alto, CA) equipped with a reversed-phase C18 rapid resolution column (Waters Xterra) of 3.0x15 mm and 3.5 µm particle diameter. Mobile phase A was mQ water with 0.1% formic acid, and mobile phase B consisted of LC/MS grade methanol. Analyses were carried out using a gradient of 0.1% formic acid and LC/MS grade methanol as specified in Table T2 (see ESI). The flow rate was set to 0.05 mL $min^{-1}$ and the column was maintained at a temperature of 30 °C. The injection volume was 1.0 µL. Detection of the analytes was performed with a photodiode array detector at the wavelength of 254 nm. A post time run time of 5 min to equilibrate the column at the initial gradient conditions was also considered. This HPLC system was connected to a time-of-flight mass spectrometer (6230 TOF LC/MS, Agilent Technologies) equipped with an electrospray interface under the following operating parameters: capillary 3500 V, nebulizer 20 psig, drying gas 10 L $min^{-1}$, gas temperature 325 °C, fragmentator 200 V, skimmer 65 V, OCT 1 RF Vpp 750 V. The mass axis was calibrated using the mixture provided by the manufacturer over the m/z 50-3000 range. A second orthogonal sprayer with a solution was used as a continuous calibration using the following reference masses: 121.050873 and 922.009798 m/z. Spectra were acquired over the m/z 100-3000 range at a scan rate of 2 spectra $s^{-1}$.

## 6.3. Results and discussion

### Synthesis and characterization of PEF

Two different synthesis protocols were used to obtain polymeric materials with different number average molecular weight ($M_n$). It was assumed that changing the maximum reaction temperature from 235 °C to 250 °C would promote the volatilization of the excessive EG and simultaneously accelerate the transesterification reaction (Scheme 1). Both factors together should shift the reaction equilibrium to the side of the polymer product and therefore yield a material with higher average molar mass. This assumption was confirmed by GPC indicating $M_n$ values of 5,739 g $mol^{-1}$ for the material with 235 °C maximum reaction temperature and 42,559 g $mol^{-1}$ for the polymer from the maximum reaction temperature at 250 °C.




**Scheme 1.** Synthesis of PEF with higher molecular weight (40 kDa) based on volatilization of EG.

The $^1$H-NMR spectra (Figure 1) show the 2 H atoms attached to the furan at 7.41 ppm and the 4 H atoms of the ethylene bridge at 4.83 ppm with an approximated integration ratio of 1:2 (2.00:4.07). Also in the 13C spectrum (see ESI, Figure S2) the signals can be attributed to the corresponding atoms: 64.2 ppm ($CH_2$), 120.5 ppm (CH), 146.6 ppm ($C_q$, furan) and 160.6 ppm ($C_q$, ester). The IR spectra (see ESI, Figure S3) also show the prominent functional groups of the polymer: 3116, v=CH (furan); 1733, vC=O (ester); 1575, vC=C (furan); 1266, vC-O (ester); 1044, ring breathing; 987, 859 and 831, out of plane vibrations of C-H.

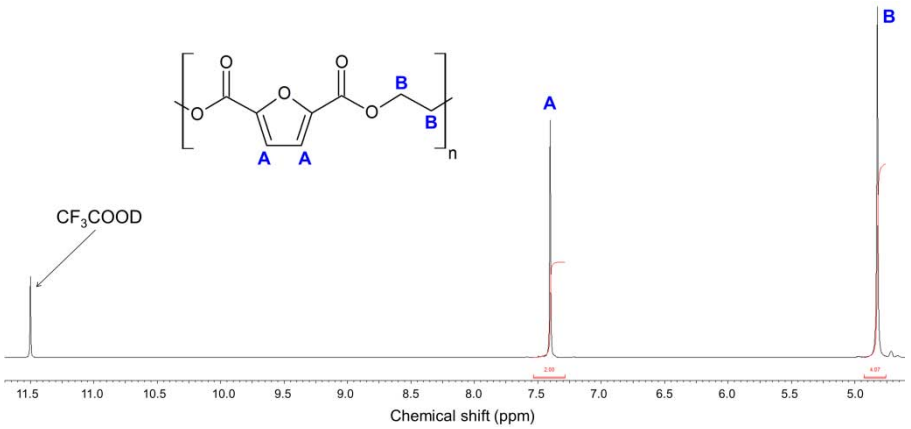



**Figure 1.** $^1$H-NMR spectra of PEF. ($CF_3COOD$): δ 7.41 (s, Ar, 2H), 4.83 (s, -Ar-COO-C$H_2$-$CH_2$-OH, 4H).

The performed $^{1}H$, $^{13}C$-NMR and FTIR spectroscopic measurements were in agreement with previous reports [18, 20] and confirmed the expected polymer structure. Additional DSC analysis showed that all obtained materials, both of low or high $M_n$, have a degree of crystallinity <1% (see ESI, Figure S4).

**Enzymatic hydrolysis of PEF**

In the next step, the enzymatic hydrolysis of the PEF with various molecular weights ($M_n$ of 6, 10 and 40 kDa) was carried out. *Thermobifida cellulosilytica* cutinase 1 (Thc_Cut1) was selected since it was previously reported to hydrolyze a variety of PET materials [2, 11].

After 8 h of incubation with Thc_Cut1, 2.0, 2.8 and 3.1 mM FDCA were released from the 6, 10 and 40 kDa PEF powders, respectively. An almost linear increase in the amount of FDCA was observed until 72 h of hydrolysis yielding 5.7, 10.2 and 13.0 mM for the three samples, respectively. Interestingly, the steepest increase was seen for the 40 kDa $M_n$ PEF samples while lower FDCA concentrations were detected for the 6 and 10 kDa powders compared to controls without enzyme.

Similar results were reported previously for hydrolysis of PET with the identical enzyme at the identical concentration of biocatalyst and conditions [2, 11]. Interestingly, the higher the molecular weight of the PEF samples the faster the hydrolysis was. While there is no information on the influence of the molecular weight on enzyme hydrolysis of other aromatic polyesters in the literature, it is well known that the crystallinity has a major influence. [33] The PEF samples synthetized in this study had a low and similar crystallinity of <1% which cannot explain the distinct hydrolysis rates. It has previously been reported that sorption of enzymes has a great impact on the hydrolysis rate of PET films [10, 11]. Probably, slow desorption from each polymer chains could explain faster hydrolysis of the larger $M_n$ samples. Based on the distribution of the detected release products, where FDCA is the major release product, Thc_Cut1 can be expected to mainly hydrolyze the PEF progressively in an exo-acting manner. Limited enzymatic hydrolysis of PEF has a potential for surface functionalization while maintaining its bulk properties, similar to what was previously described for PLA [32] and PET [33]. Applications range from packaging, medical to textile where e.g. enzymatic hydrophilization can replace drastic chemical treatments. The present results show that enzymatic PEF hydrolysis is not limited to low molecular weight materials. Obviously an optimization of the reaction conditions will

be required to adapt this process to currently developing materials based on renewable PEF.

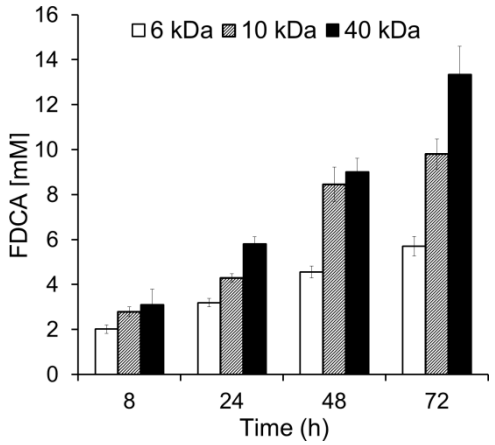
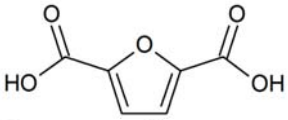
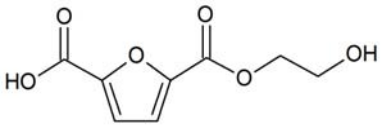
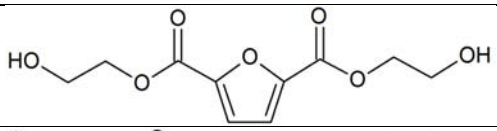
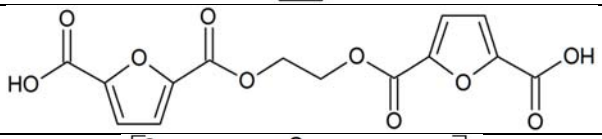
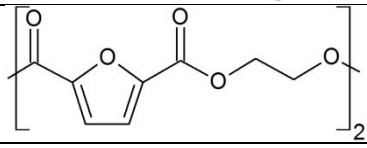



**Figure 2**. Enzymatic hydrolysis of PEF powders with different molecular weights (White: 6 kDa; Striped: 10 kDa and Black: 40 kDa) using cutinase 1 from *Thermobifida cellulosilytica* (Thc_Cut1). Detected released product: 2,5-furandicarboxylic acid (FDCA). No release products were detected in control reactions in the absence of enzyme.

## LC/TOF-MS release products analysis

LC/TOF-MS analysis was carried out in order to identify released product after enzymatic hydrolysis of PEF larger than FDCA. Indeed, after 72 h of incubation several oligomers were released from the 6 kDa PEF powder (Table 1). The most abundant product was FDCA as already seen in HPLC-DAD analysis but in addition several oligomers were detected. A similar pattern of oligomeric release products with FDCA as main product was also found for the higher $M_n$ PEF powders (data not shown). All the found oligomer masses (see ESI, Figure S5) had a Δppm<4 and scores >96%, certifying the high accuracy of the developed analytic technique. No release products were detected in the control reactions of the three substrates object of this study. Longer oligomers might be released but not detected due to their limited solubility in the reaction media. These results are consistent with previously reported data for MALDI/TOF analysis of release products from enzymatic hydrolysis of PET where oligomers with DP4 were the main reaction product [7, 34].

**Table 1.** LC/TOF-MS analysis with DAD peak assignment of the released products after PEF enzymatic hydrolysis.

| RT (min) | Compound | Structure | DAD Signal Area | Found mass ($m\,z^{-1}$) | Diff (ppm) | Score (%) |
|---|---|---|---|---|---|---|
| 6.527 | C6H4O5 |  | 1581528 | 157.0137 $(M+H)^+$ | 3.53 | 98.22 |
| 6.527 | C8H8O6 |  | 991240 | 223.0219 $(M+Na)^+$ | 3.07 | 98.34 |
| 7.777 | C10H12O7 |  | 61753 | 245.0659 $(M+H)^+$ | 1.70 | 98.37 |
| 8.343 | C14H10O10 |  | 15560 | 361.0167 $(M+Na)^+$ | 0.87 | 99.59 |
| 8.876 | C16H14O11 |  | 5477 | 383.0616 $(M+H)^+$ | 1.57 | 96.57 |

## 6.4 Conclusions

The present work is, at best of our knowledge, the first study on enzymatic hydrolysis of PEF synthesized with different molecular weights. Cutinase 1 from *Thermobifida cellulosilytica* was selected as biocatalyst due to its proven ability to hydrolyze several PET substrates [2, 8, 11]. Cutinase 1 demonstrated to be highly active onto hydrolysis of PEF powders of different $M_n$ (6, 10 and 40 kDa). Specifically, the biocatalyst showed a high hydrolytic activity on PEF for 72 h at 50 °C and demonstrated to be most active on the 40 kDa PEF powder, leading to the release of 13 mmol $L^{-1}$ of FDCA. The enzyme was also proven to release FDCA containing oligomers during hydrolysis of the three PEF powders but to a lower extend.

Future studies should focus on enzymatic surface functionalization of PEF films increasing the wettability without affecting its bulk properties, in a similar way like recently reported by our group for PLA and PET films [32, 35] in order to open perspectives for higher value applications (e.g. biomedical) of PEF polymers.

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**Notes**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The authors declare no competing financial interest.

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# 7

## General Conclusion

This thesis was part of the FP7 REFINE project which aimed the development of new skills and expertise in sustainable green materials manufacturing, technologies and applications for the development of a greener and more sustainable society. On this regard is well known that biotechnological conversion of renewable carbon into chemical building blocks for polymers and other chemicals is paving the way for the sustainable innovation of chemistry. Industrial projections indicate that, by 2025, sustainable biotechnologies will contribute to the production of a quarter of chemicals and polymers, and their success will depend not only on technological factors. Availability of renewable feedstock not competing with food is one of the issues, together with the capacity of new products to penetrate the market. Bioconversion of $CO_2$ by chemo-enzymatic routes or through microbial electrocatalysis and algal technologies and other non-conventional sustainable sources of biomass (e.g., insects) may also contribute to relieve the pressure on soil exploitation but such fields still require further exploitation in order to be industrially applicable.

In the meantime, research must be directed not only towards the replacement of existing petrol-based polymers, but also for conferring higher value and competitive functions to bio-based polymers. Biocatalysis already contributes not only to the selective modification and degradation of bio-based polymers, enabling the closure of the carbon circle, but also to the in vitro synthesis of advanced polymers, which are not accessible by chemical routes. Despite major advances achieved using modern genetic tools, efforts are still needed to not only enlarge the portfolio of biocatalysts

endowed with necessary selectivities, but also for the optimization of biocatalyzed processes moving away from conventional chemical-engineering paradigms. These integrated efforts are necessary for the success of the new generation of bio-based polymers, which must compete with well-established products, optimized through decades of chemistry research and innovation practices.

Focusing on biocatalysis as powerful tool for polyesters synthesis and functionalization, the first step of this thesis work was the development of the polycondensation of biobased monomers (namely dimethyl adipate and 1,4-butanediol) using *Thermobifida cellulosilytica* cutinase 1 (Thc_cut1) as biocatalyst. This enzyme was previously reported for the hydrolysis of poly(ethylene terephthalate) (PET), but its synthetic activity was never exploited. The results of thesis show how, in the used reaction conditions, Thc_cut1 seems to be a competitive biocatalyst when compared to the widely used *Candida antarctica* lipase B (CaLB) and with the cutinase from *Humicola insolens* (HiC). However, further studies, both experimental and computational, are needed in order to explore the full potential of this enzyme in polymer synthesis.

After exploring Thc_cut1 as novel synthetic biocatalyst, we investigated the challenging task of synthetizing aromatic-aliphatic polyesters in order to explore the potential of enzymes for the production of industrially-relevant oligoesters. In this part of the work CaLB was widely investigated varying reaction conditions and substrates, such as monomers and solvent, in order to find out the best conditions for this biocatalyzed synthesis. The polycondensation of dimethyl isophthalate with 1,10-decanediol led to the best results in terms of $M_w$ (≈1500 Da) and conversion (87% of converted monomers) after 96 h of reaction.

In a second instance, in order to expand the biocatalysis applications for polyesters functionalization and recycling purposes, we focused our attention on the processing of chemically-synthetized bio-based polymers.

Poly(lactic acid) (PLA) was selected because reported to be a biocompatible polymer with good processability properties. PLA is widely used for packaging and biomedical applications exploiting the fact that it can be easily absorbed by the human body without causing side effects. The enzymatic surface hydrolysis of PLA by *Humicola insolens* cutinase (HiC) was also studied. This part of the thesis aimed at the development of a controlled surface functionalization of this polymer for the

subsequent coupling of a moiety of interest. This preliminary work reports the CaLB-catalyzed coupling of the model molecule butyric acid but further studies are ongoing and aim the development of a superhydrophobic polymer surface and of a PLA-based controlled-release system. Nevertheless, this is the first study where an enzymatic coupling of a carboxylic moiety on a polymeric surface was achieved.

The last part of this thesis describes the enzymatic hydrolysis of poly(ethylene furanoate) (PEF), a currently investigated polyester as alternative to PET for applications that include food packaging, personal and home care containers and thermoforming equipment. PEF is expected to be commercialized in 2018 with a production range of about 300 ktons/year. At the moment the production is on a pilot-plant scale and the process technology is owned by Avantium (Geleen, The Netherlands), who developed the process together with its partners. This polyester, at the actual status of the art, seems to be really promising for food packaging applications since its barrier properties are comparable or even better than PET. The research we conducted on PEF involved the synthesis of the material with various molecular weight and its hydrolysis using Thc_cut1, in order to investigate the potential for functionalization or recycling purposes.

Biotechnological production of renewable monomers is already contributing to the replacement of petrol-based plastics with novel bio-based polymeric products such as the newly developed PEF. Improvement of the fermentation efficiency processes remains a major challenge for enlarging the array of available monomers at industrial scale, thus following the successful example of PLA.

In the future, polymer chemistry is expected not only to introduce bio-based monomers within existing productive processes but also to create new options for the plastic market starting from the most promising building blocks. On that respect, the possibility to tune polymer functional properties and bio-degradability by means of optimized biocatalysts will guide the development of new functional polymers.

# 8

# Appendix

## 8.1 Scientific publications

1. **Alessandro Pellis**, Livia Corici, Loris Sinigoi, Nicola D'Amelio, Diana Fattor, Valerio Ferrario, Cynthia Ebert, Lucia Gardossi - Towards feasible and scalable solvent-free enzymatic polycondensations: integrating robust biocatalysts with thin film reactions. *Green Chem*., **2015**, 17, 1756-1766.

2. Livia Corici, **Alessandro Pellis**, Valerio Ferrario, Cynthia Ebert, Sara Cantone, Lucia Gardossi - Understanding Potentials and Restrictions of Solvent-Free Enzymatic Polycondensation of Itaconic Acid: An Experimental and Computational Analysis. Adv. Synth. Catal., 2015, 357, 1763-1774.

3. **Alessandro Pellis**, Enrique Herrero Acero, Hansjoerg Weber, Michael Obersriebnig, Rolf Breinbauer, Ewald Srebotnik, Georg M. Guebitz - Biocatalyzed approach for the surface functionalization of poly(L-lactic acid) films using hydrolytic enzymes. *Biotechnol. J*., **2015**, 10, 1739-1749

4. **Alessandro Pellis**, Valerio Ferrario, Barbara Zartl, Martin Brandauer, Caroline Gamerith, Enrique Herrero Acero, Cynthia Ebert, Lucia Gardossi, Georg M. Guebitz - Enlarging the tools for efficient enzymatic polycondensation: structural and catalytic features of cutinase 1 from *Thermobifida cellulosilytica*. *Catal. Sci & Technol*., **2016**, DOI: 10.1039/C5CY01746G

5. **Alessandro Pellis**, Alice Guarneri, Martin Brandauer, Enrique Herrero Acero, Henricus Peerlings, Lucia Gardossi, Georg M. Guebitz - Exploring mild enzymatic sustainable routes for the synthesis of bio-degradable aromatic-aliphatic oligoesters. *Biotechnol. J*., **2016**, DOI: 10.1002/biot.201500544

6. **Alessandro Pellis**, Enrique Herrero Acero, Valerio Ferrario, Doris Ribitsch, Georg M. Guebitz, Lucia Gardossi - The closure of the cycle: enzymatic synthesis and functionalization of bio-based polyesters. *Trends Biotechnol*., **2016**, 34, 316-328.

7. **Alessandro Pellis**, Karolina Haernvall, Christian M. Pichler, Gagik Ghazaryan, Rolf Breinbauer, Georg M. Guebitz - Enzymatic hydrolysis of poly(ethylene furanoate). *J. Biotechnol*., **2016**, DOI: 10.1016/j.jbiotec.2016.02.006

8. **Alessandro Pellis**, Enrique Herrero Acero, Lucia Gardossi, Valerio Ferrario, Georg M. Guebitz - Renewable building blocks for sustainable polyesters: new biotechnological routes for greener plastics. *Polym. Int.*, **2016**, DOI: 10.1002/pi.5087

9. Caroline Gamerith, Enrique Herrero Acero, **Alessandro Pellis**, Andreas Ortner, Robert Vielnascher, Daniel Luschnig, Barbara Zartl, Karolina Haernvall, Sabine Zitzenbacher, Gernot Strohmeier, Oskar Hoff, Georg Steinkellner, K. Gruber, Doris Ribitsch, Georg M. Guebitz - Improving enzymatic polyurethane hydrolysis by tuning enzyme sorption. Polym. Degrad. Stabil., 2016, DOI:10.1016/j.polymdegradstab.2016.02.025

## 8.2 Oral presentations

1. **Alessandro Pellis**, Enrique Herrero Acero, Rolf Breinbauer, Ewald Srebotnik, Georg M. Guebitz - Two-step enzymatic functionalization of poly(L-lactic acid) films. *3rd Multistep Enzyme Catalyzed Processes Congress (MECP14)*, 07-10 April **2014**, Madrid, Spain.

2. **Alessandro Pellis**, Enrique Herrero Acero, Rolf Breinbauer, Ewald Srebotnik and Georg M. Guebitz - Lipase-catalyzed functionalization of poly(L-lactic acid) films. *7th International Congress on Biocatalysis (Biocat2014)*, 30 August-04 September **2014**, Hamburg, Germany.

3 **Alessandro Pellis**, Livia Corici, Valerio Ferrario, Enrique Herrero Acero, Cynthia Ebert, Georg M. Guebitz, Lucia Gardossi - Enzymatic Green Routes for the Synthesis and Functionalization of Polyesters. *Green Processing Technologies (EUBIS) Cost Action*, 08-09 September **2015**, Tallinn, Estonia.

4. **Alessandro Pellis**, Livia Corici, Valerio Ferrario, Enrique Herrero Acero, Cynthia Ebert, Georg M. Guebitz, Lucia Gardossi - A green route to biobased polyesters: Solvent-free thin film reactions for enzymatic polycondensations, *The international chemical congress of Pacific Basin Societies 2015 (Pacifichem 2015)*, 15-20 December **2015**, Honolulu, Hawaii, USA.

5. **Alessandro Pellis**, Enrique Herrero Acero, Hansjoerg Weber, Michael Obersriebnig, Rolf Breinbauer, Ewald Srebotnik, Georg M Guebitz - Two-step surface functionalization of poly(L-lactic acid) films with enzymes, *The international chemical congress of Pacific Basin Societies 2015 (Pacifichem 2015)*, Honolulu, Hawaii, USA, 15-20 December **2015**, Honolulu, Hawaii, USA.

## 8.3 Secondments

1. **2014**-**2015**, globally 3 months, Laboratory of Applied and Computational Biocatalysis, University of Trieste, Italy. Supervisor Prof. Lucia Gardossi.

2. 03-30 November **2014**, Biocatalysis Group, Royal Institute of Technology (KTH), Stockholm, Sweden. Supervisor Prof. Mats Martinelle.

3. 11 April-20 May **2016**, Green Chemistry Centre of Excellence, University of York, York, UK. Supervisor Dr. Thomas J. Farmer.

## 8.4 Fellowships and awards

1. **2013-2015**, Marie Curie Fellowship, Seventh Framework Programme (FP7) for Research and Technological Development of the European Commission.

2. **2016**, Founding for a Short Term Scientific Mission (STSM) from the Green Processing Technologies (EUBIS) Cost Action, EU Framework Programme Horizon 2020. Destination: Green Chemistry Centre of Excellence, University of York, York, UK.

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Valerio for teaching me the Power of Modelling and for his scientific and human support.

Prof. Rolf Breinbauer for the great scientific discussions and for his help with the chemical synthesis. Prof. Ewald Srebotnik for hosting me in his lab and the support in developing the $^{14}C$ technique.

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The Enzyme Queen Barbara for her excellent work on enzyme production & purification.

Thanks to Caro, Orti and CK for sharing with me the office and the consequent delirium deriving from that.

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At the end I wish to thank my family and all my friends who supported and helped me through the years.

## 8.6 Statutory declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used source.

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