Exploring Biocatalyzed Lactone Building Blocks Toward Biobased Polyesters

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EXPLORING BIOCATALYZED LACTONE BUILDING BLOCKS TOWARD BIOBASED POLYESTERS

DISSERTATION

to obtain the degree of Doctor at Maastricht University, on the authority
of the Rector Magnificus, Prof.dr. Rianne M. Letschert in accordance
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EXPLORING BIOCATALYZED LACTONE BUILDING BLOCKS TOWARD BIOBASED POLYESTERS

Marie Agnès Françoise Delgove

Exploring biocatalyzed lactone building blocks toward biobased polyesters
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Glossary

$[M]_{eq}$	Monomer concentration at equilibrium (M)	DPP	Diphenyl phosphate
$[M]_0$	Initial monomer concentration (M)	DSC	Differential scanning calorimetry
1,3-DL	1,3-Dioxolane	DSP	Downstream processing
1,4-D	1,4-Dioxane	DXO	1,4-Dioxan-2-one
^{13}C NMR	Carbon NMR	DXP	1,5-Dioxepan-2-one
1H NMR	Proton NMR	EtOH	Ethanol
AcCHMO	Cyclohexanone mono-oxygenase from <i>Acinetobacter calcoaceticus</i> NCIMB 9871	f_M	Molar ratio of monomer M in the monomer feed (-)
ArCHMO	Cyclohexanone mono-oxygenase from <i>Arthrobacter</i> sp. BP2	F_M	Molar ratio of monomer M in the polymer composition (-)
ACN	Acetonitrile	FU	Functional unit
ADH	Alcohol dehydrogenase	GC-FID	Gas chromatography with flame ion detection
BnOH	Benzyl alcohol	GC-MS	Gas chromatography with mass spectrometry
BNPP	Bis(4-nitrophenyl)-phosphate	GDH	Glucose dehydrogenase
BVMO	Baeyer–Villiger mono-oxygenase	GPC	Gel permeation chromatography
CHMO-Phi1	Cyclohexanone mono-oxygenase from <i>Rhodococcus</i> sp. Phi1	GWP	Global warming potential (kg CO ₂ equivalents)
CSF	Continuous substrate feeding	IPCC	Intergovernmental panel on climate change
DALY	Disability adjusted life year	ISO	International organization for standardization
DC	Denaturation capacity	k_{obs}	Observed initial reaction rate (s ⁻¹)
DFT	Density functional theory		
D_m	Dispersity index (-) [measured by GPC]		
DP_n	Degree of polymerization		

LCA	Life cycle assessment	PsCPDMO	Cyclopentadecanone monooxygenase from <i>Pseudomonas</i> sp. strain HI-70
Log P	Log of partition coefficient (-)	PTDH	Phosphite dehydrogenase
MALDI-ToF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry	p -TSA	p -toluene sulfonic acid
MANA	Monoaminoethyl- N -aminoethyl	R	Degree of randomness (-) or ideal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$)
m -CPBA	m -Chloroperbenzoic acid	RhCHMO	Cyclohexanone monooxygenase from <i>Rhodococcus</i> sp. HI-31
MeOH	Methanol	r_M	Reactivity ratio of monomer M
$M_{n, \text{GPC}}$	Molecular weight measured by GPC (kg mol^{-1})	ROP	Ring opening polymerization
$M_{n, \text{NMR}}$	Molecular weight calculated by NMR (kg mol^{-1})	RT	Room temperature
$M_{n, \text{th}}$	Theoretical molecular weight (kg mol^{-1})	SF	Sequential feed
NADPH	Nicotinamide adenine dinucleotide phosphate (cofactor)	SFPR	Substrate feeding product removal
NMR	Nuclear magnetic resonance	Sn(Oct) ₂	Tin(II) 2-ethyl-hexanoate
$P_{4-t\text{-Bu}}$	1- <i>tert</i> -butyl-4,4,4-tris-(dimethylamino)-2,2-bis[tris(dimethylamino)-phosphoranylideneamino]-2 λ^5 ,4 λ^5 -catenadi-(phosphazene)	STY	Space-time yield ($\text{g L}^{-1} \text{ h}^{-1}$)
PDF	Potentially disappeared fraction of species	$t_{1/2}$	Half-life (min)
Poly(DXO)	Poly(1,4-dioxan-2-one)	TBD	1,5,7-Triazabicyclo-[4.4.0]-dec-5-ene
Poly(DXP)	Poly(1,5-dioxepan-2-one)	T_c	Ceiling temperature ($^{\circ}\text{C}$)
pK_a	Acid dissociation constant (-)	T_g	Glass transition temperature ($^{\circ}\text{C}$) [determined by DSC]
Poly(δ -UDL)	Poly(δ -undecalactone)	THF	Tetrahydrofuran
Poly(ω -PDL)	Poly(ω -pentadecalactone)	Ti(O- n -Bu) ₄	Titanium(IV) n -butoxide
		T_m	Melting temperature ($^{\circ}\text{C}$) [measured by DSC]

T_m	Melting temperature of the crystal structure of an enzyme (°C)
TmCHMO	Cyclohexanone monooxygenase from <i>Thermocris-pum municipale</i> DSM 44069
TMCH	3,3,5-Trimethylcyclohexanone
TMCL	Mixture of β,δ -trimethyl- ε -caprolactones
TMCL-I	β,δ,δ -Trimethyl- ε -caprolactone
TMCL-II	β,β,δ -Trimethyl- ε -caprolactone
TON	Turnover number (-)
WT	Wild-type (enzyme)
ΔG_p or ΔG_r	Gibbs free energy of polymerization or reaction (J mol ⁻¹)
ΔH_m	Enthalpy of melting (J mol ⁻¹) [measured by DSC]
ΔH_p or ΔH_r	Enthalpy of polymerization or reaction (J mol ⁻¹)
ΔS_p or ΔS_r	Entropy of polymerization or reaction (J K ⁻¹ mol ⁻¹)
δ -UDL	δ -Undecalactone
δ -VL	δ -Valerolactone
ε -CL	ε -Caprolactone
ω -PDL	ω -Pentadecalactone

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Summary

Both polymers and enzymes are omnipresent in our lives. While the first ones are fully integrated in our environment, the second ones are essential to most biological functions taking place in our bodies. Enzymes are not only needed for biological processes, they are also of paramount importance for the chemical industry, where they act as catalysts for the synthesis of molecules.

The goal of this thesis is to expand the use of oxidative enzymes for the synthesis of building blocks, which are used as precursors for polymeric materials. More specifically, the application of Baeyer-Villiger monooxygenases is explored, targeting the preparation of alkyl substituted lactones by oxidation. Ring opening polymerization of these lactones affords aliphatic polyesters. Depending on the structure of the lactones, the properties of the polyesters can be tuned, thus allowing their use in different types of applications.

In **Chapter 2**, an overview of functional lactones for the preparation of aliphatic polyesters by ring opening polymerization is given. Although these lactones are currently predominantly obtained by chemical processes, it is also possible to use enzymes, namely Baeyer-Villiger monooxygenases, to synthesize them. This chapter highlights the role that these oxidative enzymes can play for the synthesis of lactones for polymers and gives the state-of-the-art regarding their application in large scale bioreactions.

Baeyer-Villiger monooxygenases are however not yet mainstream for the synthesis of lactones. One reason is that they typically suffer from poor stability, which is a major drawback for process intensification. In addition, enzymes applied to the synthesis of chemicals at large scale must be pushed to their limits in order to achieve high process metrics, for example high product concentration and high productivity. This is due to their initial function, which is to catalyze biochemical reactions in biological systems.

In order to expand the industrial applicability of Baeyer-Villiger monooxygenases, several of these enzymes were screened for the oxidation of alkyl substituted cyclic ketones in **Chapter 3**. A particularly thermostable enzyme was identified for the oxidation of 3,3,5-trimethylcyclohexanone: cyclohexanone monooxygenase from *Thermocrispum municipale*. This reaction is further investigated by reaction engineering in **Chapter 4**, where improved process metrics are targeted. Substrate feeding strategies are employed to overcome substrate inhibition while ensuring that high product concentrations are achieved. Coimmobilization of this Baeyer-Villiger monooxygenase and the

selected coenzyme is shown to be particularly relevant for the reutilization of these enzymes over several reaction cycles. Lastly, up-scaling of this reaction with soluble enzymes is achieved in the first gram-scale biocatalyzed synthesis of the alkyl substituted lactones, which are obtained as a mixture of regio-isomers.

The ring opening polymerization of these alkyl substituted regio-isomeric lactones is explored in **Chapter 5**. In addition to metal-based catalysts, organocatalysts such as bases and bifunctional catalysts are shown to be efficient for the polymerization of these lactones. A difference of reactivity upon polymerization is observed between the two regio-isomeric lactones. The most substituted lactone is shown to be more reactive for all catalysts evaluated in this chapter. Computational studies confirm that this difference of polymerization behavior is due to their thermodynamic properties, and can be predicted.

The effect of the structure of lactones upon copolymerization is assessed in **Chapter 6**. Copolyesters from alkyl substituted lactones with a more polar lactone are evaluated. The determination of the reactivity ratio indicates a tendency for random copolymers. Different copolymerization strategies were employed to avoid the formation of homopolymers and to tune the microstructure of the copolyesters. In **Chapter 7**, a potential application for copolyesters based on a biobased macrolactone is evaluated as dispersing agents in inks and coatings. The aim is to increase the solubility range by copolymerization with alkyl substituted lactones. An important decrease in crystallinity is achieved. Control over the microstructure of the copolymers is directly correlated to their crystalline properties, and thus solubility range.

The environmental impact of (bio)chemical processes is a growing concern. While most enzymatic reactions are typically perceived as being greener than their chemical counterparts, this is however usually not supported by quantified studies. In **Chapter 8**, this perception is analyzed by comparing the environmental impact of the synthesis of lactones *via* two routes, using either a Baeyer-Villiger monooxygenase or an organic oxidant. This early-stage life cycle assessment highlights the importance of laboratory scale studies for the identification of key process metrics.

Lastly, the main conclusions, outlook and recommendations of this thesis are given in **Chapter 9**. A valorization of the work presented in this thesis is given in **Chapter 10**.

CHAPTER 1

1

Introduction

1.1 Polymers as synthetic macromolecules

Polymers are macromolecules made of many repeating units. They are obtained by the polymerization of building blocks, also called monomers. The structure of a polymer depends on the structure of its monomer(s) and defines its properties. The monomer(s) structure not only influences the structure of the polymer but also the type of polymerization, and the catalysts used. The (micro)structure of a polymer is also determined by the reactivity of the (co)monomers, which in turn dictates its properties as summarized in Figure 1.1.

Figure 1.1. Relationship between monomer structure, polymer (micro)structure and potential applications with T_g glass transition temperature and T_m melting temperature.

Polymers can be found in almost every aspect of our lives. Although the vast majority of synthetic polymers are found in polymeric materials known as plastics or composites (such as packaging, medical devices, plastic parts in aircrafts or trains), polymers can also be applied in inks, coatings, or paints, in which they act as additives. In this thesis, one of the goals is to broaden the applicability of synthetic polyesters, derived from biocatalytically synthesized monomers, to be used in inks and coatings.

In addition to their structure and applications, many terms with the prefix ‘‘bio’’ are used to describe polymers: biopolymer, biobased, synthesized with biocatalysis, biocompatible and biodegradable. These terms are however not interchangeable and describe

different steps in the life of a polymer related to how it is synthesized, its application or its end-of-life. Figure 1.2 shows how these terms can be adapted to a specific class of polymers, in this case polyesters, which are the target of this thesis.

Figure 1.2. Characteristics related to the synthesis, properties or end-of-life of polyesters.

As opposed to synthetic polymers, **biopolymers** are naturally occurring polyesters made by microorganisms growing on organic waste, fatty acids, or cellulosic materials.¹ **Biobased** polymers, on the other hand, can be synthesized using conventional synthesis methods but their building blocks (monomers) are partially or fully biobased, meaning that they are synthesized from renewable resources such as ligno-cellulosic feedstocks,¹ carbohydrates,² terpenes and terpenoids.³

Biocompatible polymers have the possibility to be applied in biomedical applications because they are compatible with biological systems. This is the case of the polyesters poly(ϵ -caprolactone) and poly(1,4-dioxan-2-one).⁴ Polyesters can also be **biodegradable**. Biodegradability consists in the recycling of the polymer by depolymerization followed by the generation of water, CO₂, CH₄, and biomass by hydrolysis.⁵⁻⁶ Microorganisms, either naturally occurring or engineered, typically perform this degradation. The biodegradability of aliphatic polyesters in particular has been explored,⁷⁻⁸ although some limitations remain.⁹

Some polymers are synthesized using catalysts which are made from renewable resources, *i.e.* **biocatalysts** used either as isolated enzymes or in whole-cells.¹⁰ For polyesters, the biocatalyzed step can be at the monomer synthesis step or at the polymerization step. In the first case, diols, diacids and lactones are the main monomers which have been reported,¹¹ while in the second case the polymerization usually involves lipases¹² and takes place by polycondensation or ring opening polymerization.¹⁰ Poly(lactic acid) is an example of polyester whose corresponding monomer, lactic acid, is synthesized by fermentation of carbohydrates involving microorganisms.¹³ In this thesis, the focus is on the biocatalyzed synthesis of cyclic esters (lactones) as precursors for polyesters.

1.2 Enzymes: biological macromolecules with catalytic activity

Enzymes (also referred to as biocatalysts in this thesis) are biological macromolecules made of amino acids characterized by amide (peptide) bonds as repeating unit. The precise three-dimensional architecture of enzymes is achieved by chemical or physical interactions in their structure, such as disulfide bridges or hydrogen bonds, and is determined by the amino acid sequence of the enzyme. The catalytic activity of an enzyme is directly derived from its structure.

Enzymes accelerate chemical reactions by facilitating and stabilizing transition states in their active sites, which lowers the energy of the intermediates. The geometry of the active site is key to the catalytic activity since it determines the type of substrates that is converted but also the selectivity of the reaction. A broad range of reactions can be catalyzed by enzymes which are of interest for the chemist: *(i)* oxido-reduction, *(ii)* group transfer, *(iii)* hydrolysis reaction, *(iv)* addition or removal of groups, and *(v)* isomerization.¹⁴ Biocatalysis, *i.e.* applying enzymes to perform chemical reactions, is used at industrial scale in various sectors such as pharmaceuticals,¹⁵ food, cosmetics, polymers,¹⁶⁻¹⁷ and the synthesis of bulk, fine¹⁸ or specialty chemicals.^{11, 19}

1.3 Aim and scope of this thesis

So far, biocatalysis and polymerization have mainly been combined by applying lipases for the enzymatic polymerization of polyesters. However, there has been little attention to the application of enzymes at the monomer synthesis step for these polymers. Although the power of enzymes has clearly been identified by chemists, their application for the synthesis of chemicals at large scale remains challenging.²⁰⁻²¹ One typical bottleneck is the limited substrate and/or product concentration usually observed. This is a

consequence of the use of water as reaction medium, together with potential inhibition of substrate and/or product.²² Additionally, enzyme stability and potential use of cofactors must be taken into consideration.

The main motivation of the work presented in this thesis is to combine oxidative biocatalysis with the synthesis of monomers. More specifically, Baeyer-Villiger mono-oxygenases are applied to synthesize lactones from cyclic ketones. These lactones are used as building blocks for polyesters, for which a potential application in inks and coatings is explored. This thesis has several objectives, which are shown schematically in Figure 1.3:

Figure 1.3. Schematic overview of this thesis (DXP: 1,5-dioxepan-2-one, TMCL: β,δ -trimethyl- ϵ -caprolactones, ω -PDL: ω -pentadecalactone, ROP: ring opening polymerization, LCA: life cycle assessment).

The first objective is to apply Baeyer-Villiger monooxygenases to the synthesis of alkyl substituted lactones, and in particular β,δ -trimethyl- ϵ -caprolactones (TMCL) (**Chapters 3 and 4**). The second objective is to prepare polyesters from these lactones by first investigating homopolymerization (**Chapter 5**), then by evaluating copolymerization and differences of reactivity (**Chapter 6**), and lastly by exploring potential applications (**Chapter 7**). For this, copolymerization with two other categories of lactones, namely macrolactones and polar lactones, is investigated. Finally, the last objective is to compare the environmental impact of the biocatalytic synthesis of lactones with the corresponding chemical oxidation reaction (**Chapter 8**).

1.4 Outline of this thesis

In this thesis, the following questions are addressed:

- **Which Baeyer-Villiger monooxygenases are the most suitable for the oxidation of cyclic ketones when having the preparation of polyesters thereof in mind?**

An overview of the current literature on aliphatic polyesters, synthesis of the corresponding lactones, and Baeyer-Villiger monooxygenases is given in **Chapter 2**. In particular, the current state-of-the-art regarding (upscaled) application of Baeyer-Villiger monooxygenases for the synthesis of lactones is detailed.

- **How can reaction engineering be applied to the synthesis of alkyl substituted lactones using a Baeyer-Villiger monooxygenase? Which biocatalyst formats can be considered?**

In **Chapter 3**, several self-sufficient Baeyer-Villiger monooxygenases are screened for the synthesis of alkyl substituted lactones. A thermostable enzyme (cyclohexanone monooxygenase from *Thermocrispum municipale* TmCHMO) is selected and applied to the synthesis of TMCL. In **Chapter 4**, the reaction is further improved by applying a different cofactor recycling system. Higher product concentration and productivities are targeted by evaluating substrate and enzyme loading, possible oxygen mass transfer limitations, as well as substrate feeding strategies. Lastly, an immobilized version of the enzymes is applied and re-used over several reaction cycles to evaluate its potential.

- **Which catalysts are suitable for the polymerization of the alkyl substituted lactones TMCL? How does the position of alkyl substituents influence the polymerizability of the corresponding lactones? Can it be predicted?**

Chapter 5 investigates the ring opening polymerization of TMCL with different organocatalysts. The effect of the position of the alkyl substituents on the polymeriza-

tion behavior is considered as well as the temperature of the reaction and the initial monomer concentration. The thermodynamic parameters of the polymerization are determined. Calculations using the density functional theory (DFT) show that the difference of reactivity between the two regio-isomeric lactones can be predicted.

- **What are the kinetic requirements for the synthesis of copolymers from lactones? How does the reactivity of lactones influence the microstructure of copolymers?**

In **Chapter 6**, the copolymerization of alkyl substituted lactones with a polar lactone is investigated. The difference of reactivity is shown to influence the copolymerization behavior, and therefore the microstructure of the copolymer.

- **Are all lactones suitable for the preparation of dispersing agents for inks and coatings?**

In **Chapter 7**, a potential application of alkyl substituted lactones is explored by copolymerization with a biobased macrolactone. The objective is to improve the solubility range of macrolactone-based polyesters by decreasing their crystalline content. The effect of the microstructure of the copolyesters on their properties is investigated.

- **Is the biocatalyzed synthesis of lactones greener than its chemical counterpart, which uses chemical oxidants?**

Chapter 8 examines the perceived environmental benefits of biocatalysis. Baeyer-Villiger monooxygenases are compared to chemical oxidants in a prospective life cycle assessment (LCA). This comparison is based on laboratory scale data and quantifies the environmental impact of both synthetic routes. This chapter demonstrates how early-stage life cycle assessments can be used for the development of greener processes.

- **What is the future of applied oxidative biocatalysis for polymeric materials?**

Finally, the main conclusions of this thesis are given in **Chapter 9** together with recommendations for the use of applied biocatalysis for polymeric materials.

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CHAPTER 2

2

The role of Baeyer-Villiger monooxygenases for the synthesis of functionalized lactones for aliphatic polyesters

In this chapter, the current state-of-the-art regarding functionalized aliphatic polyesters from lactones is reviewed. Additional functionality enables broadening of the application of these polyesters, with three categories of lactones: alkyl substituted lactones, macrolactones and lactones functionalized with a heteroatom including halogens, or metals. Several synthetic routes to functionalize polyesters are described with an emphasis on the use of Baeyer-Villiger oxidation of cyclic ketones. The differences between the chemical oxidation and Baeyer-Villiger monooxygenases are outlined with the example of functionalized lactones synthesized from biobased resources. The two main limitations of these biocatalyzed reactions for upscaled synthesis, *i.e.* the thermostability of the enzyme and low product concentrations that are typically obtained, are addressed. Strategies for increasing the operational stability of Baeyer-Villiger monooxygenases and process intensification are reviewed.

2.1 Functionalized lactones for polymer applications

Poly(ϵ -caprolactone) (poly(ϵ -CL)) is a semi-crystalline aliphatic polyester with a low melting temperature ($T_m = 60\text{ }^{\circ}\text{C}$). This polyester is typically synthesized at industrial scale from the ring opening polymerization (ROP) of ϵ -caprolactone which is obtained by Baeyer-Villiger oxidation of cyclohexanone (Figure 2.1).¹ This polyester is probably the most widespread aliphatic polyester synthesized from a lactone and is considered a specialty polymer. Due to its biocompatibility, poly(ϵ -CL) is mainly applied in biomedical applications, including drug delivery and tissue engineering.² One of the most widely known applications are self-resorbable sutures from poly(ϵ -CL)-based materials, taking advantage of the bioresorbability of this polyester.

Figure 2.1. Comparison of the properties of poly(ϵ -caprolactone), which is synthesized by the ring opening polymerization (ROP) of ϵ -caprolactone after Baeyer-Villiger (BV) oxidation, and poly(γ -methyl- ϵ -caprolactone).³

While the applications of poly(ϵ -CL) are in general limited to biomedical applications, the insertion of functional groups on the structure of the monomer enables broadening the applicability of aliphatic polyesters. For example, the addition of a methyl group on the structure of the lactone decreases the glass transition temperature (T_g) by at least $6\text{ }^{\circ}\text{C}$ and completely suppresses crystallinity yielding an amorphous polyester (Figure 2.1). Thermoplastic elastomers have been prepared from poly(γ -methyl- ϵ -caprolactone), as a direct consequence of its thermal properties which are induced by the additional methyl substituent.³⁻⁵

2.1.a. Added functionality for broader applicability

In fact, additional functionalities on the structure of lactones give rise to polyesters with various properties and broader applications. Such functionalized lactones can be classified in three categories: macrolactones (with a ring size > 8), alkyl substituted

lactones and functionalized lactones bearing substituents with heteroatoms such as halogen atoms or metal atoms. In the last two categories, the focus is on functionalized δ -valerolactones (δ -VL, 6-membered ring lactones) and ϵ -caprolactones (ϵ -CL, 7-membered ring lactones). An overview of these lactones is shown in Schemes 2.1 and 2.2, with the reported thermal properties of the corresponding polyesters (T_g and T_m). This list of lactones is not meant to be extensive but rather to show the variation of structures that is present in the literature.

- **Macrolactones**

Due to their large ring size and regular structure, polyesters from unsubstituted macrolactones such as ω -undecanolactone (12-membered ring lactone), ω -dodecalactone (13), ω -pentadecalactone (16), hexadecalactone (17) and ethylene brassylate (17) are semi-crystalline.⁶⁻⁸ Poly(ω -PDL) in particular has been found to be a suitable biobased replacement for poly(ethylene).⁹ Additional functionalities making advantage of the presence of unsaturation on macrolactones, such as ambrettolide (17), allows the synthesis of polyesters with more complex architectures. One example is the synthesis of polyesters similar to (linear) low-density poly(ethylene).¹⁰

- **Alkyl substituted small lactones**

Compared to poly(δ -VL) and poly(ϵ -CL), polyesters from alkyl substituted lactones are fully amorphous with a low T_g .¹¹ Biobased alkyl substituted lactones are typically sourced from the fermentation of fatty acids (δ -decalactone or ϵ -decalactone),¹²⁻¹³ synthesized from lignin precursors,^{4, 14} or from terpenoid precursors (dihydrocarvide, carvomenthine or menthine).¹⁵⁻¹⁹ An interesting case is β -methyl- δ -VL, which is synthesized by fermentation of glucose.²⁰⁻²¹ This lactone is used for the preparation of recyclable cross-linked elastomers and polyurethanes for which the monomer can be fully recovered after pyrolysis or depolymerization,²²⁻²³ a feature that is particularly interesting for polymers in general.²⁴

Polyesters synthesized from alkyl substituted δ -VL and ϵ -CL are often referred to as elastomers, sometimes thermoset elastomers, due to the low T_g that they exhibit.^{4, 11, 25-27} Biomedical applications include drug delivery,^{3, 28-32} tissue engineering scaffolds,¹⁷ and antibiofouling materials.³³ The thermoplastic behavior of these polyesters is also exploited to make pressure-sensitive adhesives,³⁴⁻³⁵ and in polyurethanes as soft segments.^{18, 36-38} Functional polyesters from these lactones are also used as additives, *e.g.* plasticizers³⁹ or nucleating agents.⁴⁰

MACROLACTONES

ALKYL SUBSTITUTED LACTONES

Scheme 2.1. Overview of functionalized alkyl substituted δ-valerolactones (δ-VL) and ε-caprolactones (ε-CL), and functionalized macrolactones (ring size > 8).

OTHER SUBSTITUTED LACTONES

Scheme 2.2. Overview of functionalized δ -valerolactones (δ -VL) and ϵ -caprolactones (ϵ -CL) containing heteroatoms.

- **Small lactones functionalized with halogen, metal or other heteroatoms**

With the addition of functional groups including heteroatoms such as oxygen, and nitrogen on the structure of lactones, it is possible to tune the polarity of the corresponding polyesters. This is particularly useful for the preparation of micelles for drug delivery.⁶³ Some of these lactones are protected and can be further functionalized or give other functional groups after reaction.⁶⁴⁻⁶⁵ Unlike alkyl substituted lactones, some polyesters from these lactones retain crystallinity.^{50, 52, 58, 62} Another example of semi-crystalline ϵ -CL-derived polyesters are polyesters from ϵ -CL containing an unsaturation on the backbone.⁶⁶

In general, the applications of functionalized polyesters are related to their properties: the low T_g enables applications as thermoplastic elastomers, the sensitivity of the ester bond to hydrolysis gives tunable degradability, and the thermodynamic behavior of the lactones leads to potential recyclability.^{52, 67} Potential biomedical applications are claimed for polyesters synthesized from lactones of all three categories described: alkyl substituted lactones,^{5, 68} macrolactones,^{7, 69-70} and small lactones with other functionalization such as amine,⁷¹⁻⁷² ether,⁵⁹ or heteroatoms on the cycle.⁵⁰ The application scope of aliphatic polyesters is broadened due to the versatility of the functionalization of the corresponding lactone monomers.

2.1.b. From lactones to polyesters: ring opening polymerization

The ring opening polymerization (ROP) of lactones leads to the formation of (co)polyesters (Figure 2.2). Unlike polycondensation, which is another type of polymerization used for the synthesis of polyesters, ROP is a controlled polymerization. This

Figure 2.2. Formation of polyester by the ROP of lactones, with $[M]_0$ initial monomer concentration, $[M]_{eq}$ equilibrium monomer concentration, T_c ceiling temperature, k_i initiation constant rate, k_p propagation constant rate, and k_t termination constant rate.

means that it displays faster initiation rate than propagation rate and a low termination rate. In these conditions, a low dispersity with a predictable molecular weight can therefore be obtained. For the ROP of lactones, it is critical to avoid moisture since water can act both as an initiator and as an end-capping agent, therefore decreasing the targeted molecular weight and increasing the dispersity of the polymer.

The formation of polyesters by the ROP of lactones is governed by two key factors:⁷³

- **Thermodynamics:** the polymerization equilibrium must be shifted toward the formation of the polymer, meaning that the free enthalpy (also called Gibbs energy) of the polymerization should be negative. A low monomer concentration at equilibrium is preferable. This is especially the case for highly strained lactones, for which ROP results in a decrease in entropy meaning that the polymerization is entropically disfavored. Polymerization is only possible when $[M]_0 > [M]_{eq}$, which restricts the polymerization of some lactones. Additionally, lactones are characterized by a ceiling temperature T_c above which the polymerization equilibrium is in favor of the formation of the monomer, meaning that no polymer is obtained. This ceiling temperature is highly dependent on the initial monomer concentration as well as on the structure of the monomer, including its ring size, the number of substituents and their size. The ceiling temperature of some lactones will be determined in **Chapter 5**.
- **Kinetics:** there must be an existing polymerization mechanism, typically catalyzed by a catalyst, taking place in a reasonable amount of time. A distinction is made between anionic and cationic ROP, depending on the charge of the propagating species,⁷⁴ and metal alkoxides which proceed *via* a coordination-insertion mechanism (Figure 2.3). While metal alkoxides have traditionally been used for the ROP of lactones, organocatalysts are an emerging class of catalysts.^{73, 75} These small organic molecules have been answering to the demand of metal-free polyesters in critical applications such as microelectronics, food packaging, or biomedical applications. Moreover, they are typically less sensitive to oxygen and moisture compared to metal alkoxides. Organocatalysts cover a broad range of molecules including:⁷⁶ (i) Brønsted acids such as sulfonic and phosphoric acids, which follow an electrophilic activated monomer mechanism; (ii) Lewis acids; and (iii) Brønsted or Lewis bases such as phosphazenes, guanidines, and amines. In addition, lipases have proven to be efficient biocatalysts for the ROP of lactones, and in particular for macrolactones which lack ring strain and require specific catalysts for polymerization.^{6, 77} Some organocatalysts will be screened for the ROP of alkyl substituted lactones in **Chapter 5**.

Examples of mechanisms of ROP

Examples of microstructures of copolymers

Figure 2.3. Above: simplified mechanisms of the ROP of lactones with activation of initiator/chain-end by strong bases (adapted from reference ⁷⁸), dual activation mechanism by bifunctional organo-catalysts (adapted from reference ⁷⁹), and coordination-insertion mechanism of metal-based catalysts (adapted from reference ⁸⁰); Below: microstructure of copolyesters and synthesis requirements.

The ROP of several lactone comonomers leads to the formation of copolyesters. The architecture of the copolymer, also referred to as microstructure, corresponds to the arrangement of the comonomer units within the structure of the copolymer. Examples of simple architectures are random copolymers, gradient copolymers and block copolymers (Figure 2.3). Two important parameters for the synthesis of block, gradient or random

copolyesters by ROP are the reactivity ratios of the lactone comonomers and the presence of transesterification reactions, which can be induced by the catalyst. The use of organo-catalysts for the synthesis of copolymers will be demonstrated in **Chapters 6** and **7**.

2.2 Synthesis of functionalized lactones

Functionalized lactones are synthesized *via* various synthetic approaches, which can be classified in two main categories (Figure 2.4). In the first case, functionalized lactones are synthesized from cyclic molecules, either lactones or cyclic ketones. In the second case, the ring closure of functionalized starting chemicals affords the lactone. In both cases, the reaction can be chemically or enzymatically catalyzed.

Figure 2.4. Chemo-enzymatic synthetic approaches for functionalized lactones. ADH: alcohol dehydrogenase, * results in δ -functionalized- δ -VL, ** results in functionalized δ -VL.

For example, the lactonization of functionalized diols affords functionalized lactones catalyzed by Palladium on carbon (Pd/C) using oxygen as oxidant,⁸¹ while enzymatic approaches catalyze the same reaction using either laccases⁸²⁻⁸³ or alcohol dehydrogenases.⁸⁴⁻⁸⁸ Functionalized lactones, and in particular δ -functionalized δ -VL, are also synthesized by ring closure starting from $(\alpha,\beta$ -unsaturated)hydroxy esters,⁸⁹⁻⁹³ keto esters

or keto acids,⁹⁴⁻⁹⁵ and oxo-alkanals.⁹⁶ The Baeyer-Villiger oxidation of cyclic ketones remains however the typical synthesis route to synthesize functionalized lactones.

2.2.a. The chemical Baeyer-Villiger oxidation

The majority of functionalized lactones used for the synthesis of functionalized aliphatic polyesters which are shown in Schemes 2.1 and 2.2 are synthesized by Baeyer-Villiger oxidation. This reaction catalyzes the oxidation of (cyclic) ketones into esters or lactones. The first report of this reaction was in 1899 with the oxidation of terpene-based substrates such as menthone, carvomenthone, and camphor.⁹⁷ Peracids, or peroxy acids, are typically used as oxidants in combination with organic solvents, which results in the formation of an equimolar amount of organic acid waste. *Meta*-chloroperbenzoic acid (*m*-CPBA) is for example a versatile organic oxidant used for this oxidation reaction.⁹⁸ Such organic peroxides however pose safety and health issues, as demonstrated in a recent chemical plant accident involving a fire caused by the spontaneous combustion of peroxides.⁹⁹

It is well known that the chemical Baeyer-Villiger oxidation typically yields the most substituted lactone, also referred to as the normal or proximal lactone, depending on the exact position of the substituent (Figure 2.5). Alternatively, a mixture of normal and abnormal (or proximal and distal) lactones consisting of an excess of normal or proximal lactone is obtained. This is influenced by the structure of the substrate, and to some extent, by the reaction conditions.¹⁰⁰

Figure 2.5. Regio-selectivity of the Baeyer-Villiger oxidation (chemical or enzymatically catalyzed) depending on the substitution of the cyclic ketone.

Efforts toward greener chemical Baeyer-Villiger oxidations are being made with recent advances targeting:¹⁰¹

- The replacement of halogenated solvents with ionic liquids,¹⁰²⁻¹⁰⁴ compressed/ supercritical CO₂, or solvent-free reactions.¹⁰⁵⁻¹⁰⁶
- The replacement of dangerous oxidants with silicate supported hydrogen peroxide,¹⁰⁷ silica supported peracids,¹⁰⁵ and modified clays.¹⁰⁶
- The *in-situ* generation of oxidants with (urea)/oxygen peroxide/lipase systems,^{103, 108} perhydrolase,¹⁰⁹ hydrogen peroxide with Sn-beta zeolites.¹¹⁰

These reactions however still suffer from the limited regio- and enantioselectivity that characterizes chemical Baeyer-Villiger oxidations.

2.2.b. Enzymatic Baeyer-Villiger oxidation: a biotechnological alternative for the synthesis of novel lactones

Baeyer-Villiger monooxygenases (BVMO) are enzymes capable of performing the corresponding Baeyer-Villiger oxidation. BVMOs are known for their regio- and enantioselectivity on a broad range of substrates.¹¹¹ This selectivity is the consequence of the unique property of enzymes to position substrates in their active site. It can be a differentiating feature for the synthesis of abnormal or distal lactones, chemical structures which are otherwise not accessible *via* classic chemical Baeyer-Villiger oxidation.

The consequences of choosing either a chemical or enzymatic synthesis route on the structure of the lactones are for example visible with alkyl substituted ϵ -lactones from (–)-carvone and (+)-carvone (Figure 2.6). These lactones have been getting attention because of their biobased origin: (–)-carvone is the main component of mint essential oil, while its regio-isomer (+)-carvone is found in dill oil.¹¹² Both molecules are derived from the hydroxylation and oxidation of (+)- or (–)-limonene, which can be extracted from citrus peel oil.¹¹³

Shape-memory materials,¹¹⁴ polyurethane films,^{18, 36} and cross-linked materials¹⁶⁻¹⁷ are just a few applications of polymers synthesized from functional lactones derived from the oxidation of (–)-carvone. The vast majority of these lactones was prepared by chemical Baeyer-Villiger oxidation of (partially) hydrogenated (–)-carvone, yielding the normal lactones dihydrocarvide (partially hydrogenated) or carvomenthide (fully dehydrogenated).¹⁶⁻¹⁸ Different regio-isomeric lactones could however be obtained *via* biocatalytic pathways, depending on the enzyme used. While the oxidation of (2*R*, 5*R*)-(+)-dihydrocarvone catalyzed by the wild-type of a cyclohexanone monooxygenase from *Rhodococcus* sp. Phi1 (CHMO-Phi1) or from *Arthrobacter* sp. BP2 (ArCHMO) yielded

the proximal lactone, using the wild-type of a CHMO from *Acinetobacter calcoaceticus* (AcCHMO) yielded the normal lactone (+)-dihydrocarvide.¹¹⁵⁻¹¹⁷ By modifying the geometry of the active site by changing (usually replacing) amino acids, it was shown that the regio-selectivity of these BVMOs could be fully switched in the case of the oxidation of (2*R*, 5*R*)-(+)-dihydrocarvone.¹¹⁵⁻¹¹⁷ A different geometry of the active site forces a different positioning and binding of the substrate, which directly influences the side of oxygen insertion during the oxidation reaction.¹¹⁸

Figure 2.6. Chemical (red) and biocatalyzed (green) steps to the synthesis of lactones from (-)- and (+)-carvone derived from renewables. Distal lactones that are only accessible by BVMO are shown in blue. When not indicated, the enzymes are wild-types.

An original approach was taken starting from (+)-limonene contained in orange peels. In this enzymatic cascade reaction, the last step consisted in an oxidation catalyzed by the wild-type of AcCHMO which resulted in the normal lactone dihydrocarvide.¹¹⁹ The oxidation of the other enantiomer by the same enzyme also yielded the normal lactone.¹¹⁶ Contrary to the results obtained with the substrate with opposite enantiopurity ((2*R*, 5*R*)-(+)-dihydrocarvone), mutations of AcCHMO were not successful in changing the regio-selectivity. This was due to the positioning of the substrate in the active site because of the alkyl groups.¹¹⁶ Another enzyme, the wild-type of a cyclohexanone monooxygenase from *Brevibacterium* sp. (CHMO_{Brevi 2}), was however able to oxidize both enantiomers to form the distal lactones.¹²⁰ Interestingly, while the chemical Baeyer-Villiger oxidation was typically performed on racemic mixtures of the substrate (since it is not enantio-selective), some wild-type BVMOs such as ArCHMO and CHMO_{Brevi 2} display opposite regio-selectivity for opposite enantiomers of dihydrocarvone.¹²¹

2.3 Oxidative biotechnology for the synthesis of lactones: toward up-scaling

2.3.a. Baeyer-Villiger monooxygenases in details

Baeyer-Villiger monooxygenases (enzyme commission number EC 1.14.13.x) are flavin-dependent enzymes, which perform oxidation reactions. Although these oxidative enzymes have the advantage of using the cheapest oxidant available, *i.e.* oxygen from air, oxygen must be activated through the formation of a covalent bond with the flavin adenine dinucleotide (FAD) cofactor of the BVMO.¹²² The generated peroxy species are able to oxidize the substrate in a similar fashion as the chemical Baeyer-Villiger oxidation, with one atom from the oxygen-derived peroxy species being inserted in a C-C bond of the ketone substrate. Because of the generation of the oxidized form of the flavin in the protein with NAD(P)H acting as electron donor, the other atom of oxygen is released in the form of water as by-product. If no substrate is present, or if the substrate is not a suitable substrate for this enzyme, the peroxyflavin intermediate will decay and the activated oxygen will result in the formation of hydrogen peroxide (H₂O₂). This side reaction, which is known as uncoupling, is not desirable because of the negative influence of H₂O₂ on the stability of BVMOs.¹²³

For each molecule of product that is formed, one molecule of molecular oxygen is consumed and one molecule of the cofactor NADP⁺ must be regenerated (Figure 2.7). Oxygen supply and cofactor regeneration are therefore two critical parameters when applying BVMOs for the synthesis of lactones. Other critical parameters include the sta-

Figure 2.7. Parameters for reaction engineering of BVMO catalyzed synthesis of lactones from cyclic ketones related to the substrate, product, oxygen, and general parameters.

bility of the enzyme, possible substrate and/or product inhibition, and recovery of the product. These parameters will be described in more details in the next sections.

2.3.b. Biocatalyst format and cofactor regeneration

Several biocatalyst formats are available for biotransformations (Figure 2.8). The use of whole-cells, either growing in fermentation processes or non-growing, does not require any additional cofactor regeneration system since it is performed by the complete enzyme apparatus of the cells.¹²⁴ For this reason, most industrial redox biotransformations apply are performed with whole-cells.¹²⁵ When the enzyme is isolated from the cell, a cofactor regeneration system must be implemented. The main advantage of using isolated enzymes is a better control of side-reactions. However, the regeneration of the cofactor of BVMOs remains a major factor hindering their application as isolated enzymes at larger scale. Many approaches have been developed to regenerate NADPH, which is the cofactor of most BVMOs.¹²⁶⁻¹²⁹ One of the most common ways is the addition of a coenzyme with the opposite redox behavior, leading to different systems:

- Addition of **sacrificial cosubstrates** such as glucose or formate, which are oxidized by a glucose dehydrogenase (GDH) or a formate dehydrogenase, respectively.¹³⁰⁻¹³² Additionally, fusion proteins comprising the BVMO and a thermostable phosphite dehydrogenase (PTDH) were shown to be efficient, with no loss of activity compared to the separate enzymes.¹³³⁻¹³⁴

Figure 2.8. Categories of biocatalyst formats.

- **Multi-enzymatic cascade reactions**, in which the product of the reaction of the coenzyme directly contributes to the overall reaction, either as a substrate for the BVMO (linear cascade from alcohol to lactone)¹³⁵⁻¹⁴⁰ or synthesizing the same product as the BVMO (convergent cascade from alcohol and ketone to lactone).^{88, 141-142} Fusion of the BVMO with the coenzyme creates a linear cascade with a single biocatalyst.¹⁴³ This approach is particularly useful if the intermediate substrate (the ketone) is not stable in the reaction conditions, or if the BVMO suffers from substrate inhibition.
- **Alternatives** for the regeneration of NADPH include light-driven reactions¹⁴⁴ and polymersome nanoreactors,¹⁴⁵ although some limitations exist.¹⁴⁶ A more drastic approach consists in using protein engineering to switch the cofactor preference from NADPH to NADH, the latter cofactor being cheaper and more stable.^{147,130,131} NADH differs from NADPH by one phosphate group located on the ribose ring close to the adenine moiety of the cofactor.

In this thesis, the use of sacrificial cosubstrates will be investigated for the oxidation of an alkyl substituted cyclic ketone, using either a self-sufficient BVMO-PTDH biocatalyst (**Chapter 3**) or a GDH (**Chapter 4**). Two other factors also hinder the application of BVMOs at large scale, namely the stability of the biocatalyst and the requirements for large scale processes (high product concentration), which are not necessarily compatible with the reaction conditions in which enzymes were evolved to react.

2.3.c. Stability of BVMOs: limitations and opportunities

Developing processes using more stable enzymes is a key step to the adoption of biotechnology into industrial processes. Operational stability is one of the biggest challenges for the application of biocatalysts, and especially BVMOs, on reactions at large scale. Operational stability depends on thermostability, oxidative stability, pH-stability and solvent stability. Enzyme stability comprises: (i) **thermodynamic stability**, such as the melting temperature T_m which is defined as the temperature at which half of the enzyme is in its unfolded state, and (ii) **kinetic stability** such as $t_{1/2}$ which is defined as the reaction time at which the residual activity of the enzymes is halved.¹⁴⁸ While thermodynamic stability characterizes a reversible equilibrium, the kinetic stability refers to an irreversible denaturation of the enzyme.

For example, AcCHMO is the most widely studied BVMO in the literature. However, the thermostability of this enzyme is rather poor with complete loss of activity within several minutes at 45 °C ($T_m = 37$ °C),¹⁴⁹ and poor solvent tolerance with less than 10 vol % of organic solvent required to unfold half of the enzyme.¹⁵⁰ This translates in poor operational stability, therefore limiting the performances of AcCHMO-catalyzed reactions. So far, several strategies have been implemented to improve the stability of BVMOs, described in order of decreasing difficulty of implementation.

Protein engineering. The hot spots for protein engineering of BVMOs were recently reviewed, outlying the potential of protein engineering for the improvement of these biocatalysts.¹¹⁸ The introduction of stabilizing disulfide bonds has proven to be a successful approach for the thermal stabilization of AcCHMO with an increase in melting temperature of about 6 °C.¹⁵¹⁻¹⁵² Increasing the oxidative stability is another approach to target improved thermostability since higher temperatures enhance oxidation side-reactions. Hydrogen peroxide, formed as the result of uncoupling which is an undesired pathway of oxygen activation,¹²³ can be damaging to the activity of the enzyme because it oxidizes some sensitive residues. Stabilization of AcCHMO against oxidation was achieved by protein engineering targeting mutations of all sensitive residues.¹⁵³ Protein engineering however presents several drawbacks. Although mutations can increase some properties such as thermostability or oxidative stability, they are sometimes accompanied by a partial loss of activity of the enzyme.

Immobilization. The goal of enzyme immobilization consists of maintaining the activity of enzymes while making them insoluble.¹⁵⁴ For isolated BVMOs, this is typically performed *via* covalent binding, *i.e.* by chemical cross-linking between amino acids residues of the enzymes and chemical groups present on the support.^{136, 155-157} For whole-

cells expressing BVMOs, entrapment in a gel or encapsulation with a polyelectrolyte is usually the preferred technique for immobilization.¹⁵⁸⁻¹⁶⁰ The overall operational stability can be increased by immobilization due to the restriction in mobility induced by fixation. Additional benefits include the ability to re-use the biocatalyst, improved downstream processing with easier separation of the biocatalyst and a potentially decreased cost of the product, depending on the performances of the immobilized biocatalyst and the cost of immobilization.¹⁶¹ Possible diffusion limitations and loss of activity during the immobilization process may decrease the performances of the biocatalyst. The immobilization of a BVMO and the evaluation of its performances is described in **Chapter 4**.

Reaction engineering. This encompasses all parameters which are part of the reaction conditions such as the pH, oxygenation of the reaction medium, the presence of (co)-solvents, or stabilizing additives. Solvent tolerance of BVMOs can be increased by careful choice of the organic solvent (cosolvent or water-immiscible solvent), as well as its amount.^{150, 162} An important parameter to consider with BVMO-catalyzed reactions is their dependence to oxygen. On the one hand, it is often necessary to supply oxygen to the reaction mixture to ensure that the reaction is not oxygen limited. On the other hand, aeration of the reaction mixture introduces an additional interface between the aqueous medium and the gas bubbles, which is known to be detrimental to the stability of the enzyme. The effect of cosolvent on the activity of a BVMO will be evaluated in **Chapter 3** while the denaturation induced by oxygenation of the reaction medium will be discussed in **Chapter 4**. Lastly, additives such as NADPH, flavin adenine dinucleotide (FAD), superoxidase dismutase and catalase, which scavenge oxygen reactive species created by uncoupling, may help in increasing the half-life of BVMOs.¹⁶³

(Thermo)stable BVMO. The best option is maybe the choice of a stable enzyme. Careful selection of the biocatalyst can circumvent the additional steps required for immobilization and protein engineering. Some BVMOs are remarkably thermostable, with high melting temperatures and half-lives (Table 2.1). One example is the cyclohexanone monooxygenase from *Thermocrispum municipale* (TmCHMO). This BVMO is particularly interesting because it can catalyze the oxidation of a broad range of substrates.¹⁴⁹ Screening of several BVMOs targeting the oxidation of small alkyl substituted cyclic ketones will be discussed in **Chapter 3**.

2.3.d. Process intensification: challenges and strategies

Typically, processes at industrial scale are performed with high product concentration and high productivity. These processes require efficient downstream processing (or product recovery), which is not possible with dilute aqueous streams. In most industrial

Table 2.1. Properties of wild-type thermostable Baeyer-Villiger monooxygenases.

Abbreviation	Enzyme	Substrate scope	T_m (°C)	Ref
TmCHMO	Cyclohexanone monooxygenase (<i>Thermocrispum municipale</i>)	Aliphatic, aromatic and cyclic ketones, sulfides	48	149, 164
PockeMO	Polycyclic ketone monooxygenase (<i>Thermothelomyces thermophilea</i>)	Aliphatic, aromatic and cyclic ketones, sulfides, steroids	47	165-166
hFMO5	Human flavin-containing monooxygenase	Aliphatic and aromatic ketones, small substituted cyclic ketones	47	167
Cm-BVMO	Baeyer-Villiger monooxygenase (<i>Cyanidioschyzon merolae</i>)	Aliphatic and aromatic ketones, limited substrate scope for cyclic ketones	56	168
PAMO	Phenylacetone monooxygenase (<i>Thermobifida fusca</i>)	Small aromatic ketones and aromatic sulfides	61	169

biotransformations, high product concentrations above 100 g L⁻¹ are achieved.¹²⁵ Such reactions are rarely operated at product concentration below 10 g L⁻¹. The challenge of applying enzymes to industrial reactions therefore lies in pushing the enzyme's limits toward industrial requirements. Indeed, enzymes have been evolved to perform reactions in metabolic conditions, *i.e.* with low substrate concentration, stable pH and ambient temperature, which are often not compatible with industrial requirements.

As such, process intensification aims at achieving improved process performance metrics such as: (*i*) lower cost of biocatalyst, which is related to the biocatalyst stability and its format/formulation; and (*ii*) increased product concentration to ease product recovery and increase space-time yield (STY), also called productivity, which related to the size of the reactor.¹⁷⁰ This is especially important when the target product is to be produced on a large scale and with a low price.¹⁶¹ An overview of the process intensification strategies applied for upscaled BVMO-catalyzed reactions is shown in Table 2.2, with a focus on reactions targeting the synthesis of lactone as polymeric building blocks. As a comparison, the table also includes *rac*-bicyclo-[3.2.0]-hept-2-ene-6-one, which is the prototype substrate evaluated for BVMOs in the literature.

Table 2.2. Overview of upscaled syntheses of lactones with BVMOs for monomer synthesis with reaction volume of 50 mL or larger.

Product	Substrate	Enzyme (format)	Reaction conditions & performances
ε-CL	cyclohexanone	AcCHMO (growing or non-growing whole-cells, isolated enzymes)	<ul style="list-style-type: none"> • Fed-batch (non-growing cells):¹⁷¹ $V = 3 \text{ L}$, $STY = 0.79 \text{ g L}^{-1} \text{ h}^{-1}$ • Fed-batch fermentation (growing cells):¹⁷² $V = 1 \text{ L}$, $[\text{prod}] = 15.3 \text{ g L}^{-1}$, $STY = 0.9 \text{ g L}^{-1} \text{ h}^{-1}$ • Batch (isolated enzymes):¹⁴¹ $V = 0.05 \text{ L}$, $[\text{sub}] = 10 \text{ g L}^{-1}$ (convergent cascade with diol)
ε-CL	cyclohexanol	AcCHMO WT or mutant (whole-cells or isolated enzyme)	<ul style="list-style-type: none"> • Fed-batch (isolated enzymes):¹³⁸ $V = 0.05 \text{ L}$, $[\text{prod}] = 33 \text{ g L}^{-1}$ (hydroxyacid after hydrolysis) • CSF (whole-cells):¹⁴⁰ $V = 0.2 \text{ L}$, $[\text{sub}] = 20 \text{ g L}^{-1}$ (induced oligomerization with lipase A from <i>Candida antartica</i>)
ω-dodeca-lactone	cyclododecanone	PsCPDMO (growing whole-cells)	<ul style="list-style-type: none"> • Biphasic system in semi-continuous mode fermentation (growing cells):¹⁷³ $V = 3 \text{ L}$, $[\text{prod}] = 10\text{-}16 \text{ g L}^{-1}$
(R)-γ-methyl-ε-CL	cyclohexanone	CoCPMO (growing cells)	<ul style="list-style-type: none"> • SFPR (fermentation):¹⁷⁴ $V = 1 \text{ L}$, $[\text{sub}] = 15 \text{ g L}^{-1}$, 11.7 g of isolated product
δ-methyl-ε-CL	<i>rac</i> -3-methylcyclohexanone	CoCPMO (growing cells)	<ul style="list-style-type: none"> • SFPR (fermentation):¹⁷⁴ $V = 1 \text{ L}$, $[\text{sub}] = 15 \text{ g L}^{-1}$, 14.7 g of isolated product
(-)-menthide	(-)-menthone	PsCPDMO-PTDH (isolated enzymes)	<ul style="list-style-type: none"> • Batch (isolated enzymes):¹¹⁵ $V = 0.5 \text{ L}$, $< 500 \text{ mg}$ of product

Product	Substrate	Enzyme (format)	Reaction conditions & performances
3-(7-oxooxepan-2-yl)propanenitrile	3-(2-oxocyclohexyl)propanenitrile	PsCPDMO (whole cells)	<ul style="list-style-type: none"> • SFPR (whole-cells):¹⁷⁵ V = 3 L, [prod] = 8 g L⁻¹, 33 g of isolated product
(+)-dihydrocarvide	(+)-dihydrocarvone	CHMO Phi1-PTDH mutant (normal lactone) or WT (abnormal lactone)	<ul style="list-style-type: none"> • Batch (isolated enzymes):¹¹⁵ V = 0.5 L, < 300 mg of product
(-)-menthide	(-)-menthone	PSCPDMO-PTDH (isolated enzymes)	<ul style="list-style-type: none"> • Batch (isolated enzymes):¹¹⁵ V = 0.5 L, < 500 mg of product
(-)-cis-verbanone lactone	(-)-cis-verbanone	AcCHMO mutant (isolated enzymes)	<ul style="list-style-type: none"> • Batch (isolated enzymes):⁴⁸ V = 0.2 L, [sub] = 3 g L⁻¹, 24 mg of product
rac-bicyclo[3.2.0]hept-2-ene-6-one	rac-bicyclo[3.2.0]hept-2-ene-6-one	AcCHMO (growing whole-cells)	<ul style="list-style-type: none"> • SFPR (fermentation):¹⁷⁶ V = 200 L, [prod] = 4.5 g L⁻¹, 495 g of isolated product • SFPR (fermentation):¹⁷⁷ V = 50 L, 900 g of isolated product • SFPR (fermentation):¹⁷⁸ V = 1 L, STY = 1 g L⁻¹ h⁻¹, [sub] = 23 g L⁻¹

With CSF continuous substrate feeding; SFPR substrate feeding product removal; space-time yield STY = [product] / reaction time; V volume of the reaction mixture; WT wild-type; AcCHMO cyclohexanone monooxygenase from *Acinetobacter calcoaceticus*; PsCPDMO cyclopentadecanone monooxygenase from *Pseudomonas* sp. HI-70; CoCPMO cyclopentanone monooxygenase from *Comamonas* NCIMB 9872; CHMO-Phi1 cyclohexanone monooxygenase from *Rhodococcus* sp. Phi1; PTDH phosphite dehydrogenase. * CL is hydrolyzed or oligomerized during the reaction.

Non-optimized batch reactions, in which the substrate is added in one time at the beginning of the reaction, typically result in low product concentration with very low productivity, as was shown with the oxidation of (+)-dihydrocarvone and menthone (product concentration below 1 g L^{-1}).¹¹⁵ Because of substrate inhibition, only low amounts of substrate are converted, thereby resulting in low product concentrations. Substrate inhibition is a bottleneck that is often encountered in BVMO-catalyzed oxidations and which can be overcome *via* several types of substrate supply strategies.¹⁷⁹ All the strategies described below aim at keeping the substrate concentration below inhibitory levels.

Aqueous-solvent biphasic systems consist of an aqueous layer in contact with a water-immiscible organic solvent layer. The organic phase is chosen so that the substrate and product have a higher affinity for this phase compared to the aqueous phase. Substrate and product should therefore be preferentially soluble in the organic phase compared to the aqueous phase, the latter containing the enzyme and the cofactor (Figure 2.9a). In the case of biphasic systems, the organic layer acts as a substrate reservoir, slowly diffusing the substrate in the aqueous phase, and as a product sink, solubilizing the product in the organic phase after it has been formed in the aqueous phase. Criteria for the selection of a suitable solvent for the organic phase include its boiling point, and its solubility in water. Its polarity (expressed as $\log P$) can be considered to evaluate its effect of the stability of the enzyme, although some authors argue that solvent functionality is more important.¹⁸⁰

Figure 2.9. Process intensification strategies aiming at increasing product concentration with a) biphasic system with water-immiscible organic solvent (top layer), and b) CSF with possible creation of product layer due to product accumulation.

Immiscible organic solvents can however be detrimental to the activity of the enzyme either because of the presence of an interphase or because some solvent molecules present in the aqueous phase replace essential water molecules on the hydration shell of the enzyme.¹⁸¹ Biphasic systems in hexadecane were applied to the synthesis of the 13-membered ring lactone ω -dodecalactone in semi-continuous fermentation mode, for which relatively high product concentrations were achieved (10-16 g L⁻¹).¹⁷³

A simpler substrate supply strategy to avoid substrate inhibition is **continuous substrate feeding (CSF)**. In this case, the substrate is fed to the reaction mixture while the reaction is taking place so that the substrate concentration remains below inhibitory levels. CSF (or fed-batch mode, in which the substrate is added by portions rather than continuously) is adapted to reactions that do not suffer from product inhibition since product will accumulate in the aqueous phase as the substrate is fed and converted (Figure 2.9b). The advantage of fed-batch or CSF is the ease of implementation since no additional solvent is required. In the cascade reaction from cyclohexanol to ϵ -CL, AcCHMO suffers from both substrate and product inhibition. Substrate inhibition was addressed by CSF of the cyclohexanol substrate while *in-situ* product removal was applied to ϵ -CL, either by hydrolysis or by oligomerization, thereby achieving high product concentrations.^{138, 140} Fed-batch was also applied to the synthesis of ϵ -CL directly from the oxidation of cyclohexanone.¹⁷¹⁻¹⁷²

In-situ product removal (ISPR) consists of the removal of the product while the reaction is taking place, therefore maintaining low substrate and product concentration throughout the reaction.¹⁸² Product removal can be performed by the addition of a resin on which the product will preferentially adsorb, therefore decreasing the concentration of the product in the aqueous phase and avoiding product inhibition. Alternatively, the substrate may be loaded on the resin so that the resin plays a dual role: *in-situ* release of the substrate and removal of the product, also known as **substrate feeding product removal (SFPR)**. The possibilities offered by SFPR were successfully demonstrated for the oxidation of *rac*-bicyclo-[3.2.0]-hept-2-ene-6-one with AcCHMO to synthesize precursors for the pharmaceutical industry. In this case, process intensification consisted in the use of SFPR to overcome substrate inhibition in combination with investigation into the oxygen input and the reactor set-up.^{178, 183} This reaction was successfully scaled up and resulted in the first kilogram synthesis of lactones and the first 200 L-scale reaction catalyzed by a BVMO.¹⁷⁶⁻¹⁷⁷ The SFPR approach was successfully transposed to BVMO-catalyzed synthesis of functionalized lactones such as (R)- γ -methyl- ϵ -caprolactone,¹⁷⁴ δ -methyl- ϵ -caprolactone,¹⁷⁴ and a nitrile functionalized ϵ -CL¹⁷⁵ at the liter-scale.

2.4 References

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CHAPTER 3

3

Exploring the substrate scope of Baeyer-Villiger monooxygenases with self-sufficient biocatalysts

The aim of this chapter is to explore the substrate scope of several Baeyer-Villiger monooxygenases toward alkyl substituted cyclic ketones, targeting lactones for polymer applications. Self-sufficient BVMOs fused with a phosphite dehydrogenase are evaluated in screening biotransformations. The most promising combination, namely the oxidation of 3,3,5-trimethylcyclohexanone using the thermostable cyclohexanone monooxygenase from *Thermocrispum municipale* is explored further under controlled reaction conditions (oxygen input, pH, and temperature). The reaction rate and product formation of the regio-isomeric lactones are improved in order to overcome substrate inhibition and poor product solubility. The effect of cosolvent, the concentration of the cofactor NADP⁺, and the biocatalyst loading are evaluated. A specific regioselective behavior upon the formation of the regio-isomeric lactones is investigated.

Keywords: substrate screening • self-sufficient enzyme • lactones • regioselectivity • reaction engineering

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3.1 Introduction

Baeyer-Villiger monooxygenases (BVMOs) typically display a broad substrate scope which includes cyclic ketones containing a heteroatom,¹⁻² macrocyclic ketones,³⁻⁶ and substituted derivatives of various ring sizes.⁷⁻¹¹ In this chapter, the substrate scope of several BVMOs was screened with alkyl substituted cyclic ketones, and in particular terpene-bases substrates. The goal is to explore BVMO / substrate combinations that can be applied to the biocatalyzed synthesis of lactones for aliphatic polyesters (see Table 2.2 in **Chapter 2** for an overview of existing BVMO-catalyzed reactions at large scale for monomer synthesis). As shown in **Chapter 2**, alkyl substituted lactones are particularly interesting for aliphatic polyester synthesis because of the presence of alkyl substituents, which typically give low glass transition temperatures (see Scheme 2.1 for an overview).

While some BVMOs are active toward some alkyl substituted cyclic ketones,¹² no comparative study with such substrates has been performed. The successful substrate/biocatalyst combinations which have been reported were screened with various reaction conditions (pH, temperature, substrate and enzyme concentration, cosolvent, enzyme format – whole cells *vs.* purified biocatalyst). However, it is well known that these conditions can influence the kinetics of the bioconversions. Additionally, it has been demonstrated that enantio- and regioselectivity of BVMOs can be influenced by the presence of cosolvent,¹³ as well as by the substrate concentration.¹⁴

In order to be able to apply BVMOs in bioconversions, the regeneration of their NADPH cofactor must be ensured, since it is costly and prone to degradation.¹⁵ Phosphite dehydrogenase (PTDH) has the advantage of being able to use phosphite as a cheap sacrificial substrate, while being thermostable and selective for NAD(P)⁺.¹⁶ As such, self-sufficient biocatalysts consisting of a BVMO fused with a PTDH were employed in this chapter.

3.2 Results and discussion

3.2.a. Specificities of BVMOs

Four biocatalysts were selected for this comparative screening in order to explore their substrate scope: three cyclohexanone monooxygenases (CHMOs), as representatives of versatile BVMOs active on small molecules, and PsCPDMO, a member of a subclass of BVMOs most active on more bulky substrates. The phylogenetic relationship between these BVMOs is shown in Figure 3.1. The characteristics of the enzymes is detailed in Table 3.1.

Figure 3.1. Representatives of different BVMO subclasses and their phylogenetic relationships are shown. Available crystal structures are indicated by a blue crystal symbol and the BVMOs selected for screening are marked in red. The tree was generated using PhyML, applying a maximum-likelihood method with 500 bootstraps (support values shown at the nodes).¹⁷ MO: monooxygenase.

AcCHMO is the most studied BVMO but its tolerance toward organic solvents is quite poor (no activity was reported after 25 min in 14 % v v⁻¹ acetonitrile at 20 °C and full deactivation within 24 h was reported in 5 % methanol at 25 °C).^{13, 18} This is accompanied by a poor thermostability, since all activity is lost after a few minutes at 45 °C.¹³ AcCHMO has been shown to be active on (+)-menthone as well as on jasmatone, with full regioselectivity toward the normal lactone in both cases and enantioselectivity for the former.^{10, 19}

RhCHMO became the prototype CHMO in structural studies since its crystal structure was solved, but it was reported to be very similar to AcCHMO in its substrate scope, covering a broad range of alkyl substituted cyclohexanone derivatives.¹⁰ More specifically, this BVMO catalyzes the oxidation of jasmatone, yielding the normal lactone exclusively, and of 3,3,5-trimethylcyclohexanone, yielding a mixture of both regioisomers (50:50). A particularly short half-life of $t_{1/2} < 1$ min in 14 % v v⁻¹ acetonitrile at 20 °C was measured for RhCHMO, despite the melting temperature of the protein being the same as that of AcCHMO ($T_m = 37$ °C).

Table 3.1. Characteristics of the BVMOs used in this chapter for screening.

Abbreviation	AcCHMO	RhCHMO	TmCHMO	PsCPDMO
Full name	<i>Acinetobacter calcoaceticus</i> NCIMB 9871	<i>Rhodococcus</i> sp. HI-31	<i>Thermocrispum municipale</i> DSM 44069	<i>Pseudomonas</i> sp. strain HI-70
Reference	¹⁹ , this work	¹⁰ , this work	¹³ , this work	⁴
Typical substrate	Cyclohexanone	Cyclohexanone	Cyclohexanone	Bulky substrates
pH range	pH optimum 8.5-9 ²⁰	Not determined	Not determined	Optimum at pH 9
Temperature range	Not determined	Not determined	Not determined	Optimum at 40 °C
T _m (°C)	37 (at pH 7-9) ¹³	37 (at pH 7-9) ¹⁰	48 (at pH 7.0) ¹³	Not available
Crystal structure	Not available	Available ¹⁰	Available ¹³	Not available
Solvent resistance	Poor ^{6,13}	Very poor ($t_{1/2} < 1$ min in 14 % v v ⁻¹ acetonitrile at 20 °C)	Very robust (79 % residual activity after 20 h in 14 % v v ⁻¹ acetonitrile at 20 °C)	Not determined
Thermo-stability	Poor (no more residual activity after a few min at 45 °C ¹³ and 70 % residual activity after 5.5 h at 30 °C)	Very poor (2 % residual activity after 0.5 h at 45 °C and 33 % residual activity after 5.5 h at 30 °C)	Good (58 % residual activity after 5.5 h at 45 °C) ¹³	Not determined

By contrast, TmCHMO, a recently discovered thermostable cyclohexanone monooxygenase, presents better temperature and solvent stability, while its substrate acceptance of alkyl substituted cyclic ketones has not been studied.¹³ Although TmCHMO can oxidize small sized cyclic ketones as well as linear ketones,¹³ its substrate scope toward alkyl substituted cyclic ketones remains however unknown.

Lastly, the substrate scope of a cyclopentadecanone monooxygenase from *Pseudomonas* sp. strain HI-70 (PsCPDMO; EC 1.14.13) was also screened. Although an optimum temperature of 40 °C was determined for this BVMO, little is known about its melting temperature, thermostability, and solvent resistance. While the typical substrate of AcCHMO, RhCHMO, and TmCHMO is cyclohexanone, PsCPDMO is active on

bulkier substrates such as steroids and macrocycles.^{4,21} Alkyl substituted substrates for this enzymes include both (+)- and (-)-menthone which are converted with high conversions, yielding the normal lactone only.³ 3,3,5-Trimethylcyclohexanone is also accepted as substrate by the enzyme.³

3.2.b. Effect of 1,4-dioxane on the activity with typical substrates

Because the water solubility of the substrates is in general limited, 1,4-dioxane was chosen as cosolvent for the preliminary screening of BVMOs on diverse ketone substrates. The effect of the cosolvent on the activity of the BVMOs on their typical substrate (cyclohexanone for AcCHMO, RhCHMO, and TmCHMO, and cyclopentadecanone for PsCPDMO) was evaluated after 20 minutes in the presence and absence of cosolvent (except for cyclopentadecanone whose water solubility is too limited) (Figure 3.2). Activities were measured by spectrophotometric assay by monitoring the decrease of absorbance at 340 nm due to the consumption of NADPH.

Figure 3.2. Activity of BVMOs (k_{obs}) measured after 20 minutes with model substrates: a) 5 mM cyclohexanone, and b) 1 mM cyclopentadecanone. The activity in the absence of cosolvent was not measured on cyclopentadecanone for PsCPDMO due to the limiting water solubility of the substrate.

AcCHMO and RhCHMO display high activity toward cyclohexanone in the absence of cosolvent ($k_{obs} = 13.0 \text{ s}^{-1}$ and 9.7 s^{-1} , respectively). However, a significant decrease in activity in the presence of 10 % v/v 1,4-dioxane was observed for both enzymes after 20 minutes (70 % and 50 % decrease respectively), therefore demonstrating the poor

solvent resistance of both enzymes. Although the activity of TmCHMO toward cyclohexanone was lower compared to the other CHMOs ($k_{\text{obs}} = 1.2 \text{ s}^{-1}$), it was decreased by only 15 % after 20 minutes with 10 % v/v 1,4-dioxane, which is in accordance with the robustness of TmCHMO reported by Romero *et al.*¹³ Cyclopentadecanone was chosen as substrate to measure the activity of PsCPDMO. Because of the high hydrophobicity and very low water solubility of this macrocyclic ketone ($113 \pm 21 \text{ }\mu\text{M}$),²² the activities could only be measured in the presence of cosolvent. Although the activity of PsCPDMO was low ($k_{\text{obs}} = 2.2 \text{ s}^{-1}$), it is in the same order of magnitude as the activities of the CHMOs in the presence of the cosolvent.

3.2.c. Screening of self-sufficient biotransformations (BVMO-PTDH)

In this chapter, alkyl substituted cyclic ketones were selected for screening, with a majority of terpene-based substrates. Terpenes are a family of compounds that can be extracted from wood rosin.²³ They are also a major constituent of some essential oils. In particular, menthone is derived from menthol which can be found in the essential oils of *Mentha arvensis* (wild mint) and *Mentha piperita* (peppermint).²⁴ Another terpene-based cyclic ketone, thujone, can be sourced from cedar wood²⁵ and is present in its α - and β -forms in more than 50 % of the essential oil of *Salvia officinalis* L. (dalmatian sage).²⁶ Dalmatian sage also contains non-negligible amounts of camphor.²⁶ This bicyclic ketone has been synthesized from turpentine at industrial scale.²⁷ (–)-Carvone can be obtained from the extraction and purification of the essential oil of *Mentha spicata* (spearmint) while (+)-carvone can be found in the essential oils of *Carum carvi* (caraway) and *Anethum graveolens* (dill).²⁸ Jasmatone, a cyclopentanone derivative with a $-\text{C}_6\text{H}_{13}$ alkyl substituent, is present in the essential oil of *Anisomeles indica* Kuntze.²⁹ Lastly, two other alkyl substituted substrates with similar structures were tested on the selected BVMOs: isophorone which is prepared from the aldol condensation of acetone,³⁰ and its hydrogenated counterpart 3,3,5-trimethylcyclohexanone. These substrates may be produced from renewable resources since acetone is a by-product of the synthesis of bio-ethanol by acetone-butanol-ethanol fermentation of lignocellulose.³¹⁻³²

A wide range of cyclic ketones was screened, namely a mixture of (+),(–)-menthone **1**, α,β -thujone **2**, (+)-camphor **3**, isophorone **4**, 3,3,5-trimethylcyclohexanone **5**, jasmatone **6**, and (–)-carvone **7**. Bioconversions were performed using self-sufficient PTDH-fused BVMOs to regenerate the NADPH cofactor (above Table 3.2). The degree of conversion was measured by GC-FID and the oxidized products were analyzed using GC-MS (Table 3.2). Since the substrates have asymmetric alkyl substituents, two regio-

isomeric lactones can be formed. In the case of the substituent directly located next to the ketone, the biocatalyst can show regioselectivity toward either the normal (most substituted) or abnormal (least substituted) lactone. When the substituent is positioned further on the cyclic ketone, either the proximal (substituent close to ester) or the distal lactone (substituent far from the ester) are expected.

Table 3.2. Screening of bioconversions of cyclic ketones with BVMO-PTDH.

Substrate	Products ^a	BVMO	Conv. ^b	Ratio n/p:a/d ^c	Reported as substrate?
 1	 1a	AcCHMO	+	n.d.	(+)-menthone only ³³
		RhCHMO	+	n.d.	This work
		TmCHMO	+	n.d.	This work
		PsCPDMO	++	100:0	Yes ³
 2	 2a	AcCHMO	+	n.d.	This work
		RhCHMO	+	50:50	This work
		TmCHMO	+	70:30	This work
	 2b	PsCPDMO	+	84:16	This work
 3	 3a	AcCHMO	-	n.d.	Not a substrate* ⁶
		RhCHMO	-	n.d.	This work
		TmCHMO	-	n.d.	This work
		PsCPDMO	-	n.d.	Not a substrate* ⁶
 4	 4a	AcCHMO	-	n.d.	This work
		RhCHMO	-	n.d.	This work
		TmCHMO	-	n.d.	This work
		PsCPDMO	-	n.d.	This work

Substrate	Products ^a	BVMO	Conv. ^b	Ratio n/p:a/d ^c	Reported as substrate?
 5	 5a 5b	AcCHMO	+	64:36	This work
		RhCHMO	++	44:56	Yes ¹⁰
		TmCHMO	+++	46:54	This work
		PsCPDMO	+	38:62	Yes ³
 6	 6a	AcCHMO	++	100:0	Yes ¹⁰
		RhCHMO	++	100:0	Yes ¹⁰
		TmCHMO	+++	100:0	This work
		PsCPDMO	+++	100:0	Yes ⁶
 7	 7a	AcCHMO	-	n.d.	Yes ¹⁰ / not a substrate* ¹⁹
		RhCHMO	-	n.d.	This work
		TmCHMO	-	n.d.	This work
		PsCPDMO	-	n.d.	This work

^a If no conversion was observed, the structure of the expected normal or proximal lactone product is shown. ^b Degree of conversion determined by GC-FID with 100 %, +++, > 50 %, ++, 1-50 %, +, 0 %, -. ^c Ratio of normal/proximal:abnormal/distal lactone product. The structure of the product was determined by comparison with lactones synthesized by chemical Baeyer-Villiger oxidation ((+),(-)-menthone and 3,3,5-trimethylcyclohexanone) or commercially available lactone (jasmatone). n.d. not determined: no oxidized product could be detected with GC-MS. * This substrate was reported as not belonging to the substrate scope of the enzyme.

Firstly, with AcCHMO, RhCHMO, TmCHMO, and PsCPDMO, no product was observed for the biotransformations of the unsaturated ketones isophorone **4** and (-)-carvone **7**. On the other hand, the hydrogenated counterpart of isophorone, 3,3,5-trimethylcyclohexanone **5**, could be oxidized by all tested BVMOs. The absence of conversion for isophorone **4** and (-)-carvone **7** is attributed to the presence of the unsaturation, therefore confirming the inactivity of AcCHMO toward α,β -unsaturated cyclic ketones like most BVMOs, with very few exceptions.³⁴ AcCHMO is able to convert substituted cycloketones when the unsaturation is located on the substituents.³⁵ Similarly, the biotransformation of (+)-camphor **3** did not result in lactone formation, maybe due to the steric hindrance of the bridged-substrate.

PsCPDMO demonstrated excellent regioselectivity toward (+)- and (-)-menthone **1** with formation of the normal lactone **1a** exclusively with full conversion. This is in agreement with the general preference of PsCPDMO for bulky cyclic ketones,³⁻⁴ although this enzyme showed limited acceptance for the smaller substrates α,β -thujone **2** and

3,3,5-trimethylcyclohexanone **5**. With AcCHMO, RhCHMO, and TmCHMO as biocatalysts, no significant conversions of (+),(-)-menthone was observed. PsCPDMO also showed excellent regioselectivity with jasmatone **6** thereby producing exclusively the normal lactone (δ -undecalactone **6a**). Although this alkyl substituted ketone has already been reported as a substrate by AcCHMO and RhCHMO,¹⁰ this work shows that it can also be converted to the normal lactone exclusively by TmCHMO and PsCPDMO, with full substrate conversion in both cases.

The mixture of α,β -thujone **2** could be oxidized by AcCHMO, RhCHMO, TmCHMO, and PsCPDMO although the biotransformations resulted in low degrees of conversion for all enzymes. This ketone has not been reported yet as a substrate for the tested CHMOs. Interestingly, while RhCHMO did not show regioselectivity (50:50 of both regioisomers), TmCHMO and PsCPDMO exhibited a preference for the normal lactone **2a** over the distal lactone **2b** (70:30 and 84:16 **2a:2b**, respectively). Although it has been demonstrated that the regioselectivity of RhCHMO is dictated by the tight-binding structure of the enzyme,³⁶ it is not yet possible to predict regioselectivity depending on the structure of the substrate. Similarly, although TmCHMO displays a compact ligand-binding site cavity which is consistent with the preference of the enzyme for small sized substrates,¹³ it is not yet possible to foresee if one of the regio-isomeric lactones will be favored.

3,3,5-Trimethylcyclohexanone **5** was successfully converted by all four BVMOs evaluated. This is the first report of the oxidation of this substrate by AcCHMO and TmCHMO. The biotransformation reached full conversion with the latter enzyme, making it a promising BVMO / substrate combination. The tested BVMOs did not seem to exhibit strong regioselectivity for this substrate since mixtures of proximal and distal lactones **5a** and **5b** were obtained with ratios close to 50:50. Since the substituents are relatively small and located one position further away from the ketone group (in β - and δ -position of the lactones), they seem to have little effect on the side of oxygen insertion during oxidation unlike other substituted ketones such as (+),(-)-menthone **1** and jasmatone **6**.

3.2.d. Self-sufficient TmCHMO for the synthesis of TMCL:

3,3,5-Trimethylcyclohexanone (TMCH) was selected as alkyl substituted substrate for further investigation. The first reason is that full conversion was achieved with TmCHMO, which is promising for up-scaling syntheses. The second reason is that, so far, little work has been reported on the polymerization of the corresponding lactones, namely the mixture of the distal lactone β,β,δ -trimethyl- ϵ -caprolactone TMCL-II (least-

substituted δ -position), and the proximal lactone β,δ,δ -trimethyl- ε -caprolactone TMCL-I (most-substituted δ -position) (Scheme 3.1). The robustness of TmCHMO, *i.e.* its solvent resistance and thermostability, is expected to be critical for the biocatalyzed application of a BVMO for lactone monomer synthesis.

Scheme 3.1. Biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone (TMCH) to distal lactone TMCL-II and proximal lactone TMCL-I using a self-sufficient TmCHMO-PTDH fused biocatalyst.

It should be noted that no resolution of the racemic mixture of the substrate TMCH was observed since both enantiomers were converted by TmCHMO with conversions higher than 50 %. Additionally, chirality of the monomers is not expected to be critical for polymerization for the targeted applications.

- **Substrate loading: substrate and/or product inhibition**

In order to investigate the best reaction conditions to perform enzymatic oxidations in a 30 mL reaction medium, several parameters and their effects were investigated, namely the substrate loading, possible oxygen limitations *via* biocatalyst loading experiments, the effect of the cosolvent on the reaction rate, and the concentration of NADP⁺. Possible substrate inhibition effects were investigated by performing biocatalysis reactions with an increasing initial loading of the substrate TMCH from 2 to 75 mM, using 10 % v v⁻¹ acetonitrile to solubilize the substrate and the formed products (Table 3.3). This solvent was chosen given the very robust solvent resistance of TmCHMO to acetonitrile (see Table 3.1).

Up to 8 mM substrate loading, full substrate conversion was obtained within 2.5 h, with very good mass balance and in solution yield (> 95 %), except for the 2 mM substrate loading which seems to be close to the limiting lower concentration for analysis. Although an initial substrate loading of about 18 mM resulted in the highest in solution product concentration (2.3 g L⁻¹), the corresponding STY was impacted by the long reaction time required for close to complete conversion of the substrate (Figure 3.3a).

For substrate concentrations higher than 18 mM, the reaction time increased as a result of substrate inhibition. Although conversions higher than 85 % were observed, the product concentrations remained low and were accompanied by a poor mass balance (Figure 3.3b, Table 3.3).

Table 3.3. Effect of substrate concentration on conversion, specific activity, product concentration, yield, and space-time yield (STY). Reaction conditions: [substrate] = 2-75 mM, 10 % v v⁻¹ acetonitrile, [TmCHMO-PTDH] = 4 μ M, [NADP⁺] = 250 μ M.

[sub] ^a (mM)	Time (h)	Conv. ^a (%)	Specific activity ^b (μ mol _{sub} min ⁻¹ μ mol _e ⁻¹)	[prod] ^a (mM)	Yield (%)	STY _{th} ^c (g L ⁻¹ h ⁻¹)	STY _{exp} ^d (g L ⁻¹ h ⁻¹)
2	0.5	100	20.4	1.2	> 50	0.65	0.37
6	1.5	100	21.7	5.9	> 95	0.60	0.61
8	2.5	100	17.7	7.6	> 95	0.49	0.47
18	23.5	94	21.2	14.6	95	0.18	0.10
50	24	86	n.d.	5.9	86	0.30	0.04
75	24	85	n.d.	6.6	85	0.49	0.04

^a Conversion and final product concentration calculated from GC-FID analysis with calibration curves. ^b Amount of substrate converted per min per amount of enzyme (μ mol_{substrate} min⁻¹ μ mol⁻¹_{enzyme}) calculated by linear regression on the initial reaction rate by plotting the substrate concentration vs. time, with [TmCHMO-PTDH] = 4 μ M. ^c Theoretical space-time yield $STY_{th} = [\text{substrate}]_{\text{initial}} \times M_s / (V \times t)$ with M_s molar mass of TMCH (140.2 g mol⁻¹), V volume (30 mL), and t total reaction time. ^d Experimental $STY_{exp} = [\text{product}]_{\text{final}} \times M_p / (V \times t)$ with M_p molar mass of TMCL (156.2 g mol⁻¹), V volume (30 mL), and t total reaction time. n.d. not determined.

Figure 3.3. Kinetics of the biocatalyzed reactions with decreasing substrate concentration with a) substrate conversion vs. time, and b) product concentration vs. time. Reaction conditions: [substrate] = 2-75 mM, 10 % v v⁻¹ acetonitrile, [TmCHMO-PTDH] = 4 μ M, [NADP⁺] = 250 μ M.

- **Stability of the substrate and products**

To investigate whether the poor mass balance could be attributed to degradation of the substrate or products, their stability was analyzed in buffer and in the absence of biocatalyst, where no instability was observed (Figure 3.4). The loss of product in the aqueous phase was therefore attributed to poor water solubility of the lactones for concentrations higher than 18 mM. Additionally, the specific activity is similar for a substrate concentration between 2 and 18 mM, which indicates that no substrate inhibition can be observed for substrate loadings lower than 18 mM (Table 3.3). For further investigation of the optimum reaction conditions, a substrate concentration of 10 mM was thus chosen.

Figure 3.4. Stability of the substrate TMCH and products TMCL (analyzed separately) in typical bioconversion reaction conditions without enzyme (the normalized area from GC-FID analysis as a function of time is shown).

- **Oxygen limitation investigation: biocatalyst loading**

Stoichiometric oxygen is required to oxidize the ketone TMCH to the lactone products TMCL (Scheme 3.1). To that end, possible oxygen limitations were evaluated by changing the biocatalyst loading. In the case of a reaction which is not oxygen-limited, increasing the biocatalyst loading should result in an increased reaction rate. However, when oxygen is the limiting reactant, the addition of more biocatalyst will not increase the reaction rate. In order to compare the rates at various biocatalyst loadings, the substrate conversions were plotted against a normalized x-axis, taking into account the enzyme concentration (Figure 3.5).

Figure 3.5. Substrate conversion as a function of time $\times$ [enzyme] (normalized x-axis) for increasing TmCHMO-PTDH concentration. Reactions performed with [substrate] = 10 mM, 10 % v v⁻¹ acetonitrile, [NADP⁺] = 250 μ M and [phosphite] = 125 mM.

This biocatalyst loading series clearly indicates that no oxygen limitation occurs because the specific activity was similar for decreasing biocatalyst concentration, although a small loss of activity was observed for the lowest biocatalyst concentration ([TmCHMO-PTDH] = 0.64 μ M). As such, a concentration of 4 μ M of biocatalyst was thus selected for further experiments.

• Effect of cosolvent on reaction rate

The use of a cosolvent is required to solubilize the substrate and products, given their hydrophobic character ($\log P = 2.56$ for TMCH, 2.14 and 2.20 for TMCL-II and TMCL-I, respectively, P partition coefficient, calculated as $\log P_{\text{oct/wat}} = \log ([\text{substrate}]_{\text{octanol}} / [\text{substrate}]_{\text{unionized water}})$ according to ChemDraw Professional 15.1). The substrate and products are fully miscible in many organic solvents at a concentration up to 50 g L⁻¹ (see the list of organic solvents in the experimental section). The effect of a series of water-miscible cosolvents at 10 % v v⁻¹ on the conversion was evaluated. The substrate and product concentrations of biocatalyzed oxidations with different cosolvents as a function of time are given in Figure 3.6a-f.

Methanol displayed the highest specific activity, which is consistent with its low denaturation capacity (DC) (Figure 3.7).³⁷ Conversely, 1,4-dioxane, which has the highest DC of all the cosolvents studied, slowed the reaction rate by a factor of 7. Both acetonitrile and ethanol had similar effects on the reaction rate. Using methanol allowed full substrate conversion within 1 h, whereas close to 3 h was required for 1,3-dioxolane, acetonitrile, or ethanol. Overall, a clear correlation could be established between the DC of the cosolvents and their effect on the specific activity (Figure 3.7b).

Figure 3.6. Kinetics of biotransformations with 10 % v v⁻¹ cosolvent and BVMO-PTDH with substrate TMCH (squares), lactone TMCL-I (full circles), lactone TMCL-II (empty circles), and the sum of the lactones (stars). Reactions performed with [substrate] = 10 mM, [NADP⁺] = 250 μ M and [phosphite] = 125 mM.

Figure 3.7. Effect of co-solvent (10 % v v⁻¹) on bioconversions with a) substrate conversion as a function of time, and b) specific activity of substrate shown as bars on the left y-axis, and denaturation capacity (right y-axis, values from reference ³⁷) of the cosolvents (blue squares with dotted line) on the right y-axis. The specific activity of substrate conversion was calculated by linear regression on the initial kinetics of the biotransformations (first 40 min for methanol, 60 min for 1,3-dioxolane, and 90 min for other co-solvents). Reaction performed with [substrate] = 10 mM, 10 % v v⁻¹ co-solvent, [TmCHMO-PTDH] = 4 μ M, [NADP⁺] = 250 μ M, [phosphite] = 125 mM.

• Effect of NADP⁺ concentration on the reaction rate

Decreasing the NADP⁺ concentration from 250 μ M to 100 μ M and 50 μ M resulted in slower kinetics (Figure 3.8). More importantly, the final conversions were very similar, independent of the NADP⁺ concentration. The faster kinetics at higher cofactor concentration may be due to improved enzyme stability. The half-life of some BVMOs, including AcCHMO, was shown to increase in the presence of larger available amount of

Figure 3.8. Substrate conversion as a function of time with cosolvent a) acetonitrile and b) methanol. Reactions conditions: [substrate] = 10 mM, 10 % v v⁻¹ cosolvent, [TmCHMO-PTDH] = 4 μ M, and [phosphite] = 125 mM.

NADPH.³⁸ In the case of the reaction studied in this chapter, NADPH is generated *in situ* by the PTDH which is fused to the BVMO. For this reason, a NADP⁺ concentration of 250 μ M was chosen for further investigation of the biocatalyzed oxidation.

- **Ratio of regio-isomeric lactones: influence of cosolvent and substrate feeding**

The ratio of regio-isomers resulting from the oxidation of TMCH by TmCHMO-PTDH was found to change considerably during the course of the biocatalyzed reaction. At low substrate conversion, the formation of the distal lactone TMCL-II was favored (up to 75:25 TMCL-II:TMCL-I at 10 % conversion) while at full substrate conversion, the ratio equilibrated to 55:45 (TMCL-II:TMCL-I). This phenomenon was found to be cosolvent independent for the range of cosolvents evaluated, which includes alcohols, ethers and nitrile (Figure 3.9a). A similar change in the ratio of the regio-isomers during the course of the reaction was also observed when using AcCHMO, whereas it remained stable when the reaction was catalyzed by RhCHMO, both enzymes being fused to PTDH (Figure 3.9b).

Interestingly, the formation of each regio-isomer was linear over time, independent of both the cosolvent and the enzyme (Figure 3.6). The rate of formation of the distal lactone TMCL-II was initially faster than that of the proximal lactone TMCL-I. However, both regio-isomeric lactones were produced from the start of the reaction. It is therefore unlikely that the change in the ratio of regio-isomers is due to product inhibition induced by one of the lactones.

Moreover, the ratio of lactone formation seems to be independent of the substrate and product concentration. The same pattern was indeed observed when comparing TmCHMO-PTDH catalyzed oxidations with different substrate supply, namely initial substrate loading and continuous substrate feeding (CSF), in which the ketone concentration remained very low throughout the reaction (Figure 3.9c-d). This change in the ratio of regio-isomeric products over time is thus enzyme dependent and cosolvent independent (at least for TmCHMO-PTDH).

In order to verify that this phenomenon was not intrinsic to Baeyer-Villiger oxidations, the ratio of regio-isomeric products formed during chemical Baeyer-Villiger oxidation of TMCH was studied, using a comparable substrate concentration (20 mM) (Figure 3.10). Interestingly, the chemical Baeyer-Villiger was found to favor the formation of the proximal lactone TMCL-I, while the BVMOs favored the formation of the distal lactone TMCL-II (45:55 and 55:45 TMCL-II:TMCL-I, respectively). Unlike its biocatalytic

counterpart, the chemical Baeyer-Villiger oxidation does not show any clear correlation between substrate conversion and ratio of regio-isomeric products formed, at least under the present experimental conditions.³⁹

Figure 3.9. Composition of lactone products with a) TmCHMO-PTDH in the presence of 10 % v⁻¹ co-solvent, b) TmCHMO-PTDH, AcCHMO-PTDH, and RhCHMO-PTDH in the presence of 10 % v⁻¹ methanol, c) several substrate feedings as a function of substrate concentration, and d) several substrate feedings as a function of product concentration. Empty symbols indicate the distal lactone TMCL-II, and full symbols indicate the proximal lactone TMCL-I. Reactions conditions: [substrate] = 10 mM, 10 % v⁻¹ cosolvent (acetonitrile for batch reactions and methanol for CSF), 10 % v⁻¹ BVMO-PTDH, [NADP⁺] = 250 μM, and [phosphite] = 125 mM. Continuous substrate feeding (CSF) was performed at 10 mM h⁻¹ feeding rate until a total concentration of 50 mM.

Figure 3.10. Composition of the products for the chemical Baeyer-Villiger oxidation of TMCH (substrate concentration of 20 mM). Empty circles indicate lactone TMCL-II and full circles indicate lactone TMCL-I.

3.2.e. Strategies to improve the process metrics of the biocatalyzed oxidations

As shown previously, this biocatalyzed oxidation suffers from substrate inhibition at high substrate loading while low substrate concentrations only afford lower product concentrations (Table 3.3). Consequently, strategies to increase the substrate and product concentrations while maintaining a limited reaction time were investigated in order to improve the productivity of TmCHMO catalyzed oxidations. These strategies include continuous substrate feeding (CSF) on the one hand and the use of an organic-aqueous biphasic catalytic system on the other hand (examples of BVMO-catalyzed reactions with these substrate feeding strategies are detailed in **Chapter 2**). In the case of CSF, the substrate concentration is maintained low throughout the reaction by gradual addition of the substrate while allowing for product accumulation. In the case of the biphasic reactions, in which a water-immiscible organic solvent forms as second phase where both the substrate and the product are soluble, the organic layer acts as a substrate reservoir and product sink. Both strategies aim at avoiding substrate inhibition while maintaining a high product concentration, and are typical strategies applied for bioprocess intensification.⁴⁰

CSF enzymatic reactions with feeding rates of 10 and 20 mM h⁻¹ were performed. Methanol was chosen as cosolvent to allow for sufficient product solubility, since this cosolvent was shown to give the highest specific activity (Figure 3.7). Additionally, TmCHMO is particularly tolerant to this cosolvent since this biocatalyst can retain 96 %

Figure 3.11. Biocatalyzed reactions with CSF with feeding rate of a) 10 mM h^{-1} over 5 h, b) 20 mM h^{-1} over 3 h, and c) 10 mM h^{-1} over 5 h with an initial product concentration of 40 mM. The mass balance is calculated as the sum of the substrate and product concentrations. Reaction conditions: 10 % v v⁻¹ methanol, [TmCHMO-PTDH] = 4 μM , [NADP⁺] = 250 μM .

of conversion yield in the presence of 30 % v v⁻¹ methanol for the conversion of 2-butanone.¹³ While the substrate was almost instantaneously converted when fed into the reaction mixture at a rate of 10 mM h^{-1} , substrate accumulation was observed when the feeding rate was doubled to 20 mM h^{-1} (Figure 3.11a-b). Additionally, product concentration followed the substrate feed in the first case, indicating immediate oxidation of the substrate. However, this was not the case when the substrate feed was doubled, although the substrate concentration decreased as soon as the feed of substrate was stopped. This increase in conversion after feeding of the substrate has stopped indicates that the substrate accumulation throughout the first 3 h of the reaction was due to the substrate feeding rate being above the maximum volumetric biocatalyst activity. An enzyme-catalyzed reaction containing an initial product concentration of 40 mM indicated that product inhibition occurs for product concentrations between 20 mM and 40 mM since

substrate conversion did not follow substrate feed and did not result in further product formation (Figure 3.11c).

One strategy to prevent product inhibition is to ensure that the product concentration remains low in the aqueous phase, where the biocatalyst is present. A biphasic system was therefore implemented to increase substrate loading and avoid substrate and/or product inhibition. The tolerance of TmCHMO-PTDH to toluene was studied by measuring the remaining activity of the biocatalyst in the presence of toluene. It was shown that 33 % v v⁻¹ toluene resulted in a similar remaining enzyme activity compared to in the presence of buffer only (Figure 3.12).

Figure 3.12. Stability of TmCHMO-PTDH in a toluene / phosphate buffer biphasic system with 33 % v v⁻¹ toluene at 30 °C (black circles) with remaining activity as a function of time. A blank was measured without toluene (empty circles). The activity was measured by measuring the NADPH consumption by spectrophotometry.

A biocatalyzed reaction with 33 % v v⁻¹ toluene as second phase was performed, *i.e.* with 30 mL of aqueous phase and 15 mL of toluene. The concentration of the substrate and products were measured in both phases (Figure 3.13). Although the majority of the substrate and products was located in the toluene phase, a non-negligible amount was also measured in the aqueous phase. Partitioning of the ketone and lactones was therefore not as straightforward as expected, despite what the log *P* values would suggest (log *P*_{toluene} = 2.52), which complicated product recovery. Of all the strategies evaluated, including CSF and a biphasic system with toluene as second phase, the latter resulted in the highest product concentration (11.6 g L⁻¹ in total), due to the high initial substrate loading of 100 mM in the toluene phase. High conversion could only be obtained after 24 h, which resulted in a decreased productivity (0.48 g L⁻¹ h⁻¹) (Table 3.4).

Figure 3.13. Biphasic reactions with toluene (33 % v v⁻¹) with a) substrate concentration and substrate conversion (red dashed line, right y-axis), and b) production concentration as a function of time. Reactions conditions: [substrate] = 100 mM, [TmCHMO-PTDH] = 4 μ M, [NADP⁺] = 250 μ M.

This is probably the result of the protein denaturation observed because of the increased interphase between the organic and aqueous phase, which decreased the amount of active biocatalyst. Additionally, this denatured protein present at the interphase is suspected to be a reservoir for non-isolated substrate and product during normal product recovery procedures.

The process metrics of some batch reactions as well as of the CSF and biphasic systems are shown in Table 3.4. Although the batch reactions resulted in fast reactions with full substrate conversion and a high STY (1.71 g L⁻¹ h⁻¹ for 10 % v v⁻¹ methanol), these reactions afforded low product concentrations (< 2 g L⁻¹) and limited turnover number (TON < 28 000) due to the low initial substrate loading. The combination of the low product concentration with low productivity limits the possibility of upscaled biocatalyzed oxidations.⁴¹ The biphasic system in toluene afforded the highest product concentration with 11.6 g L⁻¹ as well as the highest biocatalyst yield, and turnover number (TON = 185 375). However, the longer reaction time required for full substrate conversion lowered the productivity of the biphasic system (0.48 g L⁻¹ h⁻¹). Moreover, this strategy requires a more complex product recovery procedure since the substrate and products are present across both phases.

A continuous substrate feed at 10 mM h⁻¹ with a total reaction time of 6 h (Table 3.4, entry 5) seems to be the best strategy to afford both a higher product concentration (7.2 g L⁻¹) and a higher productivity (1.20 g L⁻¹ h⁻¹) while maintaining a good biocatalyst loading and turnover number (TON = 115 000).

Table 3.4. Comparison of the process metrics of the best performing biocatalyzed oxidations.

	Reaction type	Solvent	[sub] _i ^a (mM)	Time (h)	Conv. ^b (%)	[prod] _{exp} ^b (g L ⁻¹)
1	Batch	ACN	8	2.5	100	1.2
2	Batch	MeOH	11	1	100	1.7
3	Biphasic	Toluene	77	24	85	11.6
4	CSF (10 mM h ⁻¹)	MeOH	20	2 + 1 ^f	100	1.8
5	CSF (10 mM h ⁻¹)	MeOH	50	5 + 1 ^f	100	7.2
6	CSF (20 mM h ⁻¹)	MeOH	60	3 + 21 ^f	100	8.7
	Reaction type (continued)	Solvent (continued)	Biocat yield ^c (g _{prod} /g _{enz})	STY _{exp} ^d (g L ⁻¹ h ⁻¹)	TON ^e	Yield (%)
1	Batch	ACN	4.3	0.46	18 375	67
2	Batch	MeOH	6.4	1.71	27 475	n.d.
3	Biphasic	Toluene	42.8	0.48	185 375	42 ^g
4	CSF (10 mM h ⁻¹)	MeOH	6.7	0.61	29 100	n.d.
5	CSF (10 mM h ⁻¹)	MeOH	26.6	1.20	115 000	47
6	CSF (20 mM h ⁻¹)	MeOH	32.1	0.36	138 725	35

^a Initial substrate concentration as measured by GC-FID or total substrate concentration added by CSF. ^b Conversion and final product concentration calculated from GC-FID. ^c Calculated from the concentration of TmCHMO-PTDH ($M_{\text{TmCHMO-PTDH}} = 100 \text{ kg mol}^{-1}$). ^d Experimental space time yield $\text{STY}_{\text{exp}} = [\text{prod}]_{\text{exp}} / \text{reaction time}$. ^e Turnover number $\text{TON} = [\text{prod}]_{\text{exp}} / [\text{TmCHMO-PTDH}]$. ^f Time of CSF + time left to react without substrate feeding. ^g 38 % from the toluene phase and 5 % from the aqueous phase. Reaction conditions: $[\text{TmCHMO-PTDH}] = 4 \text{ }\mu\text{M}$, $[\text{NADP}^+] = 250 \text{ }\mu\text{M}$, 10 % v v⁻¹ (cosolvent) or 33 % v v⁻¹ (toluene). n.d. not determined.

3.3 Conclusions

The aim of this chapter was to explore the substrate scope of several BVMOs with alkyl substituted cyclic ketones since the corresponding lactones are of interest for the synthesis of alkyl substituted polyesters. Four self-sufficient BVMO-PTDHs were selected as biocatalysts for screening and new BVMO / substrate combinations are

reported. In particular, full conversion was observed for the oxidation of 3,3,5-trimethylcyclohexanone with the thermostable TmCHMO. This combination was further explored with small scale bioconversions (30 mL). Low product concentration was obtained with batch reactions, which was attributed to substrate inhibition and poor solubility. Methanol was identified as the cosolvent maintaining the higher reaction rate. Substrate inhibition and limited product solubility were overcome using two substrate feeding strategies, namely CSF and a biphasic system with 33 % v v⁻¹ toluene, which increased the product concentration as well as the productivity. Further improvements are required to increase the product concentration.

3.4 Experimental section

Starting materials. *L*-menthone (**1**; > 96 %, Sigma-Aldrich), α,β -thujone (**2**; 80 %, Sigma-Aldrich), (+)-camphor (**3**; 97 %, Sigma-Aldrich), isophorone (**4**; 98 %, Acros), 3,3,5-trimethylcyclohexanone (**5** or TMCH; 98 %, Sigma-Aldrich), jasmatone (**6**; 97 %, Alfa Aesar), (-)-carvone (**7**; 98 %, Sigma-Aldrich), cyclododecanone (> 99 %, TCI), sodium phosphite dibasic pentahydrate (> 98 %, Sigma-Aldrich), dichloromethane (Biosolve), hexadecane (99.5 %, TCI), 1,3-dioxolane (Acros), 1,4-dioxane (Sigma-Aldrich), acetonitrile (Biosolve), ethanol (Biosolve), ethyl acetate (Biosolve), and methanol (Biosolve) were used as received. Cyclopentadecanone was kindly supplied by Givaudan. The mixture of menthone isomers consisted of (-)-menthone (83 %) and (+)-menthone (17 %) as measured by GC-FID. The mixture of α,β -thujone consisted of (-)- α -thujone (70 %), (+)- β -thujone (12 %), fenchone (15 %) and (+)-camphor (3 %) as measured by GC-FID. Phosphate buffer (25 mM) at pH 8.5 was prepared from stock solutions of K₂HPO₄ and KH₂PO₄. β -Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH; 95 %, Alfa Aesar) and β -nicotinamide adenine dinucleotide phosphate disodium salt (NADP⁺; 97 %, Alfa Aesar) were stored at - 20 °C and solutions in buffer were prepared fresh prior to use.

¹H and ¹³C NMR. Proton (¹H) and carbon (¹³C) NMR spectra were recorded on a Bruker DPX-300 MHz apparatus at ambient probe temperature in CDCl₃. ¹H NMR experiments (300 MHz) were recorded with 16 scans and ¹³C NMR experiments (75 MHz) were recorded with 1024 scans. Chemical shifts were reported in ppm.

Enzymes production for screening (purified enzymes). AcCHMO, RhCHMO, TmCHMO, and PsCPDMO were prepared as purified His-PTDH-fusion proteins as described in the literature.^{6, 13} *E. coli* NEB 10- β cells transformed with the pCRE-BVMO plasmid were grown for 40 h at 24 °C shaking in Terrific Broth TB medium supplemented with ampicillin and 0.02 % *L*-arabinose. Cells were harvested, resuspended in Tris/HCl buffer at pH 7.5, and sonicated to create cell free extract. Purification of the enzyme was performed *via* Nickel-Sepharose affinity chromatography. Elution was achieved with 500 mM imidazole and the protein was subsequently applied on a desalting column before shock freezing in liquid nitrogen and storage at - 80 °C.

Biocatalyst production for bioconversions (cell-free extracts CFE). TmCHMO-PTDH, AcCHMO-PTDH and RhCHMO-PTDH were prepared as His-PTDH-fusion proteins in cell-free extracts (CFE). *E. coli* NEB 10- β cells transformed with the corresponding pCRE-BVMO plasmid in Terrific Broth TB medium were preincubated overnight at 37 °C. The medium was then supplemented with ampicillin and *L*-arabinose (0.02 %) and the cells were grown for 36 h at 24 °C. The culture medium to flask ratio was 1/10 and the culture medium was shaken at 135 rpm during fermentation. After growth, the cells were harvested by ultracentrifugation (4 °C, 6000 rpm, 15 min) and resuspended in Tris/HCl buffer (50 mM, pH 7.5). The cells were disrupted by sonication. Cell-free extracts (CFE) were obtained after a centrifugation step (4 °C, 15000 rpm, 45 min) and were stored at - 80 °C. The BVMO concentration in each CFE was estimated based on overexpression levels (evaluated from SDS-PAGE, normalized by volume) and total protein concentrations (measured by Bradford assay).

Estimation of the overexpression level by SDS-PAGE. Sodium dodecyl-polyacrylamide gel electrophoresis was performed with the CFE after sonication and each supernatant and resuspended pellet after ultracentrifugation following sonication (10 μ L samples). The samples were incubated at 95 °C for 5 min after addition of SDS loading dye. After centrifugation, the samples were loaded onto an SDS-PAGE gel (GenScript, USA) with a protein ladder of 10 to 200 kDa (PageRuler, pre-stained Thermofischer). The gels were run at 120 V on a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad). After the run, the gel was rinsed with water and stained with Coomassie InstantBlue™. The overexpression levels were estimated to 10-20 % for TmCHMO-PTDH, 20-30 % for AcCHMO-PTDH and 10-20 % for RhCHMO-PTDH.

Total protein concentration determination by Bradford assay. Samples containing 15 μ L of CFE (diluted 1/100 v v⁻¹ in Tris/HCl buffer at 50 mM and pH 7.5) and 150 μ L of Bradford reagent (Bio-Rad) were prepared in triplicate. Absorbance was measured at 595 nm in a 96-well microtiter plate with a Multiskan GO microplate spectrophotometer at 22 °C. A calibration curve was made with bovine serum albumin (Sigma-Aldrich). The total protein concentration of the CFE was determined as 13.2 ± 2.0 mg mL⁻¹ for TmCHMO-PTDH, 12.5 ± 1.4 mg mL⁻¹ for AcCHMO-PTDH, and 14.2 ± 1.2 mg mL⁻¹ for RhCHMO-PTDH.

Determination of the concentration of active BVMO by spectrophotometry (biocatalysts for bioconversions). The active BVMO concentration was measured using a flavin-reduction method.⁴² The absorbance spectrum of the BVMOs in their oxidized form (CFE diluted 1/10 in Tris/HCl buffer at 50 mM and pH 7.5) was measured from 300 to 700 nm with a JASCO V-330 spectrophotometer. NADPH (500 μ M) was added and the samples were incubated at room temperature for 10 min to afford the BVMOs in their reduced form. The absorbance spectrum of the BVMOs was measured again. The concentrations of active BVMO in CFE were determined as $[\text{BVMO}] = (A_{440, \text{ox}} - A_{440, \text{red}}) / \epsilon_{440, \text{diff}}$ ($\epsilon_{440, \text{diff}} = 10.15 \text{ mM}^{-1} \text{ cm}^{-1}$): $[\text{TmCHMO-PTDH}] = 37 \mu\text{M}$, $[\text{AcCHMO-PTDH}] = 96 \mu\text{M}$, and $[\text{RhCHMO-PTDH}] = 74 \mu\text{M}$.

Determination of enzyme long-term stability and stability in acetonitrile. The long term stability and the stability in acetonitrile were determined based on a procedure from Romero *et al.*¹³ The enzymes (10 μM) were incubated in Tris/HCl buffer (50 mM) at pH 7.0 for 16 h with a final volume of 400 μL . For long term stability determination, the enzymes were incubated at 30 $^{\circ}\text{C}$ or 45 $^{\circ}\text{C}$. For the determination of the stability in acetonitrile, the enzymes were incubated at 20 $^{\circ}\text{C}$ in the presence of 14 % v v⁻¹ acetonitrile. Aliquots were taken to determine the enzyme activity using the NADPH consumption assay described by Romero *et al.*¹³ with cyclohexanone as substrate. Reaction mixtures (total volume of 1 mL) contained 0.1 μM of enzyme, 150 μM of NADPH and 30 μM of cyclohexanone in air-saturated Tris/HCl buffer (50 mM) at pH 7.0 and at 25 $^{\circ}\text{C}$. Activities were measured in duplicate.

Analysis of the reaction mixture by GC-FID analysis (conversion). Gas Chromatography (GC) analyses were performed using a Shimadzu GC-2010 Plus Gas Chromatograph with a hydrogen flame-ionization detector (FID), and a Supelco SPB-1 capillary column (30 m $\times$ 0.25 μm with 0.25 mm inner diameter). The sample was injected at 250 $^{\circ}\text{C}$ (split ratio of 10, 2 μL injection volume). The temperature program of the oven was: temperature maintained at 60 $^{\circ}\text{C}$ for 2 minutes, increased to 200 $^{\circ}\text{C}$ at a heating rate of 15 $^{\circ}\text{C min}^{-1}$ (10 $^{\circ}\text{C min}^{-1}$ for screening), maintained at 200 $^{\circ}\text{C}$ for 2 minutes, increased to 320 $^{\circ}\text{C}$ at a heating rate of 20 $^{\circ}\text{C min}^{-1}$, and maintained at 300 $^{\circ}\text{C}$ for 5 minutes. Retention times of the products were identified with commercially available chemicals or lactones synthesized by chemical Baeyer-Villiger oxidation. For biocatalyzed reactions, aliquots of the reaction mixture (50 μL) were taken and mixed with acetonitrile (950 μL) containing hexadecane at a concentration of 100 μM as external standard. The sample was centrifuged, using an Eppendorf centrifuge 5424, to remove precipitated protein and analyzed by GC-FID. Concentration of substrate and lactones were determined using calibration curves.

Determination of the mass of products by GC-MS analysis. Mass spectrometry analyses were performed on a Shimadzu GC-2010 Plus Gas Chromatograph system, equipped with an AOC-20i auto-injector system, a Shimadzu GC-MS-QP2010 ultra mass spectrometer detector, and a Supelco SPB-1 capillary column (30 m $\times$ 0.25 μm with 0.25 mm inner diameter, for screening only) or a SH-Rxi-5 ms capillary column (30 m $\times$ 0.25 μm $\times$ 0.25 mm inner diameter, for bioconversions only). The temperature program was: starting temperature of 60 $^{\circ}\text{C}$ held for 2 min, temperature increased to 200 $^{\circ}\text{C}$ with a heating rate of 15 $^{\circ}\text{C min}^{-1}$ (10 $^{\circ}\text{C min}^{-1}$ for screening), and temperature finally increased to 320 $^{\circ}\text{C}$ at a heating rate of 20 $^{\circ}\text{C min}^{-1}$. Between each temperature increase, a 2-min hold time was implemented. A mass/charge (m/z) range of 35 to 750 Da was analyzed. Fragmentation patterns were compared to those of commercially available lactones (δ -undecalactone) or to lactones synthesized by chemical Baeyer-Villiger oxidation according to procedures reported in the literature (TMCL,⁴³ α,β -thujone lactone,⁴⁴ and menthicle⁴⁴).

GC-MS fragmentation patterns of the products (m/z): menthicle **1a** ($\text{C}_{10}\text{H}_{18}\text{O}_2$): 166, 127, 99, 81, 69, 55, 41, 39. Thujone lactone **2a** ($\text{C}_{10}\text{H}_{16}\text{O}_2$): 154, 139, 121, 112, 97, 83, 69, 55, 41, 39. Thujone lactone **2b** ($\text{C}_{10}\text{H}_{16}\text{O}_2$): 154, 139, 121, 111, 97, 83, 69, 55, 41, 39. β,δ,δ -Trimethyl- ϵ -caprolactone **5a** TMCL-I ($\text{C}_9\text{H}_{16}\text{O}_2$): 156, 126, 111, 99, 83, 69, 56, 41, 39. β,β,δ -Trimethyl- ϵ -capro-

lactone **5b** TMCL-II (C₉H₁₆O₂): 126, 111, 108, 93, 83, 69, 56, 41, 39. δ -Undecalactone **6a** (C₁₁H₂₀O₂): 166, 148, 133, 114, 99, 84, 71, 55, 41, 39.

Activity of BVMOs in the presence of 1,4-dioxane for screening. The activity of the BVMOs on substrates was measured by spectrophotometry by monitoring NADPH consumption corresponding to a decrease in absorbance at 340 nm using a Multiskan GO microplate spectrophotometer from Thermo Scientific. Reaction mixtures were placed in a 96-well plate and consisted of [substrate] = 5 mM in 10 % v v⁻¹ 1,4-dioxane, [NADPH] = 800 μ M, [BVMO-PTDH] = 0.5 μ M in Tris/HCl buffer at pH = 8.5. Activity was measured at 22 °C except for PsCPDMO (28 °C). Initial reaction rates (k_{obs} in s⁻¹) were calculated as $k_{\text{obs}} = (dA_{340}/dt) / (\epsilon_{340} \cdot [\text{BVMO-PTDH}] \cdot \ell)$ with $\ell = 0.5$ cm and $\epsilon_{340, \text{NADPH}} = 6.22 \text{ L mmol}^{-1} \text{ cm}^{-1}$. Initial reaction rates were measured in triplicate.

Small scale bioconversions for substrate screening. Bioconversions were performed with a total volume of 2 mL with [ketone] = 15 mM for substrate **3** and 50 mM for the other substrates, in 10 % v v⁻¹ 1,4-dioxane, [NADPH or NADP⁺] = 100 μ M, [BVMO-PTDH] = 0.5 μ M (AcCHMO, RhCHMO, TmCHMO or PsCPDMO), [HPO₃²⁻] = 2.5 eq compared to the substrate. The bioconversions were performed in phosphate buffer (25 mM) at pH 8.0. The reaction mixtures were left to incubate at 20 °C (AcCHMO, RhCHMO) or 30 °C (TmCHMO, PsCPDMO) for 24 h. Degrees of conversions were measured by GC-FID. Samples for GC-FID analysis were prepared by dissolving part of the reaction mixture in acetonitrile (1/1 v v⁻¹). The structure of the products was analyzed by GC-MS after extraction with dichloromethane (1/1 v v⁻¹).

Solubility of TMCH and TMCL in organic solvents. The solubility of the substrate and products was assessed by dissolving 50 mg in 1 mL of solvent (357 mM for TMCH, 321 mM for the mixture of TMCL-I and TMCL-II) at room temperature. The solubility was assessed after 24 h by visual inspection. The solvents tested were acetic acid, acetone, acetonitrile, benzonitrile, benzyl alcohol, 2-butanol, γ -butyrolactone, chlorobenzene, chloroform, *m*-cresol, cyclohexane, cyclohexanone, di-*n*-butyl ether, diethyl amine, diethylene glycol, *N*-*N*-dimethyl acetamide, dimethyl formamide, dimethyl sulfoxide, ethanol, ethyl acetate, ethyl benzene, ethylene glycol, ethylene glycol mono-methyl ether, formamide, hexane, isobutyl alcohol, methanol, methyl ethyl ketone, *N*-methyl-2-pyrrolidone, dichloromethane, nitrobenzene, 1-octanol, 1-pentanol, 1-propanol, propylene carbonate, 1-methoxy-2-propanol, tetrahydrofuran, toluene, valeronitrile, water, *N*-methyl formamide, 2-phenyl ethanol, 1,3-dioxolane, and 1,4-dioxane.

Stability of TMCH and TMCL in typical reaction medium. Two reaction mixtures containing either 50 mM of TMCH or the mixture of lactones TMCL (as synthesized by chemical Baeyer-Villiger oxidation using *m*-chloroperbenzoic acid as the oxidant³⁸), 10 % v v⁻¹ 1,4-dioxane, 100 μ M of NADPH, 125 mM of phosphite were prepared. The medium was prepared with phosphate buffer (25 mM) at pH 8.0. The reaction mixtures were left to incubate at 30 °C in a flask shaking apparatus. The stability of the substrate and products were evaluated by measuring the remaining amount by GC-FID. Samples were prepared by diluting aliquots of the reaction mixture in acetonitrile (1/1 v v⁻¹).

Bioreactor set-up for bioconversions. Biocatalyzed reactions were performed with a Metrohm 877 Titrino plus titration apparatus. The reactor was a double-walled vessel with a volume of 90 mL whose temperature was kept constant at 30 °C. Water-saturated air was bubbled through the reaction mixture at a rate of 8 mL min⁻¹ with a Vögtlin red-y flow controller. The pH was maintained at 8.0 by automatic titration of NaOH (1 M, Merck).

Typical biocatalyzed reactions with single batch addition. The reaction vessel was loaded with NADP⁺ (250 μM), 2-50 mM of TMCH (8.4 to 210.3 mg), phosphite (125 mM), and 10 % v v⁻¹ co-solvent. The stock solutions were prepared in a phosphate buffer at 25 mM at pH 8.0. The reaction mixture was stirred at 500 rpm and air was bubbled through at a rate of 8 mL min⁻¹. The TmCHMO-PTDH CFE was added to give an enzyme concentration of 4 μM (3 mL, 10 % v v⁻¹) to start the reaction. Kinetics was followed by GC-FID analysis.

Typical biocatalyzed reactions with continuous substrate feeding (CSF). The reaction vessel was loaded with NADP⁺ (250 μM), phosphite (125 mM) and 10 % v v⁻¹ methanol, in phosphate buffer at 25 mM at pH 8.0. The reaction mixture was stirred at 500 rpm and air was bubbled through at a rate of 8 mL min⁻¹. The TmCHMO-PTDH CFE was added to give an enzyme concentration of 4 μM (3 mL, 10 % v v⁻¹). Pure TMCH was fed continuously using a syringe pump (KDS Legato 110) with a feeding rate of 10 or 20 mM h⁻¹ (42.1 or 84.1 mg h⁻¹) for 2, 3 or 5 h. Kinetics was followed by GC-FID analysis.

Typical biocatalyzed reactions with buffer/toluene biphasic system. The reaction vessel was loaded with NADP⁺ (250 μM), and phosphite (125 mM) in phosphate buffer at 25 mM at pH 8.0 (volume of the aqueous phase 30 mL) and stirred at 500 rpm. TMCH (210.3 mg) was dissolved in toluene (15 mL) to give a solution of 100 mM substrate in the toluene phase (33 % v v⁻¹). The solution was added to the reaction medium. Air was bubbled through the reaction medium at a rate of 8 mL min⁻¹. The TmCHMO-PTDH CFE was added (3 mL, 10 % v v⁻¹, final concentration 4 μM) to start the reaction. Kinetics was followed by GC-FID analysis. Stirring was stopped prior to each sampling point in order to achieve phase separation and take a sample from both the aqueous and the toluene phase. The reaction was stopped after 24 h.

Determination of the stability of TmCHMO-PTDH to toluene as second phase. TmCHMO-PTDH (250 μL of CFE) was incubated at 30 °C for 24 h in a reaction mixture consisting of 33 % v v⁻¹ toluene (1.25 mL) in phosphate buffer (2.25 mL) at 25 mM and pH 8.0. The remaining activity was measured by spectrophotometric assay in a 96-well plate with a Multiskan GO microplate spectrophotometer at 30 °C. The NADPH consumption of mixtures containing sample from the incubated enzyme (20 μL), 500 μM NADPH (160 μL) and 50 mM cyclohexanone (20 μL) was measured at 340 nm. The remaining activity was measured relative to the initial k_{obs} calculated as $k_{\text{obs}} = (dA_{340}/dt) / ([\text{TmCHMO-PTDH}] \times \epsilon_{340} \times \ell)$ with A absorbance, ϵ_{340} extinction coefficient of NADPH at 340 nm (6.22 mM⁻¹ cm⁻¹), $[\text{TmCHMO-PTDH}] = 37 \mu\text{M}$, and $\ell = 0.5 \text{ cm}$.

Product recovery procedure of the biocatalyzed reactions. Reaction mixtures consisting of one phase were centrifuged to separate precipitated protein. The supernatant solution was extracted with

ethyl acetate (3 × 30 mL). The combined organic layers were dried over MgSO₄. The solvent was removed under rotary evaporation to afford a colorless to light-yellow liquid (110 mg, 47 % yield). In the case of the biphasic system, the phases were centrifuged separately to remove precipitated protein. The solvent in the toluene phase was removed in *vacuo* to afford a colorless to light yellow liquid (88.8 mg, 38 % yield). The aqueous phase was extracted with ethyl acetate (3 × 30 mL). The solutions were dried over MgSO₄ and ethyl acetate was removed under rotary evaporation to afford a colorless to light-yellow liquid (11.1 mg, 5 % yield). **¹H NMR (300 MHz, CDCl₃)** of the regio-isomeric lactones β,δ,δ-trimethyl-ε-caprolactone TMCL-I and β,β,δ-trimethyl-ε-caprolactone TMCL-II: δ (ppm) = 0.85 (s, 6H); 0.94 (s, 6H); 0.98 (s, 6H); 1.04–1.28 (m, 2H); 1.47–1.63 (m, 2H); 1.82–2.12 (m, 2H); 2.25–2.47 (m, 3H); 2.61–2.72 (m, 1H); 3.64–3.74 (m, 1H); 3.80–4.04 (m, 3H). **¹³C NMR (75 MHz, CDCl₃)**: δ (ppm) = 17.9, 21.2, 23.0, 23.8, 25.3, 27.2, 29.6, 30.4, 32.3, 33.1, 40.9, 45.7, 50.5, 50.8, 73.3, 75.6, 173.0, 173.7.

Kinetics of the chemical Baeyer–Villiger oxidation of TMCH. TMCH (0.56 g, 4 mmol, 1 eq) was dissolved in dichloromethane (200 mL) to a concentration of 20 mM. *m*-Chloroperbenzoic acid (70 %, Sigma-Aldrich) (1.79 g, 8 mmol, 2 eq) was added in one batch. The solution was stirred at room temperature for 28 days. Kinetics was followed by taking aliquots of the reaction mixture (1 mL) and were directly analyzed by GC-FID.

3.5 References

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CHAPTER 4

Application of a thermostable Baeyer-Villiger monooxygenase for the synthesis of monomer building blocks

4

In this chapter, the most promising BVMO / ketone combination identified in **Chapter 3** is further explored. The oxidation of 3,3,5-trimethylcyclohexanone with TmCHMO is investigated with a glucose dehydrogenase as coenzyme. Improved process metrics are targeted, namely increased conversion, product concentration and space-time yield. The biocatalyst loading is improved, and possible oxygen limitations are investigated. Product concentration and productivity are increased by keeping the substrate concentration below inhibitory level using continuous substrate feeding. This substrate feeding strategy is evaluated against two biphasic reactions using either toluene or *n*-butyl acetate as water-immiscible organic solvents. The reaction is upscaled to the gram-scale synthesis of the product by applying the CSF strategy, which gives the best process metrics. Additionally, this chapter reports the covalent immobilization of TmCHMO and GDH on a amino-functionalized agarose support, in order to increase the stability of the enzymes. The performances of the immobilized biocatalysts are investigated in reutilization reactions with up to 15 cycles.

Keywords: applied biocatalysis • up-scaling • process metrics • product concentration • immobilization

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4.1 Introduction

The goal of this chapter is to optimize the biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone (TMCH) with a cyclohexanone monooxygenase from *Thermocrispum municipale* (TmCHMO). This enzyme / substrate combination was selected because, although the oxidation of TMCH has been reported for some BVMOs, it has not yet been applied to a large scale synthesis. TmCHMO is particularly relevant for the upscaled synthesis of the alkyl substituted lactones β,δ -trimethyl- ϵ -caprolactones (TMCL) because of its high solvent resistance and thermostability. These properties are expected to be crucial for the improvement of the process metrics of the reaction. In particular, high product concentration and productivities, close to those reported for existing upscaled BVMO processes described in **Chapter 2**, are targeted.

While the previous chapter explored the use of a self-sufficient biocatalyst, this chapter focuses on applying a glucose dehydrogenase (GDH) as coenzyme, which uses glucose as sacrificial cosubstrate (Scheme 4.1). There are several reasons for this choice of cofactor regeneration system. The first one is that phosphite dehydrogenase, the coenzyme used for self-sufficient fusions, generates phosphate as coproduct, which is problematic for water treatment when applying the reaction at large scale. The second reason is that using a separate coenzyme allows for more flexibility because the ratio of enzymes can be tuned, thereby improving the biocatalyst loading.

Scheme 4.1. Biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone **TMCH** to a mixture of β,δ -trimethyl- ϵ -caprolactones **TMCL** (β,β,δ -trimethyl- ϵ -caprolactone **TMCL-II** and β,δ,δ -trimethyl- ϵ -caprolactone **TMCL-I**) in a two-enzyme system using a GDH to regenerate NADPH with glucose as sacrificial cosubstrate.

The major difference with the fused TmCHMO-PTDH biocatalyst used in **Chapter 3** is that pH control is essential when applying GDH as coenzyme for cofactor regeneration. This is because, for each molecule of substrate that is oxidized, one molecule of co-product gluconolactone is formed (Scheme 4.1). Spontaneous hydrolysis results in the formation of gluconic acid, which decreases the pH of the reaction. The pH of the reaction must therefore be maintained at 8 by autotitration, leading to the formation of gluconate as coproduct.

4.2 Results and discussion

4.2.a. Reaction engineering with TmCHMO/GDH as soluble biocatalysts

- **Biocatalyst loading**

The stability of the TmCHMO biocatalyst was evaluated by performing bioconversions with decreasing biocatalyst loading. These reactions were performed in a fed-batch manner, meaning that about 10 mM of substrate was added stepwise every hour. This time interval was chosen to give the biocatalyst enough time to fully convert the substrate, so that no substrate accumulation was observed. It has indeed been shown in **Chapter 3** that this reaction suffers from substrate inhibition. Additionally, the reactions were performed using methanol as cosolvent since it was identified in the previous chapter being an efficient cosolvent to solubilize the substrate while maintaining a high biocatalytic activity compared to other cosolvents.

Under these hourly substrate additions, the reaction progress of the bioconversions was found to be independent of the decrease in the load of biocatalyst, from a range of 10 % v v⁻¹ to 1.25 % v v⁻¹ of TmCHMO (Figure 4.1). For all reactions, the substrate was fully converted within one hour, independent of the TmCHMO concentration. This indicates good stability of the enzyme, at least within the time frame of the reaction. Moreover, the product accumulation as measured in the aqueous phase followed the total amount of substrate fed, indicating good efficiency of the enzyme.

Interestingly, it was observed that the substrate acted as anti-foaming agent, with foaming only observed when there was no more substrate available in the reaction mixture. As long as there was substrate, foaming was almost suppressed, preventing the possible air / protein interphase pathway of protein deactivation to take place.

Figure 4.1. Concentration of substrate and product as a function of time for increasing TmCHMO biocatalyst loading. The arrows indicate batch additions of 10 mM of substrate. Reaction conditions: initial [substrate] = 10 mM, 10 % v v⁻¹ methanol, [NADP⁺] = 250 μ M, [GDH] = 1 mg mL⁻¹ (except for a) 0.1 mg mL⁻¹), [glucose] = 125 mM, air flow 8 mL min⁻¹, stirring rate 500 rpm, total volume 30 mL.

The concentration of GDH was successfully decreased from 1 mg mL⁻¹ to 0.1 mg mL⁻¹ for the lowest TmCHMO concentration (1.25 % v v⁻¹), without negatively affecting either substrate conversion or product accumulation (Figure 4.1a).

The biocatalyst loading can by consequence be decreased by a factor 10 for both enzymes (TmCHMO and GDH), while maintaining full substrate conversion in the same reaction time. Based on the full conversion of 10 mM of substrate observed for all biocatalyst loading, a substrate feeding rate of at least 10 mM h⁻¹ seems to be appropriate.

• Oxygen mass transfer limitations

In BVMO-catalyzed oxidations, the oxygen supply is critical since molecular oxygen is one of the reactants in the reaction. The solubility of molecular oxygen in aqueous solutions at atmospheric pressure is very low (about 0.3 mM), while it is usually higher in organic solvents.¹ However, the solubility of oxygen in a mixture of 20 % methanol in water is not significantly increased compared to water only (0.34 mM).¹ It is therefore important to identify oxygen mass transfer limitations since they can limit reaction productivity, and therefore the overall process performances. The oxygen transfer rate is influenced by the size of the gas bubbles in the reaction mixture. The effect of stirring speed and air flow rate, which are two factors impacting the amount of dissolved oxygen, was evaluated on the reaction progress of the reaction (Figure 4.2a).

Doubling the air flow from 8 to 16 mL min⁻¹ resulted in the same increase in conversion as doubling the stirring rate from 500 to 1000 rpm did. However, the stirring rate of 1000 rpm led to a significant amount of observed denatured protein which was attributed to the increased air / aqueous solution interface, which is detrimental to the activity of the protein. Increasing the stirring rate and/or air flow resulted in similar mass balance for all reactions, indicating that there was no removal of substrate or product from the reaction mixture through the air stream (Figure 4.2b). The optimal reaction conditions for this reaction were thus identified to be an air flow rate of 16 mL min⁻¹ with a stirring speed of 500 rpm.

Figure 4.2. a) Substrate conversion, and b) product concentration, as a function of time with increasing stirring rate and air flow. Reaction conditions: [substrate] = 30 mM (22 mM for the reaction with 8 mL min⁻¹ and 500 rpm), 2.5 % v v⁻¹ TmCHMO, 10 % v v⁻¹ methanol, [NADP⁺] = 250 μM, [GDH] = 0.1 mg mL⁻¹, [glucose] = 125 mM, total volume 30 mL.

- **Increasing product concentration with continuous substrate feeding**

In order for the TmCHMO-biocatalyzed oxidation to be competitive against classical chemical Baeyer-Villiger oxidation, it is necessary to achieve high product concentrations, typically exceeding 10 g L^{-1} . Continuous substrate feeding (CSF) was therefore selected as a suitable substrate supply strategy in order to keep substrate concentrations low, and thus avoid substrate inhibition.

Potential product inhibition was evaluated by performing a reaction containing an initial 30 mM (approximately 4.7 g L^{-1}) of chemically-synthesized TMCL lactones (Figure 4.3). While the initial presence of 30 mM of product did not seem to hamper product accumulation, it resulted in lower substrate accumulation, maybe as a result of better solubility of the substrate in the aqueous phase due to the presence of the product. In these reaction conditions, no product inhibition for product concentrations lower than 30 mM was observed.

A comparable low substrate accumulation and high substrate conversion were obtained for feeding rates of 10 mM h^{-1} and 15 mM h^{-1} (Figure 4.4a). Increasing the feeding rate resulted in faster product formation, and consequently higher product concentration because of the higher total amount of substrate to be converted (Figure 4.4b).

Figure 4.3. Concentration of substrate and product as a function of time in the absence of initial product (full symbols), and with an initial product concentration of 30 mM (empty symbols). Reaction conditions: CSF of 12.5 mM h^{-1} , $2.5 \% \text{ v v}^{-1}$ TmCHMO, $10 \% \text{ v v}^{-1}$ methanol, $[\text{NADP}^+] = 250 \text{ }\mu\text{M}$, $[\text{GDH}] = 0.1 \text{ mg mL}^{-1}$, $[\text{glucose}] = 125 \text{ or } 375 \text{ mM}$, air flow 8 mL min^{-1} , stirring rate 500 rpm , total volume 30 mL .

Figure 4.4. Effect of substrate feeding rate with CSF of 10 mM h⁻¹ and 15 mM h⁻¹ with a) concentration of substrate (left axis) and substrate conversion (right axis), and b) concentration of product. Reaction conditions: 2.5 % v v⁻¹ TmCHMO, 10 % v v⁻¹ methanol, [NADP⁺] = 250 μM, [GDH] = 0.1 mg mL⁻¹, [glucose] = 125 or 375 mM, air flow 8 mL min⁻¹, stirring rate 500 rpm, total volume 30 mL.

In the case of this reaction, a reaction rate of 15 mM h⁻¹ was favored as the final product concentration was higher with a similar conversion, compared with a feed of 10 mM h⁻¹. Based on the reaction progress observed for various TmCHMO loadings (Figure 4.1), an initial biocatalyst loading of 2.5 % v v⁻¹ was selected. When the substrate was fed at a rate of 15 mM h⁻¹ for 7 h, little substrate accumulation was observed (Figure 4.5a). Feeding at such a rate overnight should allow for a total product concentration of about 37 g L⁻¹. This was however not the case as the reaction suffered from significant substrate accumulation, up to about 95 mM (about 15 g L⁻¹). As expected, increasing the reaction time increased the protein denaturation observed.

Interestingly, the same continuous overnight feeding of substrate resulted in almost no substrate accumulation when the reactor was loaded with 5 % v v⁻¹ biocatalyst instead of 2.5 % v v⁻¹ with the conversion increasing from 42 % to 85 %. Despite high substrate conversion, the product concentration in the aqueous phase remained limited to 15 g L⁻¹ in both cases, even with the presence of 10 % v v⁻¹ of methanol (Figure 4.5b). This was due to the product being located in the aqueous phase at a concentration up to 15 g L⁻¹ but also as a second phase in the reaction mixture, and to some minor extent adsorbed onto the denatured protein. The volume of the methanol cosolvent was increased from 10 % to 20 % v v⁻¹ in order to attempt to increase the solubility of the product in the aqueous phase, and therefore the overall product concentration as measured in the aqueous phase (Figure 4.6).

An increased concentration of methanol cosolvent resulted in a lower substrate accumulation and a better product formation according to the substrate feed. This is in line with the reported robustness of TmCHMO toward methanol, since the enzyme can retain 96 % of its conversion yield toward 2-butanone in the presence of 30 % methanol.² Although the addition of more methanol seemed to be beneficial to the product solubility, a cosolvent volume of 10 % was selected for further experiments in order not to complicate the product recovery procedure.

Figure 4.5. Concentration of substrate and product as a function of time for bioconversions with CSF at 15 mM h⁻¹ a) for 7 h and 16 h with 2.5 % v v⁻¹ TmCHMO, and b) with 2.5 % v v⁻¹ TmCHMO and 5 % v v⁻¹ TmCHMO for 16 h. Reaction conditions: [substrate] = 110 or 240 mM, 10 % v v⁻¹ methanol, [NADP⁺] = 250 μM, [GDH] = 0.1 mg mL⁻¹, [glucose] = 125 or 375 mM, air flow 16 mL min⁻¹, stirring rate 500 rpm, total volume 30 mL.

Figure 4.6. Substrate and product concentration with 10 % or 20 % v v⁻¹ methanol. Reaction conditions: CSF of 10 mM h⁻¹, 2.5 % v v⁻¹ TmCHMO, [NADP⁺] = 250 μM, [GDH] = 0.1 mg mL⁻¹, [glucose] = 125 or 375 mM, air flow 8 mL min⁻¹, stirring rate 500 rpm, total volume 30 mL.

Using CSF, the best reaction conditions for achieving high substrate conversion and high product concentration were thus obtained with a TmCHMO biocatalyst loading of 5 % v v⁻¹, using 10 % v v⁻¹ methanol as cosolvent, and a continuous feeding rate of 15 mM h⁻¹ for a total of 240 mM of substrate to be converted.

- **Continuous substrate feeding vs. biphasic systems**

This improved CSF reaction was evaluated against another substrate supply strategy, namely a biphasic system using either toluene or *n*-butyl acetate as water-immiscible organic phase. The objective of the water-immiscible organic phase is twofold: first acting as a substrate reservoir and decreasing the substrate concentration in the aqueous phase where the biocatalyst is present, and secondly serving as a product sink, solubilizing the formed product and ensuring high product concentration.

Toluene was selected because of the favorable solvent resistance of TmCHMO toward this solvent. No significant decrease in activity in the presence of 33 % v v⁻¹ toluene for the oxidation of cyclohexanone at 30 °C was observed (see **Chapter 3** for details). *n*-Butyl acetate was selected as a low-environmental-impact alternative.³ Additionally, both solvents have a low solubility in water, which should result in a clear partitioning of substrate and product, and a high boiling point circumventing any potential evaporation.

A biphasic system consisting of 25 % v v⁻¹ of organic solvent (*i.e.* 30 mL of aqueous phase with 10 mL of organic phase) was loaded with 7.2 mmol of substrate (1.138 mL), corresponding to the same amount of substrate that was converted in the optimized CSF system (Figure 4.7). In the biphasic systems, the substrate concentration was 180 mM for the overall reaction mixture (240 mM if all the substrate was in the aqueous phase and 720 mM if all the substrate was in the organic phase). During the reaction, the substrate and the product formed were found almost exclusively in the organic phase (Figure 4.7 b-d vs. a-c).

It was however difficult to follow the reaction progress of each phase due to the significant emulsification that occurred. It is therefore probable that the amount of substrate and product measured in the (lower) aqueous phase was overestimated due to contamination with the (upper) organic phase, while the amount in the organic phase was probably underestimated due to the emulsification. This is particularly visible with the sudden drop of product concentration in the toluene phase between 23 and 25 h (Figure 4.7b), which was due to samples of the reaction mixture containing a mix of both phases.

Figure 4.7. Kinetics of the biphasic reactions with quantity of substrate and product in mmol as a function of time with a-b) toluene, and c-d) *n*-butyl acetate. The mass balance is shown in dotted red line (right axis), calculated as $(n_{\text{sub, aq}} + n_{\text{sub, org}} + n_{\text{prod, aq}} + n_{\text{prod, org}}) / n_{\text{sub, initial}} \times 100$, with $n_{\text{sub, initial}} = 7.2$ mmol.

At the end of the reaction (26.5 hours), the reaction mixture was diluted with additional organic solvent in order to deactivate all remaining protein. This resulted in almost full solubility of the substrate and product in the organic phase (> 98 %). Analysis of the isolated products revealed that both biphasic systems had a lower substrate conversion compared to the equivalent CSF reaction, with 32 % and 43 % of initial substrate recovered for the toluene and *n*-butyl acetate systems, respectively. In contrast, the products isolated from the improved CSF reaction contained only 4 % of ketone, with a substrate conversion of 85 % (Figure 4.8).

Figure 4.8. Composition of the isolated products as analyzed for GC-FID of the reactions performed with CSF at 15 mM h^{-1} , and biphasic systems in *n*-butyl acetate or toluene. The percentage of substrate TMCH found in the isolated product is indicated. The ratio of lactone TMCL-I:TMCL-II is 38:62 for the two biphasic reactions, and 40:60 for the CSF reaction.

4.2.b. Gram-scale preparation of branched lactones

The best reaction conditions obtained using CSF were up-scaled to a 500 mL-scale reaction. The substrate was fed at a rate of 15 mM h^{-1} for 16 h, after which the reaction was left running. Unlike with the equivalent 30 mL-scale experiment, a large amount of unreacted substrate (27 % of the total amount of substrate fed) was present in the reaction after 19 h (Figures 4.5b vs. 4.9a). This was attributed to significant protein denaturation

Figure 4.9. Up-scaled biocatalyzed reaction with a) concentration of substrate and product, and b) composition based on GC-FID of the isolated products from the aqueous phase and cell-pellets (denatured enzymes). Details of the composition are given in the experimental section. Reaction conditions: 240 mM of substrate fed at 15 mM h^{-1} , $[\text{TmCHMO}] = 7.5 \text{ \% v v}^{-1}$ in total, $10 \text{ \% v v}^{-1}$ methanol, 500 mL reaction volume.

occurring during the upscaled reaction which might be due to increased air / aqueous interphase. An additional 2.5 % v v⁻¹ of TmCHMO biocatalyst was therefore added, resulting in instantaneous substrate conversion until almost all substrate was fully converted (> 99 % conversion).

A space-time yield (STY) of 1.35 g L⁻¹ h⁻¹ was obtained with the 500 mL-scale reaction, similar to the STY obtained at the 30-mL scale, despite the increased reaction time needed to convert the remaining substrate after addition of TmCHMO (Table 4.1). This extra biocatalyst loading resulted in a lower biocatalyst yield compared to the smaller reaction at 30 mL. It should be noted that the product concentration measured in the aqueous phase reached saturation at about 15 g L⁻¹, and is therefore not representative of the real total product concentration, which was about 38 g L⁻¹.

Table 4.1. Process metrics of the biocatalyzed oxidation of TMCH with TmCHMO.

Reaction type	Volume (mL)	Time (h)	Conv. ^a (%)	[product] ^b (g L ⁻¹) Total (in aqueous phase)	STY ^c (g L ⁻¹ h ⁻¹) From solution (from isolated product)
Biphasic (n-butyl acetate)	30	26.5	57 ^e	n.d.	(0.32)
Biphasic (toluene)		26.5	68 ^e	n.d.	(0.36)
CSF		24	85	31.8 (15.5)	1.33 (0.42)
CSF	500	28	> 99	37.8 (16.8)	1.35 (0.87)
Reaction type (continued)	Volume (mL)	Biocatalyst loading ^d (g _{cww} L ⁻¹)	Biocatalyst yield ^d (g _{prod} g _{cww} ⁻¹)	Isolated product (g)	Isolated yield (%)
Biphasic (n-butyl acetate)	30	25.0	0.52 ^f	0.258	22
Biphasic (toluene)		25.0	0.57 ^f	0.287	25
CSF		25.0	1.29	0.302	32
CSF	500	37.5	1.01	12.115	69

^a Final conversion. ^b Total product concentration: [product]_{total} = [substrate]_{initial} × conv × M_{TMCL} (M_{TMCL} = 156 g mol⁻¹). ^c STY = [product]_{total} / reaction time and STY = m_{isolated product} / reaction time / V_{reactor}. ^d Biocatalyst yield = [product]_{total} / biocatalyst loading. ^e Calculated from the composition of the isolated products. ^f Calculated from the isolated amount of product. n.d. not determined.

Increasing the reaction volume resulted in an increase in isolated yield with over 12 g of isolated lactones product for the 500 mL-upscaled reaction. The STY was therefore also calculated based on the amount of isolated product. In that case, increasing the reaction volume doubled the STY as a result of the higher substrate conversion and higher isolated yield. Analysis of the denatured protein at the end of the reaction revealed that it adsorbed both products and substrate, with a mixture consisting mainly of lactone products (85 %) with some substrate (12 %) (Figure 4.9b). The isolated product additionally contained low amounts of side-products, which were identified by 2D-NMR as being the corresponding methyl ester ring opened products (Figures 4.9b and 4.10).

A model reaction of chemically synthesized lactones in a reaction mixture of 50: 50 % v v⁻¹ buffer and methanol confirmed that this side-product formation was induced by the dilution of the reaction mixture with methanol to deactivate all remaining protein, and can be avoided by direct product recovery at the end of the reaction (Figure 4.11).

Figure 4.10. Heteronuclear single quantum coherence (HSQC) spectrum of the isolated products from the upscaled reaction (500 mL), containing the targeted lactone products TMCL-I and TMCL-II with the corresponding methyl ester ring opened products TMCL-I' and TMCL-II' (proton of the methyl esters ring opened products highlighted in blue).

Figure 4.11. Kinetics of the formation of the methyl ester TMCL-I' and TMCL-II' after dilution with methanol (50 % v v⁻¹). The percentage of methyl esters was calculated from GC-FID analysis.

4.2.c. Principle of enzyme immobilization

Immobilization of whole-cells or isolated enzymes is known to usually increase the operational stability of enzymes. Additionally, this biocatalyst format has several advantages including facilitating the recovery of the biocatalyst, decreasing the cost of downstream processing, and potentially decreasing the enzyme cost per kilogram of product, provided that the immobilized biocatalysts maintain their activity throughout the reuses.⁴⁻⁵ Immobilization is therefore particularly interesting for the industrial application of biocatalyzed reactions.⁶ So far, TmCHMO has only been applied in the form of a soluble biocatalyst and has not yet been immobilized.

The immobilization of an enzyme on a support can be performed *via*: (i) physical adsorption such as hydrogen bonding, (ii) formation of a covalent bond between enzyme and support, (iii) encapsulation and entrapment of the enzyme in a polymeric matrix, and (iv) cross-linking which is a combination of covalent binding and entrapment.⁷ Covalent binding was selected as method for immobilization because it gives strong binding and usually prevents leaching of the biocatalyst. In the literature, isolated BVMOs have mostly been immobilized to polymeric supports using this method.⁸ For example, a cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* (AcCHMO) was immobilized on Eupergit (polyacrylamide based supported beads) *via* covalent binding with a glucose 6-phosphate dehydrogenase for the synthesis of chiral lactone building blocks.⁹ Fusions of AcCHMO with a polyol dehydrogenase were similarly immobilized. However, a low stability on the support and a poor operational stability were reported.¹⁰

In this chapter, the goal is to immobilize TmCHMO and a glucose dehydrogenase from *Thermoplasma acidophilum* (EC 1.1.1.47) (GDH-Tac) by covalent binding, either separately or by coimmobilization on the same support. Monoaminoethyl-*N*-aminoethyl-agarose (MANA-agarose) was selected as support because it was recently identified as being suitable for the immobilization of AcCHMO-PTDH.¹¹ The support consists of highly porous cross-linked beads from agarose, which is a polysaccharide.¹² MANA-agarose was first reported more than 25 years ago for enzyme immobilization, and has the particularity of being functionalized with amine groups with low pK_a (Scheme 4.2).¹³

The principle of immobilization of enzymes on MANA-agarose is the formation of a covalent bond between the amine groups of the support and acid groups present at the surface of the enzyme, which are typically located on glutamic and aspartic residues. The formation of amide bonds in aqueous medium is promoted by the addition of 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) as coupling agent.¹⁴ Three steps are necessary: (i) ionic adsorption of the enzyme on the support, which is favored by carboxylic acid/amine ionic interactions (formation of an ammonium/carboxylate salt), (ii) formation of an amide bond promoted by EDC,¹⁴ and (iii) ionic desorption of the uncoupled enzymes.

Scheme 4.2. Principle of biocatalyst immobilization on MANA-agarose by formation of an amide covalent bond between the amine functions of the support and free acid groups of the enzyme.

Immobilization is characterized by two metrics: the immobilization yield (**Eq 4.1**) and the retained activity (**Eq 4.2**). While the immobilization yield quantifies the amount of enzymes covalently attached to the support, the retained activity quantifies the enzymatic activity of the immobilized enzyme. This difference is important because a loss of enzymatic activity may occur during immobilization.

$$\text{Immobilization yield (\%)} = \frac{\text{Initial supernatant activity} - \text{Final supernatant activity}}{\text{Initial supernatant activity}} \times 100 \quad (\text{Eq 4.1})$$

$$\text{Retained activity (\%)} = \frac{\text{Final suspension activity} - \text{Final supernatant activity}}{\text{Initial supernatant activity}} \times 100 \quad (\text{Eq 4.2})$$

• Immobilization of TmCHMO on MANA-agarose support

Coupling was performed in a slightly acidic medium (pH 6) in order to increase the nucleophilic strength of the carboxylic acids,¹³ and to promote ionic adsorption. The amide bond obtained by coupling was shown to be stable up to pH 8.¹⁴ AcCHMO was reported to be successfully immobilized on MANA-agarose, for which a high immobilization yield and retained activity were obtained using an EDC concentration of 25 mM (Figure 4.12a).¹¹ The immobilization of TmCHMO was therefore attempted with the same EDC concentration. Although the enzyme was fully adsorbed on the support after 15 min, lower immobilization yield and retained activity were obtained compared to AcCHMO (Figure 4.12a)

Figure 4.12. Immobilization yield (left axis) and retained activity (right axis) as a function of the EDC concentration for the immobilization of a) TmCHMO (with AcCHMO as reference), and b) GDH-Tac.

Table 4.2. Overview of the characterization of the immobilization of TmCHMO and GDH-Tac on MANA-agarose support under optimum conditions.

Enzyme	EDC (mM)	Offered enzyme load* (per g support)	Immobilization yield (%)	Retained activity (%)	U per g support
TmCHMO	35	5 U (8 mg TmCHMO)	93.0 ± 0.4	62.4 ± 2.1	3.1 ± 0.1
GDH-Tac	10	5 U (3.7 mg GDH-Tac)	78.7 ± 3.0	57.1 ± 2.0	2.9 ± 0.14
Coimmobilized TmCHMO and GDH-Tac	10	5 U for each enzyme	TmCHMO: 79.4 ± 2.3 GDH-Tac: 96.5 ± 0.4	TmCHMO: 12.9 ± 1.6 GDH-Tac: 48.2 ± 6.5	TmCHMO: 0.7 ± 0.1 GDH-Tac: 2.4 ± 0.5

Error values correspond to standard deviation (n = 2). * No substrate transfer limitations were found at this enzymatic load. U enzyme unit ($1 \mu\text{mol min}^{-1}$).

The low immobilization yield could indicate that the EDC concentration was too low to ensure a complete covalent attachment of TmCHMO. The concentration of EDC was thus increased to 35 mM, which resulted in a higher immobilization yield and retained activity, similar to what was reported for AcCHMO. This concentration was selected as the most appropriate concentration of EDC for TmCHMO immobilization in MANA-agarose (Table 4.2).

• Immobilization of GDH-Tac on MANA-agarose support

The successful immobilization of GDH-Tac on MANA-agarose has been reported.¹⁵ The immobilization of GDH-Tac was studied at different concentrations in order to find compatible conditions for the subsequent coimmobilization of GDH-Tac and TmCHMO. Using 10 mM of EDC, an immobilization yield and a retained activity of $78.7 \pm 3.0 \%$ and $57.1 \pm 2.0 \%$, respectively, were obtained (Figure 4.12b).

Higher concentrations of EDC, which are required for the immobilization of TmCHMO, however led to lower retained activities for GDH-Tac (Figure 4.12b). This indicates that GDH-Tac could be deactivated at EDC concentration higher than 10 mM. This negative effect of EDC on the enzymatic activity has been already reported for other enzymes.¹⁶ In general, loss of enzymatic activity can be attributed to several factors such

as: (i) side reactions during coupling (with tyrosine, or thiol containing amino acids such as cysteine),¹⁷ (ii) possible distortion of the protein structure due to multiple-point attachment,¹⁸ and (iii) diffusion limitations of the substrate due to the support which slows down the observed reaction rate. Consequently, the optimum EDC concentration for the immobilization of TmCHMO (35 mM) was not tested for the immobilization of GDH-Tac given the negative effect of high EDC concentrations on the latter enzyme.

When lower concentration of EDC (1, 3 and 5 mM) were used, lower immobilization yields and lower retained activities were obtained (Figure 4.12b). This is probably due to an incomplete covalent attachment of GDH-Tac on the support. An EDC concentration of 10 mM was therefore selected as the most appropriate concentration for the immobilization of GDH-Tac on MANA-agarose support.

• Coimmobilization of TmCHMO and GDH-Tac

For the coimmobilization of TmCHMO and GDH-Tac, two EDC concentrations were tested, 10 and 20 mM (Figure 4.13). This concentration range was mostly determined by the results obtained from the immobilization of GDH-Tac. The retained activity of GDH-Tac was 19 % lower when using 20 mM EDC compared to 10 mM of EDC although similar immobilization yields were obtained. This would indicate possible chemical side reactions between the amine groups of the support and the amino acids of the enzyme induced by an excess of EDC.¹⁴ An increase from 10 to 20 mM EDC did not improve significantly the obtained immobilization yield for TmCHMO. Finally, an EDC concentration of 10 mM was selected for coimmobilization. An overview of the immobilized biocatalysts used for the rest of the study is shown in Table 4.2.

Figure 4.13. Immobilization yield (left axis) and retained activity (right axis) during coimmobilization of TmCHMO and GDH-Tac.

4.2.d. Reutilization of the immobilized biocatalysts and comparison with the soluble enzymes

The performances of the immobilized biocatalysts were evaluated for the oxidation of TMCH to the alkyl substituted lactones TMCL (Scheme 4.1). In total, three biocatalyst formats were examined for TmCHMO and GDH-Tac: soluble enzymes, enzymes immobilized separately, and coimmobilized enzymes. The performances of the immobilized enzymes were evaluated in over 15 repeated biooxidation cycles, in which the immobilized biocatalysts were re-used each time, and compared to the corresponding soluble enzymes.

• Soluble enzymes with repeated substrate addition

The oxidation of TMCH was first performed with soluble TmCHMO and soluble GDH-Tac using a TmCHMO load of 1.07 mg mL^{-1} and an enzyme ratio of 1:2 (mg TmCHMO/mg GDH-Tac). These soluble enzymes are cell-free extracts containing 34 mg mL^{-1} of TmCHMO and 46 mg mL^{-1} of GDH-Tac. Full conversion of 10 mM of substrate was achieved within 1 h (Figure 4.14). Once the initial substrate was completely reacted, a fed-batch strategy was applied by supplying an additional 10 mM of substrate to the reaction mixture every hour up to a total of 150 mM of substrate.

Figure 4.14. Reaction course of the conversion of TMCH with soluble TmCHMO and soluble GDH-Tac (TmCHMO/GDH-Tac 1:2) with the concentration of substrate and product (left axis), and the volume of NaOH added (right axis). The total amount of substrate accumulated is shown (pink dotted line). The black dotted line indicates the initial rate of NaOH addition.

While the substrate was fully converted in 1 h for the first 3 substrate additions, the accumulation of unreacted substrate was observed for the rest of the reaction until a final substrate concentration of about 100 mM (Figure 4.14). This change in the enzymatic reaction rate was directly correlated to the amount of base needed to maintain the pH of the reaction to a constant value of 8, which is related to the amount of gluconic acid co-product formed and therefore the amount of substrate converted (Figure 4.14).

The conversion for each addition of substrate was calculated (Figure 4.15). A sharp decrease in conversion was observed after 4 sequential substrate addition until an average conversion of about 10 % was observed. This was attributed to the loss of enzymatic activity during the reaction, but substrate inhibition of TmCHMO as a consequence of substrate accumulation in the reaction mixture probably also played a role. Process metrics were analyzed for the fed-batch strategy using soluble enzymes. The total reaction time after 14 additions was 14.4 h with a final product amount of 0.308 g and a final unreacted substrate amount of 0.423 g. The biocatalyst yields reached 9.6 and 4.7 mg of product per mg of TmCHMO and GDH-Tac, respectively.

Figure 4.15. Sequential additions of substrate for the reaction with soluble TmCHMO and soluble GDH-Tac (TmCHMO/GDH-Tac 1:2) with conversion as a function of the number of substrate additions (conversion = $1 - ([\text{sub}]_f / [\text{sub}]_i)$ with $[\text{sub}]_f$ the substrate concentration before the next addition of substrate and $[\text{sub}]_i$ the substrate concentration after the last addition of substrate).

• Re-cycles with immobilized TmCHMO and GDH-Tac

The performance of TmCHMO and GDH-Tac, which were separately immobilized at high enzymatic loads, was also studied. The TmCHMO immobilized derivative contained 20 mg of TmCHMO per g of support, while the GDH-Tac derivative contained 29 mg of GDH-Tac per g of support. Aiming to compare the results with the soluble enzymes,

reactions with separately immobilized enzymes were carried out using the same load of TmCHMO (1.07 mg TmCHMO per mL of reaction). The ratio of TmCHMO/GDH-Tac was slightly lower (1.0:1.5) since it is determined by the maximum amount of immobilized derivative that can be used (10 % v v⁻¹ of support) to ensure a proper suspension and mixing, as well as the enzymes load per mg of support obtained during the immobilization processes.

Separately immobilized derivatives were used in the biooxidation reaction, where the first cycle took about 1.33 hour to reach full substrate conversion (Figure 4.16a). The increase in reaction time during the first cycle could be related to: (i) the lower amount of loaded GDH-Tac with the immobilized enzymes (the cofactor regeneration reaction could be the limiting step), (ii) diffusion limitations of the NADP(H) cofactor between the bead particles containing TmCHMO and GDH-Tac, and (iii) oxygen, glucose or TMCH mass transfer limitations due to potential hindered diffusion of these molecules in the support particles.

The operational stability of the biocatalysts was studied by re-using the same immobilized enzymes over several reactions or cycles. At the end of the reaction, both immobilized enzymes were recovered by filtrating the reaction mixture. These enzymes were reused in a new reaction for conversion of the substrate, using the same reaction conditions for all cycles. In total, the immobilized enzymes were reused up to 15 times aiming to compare the results with the data obtained using soluble enzymes for which 14 additions were carried out (Figure 4.16b). Full conversion was obtained for the first 5 cycles, after which the conversion slowly started to decrease.

Figure 4.16. Reuses of TmCHMO and GDH-Tac immobilized on separate supports (TmCHMO/GDH-Tac 1.0:1.5) with a) reaction profile for cycles 1, 6, 10, 15; and b) substrate conversion after 1.33 hour for all cycles. The vertical dotted lines indicate overnight storage of the immobilized enzymes in buffer solution.

Table 4.3. Overview of the performances of TmCHMO and GDH-Tac biocatalysts for the oxidation of TMCH.

Biocatalyst format	Ratio TmCHMO :GDH-Tac (mg:mg)	Total reaction time (h)	Product formed ^a (g)	Unreacted substrate ^a (g)
Soluble	1.0:2.0	14.4	0.308	0.423
Immobilized	1.0:1.5	20.0	0.422	0.199
Coimmobilized	1.0:0.5	17.5	0.538	0.138
Biocatalyst format	Average conv. ^b (%)	Biocatalyst yield ^c (mg product mg biocatalyst ⁻¹)		
		TmCHMO	GDH-Tac	Total
Soluble	51	9.6	4.7	3.1
Immobilized	73	13.1	9.1	5.4
Coimmobilized	83	16.8	34.0	11.2

^a Cumulated amount of product and unreacted substrate (sum of each cycle for the immobilized enzymes and value measured at the end of the reaction for the soluble enzymes). ^b Average conversion calculated for 15 cycles for the immobilized enzymes and for 14 substrate additions for the soluble enzymes. ^c Biocatalyst yield = mass of product / mass of biocatalyst (TmCHMO, GDH-Tac or TmCHMO + GDH-Tac).

The process metrics obtained using separately immobilized biocatalysts are shown in Table 4.3. Even though the total reaction time of the process was 1.4-fold higher, the average final product amount increased by 37 %. Moreover, the use of separately immobilized enzymes also improves the process performance by reducing by 2.1-fold the final unreacted substrate amount and increasing the TmCHMO biocatalyst yield by 36 %. The overall biocatalyst yield is increased by 74 % due to the better performances obtained with the separately immobilized biocatalysts, despite the lower GDH-Tac biocatalyst loading (70 % of the GDH-Tac loading of the reaction with the soluble enzymes).

• Re-cycles with coimmobilized TmCHMO and GDH-Tac

The performance of the enzymes that were coimmobilized at high loads was also studied (18.4 mg of TmCHMO per g of support and 9.1 mg of GDH-Tac per g of support). In order to compare the coimmobilized biocatalysts with the ones immobilized separately and the soluble enzymes, the amount of coimmobilized support used in the oxidation reaction was calculated so that the same amount of TmCHMO was applied in all cases (1.07 mg mL⁻¹). The ratio TmCHMO/GDH-Tac in this case (1.0:0.5) was determined by the ratio obtained during the coimmobilization process, where both enzymes compete for the same support.

For this bioconversion, the reaction time of one cycle was 1.17 h until full conversion of the substrate was achieved, 17 % higher compared to the soluble enzymes (Figure 4.17a). The higher reaction time compared to the soluble enzymes could be due to the lower GDH-Tac load or to mass diffusion restrictions, as already mentioned with the separately immobilized enzymes. However, even though a lower TmCHMO/GDH-Tac ratio was used when coimmobilized derivatives were used (1.0:0.5) compared to the separately immobilized enzymes (1.0:1.5), the reaction time was 12 % lower. Thus, the reduction of the reaction time of the coimmobilized derivative compared to the separately immobilized biocatalyst probably indicates that NADP(H) cofactor diffusional restrictions between bead particles is likely the main cause of reaction time increase with separately immobilized derivatives.

Operational stability studies were carried out with the coimmobilized derivatives during 15 cycles (Figure 4.17b). Compared to the biocatalysts immobilized separately, the coimmobilized biocatalysts performed much better with the reuses. A substrate conversion of 58 % was achieved for the last cycle (cycle 15) compared to 39 % substrate conversion obtained for the same cycle with the biocatalysts immobilized separately.

Figure 4.17. Reuses of coimmobilized TmCHMO and GDH-Tac (TmCHMO/GDH-Tac 1.0:0.5) with a) reaction profile for cycles 1, 6, 10, 15; and b) substrate conversion after 1.17 hour for all cycles. The vertical dotted lines indicate overnight storage of the immobilized enzymes in buffer solution.

• Overview of the performances of immobilized enzymes

Regarding the process metrics, coimmobilization in particular proved to be the best option of this biotransformation with higher average conversion over all reutilization cycles (83 %) despite the lower concentration of GDH-Tac in the reaction (Table 4.3). The highest biocatalyst yields and final average product amounts were achieved with the

coimmobilized enzymes. Compared to the separately immobilized enzymes, all process metric analyzed were improved: a 1.14-fold decrease in total process time, a 1.3-fold increase in the final average product amount, a 1.4-fold decrease in the amount of unreacted substrate, a 1.1-fold increase in average conversion, and a 1.3-fold increase in TmCHMO biocatalyst yield. The GDH-Tac biocatalyst yield was improved by 3.7-fold because the experiment with the coimmobilized enzymes achieved the best performances with the lowest GDH-Tac loading. Compared to the soluble enzymes for which a fed-batch strategy was applied, even though the total process time was slightly increased, the final average amount of product formed was improved by 1.7-fold, the unreacted substrate amount decreased by 3.1-fold, the average final conversion was increased by 1.6-fold, and the total biocatalyst yield was 3.6-fold higher. These values demonstrate the better performances of the coimmobilized enzymes in the target reaction studied compared to separately immobilized enzymes.

4.3 Conclusions

The goal of this chapter was to optimize the TmCHMO-biocatalyzed oxidation of TMCH, for which the cofactor regeneration was ensured by the use of GDH as coenzyme. The reaction was first optimized at 30 mL scale using soluble enzymes. The TmCHMO and GDH biocatalyst loadings could be reduced by almost a factor 10, without any loss of activity during fed-batch experiments. Possible oxygen limitation was investigated by changing the amount of dissolved oxygen present in the reaction mixture. The fastest reaction progress was obtained with increased air flow and reduced stirring rate, which also resulted in a favorable foaming reduction.

CSF was applied to keep the substrate concentration below inhibitory level. Compared to the results obtained in **Chapter 3** with the fused TmCHMO-PTDH biocatalyst, the product concentration was increased by a factor 4. These results were achieved with a substrate feeding rate of 15 mM h^{-1} and optimization of the biocatalyst loading as well as the time of substrate feed. This reaction was evaluated against two biphasic systems (in toluene and *n*-butyl acetate), for which significant amounts of unreacted substrate were found. This was attributed to premature protein denaturation induced by foaming and increased interphase between the aqueous phase and the organic solvent. The first gram-scale TmCHMO-biocatalyzed oxidation of TMCH was performed by upscaling the CSF reaction at 500 mL scale. A product concentration of about 38 g L^{-1} and a productivity of $1.35 \text{ g L}^{-1} \text{ h}^{-1}$ were achieved. In order to improve the process performance metrics of the reaction, the use of pure oxygen instead of air should be considered. Additionally, other enzyme formulations such as whole cells or (sonicated) fermentation broth could

be applied. Lastly, the reaction rate could potentially be increased by investigating other GDHs.

In addition, TmCHMO was for the first time successfully immobilized on a MANA-agarose support with the coenzyme GDH-Tac to ensure cofactor regeneration. The enzymes displayed good retention of activity in repeated reutilizations for the oxidation of the substrate, whether immobilized separately or coimmobilized. The latter biocatalyst format proved to be the most efficient, achieving 83 % conversion in average over 15 reutilization cycles. The higher reaction rate was attributed to more efficient diffusion of the NADP(H) cofactor between the two enzymes when coimmobilized on the same support.

4.4 Experimental section

Chemicals. 3,3,5-trimethylcyclohexanone (TMCH; 98 %, Sigma-Aldrich), glucose (> 99 %, Alfa Aesar), sodium phosphite dibasic pentahydrate (> 98 %, Sigma-Aldrich), hexadecane (99.5 %, TCI), methanol (Biosolve), *n*-butyl acetate (99.5 %, Alfa Aesar), toluene (Biosolve), ethyl acetate (Biosolve) and Celite (Acros Organics) were used as received. β -Nicotinamide adenine dinucleotide phosphate disodium salt (NADP⁺; 97 %, Alfa Aesar) and glucose dehydrogenase (GDH-105; Codexis) were stored at - 20 °C and solutions in phosphate buffer (25 mM, pH 8.0) were prepared fresh prior to use. *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC; $\geq$ 97 %, Sigma-Aldrich) was stored at - 20 °C. High-density aminoethyl 4BCL monoaminoethyl-*N*-aminoethyl functionalized agarose (MANA-agarose, density: 1.07 g mL⁻¹, Agarose Beads Technologies) was stored at 4 °C. The TMCL lactones used as standard were synthesized by chemical Baeyer-Villiger oxidation, which was performed according to a procedure reported in the literature.¹⁹

Recombinant production of TmCHMO and GDH-Tac in *Escherichia coli* (preparation of cell-free extracts). Cyclohexanone monooxygenase from *Thermocrispum municipale* (TmCHMO) and glucose dehydrogenase from *Thermoplasma acidophilum* (GDH-Tac) were recombinantly produced in *Escherichia coli* in a 20 L batch fermentor employing an *E. coli* K12 derivative and a pBAD/myc-HisC based expression vector. A 500 mL pre-culture prepared in standard Luria-Bertani (LB) medium and supplemented with 100 μ g mL⁻¹ neomycin was used to inoculate 20 kg of main culture medium. The fermentation was performed using terrific broth (TB) medium with glycerol. Pre-sterilized *L*-arabinose was added to the fermenter to a final concentration of 0.02 % w w⁻¹ after 2.5 h of inoculation. The cell material was harvested by centrifugation 4 h after inoculation of the fermenter. A cell free extract of the protein was prepared by adding 2 weight equivalents of potassium phosphate buffer (100 mM, pH 7.0) to 1 weight equivalent of *E. coli* wet cells. The cell suspension was sonicated with an ultrasound probe for 20 min with cooling on ice and then centrifuged to remove the cell-debris.

Determination of the TmCHMO and GDH-Tac enzyme content by Bradford assay and SDS-PAGE (soluble enzymes). The cell-free extracts containing TmCHMO and GDH-Tac were pre-clarified by centrifugation (3220 g for 15 min), and the total protein content was determined by means of a Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) using bovine serum albumin as standard. Enzyme content was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage 12 %, Invitrogen, USA) ran in a Mini-PROTEAN II apparatus (BioRad, USA) as described in the literature.²⁰ Low range protein markers were used for subunit molecular mass determination. Gels were stained using Coomassie G250 colloidal stain solution (34 % v v⁻¹ ethanol, 2 % v v⁻¹ H₃PO₄, 17 % w v⁻¹ NH₄SO₄ and 0.066 % Coomassie G250) and Image LABTM software (BioRad, USA) was used for image processing.

Determination of the activity of TmCHMO and GDH-Tac (soluble enzymes). TmCHMO activity was determined spectrophotometrically following NADPH consumption at 340 nm with cyclohexanone as a substrate ($\epsilon_{\text{NADPH}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). The mixture contained Tris/HCl (50 mM, pH 8.5), cyclohexanone (0.5 mM), NADPH (0.1 mM). One unit of TmCHMO activity (U) was defined as the amount of enzyme required to catalyze the conversion of 1 μmol of NADPH to NADP⁺ per min at 20 °C and pH 8.5.¹¹ GDH-Tac activity was determined spectrophotometrically at 340 nm following the NADPH (400 μM) formation using glucose (200 mM) as substrate in sodium phosphate buffer (100 mM, pH 8.0).¹⁵ The production of NADPH by unspecific enzymes present in the cell-free extract was determined by performing a blank reaction. This production rate was subtracted from the measurement with glucose. One unit of GDH-Tac activity (U) was defined as the enzyme required to convert 1 μmol of NADP⁺ per min at 30 °C. The absorbance was recorded using a spectrophotometer Cary 50 Bio UV-visible (Palo Alto, USA).

Preparation of immobilized TmCHMO and immobilized GDH-Tac. The **immobilization of TmCHMO** was carried out by suspending the support in 25 mM 2-*N*-(morpholino)ethanesulfonic acid (MES) buffer (pH 6.0). The enzyme was added to the suspension and left to adsorb ionically to the support for 0.25 h. EDC was added to final concentrations of 25 or 35 mM and incubated for 2 h. NaCl was added to a final concentration of 1 M and incubated for 1 h. The immobilized derivative was washed with distilled water and filtered. The **immobilization of GDH-Tac** was performed in 50 mM sodium phosphate buffer (pH 6.0). The enzyme was added to the suspension and left to adsorb ionically to the support for 0.25 h. EDC was added to final concentrations of 1, 3, 5, 10 or 15 mM and incubated for 1h. NaCl was added to a final concentration of 0.5 M and incubated for 0.5 h. The immobilized derivative was washed with 100 mM sodium phosphate buffer (pH 8.0) and filtered. The **coimmobilization of TmCHMO and GDH-Tac** was performed in 50 mM sodium phosphate buffer (pH 6.0). The enzyme were added to the suspension and left to adsorb ionically to the support for 0.25 h. EDC was added to final concentrations of 10 or 20 mM and incubated 1 h. NaCl was added to a final concentration of 1 M. The derivative was washed with distilled water and filtered. TmCHMO and GDH-Tac immobilized on MANA-agarose were stored at 4 °C prior to use.

Bioreactor set-up. Biocatalyzed reactions were performed in the same set-up as described in **Chapter 3**. In the case of biocatalyzed reactions with a total volume of 30 mL, the reactor was a double-walled vessel with a volume of 90 mL and with the temperature kept constant at 30 °C. In the case of the biocatalyzed reaction with a total volume of 500 mL, the reactor was a double-walled vessel with a volume of 1 L and with the temperature kept constant at 30 °C. The air flow was provided with a sintered frit (1.3 cm diameter $\times$ 2 cm length). The mixture was stirred with a IKA Eurostar Power control visc overhead stirrer equipped with a 4-blade glass propeller stirrer (5.5 cm diameter).

Determination of the reaction progress for biocatalyzed reactions using GC-FID. The substrate and product concentration were determined by GC-FID analysis in triplicate as described in **Chapter 3**. The following retention times were observed for kinetics samples measured from the reaction mixture: 6.58 min for the substrate TMCH, 9.18 min for the lactone TMCL-I, and 9.29 min for the lactone TMCL-II. Isolated products after product recovery were measured with a modified method in which the temperature was increased from 60 °C to 200 °C at 10 °C min⁻¹ (sample injected at 300 °C with a split ratio of 100, 1 μ L injection volume). The following retention times were observed: 7.42 min for the substrate TMCH, 11.16 min for the lactone TMCL-I, 11.36 min for the lactone TMCL-II, and 11.74 for the methyl esters TMCL-I' and TMCL-II'.

Typical biocatalyzed reactions with continuous substrate feeding (CSF) at 30 mL scale. Unless stated otherwise, the reaction vessel was loaded with NADP⁺ (250 μ M), glucose (375 mM), GDH-105 (0.1 mg mL⁻¹) and 10 % v v⁻¹ methanol, in phosphate buffer (25 mM, pH 8.0). The reaction mixture was stirred at 500 rpm and air was bubbled through at a rate of 16 mL min⁻¹. The TmCHMO biocatalyst was added (1.5 mL, 5 % v v⁻¹). Pure TMCH was fed continuously using a syringe pump (KDS Legato 110) with a feeding rate of 15 mM h⁻¹ for 16 hours for a total substrate volume of 1.138 mL (7.2 mmol, 240 mM). Reaction progress was followed by GC-FID analysis. The reaction was stopped after 24 h by addition of methanol (30 mL). The denatured protein was separated from the reaction mixture by centrifugation (5000 rpm, 20 min), and filtration of the supernatant over Celite followed by saturation with NaCl. Methanol was removed under rotary evaporation, and the remaining aqueous phase was extracted with ethyl acetate (3 $\times$ 30 mL). The organic phase was dried over MgSO₄ and solvent was removed by rotary evaporation to afford a colorless oil (0.302 g, 32 % isolated yield).

Typical biocatalyzed reactions in biphasic reactions. The reaction vessel was loaded with NADP⁺ (250 μ M), glucose (375 mM), and GDH-105 (0.1 mg mL⁻¹) in phosphate buffer (25 mM, pH 8.0). The reaction mixture was stirred at 250 rpm and air was bubbled through at a rate of 16 mL min⁻¹. TMCH (7.2 mmol, 1.138 mL) was dissolved in either toluene or *n*-butyl acetate (10 mL) and the organic phase was added to the aqueous phase. The TmCHMO biocatalyst was added (1.5 mL, 5 % v v⁻¹) to start the reaction. Reaction progress was followed by GC-FID analysis taking samples of both the aqueous and organic phases. The reaction was stopped after 26.5 hours by addition of organic solvent (30 mL, same solvent as that of the organic phase). The two phases were separated by centrifugation (5000 rpm, 30 min), and the organic phase was dried over MgSO₄ and removed

under rotary evaporation to afford a colorless oil (toluene as the organic phase: 0.409 g, 25 % yield; *n*-butyl acetate as the organic phase: 0.432 g, 22 % yield).

Typical biocatalyzed reactions with continuous substrate feeding (CSF) at 500 mL scale. The reaction vessel was loaded with NADP⁺ (250 μ M), glucose (375 mM), GDH-105 (0.1 mg mL⁻¹), TMCH (0.394 mL, 5 mM), and 10 % v v⁻¹ methanol, in phosphate buffer (25 mM, pH 8.0). The reaction mixture was stirred at 400 rpm and air was bubbled through at a rate of 30 mL min⁻¹. The TmCHMO biocatalyst was added (25 mL, 5 % v v⁻¹). Pure TMCH was fed continuously using a syringe pump (KDS Legato 110) with a feeding rate of 15 mM h⁻¹ for 16 hours for a total substrate volume of 18.912 mL (120 mmol, 240 mM). Reaction progress was followed by GC-FID analysis. Additional TmCHMO biocatalyst was added after 22 h (12.5 mL, 2.5 % v v⁻¹). The reaction was stopped after 28 h by addition of methanol (500 mL). The denatured protein was separated from the reaction mixture by centrifugation (5000 rpm, 20 min), washed with ethyl acetate (24 $\times$ 30 mL, 720 mL of ethyl acetate in total), and separated by centrifugation (5000 rpm, 20 min). The ethyl acetate solution was dried over MgSO₄, and the solvent removed under rotary evaporation to afford a yellow oil (0.364 g, 1.6 % yield). The mixture as analyzed by GC-FID consisted of the desired products TMCL-I and TMCL-II (84.15 %), the substrate TMCH (11.73 %), and the methyl esters TMCL-I' and TMCL-II' (4.12 %). Methanol contained in the supernatant was removed under rotary evaporation, and the remaining aqueous solution was filtered over Celite followed by saturation with NaCl. The aqueous solution was extracted with ethyl acetate (3 $\times$ 600 mL). The organic phase was dried over MgSO₄ and the solvent was removed by rotary evaporation to afford a yellow oil (13.023 g, 64.9 % yield) which consisted of the desired products TMCL-I and TMCL-II (93.0 %), the substrate TMCH (0.2 %), and the methyl esters TMCL-I' and TMCL-II' (6.8 %), as analyzed by GC-FID.

¹H and ¹³C NMR. Proton (¹H) and carbon (¹³C) NMR spectra were recorded according to the description of **Chapter 3**. Heteronuclear single quantum coherence spectroscopy experiments (HSQC) were recorded with 4 scans.

Kinetics of the formation of methyl esters TMCL-I' and TMCL-II' (model reaction). A mixture of lactones TMCL-I and TMCL-II prepared by chemical Baeyer-Villiger oxidation (1.055 mL) was dissolved in phosphate buffer (25 mM, pH 8.0) (25.945 mL) and methanol (3 mL). Additional methanol was added (30 mL) and the reaction mixture was left to react at room temperature, without stirring. The kinetics of the formation of the methyl esters was followed on GC-FID. Samples for analysis consisted of 300 μ L of reaction mixture diluted in 700 μ L of acetonitrile.

Bioreaction with repeated substrate addition (soluble TmCHMO and GDH-Tac biocatalysts). The reaction vessel was loaded with TMCH (10 mM, 47.4 μ L), NADP⁺ (250 μ M, 5.9 mg), glucose (350 mM), and 10 % v v⁻¹ of methanol for a total reaction volume of 30 mL. The reaction was started by the addition of a 3.07 % v v⁻¹ of soluble TmCHMO (0.921 mL of CFE containing 32.1 mg TmCHMO) and 4.87 % v v⁻¹ of soluble GDH-Tac (1.422 mL of CFE containing 65.8 mg soluble GDH-Tac). An additional 10 mM of substrate (47.4 μ L) was added every hour until a total of

140 mM of substrate. The reaction was stirred at 500 rpm, and air was bubbled in the reaction volume at a rate of 8 mL min⁻¹.

Reusability of immobilized TmCHMO and GDH-Tac biocatalysts. The reaction vessel was loaded with TMCH (10 mM, 47.4 µL), NADP⁺ (250 µM, 5.9 mg), glucose (350 mM), and 10 % v v⁻¹ of methanol for a total reaction volume of 30 mL. The reaction was started by the addition of 5 % v v⁻¹ of immobilized TmCHMO (20 mg TmCHMO per g support, 1.605 g of supported enzyme corresponding to 32.1 mg TmCHMO) and 5 % v v⁻¹ of immobilized GDH-Tac (29 mg GDH-Tac per g support, 1.605 g supported enzyme corresponding to 46.5 mg GDH-Tac). The reaction was stirred at 500 rpm, and air was bubbled in the reaction volume at a rate of 8 mL min⁻¹. The substrate and product concentration were determined by GC-FID analysis in triplicate. At the end of the reaction, the immobilized TmCHMO and immobilized GDH-Tac were filtered and washed with buffer. New reaction medium containing TMCH (10 mM), NADP⁺ (250 µM), glucose (30 mM) and 10 % v v⁻¹ of methanol was prepared; to which the immobilized TmCHMO and immobilized GDH-Tac rinsed with buffer were added to start the reaction. The supported enzymes were stored at 4 °C overnight after cycles 5 and 10.

Reusability of coimmobilized TmCHMO and GDH-Tac biocatalysts. The reactions were performed in a similar fashion as for the immobilized TmCHMO and GDH-Tac biocatalyst. The biocatalyst concentration was 5.4 % v v⁻¹ (18.4 mg TmCHMO and 9.1 mg GDH-Tac g⁻¹ support, 1.74 g of supported coimmobilized enzymes corresponding to 32.1 mg of TmCHMO and 15.83 mg of GDH-Tac).

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CHAPTER 5

Ring opening polymerization of alkyl substituted lactones: catalysts, reactivity & thermodynamics

Organocatalysts are increasingly gaining attention for the ring opening polymerization (ROP) of lactones due to numerous advantages. In this chapter, the goal is to expand the scope of catalysts for the ROP of alkyl substituted caprolactones: a mixture of β,δ -trimethyl- ϵ -caprolactones (TMCL). Several organocatalysts were evaluated, with a focus on bifunctional organophosphorus catalysts including two novel categories, namely phosphonic acids and phosphinic acids. The effects of initial monomer concentration, temperature, and catalyst loading on the monomer conversion at equilibrium and the reaction rate were addressed. During polymerization, higher conversion and reaction rate were observed for the most substituted lactone TMCL-I (proximal lactone). This difference in reactivity between the two regio-isomeric lactones was greater for lower reaction temperatures. Density functional theory (DFT) calculations on the ring opening of the lactones demonstrated that the difference of reactivity can be attributed to a lower enthalpic contribution of the proximal lactone making the ring opening of this lactone more energetically favored. The difference in calculated Gibbs free energy was found to increase with lower temperature.

Keywords: ring opening polymerization • organocatalyst • thermodynamics • reactivity • DFT calculations

5.1 Introduction

Organocatalysis, *i.e.* the use of metal-free molecules as catalysts, has been receiving increasing attention for the ring opening polymerization (ROP) of a broad range of cyclic monomers including lactide and (un)substituted lactones of various ring size.¹⁻³ These catalysts give low dispersities as a result of controlled polymerization. Additional advantages are lower reaction temperature compared to metal-based catalysts, and tolerance to water and oxygen.² In general, precise macromolecular architecture can be obtained with organocatalysts due to the absence of side-reactions.⁴ H-bonding organocatalysts in particular have attracted attention due to the versatility of their structure and mechanism.² Two main mechanisms are reported for the organocatalyzed ROP of lactones:

- (i) **Activation of the initiator/chain-end:** the formation of an H-bond between the organobase and the initiator/chain-end increases the nucleophilicity of the latter and facilitates nucleophilic attack on the carbonyl bond of the monomer. This mechanism was reported for strong bases such as the phosphazene superbases series.⁵⁻⁶
- (ii) **Bifunctional activation of the monomer and initiator/chain-end:** two H-bonds are involved. The first one aims at activating the initiator/chain-end by increasing its nucleophilicity while the second activates the monomer by H-bonding of the carbonyl bond which increases its electrophilicity. This dual activation mechanism requires catalysts that have both an acid and basic function. This is the case of catalytic systems consisting of an acid/base pair, for example alkoxide/cyclic amides,⁷ (sulfon)amide/amines,⁸ phenol/amines,⁹ or thiourea/amines,¹⁰ for which the mechanism depends on the pK_a of the pair.¹¹ Bifunctional catalysts, which have the advantage of bearing both functions, operate *via* a concerted mechanism. Sulfonic acids,¹²⁻¹³ (imido)phosphoric acids,¹⁴⁻¹⁵ and guanidines such as 1,5,7-triazabicyclo[4.4.0]dec-5-ene¹⁶ (TBD) are just a few examples of bifunctional catalysts. These organocatalysts typically exhibit a ‘proton shuttle behavior’, that is characterized by the transfer of a proton from the catalyst to the initiator or monomer and back to the catalyst, as elucidated by computational calculations using the density functional theory (DFT).¹⁷⁻¹⁹

Alkyl substituted lactones are particularly relevant for the synthesis of aliphatic polyesters. As was shown in **Chapter 2**, lactones with additional alkyl substituents give polyesters which are usually amorphous with glass transition temperatures (T_g) below room temperature,²⁰ while polyesters from unsubstituted lactones such as δ -valerolactone

or ϵ -caprolactone are typically semi-crystalline. Substituents also have an effect on the reactivity of lactones, which is directly related to their structure, therefore influencing their thermodynamic properties. The number, size and position of substituents all influence the reactivity of lactones during ROP, in addition to the ring size of the lactone.²¹ A comprehensive study on δ -valerolactones with various degree of substitution demonstrated that the reaction rate is particularly influenced by the position of the substituents.²⁰

Most alkyl substituted lactones studied for ROP are monosubstituted, with the exception of some biobased lactones such as menthide²² and carvomenthide.²³ The polymerization of lactones with several substituents such as β,δ -trimethyl- ϵ -caprolactones (TMCL) has been less investigated. This mixture of methyl substituted lactones can be synthesized chemically or enzymatically by Baeyer-Villiger oxidation as demonstrated in **Chapters 3** and **4** (Scheme 5.1).

So far, very few catalysts are known to polymerize TMCL. In fact, metal-based catalysts such as tin octoate ($\text{Sn}(\text{Oct})_2$) and titanium butoxide ($\text{Ti}(n\text{-OBu})_4$) are the only two catalysts reported for the homopolymerization of TMCL (Scheme 5.2).²⁴ These catalysts proceed *via* a coordination-insertion mechanism (see Figure 2.3 for the mechanism).²⁵

Scheme 5.1. Synthesis of the mixture of β,δ -trimethyl- ϵ -caprolactones (TMCL) by Baeyer-Villiger oxidation and ROP of TMCL. The monosubstituted position is indicated with a blue ball and the disubstituted position with a red ball.

Organocatalysts evaluated in this chapter:

Superbase organocatalysts

P₄-t-Bu phosphazene
 $pK_a, \text{MeCN} = 42.7^{26}$

P₂-Et phosphazene
 $pK_a, \text{MeCN} = 32.9^{26}$

Bifunctional organocatalysts

1,5,7-triazobicyclo-
[4.4.0]dec-5-ene
TBD
 $pK_a, \text{MeCN} = 26^{27}$

p-toluene sulfonic acid
p-TSA
 $pK_a = -2.8^{28}$

Phosphoric acids

bis(4-nitrophenyl)phosphate
P1 (BNPP)
 $pK_a, \text{DMSO} = 1.77^{29}$

dimethyl phosphate
P2
 $pK_a = 1.29^{30}$

diphenyl phosphate
P3 (DPP)
 $pK_a = 2.04^{31}$

Phosphonic acids

methyl phosphonic acid
P4
 $pK_{a1} = 1.50^{32}$

phenylphosphonic acid
P5
 $pK_{a1} = 1.83^{33}$

pinacolyl methylphosphonate
P6
 $pK_{a1} = 2.34, 2.50^{34-35}$

ethyl methylphosphonate
P7
 $pK_{a1} = 2.19, 2.76^{34-35}$

t-butylphosphonic acid
P8
 $pK_{a1} = 2.79^{36}$

Phosphinic acids

bis(pentafluorophenyl)-
phosphinic acid
P9

diphenyl phosphinic acid
P10
 $pK_a = 2.32^{37}$

dimethyl phosphinic acid
P11
 $pK_a = 3.08^{36}$

Metal-based catalysts from previous work²⁴:

Tin(II)-2-ethylhexanoate
Sn(Oct)₂

Ti(IV)-*n*-butoxide
Ti(O-*n*-Bu)₄

Scheme 5.2. Catalysts investigated for the polymerization of TMCL. When not specified, the pK_a values were measured in aqueous solutions. The pK_a values of **P4-P8** correspond to the first deprotonation.

The goal of this chapter is therefore to explore organocatalysts for the ROP of TMCL using superbases and bifunctional organoacids and organobases of various strength (Scheme 5.2). One class of organocatalysts in particular is investigated: organophosphorus compounds. Inspired by the catalyst activity in ROP of some phosphoric acids such as diphenyl phosphate (DPP),^{20, 38} *p*-nitrodiphenyl phosphate (BNPP),²⁹ and other aromatic and cycloaliphatic derivatives,³⁹⁻⁴⁰ the potential of phosphonic acids and phosphinic acids as catalysts for the ROP of TMCL is explored (Scheme 5.2).

Phosphoric acids have been reported to proceed *via* a dual activation mechanism involving a cationic activated monomer (see Figure 2.3).^{17, 20} The mechanism comprises the formation of a strong H-bond between the hydrogen donor P-OH group, and the carbonyl of the lactone, making the monomer more electrophilic. In addition, a weak H-bond between the basic phosphoryl P=O moiety and the hydroxyl group of the initiator/propagating center is formed, which increases the nucleophilicity of the latter. Cooperative activation of monomer and initiator/chain-end leads to the formation of an 8-membered ring structure with the proton of the acid catalyst acting as a proton shuttle.¹⁷

Phosphonic acids and phosphinic acids, which also possess these two moieties, have however so far not been reported for the ROP of lactones. For similar substitution groups, the pK_a is expected to decrease with the number of P-OR bonds (R = alkyl, aryl, or H). Consequently, it is predicted that the activity in ROP follows the strength in acidity, from phosphinic acid to phosphonic acid and phosphoric acid, with the latter being the most active (Scheme 5.2). Additionally, the pK_a is expected to decrease further with electro-deficient substitution groups, which leads to higher catalytic activity.

5.2 Results and discussion

The alkyl substituted lactones which are investigated in this work are synthesized by Baeyer-Villiger oxidation, yielding a mixture of two regio-isomeric lactones (Scheme 5.1). TMCL-I is the most substituted lactone (proximal lactone), with two methyl groups on the δ -position and one methyl on the β -position. TMCL-II is the least substituted lactone (distal lactone), with one methyl group on the δ -position and two methyl groups on the β -position. The composition, as determined by NMR analysis, shows that the chemical synthesis favors the formation of the most substituted lactone with a ratio of 55:45 (TMCL-I:TMCL-II) (Figure 5.1). During polymerization, the conversion cannot be determined by ¹H NMR due to overlapping peaks of the lactones and polymer (Figure 5.2).

Figure 5.1. NMR spectra of TMCL in CDCl_3 with a) ^1H NMR and b) ^{13}C NMR.

The conversion was therefore determined by GC-FID based on the remaining amount of monomer in the reaction mixture relative to the amount of hexadecane that was used as internal standard (Figure 5.3). The ROP of TMCL gives an amorphous polyester with

Figure 5.2. NMR spectra of poly(TMCL) in CDCl_3 with a) ^1H NMR and b) ^{13}C NMR.

a low glass transition temperature (T_g around -63°C for poly(TMCL) of 2.5 kg mol^{-1} as determined by DSC – it should be noted that the polymer still contained some monomer).

Figure 5.3. Example of GC-FID trace of the mixture of TMCL-I and TMCL-II during polymerization with the composition at the start of the polymerization and during the polymerization.

5.2.a. Effect of temperature and initial monomer concentration

The ROP of TMCL was first evaluated with catalysts that are reported to polymerize unsubstituted lactones (Table 5.1). TMCL can be polymerized in bulk by several categories of catalysts over a broad range of temperatures. While the organocatalysts, namely *p*-TSA, TBD and *P*₄-*t*-Bu, were active at room temperature this was not the case of Sn(Oct)₂ which could only catalyze the ROP of TMCL at 130 °C. Ti(*n*-OBu)₄, which was used with less than 1 equivalent because of the mechanism of this metal-based catalyst, was also able to polymerize TMCL at 130 °C. The most acidic catalyst of this

Table 5.1. ROP of TMCL with bifunctional catalysts, superbases, and metal-based catalysts.

	Catalyst	Ratio I/TMCL/C ^a	Time ^b (h)	<i>T</i> (°C)	[TMCL] ₀ (mol L ⁻¹)	Conv. ^c (%)	<i>M</i> _{n,GPC} ^d (kg mol ⁻¹)	<i>Đ</i> _M ^d
1	<i>p</i> -TSA	1/30/1	72	23	5.29	61	3.2	1.37
2	TBD	1/30/1	5	30	5.29	30	3.1	1.72
3	<i>P</i> ₂ -Et	1/30/1	5.3	23	5.29	71	5.3	1.24
4	<i>P</i> ₄ - <i>t</i> -Bu	1/30/1	2	30	5.29	n.d.	5.4	1.94
5		1/30/1	1	30	2.01 ^e	35	2.3	1.76
6		1/30/1	5	- 20	2.01 ^e	59	3.8	1.77
7		1/30/1	22	- 50	2.01 ^e	71	5.3	2.15
8		1/30/1	22	- 20	3.99 ^e	85	5.5	1.96
9	Sn(Oct) ₂	1/30/1	22	25	5.29	0	-	-
10		1/30/1	6	130	5.29	n.d.	3.9	1.28
11	Ti(<i>n</i> -OBu) ₄	1/30/0.42	30.5	130	5.29	n.d.	3.3	1.56

^a I initiator (BnOH), C catalyst. ^b Reaction time at maximum conversion. ^c Average conversion of TMCL-I and TMCL-II calculated by GC-FID. ^d Measured by GPC in THF with polystyrene standards. ^e THF as solvent. n.d. not determined.

selection, *p*-TSA, gave a relatively high conversion and low dispersity. When replaced by the base TBD, a faster reaction time was observed but only half of the conversion could be achieved. The highest conversion and polymerization rates were obtained with the P_4 -*t*-Bu phosphazene superbase with conversions up to 85 %. This is not surprising giving the extreme basicity, and therefore activity, of this catalyst, which has been reported to polymerize thermodynamically challenging lactones such as γ -butyrolactone.⁴¹⁻⁴²

This increased activity however led to high dispersities due to side-reactions ($1.76 < D_M < 2.15$) (Figure 5.4a). Because P_4 -*t*-Bu-catalyzed ROP takes place according to a chain-end activation mechanism, reactions such as back-biting lead to the formation of macrocycles whose presence was confirmed by MALDI-ToF MS (Figure 5.4b-c). In comparison, P_2 -Et, which is another phosphazene superbase, gave comparable conversions at room temperature with a relatively low dispersity as a consequence of its lower basicity (71 % conversion) (Table 5.1, entry 3).

Figure 5.4. P_4 -*t*-Bu catalyzed ROP of TMCL (Table 5.1, entry 4) with a) GPC, b) and c) MALDI-ToF MS spectrum, with benzyl alcohol initiated structures (blue) and macrocyclic structures (red).

Because the ROP of lactones is thermodynamically driven, the monomer conversion at equilibrium is influenced by two factors, namely the reaction temperature and the initial monomer concentration.²¹ From the polymerizations performed at various temperatures using $P_4-t\text{-Bu}$ as catalyst, it is clear that higher monomer conversions at equilibrium are achieved by decreasing the reaction temperature (Table 5.1, entries **5-7**). The conversion was almost doubled by decreasing the temperature by 80 °C, from 30 °C to - 50 °C. Such reaction temperatures require the use of a solvent, without which the reaction temperature is limited by the freezing temperature of the monomer. However, decreasing the initial monomer concentration results in lower monomer conversion at equilibrium, as shown with the two polymerizations performed at - 20 °C (Table 5.1, entries **6** and **8**). In the case of TMCL, the highest conversion was achieved by doubling the initial monomer concentration to 4 M (Table 5.1, entry **8**).

5.2.b. Organophosphorus catalysts

- **Screening of phosphoric, phosphonic and phosphinic acids**

Organophosphorus catalysts were evaluated for the ROP of TMCL. Polymerizations were performed in bulk at room temperature. Only some of the most acidic catalysts, namely **P3** (DPP) and the fluorosubstituted phosphinic acid **P9**, were able to catalyze ROP under these conditions (Table 5.2, entries **5-7** and **19**). For all other organophosphorus catalysts evaluated, the polymerization did not proceed at this temperature. This was due to a lack of initiation since benzyl alcohol, which is the initiator, did not react according to kinetics measured by GC-FID. In order to enable initiation, polymerizations were performed at 80 °C. At this temperature, all catalysts were able to promote initiation of benzyl alcohol. Most of the initiator reacted within one day, after which the reaction temperature was slowly decreased to room temperature. Decreasing the temperature to room temperature enables comparing the conversion obtained between the reactions initiated at 80 °C and the reactions performed at room temperature only. Indeed, monomer conversion depends on the temperature of the reaction, with lower reaction temperatures favoring higher monomer conversions.²¹ This effect is explained in more details in the next sections and the difference of conversion for the polymerization of TMCL is shown in Figure 5.10a. All catalysts were active at 80 °C. Although the conversion could not always be determined, the formation of a polymer was confirmed by GPC. Relatively low dispersities (typically $D_M < 1.35$) were obtained with the organophosphorus catalysts evaluated in this chapter.

Phosphoric acids are clearly more reactive than the corresponding phosphinic acids with similar structures: the dimethoxy substituted phosphate **P2** is more active than the

dimethyl substituted phosphinic acid **P11**, similarly the diphenoxy substituted **P3** (DPP) is more active than the diphenyl substituted phosphinic acid **P10**. This is due to the increased acidity (*i.e.* lower pK_a values) of phosphoric acids compared to phosphinic acids, which is a consequence of the strong - I inductive effect of RO- groups compared to weak + I inductive effect of alkyl groups.⁴³⁻⁴⁴ Indeed, acidity is enhanced by the presence of electron-withdrawing groups, by effect of charge stabilization of the corresponding conjugate base.

Table 5.2. ROP of TMCL catalyzed by organophosphorus catalysts.

	Catalyst	Ratio I/TMCL/C ^a	Time ^b (h)	T ^c (°C)	Conv. ^d (%)	M _{n, GPC} ^e (kg mol ⁻¹)	D _M ^e
1	P1	1/30/1	2	23	6	-	-
2	(BNPP)	1/30/1	2	80→23	52	4.8	1.70
3	P2	1/30/1	68	23	3	-	-
4		1/30/1	22	80→23	38	3.9	1.39
5		1/30/0.5	73	23	54	2.4	1.28
6	P3	1/30/1	101	23	72	3.9	1.23
7	(DPP)	1/30/2	53	23	70	3.8	1.29
8		1/30/1	25.5	80→23	70	4.3	1.70
9	P4	1/30/1	20	23	0	-	-
10		1/30/1	21	80→23	n.d.	1.1	1.28
11	P5	1/30/1	6	23	0	-	-
12		1/30/1	25	80→23	n.d.	2.1	1.23
13	P6	1/30/1	20	23	0	-	-
14		1/30/1	28	80→23	n.d.	1.3	1.26
15	P7	1/30/1	1	23	0	-	-
16		1/30/1	45.5	80→23	n.d.	1.7	1.28
17	P8	1/30/1	2	23	0	-	-
18		1/30/1	28	80→23	n.d.	1.1	1.34
19	P9	1/30/1	48	23	66	3.1	1.34
20		1/30/1	2	80→23	56	3.6	1.38
21	P10	1/30/1	6	23	0	-	-
22		1/30/1	45.5	80→23	n.d.	1.8	1.30
23	P11	1/30/1	1	23	0	-	-
24		1/30/1	27	80→23	n.d.	0.6	1.58

All polymerizations were performed in bulk ([TMCL]₀ = 5.29 M). ^a I initiator (BnOH), C catalyst. ^b Reaction time at maximum conversion. ^c 80→23: temperature of 80 °C for about one day then decreased to room temperature. ^d Average conversion of TMCL-I and TMCL-II calculated by GC-FID. ^e Measured by GPC in THF with polystyrene standards. n.d. not determined.

Although the reactivity in ROP is expected to be directly related to the acidity of the organocatalyst, *i.e.* phosphoric acids > phosphonic acids > phosphinic acids, it is not possible to draw a more general trend for the catalysts evaluated in this work. One reason may be that the acidity range of the organophosphorus catalysts selected is rather limited ($1 < \text{p}K_{\text{a}} < 3.08$) compared to organocatalysts which are in general either strong acids such as *p*-TSA ($\text{p}K_{\text{a}} = -2.8$) or superstrong bases such as $\text{P}_4\text{-}t\text{-Bu}$ ($\text{p}K_{\text{a}} = 30.25$).

The effect of the catalyst loading on the reaction rate was studied by varying the amount of **P3** (DPP) from 0.5 to 2 equivalents, relative to the amount of initiator (Table 5.2, entries **5-7**). Although the amount of catalyst did not change the monomer conversion at equilibrium (about 70 % for all reactions), an increased catalyst loading resulted in an increased reaction rate (Figure 5.5). The low dispersities obtained for all catalyst loadings indicates that transesterification reactions are limited with this catalyst, which is probably related to its bifunctional activation mechanism. The first order kinetics suggests that increasing the catalyst loading also increases termination, as can be seen from the deviation from the linear first order kinetics (Figure 5.5b).

Figure 5.5. Effect of the P3 (DPP) catalyst loading on the polymerization kinetics (Table 5.2, entries **5-7**) with a) monomer conversion, and b) $\ln([M]_0/[M])$ as a function of time (the dotted line indicates the initial reaction rate).

• Mechanism investigation

In order to confirm that the organophosphorus catalysts evaluated in this work are bifunctional catalysts, the mechanism was investigated by NMR (Scheme 5.3). The alcohol proton of benzyl alcohol was shown to be deshielded in the presence of the phosphinic acid catalyst **P9** by ^1H NMR. Shifts of 0.85 ppm and 0.90 ppm were observed at room temperature and at 80 °C, respectively (Figure 5.6a-c). Polymerization of TMCL catalyzed by **P9** was observed for both these temperatures (Table 5.2, entries **19** and **20**).

The position of the carbonyl carbon of the TMCL lactones was also shifted downfield in the presence of the catalyst as was observed by ^{13}C NMR (Figure 5.6b-d). This shift was slightly increased from room temperature to 80 °C. Interestingly, a higher shift was obser-

Scheme 5.3. Investigation of the H-bonding between the phosphinic acid **P9**, benzyl alcohol and TMCL (^1H and ^{13}C NMR performed in $\text{C}_2\text{D}_2\text{Cl}_4$).

Figure 5.6. Dual activation of the catalyst **P9** with a) ^1H NMR at RT, b) ^{13}C NMR at RT, c) ^1H NMR at 80 °C, d) ^{13}C NMR at 80 °C in $\text{C}_2\text{D}_2\text{Cl}_4$, with interaction with benzyl alcohol and TMCL.

ved for TMCL-II in both cases. These experiments suggest that the phosphinic acid **P9** proceeds *via* a bifunctional mechanism involving two H-bonds, the first one to deprotonate the initiator/end-chain, and the second one to increase the electrophilicity of the carbonyl of the lactones.

5.2.c. Reactivity of TMCL: effect of the position of the alkyl substituents

It is well known that the reactivity of lactones upon ROP is influenced by their structure.²¹ Kinetic studies of alkyl-substituted δ -valerolactones showed that the substitution position has an important effect on the reaction rate.²⁰ Although both regioisomeric lactones TMCL-I and TMCL-II only differ by the position of one methyl group (in β - or δ -position), they display different reactivity. The higher reactivity of TMCL-I (most substituted lactone) was already observed for two metal-based catalysts but only for specific reaction conditions (130 °C in bulk, with an initial ratio of lactones of 70:30 TMCL-I:TMCL-II).²⁴ During polymerization, the composition of the remaining monomers changes as a function of the average TMCL conversion. This behavior was observed with several types of catalyst, including a strong base as well as bifunctional organo-catalysts of various acidity and basicity (Figure 5.7a).

The monomer mixture becomes enriched in TMCL-II as the polymerization proceeds, meaning that TMCL-I is more incorporated in the polymer (Figure 5.7a). While the initial

Figure 5.7. a) Remaining TMCL monomer composition as function of the average TMCL conversion with TMCL-I (full symbols) and TMCL-II (empty symbols) for polymerizations performed at 30 °C (Table 5.1, entry 2 and 8; Table 5.2, entry 6) , and b) composition in remaining TMCL-I monomer at equilibrium as a function of the reaction temperature (Table 5.1, entries 5-7 and 8 for P_4 -*t*-Bu, conversions are shown in Figure 5.10 for DPP).

ratio was slightly in favor of the most substituted lactone (55:45 TMCL-I:TMCL-II), the monomer mixture was enriched in the least reactive lactone upon polymerization (TMCL-II) until equilibrium was reached. This polymerization behavior, namely the higher reactivity of TMCL-I compared to TMCL-II, was observed for all catalysts evaluated. In particular, higher conversions were observed for this lactone, as is visible from the reaction profiles shown in Figure 5.8.

Figure 5.8. Polymerization profiles of TMCL catalyzed by a) **P3** (DPP) (23 °C, [TMCL]₀ = 5.29 M; Table 5.2, entry **6**), b) **P4-*t*-Bu** phosphazene (-20 °C, [TMCL]₀ = 4 M in THF; Table 5.2, entry **8**), and c) **TBD** (30 °C, [TMCL]₀ = 5.29 M; Table 5.2, entry **2**).

These experiments show that a difference of reactivity was observed for different types of catalysts (metal-based catalysts, superbases and bifunctional organocatalysts), and therefore different types of ROP mechanisms. The extent to which TMCL-I is more reactive depends on the catalyst used and the temperature. Interestingly, the difference of reactivity between the two regio-isomeric lactones was greater at lower temperature (Figure 5.7b). For the same reaction conditions (catalyst, initial monomer concentration, and initial ratio of lactones), the conversion of TMCL-I was substantially increased when

the temperature was decreased, meaning that less TMCL-I was observed in the remaining monomer reaction mixture. This could be due to the higher thermal energy decreasing the energy of the transition state of both lactones, thereby decreasing the difference between them. Moreover, it seems that the difference of reactivity is further accentuated by increasing the initial monomer concentration since the P_4 -*t*-Bu-catalyzed polymerization performed at - 20 °C gives a lower percentage of TMCL-I at equilibrium for $[TMCL]_0 = 4$ M compared to 2 M (Figure 5.9). The lower amount of TMCL-I at equilibrium at lower temperature could be due to an intrinsic difference of reactivity of the two lactones depending on the temperature. It could also be the consequence of the higher monomer conversion at equilibrium that can be achieved at low temperature.

Figure 5.9. Amount of TMCL-I in remaining monomer mixture at equilibrium as a function of monomer conversion at equilibrium and temperature (Table 5.1, entries **5-7** and **8** for P_4 -*t*-Bu, conversions are shown in Figure 5.10 for DPP).

5.2.d. Determination of the thermodynamic parameters of the ROP of TMCL

It is well known that ROP is a reversible reaction whose equilibrium is governed by thermodynamics. During polymerization, the monomer conversion at equilibrium is dependent on the temperature, and the initial monomer concentration, as is shown by the experiments discussed in this chapter. For a given monomers, an increase in temperature results in a decrease in the monomer conversion at equilibrium and thus an increase in monomer concentration at equilibrium. The ceiling temperature T_c is defined as the temperature at which the monomer concentration at equilibrium is equal to the initial monomer concentration. At this temperature, the polymerization and depolymerization rates are equal meaning that polymerization is as favorable as depolymerization, resulting

in no observable monomer conversion. Below the T_c , polymerization takes place until the monomer concentration reaches the monomer concentration at equilibrium. Above the T_c , polymerization is thermodynamically unfavored. The T_c is highly dependent on the initial monomer concentration, with higher monomer conversions being reached for concentrated polymerizations, preferably in bulk. This ceiling temperature is applicable to monomers with a negative enthalpy of polymerization (ΔH_p) and a negative entropy of polymerization (ΔS_p), *i.e.* lactones that are strained but suffer from a loss of conformational freedom upon polymerization.^{21, 45}

The structure of lactones has a crucial effect on the thermodynamic properties during ROP because it influences the T_c and the monomer concentration at equilibrium. The ring size, degree of substitution and position of the substituents are particularly important.²¹ The effect of substitution on T_c was demonstrated with a series of δ -substituted-valerolactones.²⁰ While an increase in the alkyl chain length resulted in a moderate decrease in T_c , the position of the methyl substituent had a dramatic effect with the T_c , increasing from 170 °C to 560 °C when the methyl substituent was shifted from the δ -position to the α -position. The ceiling temperature of the mixture of TMCL-I and TMCL-II was determined by measuring the monomer conversion at equilibrium of polymerizations performed in bulk at different temperatures (Figure 5.10a).

As expected, the monomer conversion at equilibrium increased when the temperature decreased. The thermodynamic parameters, namely ΔH_p the enthalpy of polymerization, ΔS_p the entropy of polymerization, and T_c the ceiling temperature, are determined by a Van't Hoff analysis.²¹ ΔH_p and ΔS_p are deduced from the equation giving the monomer concentration at equilibrium (Figure 5.10b) (**Eq 5.1**):

(Eq 5.1)

$$R \cdot \ln\left(\frac{[M]_{eq}}{[M]_{ss}}\right) = \frac{\Delta H_p^0}{T} - \Delta S_p^0$$

With T the temperature at which the polymerization is conducted, and $[M]_{ss}$ the standard state monomer concentration.

Several assumptions were made to determine ΔH_p and ΔS_p :

- The reaction volume does not change upon polymerization.
- The ring strain, which is a measure of the enthalpy of polymerization (ΔH_p), remains unaffected by the monomer concentration and solvent, meaning that $\Delta H_p^0 = \Delta H_p$.
- The entropy and enthalpy of polymerization (ΔS_p and ΔH_p) remain constant over a large range of temperatures.
- The reactivity of an active chain-end does not depend on the degree of polymerization.

Figure 5.10. Determination of the thermodynamic parameters of TMCL with a) monomer conversion at equilibrium as a function of the temperature (in bulk), b) van 't Hoff analysis with ΔH_p determined from the slope and ΔS_p determined from the intersection with the y-axis (in bulk), and c) extrapolated conversion at equilibrium as a function of the temperature for polymerizations in bulk (full line) and at [TMCL]₀ = 1 M (dotted line). Reaction conditions: benzyl alcohol/monomer/DPP 1/30/1. Conversion determined by GC-FID using hexadecane as internal standard.

The T_c was extrapolated from a theoretical conversion-temperature curve, which depends on ΔH_p and ΔS_p determined experimentally, and which extends beyond the temperature range that was considered experimentally (Figure 5.10c). The ΔH_p , ΔS_p , and T_c values for bulk polymerizations and polymerizations at 1 M are given in Table 5.3 for TMCL. The thermodynamic properties of other relevant lactones as reported by the literature are indicated. The ΔS_p of polymerization with $[M]_0 = 1$ M can be extrapolated using the equation below (Eq 5.2):

(Eq 5.2)

$$\Delta S_p^0 = \Delta S_p - R \ln[M]$$

The negative ΔH_p and ΔS_p are quite typical of ROP of alkyl substituted lactones.²⁰⁻²¹ They confirm that ROP is driven by enthalpy, which is the consequence of the release of ring strain upon ring opening, and disfavored by entropy. As expected, the ceiling temperature

in bulk is higher than that at lower initial monomer concentrations. For TMCL, the T_c at 1 M is about 34 °C while it reaches 302 °C in bulk (Figure 5.10c). This outlines the importance of performing polymerizations with the highest initial monomer concentration possible. It should be noted that the thermodynamic behavior as shown in Figure 5.10c is however limited in practice. It is indeed not possible to perform the polymerization in bulk below the freezing temperature of the lactones. Similarly, the upper limit in temperature is the thermal degradation of the lactones.

Table 5.3. Thermodynamic properties of TMCL at bulk concentration and at 1 M (extrapolated) as compared to other monomers. δ -UDL, δ -undecalactone; δ -VL, δ -valerolactone; ϵ -CL, ϵ -caprolactone.

Monomer	[M] ₀ (mol L ⁻¹) ^a	State ^b	ΔH_p (kJ mol ⁻¹)	ΔS_p (J mol ⁻¹ K ⁻¹)	T_c (°C)	Ref
TMCL	5.29	l/a	- 9.1	- 15.8	302	This work
	1	s/s	- 9.1	- 29.7	34	This work
Menthide	1	s/s	- 16.8 ± 1.6	- 27.4 ± 4.6	334	²²
ϵ-CL	8.7	l/c	- 28.8	- 35.9	530	⁴⁵
	1	s/s	- 28.8	- 53.9	261	⁴⁵
δ-UDL	5.17	l/a	- 16.9	- 41.3	135	This work
	1	s/s	- 16.9 - 18.4 ± 0.5	- 54.9 - 58 ± 2	34 44	This work ²⁰
δ-VL	10	l/c	- 12.2	- 9.5	1018	⁴⁶
	1	s/s	- 12.2	- 28.6	153	⁴⁶

^a The values at 1 M were extrapolated from the values in bulk using the change of entropy as a function of the temperature (Eq 5.2). ^b State of the monomer/polymer: l liquid monomer, s solid monomer, a amorphous polymer, c semi-crystalline polymer, and s polymer and monomer in solution. The determination of the thermodynamic parameters of δ -UDL is described in Appendix A.

5.2.e. Computational studies (DFT calculations): reactivity of the lactones and thermodynamics

A computational study was performed in order to gain insight into the apparent higher reactivity of TMCL-I, which is more pronounced with lower temperature. The theoretical study was carried out using a density functional theory (DFT). In order to model the ring opening of the lactones, the most stable conformer of each lactone was first identified. Four stable conformers were selected, whose ring possess a chair-like conformation (conformers C and C') or a boat-like conformation (conformers B and B') (Figure 5.11). The atomic coordinates of the optimized geometries of all conformers are given in **Appendix B**. The chair-like and boat-like structures are the 7-membered ring analogues of the chair and boat conformation of the cyclohexane ring. In general, the stability of conformers is related to the stability of the ring, which is influenced by the interactions between groups on the ring. In this case, two types of interactions influence the stability: repulsion between methyl substituents, and repulsion between methyl substituents and the ester group.

According to the calculations, both lactones readily adopt a chair-like conformation. In conformation C, the ester group and one of the methyl groups are pointing in the same direction while the two other methyl groups are in pseudo-equatorial position, pointing

Figure 5.11. Suggested potential energy surfaces for a) TMCL-I and b) TMCL-II with optimized structures of four stable conformers (chair-like conformers C and C', boat-like conformers B and B'). The values indicated are calculated potential energies of each conformer normalized toward the conformer with the lowest energy for each lactone (conformer C). The line is for visual guidance only.

away from the ring. This conformation is the most stable of all the conformations evaluated. In the other chair-like conformation (conformer C'), two methyl substituents are in pseudo-axial position. The shorter distance between these substituents induces a higher steric hindrance, which is why conformation C' has a higher energy. This conformation is of similar energy compared to the boat-like conformation (B). In this conformation, the two methyl substituents are in pseudo-equatorial position, pointing away from the ring. The last conformation is noticeably of higher energy compared to all other configurations (conformer B'). This is the boat-like conformation in which the same two methyl substituents are this time in pseudo-axial position, an effect that is particularly destabilizing for the lactone.

The ring opening of the lactones TMCL-I and TMCL-II with methanol as nucleophile was simulated at different temperatures in order to evaluate the influence of the position of the methyl substituents on the reactivity of the lactones as well as the influence of temperature on the thermodynamics of the reaction. For this reaction, the substrate consisted of the conformer C of each lactone and methanol, and the product consisted of the methyl ester in the extended geometry (Scheme 5.4). The atomic coordinates of the extended esters can be found in **Appendix B**.

Scheme 5.4. Reaction evaluated by DFT calculations: ring opening of TMCL-I and TMCL-II with methanol to give the corresponding methyl esters. TMCL-I and TMCL-II are in conformation C and the methyl ester product is in an extended conformation.

The ring opening of TMCL-I and TMCL-II are enthalpically favored ($\Delta H_{\text{reaction}} < 0$) but entropically disfavored ($\Delta S_{\text{reaction}} < 0$) over the temperature range evaluated (- 50 °C to 150 °C) (Figure 5.12a-b). The negative enthalpy is related to the release of ring strain as a consequence of the ring opening of the lactones while the negative entropy is a

measure of the increased order of the system, from lactone to ring opened product in the case of this study, or from monomer to polymer in the case of polymerization. The ring opening of TMCL-I and TMCL-II is therefore enthalpy-driven despite the loss of entropy upon ring opening. These negative enthalpy and entropy are quite typical of medium-sized lactones (4- to 7-membered ring lactones, with the exception of 5-membered ring lactones which are lacking ring strain).⁴⁵ Alkyl substituted lactones such as substituted δ -valerolactones and menthide follow the same trend (Table 5.3).^{20, 22}

Figure 5.12. Calculated thermodynamic values for the ring opening of TMCL-I and TMCL-II with methanol in the gas phase with a) enthalpy of the reaction ($\Delta H_{\text{reaction}}$), b) the entropic contribution of the reaction ($T\Delta S_{\text{reaction}}$), and c) Gibbs free energy ($\Delta G_{\text{reaction}}$).

These results are also in line with the experimentally determined thermodynamic parameters of the mixture of TMCL-I and TMCL-II. For lactones of the ring size of TMCL, the ring strain is the result of a combination of several strains, including:⁴⁷

- The *angle strain* which is the bond angle deformation deviating from the ideal angle of 109° due to the cyclic structure (cisoid conformation of the ester bond required for the formation of the ring);

- The *conformational strain*, which is the consequence of eclipsed conformations due to inability of C-C bonds to rotate because of the ring structure;
- The *transannular strain*, which comes from unfavorable non-bonding interactions between non-adjacent substituents on the ring.

The ring opening of TMCL-I is more enthalpically favored than that of TMCL-II ($\Delta H_{\text{reaction, TMCL-I}} < \Delta H_{\text{reaction, TMCL-II}}$) (Figure 5.12a). This is attributed to the general larger influence of methyl substituents located on the δ -position compared to the β -position on the ring strain. Indeed, the determination of the thermodynamic parameters of a series of alkyl monosubstituted δ -valerolactones demonstrated that the position of the alkyl substituent has a higher impact on the increase in ring strain (and therefore increase the absolute value of ΔH_p) than the size of this substituent.²⁰ More specifically, methyl substituted valerolactones are increasingly more strained when the substituent is positioned from the α -position to the δ -position (from abnormal/distal lactone to normal/proximal lactone).²⁰ In most ROP of medium sized (substituted) lactones, the entropic change is thermodynamically unfavorable ($\Delta S_p < 0$). From an entropic point of view, the ring opening of TMCL-II is slightly advantaged compared to TMCL-I ($\Delta S_{\text{reaction, TMCL-I}} < \Delta S_{\text{reaction, TMCL-II}}$).

It is important to note that the DFT calculations are performed in the gas phase, which can be considered as an extremely diluted system. ROP is very sensitive to monomer concentration, since the absolute value of ΔS_p increases when the initial monomer concentration decreases. It is therefore not surprising that the calculated absolute values for $\Delta S_{\text{reaction}}$ are very high. As a consequence, a positive Gibbs free energy is obtained, meaning that the reaction in the gas phase is very unlikely (Figure 5.12c), while experimentally polymerization in bulk was proven to be successful. This is consistent with the fact that ROP is favored by high initial monomer concentrations ($[M]_0 > [M]_{\text{eq}}$). The Gibbs free energy clearly shows that the ring opening of the lactones is becoming more unfavorable for higher temperatures (Figure 5.12c). Based on the equation below (Eq 5.3),

(Eq 5.3)

$$\Delta G_{\text{reaction}} = \Delta H_{\text{reaction}} - T \cdot \Delta S_{\text{reaction}}$$

it was calculated that the ring opening of TMCL in a very diluted system (gas phase) would only become spontaneous for temperatures lower than - 134 °C for TMCL-II and lower than - 100 °C for TMCL-I. It should however be noted that these temperatures are based solely on theoretical thermodynamic considerations, and are only valid for very diluted systems. Additionally, such values do not account for the actual feasibility of the

reaction given the freezing temperature of the reaction mixture, the reactivity of catalysts at such low temperatures, *etc.*

Based on experimental polymerizations, it was observed that the percentage of TMCL-I in the monomer composition decreased during polymerization (Figure 5.7a). This was attributed to a higher reactivity of TMCL-I compared to TMCL-II. The DFT calculations confirm this difference of reactivity since the ring opening of TMCL-I is more favored than that of TMCL-II ($\Delta G_{\text{reaction, TMCL-I}} < \Delta G_{\text{reaction, TMCL-II}}$). Additionally, the amount of TMCL-I in the monomer concentration at equilibrium was observed to be lower at low reaction temperatures (Figure 5.7b). The calculated difference of Gibbs free energy is again in agreement with these experimental results. At low temperature (-50 °C), the difference of Gibbs free energy between TMCL-I and TMCL-II is 3.7 kJ mol⁻¹ and decreases with higher temperature to reach 2.5 kJ mol⁻¹ at 150 °C. This means that the difference in reactivity becomes progressively higher with lower temperatures. The difference in reactivity between the lactones at different temperatures, which is solely due to a change of position of one methyl group – either in β -position or in δ -position, is attributed to the enthalpy advantage of TMCL-I. More specifically, this difference of reactivity stems from the fact that the increase in entropy of TMCL-II at higher temperature ($1.33 < T\Delta S_{\text{reaction, TMCL-II}} - T\Delta S_{\text{reaction, TMCL-I}} < 2.55$ kJ mol⁻¹ between -50 °C and 150 °C, respectively) cannot compensate the important enthalpic advantage of TMCL-I, which remains quite constant independently of the temperature ($5.00 < \Delta H_{\text{reaction, TMCL-II}} - \Delta H_{\text{reaction, TMCL-I}} < 5.05$ kJ mol⁻¹ between -50 °C and 150 °C, respectively). In other words, TMCL-I is more reactive over the temperature range considered because the difference of ring strain in its favor is higher than the difference of entropic contribution in its disfavor, compared to TMCL-II.

5.3 Conclusions

The goal of this work was to investigate organocatalysts for the ROP of a mixture of β,δ -trimethyl- ϵ -caprolactones (TMCL). Several organophosphorus catalysts were evaluated with in particular two novel categories: phosphonic acids and phosphinic acids. While the most acidic organophosphorus catalysts were active even at room temperature, an initial temperature of 80 °C was needed for most of them to enable initiation. It was observed that, for all catalyst categories (superbases such as phosphazene, bifunctional organocatalysts such as TBD, *p*-TSA or organophosphorus catalysts), the TMCL monomer conversion at equilibrium increases with higher initial monomer concentrations and with lower reaction temperatures. The amount of remaining TMCL-I (percentage of TMCL-I in the monomer mixture), which is the proximal regio-isomeric

lactone, decreased during the polymerization as the average conversion increased. Lower reaction temperatures resulted in lower percentage of remaining TMCL-I, implying a higher reactivity of this regio-isomer. These experimental observations were confirmed by DFT calculations. In particular, the release of ring strain is higher for TMCL-I than for TMCL-II upon ring opening (lower $\Delta H_{\text{reaction}}$). This means that the presence of a methyl substituent at the δ -position has a higher influence on the ring strain than at the β -position. The ring opening of TMCL-I is more thermodynamically favorable than that of TMCL-II because it has a lower Gibbs free energy. The difference of $\Delta G_{\text{reaction}}$ between the lactones is higher at lower temperature due to the fact that the high difference in enthalpy in favor of TMCL-I is not compensated by the increased entropy in favor of TMCL-II at higher temperature.

5.4 Experimental section

Chemicals. Anhydrous tetrahydrofuran (THF) was purified using a MBraun SPS-compact solvent purification system. Benzyl alcohol (BnOH; 99 %, Alfa Aesar) and TMCL were distilled over CaH₂ prior to use (BnOH: 2.10⁻² mbar, 90-95 °C; TMCL: 1.10⁻³ mbar, 95-105 °C). *p*-Toluene sulfonic acid (*p*-TSA; < 99 %, Acros Organic), bis(4-nitrophenyl)phosphate (**P1** or BNPP; 99 %, Sigma-Aldrich), dimethyl phosphate (**P2**; Aldrich^{CPR}), diphenyl phosphate (**P3** or DPP; > 99 %, TCI), methyl phosphonic acid (**P4**; 98 %, Sigma-Aldrich), phenyl phosphonic acid (**P5**; 98 %, Sigma-Aldrich), pinacolyl methyl-phosphonate (**P6**; 97 %, Sigma-Aldrich), ethyl methyl phosphonate (**P7**; 98 %, Sigma-Aldrich), *t*-butylphosphonic acid (**P8**; 98 %, Sigma-Aldrich), diphenyl phosphinic acid (**P10**; > 98 %, Sigma-Aldrich), and dimethyl phosphinic acid (**P11**; 97 %, Sigma-Aldrich) were freeze-dried prior to use. Bis(pentafluorophenyl)-phosphinic acid **P9** was synthesized from tris(pentafluorophenyl)phosphine (97 %, Sigma-Aldrich) following a procedure reported in the literature,⁴⁸ and freeze-dried prior to use. 1-*Tert*-butyl-4,4,4-tris(dimethylamino)-2,2-bis[tris(dimethylamino)-phosphoranylideneamino]-2 λ^5 ,4 λ^5 -catenadi(phosphazene) (P4-*t*-Bu solution at 0.8 M in hexanes, Sigma-Aldrich) was vacuum dried prior to use. Tin(II) 2-ethylhexanoate (Sn(Oct)₂; 95 %, Sigma-Aldrich), titanium(IV) butoxide (Ti(*n*-BuO)₄; 97 %, Sigma-Aldrich), 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD; 98 %, Sigma-Aldrich), 1-ethyl-2,2,4,4,4-pentakis(dimethyl-amino)-2 λ^5 ,4 λ^5 -catenadi-phosphazene (P2-Et phosphazene; > 98 %, Sigma-Aldrich) and hexadecane (< 99.5 %, TCI) were used as received.

¹H and ¹³C NMR. NMR spectra were recorded as described in Chapter 3. For the ROP mechanism investigation, samples were measured in C₂D₂Cl₄ with 64 scans for ¹H NMR and 512 scans for ¹³C NMR, at room temperature and at 80 °C.

GC-FID. Gas chromatography with flame ion detection (GC-FID) measurements were performed on a Shimadzu GC-2010 equipped with a Supelco SPB-1 capillary column (30 m × 0.25 mm × 0.25 μ m film thickness) or a SH-RXI-1ms column (30 m × 0.25 mm × 0.25 μ m film thickness). The temperature program was as follows: an initial temperature of 80 °C was maintained for 3 min,

then increased to 140 °C with a heating rate of 10 °C min⁻¹. This temperature was maintained for 1 min, and further increased to 300 °C with a heating rate of 20 °C min⁻¹ and was maintained at 300 °C for 5 min. Hexadecane was used as internal standard to determine the conversion of TMCL.

DSC. Differential scanning calorimetry (DSC) spectra were recorded on a Netzsch Polyma 2014 DSC. DSC data were measured with heating/cooling rates of 50 °C min⁻¹ under a nitrogen flow of 20 mL min⁻¹. DSC heating and cooling cycles were performed from - 70 °C to 100 °C in duplicate. The glass transition value reported corresponds to the second heating cycle.

GPC-THF. Gel permeation chromatography (GPC-THF) was performed at 30 °C using a Waters GPC equipped with a Waters 2414 refractive index detector. THF was used as eluent at a flow rate of 1 mL min⁻¹. Three linear columns were used (Styragel HR1, Styragel HR4 and Styragel HR5). Molecular masses are given relative to polystyrene standards.

MALDI-ToF MS. Matrix-assisted laser desorption/ionization time-of-flight mass spectra were recorded on a Bruker UltrafleXtreme spectrometer with a 355 nm Nd:YAG laser (2 kHz repetition pulse/Smartbeam-IITM) and a grounded steel plate. Trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]-malononitrile (> 98 %, Sigma-Aldrich) was used as matrix (20 mg mL⁻¹ in THF), potassium trifluoroacetate (> 98 %, Sigma-Aldrich) was used a cationization agent (10 mg mL⁻¹ in THF). The polymers were dissolved in THF (10 mg mL⁻¹). Solutions of matrix, salt and polymer were mixed in volumetric ratios of 200:10:30, respectively. All mass spectra were collected in the reflector mode. Poly(ethylene glycol) standards with M_n equal to 5 000, 10 000 and 15 000 g mol⁻¹ were used for calibration. Data was processed using the FlexAnalysis (Bruker Daltonics) software package.

Synthesis of the mixture of β,δ,δ -trimethyl- ϵ -caprolactone (TMCL-I) and β,β,δ -trimethyl- ϵ -caprolactone (TMCL-II) monomer (TMCL). The monomer mixture was synthesized by Baeyer-Villiger oxidation of 3,3,5-trimethylcyclohexanone following a modified version of a procedure reported in the literature.⁴⁹ 3,3,5-Trimethylcyclohexanone (86.6 g, 0.62 mol, 1 eq) was dissolved in dichloromethane (1.4 L) and *m*-chloroperbenzoic acid (207.6 g, 0.92 mol, 1.5 eq) was added in portions within 30 min at room temperature. The mixture was left stirring until full conversion was reached (2 days) and the precipitate was removed by filtration. The filtrate was washed with basic aqueous solution at 7 % NaOH (2 $\times$ 250 mL) and with brine (2 $\times$ 250 mL). The solvent was removed by evaporation and the lactone was purified and dried by vacuum distillation (1.10⁻³ mbar, 95-105 °C) over CaH₂. TMCL was obtained as a colorless liquid (66 g, 77 % yield).

Typical ROP of TMCL. In a vacuum-dried flask, BnOH (20 μ L, 0.2 mmol, 1 eq) was added to a mixture of TMCL (1 mL, 5.9 mmol, 30 eq) and hexadecane as internal standard (typically 5 mol % relative to the amount of TMCL). The mixture was left to reach the desired reaction temperature. Polymerizations at room temperature were performed in a water bath. Polymerizations at higher temperature were performed in an oil bath. Polymerizations at - 20 and - 50 °C were performed in a double wall flask and cooled down with a Julabo FP89-HL ultra-low refrigerated-heating circulator. The polymerization was started by adding the catalyst (0.2 mmol, 1 eq) with a funnel to

the reaction mixture at the desired temperature under nitrogen atmosphere. Conversion was determined by GC-FID. Samples were prepared by dissolving an aliquot of the reaction mixture in chloroform containing either triethylamine for polymerizations performed with organoacids, or acetic anhydride for polymerizations performed with organobases and metal-based catalysts. When $P_4-t\text{-Bu}$ phosphazene was employed as catalyst, a mixture consisting of TMCL, $BnOH$ and hexadecane was thermostated at the desired reaction temperature and then added to the dried catalyst to start the polymerization.

Determination of the thermodynamic parameters of TMCL. The polymerization were performed from 25 °C to 170 °C using DPP as catalyst ($BnOH$ 1eq, TMCL 30 eq, DPP 1 eq, hexadecane 5 mol %) as described in the previous paragraph. The reaction was left to react until the equilibrium monomer conversion was reached as monitored by GC-FID.

DFT calculations. Geometry optimization and frequency calculations were performed using a density functional theory (DFT) method with diffusion functions on heavy atoms and polarization functions on hydrogen using the B3-LYP/6-31G (d,p) basis set with the Gaussian 09 and GaussView 05 software package.⁵⁰ The substrates and products were optimized to the minimum structure with 0 negative frequency. The list of atomic coordinates is given in **Appendix B**.

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CHAPTER 6

Copolymerizing lactones: reactivity differences and influence on the copolymerization

In this chapter, the potential of the copolymerization of lactones with various structures is investigated by ring opening polymerization. Lactones are selected based on their solubility profiles: the alkyl substituted δ -undecalactone and the mixture of β,δ -trimethyl- ϵ -caprolactones, for which the enzymatic preparation was established in **Chapters 3** and **4**, and whose kinetics and thermodynamics was investigated in **Chapter 5**. These two monomers are copolymerized in combination with the more polar 1,5-dioxepan-2-one. The compatibility of several catalysts with these lactones is evaluated by investigating their reaction kinetics. The reactivity differences are determined by measuring the reactivity ratios in order to understand the topology obtained during copolymerization.

Keywords: microstructure • kinetics • alkyl substituted lactone • copolymerization • reactivity ratio

6.1 Introduction

Macromolecular architecture refers to the way monomers are assembled together upon polymerization. As outlined in **Chapter 1** (Figure 1.1), macromolecular architecture is directly related to the synthesis procedure of the polymerization and it influences the properties of the copolymer. In particular, the microstructure of copolyesters contributes to defining their properties and applications. For example, amphiphilic block copolymers, which are composed of a hydrophobic block and a hydrophilic block, can self-assemble to form nanoparticles, micelles and polymersomes.¹ Polyesters from ϵ -caprolactone derivatives of varying properties have for example been investigated for the preparation of micellar drug carriers for pharmaceutical formulations.² Another example outlying the relationship between microstructure and properties is the tunable degradation of block copolyesters, which depends on the hydrophobic / hydrophilic ratio of the blocks as well as their respective crystallinity.³ As such, it is important to evaluate the microstructure of copolymers. The relationship between the organocatalysts used upon ring opening copolymerization (ROP) and macromolecular structure has recently been reviewed.⁴

In this chapter, several lactones were considered for the synthesis of copolyesters, which were selected based on the solubility and thermal properties of the corresponding polyesters. The solubility range was assessed using the Hansen solubility parameters (Figure 6.1). The homopolymers were dissolved in solvents, which are classified in categories based on their dispersive forces (δ_D), polar forces (δ_P) and hydrogen bonding (δ_H) contributions between molecules, according to the definition of Hansen.⁵ The details of the solvents are given in Table 6.1. Two polyesters have an extremely limited solubility sphere, namely poly(ω -pentadecalactone) (poly(ω -PDL)) and poly(1,4-dioxan-2-one) (poly(DXO)). Only a couple of solvents are able to solubilize these polyesters (Figure 6.1 below). This is a consequence of their high crystalline content and melting temperature ($T_{m, \text{poly(DXO)}} = 110\text{ }^\circ\text{C}$,⁶ $T_{m, \text{poly}(\omega\text{-PDL})} = 97\text{ }^\circ\text{C}$ ⁷). These polymers were therefore not selected further given their semi-crystalline properties and limited solubility range.

On the other hand, amorphous polyesters with low glass transition temperatures have a broad solubility range (Figure 6.1 above). As expected, the solubility sphere of poly(1,5-dioxepan-2-one) (poly(DXP), $T_g = -39\text{ }^\circ\text{C}$)⁸ lies in the region of polar aprotic solvents, and also comprises a few polar protic solvents. Two additional polar protic solvents, which are located outside the solubility sphere, are able to solubilize poly(DXP), namely formamide and methanol. Both of them have a high H-bonding contribution (δ_H) and polarity contribution (δ_P). The solubility spheres of the two alkyl substituted polyesters poly(TMCL) (see **Chapter 5** for thermal properties) and poly(δ -UDL) ($T_g = -53\text{ }^\circ\text{C}$)⁹ are quite similar.

Figure 6.1. Hansen solubility range of the selected polyesters (above) and polyesters with limited solubility (below). D, P, and H indicate the δ_D , δ_P and δ_H position of the middle of the solubility sphere. R indicates the radius of the solubility sphere. Red dots indicate non-solvents, blue dots indicate solvents in which the polymer is soluble. Poly(DXP): poly(1,5-dioxepan-2-one), poly(TMCL): poly(β , δ -trimethyl- ϵ -caprolactones), poly(δ -UDL): poly(δ -undecalactone), poly(DXO): poly(1,4-dioxan-2-one), poly(ω -PDL): poly(ω -pentadecalactone). The list of solvents is given in Table 6.1.

Both these solubility spheres comprise some polar aprotic solvents. Compared to poly(DXP), the solubility spheres are shifted toward more apolar solvents such as hexane and toluene. This is due to the presence of apolar alkyl substituents on the backbone of the polyesters. So far, copolymers from δ -UDL and TMCL have received little attention. The goal of this chapter is therefore to evaluate the copolymerization of these lactones with DXP (Scheme 6.1). Because of the difference of solvophilicity, it is expected that the properties of the resulting copolyesters may be tuned, therefore potentially enabling applications as dispersants or encapsulating agents in emulsions, dispersions, coatings, *etc.*

Scheme 6.1. Copolymerization of a mixture of β,δ -trimethyl- ϵ -caprolactones (TMCL) or δ -undecalactone (δ -UDL) with 1,5-dioxepan-2-one (DXP).

Table 6.1. Detailed solubility of polyesters evaluated for 43 solvents (1 indicates full solubility, 0 indicates no solubility and 0.5 indicates partial solubility of the polyester).

Solvent	Poly(DXP)	Poly(TMCL)	Poly(δ -UDL)	Poly(DXO)	Poly(ω -PDL)
1-Octanol	0	1	1	0	0
1-Pentanol	0	1	1	0	0
1-Propanol	0	1	1	0	0
2-Butanol	0	1	1	0	0
2-Phenyl Ethanol	1	1	1	0	0
Acetic Acid	1	1	1	0	0
Acetone	1	1	1	0	0
Acetonitrile	1	0	0	0	0
Benzonitrile	1	1	1	0	0
Benzyl Alcohol	1	1	1	0	0
Chlorobenzene	1	1	1	0	0
Chloroform	1	1	1	0	1
Cyclohexane	0	0	1	0	0
Cyclohexanone	1	1	1	0	0
Diethyl Amine	0	1	1	0	0
Diethylene Glycol	0	0	0	0	0
Dimethyl Formamide (DMF)	1	1	1	0	0
Dimethyl Sulfoxide (DMSO)	1	1	0	0	0
Di- <i>n</i> -Butyl Ether	0	0	1	0	0
Ethanol	0	0	1	0	0
Ethyl Acetate	1	1	1	0	0
Ethyl Benzene	0	1	1	0	0
Ethylene Glycol	0	0	0	0	0
Ethylene Glycol Monomethyl Ether	1	0	0.5	0	0
Formamide	1	0	0	0	0
Hexane	0	0	1	0	0
Hexafluoro-2-propanol (HFIP)	1	1	1	1	1
Isobutyl Alcohol	0	1	1	0	0
<i>m</i> -Cresol	1	1	1	1	0
Methanol	1	0	0	0	0

Solvent	Poly(DXP)	Poly(TMCL)	Poly(δ -UDL)	Poly(DXO)	Poly(ω -PDL)
Methyl Ethyl Ketone (MEK)	1	1	1	0	0
Dichloromethane	1	1	1	0	0
<i>N,N</i> -Dimethyl Acetamide	1	1	1	0	0
Nitrobenzene	1	1	1	0	0
<i>N</i> -Methyl Formamide	1	0	0	0	0
<i>N</i> -Methyl-2-Pyrrolidone (NMP)	1	1	1	0	0
Propylene Carbonate	1	0	0	0	0
Propylene Glycol Monomethyl Ether	1	1	1	0	0
Tetrahydrofuran (THF)	1	1	1	0	0
Toluene	0	1	1	0	0
Valeronitrile	1	1	1	0	0
Water	0	0	0	0	0
γ -Butyrolactone (GBL)	1	0	0	0	0

Poly(DXP): poly(1,5-dioxepan-2-one), poly(TMCL): poly(β,δ -trimethyl- ϵ -caprolactones), poly(δ -UDL): poly(δ -undecalactone), poly(DXO): poly(1,4-dioxan-2-one), poly(ω -PDL): poly(ω -pentadecalactone).

6.2 Results and discussion

6.2.a. Kinetics of homopolymerizations of TMCL, DXP, and δ -UDL

In order to perform the copolymerization, it is necessary to ensure that a common catalyst can polymerize all the selected lactones. Based on the catalyst screening which was performed in **Chapter 5** for the polymerization of TMCL, two catalysts were selected: the bifunctional organocatalyst diphenyl phosphate (DPP), and the superbases phosphazene P_2 -Et (the structures are shown in Scheme 5.2).

- **Diphenyl phosphate (DPP) as bifunctional organocatalyst**

Homopolymerizations of DXP, δ -UDL and TMCL were performed in bulk at 30 °C using DPP (Figure 6.2). This organocatalyst was selected for its ability to avoid transesterification reactions, which typically lead to randomization of the microstructure of copolymers.¹⁰ The conversions of DXP and δ -UDL upon polymerization were determined by ^1H NMR spectroscopy by monitoring the shift of the protons in ϵ - and δ -position, respectively (Figure 6.3). The conversion of TMCL was determined by GC-FID by using an internal standard (see **Chapter 5**). The polymerization of DXP proceeded the fastest, with almost full conversion after 4.5 hours. A poly(DXP) of low dispersity was achieved ($M_n = 4.4 \text{ kg mol}^{-1}$, $D_M = 1.07$). Longer reaction times resulted in broader dispersity ($D_M = 1.30$), which were probably caused by transesterification reactions taking place once all DXP monomer has been polymerized (Figure 6.4a).

Figure 6.2. a) Kinetics of homopolymerizations of DXP (black squares), δ -UDL (blue circles), and TMCL (red triangles) catalyzed by DPP, and b) $\ln([M]_0/[M])$ as a function of time. Reaction conditions: benzyl alcohol/monomer/DPP/hexadecane) 1/30/1/(1.6), 30 °C, bulk.

Figure 6.3. Kinetics during polymerization by ^1H NMR spectroscopy with a) homopolymerization of DXP ($(\text{C}=\text{O})\text{-O-CH}_2\text{-}$ protons at 4.37 ppm for the monomer and 4.27 ppm for the polymer), and b) homopolymerization of δ -UDL (δ -methylene proton at 4.28 ppm for the monomer and 4.87 ppm for the polymer). The -CH_2 protons of benzyl alcohol at 4.72 ppm shift to 5.11 ppm once initiated.

The polymerization of the alkyl substituted monomers δ -UDL and TMCL proceeded slower in comparison to DXP. A monomer conversion at equilibrium of 84 % and 70 % was achieved after 30 h and 100 h for δ -UDL and TMCL, respectively (Figure 6.2a). For these monomers, no significant broadening of dispersity was observed after reaching monomer conversion at equilibrium although the dispersity increased with higher conversions (Figures 6.4b-c and 6.5b). This seems to indicate that the presence of the substituents in δ - and ϵ -position may limit transesterification. Another possibility is that DPP is not basic enough to activate the polymer end-chains and thereby transesterify them.

Figure 6.4. GPC spectra of homopolymers (measured in THF) as a function of reaction time for a) DXP, b) TMCL, and c) δ -UDL.

Figure 6.5. a) $M_{n,GPC}$ as a function of conversion, and b) dispersity ($\bar{D}_M$) as a function of conversion for the ROP of DXP, δ -UDL, and TMCL. The dotted lines indicate the initial reaction rates.

Although the increase in the molecular weight as measured by GPC is linear with the conversion, the kinetic plot indicates termination reactions, especially for δ -UDL (Figures 6.5a and 6.2b)

- **P₂-Et phosphazene as superbase organocatalyst**

Homopolymerizations of δ -UDL and TMCL were performed with the P₂-Et phosphazene superbase at room temperature in bulk. This catalyst was selected because it is less reactive than the P₄-*t*-Bu analogue phosphazene. The latter was shown in **Chapter 5** to give polymerizations that are more difficult to control, at least for the ROP of TMCL in bulk conditions. Replacing DPP by P₂-Et resulted in a dramatic decrease in the reaction time for the alkyl substituted lactones. Equilibrium monomer conversion was achieved within 5 h and 6 h for δ -UDL and TMCL, respectively (Figure 6.6a). Although a linear increase in molecular weight as a function of time was observed by GPC, the polymerization kinetics indicate that the reaction is not fully controlled, in which case a linear first order kinetic plot would be observed (Figures 6.6b and 6.7). The first order kinetic plot indicates termination of both polymerizations (Figure 6.6). In addition, there may be diffusion limitations when high conversions are achieved due the increased viscosity of the reaction mixture, the polymerization being performed in bulk.

Nonetheless, both polymerizations resulted in low dispersities ($\mathcal{D}_M < 1.13$ for δ -UDL and $\mathcal{D}_M < 1.31$ for TMCL) (Figure 6.7a-b). Interestingly, the dispersities did not increase significantly when the polymerizations were left to react overnight after equilibrium monomer conversion was reached (Figure 6.7). This shows that despite its extreme basicity and end-chain activation mechanism, P₂-Et does not induce transesterification in the reaction conditions that were evaluated.

While P₂-Et is beneficial for the ROP of δ -UDL and TMCL, it is unable to polymerize DXP in a controlled fashion. The ROP of DXP catalyzed by P₂-Et led to high dispersities,

Figure 6.6. Homopolymerizations of δ -UDL, TMCL, and DXP catalyzed by P₂-Et with a) kinetics, and b) $\ln([M]_0/[M])$ as a function of time. Reaction conditions: BnOH/monomer/P₂-Et/(hexadecane) 1/30/1/(1.6).

Figure 6.7. Evolution of molecular weight $M_{n,GPC}$ (left axis) and dispersity (D_M , right axis in red) as measured by GPC in THF with a) poly(δ -UDL), b) poly(TMCL), and c) poly(DXP) (as a function of time because conversion could not be determined).

starting already from $D_M = 1.81$ after a few minutes (Figure 6.7c). Although the reaction was left running for 22 h, the molecular weight as measured by GPC did not increase. This extended reaction time however led to transesterification (final dispersity of $D_M = 1.91$). In addition, the DXP monomer conversion could not be determined by ¹H NMR, which seems to indicate possible degradation upon ROP possibly due to an incompatibility between DXP and P₂-Et, at least under the reaction conditions evaluated in this chapter. DPP was therefore selected as suitable catalyst to copolymerize DXP, δ -UDL, and TMCL.

6.2.b. Reactivity ratio during copolymerization

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) is a powerful tool for (co)polymers analysis.¹¹ When analyzing copolymers, the spectra obtained can be quite complex since all the chemical distributions

are visible. In the case of copolymers, MALDI-ToF MS gives information about the composition, the total length, and, in some cases, the microstructure. Contour plots make the complex MALDI-ToF MS spectra of copolymers more accessible and represent the fingerprint of the polymer. The contour plot is a 3D-representation of a normalized matrix of the mass spectrum with the number of each monomer unit on the x/y-axis and the corresponding peak intensity on the z-axis (color strength or heat map).

The shape and the position of the contour plot may sometimes give information about the topology of the copolymer.¹²⁻¹⁴ The average chemical distribution of a copolymer is shown by a line drawn through the center of its contour plot. For random copolymers, this line crosses the origin with a constant slope while for block copolymers the line does not cross the origin. In the latter case, the contour plot displays an oval or circular shape. Alternating copolymers are characterized by a line pattern with a width of one monomer, which follows the diagonal.

One way to predict the microstructure of copolymers is to determine the reactivity ratio of the comonomers. Traditionally, these reactivity ratios have been measured by determining the chemical composition of copolymers prepared from various comonomer feed ratio. This method is however time-consuming and tedious. Recently, a method to determine these parameters from a single MALDI-ToF MS analysis of a one-pot copolymerization with a 50/50 mol % feed ratio has been reported.¹³ The measurement must be performed at low monomer conversion (typically < 15 % conversion) to avoid that any potential transesterification may take place and modify the initial microstructure, which is directly influenced by the reactivity of the comonomers, and to avoid composition drift occurring at higher conversions. This method relies on the Mayo-Lewis method, where the simplest copolymer model is represented by a first order Markov chain in which the terminal chain unit influences the propagation of monomers M₁ and M₂ (Eq 6.1).¹⁵⁻¹⁶

(Eq 6.1)

With k_{11} , k_{12} , k_{22} , k_{21} the propagation rate constants for homo- and cross-propagation.

The reactivity ratios r_1 and r_2 are defined as shown in Eq 6.2:

(Eq 6.2)

$$r_1 = \frac{k_{11}}{k_{12}} \quad r_2 = \frac{k_{22}}{k_{21}}$$

The Mayo-Lewis copolymer composition equation is shown in **Eq 6.3**:

(Eq 6.3)

$$F_1 = \frac{f_1^2(r_1 - 1) + f_1}{f_1^2(r_1 + r_2 - 2) + 2f_1(1 - r_2) + r_2}$$

$$\text{with } f_1 = \frac{[M_1]}{[M_1] + [M_2]} \text{ and } F_1 = \frac{d[M_1]}{d[M_1] + d[M_2]}$$

With f_1 the molar fraction of monomer M_1 in the monomer, F_1 the molar fraction of monomer M_1 in the polymer, $[M_1]$ and $[M_2]$ the concentrations of M_1 and M_2 , and $d[M_1]$ and $d[M_2]$ the corresponding rates of polymerization of M_1 and M_2 .

The reactivity ratio values of DXP with δ -UDL, and DXP with TMCL were determined using this approach. The MALDI-ToF MS traces are shown in Figure 6.8a-b and the corresponding contour plots (heat maps) are shown in Figure 6.8c-d. The reactivity ratio of DXP and δ -UDL are quite similar ($r_{\text{DXP}} = 1.8$ and $r_{\delta\text{-UDL}} = 1.6$). Because the reactivity ratios are higher than 1, this copolymerization may yield block-like copolymers (Table 6.2). Few homopolymers were observed on the MALDI-ToF MS spectrum. This is indicated by the fact that the major copolymer distribution is not located on the x- or y-axis, which would indicate the presence of DXP and δ -UDL homopolymers, respectively. The distribution of the copolymer on the contour plot shows that copolymerization is indeed predominant over homopolymerization of each monomer (Figure 6.8c). It is however difficult to confirm the prediction on the type of copolymer microstructure solely based on the reactivity ratio of DXP and δ -UDL. According to the reactivity ratio determination, this pair of monomer is expected to give random copolymers, with exceptionally blocky copolymer segments (Table 6.2).¹⁷

When DXP is copolymerized with TMCL, the copolymerization behavior is drastically different. In this case, the reactivity ratio of DXP is very high compared to that of TMCL ($r_{\text{DXP}} = 12.0$ and $r_{\text{TMCL}} = 0.2$). This leads to the formation of DXP homopolymer at low monomer conversion, as shown by the position of the contour plot along the DXP axis (Figure 6.8d). This is in agreement with the predicted microstructure for this type of reactivity ratio pair (Table 6.2). Besides the formation of homopolymers, it may be possible that this copolymerization also leads to the formation of block copolymers, since TMCL could potentially polymerize after DXP reaches full conversion. As such, copolymerizations of TMCL with DXP are expected to be challenging if solely copolymers are expected to be synthesized.

Figure 6.8. MALDI-ToF MS spectra of a) DXP and δ -UDL ($f_{\text{DXP}} = 0.5$, total conversion 10 %); b) DXP and TMCL ($f_{\text{DXP}} = 0.5$, total conversion 13 %); c) and d) corresponding heat maps and reactivity ratios determined upon copolymerization.

Table 6.2. Copolymer microstructure depending on the reactivity ratio of the comonomers with r_1 reactivity ratio of monomer M_1 and r_2 reactivity ratio of monomer M_2 .

Topology	r_1	r_2	Reactivity ratios
Random copolymer (ideal copolymerization)	$\frac{1}{r_2}$	$\frac{1}{r_1}$	$r_1 \times r_2 = 1$
Alternating copolymers	$r_1 \ll 1$	$r_2 \ll 1$	$r_1 \times r_2 \rightarrow 0$
Tends to M_1 homopolymer	$r_1 \gg 1$	$r_2 < 1$	$r_1 \times r_2 < 1$
Random with exceptionally blocky copolymer	$r_1 > 1$	$r_2 > 1$	$r_1 \times r_2 > 1$
Tends to block copolymer	$r_1 \gg 1$	$r_2 \gg 1$	$r_1 \times r_2 \gg 1$

The composition drift of the copolymers was predicted from the reactivity ratios. The Mayo-Lewis equation gives the monomer composition of copolymers (F_{DXP}) based on the monomer feed (f_{DXP}) (Eq 6.3). The composition drift is directly dependent on the reactivity ratio of each comonomer (Figure 6.9). As expected, the copolymer from DXP and δ -UDL is close to statistical since both monomers display similar reactivity ratios. The important difference in the reactivity ratio of DXP and TMCL results in a predicted composition drift. A predominance of DXP in the copolymer is expected, even at low feed of DXP. This is due to the higher reactivity of DXP compared to TMCL.

Figure 6.9. Composition of the copolymers as a function of the feed monomer composition based on the Mayo-Lewis equation (Eq 6.3) with f_{DXP} the molar fraction of DXP in the feed and F_{DXP} the molar fraction of DXP in the copolymer (the black dotted line indicates a perfectly random copolymer with $r_1 = r_2 = 1$).

6.2.c. One-pot copolymerizations of DXP with δ -UDL and TMCL

Based on the difference of reactivity ratio determined previously, one-pot copolymerization is expected to give a relatively random copolymer in the case of δ -UDL and DXP and a block-like copolymer with some poly(TMCL) homopolymer in the case of TMCL and DXP (Table 6.3, entries 1-2). The conversion rate of DXP was much slower in presence of δ -UDL or TMCL as comonomer compared to the homopolymerization kinetics (Figure 6.10a-c vs. 6.2a). As expected, both δ -UDL and DXP displayed similar reactivity during copolymerization, despite the monomer equilibrium conversion of δ -UDL being lower than that of DXP (83 % compared to full conversion). It is probable that both monomers are partially incorporated together in the copolymer (random-like microstructure) and not sequentially (block-like structure) based on their simultaneous conversions. The composition of the remaining monomer as a function of conversion confirms that both DXP and δ -UDL are being converted in a similar fashion (Figure 6.10b).

A discrepancy in the composition of the copolymers was observed compared to what is predicted by the Mayo-Lewis equation. The obtained polymer had a lower content of DXP when TMCL was used as comonomer, while a higher content of DXP was measured when δ -UDL was used as comonomer. This is explained by the composition drift during copolymerization. As DXP is converted, the concentration of TMCL in the remaining monomer increases. As shown in **Chapter 5**, the thermodynamics of the polymerization are related to the monomer concentration, with high monomer concentrations leading to higher monomer conversion.

Table 6.3. Copolymerization of DXP with comonomer M (δ -UDL or TMCL).

Synthesis	M	f_{DXP}	DP_n (DXP/M) (feed)	$F_{\text{DXP, th}}^a$	$F_{\text{DXP, exp}}^b$	DXP conv. ^c (%)	M conv. ^c (%)
1 One-pot	TMCL	0.50	30/30	0.92	0.77	100	72
2 One-pot	δ -UDL	0.50	30/30	0.52	0.60	100	83
3 SF (DXP first)	TMCL	0.33	30/60	0.84	0.64	100	56
4 SF (δ -UDL first)	δ -UDL	0.50	16/16	0.52	0.69	100	85
5 SF (DXP first)		0.50	16/16	0.52	0.62	100	79
6 SF (DXP first)**		0.44	16/20	0.44	0.64	100	85
Synthesis (continued)	M	f_{DXP}	DP_n (DXP/M) (feed)	$M_{n, \text{th, conv}}^d$ (kg mol ⁻¹)	$M_{n, \text{NMR}}^e$	$M_{n, \text{GPC}}^f$ (kg mol ⁻¹)	$\bar{D}_M^f$
1 One-pot	TMCL	0.50	30/30	7.0	4.7	5.9	1.32
2 One-pot	δ -UDL	0.50	30/30	8.2	6.9	9.3	1.20
3 SF (DXP first)	TMCL	0.33	30/60	10.0*	6.4	5.3	1.59
4 SF (δ -UDL first)	δ -UDL	0.50	16/16	4.3	4.7	5.0	1.22
5 SF (DXP first)		0.50	16/16	4.2	3.4	4.9	1.29
6 SF (DXP first)**		0.44	16/20	5.0	3.5	4.4	1.24

^a Theoretical composition determined based on the Mayo-Lewis equation (**Eq 6.3**). ^b Calculated from the copolymer composition (¹H NMR analysis after purification). ^c DXP and δ -UDL conversion by ¹H NMR, TMCL conversion by GC-FID. ^d Theoretical molecular mass of the copolymer: $M_{n, \text{th, conv}} = 108 + (DP_n, \text{DXP} \times 116) + (DP_n, \text{M} \times M_M)$ with $M_M = 184 \text{ g mol}^{-1}$ for δ -UDL and 156 g mol^{-1} for TMCL. ^e Measured by ¹H NMR using the signal of initiated BnOH at 5.10 ppm. ^f Determined by GPC in THF against polystyrene standards. * Based on the total conversion. ** Second step performed at -10 °C. Unless indicated otherwise, all reactions were performed at 30 °C using DPP as catalyst and benzyl alcohol as initiator (1/1 ratio). SF sequential feed.

This explains why more TMCL is incorporated than expected from the determination of the Mayo-Lewis equation of this comonomer pair. The kinetics of the copolymerizations of DXP with TMCL was similar to the kinetics observed for each separate homopolymerization (Figure 6.10c vs. Figure 6.2a). About half of the conversion of TMCL took place when full conversion of DXP was achieved (after 20 h of reaction time), indicating the possible formation of a TMCL block. The composition of monomer as a function of conversion clearly confirms that the difference of reactivity ratio of both monomers results in delayed polymerization of TMCL during one-pot copolymerization (Figure 6.10d).

Figure 6.10. One-pot copolymerization of DXP with δ -UDL (above; Table 6.2, entry **1**) and TMCL (below; Table 6.2, entry **2**), with a-c) monomer conversion as a function of time, and b-d) remaining monomer composition as a function of conversion. Reaction conditions: BnOH/DEXP/ δ -UDL or TMCL/DPP/(hexadecane) 1/30/30/1/(1.6).

The microstructure of the copolymers was assessed by analyzing the diad distribution by semi-quantitative ^{13}C NMR spectroscopy, based on the carbonyl signal of each monomer repeating unit. An example of the diad distributions for each copolymerization is shown in Figure 6.11. The measured diad distribution was compared to the theoretical

diad distribution, which is calculated based on the monomer conversion and the targeted degree of polymerization. The probability to have a DXP unit (P_{DXP}) depends on the theoretical degree of polymerization ($DP_{\text{n,th}}$) and is corrected with the monomer conversion ($\text{conv}(\text{DXP})$) shown in **Eq 6.4**:

$$P_{\text{DXP}} = \frac{DP_{\text{n,th,DXP}} \cdot \text{conv}(\text{DXP})}{DP_{\text{n,th,DXP}} \cdot \text{conv}(\text{DXP}) + DP_{\text{n,th,M}} \cdot \text{conv}(\text{M})} \quad (\text{Eq 6.4})$$

$$P_{\text{M}} = \frac{DP_{\text{n,th,M}} \cdot \text{conv}(\text{M})}{DP_{\text{n,th,DXP}} \cdot \text{conv}(\text{DXP}) + DP_{\text{n,th,M}} \cdot \text{conv}(\text{M})}$$

The theoretical diad distributions correspond to the product of the probabilities:

$$\text{Diad (DXP}^* - \text{DXP)}: P_{\text{DXP}}^2$$

$$\text{Diad (M}^* - \text{M)}: P_{\text{M}}^2$$

$$\text{Diad (DXP}^* - \text{M)} \text{ and diad (M}^* - \text{DXP)}: P_{\text{DXP}} \cdot P_{\text{M}}$$

The randomness character of copolymers is calculated as shown in **Eq 6.5**:¹⁸

(Eq 6.5)

$$l_{\text{DXP}} = \frac{\text{diad (DXP}^* - \text{DXP)} + \frac{1}{2} \text{diad (DXP}^* - \text{M)}}{\frac{1}{2} \text{diad (DXP}^* - \text{M)}} = \frac{2 (\text{DXP})}{\text{diad (DXP}^* - \text{M)}}$$

$$(l_{\text{DXP}})_{\text{random}} = \frac{1}{(\text{DXP})} \quad R = \frac{(l_{\text{DXP}})_{\text{random}}}{l_{\text{DXP}}}$$

With R randomness character, l_i number-average sequence length, $l_{\text{r,random}}$ the Bernoullian random number-average sequence length, and (DXP) molar fraction of DXP.

Analysis of the diads clearly indicates that the microstructure of copolymers obtained by one-pot polymerization corresponds relatively well to the expected microstructures based on the analysis of their reactivity ratios (Table 6.4, entries **1-2**). Although the percentage of diads indicating a block structure (diads DXP*-DXP and M*-M) is relatively high, the degree of randomness indicates that the copolymers have a random-like structure ($R = 0.48$ and 0.57 for δ -UDL and TMCL, respectively). This is explained by the fact that the polymer consists of a mixture of copolymers and homopolymer, as evidenced by the contour plot obtained by MALDI-ToF MS analysis (Figure 6.12a-b). The tendency for poly(DXP) homopolymerization is particularly true for the copolymerization with TMCL and is directly related to the difference of reactivity between DXP

and TMCL, which favors the addition of a DXP monomer unit on a growing chain with DXP as last unit.

Figure 6.11. ^{13}C NMR spectrum of the carbonyl signals of copolymers obtained from one-pot synthesis, for a) poly(DXP-*co*-TMCL) with the two regio-isomers of TMCL monomer (TMCL I: 174.70 ppm, TMCL II: 174.02 ppm), diad TMCL*-TMCL at 172.91 ppm and 172.25 ppm, diad TMCL*-DXP at 172.76 ppm and 172.22 ppm, diad DXP*-TMCL at 171.40 ppm, diad DXP*-DXP at 171.35 ppm; and b) poly(DXP-*co*- δ -UDL) copolymer with diad δ -UDL*-DXP at 173.23 ppm, diad δ -UDL*- δ -UDL at 173.06 ppm, δ -UDL monomer at 171.99 ppm, diad DXP*-DXP at 171.31 ppm, and diad DXP*- δ -UDL at 171.13 ppm.

Table 6.4. Characterization of the microstructure (diad distribution) of the copolymers.

Addition		M	Diad distribution: experimental ^a (theoretical) ^b				<i>R</i> ^c	
			DXP*-DXP	DXP*-M	M*-DXP	M*-M	DXP	M
1	One-pot	TMCL	46 (34)	16 (24)	15 (24)	23 (18)	0.63	0.57
2	One-pot	δ-UDL	44 (30)	18 (25)	10 (25)	28 (21)	0.64	0.48
3	SF (DXP first)	TMCL	39 (22)	0 (25)	0 (25)	61 (24)	0	0
4	SF (δ-UDL first)	δ-UDL	56 (29)	5 (25)	0 (25)	39 (21)	0.18	0
5	SF (DXP first)		53 (31)	5 (25)	3 (25)	39 (19)	0.20	0.13
6	SF (DXP first)**		55 (24)	2 (25)	5 (25)	38 (27)	0.07	0.27

^a Determined by semi-quantitative ^{13}C NMR spectroscopy with * defining the carbonyl analyzed.

^b Theoretical diad distribution for a statistically random copolymer in parentheses. ^c R degree of randomness with $R \rightarrow 0$ indicating a block copolymer, and $R \rightarrow 1$ indicating a random copolymer.

** Second step performed at -10°C .

Figure 6.12. Contour plot from MALDI-ToF MS of the copolymers with a) poly(DXP-*co*-TMCL) synthesized in one-pot (Table 6.3-6.4, entry 1); b) poly(DXP-*co*- δ -UDL) synthesized in one-pot (Table 6.3-6.4, entry 2); c) poly(DXP-*co*-TMCL) by sequential TMCL addition (after the first addition) (Table 6.3-6.4, entry 3); d) poly(δ -UDL-*co*-DXP) with sequential DXP addition (1/1 δ -UDL/DXP) (Table 6.3-6.4, entry 4); e) poly(DXP-*co*- δ -UDL) with sequential δ -UDL addition (1/1 DXP/ δ -UDL) (Table 6.3-6.4, entry 5); and f) poly(DXP-*co*- δ -UDL) with sequential δ -UDL addition (1/1.25 DXP/ δ -UDL) (Table 6.3-6.4, entry 6).

6.2.d. Copolymerization by sequential feeding

In order to investigate the effect of the synthesis procedure on the topology of the polymers, a sequential feed strategy in two steps was applied. The goal was to attempt to synthesize a first block by letting one monomer polymerize, and then add the comonomer to form the second block. Given the high reactivity of DXP compared to TMCL, the alkyl substituted lactone was added as second monomer once full conversion of DXP was achieved (Table 6.3, entry **3**). Although the diad distribution seems to indicate that a block copolymer was formed, a mixture of poly(DXP) homopolymer and copolymers was observed in MALDI-ToF MS (Table 6.4, entry **3** and Figure 6.12c). This may be due to termination of the growing poly(DXP) homopolymer induced by the presence of traces of water during the addition of TMCL in the second step. A second addition of TMCL led to further TMCL conversion with a slower conversion rate compared to the first addition (Figure 6.13a). This second addition however mainly resulted in the formation of poly(TMCL) homopolymers as was observed by MALDI-ToF MS therefore indicating either termination of the poly(DXP) macromolecule or the inability for poly(DXP) to add TMCL monomer units due to reactivity differences (Figure 6.13b).

The reactivity ratios of DXP and δ -UDL being similar, two comonomer addition orders were considered for this copolymerization (Table 6.3, entries **4-5**). Both reactions resulted in similar monomer composition (as determined by ^1H NMR) and diad distribution (Table 6.4, entries **4-5**). The contour plots clearly show that the order of addition has

Figure 6.13. Sequential feed copolymerization of DXP with TMCL (Table 6.3/6.4, entry **3**) with a) TMCL conversion as a function of time, and b) MALDI-ToF MS after the second TMCL addition. Each addition corresponds to a DP_n of 30. The TMCL conversion was calculated for each separate addition based on GC-FID measurements. Reaction conditions: BnOH/DXP/TMCL/DPP/hexadecane 1/30/60/1/1.6.

a strong influence on the polymerization (Figure 6.12d-e). Indeed, a majority of poly-(δ -UDL) homopolymer was observed when it was polymerized first while polymerizing DXP first resulted in a majority of poly(DXP) homopolymer. Adding δ -UDL second is however beneficial because it results in some copolymerization which was not the case when DXP was added second. This is visible from the distribution that is located above the distribution against the x-axis (Figure 6.12e). It was attempted to increase the amount of δ -UDL incorporated in the copolymer by decreasing the temperature to -10 °C during the second step of the synthesis (Table 6.3, entry 6). The reason is that, as demonstrated in **Chapter 5**, higher conversions are obtained at lower reaction temperatures (Figure 5.10a). Although the conversion was slightly improved, the copolymer composition and the microstructure were not affected (Table 6.4, entry 6 and Figure 6.12f).

6.3 Conclusions

The copolymerization of δ -UDL and TMCL with DXP was investigated in this chapter. The lactones were selected because they yield amorphous polyesters which display broad solubility ranges. DPP was identified as a suitable catalyst for the copolymerization of these lactones since it is able to polymerize all the lactones investigated. This was not the case of the P₂-Et phosphazene superbase, which failed to polymerize DXP in a controlled manner. δ -UDL and DXP were shown to have similar reactivity ratios as determined by MALDI-ToF MS analysis. For this reason, one-pot copolymerization led to rather random copolyesters for δ -UDL. TMCL and DXP were shown to have distinctly different reactivity ratios, although the microstructure cannot be predicted from the reactivity ratio alone. The copolymerization of these lactones led to the formation of a majority poly(TMCL) homopolymer.

In order to potentially tune the polymerization behavior of the comonomers, copolymerizations with sequential addition of monomers were investigated. In this case, some homopolymer from the first monomer was observed, potentially resulting from termination induced by traces of water present in the second monomer. The order of addition of the comonomer was found to be of importance because it resulted in different polymer structures. More specifically, less homopolymers were observed when the alkyl substituted lactones were added to DXP.

6.4 Experimental section

Chemicals. Benzyl alcohol (BnOH; ≥ 99.0 %, Sigma-Aldrich), *m*-perbenzoic acid (*m*-CPBA; ≤ 77 %, Sigma-Aldrich), hexadecane (> 99.5 %, TCI), and diphenyl phosphate (DPP; > 99.0 %, TCI) were used as received. δ -Undecalactone (δ -UDL; > 97 %, Sigma-Aldrich) and β,δ -trimethyl-

ϵ -caprolactones (TMCL, synthesized as described in **Chapter 5**) were distilled over CaH_2 under reduced pressure (δ -UDL: 1.10^{-2} mbar, 160°C ; TMCL: 1.10^{-3} mbar, 95 – 105°C). 1,5-Dioxepan-2-one (DXP; $> 98.0\%$, TCI) was recrystallized from dry diethylether and distilled over CaH_2 under reduced pressure (1.10^{-3} mbar, 160°C). All monomers and catalysts were stored under nitrogen atmosphere. Solvents were obtained from Biosolve and were used as received.

^1H NMR and ^{13}C NMR analysis spectra were recorded as described in **Chapter 3**. Samples to follow the kinetics of the (co)polymerization with DXP and δ -UDL were prepared by dissolving the reaction mixture in CDCl_3 with about one drop of triethylamine to quench the reaction mixture.

GPC-THF. Molecular weight determination was performed by gel permeation chromatography as described in **Chapter 5**.

GC-FID. The kinetics of the (co)polymerizations with TMCL was determined by gas chromatography with flame ion detection (GC-FID) measurements performed as described in **Chapter 5**. The TMCL conversion was following by adding an internal standard to the reaction mixtures (hexadecane). Samples for kinetics were prepared by dissolving part of the reaction mixture in chloroform containing about one drop of triethylamine to quench the reaction.

MALDI-ToF MS. Matrix-assisted laser desorption/ionization time-of-flight mass spectra were recorded as described in **Chapter 5**. Contour plots were obtained with COCONUT version 1.4.2.¹⁹

DSC. Differential scanning calorimetry spectra were recorded as described in **Chapter 5**.

Determination of the Hansen solubility parameters. The solubility of the polymers was assessed using the Hansen solubility parameters by dissolving 50 mg of polymer in 1 mL of solvent at room temperature. Solubility was assessed after 24 h. The list of solvents is given in Table 6.1. The Hansen solubility spheres were obtained using the software HSPiP.

Typical procedure for the homopolymerization of TMCL, DXP or δ -UDL. TMCL (1.0 mL, 5.9 mmol, 30 eq) and BnOH (20 μL , 0.2 mmol, 1 eq) were mixed under nitrogen atmosphere. Hexadecane (90 μL , 0.3 mmol) was added to about 5 mol % relative to the monomer in the case of the polymerization of TMCL. DPP (48.3 mg, 0.2 mmol, 1 eq) was added to start the reaction. The conversion of TMCL was monitored by GC-FID, and the conversion of DXP or δ -UDL was monitored by ^1H NMR. When the equilibrium conversion was reached, the reaction was quenched by diluting the polymer in chloroform and adding excess triethylamine. The polymers were precipitated in cold hexane (-20°C) and dried *in vacuo* at 35°C to obtain a viscous liquid.

Typical synthesis of copolymers of DXP and TMCL using a one-pot strategy. BnOH (40 μL , 0.4 mmol, 1 eq), δ -UDL (2.2 mL, 11.8 mmol, 30 eq), and DXP (1.365 g, 11.8 mmol, 30 eq) were placed in a flask under nitrogen atmosphere. In the case of copolymerization with TMCL, δ -UDL was replaced by TMCL (2.0 mL, 11.8 mmol, 30 eq) with hexadecane (180 μL , 0.6 mol) used as internal standard to measure the conversion. DPP (100 mg, 0.4 mmol, 1 eq) was added to start the

polymerization. The conversion of TMCL was monitored by GC-FID, and the conversion of DXP or δ -UDL was monitored by ^1H NMR. When the equilibrium conversion was reached, the reaction was quenched by diluting the polymer in chloroform and adding excess triethylamine. The polymers were precipitated in cold hexane ($-20\text{ }^\circ\text{C}$) and dried in *vacuo* at $35\text{ }^\circ\text{C}$ to obtain a viscous liquid.

Typical synthesis of copolymers using sequential feeding strategy (SF). BnOH ($35\text{ }\mu\text{L}$, 0.3 mmol , 1 eq) and DXP (0.61 g , 5.3 mmol , 16 eq) were placed in a flask under nitrogen atmosphere. DPP (85.5 mg , 0.3 mmol , 1 eq) was added to start the polymerization. The conversion of DXP and δ -UDL was monitored by ^1H NMR. When full conversion of DXP was achieved after 4.5 h at room temperature, δ -UDL (1.0 mL , 5.3 mmol , 16 eq) was added to the reaction mixture. When the equilibrium conversion was reached, the reaction was quenched by diluting the polymer in chloroform and adding excess triethylamine. The polymers were precipitated in cold hexane ($-20\text{ }^\circ\text{C}$) and dried in *vacuo* at $35\text{ }^\circ\text{C}$ to obtain a viscous liquid.

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CHAPTER 7

Copolymers for dispersing agents: expanding the solubility range by tuning the microstructure

In this chapter, the aim is to explore the use of a macrolactone and alkyl substituted lactones for applications in inks and coatings. More specifically, ω -pentadecalactone is copolymerized with the alkyl substituted lactone δ -undecalactone in order to increase the solubility range of the resulting copolyester. The copolyester microstructure was assessed according to its diad distribution together with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS). Several feed compositions and synthesis strategies were evaluated (one-pot, addition of the macrolactone first, or addition of the alkyl substituted lactone first). Crystallinity of the copolyesters was assessed by differential scanning calorimetry. The solubility range of the copolyesters was evaluated with the Hansen solubility parameters analysis. Finally, the effect of the ring size and position of the substituents of the lactone comonomer was investigated by copolymerization with β,δ -trimethyl- ϵ -caprolactones (TMCL).

Keywords: macrolactone • alkyl substituted lactone • random copolymer • microstructure • solubility

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7.1 Introduction

In this chapter, poly(ω -pentadecalactone) (poly(ω -PDL)) is intended to be used as a component of dispersing agents for hydrophobic coating formulations. This polyester is synthesized from the biobased monomer ω -pentadecalactone. This macrolactone can be extracted from plants¹⁻² and plant essential oils³ as well as synthesized from renewable C₁₅ fatty acids.⁴ The potential of ω -PDL as biobased replacement for linear low density polyethylene has been explored due to the high crystallinity and melting temperature of poly(ω -PDL).⁵ As a consequence, its solubility range is extremely limited as was shown in **Chapter 6** (Figure 6.1).

Dispersing agents are added as additives to coating formulations containing pigments to ensure colloidal stability such that pigments stay dispersed in the continuous phase of the coating system (medium). Polymeric dispersing agents are typically amphiphilic copolymers with two types of segments. While the pigmentophilic chains show affinity for the pigment, the matrixophilic chains are soluble in the continuous phase and extend into it to promote steric stabilization (Scheme 7.1).

Scheme 7.1. Schematic representation of a polymeric dispersing agent for pigment based on an aliphatic polyester. A schematic representation of poly(ethylene imine) is shown. With I initiator.

Polyesters are commonly used as matrixophilic chains for polymeric dispersing agents.⁶ Their polarity has to be tuned depending on the coating medium. Due to its hydrophobicity, poly(ω -PDL) is expected to be a good matrixophilic chain for hydrophobic coating media. However, its high crystallinity severely hampers its solubility in most organic solvents. By consequence, poly(ω -PDL) as such cannot be used as matrixophilic chain for dispersing agents. It is well known that the polymerization of a lactone with a comonomer is one method to reduce the crystalline content of a copolymer

and thus improve its solubility.⁷ The microstructure of copolyesters then plays an important role in disrupting interactions within the polymer chains and therefore preventing packing of the chains.

Previous studies from the literature demonstrate that the microstructure of ω -PDL-based copolyesters synthesized in one-pot is dependent on the structure of the comonomer. When copolymerized with unsubstituted lactones such as δ -valerolactone,⁸ ϵ -caprolactone^{5, 8-9} or 1,4-dioxan-2-one,¹⁰ ω -PDL-based copolymers displayed a near random order. However, crystallinity was retained over the whole composition range.⁸ As was reviewed in **Chapter 2**, substituted lactones such as menthilde, ϵ -decalactone, dihydrocarvide and δ -hexalactone yield amorphous homopolyesters because of the alkyl substituents preventing crystallization (respective glass transitions are $T_g = 22\text{ }^\circ\text{C}$, $-53\text{ }^\circ\text{C}$, $-20\text{ }^\circ\text{C}$ and $-38\text{ }^\circ\text{C}$).¹¹⁻¹⁴ Despite these alkyl substituents, crystalline copolyesters with ω -PDL were obtained, even at low ω -PDL ratio, because of the block-like structure of the copolyester (except for menthilde/ ω -PDL 90/10¹⁵).^{5, 9, 15-18}

So far, the synthesis of random ω -PDL-based copolyesters using alkyl substituted lactones has proven unsuccessful. The main reason is the difference of reactivity between ω -PDL and these lactones. Furthermore, transesterification side reactions, which increase randomness of the structure of the copolyesters, are blocked by the position of the alkyl substituent of the alkyl substituted lactone comonomers. As a result, block-like structure remains an obstacle for the solubility range of ω -PDL-based copolyesters.

Scheme 7.2. Copolymerization of ω -PDL with either δ -UDL or TMCL using 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) as catalyst and benzyl alcohol as initiator.

The aim of this chapter is thus to increase the solubility range of ω -PDL-based copolyesters by using alkyl substituted lactones as comonomers, δ -undecalactone (δ -UDL) and a mixture of β,δ -trimethyl- ϵ -caprolactones (TMCL) (Scheme 7.2). Random copolyesters are targeted in order to suppress the crystallinity of poly(ω -PDL), and to broaden the solubility range of ω -PDL-based copolyesters.

7.2 Results and discussion

7.2.a. Copolymerization reaction conditions

In order to synthesize a ω -PDL-based copolyester of improved solubility, the copolyester should have a decreased crystallinity. Small sized lactones with alkyl substituents were thus considered as comonomers. For example, δ -UDL, which is a δ -valerolactone derivative with a long aliphatic $-C_6H_{13}$ alkyl chain in δ -position, was chosen as comonomer.¹⁹ Random copolymer structure should be preferred over block-like structure which may retain the crystallinity of ω -PDL.⁷ The monomers should have similar reactivity in order to obtain a random structure of the copolyester.

Upon homopolymerization at 75 °C, ω -PDL reaches full monomer conversion after 20 h (Figure 7.1a). Moreover, its conversion increases with the reaction temperature (Figure 7.2). This is consistent with the fact that this macrolactone polymerizes *via* a transesterification polymerization because of the lack of ring strain of its structure. On the other hand, upon polymerization at 75 °C, δ -UDL conversion reaches equilibrium within 30 min to about 60 % conversion (Figure 7.1a). As highlighted in **Chapter 5**, the ROP of small strained lactones is dependent on thermodynamics.²⁰ Monomer conversion decreases as the temperature increases until the ceiling temperature (T_c) is reached, after which no conversion is observed (Figure 7.2). A T_c for 135 °C was determined for δ -UDL in bulk (Table 5.3, **Appendix A**).

In order to copolymerize ω -PDL, which is a strainless macrolactone requiring high reaction temperatures, and δ -UDL, which is a small strained lactone whose conversion increases with lower temperatures, an intermediate temperature of 75 °C was chosen (Figure 7.2). During copolymerization of ω -PDL with δ -UDL in one-pot, both monomers polymerized slower compared to their respective homopolymerizations (Figure 7.1a-b). δ -UDL however polymerized faster than ω -PDL. Consequently, one-pot copolymerization of ω -PDL with δ -UDL is likely to afford copolyesters of block-like structure, with δ -UDL homopolymerizing first followed by increased incorporation of ω -PDL over time.

Figure 7.1. Monomer conversion as a function of time for polymerizations at 75 °C of ω -PDL and δ -UDL for a) homopolymerization; and copolymerizations with a feed ratio of $f_{\omega\text{-PDL}} = 0.5$ (total $DP_n = 16$) with b) one-pot synthesis, c) ω -PDL added to δ -UDL over 30 min, and d) δ -UDL added to ω -PDL over 30 min.

Figure 7.2. Temperature dependence of bulk homopolymerizations with ω -PDL and δ -UDL conversions after 60 min (BnOH/ ω -PDL/TBD 1/20/1 and BnOH/ δ -UDL/TBD 1/15/1).

7.2.b. Copolymerizations of ω -PDL with δ -UDL

In order to avoid block-like structures due to the difference of reactivity of the comonomers, copolymerizations with different monomer addition strategies were investigated (one-pot, gradual addition of ω -PDL to δ -UDL and gradual addition of δ -UDL to ω -PDL) with varying ω -PDL feed ratios ($f_{\omega\text{-PDL}}$). It was expected that by manipulating the conversion vs. time behavior of the monomers, a random monomer distribution could be induced (Table 7.1). In particular, it was hypothesized that addition of the faster monomer (δ -UDL) to the slower comonomer (ω -PDL) was the strategy most likely to yield copolymers of random monomer distribution.

A discrepancy between the ω -PDL feed ratio ($f_{\omega\text{-PDL}}$) and the ω -PDL molar ratio ($F_{\omega\text{-PDL}}$) in the prepared copolyesters was observed (Table 7.1). In particular, the targeted DP_n was approximately reached for δ -UDL while it was higher than targeted for ω -PDL. During homopolymerizations at 75 °C, initiation efficiency (calculated from ^1H NMR using Ar-CH₂- protons of benzyl alcohol (BnOH) at 4.68 ppm and at 5.10 ppm for initiated Ar-CH₂-O-(C=O)) was higher for δ -UDL than for ω -PDL, with 96 % and 89 % efficiency respectively. During copolymerizations, the overall initiation efficiency was also lower than 100 % (Table 7.1). As a consequence, the targeted DP_n was approximately reached for δ -UDL while no full δ -UDL conversion was obtained. For ω -PDL, overall higher conversions were observed than for δ -UDL. This, in combination with the lack of full initiation efficiency, resulted in higher measured $DP_{n,\text{NMR}}$ than targeted. Accordingly, the molecular weight of the copolymers as measured by ^1H NMR was systematically higher than the theoretical molecular weight calculated based on the feed ratio and conversion of the monomers. Examples of the ^1H NMR spectra of the purified copolyesters are shown in Figure 7.3.

The synthesis strategy did not however influence the final conversion ω -PDL, which remained > 95 % for all copolymerization methods (Table 7.1), probably due to the transesterification polymerization that ω -PDL undergoes. The conversion of δ -UDL, on the other hand, was lower when δ -UDL was gradually added to ω -PDL compared to the two other synthesis strategies. It is known that the monomer conversion at equilibrium of strained lactones increases with initial monomer concentration at a given temperature.²⁰ When δ -UDL is gradually added to ω -PDL, the δ -UDL concentration remains lower than during one-pot synthesis, therefore reaching lower conversions. Decreasing the speed of addition of the comonomer was shown to increase the final monomer conversion (Table 7.1, entries **8** vs. **9** and **12** vs. **13**).

Table 7.1. Copolymerizations of ω -PDL with comonomer M (δ -UDL or TMCL) using one-pot synthesis or gradual monomer addition as function of the ω -PDL feed ratio ($f_{\omega\text{-PDL}}$).

	Addition	M	$f_{\omega\text{-PDL}}$	$DP_{n,\text{NMR}}^a$ $\omega\text{-PDL}/M^a$	$F_{\omega\text{-PDL}}^b$	Add. time (h)	Ini. eff. ^c (%)	Conv ^d (%)	
								$\omega\text{-PDL}$	M
1	One-pot	δ -UDL	0.25	7/12	0.35	/	82	95	78
2		TMCL	0.25 ^h	0/0	0	/	0	0	0
3		δ -UDL	0.50	12/7	0.63	/	88	100	83
4		TMCL	0.50	13/8	0.62	/	83	99	61
5		δ -UDL	0.75	24/4	0.86	/	75	97	85
6		TMCL	0.75	18/4	0.81	/	78	96	38
7	ω -PDL added to	δ -UDL	0.25	8/11	0.44	4	79	99	85
8			0.50	15/7	0.69	4	82	100	84
9			0.50	16/7	0.69	0.5	80	100	67
10		δ -UDL	0.75	23/3	0.89	4	80	99	78
11		δ -UDL	0.25	9/14	0.39	4	90	98	64
12			0.50	12/9	0.59	4	85	100	90
13			0.50	6/3	0.89	0.5	90	43	43
14	ω -PDL		0.75	19/4	0.84	4	63	98	75
	Addition (continued)	M	$f_{\omega\text{-PDL}}$	$M_{n,\text{th}}^e$ (kg mol ⁻¹)	$M_{n,\text{NMR}}^f$ (kg mol ⁻¹)		$M_{n,\text{GPC}}^g$ (kg mol ⁻¹)	$\bar{D}_M^g$	
1	One-pot	δ -UDL	0.25	2.7	4.0		9.8	1.54	
2		TMCL	0.25 ^h	0	0.0		-	-	
3		δ -UDL	0.50	3.3	4.3		10.2	1.64	
4		TMCL	0.50	2.8	4.7		6.9	1.80	
5		δ -UDL	0.75	3.5	6.6		6.6	1.60	
6		TMCL	0.75	3.1	5.1		6.2	1.69	
7	ω -PDL added to	δ -UDL	0.25	2.9	4.1		10.5	1.42	
8			0.50	3.3	5.0		6.4*	2.21	
9			0.50	3.0	5.2		5.0*	2.09	
10		δ -UDL	0.75	3.5	6.2		7.1*	1.87	
11		δ -UDL	0.25	2.5	4.8		10.3	1.37	
12			0.50	3.4	4.6		8.2	1.54	
13			0.50	1.6	2.1		2.4*	1.68	
14	ω -PDL		0.75	3.5	5.4		5.0*	1.43	

^a Determined by ¹H NMR on the purified polymers using the signals of Ar-CH₂-O-C=O (BnOH, 5.10 ppm), CH₂O-C=O (ω -PDL, 4.03 ppm), CH(C₆H₁₃)-O-C=O (δ -UDL, 4.86 ppm) and -CH₃ (TMCL, 0.80-1.05 ppm). ^b $F_{\omega\text{-PDL}} = DP_{n,\omega\text{-PDL}}/(DP_{n,M} + DP_{n,\omega\text{-PDL}})$. ^c Initiator efficiency determined by ¹H NMR on the crude polymers after 20 h (Ar-CH₂-O-(C=O) at 5.10 ppm and Ar-CH₂-OH at 4.68 ppm). ^d Determined after 20 h by ¹H NMR (ω -PDL and δ -UDL) and by GC-FID (TMCL).

^e $M_{n,\text{th}} = 108.1 + (DP_{n,\text{th},\omega\text{-PDL}} \times \% \text{ conv}_{\omega\text{-PDL}}/100 \times 240.0) + (DP_{n,\text{th},\delta\text{-UDL or TMCL}} \times \% \text{ conv}_{\delta\text{-UDL or TMCL}}/100 \times M_M)$. ^f $M_{n,\text{NMR}} = 108.1 + (240.0 \times DP_{n,\text{NMR},\omega\text{-PDL}}) + (M_M \times DP_{n,\text{NMR},\delta\text{-UDL or TMCL}})$.

^g Determined by GPC in THF against polystyrene standards. ^h No polymer was obtained. * Not fully dissolved in THF. Reaction conditions: BnOH/ ω -PDL+M/TBD 1/16/1, bulk, 20 h at 75 °C.

Figure 7.3. ^1H NMR spectrum of purified copolymers with a) poly(ω -PDL-*co*- δ -UDL) (Table 7.1, entry **3**), and b) poly(ω -PDL-*co*-TMCL) (Table 7.1, entry **6**).

It was hypothesized that gradual addition of δ -UDL to ω -PDL would avoid composition drift and ensure a more random monomer distribution of the poly(ω -PDL-*co*- δ -UDL). As expected, the addition of δ -UDL to ω -PDL resulted in similar conversions of

both monomers in contrast to the one-pot polymerization (Figure 7.1b vs. 7.1d). As could be expected for the contrary case of our hypothesis, the addition of ω -PDL to δ -UDL, *i.e.* addition of the slow lactone to the fast lactone resulted in a much lower conversion rate of ω -PDL and a higher conversion rate of δ -UDL, compared to the one-pot polymerization (Figure 7.1c vs. 7.1b). Comparison of the synthesis strategies thus shows that addition of δ -UDL to ω -PDL leads to a more similar conversion vs. time behavior of both lactones compared to one-pot polymerization.

7.2.c. Microstructure of poly(ω -PDL-*co*- δ -UDL)

The influence of the monomer addition strategy on the monomer distribution in the copolymers was investigated using ^{13}C NMR and MALDI-ToF MS. The monomer distribution within the copolyesters was assessed using semi-quantitative ^{13}C NMR spectroscopy (Table 7.2).²¹ As shown in Figure 7.4a, four diad distributions were observed: ω -PDL* ω -PDL and δ -UDL* δ -UDL corresponding to ω -PDL and δ -UDL blocks as well as ω -PDL* δ -UDL and δ -UDL* ω -PDL which correspond to random structures. The diads involving δ -UDL were sometimes doubled due to the stereochemistry on the δ -position. Because ω -PDL and δ -UDL did not show full monomer conversion, the theoretical diad distribution calculated for a statistically random copolymer (values indicated in between parentheses in Table 7.2) was corrected taking into account the final monomer conversions (see calculations in Chapter 6, Eq 6.4).

Figure 7.4. Typical diad distribution determined by ^{13}C NMR of a) poly(ω -PDL-*co*- δ -UDL) with ω -PDL* ω -PDL (174.11 ppm), ω -PDL* δ -UDL (173.76 ppm), δ -UDL* ω -PDL (173.58 ppm) and δ -UDL* δ -UDL (173.23 ppm) and b) poly(ω -PDL-*co*-TMCL) with ω -PDL* ω -PDL (174.14 ppm), ω -PDL* TMCL (174.09 ppm), TMCL * ω -PDL (174.04 ppm), TMCL * TMCL (173.03 ppm) and TMCL * TMCL (172.33 ppm).

Table 7.2. Monomer sequencing of ω -PDL-based copolyesters based on their experimental diad distribution as determined by ^{13}C NMR.

	Synthesis	M	$F_{\omega\text{-PDL}}^a$	Diad distribution: experimental ^b (theoretical) ^c				Sequencing
				$\omega\text{-PDL}^*$ - $\omega\text{-PDL}$	$\omega\text{-PDL}^*$ -M	M*- $\omega\text{-PDL}$	M*-M	
1		δ -UDL	0.35	16 (8)	17 (21)	17 (21)	50 (51)	Random-like
2		TMCL	-	-	-	-	-	-
3	One-pot	δ -UDL	0.63	41 (30)	26 (25)	16 (25)	16 (20)	Block-like with alternating
4		TMCL	0.62	41 (38)	15 (24)	17 (24)	27 (15)	Block-like with alternating
5		δ -UDL	0.86	73 (52)	14 (20)	7 (20)	6 (8)	ω -PDL block-like
6		TMCL	0.81	64 (79)	11 (10)	10 (10)	16 (1)	ω -PDL block-like
7	ω -PDL added to δ -UDL	δ -UDL	0.44	30 (8)	21 (20)	17 (20)	32 (52)	Random-like
8			0.69	55 (30)	16 (25)	15 (25)	14 (21)	Block-like with alternating
9			0.69	5 (36)	22 (24)	16 (24)	11 (16)	Block-like with alternating
10			0.89	100 (63)	0 (16)	0 (16)	0 (4)	ω -PDL block-like
11	δ -UDL added to ω -PDL	δ -UDL	0.39	21 (11)	19 (22)	16 (22)	45 (44)	Random-like
12			0.59	44 (28)	25 (25)	14 (25)	1 (22)	Block-like with alternating
13			0.89	5 (25)	14 (25)	20 (25)	12 (25)	ω -PDL block-like
14			0.84	8 (63)	12 (16)	5 (16)	0 (4)	ω -PDL block-like

^a Determined by ^1H NMR. ^b Determined by semi-quantitative ^{13}C NMR spectroscopy, with * defining the carbonyl analyzed. ^c Theoretical diad distribution for a statistically random copolymer in parentheses (details of calculations in Eq 6.4).

Increasing the ω -PDL feed ratio led to increased $\omega\text{-PDL}^*\text{-}\omega\text{-PDL}$ diads compared to the theoretical diad distribution calculated for statistically random copolymers, independently of the synthesis strategy. As a consequence, the same change of microstructure was observed when increasing the ω -PDL molar ratio for each synthesis strategy. At low ω -PDL molar ratio ($F_{\omega\text{-PDL}} < 0.44$), the copolymers displayed random or random-like microstructures. Microstructures become block-like with alternating

sequences for $0.59 < F_{\omega\text{-PDL}} < 0.69$. Lastly, $F_{\omega\text{-PDL}} > 0.84$ resulted in a ω -PDL block-like structure. It should be noted that shortening the comonomer addition time for the addition of δ -UDL to ω -PDL from 4 h to 30 min increased the $F_{\omega\text{-PDL}}$, which resulted in a ω -PDL block-like structure rather than a block-like with alternating sequence microstructure (Table 7.2, entry **12** vs. **13**).

ω -PDL rich copolyesters synthesized with $f_{\omega\text{-PDL}} = 0.75$ (feed ratio of ω -PDL/ δ -UDL 12/4; Table 7.2, entries **5**, **10** and **14**) displayed a clear ω -PDL block-like structure independent of the synthesis strategy ($73\% < \omega\text{-PDL}^*\text{-}\omega\text{-PDL diad} < 100\%$). For these copolymers, the δ -UDL* δ -UDL diad was either very low (one-pot synthesis, entry **5**) or (almost) not observed (addition strategies, entries **10** and **14**). When ω -PDL was added to δ -UDL, only the $\omega\text{-PDL}^*\text{-}\omega\text{-PDL}$ distribution could be observed, despite the conversion of δ -UDL and a DP_n of δ -UDL of 3 (Table 7.1 and 7.2, entry **10**). On the other hand, the δ -UDL-rich copolymers synthesized with $f_{\omega\text{-PDL}} = 0.25$ (ω -PDL/ δ -UDL feed ratio of 4/12; Table 7.2, entries **1**, **7** and **11**) showed good correlation between the measured diad distribution and the theoretical random diad distribution. They were thus considered as random-like copolymers. In the case of the copolymer prepared by addition of ω -PDL to δ -UDL, the correlation between the measured and the theoretical diad distribution was not as good compared to the other synthesis strategies but a good distribution of all the diads was observed despite the increased $F_{\omega\text{-PDL}}$ induced by this synthesis strategy.

Copolymers synthesized from an equal amount of comonomers, *i.e.* with $f_{\omega\text{-PDL}} = 0.5$, were actually ω -PDL-rich with $F_{\omega\text{-PDL}} > 0.5$ (Table 7.2, entries **3**, **8**, **9**, **12**, and **13**). The δ -UDL* δ -UDL diad distribution of these copolymers was always lower than expected due to the steric hindrance of the alkyl substituent on the δ -carbon of δ -UDL.¹⁵ Consequently, the microstructure of these copolymers was that of block-like copolymers with some alternating patterns as shown by the $\omega\text{-PDL}^*\text{-}\delta\text{-UDL}$ and $\delta\text{-UDL}^*\text{-}\omega\text{-PDL}$ diad distributions. As expected, the addition of ω -PDL to δ -UDL led to more block-like copolymers than the other synthesis strategies, with higher $\omega\text{-PDL}^*\text{-}\omega\text{-PDL}$ and lower $\delta\text{-UDL}^*\text{-}\delta\text{-UDL}$ diad distributions than theoretically calculated (Table 7.2, entries **7**, **8**, **9** and **10**). On the contrary, one-pot polymerization and addition of δ -UDL to ω -PDL both resulted in copolymers with diad distributions closer to what was expected.

7.2.d. Composition and topology of poly(ω -PDL-*co*- δ -UDL) with MALDI-ToF MS

The average chemical distribution of a copolymer is shown by a line drawn through the center of its contour plot (Figure 7.5). As described in **Chapter 6**, random copolymers are characterized by a line crossing the origin with a constant slope while block

copolymers are characterized by a line that does not cross the origin. In the latter case, the contour plot displays an oval or circular shape. None of the white dotted lines in the contour plots in Figure 7.5 goes through the origin, nor parallel to the axis, which confirms the block-like character of the corresponding copolymers rather than random or purely block. The structure of the corresponding MALDI-ToF MS spectra are given in Figure 7.6. The *average chemical composition* of the poly(ω -PDL-*co*- δ -UDL) copolymers can also be extracted from MALDI-ToF MS contour analysis.

The contour plots of the poly(ω -PDL-*co*- δ -UDL) synthesized with $f_{\omega\text{-PDL}} = 0.75$ clearly indicate that the sequences display a majority of ω -PDL units. This is especially the case for the copolymer prepared by one-pot synthesis (Figure 7.5a) whose contour plot is shifted below the diagonal indicating a high ω -PDL content in the polymer. When ω -PDL was added to δ -UDL ($f_{\omega\text{-PDL}} = 0.75$) the copolymer actually consisted of a mixture of sequences with high ω -PDL content as well as (cyclic) poly(ω -PDL) homopolymers (Figure 7.6b). It is therefore not surprising that the diad distributions involving δ -UDL could not be observed by ^{13}C NMR (Table 7.2, entry **10**). The contour plot of this copolymer clearly indicates that it is mainly composed of linear ω -PDL-rich sequences as well as cyclic sequences (Figure 7.5b-c).

The copolymer resulting from the addition of δ -UDL to ω -PDL with $f_{\omega\text{-PDL}} = 0.75$ comprises sequences with high δ -UDL content as can be seen from the shift above the diagonal on the contour plot (Figure 7.5d). The addition of δ -UDL to ω -PDL resulted in a copolymer with a broader dispersity in the sequences compared to one-pot synthesis (Figure 7.5d *vs.* Figure 7.5a). When the comonomer feed ratio was decreased to $f_{\omega\text{-PDL}} = 0.5$ during one-pot synthesis, the contour plot was symmetrical with respect to the diagonal (Figure 7.5e). This symmetry indicates that the copolymer should have a ω -PDL molar ratio ($F_{\omega\text{-PDL}}$) of about 0.5, which is close to what was measured by ^1H NMR (Table 7.1, entry **3**).

Decreasing further the comonomer feed ratio to $f_{\omega\text{-PDL}} = 0.25$ for one-pot synthesis led to a contour plot whose average chemical composition is close to the δ -UDL axis (Figure 7.5g). Additionally, this polymer also contained some δ -UDL homopolymers, which was to be expected given the high δ -UDL content of the copolymer and the high δ -UDL* δ -UDL diad (Figure 7.6f and Table 7.2, entry **1**).

Figure 7.5. Contour plots of the MALDI-ToF MS spectra of copolymers a) entry **5**, b) entry **10**, c) entry **10** (cyclics), d) entry **14**, e) entry **3**, f) entry **4**, and g) entry **1**. The white dotted line indicates the average chemical composition of a random copolymer. The distribution represented is BnO- $M_{1,n}$ -co- $M_{2,m}$ -H, K^+ , with M_1 and M_2 the two monomers, except for c) which represents cyclic structures.

Figure 7.6. MALDI-ToF MS spectra of a) entry 5, b) entry 10, c) entry 14, d) entry 3, e) entry 4, and f) entry 1. Squares indicate poly(ω -PDL) homopolymer (black for linear and empty for cyclics) and black stars indicate poly(δ -UDL) homopolymer. The symbols indicate the range of values of ω -PDL molar ratio ($F_{\omega\text{-PDL}} > 0.7$: black triangles, $0.3 < F_{\omega\text{-PDL}} < 0.7$: black circles, $F_{\omega\text{-PDL}} > 0.3$: empty circles).

7.2.e. Thermal behavior of the copolyesters

The thermal behavior of the copolyesters was assessed by measuring the enthalpy of melting ΔH_m and melting temperature T_m with differential scanning calorimetry (DSC). Thermographs of the copolymers are shown in Figure 7.7. T_m and ΔH_m values are given in Table 7.3.

Figure 7.7. DSC thermographs (2nd heating) of poly(ω -PDL-*co*- δ -UDL) synthesized in one-pot (black full line), with ω -PDL addition to δ -UDL (black dashed line) and with δ -UDL addition to ω -PDL (black dotted line) with $f_{\omega\text{-PDL}}$ feed ratios of a) 0.25, b) 0.50, and c) 0.75; d) poly(ω -PDL-*co*-TMCL) synthesized in one-pot with $f_{\omega\text{-PDL}} = 0.50$ (full line) and $f_{\omega\text{-PDL}} = 0.75$ (blue dashed line).

Increasing the ω -PDL content increased the crystallinity of poly(ω -PDL-*co*- δ -UDL)s (Figure 7.8a). Residual crystallinity can be attributed to crystallization of ω -PDL block-like regions in a δ -UDL-rich environment, poly(δ -UDL) being an amorphous polyester ($T_g = -53$ °C).²² Interestingly, the synthesis strategy had the most influence on ω -PDL-rich copolyesters ($F_{\omega\text{-PDL}} > 0.80$). For these copolymers, the lowest crystallinity was achieved for copolymers synthesized by addition of ω -PDL to δ -UDL ($\Delta H_m = 9$ J mol⁻¹).

Table 7.3. Thermal analysis of the copolyesters by DSC.

	Synthesis	M	$F_{\omega\text{-PDL}}^a$	T_m^b (°C)	ΔH_m^b (J g ⁻¹)
1	One-pot	δ -UDL	0.35	45	5
2		TMCL	-	-	-
3		δ -UDL	0.63	61/ 67	30
4		TMCL	0.62	57/ 64	3
5		δ -UDL	0.86	86	49
6		TMCL	0.81	82	6
7	ω -PDL added to δ -UDL	δ -UDL	0.44	48	9
8			0.69	70/ 75	23
9			0.69	67/ 71	23
10			0.89	83	9
11	δ -UDL added to ω -PDL	δ -UDL	0.39	50	8
12			0.59	60/ 67	10
13			0.89	63/ 68	12
14			0.84	70/ 72	24

^a Determined by ¹H NMR. ^b Determined by DSC with heating rate of 10 °C min⁻¹. T_m measured at the top of the melting transition. Two T_m values indicated when several melting transitions were observed (see Figure 7.7 for thermographs).

The sharp decrease in crystallinity of the copolymers synthesized by addition of δ -UDL to ω -PDL can in fact be attributed to the presence of δ -UDL-rich sequences ($F_{\omega\text{-PDL}} < 0.3$) as shown by MALDI-ToF MS (Figures 7.5d and 7.6c). These δ -UDL-rich sequences are not present when the copolymer was synthesized by addition of ω -PDL to δ -UDL (Figure 7.6b). The one-pot synthesis yielded copolymers with the highest crystallinity for various ω -PDL molar ratios. This is consistent with the fact that these copolymers comprise sequences with a similar number of ω -PDL and δ -UDL units, *i.e.* that they are not especially δ -UDL-rich, which contributes to lowering the crystallinity due to the presence of this amorphous section.

Interestingly, a decrease in crystallinity was observed for increasing δ -UDL molar ratios despite the fact that the copolymers do not have a purely random microstructure. The presence of poly(δ -UDL) blocks in poly(ω -PDL-*co*- δ -UDL) with $f_{\omega\text{-PDL}} = 0.25$ (Table 7.2, entries **1**, **7** and **11**) as shown by the δ -UDL*- δ -UDL diad (between 32 % and 50 %) helped in lowering the overall crystallinity and the melting temperature of the copolymers because of the amorphous nature of this block.

Figure 7.8. Thermal behavior (DSC) of ω -PDL copolyesters as a function of the ω -PDL molar ratio ($F_{\omega\text{-PDL}}$) with a) melting enthalpy ΔH_m , and b) melting temperature T_m (top values, average was taken in the case of two melting points). DSC was measured with a heating rate of 10 °C min⁻¹ for the second heating run.

All of the copolyesters synthesized by addition of δ -UDL to ω -PDL displayed a double melting peak (Figure 7.7a-c) due to the presence of several microstructures. Because the gradual addition of δ -UDL does not favor high δ -UDL conversion due to the lowered δ -UDL concentration, the copolyesters presented ω -PDL block-like regions as well as mixed ω -PDL- δ -UDL regions which show different thermal behaviors resulting in two melting temperatures corresponding to different regions.

The melting temperature of all poly(ω -PDL-*co*- δ -UDL) was decreased compared to pure poly(ω -PDL), which has a high melting temperature ($T_m = 86$ °C). Crystallinity is caused by ω -PDL-rich regions due to the high regularity of the structure of the monomer. Because of the presence of the alkyl substituted δ -UDL, all the ω -PDL units are not able to crystallize since the alkyl substituent and the absence of stereoselectivity on the δ -position prevent polymer chain packing. As a consequence, the melting temperature is lowered because the composition of the crystallites is modified.

7.2.f. Hansen solubility of the copolyesters

The solubility range of ω -PDL-based copolyesters was assessed using the Hansen solubility parameters, by dissolving them in solvents classified in categories based on their dispersive forces (δ_D), polar forces (δ_P) and hydrogen bonding (δ_H) contributions between molecules, according to the definition of Hansen.²³ Solubility spheres of ω -PDL-based copolyesters of increased size (*i.e.* the polymers are soluble in more solvents) were obtained for increased δ -UDL molar ratio (Figure 7.9).

Figure 7.9. Hansen solubility spheres of ω -PDL-based polyesters synthesized in one-pot with increasing F_M comonomer molar ratio ($M = \delta$ -UDL above and TMCL below) with a) poly(ω -PDL), b) poly(ω -PDL₁₂-co- δ -UDL₇), c) poly(ω -PDL₇-co- δ -UDL₁₂), d) poly(δ -UDL), e) poly(ω -PDL₁₈-co-TMCL₄), f) poly(ω -PDL₁₃-co-TMCL₈), and g) poly(TMCL).

The solubility sphere of pure poly(ω -PDL) is very limited, it can only be dissolved in chloroform and hexafluoroisopropanol (HFIP). This is due to the large apolar segments of poly(ω -PDL) in between the ester groups which give to the polyester its high crystallinity, thus restricting its solubility range. On the contrary, the solubility sphere of poly(δ -UDL) is larger. This polyester can be dissolved in some polar protic solvents such as alcohols from ethanol to octanol, as well as some polar aprotic solvents. Because of the amorphous behavior of poly(δ -UDL) at room temperature, solubility is not hindered. Moreover, the small ring size of δ -UDL ensures high frequency of ester groups within the polyester, which interact with polar solvents, while the long alkyl chain ($-\text{C}_6\text{H}_{13}$) enables solubility with apolar solvents with high dispersive forces. The higher entropy of the branched poly(δ -UDL) compared to poly(ω -PDL) also explains the broader solubility sphere.

Increasing the δ -UDL molar ratio of PDL-based copolyesters up to $F_{\delta\text{-UDL}} = 0.37$ retains the ability of poly(ω -PDL₁₂-co- δ -UDL₇) to be dissolved in non-polar solvents (ethyl benzene, chlorobenzene and *m*-cresol). Additionally, it enables broadening the solubility range toward borderline aprotic solvents such as tetrahydrofuran. Increasing further the δ -UDL content to $F_{\delta\text{-UDL}} = 0.65$, and therefore the oxygen to carbon balance,

allows solubility of poly(ω -PDL₇-*co*- δ -UDL₁₂) in more polar aprotic solvents such as ethyl acetate and diethyl amine. However, the presence of the long alkyl segment between ester groups from the ω -PDL monomer prevents solubility of the copolyester in polar protic solvent, unlike pure poly(δ -UDL).

7.2.g. Effect of comonomer structure: δ -UDL vs. TMCL

It is expected that the solubility range of ω -PDL-based copolyesters can be improved if the copolyesters display a random structure rather than a block-like structure because it would decrease crystallinity. It was hypothesized that a broader solubility range could be obtained with TMCL as comonomer, which is a mixture of β,δ -trimethyl- ϵ -caprolactones (see **Chapter 5**), because transesterification would not be blocked by the position of the substituent as could be observed for δ -UDL (Scheme 7.2). The copolyester structure is expected to be less block-like as a consequence of randomization.

The homopolymerization of TMCL at 75 °C (BnOH/TMCL/TBD 1/20/1) was not successful. It was shown in **Chapter 5** that the TBD-catalyzed ROP of TMCL at 30 °C reached a conversion of only 30 %. Moreover, higher reaction temperatures were even more unfavorable for TMCL polymerization. Although higher conversions were obtained with other catalysts, these organocatalysts are not reported to polymerize ω -PDL, due to the lack of ring strain of the macrolactone. Attempts to synthesize a poly(ω -PDL-*co*-TMCL) with $f_{\omega\text{-PDL}} = 0.25$ remained also unsuccessful, probably for the same reason (Table 7.1, entry **2**). However, two poly(ω -PDL-*co*-TMCL) with $f_{\omega\text{-PDL}} \geq 0.5$ could be synthesized using the one-pot synthesis strategy (Table 7.1, entries **4** and **6**).

Kinetics studies of the copolymerization of ω -PDL and TMCL show that both monomers are built in the copolymer at a similar rate up to 30 % conversion, after which the conversion of ω -PDL slows down compared to that of TMCL (Figure 7.10). After about 1 h, the conversion of TMCL however reaches a plateau at 60 % conversion while ω -PDL continues polymerizing until full ω -PDL conversion is achieved. Consequently, it is expected that the copolymer will contain a ω -PDL block which is formed during the last phase of the copolymerization.

The microstructure of poly(ω -PDL-*co*-TMCL) as analyzed by ¹³C NMR confirms that again block-like copolymers with high contents of ω -PDL*- ω -PDL diads are obtained as was the case with δ -UDL as comonomer (block-like with alternating sequences for entries **3** and **4** and ω -PDL block-like for entries **5** and **6**). However, an increase in the TMCL*-TMCL diads was seen compared to the theoretical diad distribution (Figure 7.4b,

Figure 7.10. Monomer conversion as a function of time of one-pot copolymerization at 75 °C with $f_{\omega\text{-PDL}} = 0.50$ with $\omega\text{-PDL}$ and $\delta\text{-UDL}$ (empty symbols) or $\omega\text{-PDL}$ with TMCL (full symbols).

Table 7.2, entries **4** and **6**) of a statistical random copolymer while using $\delta\text{-UDL}$ resulted in the opposite trend with a lower amount of $\delta\text{-UDL}^*\text{-}\delta\text{-UDL}$ diad than expected. Interestingly, using TMCL rather than $\delta\text{-UDL}$ as comonomer resulted in copolymers of broader compositional dispersity as can be seen on the MALDI-ToF MS contour plots (Figure 7.5e vs. f). This is in correlation with the dispersities measured by GPC ($\mathcal{D}_M = 1.80$ vs. 1.64 for $f_{\omega\text{-PDL}} = 0.5$, and 1.69 vs. 1.60 for $f_{\omega\text{-PDL}} = 0.75$).

Although the melting temperature of poly($\omega\text{-PDL-co-TMCL}$) copolymers remained similar to that of copolymers prepared with $\delta\text{-UDL}$, the crystallinity was drastically lowered for comparable high $\omega\text{-PDL}$ ratios (Figure 7.8). This is due to the increased amorphous domains related to the TMCL blocks compared to $\delta\text{-UDL}$ -based copolymers. Indeed, replacing $\delta\text{-UDL}$ by TMCL resulted in more TMCL $^*\text{-TMCL}$ diads, which indicates that blocky domains of alkyl substituted comonomer are obtained (Table 7.2, entries **4** and **6**). This amorphous block counterbalances the effect of the crystalline $\omega\text{-PDL}$ block. MALDI-ToF MS analysis confirmed that the decrease in crystallinity was due to the successful insertion of TMCL and $\omega\text{-PDL}$ in a copolymer and not to the presence of amorphous poly(TMCL) homopolymer (Figures 7.5e and 7.6e). The absence of $\delta\text{-UDL}$ homopolymers was also confirmed for the corresponding poly($\omega\text{-PDL-co-}\delta\text{-UDL}$) (Figures 7.5d and 7.6d).

The solubility range displayed by poly($\omega\text{-PDL-co-TMCL}$) was comparable to that of poly($\omega\text{-PDL-co-}\delta\text{-UDL}$) of similar $\omega\text{-PDL}$ molar ratio, with solubility in non-polar solvents and borderline aprotic solvents such as tetrahydrofuran for the highest comonomer molar ratio (Figure 7.9). Nevertheless, decreasing the TMCL molar ratio to only $F_{\omega\text{-PDL}} = 0.19$ resulted in limited solubility. Compared to pure poly($\omega\text{-PDL}$), the

solubility range of poly(ω -PDL-*co*-TMCL) was increased only with *m*-cresol. This is however expected because of the block-like structure of the copolymer which makes the copolyester comparable to a pure poly(ω -PDL).

7.3 Conclusions

The goal of this chapter was to increase the solubility range of ω -PDL-based copolyesters by copolymerization with alkyl substituted lactones, such as δ -UDL or TMCL. In order to disrupt the high crystallinity of poly(ω -PDL), it was preferable that the copolymers display a random-like microstructure.

Despite gradual monomer addition, poly(ω -PDL-*co*- δ -UDL)s displayed a block-like structure, because transesterification side reactions are blocked by the δ -position of the substituent of δ -UDL. Random-like copolymers were however achieved by decreasing the ω -PDL molar content. The presence of δ -UDL-rich or ω -PDL-rich sequences was confirmed by MALDI-ToF MS depending on the monomer addition technique. Crystallinity was however significantly decreased even for copolyesters with high ω -PDL molar ratios. This considerable decrease in crystallinity of the copolyesters was attributed to the lack of stereoselectivity of the alkyl substituent of δ -UDL hampering chain packing. The melting temperature T_m remained relatively high because of crystalline ω -PDL-rich regions in an environment of amorphous δ -UDL-rich regions.

Solubility of poly(ω -PDL-*co*- δ -UDL) was greatly improved compared to pure poly(ω -PDL). The copolyesters were soluble in some apolar solvents as well as polar aprotic solvents. Increasing the δ -UDL content resulted in broader solubility due to the high frequency of ester groups thanks to the small ring size of δ -UDL coupled with the long alkyl chain of δ -UDL and the very regular apolar segment of ω -PDL. Additionally, the crystallinity of ω -PDL-based copolyesters was drastically decreased with as little as 20 % molar ratio of comonomer by using TMCL. The increased solubility range of δ -UDL- and TMCL-based copolyesters should enable the use of poly(ω -PDL) as polymer in applications such as dispersing agents in coating formulation which require a broader solubility range than that of pure poly(ω -PDL).

7.4 Experimental section

Materials. Benzyl alcohol (BnOH; 99 %, Alfa Aesar) was used as received. ω -Pentadecalactone (ω -PDL; 98 %, FG, Sigma-Aldrich) and TMCL (synthesized as described in **Chapter 5**) were distilled under reduced pressure over CaH_2 before use (ω -PDL: 1.10^{-3} mbar, 150-160 °C; TMCL: 1.10^{-3} mbar, 95-105 °C). 1,5,7-Triazabicyclo[4.4.0]dec-5-ene (TBD; 98 %, Sigma-Aldrich) was

used as received. δ -Undecalactone (δ -UDL; 97 %, FG, Sigma-Aldrich) was stored on molecular sieves (3 Å) prior to use. Decane (> 99 %, Acros) were used as received. Toluene was dried over Na/benzophenone before use. All other solvents used were supplied from Biosolve and used as received. All glassware was oven-dried at 150 °C prior to use.

^1H and ^{13}C NMR analysis (CDCl_3). Proton (^1H) and carbon (^{13}C) NMR spectra were recorded as described in **Chapter 3**.

GC-FID measurements. Gas chromatography with flame ionization detection (GC-FID) measurements were performed as described in **Chapter 5**. Samples were prepared by dissolving part of the reaction mixture which contained 5 mol % decane relative to the monomers as internal standard in about 1.5 mL of dichloromethane.

MALDI-ToF MS measurements. Matrix-assisted laser desorption/ionization time-of-flight mass spectra were recorded as described in **Chapter 5** except that the polymer samples were dissolved in chloroform. Contour plots were obtained with COCONUT version 1.4.2.²⁴

GPC analysis. Gel permeation chromatography (GPC-THF) was performed as described in **Chapter 5**.

DSC analysis. Differential scanning calorimetry spectra were recorded on a Netzsch Polyma 2014 DSC. DSC data was obtained from about 5 mg of purified polymer at heating/cooling rates of 10 °C min⁻¹ under a nitrogen flow of 20 mL min⁻¹. Indium, zinc, tin and bismuth were used as standards for temperature and enthalpy calibration. DSC heating and cooling cycles were performed from - 70 °C to 140 °C in triplicates. The melting temperatures (top value) and the enthalpy values reported correspond to the second heating cycle.

Hansen solubility parameters. Solubility of the polymers was assessed as described in **Chapter 6**.

Typical homopolymer synthesis. In a 5 mL three-neck round-bottom flask, ω -PDL (2.0 g, 8.3 mmol, 20 eq) and BnOH (43 μL , 0.4 mmol, 1 eq) were added under nitrogen atmosphere. The flask was heated to the reaction temperature (40, 50, 75, 100 or 125 °C). TBD (58 mg, 0.4 mmol, 1 eq) was added after 10 minutes to start the reaction. Conversion was determined by ^1H NMR in CDCl_3 . The reaction was stopped by exposing the reaction mixture to air and let it cool down to room temperature. The polymer was purified by dissolving in chloroform (10 mL) and precipitating in cold methanol (100 mL). The polymer was obtained as a white powder after drying *in vacuo* at room temperature overnight.

Typical one-pot synthesis of poly(ω -PDL-co- δ -UDL). In a 10 mL three neck round-bottom flask, ω -PDL (3.72 g, 15.5 mmol, 8 eq), δ -UDL (3.0 mL, 15.4 mmol, 8 eq) and BnOH (200 μL , 2 mmol, 1 eq) were added under nitrogen atmosphere. The flask was heated to 75 °C. TBD (269 mg, 2 mmol, 1 eq) was added after 10 minutes to start the reaction. Conversion was determined by ^1H NMR in CDCl_3 . After 20 h, the reaction was stopped by exposing the reaction mixture to air and let it cool down to room temperature. The polymer was purified by dissolving in chloroform (15 mL) and

precipitating in cold methanol (150 mL). The polymer was obtained as a white solid after drying in *vacuo* at room temperature overnight.

Typical copolymerization of poly(ω -PDL-co- δ -UDL) with gradual ω -PDL addition. In a 10 mL three neck round-bottom flask, δ -UDL (3.0 mL, 15.4 mmol, 8 eq) and BnOH (200 μ L, 2 mmol, 1 eq) were added under nitrogen atmosphere. The flask was heated to 75 °C. TBD (269 mg, 2 mmol, 1 eq) was added after 10 minutes to start the reaction. ω -PDL (3.72 g, 15.5 mmol, 8 eq) was dissolved in dry toluene at a concentration of 10 M and gradually added over 0.5 h or 4 h to the reaction mixture using a KDS Legato 110 single syringe pump. Conversion was determined by ^1H NMR in CDCl_3 . After 20 h, the reaction was stopped by exposing the reaction mixture to air and let it cool down to room temperature. The polymer was purified by dissolving in chloroform (15 mL) and precipitating in cold methanol (150 mL). The polymer was obtained as a white solid after drying in *vacuo* at room temperature overnight.

Typical copolymerization of poly(ω -PDL-co- δ -UDL) with gradual δ -UDL addition. In a 10 mL three neck round-bottom flask, ω -PDL (3.72 g, 15.5 mmol, 8 eq) and BnOH (200 μ L, 2 mmol, 1 eq) were added under nitrogen atmosphere. The flask was heated to 75 °C. TBD (269 mg, 2 mmol, 1 eq) was added after 10 minutes to start the reaction. δ -UDL (3.0 mL, 15.4 mmol, 8 eq) was gradually added over 0.5 h or 4 h to the reaction mixture using a KDS Legato 110 single syringe pump. Conversion was determined by ^1H NMR in CDCl_3 . After 20 h, the reaction was stopped by exposing the reaction mixture to air and let it cool down to room temperature. The polymer was purified by dissolving in chloroform (15 mL) and precipitating in cold methanol (150 mL). The polymer was obtained as a white solid after drying in *vacuo* at room temperature overnight.

Typical copolymerization of poly(ω -PDL-co-TMCL) in one-pot. In a 5 mL three-neck round-bottom flask, ω -PDL (2.83 g, 11.8 mmol, 8 eq), TMCL (2.0 mL, 11.8 mmol, 8 eq) and BnOH (152 μ L, 1.5 mmol, 1 eq) were added under nitrogen atmosphere. The flask was placed in an oil bath at 75 °C. TBD (204 mg, 1.5 mmol, 1 eq) was added after 10 minutes to start the reaction. Conversion was determined by GC-FID using decane as internal standard (5 mol % relative to the monomers). The reaction was stopped by exposing the reaction mixture to air and let it cool down to room temperature. The polymer was dissolved in chloroform (10 mL) and washed with an acidified aqueous solution (1 $\times$ 10 mL) and then with distilled water (1 $\times$ 10 mL). The polymer was then precipitated from cold methanol (100 mL). The polymer was obtained as a white powder after drying in *vacuo* at room temperature overnight.

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CHAPTER 8

A prospective life-cycle assessment (LCA) of monomer synthesis: comparison of biocatalytic and oxidative chemistry

The environmental impact of chemicals is a growing concern. While biotechnological processes are perceived as being greener than chemical processes, Life Cycle Assessments (LCAs) can provide quantitative evidence. In this chapter, an LCA was performed to compare the chemical and biochemical synthesis of β,δ -trimethyl- ϵ -caprolactones obtained by Baeyer-Villiger oxidation. The LCA is prospective (based on experiments at a small scale with primary data) since the process is at an early stage, as described with the optimization performed in **Chapter 4**. The results show the enzymatic synthesis has a lower environmental impact but that the difference is however not significant between the two synthetic routes at this scale. Key process performance metrics affecting the environmental impact were evaluated by performing a sensitivity analysis. Parameters such as the electricity production source, recycling of solvents and coproduct, reuse of the enzymes, and replacement of the chemical oxidant by greener alternatives were also assessed. The study shows that comparative LCAs can be used to usefully support decisions at an early-stage of process development.

Keywords: sustainable chemistry • early-stage • comparative LCA • cradle-to-gate • process performance metrics

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8.1 Introduction

Enzymatic reactions are typically perceived, and sometimes claimed, as being greener than the corresponding chemical conversions.¹ Their assumed environmental benefits are an incentive for the development of such processes. This contributes to the growing importance of industrial biocatalysis for the synthesis of molecules used as pharmaceuticals, flavors & fragrances, and bulk chemicals.²⁻³ Typical green features associated with the use of enzymes are correlated with the green chemistry principles (Table 8.1).⁴⁻⁵ Enzymatic reactions are usually performed under mild reaction conditions, at low reaction temperature and at ambient pressure. Enzymes operate using water as reaction medium, thereby reducing the use of organic solvents. Additionally, enzymes are biobased, since they are produced from renewable resources in fermentation processes, and biodegradable. More importantly, the major advantages of enzymes is their regio- and enantioselectivity, which results in more straightforward synthetic routes and avoids multiple protection/deprotection steps which generate waste.⁶⁻⁷

There is however some criticism concerning the environmental benefits of enzymatic reactions.⁸ The use of water as reaction medium typically leads to dilute reaction streams since many compounds are poorly water-soluble and may also be inhibitory at higher concentrations. This results in an increased solvent use for product isolation during downstream processing (DSP).⁹ Since water is the solvent, this also comes with costly technology for concentration, on account of its high boiling point. Another concern is the

Table 8.1. Relationship between green chemistry principles and biocatalysis (adapted from reference;⁷ principle n° 4 “safer products by design” is not considered).

	Green chemistry principle	Biocatalysis
1	Waste prevention	Significantly reduced waste
2	Atom economy	More atom and step-economical (selectivity)
3	Less hazardous syntheses	General low toxicity
5	Safer solvents and auxiliaries	Reactions usually performed in water
6	Energy efficiency	Mild conditions (energy-efficient)
7	Renewable feedstocks	Enzymes are renewable Some renewable feedstocks for fermentations
8	Reduced derivatizations	No protection/deprotection steps (selectivity)
9	Catalysis	Enzymes are biocatalysts
12	Safer processes	Mild and safe reaction conditions

high energy consumption associated with the production of enzymes, in particular if they are further purified and immobilized.¹⁰⁻¹¹ Additionally, although oxidative biocatalysis allows the replacement of toxic oxidants by molecular oxygen, it raises other concerns such as the generation of waste associated with the use of cosubstrate to regenerate cofactors, especially when applying isolated enzymes,⁸ as was outlined in **Chapter 2** for the case of Baeyer-Villiger monooxygenases (BVMOs).

In order to quantify and compare the environmental impact of chemical reactions, some green process metrics have already been developed. The most commonly used is the E factor, which is a measure of the amount of waste generated by a reaction (mass of waste / mass of product).¹²⁻¹³ The comparison of the environmental impact of two processes, for example chemical and enzymatic, is however limited due to the fact that the E factor does not take into consideration the type of waste that is generated, nor the energy consumption of each process.⁸

In order to obtain a more accurate quantification of the environmental impact, life cycle assessments (LCAs) are being more widely performed by the scientific community.¹⁴ An LCA is a standardized and internationally recognized tool specifically designed to quantify the environmental impact of a product or service, taking into account its entire life cycle (ISO14044 : 2006).¹⁵ Several LCAs have been applied to chemical processes (both with biobased and fossil based processes),¹⁶ and some of them have demonstrated unexpected results. For example, some biobased polymers and biobased solvents have a higher environmental impact than their fossil-based counterparts.¹⁷⁻¹⁸ Such counterintuitive results demonstrate the need to perform a LCA before claiming the sustainability or green aspects of a given reaction or product synthesis. Comparative LCAs are especially useful to identify the most sustainable synthetic route to a given product, when several routes are possible. The sustainability of enzymatic reactions, as opposed to chemical routes, could for example be claimed for the industrial synthesis of a bulk chemical (the ester myristyl myristate),¹⁹ and a pharmaceutical molecule (the building block 7-aminocephalosporanic acid).²⁰

The quality and comprehensive character of an LCA depends on the reliability of the data, which should also be as representative and as complete as possible.²¹ Conventionally, LCAs have been based on retrospective data from existing processes. In order to provide a quantitative analysis of the environmental impact of a process that has not yet been implemented at industrial scale, a prospective approach has been developed in the recent years.²² Prospective LCAs, which are performed on different development stages of processes such as laboratory scale, simulations, and pilot plants, enable the identification of key steps in the process that require focus for improvement and provide guidance

regarding process up-scaling.²² Although the limitation of prospective LCAs is that they cannot provide an absolute quantification of the environmental impact, due to the type of data on which they are based, they are particularly relevant for the comparison of early-stage processes in order to identify the most advantageous route for upscaling from an environmental perspective. For example, comparative early-stage LCAs have proven useful for the selection of raw chemicals.²³⁻²⁴ It appears that accounting for the energy consumption may be crucial since it plays an important role in LCA and is very dependent on the scale of operation.²⁵

The goal of the current study is to compare the environmental impact of two early-stage synthetic routes for the preparation of the alkyl substituted β,δ -trimethyl- ϵ -caprolactones (TMCL). These cyclic esters were obtained by Baeyer-Villiger oxidation, inserting an oxygen atom in the C-C bond of a ketone (see **Chapter 2** for more details on this reaction).²⁶ The lactones were demonstrated to be of interest for the synthesis of homopolyesters (**Chapter 5**) and copolyesters (**Chapters 6 and 7**). Although it has been known for many years that organic peracids are efficient oxidants for the Baeyer-Villiger oxidation,²⁷ recent research focuses on the development of greener oxidants.²⁸⁻³⁰ The desire to replace toxic oxidants by molecular oxygen is one of the most important motivations for the development of industrial oxidative biocatalysis. Biocatalytic Baeyer-Villiger oxidations are enabled by the use of Baeyer-Villiger monooxygenases, which catalyze the reaction using molecular oxygen as oxidant. These enzymes were screened in **Chapter 3** and the enzymatic synthesis of TMCL was the focus of **Chapters 3 and 4**. Although the range of esters and lactones that can be synthesized using Baeyer-Villiger monooxygenases is increasing, including for example (substituted) lactones of various ring sizes, steroids and bicyclic compounds,³¹ these biocatalysts have not yet been reported at truly industrial scale. Consequently, these reactions are still carried out at laboratory or pilot-plant scale, with a few exceptions (see **Chapter 2** for an overview).³²

TMCL can be synthesized *via* two synthetic routes from the same cyclic ketone 3,3,5-trimethylcyclohexanone (TMCH) (Figure 8.1). The first route is a chemical Baeyer-Villiger oxidation using the peracid *m*-chloroperbenzoic acid (*m*-CPBA) as chemical oxidant.³³ The second route is an enzymatic oxidation with oxygen as oxidant, catalyzed by a Baeyer-Villiger monooxygenase from *Thermocrispum municipale* (TmCHMO).³⁴ Because the product can be used for several types of polymers (as plasticizers, or dispersants for inks and coatings),^{33, 35-36} which have different applications, the end-of-life of the product is not included in this LCA. The studied boundaries of this assessment are therefore cradle-to-gate as shown in Figure 8.1. This LCA is based on primary data for the two syntheses, which comprises the synthesis of the product by oxidation and the product isolation procedure. The substrate, the chemical oxidant and the enzymes were also

Boundaries studied: comparative cradle-to-gate LCA

Figure 8.1. Boundaries studied for the synthesis of the product (TMCL): comparative cradle-to-gate assessment comprising (1) the synthesis of the substrate (TMCH), (2) the synthesis of the chemical oxidant, (3) the synthesis of the product using a chemical oxidant, (4) the enzyme preparation, and (5) the enzymatic synthesis of the product using a Baeyer-Villiger monooxygenase (BVMO). All reactions include downstream processing (DSP).

modelled and included in the study. It is worth noting that this comparative LCA is a worst case scenario for the comparison of this type of enzymatic reaction with its chemical counterpart because the main advantage of biocatalyst, and Baeyer-Villiger monooxygenases in particular, which is their regio- and enantioselectivity, is not exploited in this case. This LCA evaluates the synthesis of a monomer, which can be considered as a bulk chemical – with high production volume and low price, for which enzymatic reactions need to be particularly efficient to be competitive. This means that the enzymatic reaction has been further developed to improve its process performance metrics to achieve higher space-time yield by increasing the product concentration and lowering the reaction time.³⁷ These improvements are also expected to contribute to reducing the environmental impact of the biocatalytic reaction.

8.2 Methodology

The present LCA was performed in accordance with the ISO standard (ISO 14044: 2006). The methodology followed the LCA framework and is described below.

- **Goal, scope definition, function, and functional unit**

This study is a comparative LCA between two synthetic routes for the same lactone product, TMCL. Both syntheses were performed at laboratory scale starting from the same substrate, TMCH. The function of the study is the synthesis and purification of the TMCL product. The functional unit was selected according to the product category rules (PCR) of the environmental product declaration (EDP) which suggests using a physical reference.³⁸ A functional unit of 1 g of TMCL product was chosen since it is representative of the laboratory scale experiments.

- **System description and boundaries**

As mentioned previously, the boundaries of this comparative LCA are cradle-to-gate since there are several applications for the product evaluated. The study includes the synthesis and purification of the product TMCL, which is synthesized either chemically using *m*-CPBA as chemical oxidant (Figure 8.2), or enzymatically with a Baeyer-Villiger monooxygenase which uses molecular oxygen as oxidant (Figure 8.3). In total, the life cycle inventory consists of five parts:

1) The synthesis of the substrate TMCH is common to the two synthetic routes (Figures 8.2 and 8.3). The base-catalyzed aldol condensation of acetone gives isophorone (step **1a**), which is then hydrogenated in supercritical CO₂ with a Palladium catalyst to produce TMCH (step **1b**). Many catalysts and experimental procedures have been reported for the preparation of isophorone from acetone.³⁹⁻⁴¹ Although some procedures report better selectivity, the procedure selected for this study gave the most complete information over the mass balance.⁴²

2) The synthesis of the chemical oxidant *m*-CPBA is performed in two steps (Figure 8.2).⁴³⁻⁴⁴ In step **2a**, *m*-chlorobenzoic acid is chlorinated using thionyl chloride. The corresponding peracid *m*-CPBA is then formed in step **2b** by nucleophilic substitution with hydrogen peroxide.

3) The synthesis of the product TMCL by chemical oxidation is performed using *m*-CPBA (Figure 8.2).³³ The reaction is performed in dichloromethane using an excess of peracid, with *m*-chlorobenzoic being formed as coproduct. Since this acid is also the pre-

Figure 8.2. Process flowsheet for the chemical synthesis of the product TMCL (3), describing the synthesis of the substrate (1), and the synthesis of the chemical oxidant *m*-CPBA (2). Reaction conditions: **1a** base-catalyzed aldol condensation (KOH, 90 °C, 20h), **1b** Pd-catalyzed hydrogenation (supercritical CO₂, 104-116 °C), **2a** chlorination (SOCl₂, 70 °C, 4h), **2b** nucleophilic reaction with hydrogen peroxide (H₂O₂, aq. NaOH, dioxane, MgSO₄, dioxane, 15 min), **3** chemical Baeyer-Villiger oxidation, RT, 72 h. Electricity consumptions are indicated as E_i (see Table 8.2 for details). Dotted arrows indicate potential recycled streams (in the sensitivity analysis only).

cursor of *m*-CPBA, this chemical is isolated by filtration and recycled. Because of the excess oxidant, the DSP steps require its neutralization by extraction with a sulfite, followed by base extraction to remove the remaining acid coproduct. Finally, the organic phase is washed with brine, and the solvent is removed under rotary evaporation. The TMCL product is recovered as an oil.

4) The preparation of the oxidizing enzyme, TmCHMO, is included in this LCA (Figure 8.3).^{37, 45} In this study, the enzyme is used as a cell-free extract, prepared in phosphate buffer. Enzyme preparation consists of a pre-incubation and an incubation step using a source of amino acids, minerals, glycerol as carbon source, and water for the fer-

Figure 8.3. Process flowsheet for the enzymatic synthesis of the product TMCL (5), describing the synthesis of the substrate TMCH (1), and the preparation of the enzyme (4). Reaction conditions: **1a** base-catalyzed aldol condensation (KOH, 90 °C, 20h), **1b** Pd-catalyzed hydrogenation (supercritical CO₂, 104-116 °C), **5** enzymatic Baeyer-Villiger oxidation (30 °C, 28 h) with **5a** oxidation, **5b** cofactor regeneration, and **5c** spontaneous hydrolysis of the coproduct. Electricity consumptions are indicated as E_i' and E_i''' (see Table 8.2 for details). Dotted arrows indicate potential recycled streams (in the sensitivity analysis only).

mentation broth. The resultant *E.Coli* K12 derivative cells are then separated from the broth by centrifugation and re-suspended in a phosphate buffer, after which they are subjected to cell lysis to create a cell-free extract. The cell debris are separated by centrifugation to obtain the enzymes in buffer solution.

5) The synthesis of the product TMCL by enzymatic oxidation is performed using TmCHMO as oxidizing enzyme in phosphate buffer.³⁷ Methanol is used as cosolvent to aid the solubility of the substrate TMCH. The reaction requires the use of nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor (step **5a**). Because of its price and relatively low stability, this cofactor is used in low amounts and is regenerated using a glucose dehydrogenase (GDH) as coenzyme with glucose as cosubstrate (step **5b**). This reaction leads to the formation of gluconolactone, which spontaneously hydrolyzes in the aqueous medium to form gluconic acid resulting in a decreased pH of the reaction (step **5c**). In order to maintain the pH constant, the reaction is autotitrated with a base solution thereby forming gluconate as a coproduct. In the DSP, the enzymes are first deactivated by addition of methanol and then separated by centrifugation from the reaction mixture, which is then concentrated by rotary evaporation to remove methanol. The product is extracted from the aqueous solution by extraction with ethyl acetate after saturation with sodium chloride to afford an oil.

- **Data collection**

In this LCA, priority was given to primary data from laboratory scale experiments. The data used are divided in several categories, in order of importance:

(i) Primary data from laboratory scale experiments, which were reported in **Chapter 4**. This is the case for the chemical and enzymatic syntheses of the product as well as the enzyme preparation.^{33, 37} Given the importance of accurate energy consumption data,⁴⁶ the electricity consumption was measured with laboratory equipments used in the syntheses (the details of the electricity consumptions are given in Table 8.2).

(ii) Secondary data modelled based on the literature were used for chemicals specific to this study: the substrate TMCH and the oxidant *m*-CPBA. All these data were based on laboratory scale experiments, except for step **1b**. In this step, the data were based on experiments performed at pilot plant scale for which the electricity consumption was estimated.^{42-44, 47}

(iii) Secondary data from the Ecoinvent v3.2 database (Ecoinvent Center, St-Gallen, Switzerland) were used for basic chemicals such as solvents, acids, bases, inorganic salts, *etc.* These data were obtained from the global market, or from the European market where

not available, in order to make sure the data are more representative of the context of this study. Likewise, the data from the electricity production were taken as an average of the European energy grid mix with low voltage.

(iv) Data from alternative chemicals were used for chemicals which were not available in the *Ecoinvent* database and were replaced by chemicals with equivalent functions (*L*-arabinose: glucose, Tryptone: soybean meal, yeast extract: Fodder yeast, *m*-chloro-benzoic acid: benzoic acid, potassium sulfate: sodium sulfate, sodium metabisulfite: sodium sulfite, sodium bicarbonate: sodium carbonate, GDH enzyme: enzyme production from potato starch).

(v) Suppressed data: a cut-off rule was applied to chemicals which were present in negligible weight percentages compared to the total chemical input of a given reaction ($< 0.05\%$ for NADP^+ , $< 0.004\%$ for ampicillin, 0.0002% for Pd supported catalyst).

Table 8.2. Details of the electricity consumption for each synthesis step.

Synthesis	Operation	Electricity consumption
(1) Synthesis of TMCH	Stirring of the reaction	2.002 kWh
	Reaction under pressure in autoclave	0.002 kWh
(2) Synthesis of <i>m</i>-CPBA	Stirring and heating	0.606 kWh
	Removal of solvent by rotary evaporation	0.018 kWh
(3) Chemical synthesis of TMCL	Stirring of the reaction	$E_1 = 17.150$ kWh
	Filtration of side-product with a vacuum pump	$E_2 = 0.017$ kWh
	Removal of solvent by rotary evaporation	$E_3 = 0.399$ kWh
(4) Enzymes preparation	Pre-incubation	$E_1'' = 3.154$ kWh
	Incubation	$E_2'' = 2.456$ kWh
	Cell separation by centrifugation	$E_3'' = 0.277$ kWh
	Cell sonication	$E_4'' = 0.007$ kWh
	Enzyme separation by centrifugation	$E_5'' = 0.832$ kWh
(5) Enzymatic synthesis of TMCL	Temperature and pH control, stirring of the reaction, continuous addition of substrate	$E_1' = 2.575$ kWh
	Removal of protein by centrifugation	$E_2' + E_3' = 0.443$ kWh
	Removal of solvent by rotary evaporation	$E_4' = 0.083$ kWh
		$E_5' = 0.018$ kWh
	Enzyme separation by ultrafiltration ⁴⁸	$E_6' = 0.011$ kWh

- **Data quality**

The pedigree matrix is representative of the quality of the data, in particular its geographical and temporal correlation as well as completeness and technological level (Table 8.3).⁴⁹ The matrix allows determination of the uncertainty for data following a log-normal distribution. The distribution of emissions in the environment is often log-normal.⁵⁰ For all chemicals, the following pedigree matrix was applied (basic uncertainty of 1.07):

Table 8.3. Pedigree matrix (all chemicals).

Category	Description
Reliability	Qualified estimate
Completeness	Representative data from only one site relevant to the market considered
Temporal correlation	Age of data unknown or more than 15 years of difference to the time period of the dataset
Geographical correlation	Average data from larger area in which area under study is included
Further technological correlation	Data on related processes on laboratory scale or from different technology
Sample size	Unknown

For the enzyme production from potato starch (as replacement for GDH), which is from the *Ecoinvent* database, the following pedigree matrix was applied (basic uncertainty of 1.5) (Table 8.4):

Table 8.4. Pedigree matrix of potato starch enzyme.

Category	Description
Reliability	Verified data based on measurements
Completeness	Representativeness unknown or data from a small number of sites and from shorter periods
Temporal correlation	Age of data unknown or more than 15 years of difference to the time period of the dataset
Geographical correlation	Average data from larger area in which area under study is included
Further technological correlation	Data on related processes on laboratory scale or from different technology
Sample size	Unknown

- **Methods and environmental impacts assessed**

The two synthetic routes were modelled in *Simapro V8* (PRé consultant, NL). Environmental impacts were calculated with the *IMPACT 2002+ V2.14* method which covers the major environmental effects.⁵¹ Only the end-point categories are presented in this study but mid-points were used for the Life Cycle Impact Assessment (LCIA) interpretations. The climate change impact was calculated with the *IPCC GWP 100a* mid-point method according to the updated method from the Intergovernmental Panel on Climate Change (IPCC).⁵² The water intensity was calculated based on the mass balance of the synthesis and product isolation as all data were available.

8.3 Analysis of the main scenario

The potential environmental impacts of the chemical and enzymatic syntheses were compared for five different end-point categories relative to the functional unit (which is 1 g of TMCL product):

- **Carbon emissions or climate change impact**, which is a measure of the increasing temperature in the lower atmosphere as a result of the emission of greenhouse gases such as carbon dioxide, methane, nitrogen dioxide being emitted in the air. Emissions are converted in CO₂ equivalent using the global warming potential (GWP) provided by the IPCC as conversion factor, expressed in kg of CO₂ equivalents;
- **Water intensity**, which measures the amount of water used for the synthesis and product isolation, expressed in g of water;
- **Damage to human health**, which is an aggregation of toxicity to humans, respiratory effects, ionizing radiation, ozone layer depletion and photochemical oxidation. It is expressed in disability adjusted life year (DALY);
- **Resources**, which combines the energy from mineral extraction and non-renewable resources, expressed in MJ primary;
- **Ecosystem quality**, which includes several factors such as ecotoxicity, acidification, eutrophication, and land use. It is expressed in potentially disappeared fraction of species per m² per year (PDF m⁻² yr⁻¹).

The relative environmental impact of the chemical and enzymatic synthesis for four impact categories is shown in Figure 8.4. The uncertainties were calculated in *Simapro* using a Monte-Carlo simulation method and are represented by the error bars. The absolute values for the environmental impact and the uncertainties are given in Table 8.5. The uncertainty is quite high with relative standard deviations around 50 % on average.

Because the LCA is based on data from laboratory experiments performed at one location, the uncertainty is higher than for a LCA performed on industrially implemented processes running at several sites over a long time period.

Figure 8.4. Contribution distribution of four environmental impact categories for a) chemical synthesis, and b) enzymatic synthesis. The energy contribution is the electricity used of the oxidation synthesis only. The percentages of contributions lower than 3 % are not indicated. The total values for each impact category are indicated below the x-axis.

Table 8.5. Impact category values and uncertainty values for the chemical and enzymatic syntheses.

Chemical synthesis					
		Impact	Standard deviation	2.5 th percentile	97.5 th percentile
Ecosystem quality	PDF.m ⁻² .yr ⁻¹	0.28	0.12	0.13	0.58
Human health	DALY	1.20.10 ⁻⁶	3.91.10 ⁻⁷	6.42.10 ⁻⁷	2.30.10 ⁻⁶
Resources	MJ primary	32.47	12.69	15.73	66.99
Climate change (GWP)	kg CO ₂ eq	1.65	0.59	0.83	3.30
Enzymatic synthesis					
		Impact	Standard deviation	2.5 th percentile	97.5 th percentile
Ecosystem quality	PDF.m ⁻² .yr ⁻¹	0.30	0.15	0.13	0.65
Human health	DALY	1.17.10 ⁻⁶	5.00.10 ⁻⁷	5.47.10 ⁻⁷	2.25.10 ⁻⁶
Resources	MJ primary	36.35	16.68	16.63	76.71
Climate change (GWP)	kg CO ₂ eq	1.64	0.67	0.78	3.34

The contribution inputs of the chemicals and energy (electricity) consumption are quite similar for all impact categories considered (Figure 8.4). It should be noted that the electricity consumption shown in Figure 8.4 only represents the electricity required for the oxidation synthesis, and that the impact of the chemicals (chemical oxidant, enzyme, substrate) takes into account the electricity required for their synthesis. Chemical and enzymatic syntheses have an almost identical environmental impact when no chemical is recycled (less than 0.4 % difference in favor of the enzymatic synthesis).

The contributions of the substrate, electricity, chemical oxidant and enzyme preparation are quite constant independently of the environmental category that is considered. The only exception is the increased impact made on human health, which is mainly due to the respiratory organics. This is attributed to the use of organic solvent, which is associated with some health risks such as dizziness (ethyl acetate), toxicity upon ingestion and inhalation (methanol), and potential carcinogenic effects (dichloromethane). Additionally, the synthesis of *m*-CPBA requires the use of thionyl chloride, which is toxic and can cause severe burns and eye damage, and dioxane, which is a suspected carcinogenic solvent. While 10 % of the climate change impact is caused by the use of organic solvent for the chemical synthesis, this impact is doubled for the enzymatic synthesis due to the use of ethyl acetate for DSP. This is one of the disadvantages of enzymatic reactions, in which the product often displays limited water solubility, thereby requiring larger amounts of organic solvent for extraction. As such, careful solvent selection combined with solvent recycling has been identified as crucial for industrial processes.⁵³ The effect of solvent recycling is investigated in the sensitivity analysis in the next section.

The rest of the study focuses on the comparison of climate change impacts of both oxidation syntheses of the product as climate change is in general the main environmental concern. Moreover, the contribution of each input to the GWP is quite representative of the distribution of the other impact categories (Figure 8.4). Because this study was based on laboratory scale experiments, the energy consumption is from electricity only. It should be noted that this differs from reactions carried out at industrial scale, for which the energy source is not exclusively electricity (with heat exchangers for example). Energy consumption in laboratory scale processes is usually higher than the corresponding optimized reactions performed at industrial scale (per unit of product).²⁵

Consequently, the contributions to climate change of both reactions were also detailed further, grouping the total electricity required for the entire process (Figure 8.5a). In both

the chemical and enzymatic oxidation routes, the largest impact is made by the total electricity consumption, for the oxidation step itself but also for the synthesis of the chemicals required for this reaction. The electricity consumption contributes up to 80 % of the GWP from the chemical synthesis and up to 76 % of the GWP from the enzymatic synthesis.

Figure 8.5. Performances of the chemical and enzymatic reactions with a) climate change (GWP), and b) water intensity. The values on top of the columns indicate the total GWP and total water intensity. Stripped bars indicate contribution due to electricity consumption. The error bar indicates the standard deviations for climate change.

This difference in electricity consumption for the same product synthesized chemically and enzymatically is due to three aspects. The first one is the increased electricity consumption of the oxidation step itself, which is almost 4 times higher for the chemical reaction, which is directly related to the increased reaction time (72 h compared to 28 h for the enzymatic reaction). The second aspect is that the electricity needed to synthesize the TMCH substrate is higher for the enzymatic reaction because, although both reactions have a substrate conversion higher than 99 %, the isolated yield of the enzymatic reaction is lower (68 % compared to 90 % for the chemical synthesis). Consequently, more substrate is needed to obtain the same quantity of product after DSP.

Lastly, the contribution of the synthesis of the chemical oxidant, *m*-CPBA, is similar to that of the enzyme preparation (less than 17 % of the total for each synthesis considering the climate change impact category). However, while half of the impact made by the chemical oxidant is due to the chemicals needed for its preparation, the electricity consumption required for the preparation of the enzymes represents almost all of its impact, with only 2 % attributed to the chemicals. More specifically, the electricity consumption for (pre)-incubation represents the largest contribution while 16 % of the GWP is due to formulation of the enzyme.

The prominent contribution of electricity in the GWP of the synthesis is quite typical of an early-stage LCA.²⁴ Clearly, the electricity consumption of laboratory data, on which early-stage LCAs are based, is not representative of the energy consumption of the same optimized process running at industrial scale.²⁵ There are several reasons for this difference in scaling: (i) the electricity consumption of laboratory equipment is usually not a factor that is taken into account when developing a reaction at laboratory scale, thereby resulting in a higher contribution of the energy consumption for early-stage LCAs; (ii) such equipment usually suffers from an increased energy consumption at start-up, which increases the average energy consumption of the process;⁵⁴ and (iii) most importantly, the equipment used for large scale processes differs from that used at laboratory scale, and is usually more energy-efficient.

This shows the importance of scale in LCAs, especially regarding equipment and their energy efficiency. It also demonstrates the need to take the electricity consumption into account for early-stage LCAs. Several ways are possible to account for the electricity consumption: it can be measured from the actual equipment, which is time consuming but the most accurate. It can also be estimated from similar equipment or calculated based on thermodynamic properties. For laboratory scale LCAs, it appears that measuring the actual electricity consumption of apparatus used becomes crucial, since the energy consumption will typically represent an important part of the environmental impact.

Another important environmental impact that is particularly relevant for enzymatic reactions is the water intensity, *i.e.* the quantity of water needed for the synthesis and DSP.⁵⁵ Water is typically the reaction medium of enzymatic reactions. Compared to the corresponding chemical reactions, biocatalytic reactions are therefore expected to have a higher water intensity. However, it is important to take into account DSP because the use of *m*-CPBA as the chemical oxidant in the corresponding chemical synthesis induces several washing steps to neutralize the remaining oxidant and remove any acid coproduct. As a result, the water intensity of the chemical synthesis is in reality higher than that of the enzymatic synthesis, despite the use of an organic solvent as reaction medium, in contrast to the enzymatic synthesis for which water is the reaction medium (Figure 8.5b). The water intensity of the enzymatic reaction is 63 % lower compared to the chemical reaction. About half of this water intensity is due to the preparation of the enzymes, the other half represents the water used as the reaction medium. Surprisingly, the synthesis of the chemical oxidant is more water intensive than the preparation of the enzymes, although the latter are obtained through fermentation in aqueous medium. Because no water is used for the synthesis and purification of the TMCH substrate, the water intensity values are independent of the conversion and isolated yield of the both oxidation reactions.

8.4 Sensitivity analysis

In the base-case scenario, whose results are presented above, the electricity was sourced from Europe, the peracid *m*-CPBA was selected as the chemical oxidant for the chemical synthesis, and the enzymes used in the biocatalyzed reactions were cell-free extracts used for one reaction only. In both cases, no chemical was recycled (see process flowsheets in Figures 8.2 and 8.3). A sensitivity analysis was performed on these parameters to evaluate their influence on the environmental impact and to test the robustness of the assumptions made in the methodology.

8.4.a. Effect of electricity source

Electricity consumption was shown to be the largest contributor to the synthesis of the product in both cases, with the current average European Union EU electricity mix as electricity source. As such, different scenarios regarding the source of electricity were also evaluated (Figure 8.6). The GWP for both reactions was compared with mostly fossil-based electricity, and electricity from renewable resources.

Figure 8.6. Evolution of climate change (GWP) for the chemical and enzymatic synthesis as a function of the electricity source, with average from Europe (EU, 0.49 kg CO₂ eq kWh⁻¹), the Netherlands (NL, 0.55 kg CO₂ eq kWh⁻¹), and Norway (NO, 0.04 kg CO₂ eq kWh⁻¹) (*Ecoinvent* v3.2). The percentage on top of the columns indicates the difference of GWP compared to the EU electricity source.

Changing the electricity source from the EU to the Netherlands resulted in an increase in the GWP of 4 to 5 %. This is due to the increased carbon intensity of the Dutch electricity grid mix, which is mainly produced using natural gas and coal, compared to average electricity from Europe which is a mix of combustible fuels, nuclear energy, and renewable energy such as wind and hydroelectric power. A dramatic decrease in environ-

mental impact was observed when the electricity source was replaced by greener electricity from Norway, which is mainly from hydroelectric power. In this case, the GWP of the enzymatic and chemical reactions could be decreased by 71 and 75 %, respectively. This is of course correlated with the high contribution of electricity consumption for the climate change impact of the chemical and enzymatic reactions. It furthermore indicates that the geographical location of industrial (bio)chemical reactions also contributes to their environmental impact.

8.4.b. Effect of recycling efficiency of solvents and coproduct streams

In both reactions, several streams can be recycled: solvents, and the coproduct of the chemical synthesis, *m*-chlorobenzoic acid, which is the precursor of the chemical oxidant *m*-CPBA. This coproduct can be recycled for the synthesis of *m*-CPBA because it is isolated during the filtration step of product purification (Figure 8.2). Similarly, dichloromethane can be recycled as the reaction medium after the solvent removal step during product isolation. In the enzymatic synthesis, two solvent streams can be recycled: methanol and ethyl acetate, which are both used during DSP and evaporated (Figure 8.3). Dichloromethane is the solvent whose production results in the highest climate change contribution (3.50 kg CO₂ eq kg⁻¹ vs. 2.56 kg CO₂ eq kg⁻¹ for ethyl acetate). However, the enzymatic synthesis requires at least twice the amount of solvent for the DSP. Consequently, recycling the solvent stream has a more drastic effect on the GWP of the enzymatic synthesis (Figure 8.7a).

When dichloromethane and *m*-chlorobenzoic acid were recycled with 90 % efficiency, the GWP of the chemical synthesis decreased by about 9 % compared to when no recycling was implemented. Improving further the recycling efficiency to 95 % results in a slight improvement, with 9.6 % decrease in GWP compared to no recycling. Interestingly, it is the recycling of the solvents that allows the enzymatic synthesis to be of lower environmental impact than the chemical synthesis. The environmental impact could be reduced by almost 20 % with a recycling efficiency of solvents of 95 %. Overall, recycling of solvents has a significant impact on the syntheses because of the high environmental impact of organic solvents (Figure 8.8). For the rest of the sensitivity analysis, a recycling efficiency of 90 % was applied.

Figure 8.7. a) Climate change (GWP) as a function of the recycling efficiency of coproduct (*m*-chlorobenzoic acid) and solvent for the chemical and enzymatic syntheses, and b) comparison of the total GWP of the syntheses with 90 % recycling efficiency of solvents with the replacement with peracetic acid (chemical synthesis) and reutilization of the enzyme (enzymatic synthesis) with either whole-cells in buffer (total GWP = 1.080 kg CO₂ eq per g of TMCL product) or whole-cells in fermentation broth (total GWP = 1.079 kg CO₂ eq per g of TMCL product). The values on top of the bars indicate the total GWP.

Figure 8.8. Contribution of chemicals to the climate change (GWP) of the synthesis of the product when no recycling of chemicals is considered with a) chemical synthesis, and b) enzymatic synthesis. Stripes indicate chemicals used for downstream processing. Stars indicate chemicals with negligible contributions. The values in parenthesis indicate the climate change for the synthesis of 1 kg of the chemicals with the largest contributions (as expressed in kg CO₂ eq per kg of product).

8.4.c. Effect of enzyme recycling

Another industrially relevant alternative to decrease the GWP of the enzymatic reaction is to re-use the enzyme over several reaction cycles. For established industrial processes, biocatalysts can be recycled as many as 200 times.⁵⁶ A reusability of 10 times with 2 % of loss of biocatalytic activity per reuse was assumed. This is a realistic estimation of the performance of this particular enzyme, based on direct experimental studies on its stability and reusability when immobilized, as shown in **Chapter 4**. In order to model the reuse of the enzyme, the enzyme preparation procedure and the product isolation step must be adapted appropriately since the standard procedure for DSP of the enzymatic reaction includes the deactivation of all remaining enzyme by addition of methanol (see standard procedure in Figure 8.3 and adapted procedure in Figure 8.9).

Figure 8.9. Alternative process flowsheet for the enzymatic synthesis of the product TMCL with recycling of the enzyme with whole-cells in fermentation broth (4)', and whole-cells in buffer (4)''. See Table 8.2 for the electricity consumptions. Dotted arrows indicate recycling streams.

The enzyme can potentially be recycled when used in the form of whole-cells, which are separated from the reaction mixture by ultrafiltration, after which the product can be directly extracted from the reaction mixture. The electricity consumption for ultrafiltration was calculated based on data from the literature.⁴⁸ Two whole-cell preparation procedures were tested: in buffer solution or in fermentation broth. The latter option has the advantage of being more energy efficient because one centrifugation step can be omitted, although this biocatalyst format may ultimately prove more challenging due to possible side-reactions. The alternative flowsheet of the enzymatic reaction with recycled whole-cells is shown in Figure 8.9.

Re-using the enzymes reduces the GWP by about 20 % (Figure 8.7b). In this case, the contribution of the enzyme to the total GWP of the enzymatic synthesis drops to less than 3 % while it accounts for about 19 % of the total GWP when it is not recycled. It should also be noted that the reusability of the enzymes induces lower electricity consumption due to the simplification of the DSP steps.

8.4.d. Effect of oxidant type

Although *m*-CPBA is an effective chemical oxidant, it displays several disadvantages including safety issues related to its flammability and shock sensitivity, making it potentially explosive.²⁷ Additionally, its use generates one equivalent of coproduct, *m*-chlorobenzoic acid, which can be recycled as detailed above. It is typically used in excess, which requires quenching of any unreacted oxidant leading to a more complex DSP. Consequently, greener alternatives such as hydrogen peroxide, peracetic acid, and of course oxygen have been identified.²⁸ When replacing *m*-CPBA with peracetic acid, no recycling of the coproduct, acetic acid in this case, was modeled because its isolation from the aqueous medium would require additional steps after extraction with ethyl acetate (see alternative flowsheet in Figure 8.10).

The synthesis of peracetic acid was modelled based on data from the literature.⁵⁷ Using peracetic acid instead of *m*-CPBA leads to a decrease of about 18 % of the GWP of the chemical synthesis (Figure 8.7b). Despite the lack of recycling for peracetic acid, the contribution of this oxidant to the total GWP of the chemical synthesis is negligible (less than 1 %). This is due to the fact that the synthesis of peracetic acid is more efficient than that of *m*-CPBA since it is synthesized in a single step, uses less chemicals (water, hydrogen peroxide, acetic acid, and sulfuric acid only), and the production of these chemicals has a low impact on the total climate change contribution.

Figure 8.10. Alternative process flowsheet for the chemical synthesis of the product TMCL using peracetic acid as oxidant (2)'. See Table 8.2 for the details of the electricity consumptions. Dotted arrows indicate recycling streams. Reaction conditions of 2: H₂O₂, H₂SO₄ aq, 4 h.

8.5 Key process performance metrics influencing the environmental impact

The electricity consumption, as well as the amount of substrate, are crucial parameters for the environmental impact of the synthesis of the product. These two parameters are directly correlated with the reaction time, the substrate conversion and the yield of the reaction, which are typical process metrics investigated for process intensification of biocatalytic reactions.⁵⁶ The enzymatic reaction has a lower isolated yield compared to the chemical reaction (65 % vs. 90 %), which results in a higher amount of substrate needed for the synthesis of the same amount of product. This effect is for example visible in Figure 8.5a. However, the chemical synthesis is less efficient and requires 3 days to reach full conversion (Figure 8.11), while the enzymatic oxidation is completed within 28 h (see **Chapter 4** for details of the kinetics of the enzymatic reaction).

Figure 8.11. Conversion of the chemical reaction followed by GC-FID (the measurements were performed as described in **Chapter 4**). Reaction conditions: *m*-CPBA (2 eq), TMCH in dichloromethane (200 mM), room temperature.

In order to use laboratory scale LCA as a tool to help improve reactions while keeping their environmental impact in mind, the GWP of both reactions was calculated as a function of the isolated yield and the reaction time (Figure 8.12). The isolated yield is calculated assuming full substrate conversion. When both reactions have the same process metrics (reaction time, conversion, and isolated yield) and same recycling efficiency, the environmental impact of the enzymatic reaction is lower than that of the chemical reaction independent of the efficiency of the reaction (Figure 8.12a-b). The reaction time is the most important factor to consider when reducing the environmental impact of the chemical reaction, while the most important factor for the enzymatic reaction is the isolated yield.

Interestingly, the GWP of the chemical reaction would equal that of the enzymatic reaction if the reaction time of the chemical reaction was more than halved while the isolated yield of the enzymatic reaction was increased to about 80 %, which can easily be obtained (Figure 8.12a-b). This decrease in reaction time of the chemical reaction however seems hardly feasible if full substrate conversion is to be achieved.

An optimistic scenario in which the solvents, coproduct and enzymes are recycled was considered. This scenario does not consider the use of energy from more renewable resources, which would drastically decrease the environmental impact of both reactions as shown in Figure 8.6, because the energy contribution is overrepresented at laboratory scale. When both reactions are optimized in terms of recycling, the enzymatic synthesis is clearly of lower environmental impact (Figure 8.12c-d). If the reaction time of the

chemical synthesis could be reduced with an improvement of the isolated yield, a GWP lower than 1.25 kg CO₂ equivalents per g of product could be obtained. On the other hand, the isolated yield of the enzymatic synthesis can easily be improved to a level similar to that of the chemical reaction, which would result in a GWP beneath 0.80 kg CO₂ equivalents per g of product for the same reaction time. This shows that the improvement of the environmental impact of (bio)chemical reactions differs per reaction. While a reduction of the reaction time impacts the chemical reaction, recycling of solvents and enzyme is crucial for the enzymatic reaction.

Figure 8.12. Evolution of climate change (GWP) in kg CO₂ eq per g of product as a function of the reaction time and the isolated yield for a) the chemical synthesis with *m*-CPBA with no recycling, b) the enzymatic synthesis with no recycling, c) the chemical synthesis with *m*-CPBA with 90 % recycling efficiency of solvents and coproduct, and d) the enzymatic synthesis with 90 % recycling efficiency of solvents and reuses of enzyme (10 cycles with 2 % loss). The intersection of the dotted lines indicates the current isolated yield and reaction time (base-case scenario).

8.6 Conclusions

The environmental impact of the enzymatic synthesis of TMCL was evaluated and compared to its chemical equivalent in an early-stage cradle-to-gate LCA. This comparative LCA demonstrates that, at laboratory scale, the enzymatic and chemical synthesis have very similar environmental impacts when no recycling of solvents or co-product is considered. It is in fact the recycling of solvents and enzyme that makes the enzymatic synthesis of lower environmental impact compared to the chemical synthesis. This is due to the higher recycling potential of the enzymatic synthesis, which is more solvent-intensive because the product isolation requires extractions from the aqueous phase with an organic solvent. It should be noted that, although recycling can be advantageous, it can also be limited in some cases by economic considerations due to potential increase in the complexity of the process or energy requirements in order to achieve increased recycling efficiencies. The enzymatic reaction was however more competitive when considering the water intensity since it resulted in less than half the water consumption, despite water being used as the reaction medium for the enzyme preparation and the oxidative reaction itself.

A sensitivity analysis performed on the source of the electricity showed that the total climate change impact of the chemical and biocatalyzed reactions can be decreased by 71 % to 75 %, respectively, by using renewable electricity. This drastic decrease shows the importance of the electricity consumption to the environmental impact of both reactions, and to early-stage LCAs in general. Several factors contributed to decreasing further the environmental impact of the reactions, namely recycling of solvent and co-product streams, recycling of the enzyme as whole-cells and replacing the chemical oxidant with peracetic acid. In the two latter cases, the contribution of the oxidant (chemical or enzymatic) was almost negligible with less than 1 % contribution of the total climate change of the oxidation.

Prospective LCAs based on laboratory scale data can also be used as a tool for the improvement of enzymatic reactions. The reduction of the reaction time (correlated with the amount of electricity consumed) and the improvement of the substrate conversion and isolated yield have a greater influence on the environmental impact of the chemical reactions than the recycling of solvent streams. For the enzymatic synthesis, the recycling of solvents and enzymes influences its environmental impact the most. These parameters help to evaluate the improvements of key process performance metrics, such as conversion, isolated yield and space-time yield, which are necessary for enzymatic reactions to be implemented as industrial processes. This optimistic scenario did not take into account replacing the electricity by renewable electricity because of the overrepre-

sensation of electricity in the data based on laboratory scale experiments. Such an analysis performed on data from pilot plant scale or simulated data at industrial scale would help further in identifying crucial parameters influencing the environmental impact.

The key learnings from this comparative cradle-to-gate LCA at laboratory scale are: (i) the importance of scale in LCA, and its impact of the energy consumption, and (ii) the use of such LCAs as learning tools to compare the environmental performances of chemical and enzymatic reactions, even at a prospective scale. This tool should enable targeting of the key process performance metrics, which can make an important difference when optimizing enzymatic reactions. This work can hopefully inspire early-stage comparative cradle-to-gate LCAs as to the development of greener processes, and in particular biocatalytic processes.

8.7 References

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CHAPTER 9

Conclusions and outlook

The goal of this thesis was to explore the applicability of Baeyer-Villiger mono-oxygenases (BVMOs) at large scale for the synthesis of lactones, which are used as precursors for polyesters for various applications. This thesis had several aims, including:

- The identification of a robust BVMO with enough operating stability for applied biocatalysis;
- The evaluation of several biocatalyst formats, including immobilization and self-sufficient enzymes;
- The application of the selected BVMO to the synthesis of alkyl substituted lactones, yielding high product concentration and productivity;
- Understanding the thermodynamic properties of these alkyl substituted lactones upon ring opening polymerization and screening organocatalysts for their polymerization;
- Evaluating their potential application in inks and coatings as dispersing agents;
- Comparing the environmental impact of biocatalysis with that of the corresponding chemical oxidation for the synthesis of alkyl substituted lactones.

This chapter highlights the challenges of this research as well as the most important findings. Future perspectives regarding biocatalysis for monomer synthesis and the polymers thereof are given.

9.1 Conclusions

In this thesis, the use of BVMOs was demonstrated for the sector of medium-cost products. These products are intermediates between bulk products (typically < 5 € kg⁻¹) and specialty chemicals (typically > 100 € kg⁻¹) in terms of price but also production volume. More specifically, lactones as monomer building blocks for the preparation of aliphatic polyesters were synthesized using TmCHMO. This enzyme was identified as being the most promising wild-type BVMO for the synthesis of alkyl substituted lactones. The reasons are three-fold: TmCHMO is thermostable with a high melting temperature ($T_m = 48\text{ °C}$);¹ it has an excellent resistance to organic (co)-solvents; and it can oxidize a broad range of cyclic ketones of various ring size but also with various substituents (**Chapter 3**).

For the first time, the alkyl substituted TMCL lactones were prepared by biocatalytic oxidation. In **Chapter 3**, careful reaction engineering was employed to maximize product concentrations and space-time yield. These two process performance metrics are particularly important due to the tendency of enzymes to suffer from substrate inhibition, which was observed for TmCHMO with the ketone precursor of TMCL. This specific

limitation was overcome by using continuous substrate feeding and biphasic systems with water-immiscible organic solvents. In **Chapter 4**, TmCHMO was successfully immobilized together with a glucose dehydrogenase. The coimmobilization of these enzymes proved to be the best performing system when re-utilizing the enzymes over several reaction cycles. The best reaction conditions using continuous substrate feeding were applied to the gram-scale synthesis of TMCL. A product concentration of 38 g L^{-1} and a productivity of $1.35 \text{ g L}^{-1} \text{ h}^{-1}$ were achieved, which is among the highest for upscaled reactions using a BVMO, as reviewed in **Chapter 2**.

While metal-based catalysts were so far the only catalysts known to polymerize TMCL, the catalyst scope for these lactones was explored with organocatalysts in **Chapter 5**. It was demonstrated that bifunctional organocatalysts such as organophosphorus catalysts are able to polymerize these lactones, and in particular phosphinic and phosphonic acids, which had not been reported before. Interestingly, the two regio-isomeric lactones TMCL display different polymerization behavior, with the proximal lactone (*i.e.* the most substituted regio-isomer TMCL-I) polymerizing faster and reaching higher monomer conversion. This emphasizes the importance of the structure of lactones for their thermodynamic properties which govern ring opening polymerization. DFT computational studies provided insight in this difference of behavior and demonstrated that the difference in reactivity in favor of the most substituted lactone can be predicted.

Chapters 6 and 7 emphasize the relationship between monomer structure, polymer (micro)structure and applications. In **Chapter 6**, the copolymerization of TMCL and δ -UDL, which are two alkyl substituted lactones, with a more polar lactone (DXP), was investigated. The determination of the reactivity ratios of the monomers offered understanding in the microstructure obtained upon copolymerization. The different copolymerization strategies that were investigated, namely one-pot polymerization and sequential feed, were found to result in different structures of (co)polymers.

In **Chapter 7**, the potential of macrolactones as dispersing agents was explored with the aim of broadening the solubility range of the corresponding copolyesters by decreasing the crystalline content by copolymerization with alkyl substituted lactones. Although the copolyesters displayed a very low crystalline content, their microstructure was quite block-like, rather than random as targeted. Lactones with different structures and therefore reactivities are expected to be able to give random copolymers. Ultimately, the solubility range of the copolyesters was too limited for the envisioned application.

Lastly, the perceived environmental benefit of biocatalysis was challenged in a prospective life-cycle assessment (LCA) in **Chapter 8**. The environmental impact of the

biocatalyzed synthesis of TMCL was compared to that using organic oxidants. This comparison revealed that enzymatic reactions are not necessarily more environmentally friendly than the corresponding chemical reactions. The possibility to recycle the enzymes and organic solvents has a larger impact than the use of enzymes alone. Additionally, this chapter demonstrates the use of early-stage comparative LCAs by identifying key process metrics such as the isolated yield and the reaction time, which should contribute to the development of greener processes.

9.2 Outlook and recommendations

It is believed that BVMOs have an important role to play in the field of polymeric materials. This thesis demonstrates and confirms the potential of these oxidative enzymes for the industrial synthesis of building blocks for polyesters. However, some challenges remain such as the selectivity of the enzyme, which influences the structure of the lactones and therefore their reactivity upon ring opening polymerization, and the applicability of such biocatalyzed reactions at industrial scale.

9.2.a. Biocatalysis for lactones synthesis: when to apply it?

In this thesis, oxidative biocatalysis was successfully applied to the large scale synthesis of polyesters precursors. The differentiating ability of biocatalysts to be selective has however not been fully exploited. Indeed, none of the wild-type enzymes evaluated in **Chapter 3** were found to selectively oxidize the targeted substrate. Even though TmCHMO displays an inverted regioselectivity compared to the chemical oxidant evaluated, the ratio of lactones remains close to 50:50, with a slight preference for the distal lactone (TMCL-II). As shown in **Chapter 5**, the structure of lactones is however crucial regarding their polymerizability by ring opening polymerization. A difference of structure as small as the position of one methyl group was found to be crucial for the reactivity of the TMCL lactones upon polymerization. The structure of lactones also influences their ability to form copolyesters with other lactones and the microstructure of these copolyesters.

Biocatalysis can therefore make an important impact when it enables access to lactones with structures that cannot be obtained by chemical oxidation. As was outlined in **Chapter 2** with the example of carvone-derived lactones, some BVMOs are able to produce abnormal or distal lactones. It would therefore be interesting to explore protein engineering of TmCHMO in order to tune its regioselectivity toward one or the other regio-isomer. The development of fast screening techniques is also expected to help screening the libraries generated by protein engineering. Recent results from the literature

confirm the potential of TmCHMO for protein engineering, targeting a change in selectivity. The enantioselectivity of the enzyme for the synthesis of enantiopure γ -methyl- ϵ -caprolactone could be switched while retaining the thermostability of the enzyme.² Another very promising result is the complete reversal of regioselectivity of TmCHMO which was achieved on a linear ketone.³ The abnormal lactone was obtained by mutation of the enzyme by controlling the stability of the intermediate upon oxidation. As such, BVMOs have an important role to play for the selective synthesis of tuned building blocks influencing the properties of the corresponding polymers.

9.2.b. Applicability at industrial scale

In this thesis, the TmCHMO-catalyzed synthesis of TMCL was screened in a micro-titer plate (0.2 mL), improved at 30 mL reaction volume before being upscaled with the gram-scale synthesis at 500 mL reaction volume. This reaction has been further improved and brought to the pilot plant by ROBOX project partners with a reaction volume of 100 L, thereby producing the lactones on a kilogram scale. This demonstrates the potential of BVMO-catalyzed reactions for the preparation of lactones as monomers, and shows that biotechnology has the potential to become important for polymeric materials. The overall goal of this thesis was to bring the fields of biocatalysis, polymer chemistry, and sustainable chemistry together in order to enable the full potential of enzymes.

The improvements required to make biocatalyzed processes relevant at industrial scale are identical for most enzymatic reactions, *i.e.* lower biocatalyst loading, suppressed substrate or product inhibition, increased product concentration and productivity, *etc.* Consequently, the achievements of this thesis on the TmCHMO-catalyzed synthesis of TMCL can easily be translated to other BVMO processes. Recently, efforts have been made to harmonize these improvement strategies and create guidelines for the development of biotransformations.⁴ In particular, strategies to improve the operational stability of enzymes should be taken into account. Monitoring oxygen in oxidative bioreactions, which is a cosubstrate but can also be detrimental to enzyme stability,⁵ and the careful selection of organic (co)solvents⁶⁻⁷ are just a few examples for reaction engineering. This type of guidelines is essential to the development of biotechnology because of the gap that separates screening performances at (very) small scale, and applying the enzyme at industrial scale.

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CHAPTER 10

Valorization addendum

10.1 Potential markets for lactones synthesized using biocatalysts

In this thesis, the biocatalyzed synthesis of (alkyl substituted) lactones was demonstrated. Several sectors have been identified that are relevant for the industrial development of lactones, in particular when they are obtained from biotechnological routes. Potential markets for (biobased) lactones prepared using biocatalysts include flavors & fragrances (F&F), as well as specialty polymers.

- **Flavors, fragrances and cosmetic ingredients.** This market was estimated to 26 billion dollars in 2017.¹ Examples of lactones that are relevant for this market are given in Scheme 10.1. In this sector, natural ingredients obtained from natural processes such as fermentation or biocatalyzed processes have a commercial added value. Additionally, biocatalysis is advantageous because it is enantioselective, meaning that it can enable the production of pure enantiomers. This is especially needed for lactones for which one enantiomer differs in intensity or in character. The market value of ingredients in F&F is estimated to less than 15 € kg⁻¹.²

Scheme 10.1. Examples of lactones used in flavors & fragrances with corresponding fragrances.

- **Additives in dispersions, emulsions, and inks.** So far, the vast majority of lactones used as precursors for polymers that are reported in the literature are synthesized chemically. As a consequence, the normal or proximal lactones are typically obtained. Thanks to the selectivity of enzymes, new structures of lactones can be produced. These lactones may give rise to polymers with novel properties. In addition, lactones synthesized using biocatalysis are seen as more sustainable or greener (although the environmental benefit of biocatalysis is not applicable to all processes by default and has to be proven on a case-to-case basis). As such, the corresponding additive polymers could be used as dispersing agents, emulsifiers, and encapsulators of added market value.

10.2 Transition to a more sustainable chemical industry: recyclable polymers

In 2016, 8.4 million tons of plastics were recycled in Europe while 60 million tons were produced, with a total of 335 million tons plastics produced worldwide.³ The recyclability of polymers is crucial for a circular economy. In fact, plastic recycling addresses the mismatch between the long lifetime of plastics and their short use, which results in massive amounts of plastic waste.

There are several ways to recycle a polymer, depending on the goal that is to be achieved with recycling. **Mechanical recycling** is used to reshape polymers. It can however lead to lowered mechanical properties due to potential degradation of the polymer upon recycling. Similarly, **biological degradation**, or biodegradability, does not allow recovering the starting building blocks. As such, **chemical recycling** has appeared to be an emerging recycling strategy.⁴ It consists of the depolymerization of polymeric materials under controlled conditions to recover either the starting monomers, or to upgrade them to new building blocks. Chemical recycling typically includes pyrolysis, thermodynamic recycling, and solvolysis.⁵

Because ring opening polymerization (ROP) is a thermodynamically-driven type of polymerization, polymers prepared from lactones are particularly relevant for chemical recycling. This type of recycling allows recovering the lactones as starting materials, which is considered as one of the most sustainable ways to recycle them. Bioprocesses and the integration of life cycle assessment (LCA) have both been identified as key green research areas.⁶ As such, the use of biocatalysis to synthesize monomers, the chemical recyclability of the corresponding polymers and a sustainability assessment of the processes are correlated as shown in Scheme 10.2. Although biocatalysis adheres in principles to the rules of green chemistry, its environmental benefits have to be proven on a case-to-case basis as was mentioned earlier.⁶⁻⁷

The chemical recyclability of polymers from γ -butyrolactone was among the first one to be demonstrated.⁸⁻⁹ Recycling is achieved either by non-catalyzed thermal processes or by using a catalyst at room temperature. Polymers obtained from the ROP of the functionalized α -methylene- γ -butyrolactone (Tulipalin A), which is biobased, can also be chemically recycled upon heating.¹⁰ This is due to the extremely low ceiling temperature of this monomer ($T_c = -52\text{ }^\circ\text{C}$ at 5 M), which means that the polymer is thermodynamically prone to depolymerization. β -Methyl- δ -valerolactone is another example of a monomer that is obtained from biomass whose polymers can be chemically recycled. This

Scheme 10.2. Relationship between chemical recyclability of polymers, biobased origin or use of biocatalysis and sustainability.

monomer is particularly relevant because its preparation by fermentation has been established, and is currently being commercially exploited by the company Valerian Materials.¹¹⁻¹² Examples of polymers produced from this monomer include recyclable cross-linked elastomers from polyesters¹³ and flexible polyurethane foams.¹⁴

Two other relevant monomers are functionalized ϵ -caprolactone derivatives. The first one is γ -methyl- ϵ -caprolactone, which can be obtained using biocatalysis.¹⁵ In addition, the techno-economic feasibility of its production from *p*-cresol (biobased platform chemical from lignin) was established for a potential industrial production of several kiloton per year.¹⁶ The monomer can be recovered from the cross-linked polyester by recycling *via* enzymatic hydrolysis.¹⁷ The second one, γ -carbomethoxy- ϵ -caprolactone is a monomer produced from malic acid which is a “top added-value chemical”. The corresponding polymer was shown to be chemically recycled using tin octanoate as catalyst.¹⁸

In conclusion, lactones and polymers therefrom have potential for commercialization in multiple markets. Together with a production from renewable resources and using biotechnology, their environmental advantage must be demonstrated to make them fully competitive in tomorrow’s biobased economy.

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Appendix A

The determination of the thermodynamic parameters of the ROP of δ -UDL was performed as discussed in **Chapter 5**.

Figure A1. Thermodynamic parameters of δ -UDL with a) monomer conversion at equilibrium as a function of the temperature for bulk polymerizations, and b) extrapolated conversion at equilibrium as a function of the temperature using the van't Hoff analysis for polymerization in bulk (full line) and at 1 M (dotted line). The ceiling temperature T_c is indicated. Reaction conditions: BnOH/ δ -UDL/DPP 1/30/1, $[\delta\text{-UDL}]_0 = 1\text{ M}$ or 5.17 M (bulk).

Appendix B

List of atomic coordinates as calculated by DFT in Chapter 5

TMCL-I

Conformation C

	X	Y	Z
C	-0.681775	1.287228	-0.114826
C	-1.479591	-0.031285	0.032794
C	-0.833950	-1.172416	-0.778902
C	0.810194	1.282739	0.262852
C	1.634861	0.268582	-0.568212
H	-0.759669	1.618095	-1.161719
H	-0.587389	-0.832938	-1.793363
H	0.913644	1.011482	1.321988
H	2.698542	0.504378	-0.498670
H	-1.187639	2.057642	0.481767
H	-1.543835	-1.998658	-0.872624
H	1.351731	0.345018	-1.626677
C	1.518646	-1.167131	-0.085970
O	2.444730	-1.774176	0.397675
O	0.311232	-1.788788	-0.167737
C	-1.621585	-0.443727	1.509358
H	-2.125555	0.345461	2.077567
H	-2.218303	-1.357531	1.602088
H	-0.655214	-0.638640	1.979928
C	-2.882816	0.199841	-0.566274
H	-3.519287	-0.682269	-0.438527
H	-3.377597	1.042790	-0.073059
H	-2.831238	0.427674	-1.637429
C	1.395913	2.692402	0.088194
H	2.441004	2.729818	0.411062
H	1.358118	3.006941	-0.961658
H	0.835608	3.427057	0.675744

TMCL-I

Conformation B

	X	Y	Z
C	0.790182	1.344680	0.285038
H	0.370846	1.878284	1.147265
C	1.606595	0.125994	0.835816
H	1.359210	-0.052800	1.888042
H	2.676388	0.337589	0.797293
C	-0.895606	-1.096511	0.775859
H	-0.571720	-0.740608	1.759618
H	-1.642403	-1.878085	0.941124
C	-1.496416	0.054930	-0.054100

C	-0.368044	0.936764	-0.648970
H	0.061667	0.418665	-1.516466
H	-0.829199	1.848579	-1.048628
O	0.187748	-1.758868	0.104529
C	1.421647	-1.175729	0.067783
O	2.301791	-1.719430	-0.554887
C	-2.414928	0.875617	0.869897
H	-2.935215	1.654639	0.303065
H	-3.178238	0.239763	1.333038
H	-1.856694	1.363718	1.675994
C	-2.320561	-0.529217	-1.217649
H	-3.194017	-1.076746	-0.846123
H	-2.681133	0.265708	-1.879400
H	-1.715497	-1.219039	-1.813909
C	1.728701	2.314265	-0.450193
H	1.183470	3.189061	-0.819854
H	2.526536	2.669748	0.209883
H	2.200274	1.823153	-1.308967

TMCL-I

Conformation C'

	X	Y	Z
C	0.866587	1.184148	-0.609317
C	1.473856	-0.092078	0.035430
C	0.733099	-1.361377	-0.427059
C	-0.652603	1.464840	-0.535489
C	-1.512444	0.291674	-1.068304
H	1.125492	1.149509	-1.677058
H	1.318279	-2.243884	-0.154380
H	-1.107147	-0.059641	-2.025467
H	1.397080	2.055862	-0.203895
H	0.618131	-1.366237	-1.518454
H	-2.533166	0.633781	-1.250766
C	-1.646754	-0.868567	-0.095990
O	-2.687543	-1.159348	0.444414
O	-0.535412	-1.593457	0.208466
C	2.923729	-0.215331	-0.483626
H	3.497906	0.683538	-0.236548
H	3.434844	-1.071930	-0.031433
H	2.954913	-0.338548	-1.572429
C	1.510742	-0.034336	1.574651

H	2.066847	-0.889297	1.975297
H	2.013366	0.878167	1.912064
C	0.514873	-0.061189	2.018238
C	-1.162898	1.970928	0.823282
H	-1.154897	1.197381	1.594958
H	-0.552326	2.807288	1.178793
H	-2.195777	2.323168	0.734704
H	-0.814662	2.285061	-1.248120

TMCL-I

Conformation B'

	X	Y	Z
C	-0.555781	0.492945	1.039163
C	-1.348038	-0.403738	0.045815
C	-0.409459	-0.991562	-1.026055
C	0.539078	1.467386	0.533452
C	1.625643	0.797956	-0.362103
H	-0.066265	-0.178535	1.754871
H	-0.183698	-0.251660	-1.800698
H	1.056692	1.781450	1.448949
H	2.595130	1.268478	-0.189696
H	-1.279408	1.068951	1.629861
H	-0.886555	-1.843098	-1.519531
H	1.382341	0.950607	-1.420086
C	1.820964	-0.687966	-0.106965
O	2.810818	-1.161988	0.396041
O	0.806759	-1.525177	-0.476040
C	-1.969379	-1.560548	0.855804
H	-2.570571	-1.177320	1.687372
H	-2.622928	-2.174251	0.225634
H	-1.190339	-2.208329	1.268835
C	-2.474749	0.361555	-0.674164
H	-3.023238	-0.301460	-1.353417
H	-3.194385	0.756847	0.050217
H	-2.098880	1.202111	-1.263293
C	0.017471	2.748815	-0.132991
H	0.838741	3.452894	-0.306210
H	-0.448877	2.551296	-1.103551
H	-0.722877	3.250282	0.499011

TMCL-II

Conformation C

	X	Y	Z
C	-0.824236	1.221254	-0.128090
C	-1.669613	0.014722	0.318414
C	-1.330901	-1.269351	-0.447952
C	0.716089	1.117751	-0.021851
C	1.257012	-0.103196	-0.821984
H	-1.079783	1.438132	-1.176386
H	-1.245097	-1.073247	-1.525315
H	2.333850	0.006140	-0.968378

H	-1.145078	2.100215	0.446437
H	-2.126296	-2.006342	-0.311833
H	0.792461	-0.120504	-1.816331
C	1.092440	-1.451780	-0.140055
O	2.028507	-2.093874	0.274828
O	-0.160510	-1.951406	0.033621
C	1.315747	2.387918	-0.658718
H	2.407227	2.393364	-0.570821
H	1.063069	2.459140	-1.722672
H	0.935430	3.288157	-0.163720
C	1.170624	1.040480	1.449124
H	2.261104	0.971362	1.516802
H	0.854754	1.937126	1.993535
H	0.758411	0.171064	1.969374
C	-3.164480	0.332218	0.147086
H	-3.792075	-0.479367	0.529056
H	-3.433428	1.246174	0.685352
H	-3.419513	0.485651	-0.908555
H	-1.485110	-0.194914	1.380022

TMCL-II

Conformation B

	X	Y	Z
C	-1.169373	-0.708984	0.015961
C	-1.170783	0.687154	0.728022
H	-0.879542	0.574375	1.777119
H	-2.177887	1.108417	0.719798
C	1.587545	0.418962	0.937516
H	1.087670	0.350663	1.908530
H	2.633602	0.670425	1.130100
C	1.484007	-0.908392	0.158700
C	0.188404	-1.004788	-0.673189
H	0.291143	-0.324437	-1.528327
H	0.136272	-2.012750	-1.103097
O	1.078376	1.545510	0.201044
C	-0.269833	1.708941	0.055100
O	-0.674725	2.634712	-0.606978
C	2.701960	-1.101871	-0.758766
H	3.631278	-1.144309	-0.181002
H	2.623068	-2.029941	-1.334126
H	2.784090	-0.271556	-1.468449
C	-2.249841	-0.687983	-1.084059
H	-2.238518	-1.617343	-1.664111
H	-3.248683	-0.575934	-0.648886
H	-2.089677	0.146556	-1.774363
H	1.490357	-1.710481	0.909530
C	-1.511917	-1.797797	1.048562
H	-2.470465	-1.587139	1.535527
H	-1.591420	-2.779388	0.568245
H	-0.751517	-1.870382	1.833901

TMCL-II**Conformation C'**

	X	Y	Z
C	0.461308	-1.471987	-0.470917
C	-1.079515	-1.460517	-0.329191
C	-1.733554	-0.243666	-0.992305
C	1.309295	-0.248445	-0.030456
C	0.787129	1.065517	-0.681457
H	0.675381	-1.633453	-1.536939
H	-2.809565	-0.408790	-1.091087
H	0.644005	0.906115	-1.757890
H	0.848381	-2.359395	0.046760
H	-1.328991	-0.079849	-1.998703
H	1.537907	1.850270	-0.565804
C	-0.475955	1.637074	-0.057922
O	-0.481890	2.654347	0.594584
O	-1.649432	0.966900	-0.219418
C	-1.620253	-1.686193	1.092191
H	-2.696588	-1.887605	1.062207
H	-1.132668	-2.546448	1.561308
H	-1.473501	-0.817493	1.736229
C	1.383057	-0.092212	1.501897
H	0.415611	0.136717	1.953042
H	1.760091	-1.011523	1.962803
H	2.064113	0.722026	1.770876
C	2.742059	-0.482068	-0.556418
H	3.409720	0.330172	-0.250458
H	3.152917	-1.418520	-0.163152
H	2.762948	-0.541868	-1.650494
H	-1.420603	-2.315353	-0.932560

TMCL-II**Conformation B'**

	X	Y	Z
C	-0.625382	0.512848	1.008837
C	-1.587891	-0.620735	0.572589
C	-0.917891	-1.683422	-0.316412
C	0.302785	1.224648	-0.018151
C	1.038610	0.199575	-0.940516
H	0.027403	0.084047	1.778341
H	-0.963868	-1.396080	-1.372787
H	1.964082	0.640297	-1.317244
H	-1.216747	1.281063	1.523172
H	-1.439721	-2.639071	-0.221841
H	0.415663	-0.035778	-1.809063
C	1.430580	-1.077392	-0.216954
O	2.555819	-1.327947	0.144855
O	0.438213	-1.973213	0.059594
C	-2.899834	-0.160079	-0.081952
H	-3.575446	-1.009025	-0.236946
H	-3.420264	0.562310	0.554329

H	-2.737857	0.309740	-1.056915
C	-0.459738	2.228655	-0.902375
H	0.229935	2.750644	-1.575193
H	-1.218423	1.743253	-1.522415
H	-0.960827	2.985208	-0.288524
C	1.367692	1.991454	0.795224
H	1.998896	2.597389	0.136498
H	0.896557	2.66326	1.521132
H	2.018979	1.30138	1.340579
H	-1.864214	-1.12267	1.509554

Geometries of extended products:**Methyl ester from TMCL-I**

	X	Y	Z
C	1.527100	-0.586222	-0.489784
C	0.317386	0.251563	-0.033207
H	1.374315	-1.646253	-0.243655
H	1.655859	-0.548627	-1.577665
C	-0.975062	-0.469904	-0.478474
H	-0.995535	-0.513196	-1.577439
H	-0.876901	-1.512985	-0.142629
C	-3.333712	-1.143088	-0.184798
H	-3.305707	-1.455302	-1.242202
H	-2.991801	-1.998042	0.421286
C	2.832801	-0.183772	0.168101
O	2.945496	0.438633	1.202897
C	5.180174	-0.363963	0.035333
H	5.268470	-0.812271	1.028090
H	5.908213	-0.801046	-0.647785
H	5.335387	0.714158	0.122826
O	3.892195	-0.645361	-0.537126
C	-2.330890	0.399157	1.516101
H	-1.922548	-0.424275	2.114119
H	-1.718308	1.285421	1.706191
H	-3.343522	0.602710	1.873168
O	-4.649470	-0.753160	0.194066
H	-5.236589	-1.505325	0.051777
C	-2.359835	0.038940	0.018882
C	-2.883066	1.232787	-0.806433
H	-2.289591	2.135375	-0.647791
H	-2.864630	1.004678	-1.878785
H	-3.916980	1.452591	-0.529915
H	0.349972	0.274023	1.061760
C	0.454894	1.695487	-0.540153
H	0.345481	1.745015	-1.630431
H	-0.295006	2.354946	-0.098673
H	1.434904	2.101602	-0.274348

Methyl ester from TMCL-II

	X	Y	Z
C	1.274308	-0.681759	-0.007817
C	0.089581	0.321556	0.054282
H	1.166592	-1.427753	0.792273
H	1.239735	-1.250821	-0.943834
C	-1.178076	-0.536709	-0.253149
H	-1.160180	-0.801219	-1.321174
H	-1.059854	-1.486319	0.288667
C	-2.583465	-0.003832	0.102223
C	-3.593285	-1.144552	-0.087034
H	-3.654556	-1.407315	-1.156933
H	-3.243312	-2.040258	0.451050
C	2.681408	-0.126742	0.126521
O	3.015645	0.935631	0.608480
C	4.957479	-0.644064	-0.224686
H	5.220897	-0.485971	0.824096
H	5.530133	-1.471988	-0.642243
H	5.155865	0.276883	-0.778345
O	3.577392	-1.024550	-0.350947
C	0.308420	1.416677	-1.010552
H	1.236531	1.959597	-0.816732
H	0.369132	0.979157	-2.014402
H	-0.506089	2.142749	-1.016048
C	0.003447	0.949618	1.458684
H	-0.195076	0.186305	2.220984
H	0.940337	1.449278	1.710800
H	-0.802173	1.688109	1.511920
O	-4.858991	-0.720927	0.409985
H	-5.508564	-1.400808	0.193300
C	-3.041867	1.232584	-0.684898
H	-2.502600	2.134720	-0.386707
H	-2.892907	1.091739	-1.762554
H	-4.106065	1.406208	-0.507886
H	-2.607909	0.239810	1.172163

List of publications

This thesis has resulted so far in the following publications:

Delgove M.A.F.; Wróblewska A.A.; Stouten J.; van Slagmaat C.A.M.R.; De Wildeman S.M.A.; Bernaerts K.V, Organocatalyzed ring-opening polymerization of regio-isomeric lactones: reactivity and thermodynamic considerations. *Macromolecules*, submitted.

Delgove, M. A. F.; Laurent, A.-B.; Woodley, J. M.; De Wildeman, S. M. A.; Bernaerts, K. V.; van der Meer, Y., A prospective life-cycle assessment (LCA) of monomer synthesis: Comparison of biocatalytic and oxidative chemistry. *ChemSusChem* **2019**, 12, 1349-1360.

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Other publications by the author:

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Curriculum vitae

Marie Delgove was born on the 24th of February 1991 in Ploemeur, France. In 2009, she started studying chemistry at the École Nationale Supérieure de Chimie de Rennes (ENSCR) in France. She continued her education at the École Nationale Supérieure de Chimie de Mulhouse (ENSCMu) in France. She had the opportunity to do a one-year internship at SABIC, in the Netherlands, where she gained industrial experience in the development of coatings. In 2015, she obtained her diplôme d'ingénieur chimiste at ENSCMu, and her Master of Science in Chemistry

with a specialization in polymers and materials science the same year. She performed her Master thesis at the University of Eindhoven (TU/e) in the group of Prof. Rint Sijbesma under the supervision of Dr. Hans Heuts on the topic of mechanochemistry applied to hairy polymeric particles.

She started her PhD research the same year under the supervision of Prof. Stefaan De Wildeman and Dr. Katrien Bernaerts at Maastricht University and the Aachen Maastricht Institute for Biobased Materials (AMIBM). At the interface of applied biocatalysis and polymer chemistry, she explored oxidative enzymes for the synthesis of monomers, which are used for polymers targeting several applications. During her PhD, she expended her knowledge by following the advanced course "Biocatalysis and Protein Engineering" at BioTechDelft. The most important results of her research are summarized in this dissertation.

“Tout objectif sans plan n’est qu’un souhait”

Antoine de Saint-Exupéry