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Lipase-Mediated Syntheses of Trimethylolpropane-Based Biolubricant and Cyclic Carbonate

Amin Bornadel

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Academic thesis, which by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Friday, 11 October 2013 at 10:15 a.m., in Lecture Hall C at the Center for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy in Engineering.

The Faculty opponent is Prof. dr. Ulf Hanefeld, Department of Biotechnology, Delft University of Technology, Delft, The Netherlands.

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Conventional lubricants are mineral oil based and lack biodegradability resulting in their accumulation in the environment. Synthetic esters of polyhydric alcohols and fatty acids are biodegradable and possess desirable technical properties for lubricant applications. Synthesis of TMP-trioleate from oleic acid and TMP catalyzed by commercial immobilized *Candida antarctica* lipase B, Novozym®435 (N435) was studied by varying reaction parameters. The product obtained possesses desirable pour point (-42 °C) for lubricant applications in sub-zero conditions. The biocatalyst was recycled in reactions at 70 °C for 7 batches, 24 h each, with a half-life of 94 h. The biocatalyst half-life was doubled by washing it with 2-propanol between the batches. A simplified kinetic model was developed for the lipase-catalyzed reaction in order to facilitate optimization and design of the process and minimize the amount of resources required for investigations of the process. The methodology used for the kinetic modeling is applicable for similar types of enzymatic reactions involving multi-substrate multi-product systems.

Cyclic carbonates are potential monomers for phosgene-/isocyanate-free polycarbonates and polyurethanes that have wide range of applications. Six-membered cyclic carbonates can readily undergo ring-opening polymerization to form aliphatic polycarbonates and polyurethanes and their copolymers. Six-membered cyclic carbonate with hydroxyl functional group was obtained with 75% yield using a chemoenzymatic process involving lipase B catalyzed transesterification of dimethylcarbonate (DMC) with TMP in the presence of molecular sieve to form linear TMP carbonate followed by thermal cyclization. Performing the reaction in a recirculating flow reactor, higher conversion rates were obtained compared to the batch process, the product was recovered easily without extra separation steps, and the biocatalyst and molecular sieve remained intact for reuse. *In silico* evaluations of the reaction accompanied with empirical investigations confirmed that lipase B prefers DMC as acyl-donor while TMP and its derivatives, formed during the course of the reaction, serve as acyl acceptors. The formation of TMP carbonate oligomers hence found to be non-enzymatic and intensified by heat.

Key words
Biocatalysis, solvent-free reaction, Novozym®435, TMP-trioleate, kinetic modeling, six-membered cyclic carbonate

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Popular Summary

Since the beginning of mankind, humans have managed to find ways to make use of the resources available at hand in order to survive and even to improve their livelihoods. The basic needs for food as well as other amenities were met from plant- and animal resources until the 19th century when industrial revolution changed the base of production to fossil sources. Access to coal initially followed by mineral oil and natural gas accompanied by ingenious chemistry and process development has provided us with unlimited number of products that are present in everything we use today. It has changed the way we live to an extent that it is difficult to think of a life without these products.

All the development has of course come at a cost - to the environment and health, the effects of which have started to be felt in terms of increased greenhouse gas emissions, toxic discharges to wastewaters, oceans, persistent pollutants in soil and ground water, climate change, etc. Moreover, the fossil reserves that have taken millions of years to build up, are depleting rapidly. This has led to an increased awareness and demand for alternative renewable resources, toxic-free, environment-friendly production process and nontoxic, biodegradable products. This has provided an interesting challenge to the scientists and engineers to consider the possibilities of developing new or improved products that fulfill the market demand and innovative technologies for processing a different raw material and not posing an environmental burden. Among the several alternatives, industrial biotechnology using microorganisms and their enzymes has gained increased attention for production of chemicals, materials and energy.

The studies in this thesis are targeting two product groups that reflect many of the problems stated above - lubricants and polymers (polyurethanes and polycarbonates). Lubricants are used in all mechanical equipment to reduce friction and wear and for other applications. Historically - from the time the Egyptians built pyramids - lubricants were made of vegetable oils and fats, but the modern lubricants are based on mineral oil. Spillage of the lubricants has resulted in widespread environmental problem. For the vegetable oils to work as well as the mineral oil based lubricants, they need to be modified so that the resulting product can be used without problems at very low and very high temperatures. This is possible by reacting fatty acid component of the oil with a branched molecule to give a biolubricant product that is still flowing at sub-zero temperature and is stable above in hot environments.

Polyurethanes and polycarbonates are materials with a market of several Mtonnes. They are used for making many products like coatings, foams, adhesives, transparent plastics, etc. The problem however is the use of phosgene, a toxic gas or isocyanate, a product of phosgene, in their manufacture. Even the products may have some traces of the components left in them. Thus, a phosgene/isocyanate-free route for producing these materials would be extremely attractive. One route is to use cyclic carbonates as building blocks that is discussed in this thesis.

The thesis presents the use of lipase, an enzyme that breaks down fats and oils in nature, for synthesis of biolubricants and cyclic carbonates. The processes developed do not make use of any volatile organic solvents and are run under very mild conditions compared to the chemical processes. It is shown that different approaches can be used to improve the process efficiency and to get high product yields.

List of Publications

The thesis is based on the following papers, which will be referred to by their roman numerals in the text.

I Åkerman, C.O., A. E. V. Hagström, M. A. Mollaahmad*, S. Karlsson, R. Hatti-Kaul. Biolubricant synthesis using immobilised lipase: Process optimisation of trimethylolpropane oleate production. Process Biochem, 2011. 46: 2225-2231.

II Bornadel, A., C. O. Åkerman, N. Borg, P. Adlercreutz, R. Hatti-Kaul. Kinetic modeling of lipase-catalyzed esterification reaction between oleic acid and trimethylolpropane: a simplified model for multi-substrate multi-product ping-pong mechanisms. Biotechnol Prog, in press.

III Bornadel, A., R. Hatti-Kaul, K. Sörensen, S. Lundmark, S.-H. Pyo. Optimization of a two-step process comprising lipase catalysis and thermal cyclization improves the efficiency of synthesis of six-membered cyclic carbonate from trimethylolpropane and dimethylcarbonate. Biotechnol Prog, 2013. 29: 66-73.

IV Bornadel, A., M. Ismail, R. Hatti-Kaul and S.-H. Pyo. Six-membered cyclic carbonates from trimethylolpropane: solvent-free lipase-mediated synthesis in a flow reactor and in silico evaluation of the reaction. (Submitted manuscript)

* Name changed to A. Bornadel

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My Contribution to the Papers

I I contributed by performing the experimental part, discussions and had minor contribution in writing and finalizing the manuscript.

II I developed the mathematical model, planned and performed the experimental part, developed a preliminary computer-based modeling and wrote and finalized the manuscript.

III I planned and performed the work and wrote the manuscript.

IV I planned and performed the flow reaction part and had a major contribution in writing and finalizing the manuscript.

Contents

1. Introduction

The first discoveries on enzymes and their functionalities can be traced back in time to the 19th century with the achievements of Kirchhoff, Payen and Persoz, Pasteur, Berzelius and Buchner to mention a few [1,2]. It was around the same time that the first modern oil wells were constructed and commercial extraction and refining of petroleum started to become a trend [3]. Due to the abundance and properties of this source of energy and raw materials, it did not take long until petroleum-based processes applying chemical catalysis took over and have been employed dominantly ever since by industries.

Currently there are several serious environmental issues that call for change of the global industry. There is an increasingly higher demand for different materials and products due to an ever-increasing consumption by a steadily growing population. On the other hand petroleum oil and other mineral resources are limited and using these nonrenewable resources for industrial production is not sustainable. Moreover, global warming and ecotoxicity problems, matters of great concern, are intensified by increased consumption of fossil-based fuels and chemicals. The practice of Green chemistry has been proposed to provide a solution to many of these problems [4]. This would involve the use of renewable resources as raw materials, employing reactions with low energy input, nontoxic catalysts, no organic solvent, low waste, biodegradable products, etc.

Industrial biocatalysis, once in its infancy, has grown considerably during the past decades and shown promising advancements and has in some cases already found use in a wide range of industrial applications in food-, detergent-, medical-, chemicals-, pharmaceuticals sectors, etc. [5]. The use of enzymes, which are renewable biological catalysts, enables chemical reactions to be carried out under mild conditions. These catalysts, besides being nontoxic and biodegradable, usually offer high rates and selectivities and hence can be used to carry out chemical reactions more efficiently and with minimum formation of undesirable side-products. Biocatalysis thus offers an extremely attractive mode of green chemistry [6-8].

There are currently many processes in operation worldwide, which apply toxic reagents and harsh conditions to produce products that lack biodegradability and result in environmental toxicity. Conventional methods used for production of mineral oil based lubricants and polymers are examples of those processes that have the potential to be replaced using benign alternatives, carried out in mild conditions and preferably using renewable resources, with products having environmentally compatible features such as biodegradability and nontoxicity.

However newly developed processes normally need further improvements before being able to compete with conventional and established production setups. This is also true for biocatalytic processes; enzymes are relatively more expensive, so high efficiency, stability and productivity is a prerequisite in order to be economically feasible. The process development time can be substantially shortened with a multiple approach using different process development tools – this mindset represents the framework of this thesis and examples of the different tools will be presented in the following chapters.

The work presented in the thesis was part of two research projects involving academia-industry collaborations: "*Greenchem, Speciality Chemicals from Renewable Resources*" and "*Cyclic Carbonates, a Route to Isocyanate-free Polyurethanes*", financed by The Swedish Foundation for Strategic Environmental Research [MISTRA].

Scope of the Thesis

The aim of the thesis is to evaluate industrial biocatalysis as an environmentally benign alternative to the conventional chemical processes for the synthesis of specialty chemicals with features making them suitable as replacements for currently available toxic products. This is shown by case studies on lipase catalysed syntheses of biolubricant and cyclic carbonate, which use an industrial branched polyhydric alcohol (polyol), trimethylolpropane as an acyl acceptor. Moreover different methodologies that have been used in the work in order to facilitate the design and optimization of these processes and eventually improve their productivity and economy, are presented and their roles for the development of biocatalytic processes are illustrated.

The thesis is based on four papers, two of which are published and one is in press:

Paper I and **II** deal with solvent-free lipase (immobilized *Candida antarctica* lipase B, Novozym®435) catalyzed esterification of trimethylolpropane and oleic acid for production of polyol fatty acid esters for biolubricant applications. In the first paper the process is studied using reaction parameters optimization and biocatalyst recycling in order to evaluate the process efficiency and productivity. In the second paper kinetic modeling of the reaction is performed and a mathematical model is developed for the process in order to facilitate the process optimization and design.

Paper III and **IV** deal with Novozym®435 catalyzed transesterification of trimethylolpropane and dimethylcarbonate in the production of six-membered cyclic carbonates. In **Paper III** statistical modeling, response surface methodology and reaction medium engineering approaches are employed to improve the process yield and productivity. **Paper IV** illustrates the application of flow reaction for facilitating and improving process economy and *in silico* molecular modeling for evaluation of the enzymatic process.

The following chapters give a broader perspective of the concepts presented by the papers included in the thesis. Chapter 2 provides a background about lipases and their functions and in particular lipase B as a versatile biocatalyst for chemical synthesis in non-conventional media. Chapter 3 gives an introduction to lubricants and the advantages of biolubricants over conventional mineral oil based lubricants followed by discussing the biocatalytic process used for production of TMP trioleate for biolubricant applications. The focus of Chapter 4 is on cyclic carbonates and in particular six-membered cyclic carbonates based on TMP for phosgene- and isocyanate-free production of aliphatic polycarbonates and polyurethanes. The final chapter summarizes the outcome of the work presented in the thesis in terms of benefits and existing barriers and gives perspectives for further improvements and industrialization of these processes.

2. Lipase - a versatile biocatalyst

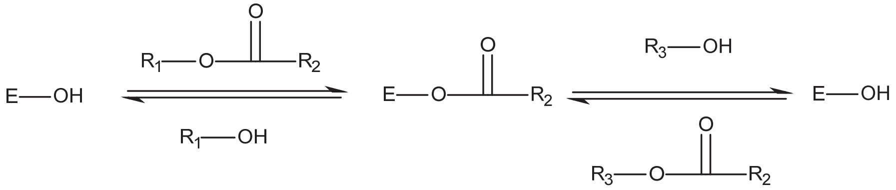
2.1 Background

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) belong to the enzyme class hydrolases. Their natural function is to catalyze hydrolysis of triacylglycerols (fats) *in vivo* and form glycerol and fatty acids. Fats have poor solubility in aqueous systems and create an interface with water. Therefore these enzymes have been evolved to work optimally in an organic-aqueous interface, a phenomenon called interfacial activation [9-11]. In fact these enzymes comprise a loop or an α-helical fragment in their molecular structure, a.k.a. the lid, which blocks the enzyme active site unless it is in physical contact with the hydrophobic phase [10]. This hypothesis is supported by 3D crystal structure imaging of the protein in open [12] and closed [13,14] forms. There are of course variations from this general pattern due to occurrence of lipases having two lids [15] or not an actual lid at all [16,17]. This behavior of lipases along with their broad substrate specificity might explain why they have been widely applied for synthesis in organic media [18] both in research laboratories and industry [19-21].

Lipases from microbial sources including bacteria, fungi and yeast have gained special attention for different applications due to easier means for their production and their less complex mechanism of action as they do not need any coenzymes [19,22]. Lipases provide a clear example for promiscuous biocatalysts [23]. They catalyze esterification, transesterification, interesterification and other reactions involving alcohols or other nucleophiles as acyl acceptor in media with low water activity that includes organic solvent systems - both non-ionic and ionic, solvent-free systems in which one of the substrates acts as solvent, and supercritical fluid systems [22,24-27]. Hence, lipases have been employed as catalysts for a variety of processes including synthesis of esters, triacylglycerols and polymers, enantiomer resolution, fatty-acid enrichment, biodiesel production, phospholipid conversion, carbohydrate modification, and increasing the lipophilicity of bioactive compounds [22].

2.2 Lipase-catalyzed reactions

The mechanism of action of a lipase involves oscillation between two modes, acylated- and free enzyme mode (Scheme 2.1). In the first step, the serine residue at the enzyme active site undergoes a nucleophilic interaction with the carbonyl group of an ester bond, leading to the formation of an acyl-enzyme intermediate, which can thereafter be attacked by another nucleophilic molecule, normally water or an alcohol but also e.g. an amine, to release a product and the free enzyme [22]. Usually the acyl-enzyme formation is followed by release of a product, water or an alcohol for esterification and transesterification reactions, respectively. However in ring-opening polymerization (ROP) of lactones and cyclic carbonates to polycaprolactones and polycarbonates for instance, there is no primary product formation and the hydroxyl terminated polymer chain directly accepts the ring-opened molecule [28]. In a reaction mixture containing more than one nucleophiles, their reaction with the lipase would depend on a number of factors including their nucleophilicity, concentration and size of the molecule. The latter influences their access to the enzyme active site. In both the reactions described in this thesis (**Papers I-IV**), the size of the polyol TMP and its derivatives formed during the reaction is the predominant factor determining their efficiency as acyl acceptors. Although the TMP esters could also potentially serve as acyl donors, *in silico* evaluations performed in **Paper IV** show that not to be the case.

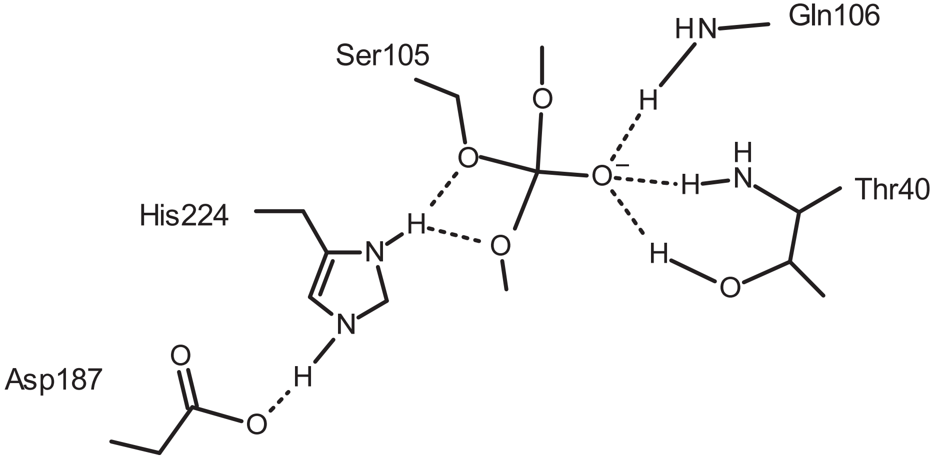


Scheme 2.1 Mechanism of lipase-catalyzed reaction involving acylation of the lipase followed by its deacylation.

2.3 *Candida antarctica* lipase B

Candida antarctica is a basidiomyceteous yeast isolated from the lake Vanda in Antarctica [29]. It produces two lipases, A and B [30]. Despite the origin of the organism producing these enzymes, these lipases are among the most thermostable ones reported so far and have found numerous applications [30,31]. *Candida antarctica* lipase A (CalA) shows a broader specificity towards secondary, tertiary and sterically hindered alcohols and a better recognition of trans fatty acids for hydrolysis of triglycerides. CalA has also been used for asymmetric synthesis of amino acids and related compounds due to chemoselectivity towards amine groups. CalA is calcium dependent and shows lower catalytic activity, hence requiring higher biocatalyst loads for catalyzing reactions [29,30].

Candida antarctica lipase B (CalB) is a well-studied and characterized enzyme, and the most commonly reported enzyme for reactions in non-conventional media [16,30-32]. It is a globular α/β type protein with approximate dimensions of 30 Å x 40 Å x 50 Å, consisting of 317 amino acids with molecular weight of 33 273 Da [16]. CalB has isoelectric point (pI) of 6.0 [33], pH optimum of 7.0 and stability in the pH range of 3.5-10.0 [30,31]. Compared to CalA, it is less thermostable but has higher specific activity. In CalB the catalytic triad is made up of Ser105, Asp187 and His224 and is located at the carboxy-terminal edge of the parallel β-sheet, which suggests that the enzyme has a similar mechanism of action as other lipases [16]. The first tetrahedral intermediate in the enzyme active site is formed by the interaction of the catalytic serine and the carbonyl group of the acyl-donor substrate, which is stabilized in the oxyanion hole by hydrogen bonding to the catalytic backbone residues, Thr40 and Gln106 (Scheme 2.2). In order for the substrate to reach the catalytic serine, it needs to travel inside along a 10 Å x 4 Å wide and 12 Å long hydrophobic channel [16]. CalB does not possess an actual lid, which is a typical feature for most lipases [16,17]. It is mainly active towards primary alcohols and is 1,3-specific towards glycerol. CalB is stereoselective for position Sn3 towards triglycerides and when the acyl chain length increases to 18 the preference switches to Sn1 position [34]. Many applications that CalB has found apart from its broad range stereo- of regio- and enantioselectivity for both hydrolysis and organic synthesis are attributed to its higher stability and catalytic activity towards different reactions [30,35-37].



Scheme 2.2 The first tetrahedral intermediate formed at the active site of CalB

2.4 Immobilized *C. antarctica* lipase B

In order to use enzymes for chemical synthesis in a water-poor environment, they are prepared by various immobilization techniques prior to their application as biocatalyst to prevent their aggregation and make them accessible for reaction with the substrates [22]. Immobilization of enzymes also facilitates their handling and reuse as well as the product purification. Sometimes immobilization is used to improve operational and storage stability of an enzyme. However, it can happen that immobilization leads to enzyme inactivation due to for instance change in the enzyme conformation through covalent binding to a resin carrier [38]. Despite the availability of various immobilization techniques [39,40], adsorption to a solid matrix including beads, surfaces or membranes is the most commonly used method. Other immobilized forms are covalent coupling, entrapment by polymer materials, cross-linked enzyme aggregates or crystals, and surfactant-based coated preparations [22,39,40]. The most commonly used immobilized preparation of CalB is the commercially available Novozym®435 (N435) from Novozymes A/S (Bagsvaerd, Denmark), in which the enzyme is immobilized by adsorption onto

Lewatit® VP OC 1600 (LANXESS AG, Cologne, Germany) beads of cross-linked polymer based on methacrylic esters (bead size: 0.32-0.45 mm). N435 preparation has even been used for the work presented in this thesis (**Paper I**, **II**, **III** and **IV**). Currently, there are even other commercial CalB preparations available e.g. CalB-CLEA, the cross-linked enzyme aggregates produced by CLEA Technologies (Delft, The Netherlands) [41], which lack a solid carrier and involve precipitation of the enzyme by e.g. high salt concentration and treatment using a cross-linking agent.

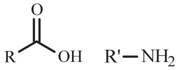
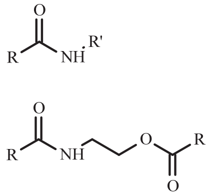
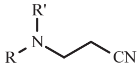
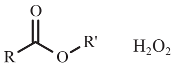
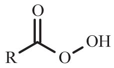
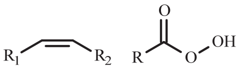
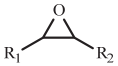
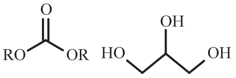
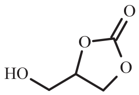
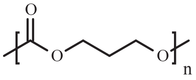
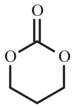
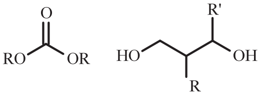
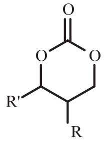
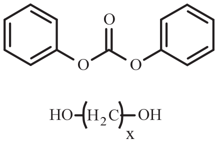
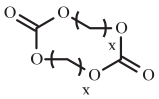
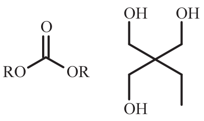
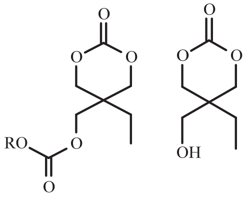
The method of immobilization, the type of matrix and linkages, and concentration of the immobilized enzyme influences the performance and stability of the biocatalyst. N435 is most efficient in hydrophobic solvents with high log P values, i.e. the ones with low or nearly zero water activity. The presence of water as a multi-layer around the enzyme active site can create a mass transport barrier and inhibit diffusion of hydrophobic substrates into the enzyme active site [42]. Moreover, the water generated during the reaction as during esterification, increases the risk of enzyme desorption from the matrix.

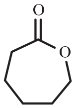
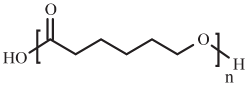
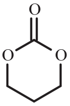
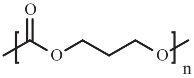
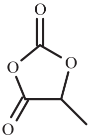
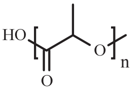
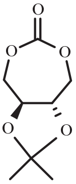
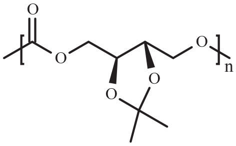
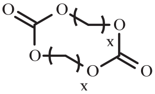
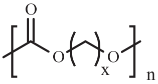
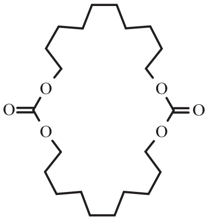
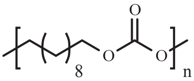
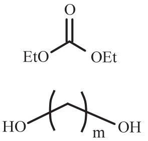
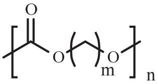
Often the reactions with immobilized preparations are carried out in a batch mode with stirring, which causes mechanical damage to the matrix and loss of active enzyme, seen also in case of N435. This can be avoided by using the biocatalyst in packed bed reactors as shown in **Paper IV**.

2.5 Novozym®435 for chemoenzymatic processes

Considering the broad substrate specificity features of lipases for biocatalysis [23], and in this context in particular, those features of CalB that make it suitable for synthesis in organic media, this enzyme has been employed for variety of reactions. In addition to its applications for esterification and transesterification in organic media, this enzyme has often been cleverly used in chemoenzymatic processes to catalyze other types of reactions. Table 2.1 lists a few examples, where commercial immobilized CalB, N435, has been used for other types of reactions.

Table 2.1 Examples of reactions other than ordinary esterification and transesterification in organic media catalyzed by Novozym®435

Reaction	Substrate	Product	Ref.
Amidation			[43,44]
Michael addition			[45]
Perhydrolysis			[43,46-49]
Epoxidation			[43,46-49]
Synthesis of cyclic carbonates			[50,51]
			[52]
			[53]
			[54]
			[55,56] Paper III & IV

Ring-opening polymerization			[57,58]
			[59]
			[60]
			[61]
			[54]
			[62]
Direct polymerization			[63,64]

2.6 Engineered *C. antarctica* lipase B

Protein engineering can be used to overcome some of the limitations of enzymes for application in biotransformation. Both rationally designed specific mutations as well as random mutations have been made. Lipase B has been investigated a lot especially by direct mutagenesis. Thermostability of CalB at 90 °C has been improved 7 fold using rational strategies for directed evolution of the enzyme [65]. Using the rational design strategy, Park and co-workers were able to improve stability of CalB in hydrophilic solvents by creating new internal hydrogen bonds in the enzyme molecule [66]. The specific activity of CalB for transeterification reactions was enhanced 6 fold by direct mutation of Leucine at position 278 to a less bulky Alanine [67]. A Ser105Ala mutant of CalB has been used for aldol addition but the rate of the reaction was low because of low frequencies of near attack complexes in the enzyme. The reaction rate was improved by a sequential aldol addition followed by Michael addition resulting in shift in the reaction equilibrium [68]. Ser105Ala CalB mutant was also used for carbon-carbon bond formation by catalyzing Michael addition of 1,3-dicarbonyl compounds to α,β-unsaturated carbonyl compounds [69]. The same CalB mutant was used for Michael addition of various thiols or diethyl amine to α,β-unsaturated carbonyl compounds [70], and for direct epoxidation of but-2-enal and 3-phenyl-prop-2-enal with hydrogen peroxide [71]. Several works have reported on rationally designed CalB mutants with increased enantioselectivity of secondary alcohols [72-74].

3. Trimethylolpropane fatty acid esters for biolubricant applications

3.1 Lubricants and biolubricants

The applications of lubricants are vast and their market size is substantial; in 2004 the global consumption of lubricants was 37.4 Mtonnes (Fig. 3.1). They play an important role in saving energy and resources and reducing emissions by lowering the friction and wear, wherever necessary. However, conventional lubricants consist mainly of mineral oil and to a small extent some different functional additives, which are added to confer the desired tribological properties (related to the science studying the mechanism of friction, lubrication, and wear of interacting surfaces that are in relative motion) e.g. high viscosity index, oxidative stability, heat and shear stability, and low volatility and pour point. The main problems with the conventional lubricants are their non-fossil origin, toxicity and poor biodegradability. It has been estimated that 50% of the lubricant that is sold around the globe end up in the ecosystem due to spillage, incidents, evaporation or similar reasons, which can cause toxic ecological effects if accumulated in the environment [75-77].

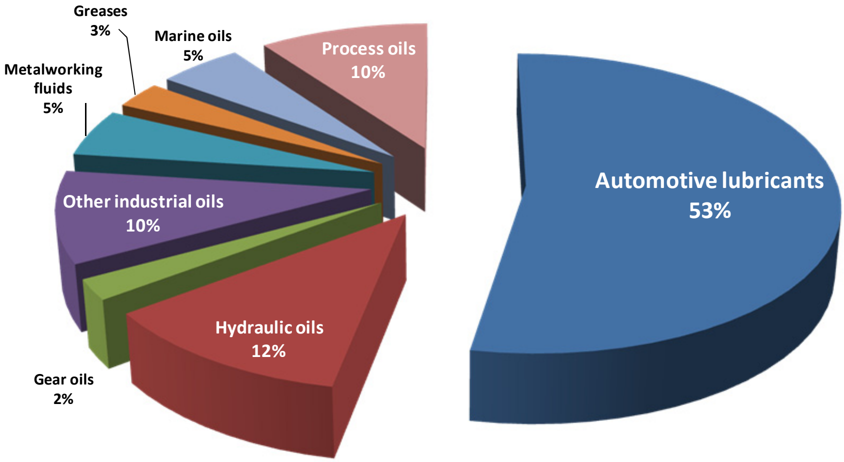


Figure 3.1 Market share of lubricants for different sectors [75].

Early developments of non-mineral oil based lubricants were based on direct applications of vegetable oils in combination with additives [78]. These lubricants although being biodegradable lacked some desirable technical properties, such as chemical stability and a wide temperature operating window [76,79]. In the following generation of biolubricants, synthetic esters of vegetable oil were developed, which modified and improved the physico-chemical properties of the base fluid [76].

Long-chain carboxylic or "fatty" acid esters with different polyhydric alcohols can result in materials of various properties, which have found numerous applications as emulsifiers, flavors, fragrances and intermediates in food, cosmetic, lubricant and polymer industries [80]. Fatty acid esters of polyols developed earlier for special applications, e.g. turbojet [81], motorcar [82] and gas turbine [83] engine oils, were known for their superior technical properties over mineral oil based lubricants [76,77]. They have particularly found applications as hydraulic fluids owing to their excellent lubricant properties, desirable heat and shear stability and viscosity features in addition to low volatility [76,84]. Moreover oleochemical esters are biodegradable and are in the category of "environmentally acceptable" lubricants [85-87]. They contain high proportion of renewable component owing to the fatty acid part coming from plant sources while the alcohol moiety currently has in most cases a petrochemical origin [88,89].

Common practices for industrial synthesis of olecochemical esters of different polyhydric alcohols, e.g. neopentyl glycol, pentaerythritol and trimethylolpropane (TMP) often involve high temperature and pressures [80] combined with solvent mediated reactions using different homo- or heterogeneous catalysts comprising alkali or other metals [8, 15 and Ref. of 15], organic and inorganic acids [80,90] or organometallic compounds [90,91]. These chemical reactions often lead to the formation of considerable amounts of by-products, which contribute to uneconomical use of raw materials and resources [92].

Several reports on lipase-catalyzed esterification of fatty acids and polyols for production of food emulsifiers [93], low-calorie fat substitutes, biodegradable detergents, paint and varnish components [80] and synthetic oils have been published.

3.2 Trimethylolpropane trioleate

Trimethylolpropane trioleate [CAS 57675-44-2] with chemical formula $C_{60}H_{110}O_6$, is a colorless or yellowish clear liquid with high boiling point, high flash point and high viscosity index. It is biodegradable and has excellent lubricating properties. It has a pour point around -50 °C (the lowest temperature at which a liquid becomes semi-solid and loses its flow characteristics), which makes it suitable for applications in sub-zero conditions [94]. TMP trioleate is an ideal base fluid for ISO 46 fire resistant hydraulic oil. It is used in formulation of biodegradable hydraulic oil, saw oil, barge engine oil, and as an effective oiliness additive for cold steel rolling, pipe drawing and other metal processing fluids [95].

As the name suggests, TMP-trioleate is produced by esterification of the polyol TMP with the fatty acid, oleic acid.

TMP, 2-ethyl-2-hydroxymethyl-1,3-propanediol, [CAS 77-99-6] with chemical formula $C_6H_{14}O_3$, is a colorless crystalline trivalent alcohol. TMP is highly soluble in water and in polar organic solvents. The three hydroxymethyl residues in the TMP molecule are bound to a tetrahedral carbon atom making the three hydroxyl groups equally capable of undergoing the normal OH group reactions. One way to synthesize TMP is by base-catalyzed aldol addition of butyraldehye with formaldehyde. TMP is the third highly used neopentyl polyhydric alcohol after pentaerythritol and neopentyl glycol. Global consumption of TMP was estimated close to 200 000 tonnes in 2012 with average annual growth rate of 3.4% during 2011-2018 [89].

TMP is used for synthesis of alkyd resins for coatings and for branching in saturated polyester resins. It is known for imparting flexibility, durability, thermal stability and chemical resistance to resins. TMP has many applications in the manufacturing of polyurethane foams, elastomers, sealants and adhesives. It is also used to make adducts with toluene diisocyanate (TDI) to lower their toxicity for coating applications. Triesters of TMP with shorter chain carboxylic acids and long chain fatty acid have applications as synthetic lubricants for engine oils, cutting and metal processing fluids and fire resistant hydraulic fluids [89].

Oleic acid (OA) is a monounsaturated omega-9 fatty acid abbreviated as 18:1 cis-9 [CAS 112-80-1]. It is an oily colorless to yellowish liquid with chemical formula $C_{18}H_{34}O_2$ and melting point and boiling point of 13 and 360 °C, respectively [96]. Oleic acid triglycerides are present in large amounts in fats and oils from various animals and plants such as olive, pecan, canola, peanut, macadamia, sunflower, grape seed, sesame, as well as chicken-, turkey- and pig fats.

OA is produced by enzymatic hydrolysis of its triglycerides or their chemical saponification in the presence of base catalyst e.g. sodium hydroxide. Oleic acid undergoes reactions of carboxylic acids and alkenes. It has different applications as emulsifier and intermediate in food, cosmetics and pharmaceuticals as well as for production of different chemicals and polymers [97].

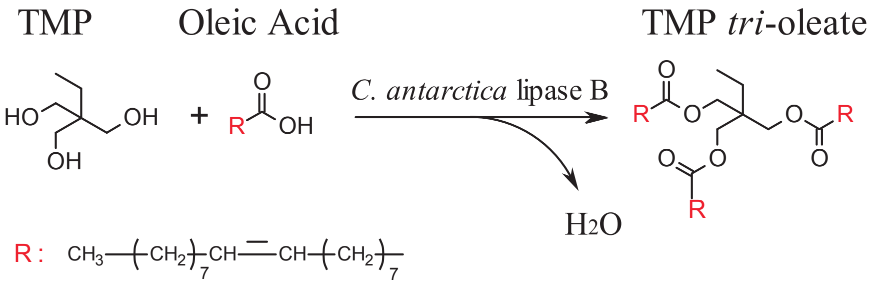
3.3 Lipase-catalyzed synthesis of TMP-oleate

Earlier efforts on lipase catalyzed synthesis of TMP-oleate have required long reaction times and incomplete conversion of TMP. Monot *et al.* (1990) reported on solvent-mediated esterification between TMP and caprylic acid catalyzed by *Rhizomucor miehei* lipase for production of TMP tricaprylate for high temperature lubricant applications [92]. They used molecular sieve to absorb the by-product water to shift the reaction equilibrium towards maximum production of the triester. After 180 h around 90% TMP conversion and 85% triester were obtained. The choice of the organic solvent, using excess amount of caprylic acid, high temperature and enzyme concentration and longer reaction time were among the parameters that most efficiently favored the conversion rate and productivity.

Linko and coworkers reported on lipase-catalyzed transesterification between TMP and rapeseed oil fatty acid methyl esters (RME) by a two step-process that required synthesis of rapeseed oil methyl esters, using methanol and alkaline catalyst, prior to the lipase catalyzed reaction [98,99]. After 24 h reaction at 58 °C and 5.3 kPa using 20% commercial immobilized *R. miehei* lipase preparation (Lipozyme IM 20), about 90% total conversion and 75% TMP triester were obtained. When 20% *Candida rugosa* lipase was used as a crude powder with 15% w/w water to maintain the enzyme activity, 73% TMP triester were obtained after 68 h at 47 °C and 2 kPa. It was therefore concluded that high loads of the biocatalyst, excess molar ratio of RME and prolonged reaction time favor the yield of the TMP triester. However, high cost of the enzyme and remaining unreacted RME requiring additional purification steps influenced process economy [100] negatively. With 40% commercial immobilized *Candida antarctica* lipase B (Novozym®435) at 58 °C and 5.3 kPa only TMP mono- and diester products were obtained after 66 h reaction [98]. The incomplete esterification was probably due to lipase inactivation by the water added to the reaction system (15 % w/w).

3.3.1 Solvent-free lipase-catalyzed synthesis of TMP-oleate

Synthesis of TMP esters from the reaction between TMP and C5-C18 carboxylic acids in a solvent-free medium using silica-sulfuric acid, Amberlyst-15 and immobilized CalB, N435 as catalysts was compared by Åkerman *et al.* (2011) [101]. Silica-sulfuric acid and Amberlyst-15 were more efficient catalysts when short chain carboxylic acids were used, while the catalytic activity of N435 was too low towards C5 acid and increased by using longer chain acids. For the synthesis of C18 esters of TMP, the biocatalytic reaction was comparable to the silica-sulfuric acid in terms of efficiency, however the latter led to a dark colored product due to carbocation of the double bond present in oleic acid (OA) (Scheme 3.1) and reaction with another OA molecule to form oligomers (a.k.a. estolide). Estolide formation lowers the quality of the final product and requires extra purification steps. The product of the reaction catalyzed by N435 was clear and showed suitable properties for application as lubricant including a low pour point (-42 °C).



Scheme 3.1 Lipase B catalyzed synthesis of TMP trioleate from OA and TMP

In order to optimize the biocatalytic process, solvent-free esterification reaction between OA and TMP catalyzed by N435 was performed under varying experimental conditions (**Paper I**). The reactions were performed in 100 g scale and under vacuum in order to shift the equilibrium towards maximum esterification.

3.3.2 Effect of reaction temperature

The reaction between OA and TMP using N435 was run at temperatures between 60 and 100 °C. The lower limit was kept above the melting point of TMP (58 °C) in order to improve the mixing of the reactants. As increasing temperature enhances the rate of chemical reactions and improves mass transport, increase in initial reaction rate, productivity and substrate conversion with increase in temperature was observed in the reaction between OA and TMP (Fig. 3.2).

On the other hand, enzymes exhibit varying thermal tolerance and undergo thermodenaturation above a certain temperature. Thermostability of an enzyme is very much affected by its secondary and tertiary structure and the interactions that exist between the different amino acids including ion pairs, hydrogen bonds and hydrophobic interactions [102]. Moreover, thermostability is also affected by the environment to which the enzyme is exposed. Higher water activity for instance can favor enzyme activity by improving its flexibility [103] but also makes it more sensitive to temperature. Immobilization of an enzyme can decrease flexibility of the enzyme and improve its thermostability [104]. N435 is known to be optimally active at very low water activity, which also increases its thermostability allowing it to be used at high temperature [105].

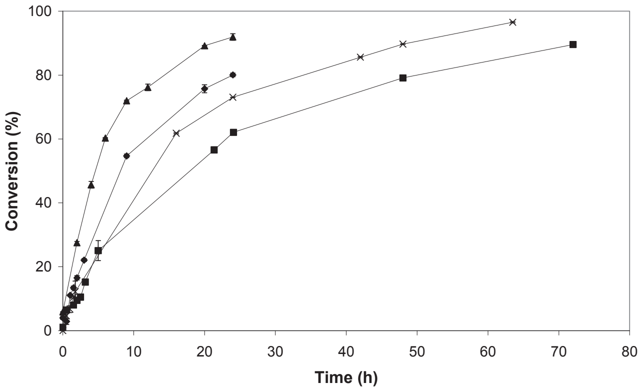


Figure 3.2 Conversion of acid groups during esterification of OA with TMP (molar ratio of 3:1), catalyzed by N435 (2 % w/w). (■) – 60 °C, (x) – 70 °C, (●) – 80 °C, and (▲) – 100 °C.

However temperatures higher than 70 °C were detrimental to the biocatalyst activity. At 100 °C no catalytic activity remained after 24 hours of using 0.5% (w/w) biocatalyst. Hence, higher enzyme loads were needed to compensate for the thermal inactivation of the biocatalyst.

The reactions with 2% (w/w) N435 at 70 and 100 °C showed that the product obtained after 64 h reaction at 70 °C contained > 90% TMP *tri*-oleate and had higher quality than the product obtained at 100 °C (Fig. 3.3), resembling the product with good lubricant properties having pour point of -42 °C suitable for application at sub-zero conditions. Moreover, the residual enzymatic activity was increased 8 fold when the operational temperature was decreased from 100 to 70 °C. This is important for improving half-life of the biocatalyst and consequently obtaining higher product amount per unit amount of the biocatalyst [106,107].

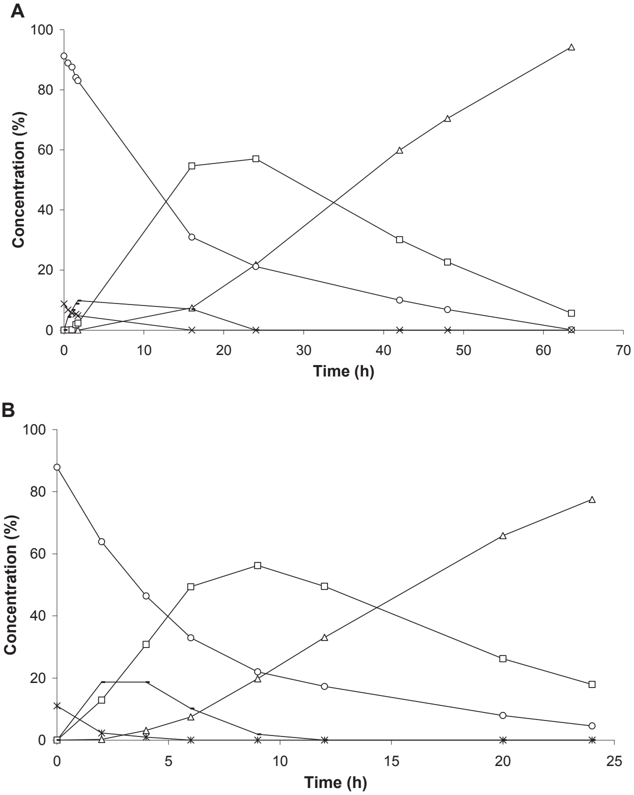


Figure 3.3 Esterification of OA with TMP (molar ratio of 3:1) at (A) 70 °C, and (B) 100 °C, catalyzed by 2 % (w/w) N435. (○) – OA, (**x**) – TMP, (**-**) – TMP monoester, (□) – TMP diester, and (Δ) – TMP triester.

3.3.3 Effect of reaction components

All components of a chemical reaction, substrates, products, solvents or impurities, influence the reaction efficiency. Kinetic of a chemical reaction is influenced by the concentration of the substrates and products; the reaction rate is generally higher at higher concentrations of substrates and lower concentrations of products due to the shift of the reaction equilibrium towards formation of more products. One way to achieve higher reaction rates is therefore by removing a product from the reaction medium by precipitation, evaporation (**Papers I** and **II**), absorption (**Papers III** and **IV**), permeation, etc.

The kinetics of lipase-catalyzed reactions can be improved by using solvents as they can facilitate solubility of hydrophobic substrates and products and shift the reaction equilibrium towards reversed hydrolysis. Using solvents however, might interfere with the catalytic performance of the enzyme [38,108]. Hydrophobic solvents with high log P value (logarithm of partition coefficient in octanol to water) are preferred for maintaining the biocatalyst stability since the molecular flexibility of the enzymes is reduced and the enzyme is maintained in a relatively rigid conformation [56]. Using organic solvents however, results in an inevitable problem of toxicity for the environment, which needs to be avoided wherever possible [4]. This is avoided by carrying out reactions without using solvents, where one of the substrates also acts as solvent for the system (**Papers I**, **II**, **III**, and **IV**).

Substrates and products can disturb the enzymatic activity by inhibiting the enzyme. Small alcohols and acids are known to be able to block CalB active site and act as inhibitors [109-111]. Water can also affect the enzymatic activity by competitive inhibition [112]. In **Paper I** and **II** running the reaction at very low water activity by continuous removal of water under vacuum showed to be very effective on both the reaction equilibrium and enzymatic activity [98]. As compared to mono-ols, TMP has a rather bulky structure and its entry to CalB active site is affected by steric hindrance owing to the neopentyl groups of the polyol [92]. CalB is shown to have a large acyl-donor site and narrow acyl-acceptor site [113], and as the size of the acyl acceptor increases by the formation of TMP-monoester and -diester, the efficiency of reaction decreases. During the course of the reaction, a mixture of TMP and its esters at varying concentrations is present with varying efficiencies as acyl-acceptor.

The effect of varying OA:TMP molar ratio in the range of 1-4.5 on the reaction was studied. At lower ratios (1:1 and 2:1) OA was almost completely consumed at all temperatures provided that the biocatalyst was not limiting (biocatalyst > 0.5% w/w). The products of these reactions were mixtures of mainly *mono*- and *di*-ester when the ratio was 1:1 and *di*- and *tri*-ester when the ratio was 2:1. Theoretically, molar ratio of 3:1 is required for complete esterification of TMP to TMP *tri*-oleate. Almost complete esterification of TMP was achieved in reactions 8 and 22 (**Paper I**, Table 2) using molar ratio 3:1 at higher temperature and biocatalyst loads. At 70 °C and 2% (w/w) biocatalyst, highest productivity was achieved at molar ratio of 3.3:1, however higher ratios (4.5:1) led to decrease in the initial reaction rate and productivity, probably because of the consequent reduced concentration of TMP. The remaining OA in the final product needs to be distilled off from the mixture through a process called deodorization in order to obtain the product with desirable properties.

3.3.4 Biocatalyst recycling

Lipases are in general more stable biocatalysts as compared to the other enzymes. As seen in **Paper I**, the immobilized CalB exhibited activity at very high temperatures although the residual activity is compromised. Besides denaturation of the enzyme caused by thermal denaturation, leaching of the enzyme from the solid carrier could be yet another cause of activity loss of the immobilized preparation [107,114]. Desorption of the enzyme from the matrix could be influenced by the medium including washing out of the soluble enzyme with water generated during the reaction, and high temperature. Furthermore, stirring of the reaction mixture leads to mechanical damage of the matrix, aggravating the problem of loss of the active enzyme. Figure 3.4 shows the residual enzyme activity when the biocatalyst was recycled for 7 batches of 24 h each. Hence better mixing can be obtained by other means e.g. by flow reaction in narrow channels.

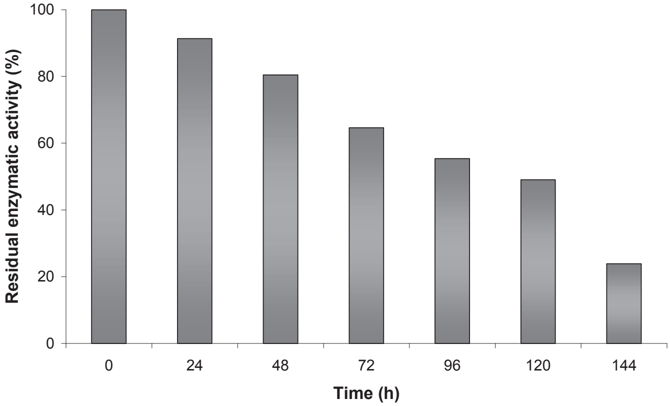


Figure 3.4 Residual enzymatic activity for the esterification reaction at 70 °C, 2 % (w/w) N435 and OA:TMP molar ratio of 3:1

The biocatalyst washed with 2-propanol in between the batches was shown to regain part of the catalytic activity (**Paper I**), which may have been lost because of the enzyme active site being blocked by the remaining substrates or products and also the unbalanced water activity of the enzyme microenvironment. The half-life of the biocatalyst was almost doubled compared with the unwashed biocatalyst (Fig. 3.5).

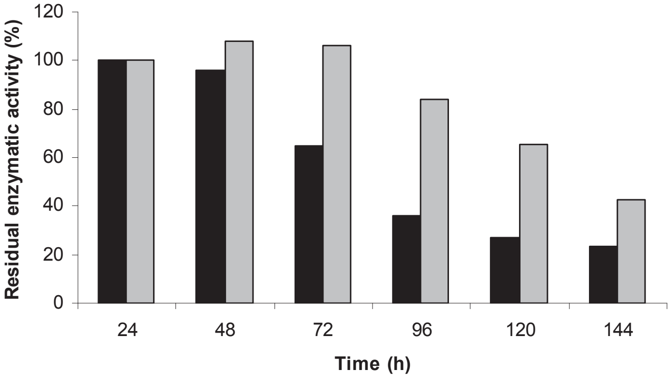


Figure 3.5 Comparison between the residual enzymatic activity when the biocatalyst was washed (gray bars) with 2-propanol between the batches and unwashed (black bars). Reaction conditions: 70 °C, 2 % (w/w) N435 and OA:TMP molar ratio of 3:1.

3.4 A simplified kinetic model for the reaction

For the reactions catalyzed by lipase discussed under section 2.2, the enzyme undergoes acylation-deacylation stages, the mechanism of which is called ping-pong (a.k.a. substituted enzyme), also followed by several other enzymes among hydrolases, transferases and oxidoreductases such as esterases, transaminases and peroxidases [115]. When two substrates and two products are involved the mechanism is commonly called ping-pong bi-bi with the general pattern shown in Scheme 3.2 [116].

$$\mathrm{E} \underset{k_{-1}}{\overset{k_1C_A}{\rightleftharpoons}} \mathrm{EA} \underset{k_{-2}C_P}{\overset{k_2}{\rightleftharpoons}} \mathrm{E}' \underset{k_{-3}}{\overset{k_3C_B}{\rightleftharpoons}} \mathrm{EQ} \underset{k_{-4}C_Q}{\overset{k_4}{\rightleftharpoons}} \mathrm{E}$$

Scheme 3.2 Enzyme catalyzed reaction following ping-pong bi-bi mechanism involving interaction of the substrate A with free enzyme, E and formation of the first intermediate, EA followed by release of the first product, P and formation of the second intermediate, EQ by nucleophilic attack of the substrate B to the acyl-enzyme, E′ and eventually release of the second product, Q and free enzyme.

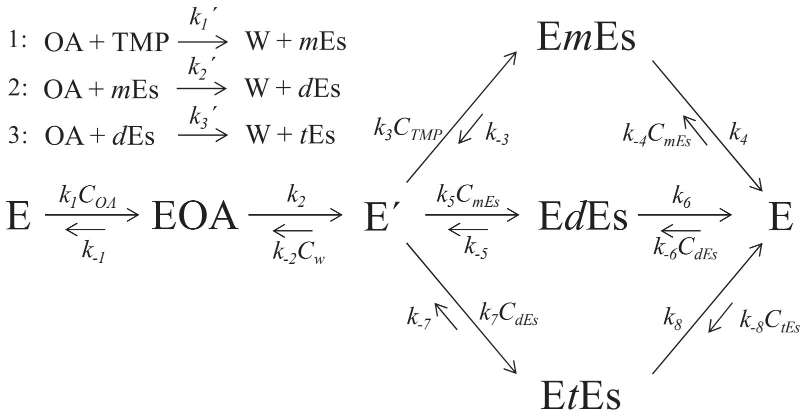
Kinetics of such reactions can be formulated as shown by Equation 3.1 containing 10 terms and 9 parameters according to Cleland's classification of kinetic parameters into *limiting rates* (V), *Michaelis constants* (K_m) and *inhibition constants* (K_i).

$$v = \frac{\dfrac{V_+ C_A C_B}{K_{iA} K_{mB}} - \dfrac{V_- C_P C_Q}{K_{iP} K_{mQ}}}{\dfrac{C_A}{K_{iA}} + \dfrac{K_{mA} C_B}{K_{iA} K_{mB}} + \dfrac{C_P}{K_{iP}} + \dfrac{K_{mP} C_Q}{K_{iP} K_{mQ}} + \dfrac{C_A C_B}{K_{iA} K_{mB}} + \dfrac{C_A C_P}{K_{iA} K_{iP}} + \dfrac{K_{mA} C_B C_Q}{K_{iA} K_{mB} K_{iQ}} + \dfrac{C_P C_Q}{K_{iP} K_{mQ}}} \qquad \textbf{Eq. 3.1}$$

When substrates with more than one functional group (e.g. polyhydric alcohols (polyols), triglycerides, dialkycarbonates etc.) are involved in similar type of reactions, the kinetics of these systems become more complex since the product of one step can act as the substrate for the subsequent step. Therefore kinetic equations for these multi-substrate multi-product systems comprise higher number of parameters and exponentially higher number of terms. For the lipase-catalyzed reaction between OA and TMP, where TMP possesses 3 hydroxyl groups leading to formation of totally 4 products, conventional approaches result in kinetic equations containing up to 27 terms and 26 parameters [117,118]. Equations of such magnitude are not practical to use and their parameters estimation is usually prone to different errors due to dealing with too many parameters with strong correlations between them [119].

The lipase catalyzed reaction performed in a non-aqueous environment shifts the reaction equilibrium to favor synthesis over hydrolysis. This is further promoted by removing a side-product e.g. water (**Paper I**), methanol (**Paper III** and **IV**), acetone [120] etc. Under these conditions and by running the reaction efficiently towards completion the effect of reversed reactions is minimized making it possible to neglect some of the rate constants in deriving the kinetic equations.

An outline of the mechanism of the lipase-catalyzed reaction between OA and TMP is shown in Scheme 3.3. Unbalanced arrows in the scheme imply the effect of the side-product removal during the course of the reaction.



Scheme 3.3 An outline of the mechanism for the reactions involved in lipase-catalyzed esterification between OA and TMP. The effect of continuous water removal during the reaction is shown by using unbalanced arrows. The reactions 1, 2 and 3 on the top left corner shows the non-enzymatic formation of the TMP oleates.

Using the steady state and negligible reversed reaction assumptions the kinetic rate equations for the different reaction components were derived (**Paper II**, supplementary material). Therefore the overall reaction rate for component *j* (*j* = *OA, TMP, mEs, dEs* and *tEs*) was the sum of enzymatic and non-enzymatic rates for that component (Equation 3.2). Non-enzymatic formation of the TMP esters taking place in parallel to the enzymatic reactions was noticed to be of some importance due to the effect of the side-product removal.

$$v_{j,tot} = v_{j,enz} + v_{j,nonenz} \qquad \textbf{Eq. 3.2}$$

Therefore by taking this approach a simplified kinetic model with up to 10 terms and 9 parameters was obtained for the lipase-catalyzed reaction between OA and

TMP. Equation 3.3 shows the kinetic equation for the enzymatic consumption of OA.

$$v_{OA,enz} = \frac{-\frac{V_1^* C_{OA} C_{TMP}}{K^*_{mTMP}} - \frac{V_2^* C_{OA} C_{mEs}}{K^*_{mmEs}} - \frac{V_3^* C_{OA} C_{dEs}}{K^*_{mdEs}}}{C_{OA} + \frac{K^*_{m1OA} C_{TMP}}{K^*_{mTMP}} + \frac{K^*_{m2OA} C_{mEs}}{K^*_{mmEs}} + \frac{K^*_{m3OA} C_{dEs}}{K^*_{mdEs}} + \frac{C_{OA} C_{TMP}}{K^*_{mTMP}} + \frac{C_{OA} C_{mEs}}{K^*_{mmEs}} + \frac{C_{OA} C_{dEs}}{K^*_{mdEs}}} \qquad \textbf{Eq. 3.3}$$

In the subsequent steps the model consisting of the rates of the consumption and/or production of the substrates and products with totally 8 independent parameters was fitted to the experimental data and the model parameters were estimated. The model was verified using different empirical data. The simplified model was shown to be capable of predicting the profile of the reactions run at different concentrations of substrates and enzyme. The procedures used for the modeling and parameters estimation are shown in Figure 3.6.

This methodology can be applied to develop simplified kinetic models for reactions that follow multi-substrate multi-product ping-pong mechanisms. The models developed can be used to facilitate the optimization of the biocatalytic processes and minimize the number of experiments and the amount of material, energy, time and manpower required for the investigations.

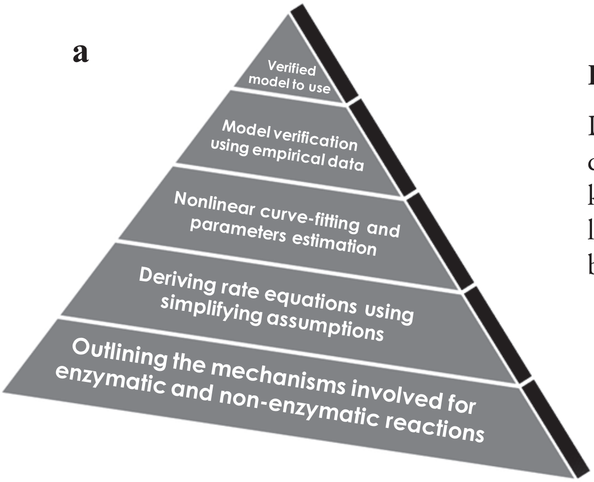


Figure 3.6a

Different steps taken for developing a verified kinetic model for the lipase-catalyzed reaction between OA and TMP

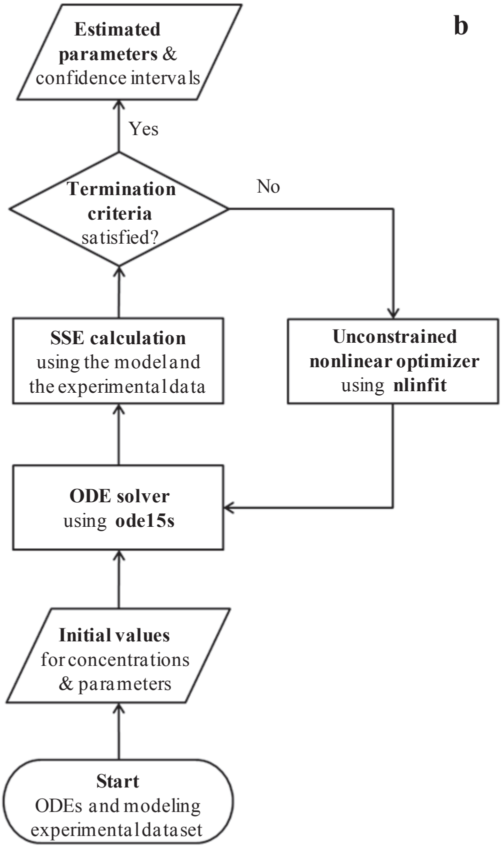


Figure 3.6b

The procedure used for non-linear curve-fitting and parameters estimation

4. Six-membered cyclic carbonate from trimethylolpropane

4.1 Polycarbonates and polyurethanes

Polycarbonates (PCs) and polyurethanes (PUs) are industrially important polymers used for a large variety of applications. PCs are impact resistant, dimensionally stable thermoplastics that have been available in the market since 1941 [121]. These polymers have optical properties superior to many types of glasses and have various applications in optics. Having excellent thermal and flame resistance and high stability to different environmental conditions, these polymers have also been used for electronic-, automotive- and construction sectors. The global market for PCs is close to worth over 6 billion USD, and is growing at 6% per annum. The carbonate linkage for these polymers is conventionally made by reaction between an alcohol and a carbonyl donor compound, usually a phosgene compound.

The most widely used aromatic polycarbonate is polybisphenol A (polyBPA) carbonate. In addition to the use of highly toxic phosgene for the production of BPA-carbonate polymers, there are now concerns about release of BPA from these resins with negative effects on human health. As a result, nowadays regulations restricting applications of these polymers are imposed in many countries [122].

Aliphatic PCs are produced by reaction between alkanediols and phosgene, triphosgene or dialkylcarbonates. [123]. Aliphatic PCs and their copolymers are biocompatible and have low toxicity and their applications particularly in the biomedical field are increasing [54]. These polymers are biodegradable and recyclable and are considered as environmentally benign [124-127]. They have also shown promising *in vivo* stability [128], which makes them very useful for long-term biological implantation purposes. They have advantage of low rate of hydrolysis in aqueous media over aliphatic polyesters such as polylactide and lactide-glycolide copolymers and moreover, contrary to polyesters, their hydrolysis does not lead to the formation of carboxylic acids, which in biological systems can increase the acidity of the surrounding tissue and elicit inflammatory response and also catalyze further degradation of the polymer [54].

Polyurethanes (PUs), currently the sixth most widespread group of industrial polymers, were first developed by Otto Bayer and colleagues in the 1940s [129]. Since then, the global market for PUs estimated at 14 Mtonnes in 2010, has been continuously growing and is predicted to reach 18 Mtonnes by 2016 [129]. The wide variety of applications that PUs have can be attributed to their versatile properties [130]. PUs of various physical states in forms of soft or rigid foams, elastomers or hard solid/flexible plastics are manufactured for numerous applications for isolation and insulation, seating and high performance coatings and adhesives. Moreover owing to their durability, biocompatibility and biostability, PUs are also used as elastomers for biomedical applications and in implantable medical devices [128,131]. Common practices for manufacturing PUs include polyaddition reaction between polyol and diisocyanate compounds using tertiary amines, e.g. 1,4-diazabicyclo[2.2.2]octane, as basic catalyst [132,133].

4.2 Phosgene and isocyanate

Carbonyl dichloride, commonly known as phosgene [CAS 75-44-5], is a colorless gas at room temperature and normal pressure (T_b = 7.48 °C at 1 atm). It was first prepared by John Davy in 1812 in the photochemical reaction between carbon monoxide and chlorine. Around 75% of the phosgene produced worldwide is used for the production of isocyanates, 20% for PCs manufacturing and approximately 5% for the synthesis of chloroformates and carbonates for pharmaceuticals and agrochemicals (Fig. 4.1). Phosgene is a highly toxic compound that was used in World War I in chemical warfare. It is an insidious poison since the odor may not be noticed. Inhaling phosgene is detrimental to the respiratory tract as it leads to pulmonary edema and disturbs normal lung function [134,135]. Therefore extensive safety precautions are required for plant design and operation to prevent exposure to phosgene.

Isocyanates are prepared from the reaction between primary amines and phosgene (Fig. 4.1) and are used in production of PUs by reaction with the hydroxyl group of diols or polyols [136]. The most frequently used isocyanate compounds for production of polyurethanes are methylene diphenyl diisocyanate (MDI) and toluene diisocyanate (TDI) accounting for more than 95% of the total and the remaining would be aliphatic and specialties including hexamethylene diisocyanate (HDI) and isophorone diisocyanate (IPDI) [133]. In addition to toxicity and the difficulties of handling and storage of phosgene for production of isocyanates, isocyanates themselves are highly reactive and hazardous and even low exposures can lead to sensitizing effects, respiratory disorders and develop allergic dermatitis and asthma [137]. Therefore, these compounds have adverse health effects on workers due to occupational exposure as well as on consumers of the PU containing products due to release of free isocyanate vapors and aerosol mists from resins [138-141]. Different organizations and authorities including the National Institute for Occupational Safety and Health (NIOSH) and the Occupational Safety and Health Administration (OSHA) have set a threshold limit value (TLV) of 0.005 ppm for full-shift workers being exposed for 8 hr and 0.02 ppm for short-term exposures to isocyanates [142-144].

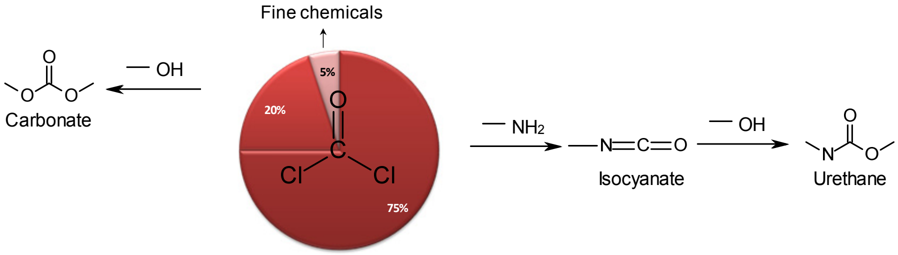


Figure 4.1 Market share of phosgene for different sectors [134]

Moreover, due to high reactivity of isocyanates, the PU produced using these compounds has complex molecular structure and lacks enzymatically cleavable linkages, and hence is resistant to biodegradation [145,146]. Owing to the high reactivity of hazardous isocyanates, great precautions are necessary with respect to equipment and safety in the application of urethane-based coatings and adhesives (paints/varnishes, glue, binders) that are of growing interest due to their excellent mechanical and optical properties for highly demanding applications. [147,148].

Thus the next generation of industrial PCs and PUs will likely require phosgene- and isocyanate-free production routes using metal-free low toxicity catalyst/initiator systems such as alcohols/diols and enzymes in environmentally benign processes. Moreover, these polymers need to be biodegradable and/or recyclable and preferably based on renewable resources to meet sustainable production criteria [121,129,133,145,149,150].

4.3 Cyclic carbonates: five- and six-membered

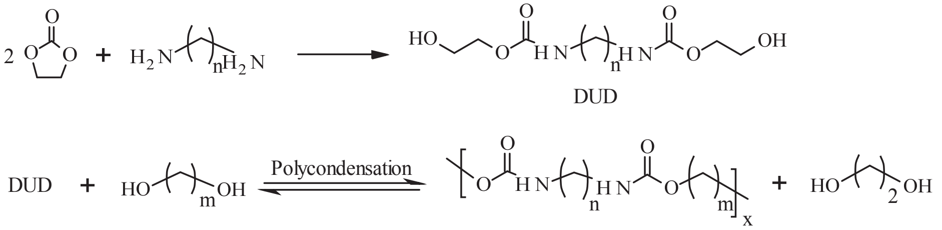
The chemistry of cyclic carbonates was explored as early as in the 1930s by Carothers and colleagues [151-153]. The first commercially available cyclic carbonates that appeared in the market over 40 years ago, were ethylene carbonate (EC) and propylene carbonate and have been used as both reactive intermediates and inert solvents for variety of applications [150]. In recent years production of glycerol carbonate, based on low price glycerol from biodiesel production, has become of interest for mainly being used as solvent for secondary batteries, coatings, cosmetics and pharmaceuticals [88,154,155]. These products are examples of five-membered cyclic carbonates (5CC) that are in general more readily prepared than six-membered ones (6CC).

Cyclic carbonates have attracted attention in recent years because of their capability of undergoing ring-opening polymerization (ROP) in bulk or solution [156,157], which opens up the possibility to use them as monomers for phosgene- and isocyanate-free production of aliphatic PCs and PUs. Ring-opening polymerization (ROP) of cyclic carbonates has been carried out using metallic compounds as catalyst [157]. However the use of metal-free nontoxic catalyst systems for ROP of the cyclic monomers is of growing interest, especially in biomedical applications. Among the alternative catalyst/initiator systems that have been used for this purpose are acids, enzymes and alcohol/diol initiators [157]. The latter offers the advantage of having a better control over the polymerization reaction by controlling the molar mass, hydrophobicity/degradability and functionalization of the final polymer material [157].

Ring-opening polymerization of 5CC is generally difficult [158] and it requires higher energy input to go beyond the so-called floor temperature (i.e. a temperature beyond which the ROP can take place when both enthalpy and entropy of polymerization are positive [159]) and is accompanied by loss of CO_2 [160]. The occurrence of decarboxylation results in the entropy of polymerization, ΔS_P becoming positive to a level that compensates for the positive enthalpy of polymerization, ΔH_P. Hence the free energy of the ROP becomes negative, $\Delta G_P < 0$ ($\Delta G_P = \Delta H_P - T\Delta S_P$) and polymerization can take place. The formation of one carbonate linkage during the polymerization is followed by decarboxylation of the subsequent carbonate to form an ether linkage. As a consequence, the fraction of carbonate to ether linkages cannot exceed 50% [161]. These ether linkages in the polymer structure are not hydrolysable but can undergo oxidative degradation through environmental stress cracking (ESC) and metal-ion oxidation (MIO) and hence negatively influence the mechanical properties of the polymer [54,128].

Six-membered cyclic carbonates, on the other hand, are thermodynamically less stable than their ring-opened form [162] and retain CO_2 during polymerization [163], which enables a smooth transformation into the corresponding polymer without volume shrinkage and loss of desirable mechanical properties of the final polymer material [164-166].

Five- and six-membered cyclic carbonates can react with diamines without using catalyst to form urethane units. A diurethanediol (DUD), 1,6-bis(hydroxyethyloxycarbonylamino)hexane was obtained at high yield (>94%) from the reaction of EC and 1,6-hexanediamine carried out without any catalyst at room temperature [167]. The polycondensation reaction between α,ω-bis(hydroxyethyloxycarbonylamine)alkane and α,ω-diols containing six or more carbon atoms per molecule ($m \geq 6$) at 150 °C using a tin catalyst led to the formation of [n,m]polyurethane (Scheme 4.1). Polyhydroxyurethanes with M_n of 20,000-30,000 were prepared by polyaddition reaction between different bis(cyclic carbonate)s and hexamethylenediamine or dodecamethylenediamine in dimethylsulfoxide or *N,N*-dimethylacetamide at 70-100 °C for 24 h [168].



Scheme 4.1 Ring-opening of EC with 1,6-hexanediamine followed by polycondensation with α,ω-diols to obtain polyurethane [167]

Six-CCs have however higher reactivity towards amines. Tomita and co-workers compared reactivity of 4-(3-butenyl)-1,3-dioxolan-2-one and 5-(2-propenyl)-1,3-dioxan-2-one with hexylamine and benzylamine in *N,N*-dimethylacetamide at 30-70 °C [169]. It was shown that the reactivity of the 6CC was 29 to 62 times higher than for the corresponding 5CC. Moreover the activation energy of the reaction of the 6CC and hexylamine (10.1 kJ/mol) was less than half of that of the corresponding 5CC and hexylamine (24.6 kJ/mol), probably due to larger ring-strain of the 6CC compared to the 5CC [169].

There are few reports on ROP of EC and TMC for production of aliphatic polycarbonates and polyurethanes [157,170]. However, applications of simple alkylene carbonates for the polymer technology might be limited due to the absence of functional groups next to the carbonate ring.

4.4 Synthesis of cyclic carbonates

Synthesis of 5CC is much easier than that of 6CC. Moreover synthesis of simple alkylene carbonates and their purification is less of a challenge in comparison with those comprising reactive groups other that the ring carbonate in their structure.

The work reported by Carothers and Van Natta in 1930 involved the reaction of diethylcarbonate with ethylene glycol, trimethylene glycol and higher (up to decamethylene) glycol. The two- and three-carbon chain glycols resulted in formation of EC and trimethylene carbonate (TMC) monomers, where longer-chain glycols led to formation of polymeric open-chain carbonates [151].

Recently different 1,3-dioxolan-2-one (5CC) derivatives containing reactive groups including vinyl moieties, esters, ethers and alcohols have been developed [171]. Among them only glycerol carbonate (GC) is commercially available. GC can be obtained from the reaction between glycerol and a carbonate source such as phosgene, dialkyl carbonate [113,172-174], alkylene carbonate [175], urea [176,177], CO_2 [178,179], CO and oxygen [180-182]. Another approach to the synthesis of GC has been the cycloaddition of CO_2 to oxirane derivatives [168,183-186].

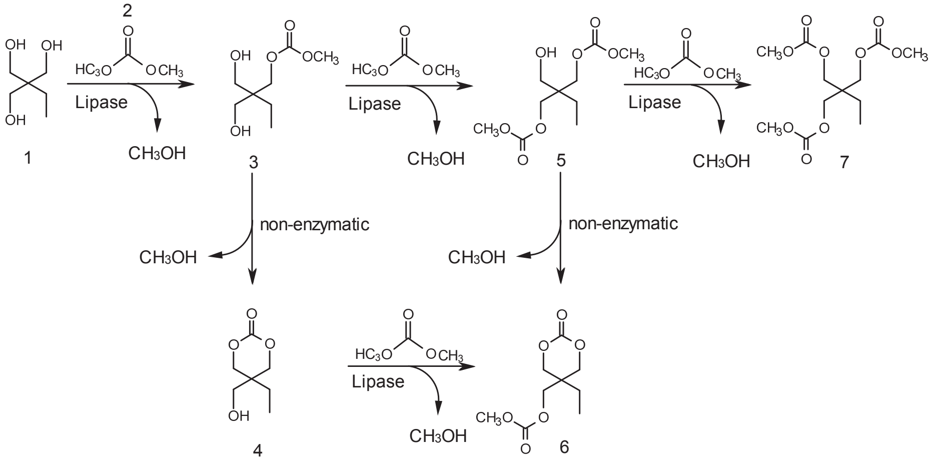
TMC, a six-membered cyclic carbonate, was synthesized by transesterification reaction between 1,3-propanediol and dialkylcarbonates using metal- or organo-catalyst [151,187,188]. Matsuo *et al.* 1998 synthesized TMC by reacting 1,3-propanediol with ethyl chloroformate in the presence of triethylamine [189]. In a different approach TMC was synthesized by cycloaddition of CO_2 to oxetane (trimethylene oxide) using vanadyl acetylacetonate as catalyst [162]. Six-membered cyclic carbonate with alkoxycarbonyloxy functional group was prepared by a multi-step process starting with dialkylcarbonate and TMP and ending with distillative depolymerization of TMP oligocarbonates at 275-300 °C under reduced pressure. However, the cyclic carbonate yield was very low and it was concluded that distillative depolymerization was not a suitable method for producing polyol cyclic carbonates due to possible cross-linking of different products [190].

In recent years biocatalytic processes for production and ring-opening polymerization of cyclic carbonates are of growing interest considering these processes generally demand less energy as enzymes work efficiently under mild reaction conditions and offer higher selectivity of desirable products. Lipase, in particular immobilized *Candida antarctica* lipase B, Novozym®435 (N435) has mainly been used in the processes as they are suitable for esterification and transesterification reactions in organic or solvent-free media media (Table 2.1).

In 2001 Matsumura and colleagues used N435 as catalyst for transformation of poly(TMC) into cyclic TMC and showed that the process can be done reversibly using the same enzyme [52]. N435-catalyzed synthesis of TMC with/without methyl substituent from dialkylcarbonate and 1,3-diol followed by ROP of cyclic products into their corresponding aliphatic PC using the same enzyme was reported by Tasaki *et al.* in 2003 [53]. Condensation of diphenylcarbonate and butane-1,4-diol and hexane-1,6-diol in toluene and chloroform to produce cyclic tetramethylene carbonate (TeMC) and hexamethylene carbonate (HMC) dimers was catalyzed by N435 and subsequently the cyclic dimers were ring-open polymerized in bulk using N435 to produce P(TeMC) and P(HMC) [54]. N435-catalyzed synthesis of GC from glycerol and DMC was carried out with high yield in organic solvent as well as in excess DMC as solvent [50,173]. In a novel approach simultaneous synthesis of biodiesel and GC was carried out using corn oil and DMC catalyzed by N435 [51]. Recently six-membered cyclic carbonates with hydroxyl and alkoxycarbonyloxy functional groups were produced by reaction between TMP and dialkylcarbonate in a two-step process [55]. In the first step catalyzed by N435, linear TMP carbonates were formed in high yield followed by thermal disproportionation of the linear products into TMP cyclic carbonates in the absence of the enzyme.

4.5 TMP cyclic carbonates

Solvent-free transesterification reaction between TMP and dialkylcarbonates catalyzed by N435 was reported by Pyo *et al.* (2011). Solubility of TMP was lower in diethylcarbonate (DEC) compared to dimethylcarbonate (DMC) and the reaction using DMC was faster [55]. The reaction between TMP and DMC was carried out at various concentration of the biocatalyst up to 40% (w/w of TMP) at 60 °C. Higher reaction rates were achieved using higher biocatalyst loads, where 90% conversion of the starting TMP was achieved within 24 h and full conversion within 48 h using more than 20% (w/w) biocatalyst. The enzymatic reaction resulted in a mixture of linear and cyclic TMP-carbonate products comprising mainly linear and cyclic *mono*-carbonated products at lower biocatalyst concentrations (2.5-5% w/w) and *di-* and *tri*-carbonated products at 20-40% (w/w) biocatalyst. In the subsequent step, the product mixture was exposed to thermal treatment at temperatures in the range of 60-80 °C in the absence of the biocatalyst in order to cyclize the linear TMP-carbonates. After thermal treatment at 80 °C for 144 h the mixture contained 85% TMP cyclic carbonates (4 and 6 in Scheme 4.2). The methanol produced during the reaction was removed by molecular sieve 4 Å to shift the reaction equilibrium towards maximum product formation and minimize the inhibition of the lipase by methanol [111].



Scheme 4.2 Pathway for synthesis of six-membered cyclic carbonates by lipase-catalyzed reaction of TMP and DMC including formation of primary linear TMP carbonates followed by thermal cyclization

N435 catalyzed reaction between TMP and DMC was also carried out in a solvent system comprising a hydrophobic and a hydrophilic solvent [56]. The binary solvent system allowed suitable conditions for lipase performance as well as the solubility of the polar substrates and products. By using a mixture of toluene-tetrahydrofuran (THF) (2:1 v/v) and lower substrates ratios (1:1 to 5:1 molar DMC:TMP), the enzymatic reactions were slowed down allowing the thermally driven formation of the TMP cyclic products to take place simultaneously. Using solvents, however prolonged the reaction time, which means lower productivity and less efficient use of the biocatalyst, and required additional steps for handling and recovery of the organic solvents.

4.6 Design of experiments for optimization of the reaction between TMP and DMC

According to the reaction pathway shown in Scheme 4.2 for the lipase-catalyzed transesterification between TMP and DMC, the individual reactions involved compete for the formation of the different products. The formation of an individual product is under the influence of different reaction parameters and its selectivity in the reaction mixture is under kinetic control [191]. Therefore, a goal oriented approach to optimization of the reaction was required for obtaining maximum reaction efficiency and maximum quality, in this case selectivity of desired cyclic product.

Statistical experimental design or design of experiments (DOE) is an organized approach for screening, optimization and robustness testing of different processes [192]. DOE gives several advantages over ordinary optimization methods that usually Consider varying One Separate factor at a Time (a.k.a. COST). In DOE experimentation strategy factors are varied simultaneously to create representative and informative experiments [193], usually leading to maximum information about the process with minimum number of experiments. Moreover DOE considers interactions that exist between different factors and lead to find the real optimum. Usually when one factor is varied at a time, experiments reach a point that no further improvement is observed, which might be far from the optimum when other factors are varied simultaneously.

DOE is normally begun by setting an objective, in this case optimization for obtaining maximum TMP cyclic carbonates, followed by defining the different factors (X variables) and responses (Y variables). It is quite common to determine the important factors and their intervals for the DOE project based on preliminary experiments.

The reaction temperature was kept constant at 60 °C, above the melting point of the TMP for obtaining high solubility in DMC and low enough to minimize thermal inactivation of the biocatalyst [55, **Paper I**]. The DMC:TMP molar ratio (X_1) was varied in the range of 10 to 30 as excess amount of the DMC is required when the reaction is carried out in a solvent-free system to avoid mass transport limitation especially when there is solid phase (the biocatalyst and molecular sieve beads) involved. The N435 amount (X_2) was varied in the range of 5-25% (w/w) as an important factor influencing the reaction efficiency and the economy of the process [194]. The reactions were monitored in the time span between 5 and 55 h (X_3), which is an important factor determining the conversion of the starting material and the selectivity of the different products. Selectivity of *mono*-carbonated products (3 and 4 in Scheme 4.2) and *di*-carbonate products (5 and 6) and the TMP conversion were the responses, Y_1, Y_2 and Y_3, respectively. A quadratic model based on a full factorial design in 3 levels was used for the DOE resulting in totally 27 experiments.

After the experiments were carried out, the data for the different responses were filled in the worksheet generated for the DOE (software program MODDE version 8.0, Umetrics AB, Umeå, Sweden). A quadratic PLS (partial least squares projections to latent structures) model [195] was fitted to the data and 4 components were calculated and the preliminary model was improved in terms of fitting and predictability (**Paper III**). The summary of the model is shown in Table 4.1 It can be seen that the percent of variation of the responses explained by the model (R^2) are very close to 1.0 showing an almost perfect fitting of the model to the data. The percent of variation of the responses predicted by the model (Q^2) being in the range of 0.9 or higher, reflect very good predictability of the model.

Once the modeling is done and the model shows characteristics of being reliable, it can be used to extract information about the importance of the different factors and model terms and their correlations with each other and with the responses. Furthermore, the model can be used to create contour plots for finding the best operating conditions.

Table 4.1 Summary of PLS model fitted to the designed experiments data obtained from solvent-free reactions between DMC and TMP catalyzed by Novozym®435

	R^2	R^2 Adj.	Q^2	SDY	RSD
Y_1^*	0.996	0.994	0.982	0.846	0.067
Y_2^*	0.988	0.979	0.942	0.580	0.084
Y_3	0.980	0.966	0.894	22.074	4.078

SDY = standard deviation of the response; RSD = the residual standard deviation; Number of observations = 21; Degrees of freedom = 12; Number of components = 4; Condition number = 6.247; Y block missing values = 0
* Logit-transformed

The model was used for finding windows of operational conditions for obtaining maximum amount of either *mono*-carbonated or *di*-carbonated products. For this purpose, "Sweet Spot" plots (MODDE jargon) were created to predict the optimum conditions for obtaining maximum amount 3+4 or 5+6 (Fig. 4.2).

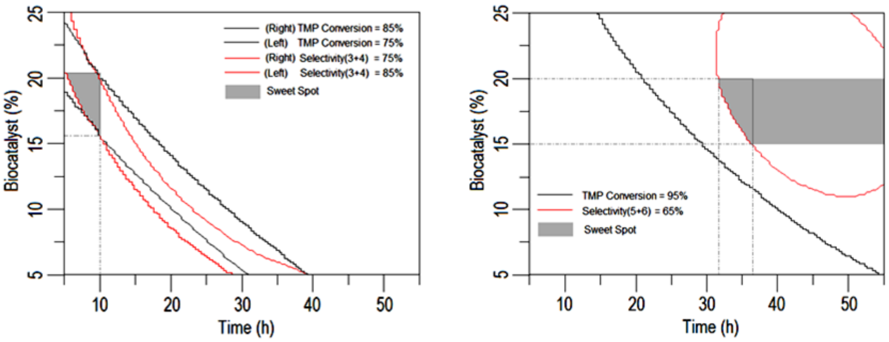


Figure 4.2 Predicted "Sweet Spot" plots based on the PLS model for obtaining high proportion of 3 and 4 (left), and 5 and 6 (right) from the solvent-free reaction between TMP and DMC catalyzed by N435

The model predicted that by running the reaction between DMC and TMP (10-30 molar excess of DMC) using 15-20% (w/w) N435, within 10 h around 75-

85% selectivity of 3+4 can be obtained when the TMP conversion is around 75-85%. Using the same conditions 65-70% yield of 5+6 can be obtained within 32-37 h, when almost all of the TMP is converted. The consistency between the model predictions and the empirical data was tested and confirmed (**Paper III**, Table 3).

The biocatalytic process was hence optimized using statistical modeling and regression analysis. By running the reaction under optimum conditions the reaction time was decreased considerably and maximum selectivity of either *mono*-carbonated products or *di*-carbonated products was achievable. When the reaction was carried out using DMC:TMP molar ratio 20:1 and 18% (w/w) N435, 65% yield of 3+4 was achieved within 6 h. The biocatalyst was recycled for consecutive batches, 10 h each (Fig. 4.3) and it was shown that 5 batches, each giving 65% yield of 3+4, could be run within totally 50 h without additional biocatalyst.

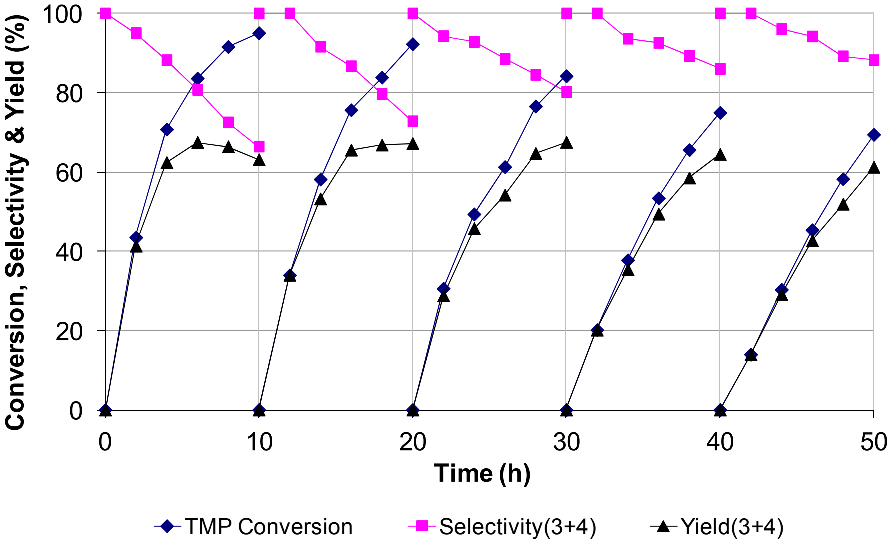


Figure 4.3 Profile of the consecutive reactions during recycling of 18% (w/w) N435 for catalyzing the reaction between TMP and DMC (20:1 molar DMC:TMP) at 60 °C

4.7 Medium engineering for thermal cyclization of linear carbonate products

TMP, DMC and different TMP carbonates are multi-functional compounds, and when present in a mixture can undergo several reactions through different possible pathways. In the presence of the biocatalyst, the DMC is the preferred acyl-donor for the lipase-catalyzed reactions (**Paper IV**) and TMP and its *mono-* and *di-*carbonated products act as nucleophiles leading to the formation of linear monomeric TMP carbonate products. Simultaneous formation of TMP cyclic products and in some cases oligomers of the different TMP carbonates are driven by heat and their formation can be influenced by the acid groups of the molecular sieve [196].

In the absence of the biocatalyst, thermal treatment of the mixture of different TMP carbonates can result in formation of different oligomers of the linear monomers and decreased formation of desirable TMP cyclic carbonates. It was experienced that the selectivity of the desirable cyclic products is very much affected by the medium and conditions of the cyclization reaction (**Paper III**).

In the absence of solvent, when the TMP carbonates mixture was exposed to heat (T ≥ 60 °C), the cyclization reaction was less efficient and required longer reaction times. The different reactions were driven mainly under the influence of the temperature, methanol evaporation rate and the concentration of the different components. Above 80 °C the oligomerization rate increased and the product obtained was a mixture of different TMP carbonate monomers and oligomers with lower selectivity of the cyclic products [55, **Paper III**].

When different solvents including DMC, THF:toluene (1:2 v/v), *tert*-butanol, *tert*-amyl alcohol and acetonitrile were used to mediate the cyclization reaction, acetonitrile showed the best results. The positive impact of acetonitrile on the cyclization reaction is probably because the small polar aprotic molecules of the solvent can solvate and separate the TMP carbonate molecules efficiently and simultaneously favor the S_N2 type cyclization reaction. The solvent ratios in the range of 20:1 (acetonitrile:TMPC v/w) efficiently suppressed the oligomerization reactions and had the best impact on the formation of the cyclic products (**Paper III**).

The reaction temperature was seen to have a great impact on the selectivity of the cyclization reaction. Usually the rate of chemical reactions increases by increasing the reaction temperature. According to the Arrhenius equation (Eq. 4.1), when there are parallel reactions involved, the reaction with higher activation energy (E_a) is more favored by increasing the temperature. This effect is illustrated in Figure 4.4 using the linear form of the Arrhenius equation (Eq. 4.2)

$$k = Ae^{(\frac{-E_a}{RT})} \qquad \textbf{Eq. 4.1}$$

$$Ln(k) = -\frac{E_a}{RT} + Ln(A) \qquad \textbf{Eq. 4.2}$$

It was observed that increasing the reaction temperature to around 80 °C increased the rate of oligomerization of TMP carbonates. However, at higher temperatures (90 °C) the cyclization reaction was accelerated and it was observed that at 110 °C the oligomerization was almost completely suppressed and high selectivity of TMP carbonates were obtained (**Paper III**, Table 4).

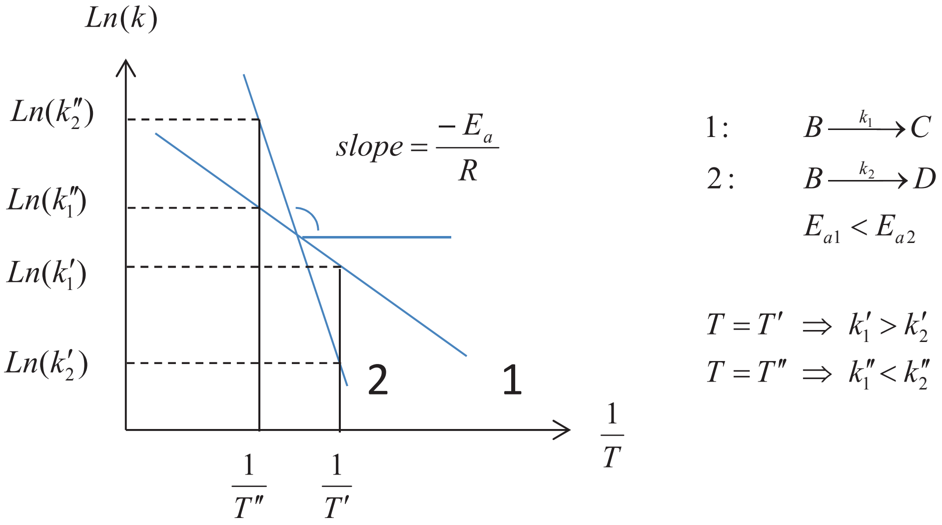


Figure 4.4 Effect of temperature on the kinetics of parallel reactions

Thus by using proper conditions and medium for the cyclization reaction it was possible to achieve about 75% overall yield of the TMP cyclic carbonate with hydroxyl functionality within 24 h. From the remaining, around 15% was TMP, which needed to be purified and reused in the process. The obtained cyclic carbonate is a highly reactive monomer capable of undergoing ring-opening polymerization per se to form aliphatic PCs or with diamines and diols to form PUs [167]. Moreover, it can be used to form adducts with polyisocyanates for reducing their toxicity for coating applications [89].

When the mixture with high proportion of *di*-carbonated products (5+6) was exposed to thermal treatment as explained above, both TMP cyclic carbonates, 4 and 6 were formed in almost equal amounts. Hence, by running the enzymatic reaction for about 24 h or slightly more to reach full conversion of the TMP and high proportion of 5+6, the final product after the thermal cyclization comprised about 75% cyclic carbonates 4 and 6. This way of running the process is probably less desirable as the enzymatic step is about 4 times longer and leads to a mixture of the TMP cyclic carbonates.

4.8 TMP cyclic carbonate production in a flow reaction

In general, batch vessel reactors offer low surface to volume ratios leading to inefficient heat and mass transport in the system, which deteriorates when it is scaled up. Hence extreme mixing systems are employed to improve the limited heat and mass transport. Immobilized enzyme preparations undergo mechanical damage in batch reactors involving agitation or mechanical stirring leading to formation of fine biocatalyst particles and leaching of the enzyme from the carrier [107,114]. Reactor engineering is among the methods used for optimization of biotransformation. It can benefit the process by allowing the biocatalyst to be used more efficiently and have higher in-process stability.

N435 biocatalyst consists of well-defined beads allowing their use efficiently in packed-bed reactors without creating clogging or back-pressure problems during the operation. For instance N435 has been used efficiently in narrow microchannels for polymerization of ε-caprolactone [58] as well as in wider tubular reactors for biodiesel production [197]. Tubular reactors offer at least several times higher surface to volume ratios compared to stirred tank reactors. The narrower the channels become the higher the surface to volume ratio is, leading to more efficient mass and heat transport in the system and higher reaction rates. Moreover, the operational stability and reusability of the biocatalyst can be improved by using it in packed-beds. Hence the problem with mechanical harm to the N435 biocatalyst and leaching of the enzyme from its carrier (**Paper I** and **III**) or formation of fine particles and separation problems (**Paper III**) caused by stirred reactions can be prevented by using packed-bed of the biocatalyst and molecular sieve.

In **Paper IV** a recirculating flow reactor with packed-bed(s) of the N435 biocatalyst and molecular sive (4Å) was designed for the reaction between TMP and DMC. Systems with different configurations of the biocatalyst and molecular sieve columns were tested for the reaction (Fig. 4.5). The system, consisting of the mixed column of the enzyme and molecular sieve (Fig. 4.5, setup C) was the most efficient setup by allowing rapid and efficient removal of the side-product methanol. For scaling up the process the mixed column can be replaced by a series of stacks of the biocatalyst and molecular sieve, which can be replaced once they lose the ability to perform their functions. The conversion rate was higher (81.6%) using the flow reactor, compared to a batch process (72%) using similar conditions during the same amount of time (12 h). Moreover the flow reactor facilitated the product recovery by avoiding extra separation steps required after the batch process. The molecular sieve and biocatalyst remained intact for subsequent regeneration and recycling.

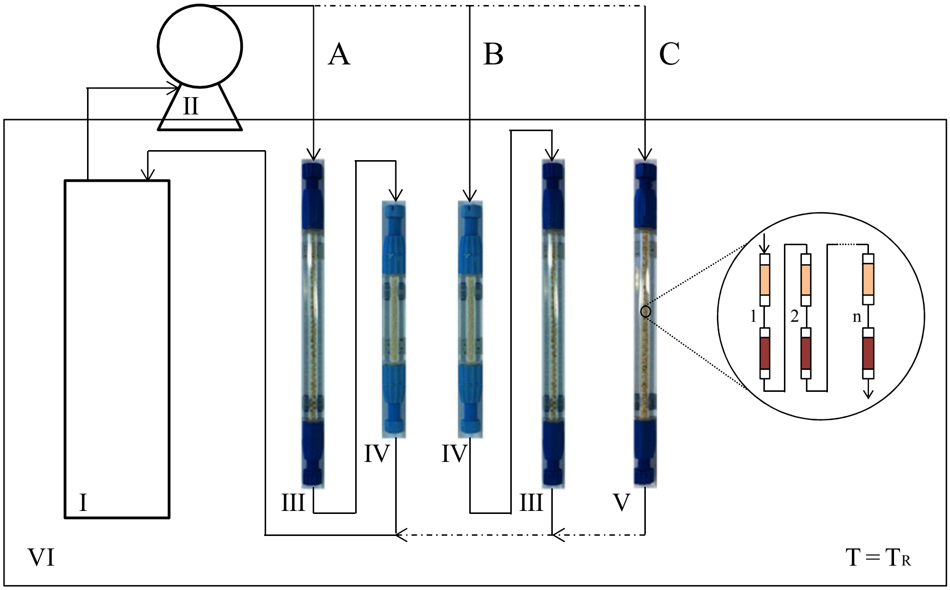
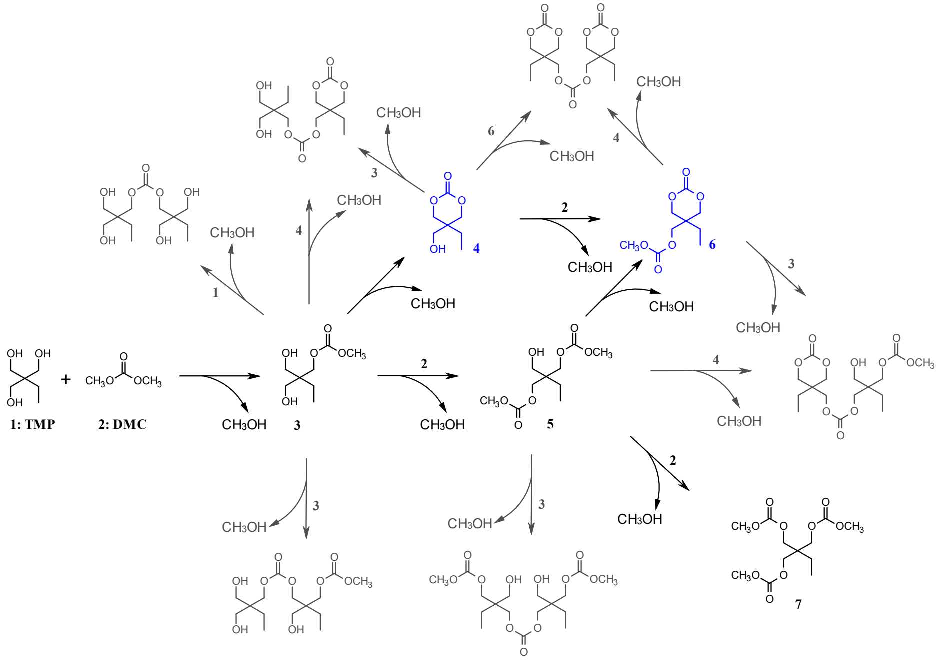


Figure 4.5 Flow reactor scheme in three different configurations A, B and C. In A and B, Novozym435 and molecular sieves are packed in separate columns with the difference that reactants flow through N435 first and then through molecular sieves column in A, and vice versa in B. In C N435 and molecular sives are mixed in a single column. Reservoir (I); pump (II); molecular sieve column (III); N435 column (IV); column with mixed N435 and molecular sieve (V); water bath at 60 °C (VI); the mixed column behaves similar to n columns of the biocatalyst and molecular sieve in series (magnified).

4.9 *In silico* evaluation of the lipase-catalyzed reaction

The reaction between DMC and TMP is clearly a multi-substrate multi-product system, potentially can result in a variety of products. Scheme 4.3 shows various possibilities for generating different monomer and dimer products. In practice the formation of different TMP carbonate dimers and oligomers during the reaction was observed to take place under certain conditions (**Paper III**, Table 4). This behavior of the reaction was seen to be influenced by the amount of the DMC/solvent, the reaction temperature and time, and the amount of the biocatalyst. It was observed that running the reaction using lower DMC/solvent amounts (< 20 times excess), in the absence of the biocatalyst for prolonged reaction times could lead to increased amounts of the oligomers in the product. While the effect of solvent and reaction temperature was investigated as discussed above, the effect of lipase B on the dimers and oligomers formation however, was unclear to us, considering that lipase B catalyzed polymerization of polyols and dialkycarbonates has been reported earlier [63,64]. Thus it was of interest to investigate if the lipase accepts even TMP carbonates as acyl-donor.



Scheme 4.3 Potential products of lipase-mediated transesterification of TMP and dimethylcarbonate (only monomers and dimers are shown)

In order to compare the DMC (compound 2 in Scheme 4.3) and *mono*-carbonated TMP, mC-TMP (compound 3), docking of these molecules to the enzyme active site was performed using PyRx virtual screening tool. Out of the 25 binding modes defined and simulated for each of the molecules, DMC showed higher binding affinity with the active site in all the modes, while mC-TMP showed binding to the active site with 50% of the modes (**Paper IV**, Table 2).

In silico simulation of the reaction allows evaluating the inter- and intra-molecular interactions using a molecular modeling approach. Molecular dynamics simulations were carried out using YASARA structure software. The simulations were performed under the reaction conditions (excess of the DMC and at 60 °C) used for the reaction and based on the serine hydrolase mechanism in order to compare the DMC (compound 2 in Scheme 4.3) and *mono*-carbonated TMP (mC-TMP) (compound 3) for the correct formation of the 1st tetrahedral intermediate (1st TH). The formation of the 1st TH in its correct form with respect to H-bonding between the acyl-donor and catalytic residues is an important step, determining whether the reaction takes place or not. Based on the results the DMC showed correct formation of H-bonds and the 1st TH, while the mC-TMP made incorrect interactions and H-bonding with catalytic and oxyanion residues and caused the His224 and Asp187 to shift from their original positions (**Paper IV**, Fig. 5).

The results from the *in silico* studies were verified using experimental investigations. In a reaction using mC-TMP in its purified form and without using DMC, catalyzed by N435 no product formation was observed. Hence, it was concluded that the formation of dimer and oligomer TMP carbonates as well as the cyclic products are spontaneous reactions promoted by other factors (such as high temperature) than the enzyme.

5. Conclusions and future perspectives

It is well known that biocatalysis can be used to make large number of products of interest to the chemical industry and can often provide a more energy efficient, environmentally benign alternative to chemical catalysis. Many conventional processes requiring harsh operational conditions, high energy and toxic reagents can be run under mild conditions without toxic materials and organic solvents using enzymes. In many cases, the use of enzymes has been shown to confer efficiency and simplicity features to chemical reactions as also shown in this thesis. However, the industrial application of biocatalysis is still limited primarily to fine chemicals and pharmaceuticals (with some exceptions) where advantage is taken of the high selectivity of enzymes and also the products demand a high price. This thesis has dealt with chemicals that can be classified as specialty chemicals for which the price range varies between 2 and 10 Euro per Kg, which implies that the process costs have to be kept low because of the difference between raw material and product costs [194]. The biocatalyst constitutes the major process cost and hence its use needs to be optimized in order to achieve high product yield per unit weight biocatalyst, which can be achieved by process optimization, recycling and improving the activity of the enzyme.

Optimization of new processes is inevitable and always requires considerable amounts of materials, manpower, time and money. Thus it is important to have tools, which minimize the amount of resources required. Developing kinetic models for multi-substrate multi-product reactions involving enzymes can be very complicated due to dealing with many substrates and products and hence kinetic parameters. However, reliable and practical kinetic models can considerably facilitate optimization and design of these processes and reduce the amount of resources required. The simplified approach to kinetic modeling of lipase-catalyzed reaction between TMP and OA shown in this thesis can be employed to facilitate developing kinetic models for similar types of reactions with multi components involved.

The enzymatic process for production of TMP cyclic carbonates presented in the thesis is a good example for using biocatalysts in chemoenzymatic processes to facilitate production of six-membered cyclic carbonates with functionalities that their production by chemical methods requires complicated processes and result in low yields. Moreover, the work illustrates that by taking organized and goal oriented approaches to the process optimization using statistical modeling the productivity and economy of the process can be significantly improved.

Reactor engineering is another important approach to improve the process efficiency and minimize the number of steps involved and hence improve the process economy. Using a flow reactor system for the reaction between TMP and DMC improved the reaction efficiency compared to the batch mode and facilitated the product recovery. Moreover the biocatalyst and molecular sieve were not damaged, which facilitated their reuse.

It is worth mentioning that the viability of using biocatalysts in industrial scale also depends on choosing a target market that is more suitable for selling higher value products. Medium-priced specialty chemicals of high quality produced by enzymatic processes can be converted to products with special applications. For instance, the synthetic esters like TMP-trioleate would also have applications in cosmetics, while aliphatic polycarbonates and polyurethanes and their co-polymers based on six-membered cyclic carbonates can be used in biomedical field due to their low toxicity and biocompatibility. Higher demand for more expensive products can also be due to their special features e.g. biodegradability and low ecotoxicity as in the case of biolubricants.

The field of biocatalysis is mature and it can be safely said that optimization of the biocatalyst as well as the bioprocess can provide competitive green routes for production of chemicals.

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