The potential of enzymatic catalysis in supercritical fluids

PROEFSCHRIFT

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Zwaantje Johanna Dijkstra

egen te Heerenveen
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Summary

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Chapter 1

Enzymatic Catalysis in Supercritical Fluids

Abstract
The aim of this thesis is to explore the potential of enzymatic catalysis in supercritical carbon dioxide. In this chapter, the main features and possibilities of enzymatic catalysis in supercritical fluids are described. The most relevant parameters including the effect of the applied immobilization method, the influence of mass transfer, and the effect of pressure and water activity on the catalytic activity are discussed. Furthermore, the outline of this thesis is given.
1.1 General introduction

The interest in industrial biocatalysis is growing due to a rapidly expanding range of possibilities. The research concerning enzymatic catalysis can be divided in two key directions: enzyme engineering and medium engineering. Enzyme engineering involves modifying existing enzymes to new functionalities, while medium engineering focuses on the environment of the reactions. These developments have opened a wide range of applications in the field of pharmaceuticals, (fine-) chemicals and intermediates. The number of biotransformation processes that have been started on an industrial scale is gradually increasing, and about 50% of these processes are applied in the pharmaceutical industry. Detailed reviews about the different processes and the applications of industrial biotransformations are available\textsuperscript{1-3}. Many of these biotransformations are performed in organic solvents. However, increased concern for the environment has lead to awareness for environmental friendly production methods. A general thought is that supercritical fluids can be used for the reduction of organic waste. An additional advantage of supercritical fluids is that separation after reaction is relatively simple which offers several process advantages. Also, supercritical carbon dioxide has the GRAS (generally regarded as safe) status, and can therefore be used in food and pharmaceutical processes without major regulatory issues. Although supercritical fluids have several attractive properties, pressurizing these kinds of systems is expensive. Therefore, biocatalytic processes in supercritical fluids should have an emphasis on the recycling of enzymes and continuous processing without the need of depressurizing the system.

1.2 Enzymes

1.2.1 General aspects

Enzymes are catalysts obtained from nature and are characterized by their specific activity. The activity is determined by a three-dimensional structure in which the catalytic center is located. Enzymes are divided in six classes, based on the type of reaction that is catalyzed (Table 1.1). Most enzymes used for catalytic oxidations
need a cofactor. This cofactor is a Cu or Fe complex, in which amino acids or polycyclic aromatic systems as flavine adenine dinucleotide, FAD, and nicotinamide adenine dinucleotide, NADH, serve as ligands.

Table 1.1 Major enzyme classes.

<table>
<thead>
<tr>
<th>Class</th>
<th>Function</th>
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<tr>
<td>Oxidoreductases</td>
<td>Catalyze oxidation-reduction reactions</td>
</tr>
<tr>
<td>Transferases</td>
<td>Catalyze group transfer reactions</td>
</tr>
<tr>
<td>Hydrolases</td>
<td>Catalyze hydrolytic reactions</td>
</tr>
<tr>
<td>Lyases</td>
<td>Catalyze reactions involving a double bond</td>
</tr>
<tr>
<td>Isomerases</td>
<td>Catalyze reactions involving isomerization</td>
</tr>
<tr>
<td>Ligases/Synthetases</td>
<td>Catalyze reactions involving joining two molecules coupled with the breakdown of a phosphate bond</td>
</tr>
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1.2.2 Candida antarctica lipase B

Lipases belong to the class of hydrolases. *Candida antarctica* lipase B (Calb) is one of the most widely used lipases because this enzyme accepts a broad spectrum of different substrates\(^4\). The crystalline structure of Calb has been resolved and shows that the enzyme has a Ser-His-Asp catalytic triad in its active site\(^5\). The structure appears to be in an 'open' conformation with a rather restricted entrance to the active site. This accounts for the substrate specificity and high degree of stereospecificity of this lipase. A schematic overview of the reaction mechanism of a transesterification reaction is given in Figure 1.1 showing a ping-pong bi-bi mechanism\(^6\text{-}^7\).
Calb has proven to be a versatile catalyst and is studied on the molecular level\textsuperscript{8-9} as well as on the level of the reaction environment\textsuperscript{10-11}.

### 1.2.3 Immobilisation methods

The main reason to use immobilized enzymes lies in the reduction in costs by enabling efficient separation, recycling, and reuse of the expensive enzymes. Other advantages of immobilized enzymes are improved enzyme performance in terms of activity, stability and selectivity. However, also dilution of the enzyme occurs since large amounts of inert material are used for attaching the enzymes, leading to a lower mass-bound productivity. Since the enzyme is attached to a carrier, the properties of the carrier (chemical and mechanical) are influencing the catalytic activity of the enzyme as well, making it even more important to find a proper immobilization method\textsuperscript{12-14}. The choice for the best immobilization method depends on the enzyme, the type of reaction and the reaction environment. Different immobilization methods can lead to different activities and stabilities under
otherwise identical circumstances. There are several ways to immobilize an enzyme, which can be roughly divided in two different groups: entrapment or binding to a carrier, see Figure 1.2.

**Figure 1.2 Different types of immobilization methods.**

Immobilization by entrapment involves the enzyme being retained in a membrane device such as a hollow fiber, polymeric network or microcapsule. In the most simple case, the ‘free’ enzyme is used. However, the immobilization is more effective when the enzyme is covalently attached to the polymer to prevent diffusion out of the carrier. Immobilization by adsorption has extensively been studied in the early stage of immobilization studies. Cross-linking the enzyme without a carrier is a technique that has received increased attention during the past years. The main advantage of this method is that the enzyme preparation consists almost entirely of enzyme. Figure 1.3 shows the preparation of four different cross-linked enzymes, with cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) as the most interesting ones. CLECs are prepared by crystallizing and cross-linking with glutaraldehyde\textsuperscript{15-16}. In this way, enzyme crystals are obtained with increased activity and stability towards organic solvents and supercritical fluids\textsuperscript{17-18}. Changing the crystallization conditions can optimize the size of the crystals. CLECs of different lipases are commercially available from Altus Biologics.
Recently, the formation of CLEAs has been explored. Instead of using enzyme crystals, enzyme aggregates are used for cross-linking. These aggregates are produced under non-denaturating conditions in order to retain the enzymatic activity. The CLEA of penicillin acylase shows comparable activities to CLECs\textsuperscript{19}. Advantages compared to the preparation of CLECs are that the preparation is much easier and more variations are possible since the enzyme conformation is not frozen, leading to more than one stable CLEA form of the enzyme\textsuperscript{20}.

### 1.2.4 Mass transfer effects

For immobilized enzymes different mass transfer effects are relevant. External mass transfer refers to molecular transport between the bulk reaction mixture and the surface of the enzyme particle through a boundary layer. The external mass transfer rate is inversely proportional to the thickness of the boundary layer around the particle (Figure 1.4). Increasing the agitation can decrease the thickness of this boundary layer. If this leads to an increase in the observed reaction rate, it is clear that external mass transfer limits the enzymatic reaction.
Internal mass transfer occurs within the pores of the enzyme and the support. The immobilization of an enzyme can affect the flexibility of the enzyme, which can also affect the accessibility of the substrates leading to a decreased internal mass transfer. Important parameters influencing internal mass transfer are the particle size, the pore size and the effective diffusion coefficient of the substrate inside the pores of the enzyme. The Thiele modulus is generally used to indicate if internal mass transfer limitation occurs\textsuperscript{21-23}:

$$\Phi = \frac{V_{\text{m}} \cdot R_p^2}{K_m \cdot D_{\text{eff}}}$$

The Thiele modulus depends on the kinetic parameters, the maximum velocity, $V_{\text{m}}$, the affinity constant, $K_m$, the radius, $R_p$, of the particle, and the effective diffusion coefficient, $D_{\text{eff}}$, of the substrate in the enzyme pores. The Thiele modulus has the following relation to the efficiency factor:

$$\eta = \frac{3}{\Phi} \cdot \left( \frac{1}{\tanh \Phi} - \frac{1}{\Phi} \right)$$
Figure 1.5 shows the relation between the Thiele modulus and the efficiency factor. The efficiency starts to decrease significantly at a Thiele modulus of 0.5. The two main ways to prevent internal mass transfer limitations are using smaller particles\textsuperscript{24} and decreasing the enzyme loading\textsuperscript{25}.

![Figure 1.5 Relation between the Thiele modulus and the efficiency factor.](image)

### 1.3 Supercritical fluids

A supercritical fluid (SCF) is defined as the state of a compound, mixture or element above the critical pressure ($P_c$) and critical temperature ($T_c$) but below the pressure required to condense it into a solid state. Above the critical point, molecular thermal energy exceeds the attractive forces between molecules and a gas-like state exists. Consequently, the properties of a supercritical fluid cover the gap between properties of gases and liquids. The diffusivity in a supercritical fluid is about a factor 100 higher as compared to a liquid, while the viscosity is up to a factor 10 lower (Table 1.2), resulting in an increased mass transfer in supercritical fluids as compared to liquids.
Table 1.2 General properties of gases, SCFs and liquids.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Gas</th>
<th>SCF</th>
<th>Liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (kg/m³)</td>
<td>1</td>
<td>100-800</td>
<td>1000</td>
</tr>
<tr>
<td>Viscosity (cP)</td>
<td>0.01</td>
<td>0.05-1</td>
<td>0.5-1</td>
</tr>
<tr>
<td>Diffusivity (m²/s)</td>
<td>1.10⁻⁵</td>
<td>1.10⁻⁷</td>
<td>1.10⁻⁹</td>
</tr>
</tbody>
</table>

For SCF the compressibility and heat capacity are higher near the critical point as compared to liquids and gases. A clear result of the high compressibility near the critical point is the strong change in density as a function of temperature and pressure (Figure 1.6). These changes in the density can effectively be used to control the solubility and separation of a solute.

![Figure 1.6 Dependence of the density of scCO₂ on the pressure for different temperatures.](image)

The four most extensively researched supercritical fluids are CO₂, ethane, ethene and water²⁶. Carbon dioxide is an attractive fluid since it has several advantages like non-toxicity, non-flammability and availability in high purity.

There is a wide interest in the use of scCO₂ because solubilities of solutes can be tuned by changing temperature and pressure. Initially, the main focus has been on supercritical extraction. The best-known example in this perspective is caffeine extraction from coffee²⁷. Other areas where (sc)CO₂ is being used are (bio-)
Chapter 1

chemical conversions, supercritical drying and particle formation. Supercritical water (SCW) has a high critical temperature and pressure compared to other supercritical fluids, but has been intensively investigated as a medium for oxidation of organic waste\textsuperscript{28-29}. Near the critical point SCW behaves like a moderately polar organic liquid, making it a good solvent for the oxidation of organic compounds resulting in the formation of simple and non-toxic compounds, like water and carbon dioxide. The critical temperature and pressure of some of the most common supercritical fluids are summarized in Table 1.3.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$T_c$ (°C)</th>
<th>$p_c$ (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO\textsubscript{2}</td>
<td>31.0</td>
<td>73.8</td>
</tr>
<tr>
<td>N\textsubscript{2}O</td>
<td>36.4</td>
<td>72.5</td>
</tr>
<tr>
<td>Ethane</td>
<td>32.3</td>
<td>48.8</td>
</tr>
<tr>
<td>Ethene</td>
<td>9.2</td>
<td>50.5</td>
</tr>
<tr>
<td>Propane</td>
<td>96.7</td>
<td>42.5</td>
</tr>
<tr>
<td>Propene</td>
<td>91.7</td>
<td>46.0</td>
</tr>
<tr>
<td>Ammonia</td>
<td>132.4</td>
<td>113.5</td>
</tr>
<tr>
<td>Methanol</td>
<td>239.5</td>
<td>81.0</td>
</tr>
<tr>
<td>Water</td>
<td>374.0</td>
<td>220.6</td>
</tr>
<tr>
<td>CHF\textsubscript{3}</td>
<td>26.2</td>
<td>48.5</td>
</tr>
<tr>
<td>CClF\textsubscript{3}</td>
<td>28.9</td>
<td>38.7</td>
</tr>
<tr>
<td>SF\textsubscript{6}</td>
<td>45.5</td>
<td>37.7</td>
</tr>
</tbody>
</table>

1.4 Enzymatic catalysis in supercritical fluids

The use of supercritical fluids as a non-aqueous solvent for enzyme-catalyzed reactions has been investigated since the 1980s\textsuperscript{30-32}. Several reviews on enzymatic reactions in supercritical fluids are available\textsuperscript{33-36}. A major class of enzymes are lipases, which are used for enantioselective (trans)-esterifications and esterifications of fatty acids. Some other enzymes used in scCO\textsubscript{2} are thermolysin\textsuperscript{37}, alkaline phosphatase\textsuperscript{32} and oxidases\textsuperscript{30,38}. More recently explored reactions are (ring-opening) polymerizations with lipases\textsuperscript{39-40}, the use of decarboxylases for CO\textsubscript{2}-fixation\textsuperscript{41} and the use of alcohol dehydrogenases\textsuperscript{42-43}. 
For enzymatic catalysis in supercritical fluids the stability and activity can be evaluated with different process parameters. The applied pressure and water activity are parameters which can have a great impact on the function of the biocatalytic activity and will therefore be discussed in more detail.

1.4.1 Pressure effects

Several studies have been performed to study the effect of the pressure on the activity and stability. For some enzymes, e.g. penicillin amidase, conformational changes are observed\textsuperscript{44-45}, while for other enzymes, e.g. alkaline phosphatise, no conformational changes are reported\textsuperscript{32}. Apart from the conformational changes, pressure may have two effects on enzymatic catalysis. First, the reaction rate can depend on the pressure according to the transition state theory and thermodynamics. For supercritical fluids the Eyring Transition-State Theory\textsuperscript{46} is used to explain the pressure effect on reaction rates:

\[
k = r \cdot \left( \frac{k_B \cdot T}{k} \right) \cdot K^*\]

The reaction rate constant, \(k\), is described as a function of a pressure and a temperature independent coefficient, \(r\), the Boltzmann constant, \(k_B\), the temperature, \(T\), the Planck constant, \(h\), and an equilibrium constant, \(K^*\), that is related to the difference in free energies between the transition-state and the reactants. Also a relation between the activation volume and the reaction rate constant has been described. In a review by Kamat\textsuperscript{34} a more detailed description of these relations is given, which can be used for predictions of pressure effects on the reaction rate in enzymatic systems\textsuperscript{47-48}.

The second effect is related to changes in the physical properties of supercritical \(\text{CO}_2\) by changing the pressure. At higher pressures, the density of the system increases, which will lead to a decrease in the mass transfer coefficient. Therefore, lower reactions rates are to be expected since diffusion limitation can occur. In only a limited number of studies an increasing reaction rate with increasing pressure has been reported\textsuperscript{49}. Other properties which have been related to changes in density and
pressure in organic solvents as well as in supercritical fluids are the partition coefficient, dielectric constant, dipole moment and \( \log P_{\text{oct}} \). Kasche has shown that chymotrypsin and trypsin undergo partial inactivation after slow pressurization, however, the amount of inactivated enzyme increased by repeating the pressurization-depressurization steps. Pressurization rate can be an important factor but at a speed of 5 bar/min most enzymes will remain active.

### 1.4.2 Structural effects

The three-dimensional structure of enzymes is very important because small changes in the surrounding of the active site can result in changes in activity. Both the type of enzyme and the source organism have influence on the activity and stability of the enzyme. For example, cholesterol oxidase from Gleocycystium has shown to retain its catalytic activity for three days, whereas the enzyme from Streptomyces losses its activity in about one hour.

Since enzymes consist of a chain of amino acids coupled by peptide bonds, many free amino-groups are available. These amine groups are known to form carbamates with CO\(_2\) (Figure 1.7). Kamat provided direct evidence of the formation of these structures with laser desorption mass spectroscopy (LS-MS). The carbamates are stable at low temperatures, however, at higher temperatures it is expected that CO\(_2\) is removed and that the activity of the enzyme is restored.

![Figure 1.7 Carbamate formation of a primary amine with carbon dioxide.](image)

### 1.4.3 Water activity

The water activity is an important parameter for the enzyme function in supercritical fluids. It is generally accepted that hydration is essential for enzymatic activity and that completely dry enzymes are inactive. Although there are different estimates of the degree of hydration required for activity, a threshold value of about 0.2 g H\(_2\)O g\(^{-1}\) enzyme is generally accepted. However, the amount of water necessary in
organic solvents can sometimes be surprisingly low\textsuperscript{58-60}. The presence of water influences the enzyme structure, and therefore enzymatic activity, through non-covalent bonding and disruption of hydrogen bonds. But it also has influence on the reaction kinetics and it can facilitate reagent diffusion. The amount of water required for optimal activity varies for different reactions. Zaks and Klibanov\textsuperscript{60} have demonstrated that it is the water bound to the enzyme that determines the catalytic activity rather than the total water content of the system. For controlling enzymatic reactions it is therefore suggested to maintain a constant water activity rather than a constant water concentration. This constant water activity can be obtained e.g. by adding molecular sieves or salt hydrates to the reaction medium\textsuperscript{61}.

1.5 Aim and outline of this thesis

The aim of this thesis is to explore the potential of enzymatic catalysis in supercritical carbon dioxide (scCO\textsubscript{2}). In particular, the activity of \textit{Candida antarctica} lipase B, Calb, in different immobilization forms is evaluated. Chapter 2 describes the catalytic activity of a cross-linked enzyme crystal (CLEC) in hexane and scCO\textsubscript{2} at different temperatures and pressures. A comparison is made between ChiroCLEC-CAB and the commercial Novozyme 435 for the transesterification reaction of (R/S)-phenyl ethanol with vinyl acetate. The focus of chapter 3 is on the long-term stability of the ChiroCLEC-CAB used in chapter two, and the importance of adjusting the water content in a continuous system. In chapter 4 a cross-linked enzyme aggregate, CLEA, is used to perform esterification reactions. The influence of water production in a continuous system is studied. Chapter 5 discusses the stability and presence of carbamates in supercritical CO\textsubscript{2}. Chapter 6 deals with the enzymatic production of polyurethanes in subcritical CO\textsubscript{2}. Finally, chapter 7 gives an outlook of the possibilities using different types of enzymes and optimizing the reaction in scCO\textsubscript{2}. All chapters are written in a way that they can be read independently.
Chapter 1

References

Chapter 1

Chapter 2

Cross-linked and Immobilized Lipase for Biocatalysis in Supercritical Carbon Dioxide

Abstract
The catalytic activity of two immobilized forms of *Candida antarctica* lipase B in supercritical carbon dioxide has been studied. In particular, the initial activity of ChiroCLEC™-CAB (cross-linked enzyme crystal of *Candida antarctica* lipase B) and of Novozyme 435 in supercritical carbon dioxide is compared with the activity obtained in hexane. The model reaction used is the enantioselective esterification of racemic 1-phenyl ethanol with vinyl acetate. In all cases, the enantioselectivity of both enzyme preparations towards (R)-1-phenyl ethanol exceeds 99%. As a function of the carbon dioxide pressure (8 - 20 MPa), the initial activity of the Novozyme 435 exhibits a maximum whereas the initial activity of the ChiroCLEC™-CAB decreases with an increase in pressure. For Novozyme 435 the initial activity obtained in supercritical carbon dioxide is always lower than the activity obtained in hexane. For the ChiroCLEC™-CAB the highest initial activity is observed for the reaction in supercritical carbon dioxide. The activity of the ChiroCLEC™-CAB is inversely proportional to the density in the supercritical region, making supercritical carbon dioxide with its low densities a highly attractive solvent for biocatalysis using ChiroCLEC™-CAB.

This chapter is based on: Z.J. Dijkstra, H. Weyten, J.T.F. Keurentjes, Cross-linked and Immobilized Lipase for Biocatalysis in Supercritical Carbon Dioxide, submitted to Enzyme and Microbial Technology
Chapter 2

2.1 Introduction

Enzymes are being used as highly selective biocatalysts in the (fine-)chemical and pharmaceutical industry. Traditionally, enzymatic catalysis is performed in aqueous buffer solutions requiring water-soluble substrates. The pioneering work of Zaks and Klibanov\(^1\), Hammond\(^2\), and Randolph\(^3\) has shown that enzymes remain active in both organic solvents and supercritical fluids. Several reviews on the performance of enzymes in supercritical fluids are available\(^4\)\(^-\)\(^6\). Supercritical fluids have properties that can be tuned by changing temperature and/or pressure. Moreover, supercritical carbon dioxide (scCO\(_2\)) is regarded as a ‘green’ solvent, which makes it a promising alternative for traditional solvents used in biocatalysis.

Lipases are a class of very stable enzymes that can convert a broad range of substrates, in particular in hydrolysis and esterification reactions. *Candida antarctica* lipase B (Calb) is one of the most active and well-known lipases. Since free lipases are not soluble in scCO\(_2\), good immobilization methods are essential. Immobilization is also important to prevent conformational changes that could occur due to chemical interaction of CO\(_2\) with the enzyme\(^7\) as well as during pressurization or depressurization\(^8\)\(^-\)\(^9\). Furthermore, improved enzyme performance in terms of activity, stability, and selectivity can be achieved by enzyme immobilization. Depending on the specific application, the preferred immobilization method can vary from immobilization on solid supports, encapsulation in aerogels, aggregation of enzymes, to immobilization on membranes\(^10\)\(^-\)\(^14\).

Novozyme 435 is an example of a widely used immobilized form of Calb in which the lipase is attached to acrylic beads\(^15\). Altus Biologics\(^16\) developed a method to form stable enzyme crystals, which are cross-linked with a bifunctional agent, like glutaraldehyde. This method produces ordered crystals that consist predominantly of the Calb enzyme, and this type of crystals are known as cross-linked enzyme crystals (CLECs). The CLECs of Subtilisin\(^17\), *Pseudomonas cepacia* lipase\(^18\), *Candida rugosa* lipase\(^19\), and *Candida antarctica* lipase B\(^20\) have been used successfully in organic solvents. The use of CLECs in supercritical fluids has been studied to a limited extent\(^21\).

An important parameter to control enzymatic reactions is the water activity. Halling\(^22\) proposed the use of salt hydrates to equilibrate non-aqueous solutions to
keep the amount of free water constant, but also molecular sieves have been used\textsuperscript{23}. In \textit{scCO}_2 the water concentration is commonly controlled off-line by drying the substrates before use or adding water to the system before the reaction is started\textsuperscript{24-25}. The main objective of this work is to study the catalytic activity of two immobilized forms of \textit{Candida antarctica} lipase B in \textit{scCO}_2. A comparison is made between ChiroCLEC\textsuperscript{TM}-CAB (cross-linked enzyme crystal of \textit{Candida antarctica} lipase B) and Novozyme 435. The model reaction used is the enantioselective esterification of (R,S)-1-phenyl ethanol with vinyl acetate, using hexane and \textit{scCO}_2 as the solvent. Both forms of the Calb enzyme are approximately 100\% enantioselective. The two immobilized forms of the Calb enzyme are compared in terms of the initial reaction rate, which has been obtained as a function of temperature and \textit{CO}_2 pressure.

![SEM image of ChiroCLEC\textsuperscript{TM}-CAB.](image)

\textbf{2.2 Experimental}

Novozyme 435 was purchased from Sigma Aldrich, and was used as received. Novozyme 435 contains about 10 wt\% of the Calb enzyme\textsuperscript{36}. The crystalline form of the Calb enzyme, ChiroCLEC\textsuperscript{TM}-CAB, was purchased from Altus Biologics, and consists for about 90 wt\% of the Calb enzyme. ChiroCLEC\textsuperscript{TM}-CAB, which was delivered as a buffered suspension, was washed at least three times with tert-amyl alcohol and was subsequently washed three times with hexane before use. In Figure 2.1 a SEM image of the ChiroCLEC\textsuperscript{TM}-CAB is presented. The ChiroCLEC\textsuperscript{TM}-CAB is an ordered octagon with a diameter and thickness of approximately 20 \(\mu\)m and 3
µm, respectively. A summary of the conditions of the different experiments is given in Table 2.1.

### Table 2.1 Overview of solvent properties and experimental conditions.

<table>
<thead>
<tr>
<th></th>
<th>Hexane</th>
<th>scCO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical temperature</td>
<td>K</td>
<td>not applicable</td>
</tr>
<tr>
<td>Critical pressure</td>
<td>MPa</td>
<td>not applicable</td>
</tr>
<tr>
<td>Density</td>
<td>g mL⁻¹</td>
<td>0.64 ⁸</td>
</tr>
<tr>
<td>(R/S)- PhEtOH</td>
<td>mM</td>
<td>100</td>
</tr>
<tr>
<td>Vinyl acetate</td>
<td>mM</td>
<td>200</td>
</tr>
<tr>
<td>Amount ChiroCLEC™-CAB</td>
<td>g L⁻¹</td>
<td>0.05 - 1.0</td>
</tr>
<tr>
<td>Concentration ChiroCLEC™-CAB</td>
<td>µM</td>
<td>1.5 - 30</td>
</tr>
<tr>
<td>Amount Novozym 435</td>
<td>g L⁻¹</td>
<td>0.05 - 1.0</td>
</tr>
<tr>
<td>Concentration Novozym 435</td>
<td>µM</td>
<td>0.14 - 2.7</td>
</tr>
</tbody>
</table>

⁸: At 0.01 MPa and 313 K.
⁹: At 10 MPa and 313 K.

#### 2.2.1 Reaction in hexane

Reactions in hexane were carried out in stirred 10 mL glass vials. In a typical experiment, 800 µmol (R,S)-1-phenyl ethanol and 0.1 mg mL⁻¹ enzyme were added to 8 mL hexane. The temperature is controlled using a heated water bath. After stirring for about 30 minutes, 1600 µmol vinyl acetate was added. During the reaction, samples of 100 µL were taken from the mixture at specific times. The samples were diluted with hexane and analyzed by gas chromatography (GC) on a β-dex or RTX-5 column with as carrier gas H₂. The oven temperature program used a starting temperature of 343 K and a final temperature of 423 K with a heating rate of 10 K min⁻¹.

#### 2.2.2 Reaction in supercritical carbon dioxide

The reactions in supercritical carbon dioxide were performed in a 100 mL stainless steel autoclave. The setup of the batch reactor is shown in Figure 2.2.
The reactor was heated electrically between 313 and 353 K, and was stirred with a magnetically coupled stirrer at 500 rpm. All experiments were carried out according to the following procedure. In a typical experiment, the autoclave was filled with 10 mg of enzyme and 10 mmol (R,S)-1-phenyl ethanol. After the desired temperature was reached, the pressure was slowly increased until 2 MPa below the final reaction pressure in the range of 8 to 20 MPa. The reactant inlet was then filled with vinyl acetate (20 mmol). The carbon dioxide used to reach the final pressure was also used to add the vinyl acetate to the autoclave. This moment was taken as the starting point of the reaction. During the reaction, samples were taken with a 6-way HPLC valve with a 100 µL sample loop. The pressure in the sample loop was released by bubbling the solution through hexane. The sample loop was subsequently rinsed with hexane and the samples were analyzed by GC.

2.3 Results and discussion

For both immobilized forms of the Calb enzyme the reaction is performed batch-wise, using hexane or scCO₂ as the solvent. First, the results obtained for the conversion in hexane are compared with the results for the conversion obtained in scCO₂ at a pressure of 8.5 MPa. Then results for the initial activity obtained in the
two different solvents will be presented, and the effect of the solvent on the activity will be discussed.

![Figure 2.3 Conversion of (R)-1-phenyl ethanol as a function of time.](image)

**Figure 2.3** Conversion of (R)-1-phenyl ethanol as a function of time.

a: ChiroCLEC\textsuperscript{TM}-CAB: (\(\triangle\)) in hexane and (\(\blacktriangle\)) in scCO\(_2\) (3 \(\mu\)M, 8.5 MPa, 313 K).

b: Novozyme 435: (\(\square\)) in hexane and (■) in scCO\(_2\) (2.7 \(\mu\)M, 8.5 MPa, 313 K).

In Figure 2.3, a typical result for the conversion of (R)-1-phenyl ethanol (R-PhEtOH) is given as a function of time, at a temperature of 313 K. In Figure 2.3 a, the results obtained in hexane and scCO\(_2\) are given for the ChiroCLEC\textsuperscript{TM}-CAB enabling a direct comparison between the two solvents which shows an increase in
activity when scCO$_2$ is used as a solvent. In Figure 2.3 b, the results for the conversion obtained with Novozyme 435 are given. For Novozyme 435 the conversion in hexane appears to be higher than the conversion in scCO$_2$. The reaction kinetics of Calb enzyme follows ping-pong bi-bi kinetics$^{26}$, however, in Figure 2.3 an exponential is fitted through the data points for the conversion. This fit is used to calculate the initial reaction rate, $V_{\text{initial}}$, which is given by the slope of the conversion curve at t=0.

### 2.3.1 Activity in hexane

The reaction with both forms of the immobilized Calb enzyme has been performed in hexane at four different enzyme concentrations, see Figure 2.4. Comparing the initial activity for different enzyme concentrations provides insight into the efficiency of the Calb enzyme. It is known that the Calb enzyme follows Michaelis-Menten kinetics, and it is, therefore, expected that the catalytic activity is independent of the enzyme concentration. For both the Novozyme 435 and the ChiroCLEC$^{\text{TM}}$-CAB, the activity is indeed constant for the four enzyme concentrations used. From Figure 2.4 it follows that, in hexane, the initial activity for the Novozyme is equal to about 6.5 (mol mol$^{-1}$ s$^{-1}$), and that the initial activity of the ChiroCLEC$^{\text{TM}}$-CAB is about 1.6 (mol mol$^{-1}$ s$^{-1}$).

It should be noted, however, that the scale of x-axis in Figure 2.4 a and 2.4 b differ by about a factor of ten. This is a result of the difference in the Calb concentration in the two immobilized forms. The Calb concentration in the ChiroCLEC$^{\text{TM}}$-CAB is about nine times higher than the Calb concentration in the Novozyme 435, see Table 2.1 and the Experimental section.
Figure 2.4 Catalytic activity as a function of the Calb concentration in hexane. 
a: ChiroCLEC™-CAB. b: Novozyme 435.

2.3.2 Activity in supercritical carbon dioxide

The esterification reaction has been performed in scCO$_2$ as a function of temperature and pressure. In Figure 2.5 the results for the initial reaction rate obtained with the ChiroCLEC™-CAB and with the Novozyme 435 are given as function of temperature, for a CO$_2$ pressure of 8.5 MPa and 10.5 MPa, respectively. For the ChiroCLEC™-CAB and the Novozyme 435 a similar trend is seen, in both cases the initial activity decreases linearly with an increase in temperature. This indicates that for this particular reaction and reaction environment the optimum temperature for
the Calb enzyme will be at a temperature below 313 K. However, to ensure supercritical conditions the temperatures used are always above the critical temperature of CO$_2$, i.e. 304 K (Table 2.1).

![Graph showing catalytic activity of ChiroCLEC$^{TM}$-CAB and Novozyme 435 in scCO$_2$ as a function of temperature.](image)

**Figure 2.5** Catalytic activity of (▲) ChiroCLEC$^{TM}$-CAB (3 µM, 8.5 MPa) and (■) Novozyme 435 (0.27µM, 10.5 MPa) in scCO$_2$ as a function of the temperature.

For the ChiroCLEC$^{TM}$-CAB the initial activity increases from about 1.6 (mol mol$^{-1}$ s$^{-1}$), in hexane, up to 6 (mol mol$^{-1}$ s$^{-1}$) in scCO$_2$. The higher activity is most likely a result of a better mass transfer in the ChiroCLEC$^{TM}$-CAB as the two substrates will diffuse faster in scCO$_2$ when compared to hexane.

The initial activity of the Novozyme 435 in hexane of about 6.5 (mol mol$^{-1}$ s$^{-1}$) is about three times higher than the activity observed in scCO$_2$. To study the effect of the static pressure on Novozyme 435, the activity of the Novozyme 435 has been obtained in hexane pressurized with argon (up to 20 MPa). No significant difference in the activity has been obtained without or with the pressurized argon, indicating that the static pressure is not the reason for the decrease in activity. The lower activity observed in scCO$_2$ for the Novozyme 435 may be the result of the swelling of the beads or can be due to changes in the conformational structure of the immobilized Calb enzyme. As a result of the swelling of the beads, breakage of the bonds between the enzyme and the bead might occur. Also, the Calb enzymes are attached to the surface of the acrylic beads in such a way that the primary amines are
exposed to CO₂. It is known that CO₂ forms carbamates with primary amines⁸, which could lead to reversible inactivation of the Calb enzyme in the presence of CO₂.

### 2.3.3 Influence of CO₂ pressure

To exploit the possibilities of supercritical CO₂, the initial activity has been measured as a function of the pressure between 8 and 20 MPa. For the reaction in scCO₂, the same experimental conditions have been applied for ChiroCLEC™-CAB and Novozyme 435. However, there is a considerable difference in the dependence of the initial activity on the pressure observed for the two forms, see Figure 2.6.

The activity of the ChiroCLEC™-CAB decreases as a function of pressure. For pressures up to about 12 MPa the activity in scCO₂ is higher than the activity in hexane, whereas for higher pressures the activity in scCO₂ becomes lower than the activity in hexane. Since cross-linking stabilizes the Calb enzyme and makes it more rigid, it is unlikely that significant conformational changes will occur. The activity obtained for the Novozyme 435 has an optimum value at about 15 MPa. Apparently, the maximum in the activity is the result of different effects occurring in the CO₂-rich environment. A similar behavior for the initial catalytic activity as function of the pressure has been reported by Overmeyer et al⁷. 

![Figure 2.6 Catalytic activity of (▲) ChiroCLEC™-CAB and (■) Novozyme 435 in scCO₂ as a function of the pressure, at 313 K.](image-url)
There are two probable reasons for this difference in activity, which both can be related to the different immobilization methods used. Novozyme 435 is prepared by immobilization of *Candida antarctica* lipase B on acrylic beads. In the presence of CO₂ swelling of the beads may occur, which will result in a more open structure with higher mass transfer rates in the beads. This can be the reason for the initial increase in activity when the pressure is increased. When the pressure is further increased the activity decreases similar to the effect observed for the ChiroCLEC™-CAB. As a result, a maximum in the activity of the Novozyme 435 is observed as a function of the CO₂ pressure. Alternatively, the observed effects can be a result of a difference in water activity at higher pressures. It can be anticipated that for the two systems the amount of water surrounding the Calb enzyme is different. However, results in literature indicate that for esterification reactions with Novozyme 435 in supercritical carbon dioxide and in solventless media the initial rate is almost independent of the water activity. Furthermore, in supercritical CO₂ (at 13 MPa and 313 K) the ratio of the water concentration to the water activity is almost constant. This would imply that the effect of the water concentration on the activity of the Calb enzyme is limited.

### 2.3.4 Density dependence

The decreasing initial activity with CO₂ pressure observed for the ChiroCLEC™-CAB is most likely a result of a decrease in mass transfer of the substrates. Mass transfer rates in scCO₂ decrease linearly with an increase in density although at higher compressibility, i.e. lower densities, there is a deviation from this linear behavior. To verify that a decrease in mass transfer rate is the reason for the decrease in activity, the initial activity is plotted as a function of CO₂ density, see Figure 2.7.
Chapter 2

Figure 2.7 Catalytic activity of (▲) ChiroCLEC™-CAB in scCO\textsubscript{2} and (△) ChiroCLEC™-CAB in hexane as a function of the density.

The initial activity shows a linear decrease with the density. The fact that for the ChiroCLEC™-CAB the initial activity depends on the density of the solvent is confirmed by the results obtained in hexane. In Figure 2.7 the activity of the ChiroCLEC™-CAB obtained in hexane is also given. The initial activity obtained in hexane coincides with the initial activity obtained in scCO\textsubscript{2}. These results for the initial activity indicate that working close to the critical CO\textsubscript{2} pressure is particularly advantageous for the ChiroCLEC™-CAB, because these low densities cannot be reached with traditional organic solvents.

2.4 Conclusions

Results are presented for an enzymatic esterification reaction in supercritical carbon dioxide using immobilized Calb. A different behavior is observed for the initial activity as a function of pressure, obtained with ChiroCLEC™-CAB and Novozyme 435. The initial activity of the ChiroCLEC™-CAB shows a decreasing behavior and the activity of the Novozyme 435 displays a maximum with an increase in pressure. Depending on the actual pressure, a difference in the order of a factor of two to three is obtained between the two immobilized forms. The highest initial activity obtained for the ChiroCLEC™-CAB is just above the pressure where CO\textsubscript{2} becomes supercritical and is comparable with the initial activity obtained for the Novozyme
435 in hexane. These results indicate that the use of CLECs in supercritical media offers an interesting combination for biocatalysis, which is in particular advantageous at low densities as a result of increased mass transfer of the solutes.

References
Chapter 3

The Effect of Water Concentration on the Activity and Stability of CLECs in Supercritical CO\textsubscript{2} in Continuous Operation

Abstract
In this study the effect of the water concentration on a crystallized enzyme of \textit{Candida antarctica} lipase B (ChiroCLEC\textsuperscript{TM}-CAB) in supercritical carbon dioxide (scCO\textsubscript{2}) is studied. The model reaction used is the enantioselective esterification of racemic 1-phenyl ethanol with vinyl acetate; the reaction is performed in scCO\textsubscript{2} at 40 °C and 90 bar in batch and in continuous operation. Kinetic parameters have been derived from continuous experiments, leading to a catalytic turnover number of 0.95 s\textsuperscript{-1}. The optimum activity is reached at low water concentrations (0.05 g L\textsuperscript{-1}). At lower concentrations, CO\textsubscript{2} is stripping water from the enzyme leading to deactivation. However, adding a small amount of water to the substrates can reverse this deactivation and the enzyme activity is restored.

3.1 Introduction

Lipases are a class of very stable enzymes that can convert a broad range of substrates, mainly in hydrolysis and esterification reactions. *Candida antarctica* lipase B (Calb) is one of the most active and well-known lipases used in various preparations. Conventionally, enzymatic catalysis is performed in aqueous buffer systems at pH~7 and room temperature. In these systems, the enzyme is dissolved and most suitable to catalyze reactions involving hydrophilic components. About 20 years ago, however, Zaks and Klibanov performed pioneering work concerning enzymatic catalysis in organic solvents. Moreover, it has been discovered that enzymes can maintain their activity in supercritical carbon dioxide (scCO₂) as well. Changing the solvent from aqueous to organic has the advantage that more apolar substrates can be used, although immobilization of the enzyme to prevent denaturation is necessary.

For the use of these enzymes in supercritical fluids, several types of reactions have been performed. This has mainly been done in a batch mode, and only a limited number of studies have been performed (semi-) continuously, mainly in tubular and packed bed reactors. In these studies mainly parameters like temperature and pressure have been varied. Nevertheless, it can be anticipated that also the water activity in the system is one of the most important parameters for controlling the reaction rate. One of the most widely used methods to control the water activity in a solvent is the addition of salt hydrates. A disadvantage of this method is that salt hydrates can also interfere with the catalytic center of the enzyme and can therefore alter the catalytic activity not only by fixing the water activity. Solid-state buffers can also be used to improve the activity, however, the effects of these buffers in scCO₂ are varying. The most common technique for controlling the water activity in organic solvents involves the use of salt hydrates, whereas in scCO₂, this is usually done by controlling the ingoing water concentration.

Whereas most of the work described in the literature on the use of lipases in scCO₂ employs carrier bound enzyme, in this study cross-linked enzyme crystals of *Candida antarctica* lipase B, ChiroCLEC™-CAB, have been used to catalyze reactions in scCO₂. The model reaction used is the enantioselective esterification of (R,S)-1-phenyl ethanol with vinyl acetate. The catalytic performance of the CLEC is
evaluated both in batch and in continuous experiments with a focus on the effect of water concentration.

3.2 Experimental

3.2.1 Batch experiments

Batch reactions in supercritical carbon dioxide were performed in a 100 mL stainless steel autoclave. The reactor was heated electrically (40 °C) and stirred with a magnetically coupled stirrer at 500 rpm. All batch experiments were carried out according to the following procedure. The reactor was filled with 10 mg of enzyme (ChiroCLEC™-CAB from Altus Biologics) and 10 mmol (R,S)-1-phenyl ethanol (Acros) and was heated until the desired temperature was reached. Because the enzyme can be sensitive to rapid changes in pressure, the reactor was slowly pressurized (at approximately 5 bar min⁻¹) by adding CO₂ (Hoekloos) until approximately 20 bar below the final reaction pressure. In the meantime, the reactant inlet was filled with 20 mmol vinyl acetate (Acros). The CO₂ required to reach the final reaction pressure (90 bar) was used to add the vinyl acetate to the reactor; this moment was taken as the starting point of the reaction. During the reaction, samples were taken with a 6-way HPLC valve with a 100-µL sample loop. The pressure in this sample loop was released by bubbling the solution through hexane. The sample loop was subsequently rinsed with hexane and the samples were analyzed with gas chromatography (GC) on an rtx-5 column with H₂ as the carrier gas.

3.2.2 Continuous experiments

The reactor setup that was used for the continuous experiments is shown in Figure 3.1. The enzyme was added to a stirred stainless steel autoclave (200 mL) connected to a separator to recover the products. The reactor was slowly pressurized at approximately 5 bar min⁻¹ and during the whole process the reactor was kept at 90 bar and 40 °C while stirring at about 500 rpm. In this setup the ChiroCLEC™-CAB catalyst was kept in the reactor using a suitable filter in the outlet of the reactor. Changing the total flow through the reactor varied the residence time in the reactor. Different water concentrations were obtained by adding salt hydrates, molecular
sieves or water to the substrates before adding the substrates to the reaction vessel. The water content was measured by Karl Fischer titration. After the separator, samples were taken and were analyzed using GC.

![Figure 3.1 Schematic high-pressure setup for the enzymatic reaction in supercritical CO\textsubscript{2} (with an optional CO\textsubscript{2}-recycle).](image)

In the continuous setup, three different amounts of enzyme were added to the reactor leading to final enzyme concentrations of 0.5, 1.0 and 5.0 mg mL\textsuperscript{-1}, respectively. The volumetric flow rate of the substrates was set to 10 vol\% of the total ingoing flow as this flow is adjusted with the pumps. This is in accordance with concentrations of 87 mM (R)-1-phenyl ethanol and 350 mM vinyl acetate in the reactor at 40 °C and 90 bar. The water concentration of the ingoing substrates (as measured with Karl Fischer) and the liquid CO\textsubscript{2} (60 ppm) lead to the calculated water concentration in the reactor.

3.3 Results and discussion

3.3.1 Comparison of batch and continuous experiments

The reaction appears to be completely enantioselective, therefore only the conversion of (R)-1-phenyl ethanol has been shown as a function of the substrate residence time in the continuous setup (Figure 3.2).
Figure 3.2 Conversion of (R)-1-phenyl ethanol with vinyl acetate as a function of time. P = 90 bar, T = 40°C. CLEC concentrations: (■) 0.5 mg mL\(^{-1}\) (▲): 1.0 mg mL\(^{-1}\) (●): 5.0 mg mL\(^{-1}\).

Figure 3.3 Catalytic activity as a function of the CLEC concentration.

From this figure initial reaction rates can be derived, leading to values of about 1.8 mmol min\(^{-1}\) mg\(^{-1}\) (Figure 3.3). However, when these values are compared to the activity of the enzyme in batch, it appears that the activity in batch conditions is approximately a factor of 3 higher. As these values are expected to be the same, two improvements of the experimental procedure in the continuous setup have been explored.
To avoid accumulation of the enzyme in the O-ring closing the reactor, a catalyst basket is introduced to contain the enzyme. The catalyst basket prevents losing the enzyme in the dead volumes of the reactor and is situated next to the stirrer. For organic solvents it has been reported that a substantial activity loss can occur in the first hour of operation when using CLECs\textsuperscript{29-30}. Although the enzyme used in these studies is subtilisin Carlsberg and not Candida antarctica lipase B it is likely that the deactivation is caused by the immobilization methods and not by the enzyme as such. Therefore, the 200 mL reactor has been extended with a sample loop making it possible to follow the conversion in time. A series of three experiments has been performed. First, a batch experiment is carried out in which the conversion is measured in time. Then, after full conversion is reached, the batch setup is changed into a continuous setup by continuously pumping CO\textsubscript{2} and substrates through the reactor at a fixed residence time. When steady state is reached, the situation is switched to batch again with the residence time of the continuous reaction as the starting point.

![Conversion of (R)-1-phenyl ethanol with vinyl acetate as a function of time with the use of a catalyst basket (●): first batch experiment (■): continuous experiment, residence time is 23 min (◊): second batch experiment.](image)

As can be seen from Figure 3.4, the activity observed in the first batch reaction is initially lower than observed in the second one. Probably, transport of substrates into
the catalyst basket is limiting. In this figure it is also shown that the activity at continuous conditions is not lower as compared to batch; the enzyme maintains its initial activity or becomes even slightly more active. Apparently, the inactivation within the first hour as observed in organic solvents does not occur in scCO₂.

3.3.2 Kinetics

For this reaction, kinetics have been evaluated using a residence time of 13.2 minutes and an enzyme concentration of 0.5 mg mL⁻¹ at a fixed vinyl acetate concentration of 350 mM and (R)-1-phenyl ethanol concentrations of 10.4, 21.2, 57.6 and 87.2 mM (Figure 3.5). From these data the initial velocities can be derived. Subsequently, the kinetic parameters are estimated assuming Michaelis-Menten kinetics.

It is known that Candida antarctica lipase B shows ping-pong bi-bi kinetics, however, if one substrate concentration is kept constant it can be treated with pseudo-one-substrate kinetics, so that the equation for the kinetics is simplified to

\[ v_{\text{ini}} = \frac{V_m \cdot [\text{Phenylethanol}]}{K_m + [\text{Phenylethanol}]} \]

Non-linear regression in Origin gives an apparent maximum velocity \( V_{\text{max}} \) of 0.013 (mM s⁻¹) and an apparent affinity constant \( K_m \) of 35.8 mM.

Figure 3.5 Michaelis-Menten plot with fixed vinyl acetate concentration. P = 90 bar, T = 40°C. The obtained parameters are \( V_m = 0.013 \) mM s⁻¹ and \( K_m = 35.8 \) mM.
The $K_m$ is used to calculate whether external diffusion limitation occurs. The efficiency $\eta$ is defined as

$$\eta = \frac{C_{si} \cdot (K_m + C_s)}{C_s \cdot (K_m + C_{si})}$$

in which $C_s$ is the concentration of (R)-1-phenyl ethanol in the bulk phase, $K_m$ is the affinity constant and $C_{si}$ is the (R)-1-phenyl ethanol concentration at the enzyme surface. With the method described by van ‘t Riet and Tramper\textsuperscript{32}, $C_{si}$ is obtained, yielding an efficiency of 0.999, indicating that no external diffusion limitation occurs. As it has been shown that diffusion in an enzyme crystal of lysozyme is slow\textsuperscript{33}, it is possible that internal diffusion limitation occurs. A general measure for the occurrence of internal diffusion limitation is the Thiele modulus:

$$\Phi = \frac{V_m \cdot R_p^2}{K_m \cdot D_{eff}}$$

in which $R_p$ is the radius of the enzyme crystal and $D_{eff}$ is the effective diffusion coefficient of the substrate in the pores. The enzyme crystal has a diameter of 20 $\mu$m, and for the effective diffusion coefficient an estimation of $10^{-8}$ m$^2$ s$^{-1}$ is made\textsuperscript{34}, yielding a Thiele modulus of $2 \times 10^3$, indicating the absence of internal diffusion limitation. Moreover, the catalytic turnover number $k_{cat}$ as estimated from the fitted $V_{max}$ is around 0.95 s$^{-1}$, calculated with a molecular weight of 33 kDa of \textit{Candida antarctica} lipase B and the assumption of 90 wt% of enzyme content in the crystal.

### 3.3.3 Water concentration

Since the water activity is one of the most important parameters to optimize the performance of the enzyme, it is useful to look at this effect. Although it would be more appropriate to consider the water activity instead of the water concentration, this quantity is practically less accessible, especially in (complex) supercritical mixtures. Therefore, the water concentration of the ingoing flow is adjusted with salt.
hydrates or molecular sieves. The effect of water concentrations between 0.05 and 2 g L\(^{-1}\) has been measured. The solid line in Figure 3.6 gives the conversions at different water concentrations obtained for a fixed residence time of 13 minutes. Clearly the lowest water concentration (0.05 g L\(^{-1}\)) gives the highest activity, a value that gradually decreases with increasing water concentration. Reducing the amount of water present in the reactor even further by placing a fixed bed of silica in the CO\(_2\) tubing shows a drastic decrease in activity (dashed line) indicating that at extremely dry conditions the enzyme is not able to keep the needed water molecules bound. The amount of water that is present in the reactor will distribute between the enzyme surface and the CO\(_2\), resulting in net water stripping from the enzyme. This effect also occurs in polar organic solvents\(^{35-36}\), where the establishment of the new water equilibrium appears to occur fast.

![Graph](image.jpg)

**Figure 3.6** Conversion of R-phenyl ethanol with vinyl acetate as function of the calculated water concentration in the reactor. CLEC concentration is 5 mg/mL, calculated residence time is 13 min. (♦): CO\(_2\) with 60 ppm water is used (□): CO\(_2\) is dried over a silica column before use.

### 3.3.4 Influence of CO\(_2\) flow

Since the water concentration in the reactor is dependent on the continuous in and outflow of CO\(_2\) and reactants, this allows for a relatively easy control. As shown in the previous paragraph, an optimum in water concentration can be found. However,
by decreasing the amount of water even more, the essential water molecules are pulled away from the enzyme causing a lower activity. The gradual effect of stripping the water by CO$_2$ can also be illustrated by varying the CO$_2$ flow.

Experiments have been performed in which a continuous reaction has been run for two hours (cycle 1). After this cycle, the reactor is flushed for 60 minutes with a high flow of CO$_2$ (20 mL min$^{-1}$). After this, the conversion is measured after two hours at the same fixed residence time again (cycle 2). This step is repeated once more (cycle 3).

![Conversion of R-phenyl ethanol with vinyl acetate after treatment with CO$_2$.](image)

Figure 3.7 Conversion of R-phenyl ethanol with vinyl acetate after treatment with CO$_2$. After each cycle the reactor is flushed for 60 min with pure CO$_2$ at 20 mL min$^{-1}$. The residence time is fixed at 25 min.

As it can be seen in Figure 3.7, the decrease in activity is tremendous: from 96 % conversion in cycle 1 to only 50 % conversion in cycle 3. Interestingly, this effect appears to be rather reversible (Figure 3.8). Since the division of water molecules between the enzyme and CO$_2$ is a rather rapid process, constant activities can be reached by continuously pumping CO$_2$ and substrates with constant water concentrations through the reactor. In this way optimal conditions can be found. In aqueous solutions the hydration state of the enzyme has been measured with FTIR, from which it has been concluded that lipase can bind up to 660 water molecules, a value corresponding to three or four hydration monolayers$^{37}$. In CO$_2$, however, the
release of the CO$_2$ pressure to atmospheric pressure will pull the water away from the enzyme, therefore, the hydration level of the enzyme in optimal supercritical CO$_2$ conditions cannot be measured.

### 3.3.5 Stability

As shown above, the activity of *Candida antarctica* lipase B in the crystallized form is proven to be high. Even though the activity of the enzyme decreases when rinsed with CO$_2$, this decrease can be reversed by the addition of water as shown in Figure 3.8. The same batch of enzyme has been used for more than two months. As dry CO$_2$ has been used in this experiment, the activity decreased within 9 days to about one third of the initial value. Subsequently, 0.5 wt% water has been added to the substrates and the activity of the enzyme increased again to about 80% of the initial activity, a procedure that could be repeated several times without any additional loss of activity. This underlines the importance of controlling the water concentration in scCO$_2$, especially in continuous operation.

![Conversion of R-phenyl ethanol with vinyl acetate as a function of number of days on stream for continuous measurements. (♦): no addition of water (★): after addition of water.](image)

**Figure 3.8** Conversion of R-phenyl ethanol with vinyl acetate as a function of number of days on stream for continuous measurements. (♦): no addition of water (★): after addition of water.

### 3.4. Conclusions

Cross-linked enzyme crystals of *Candida antarctica* lipase B appear to be very stable enzyme preparations, showing a high activity in supercritical CO$_2$ with a
turnover number of 0.95 s\(^{-1}\). It is shown that CO\(_2\) strips the necessary water from the enzyme leading to a decreased activity. However, this effect can be reversed by the addition of water to the system leading to an immediate increase in activity again.

References
Chapter 4

Stability and Activity of Enzyme Aggregates of Calb in Supercritical Carbon Dioxide

Abstract

The activity and stability of a cross-linked enzyme aggregate of Candida antarctica lipase B (CLEA-Calb) in supercritical carbon dioxide (scCO$_2$) has been studied. The model reaction used is the esterification of isoamyl alcohol with acetic acid. The catalytic performance of CLEA-Calb is evaluated both in batch and in continuous experiments, with a focus on the effect of water production upon reaction. The results of the batch experiments show a decreasing initial activity of the CLEA-Calb with an increase in pressure. Moreover, CLEAs appear to be highly stable in supercritical carbon dioxide and also remain active during continuous reactions. Suggestions for improved process designs are given for which the optimal design depends on the specific reaction requirements.

This chapter is based on: Z.J. Dijkstra, R. Merchant, J.T.F. Keurentjes, Stability and activity of enzyme aggregates in supercritical carbon dioxide, submitted to Journal of Supercritical Fluids.
4.1. Introduction

Esters of short chain acids and alcohols are known as flavor and fragrance compounds used in food, beverage, cosmetic and pharmaceutical industries. Traditionally, the production of these compounds has been achieved by chemical synthesis. Since it is known that enzymes maintain their activity in organic solvents\(^1\), several flavor esters have also been produced by free and immobilized lipases in organic solvents\(^2-5\). However, the use of organic solvents for food and health products is restricted and alternatives have to be found. Supercritical carbon dioxide, scCO\(_2\), has the GRAS (generally regarded as safe) status and can therefore be used as an alternative for organic solvents. Furthermore, scCO\(_2\) has properties that can be tuned by changing temperature and pressure allowing easy separation after the reaction. The most widely used type of enzymes in scCO\(_2\) are lipases\(^6-7\).

Lipases are rather stable enzymes that can convert a broad range of substrates, in particular in hydrolysis and esterification reactions. An important parameter to control enzymatic reactions is the water activity. Halling\(^8\) proposed the use of salt hydrates to equilibrate non-aqueous solutions to keep the amount of free water constant, but also molecular sieves can be used effectively\(^9\). A disadvantage is that salt hydrates can interfere with the catalytic center of the enzyme, and can therefore alter the catalytic activity not only by the water activity\(^9-10\). In scCO\(_2\) the water concentration is commonly controlled off-line by drying the substrates before use or adding water to the system before the reaction is started\(^11-12\).

Since free lipases are not soluble in scCO\(_2\), good immobilization methods are essential. Immobilization is also important to prevent conformational changes that could occur due to chemical interaction of CO\(_2\) with the enzyme\(^13\) as well as during pressurization or depressurization\(^14-15\). Furthermore, improved enzyme performance in terms of activity, stability, and selectivity can be achieved by enzyme immobilization. Depending on the specific application, the preferred immobilization method can vary from immobilization on solid supports, encapsulation in aerogels, aggregation of enzymes, to immobilization on membranes\(^16-20\).

Cross-linking the enzyme without a carrier is an immobilization technique that has gained considerable attention\(^21-23\). The main advantage of this method is that the
enzyme preparation consists almost entirely of enzyme. Altus Biologics has
developed a method to form stable enzyme crystals, which are cross-linked with a
bifunctional agent, like glutaraldehyde. This method produces ordered crystals that
consist predominantly of the Calb enzyme, and this type of crystals are known as
CLECs. The formation of cross-linked enzyme aggregates, CLEAs, has more
recently been explored. Instead of using enzyme crystals, enzyme aggregates are
used for cross-linking. The CLEA of penicillin acylase shows activities comparable
with the CLECs\textsuperscript{17}. The main advantage as compared to the preparation of CLECs is
that the preparation of CLEAs is easier\textsuperscript{24}.
Whereas most of the work described in the literature on the use of lipases in scCO\textsubscript{2}
employs carrier bound enzymes\textsuperscript{6-7}, in this study cross-linked enzyme aggregates of
Candida antarctica lipase B, CLEA-Calb, have been used to catalyze reactions in
scCO\textsubscript{2}. The model reaction used is the esterification of amyl alcohol with acetic
acid. The catalytic performance of the CLEA-Calb is evaluated both in batch and in
continuous experiments with a focus on the effect of water production. The
challenge in this research is to maintain a low water concentration in a continuous
system since water is produced upon reaction. The obtained results are used as a
starting point for possible optimization steps in the process design.

4.2. Experimental

4.2.1 General

The cross-linked enzyme aggregate, CLEA-Calb, was obtained from CLEA
Technologies (Delft, The Netherlands). The CLEA is delivered as a powder. Prior to
the experiments the CLEA-Calb was weighed on an electronic balance and washed
three times with tert-butanol and hexane, respectively.

4.2.2 Batch experiments

Batch reactions in supercritical carbon dioxide were performed in a 100 mL stainless
steel autoclave (Figure 4.1a). The reactor was heated electrically (313 K) and stirred
with a magnetically coupled stirrer at 500 rpm. In a typical experiment, the catalyst
basket was filled with 100 mg of CLEA-Calb and 4.5 mmol isoamyl alcohol
(Sigma-Aldrich) was added to the autoclave. Then the autoclave was heated until the desired temperature was reached. The Calb enzyme can be sensitive to rapid changes in pressure, and therefore the reactor was slowly pressurized (at approximately 0.5 MPa min \(^{-1}\)) by adding CO\(_2\) (Hoekloos) until approximately 2 MPa below the final reaction pressure. In the meantime, the reactant loop was filled with 4.5 mmol acetic acid (Sigma-Aldrich). The CO\(_2\) required to reach the final reaction pressure (11, 14 or 18 MPa) was used to add the acetic acid to the reactor; this moment was taken as the starting point of the reaction. During the reaction, samples were taken with a 6-way HPLC valve with a 500-µL sample loop. The pressure in this sample loop was released by bubbling the solution through hexane. The sample loop was subsequently rinsed with hexane and the samples were analyzed with gas chromatography (GC) on an rtx-5 column with helium as the carrier gas.

![Experimental set-up](image)

**Figure 4.1** Experimental set-up. a) batch b) continuous.
4.2.3 Continuous experiments

For the continuous reaction, the batch setup is extended with a separator and a pressure controller at the outlet of the autoclave. At the inlet, the reactant loop is removed and a high pressure liquid pump is used to add the reactants (Figure 4.1b). In a typical experiment, 500 mg CLEA-Calb is added to the catalyst basket while the reaction is performed at 11 MPa and 313 K. By changing the flow rate of the CO$_2$ and the liquid reactants, the residence time in the reactor was varied. Samples were taken with a 6-way HPLC valve and analyzed with gas chromatography similar to the batch experiments.

4.3. Results and Discussion

4.3.1 Batch experiments

In the esterification of isoamyl alcohol with acetic acid, water is formed resulting in a continuously changing microenvironment of the enzyme. The influence of fixing the water activity with salt hydrates is measured and compared with an experiment in which no salt hydrates are added. NaAc (3/0) and Na$_2$PO$_4$ (7/2) are used to fix the water activity at 0.28 and 0.73, respectively.

![Graph](image_url)

Figure 4.2 Conversion of isoamyl alcohol with acetic acid as a function of time. P = 11 MPa, T = 313 K. (■): NaAc (3/0) (▲): Na$_2$HPO$_4$ (7/2) (♦): no salt hydrate added.
Chapter 4

From Figure 4.2 it follows that there is no significant difference between these three experiments. This indicates that there is no need for the addition of salt hydrates to improve the performance of the enzyme.

The initial water concentration is varied to see whether a more hydrated state of the enzyme has a positive effect on the initial reaction rate of the reaction. Four different water concentrations have been measured at two different pressures (Figure 4.3). The optimal water concentration is obtained around 0.2 mg mL$^{-1}$ for both pressures. This indicates that the maximum solubility of water in CO$_2$, which is different for both pressures, has no influence on the performance and also not on the hydration state of the enzyme. When no extra water is added to the system, the initial activities at 11 MPa and 15 MPa are 0.41 mol mol$^{-1}$ s$^{-1}$ and 0.23 mol mol$^{-1}$ s$^{-1}$, respectively. However, when 0.45 mg mL$^{-1}$ water is added, the initial activities at 11 MPa and 15 MPa are 0.36 mol mol$^{-1}$ s$^{-1}$ and 0.28 mol mol$^{-1}$ s$^{-1}$, respectively. The difference in activity between the two pressures is reduced upon the addition of water indicating that the enzymatic activity as a function of the water concentration levels off to a constant value which will be the same for different pressures.

![Figure 4.3](image)

**Figure 4.3** Catalytic activity of CLEA-Calb as a function of initial water concentration. (■): at 11 MPa, (▲): at 15 MPa.

Furthermore, the influence of the pressure on the behavior of CLEA-Calb is evaluated. Figure 4.4 a shows the production of isoamyl acetate as a function of time
for the three different pressures. These experiments are performed without addition of salt hydrates to control the water activity, as it follows from Figure 4.2 that the addition of salt hydrates has no influence on the reaction rate. For a proper evaluation, the initial reaction rates are derived from the initial slope of these curves and expressed as a function of pressure in Figure 4.4 b. A decrease in initial activities is obtained when the pressure is increased. At a pressure above 14 MPa almost no further decrease in activity has been observed.

Figure 4.4 Catalytic activity of CLEA-Calb at different pressures, at 313 K. a) Conversion of isoamyl alcohol as a function of time. (▲): at 11 MPa (■): at 15 MPa (♦): at 18 MPa. b) Catalytic activity of CLEA-Calb as a function of pressure.
To study the stability of the CLEA-Calb in scCO$_2$, all batch experiments with an enzyme concentration of 1 mg mL$^{-1}$ have been performed with the same enzyme. After each reaction, the pressure is slowly released and the enzyme has been rinsed with hexane and left overnight in a NaAc (3/0)/hexane solution. To check whether decreases in activity are not caused by a deactivated enzyme, a typical experiment performed at 11 MPa and 313 K has been repeated after several pressurization and depressurization steps (Figure 4.5).

![Figure 4.5 Stability of CLEA-Calb. (■): initial activity (●): after one pressurization / depressurization step (×): after several pressurization / depressurization steps.](image)

No significant difference between the results for the conversion is observed. It can, therefore, be concluded that the stability of this CLEA-Calb is very high, making this a versatile immobilization method.

### 4.3.2 Continuous experiments

The results of the batch experiments where water is added to the reaction medium indicate that accumulation of water during continuous reactions might become an issue. Since CO$_2$ is continuously pumped through the reactor it should be possible to use CO$_2$ as an extraction solvent. Previous research$^{25}$ has shown that a continuous high flow of pure CO$_2$ extracts the water around the enzyme resulting in a decrease
in catalytic activity. It appeared, however, that the deactivation can be reversed by the addition of water to the substrates.

A high flowrate, however, results in a short residence time and a low conversion. Lower flowrates will result in higher conversions and, therefore, in a higher water production. Due to the low solubility of water in CO$_2$, it might be possible that the water produced upon reaction at high conversion cannot be extracted completely by the CO$_2$ flow. As a preliminary estimate, the maximum solubility of water in CO$_2$ at different pressures and constant temperature (313 K) is calculated and expressed as the maximum amount of water that can be extracted as a function of the CO$_2$ flowrate (Figure 4.6). The amount of water produced at the conditions used in the batch reaction (11 MPa) is shown as well.

![Figure 4.6 Maximum water extraction capacity at different pressures. (●): actual water production, derived from conversion plot at 11 MPa.](image)

With a residence time of about 148 minutes, corresponding to a CO$_2$ flowrate of 0.5 mL min$^{-1}$, a conversion of about 70 % is reached. Figure 4.6 shows that at this flowrate the amount of water produced can still be extracted with the CO$_2$ flow. These calculations are based on pure CO$_2$-water systems, and in the case that other more polar components are present it is expected that the solubility of water in CO$_2$ will be slightly higher. However, when conversions higher than 70 % are obtained,
i.e. at \( CO_2 \) flowrates below 0.5 mL min\(^{-1}\), water will accumulate and the conversion will drop.

Figure 4.7 shows the continuous production of isoamyl acetate at fixed residence times as a function of the running time. To show the viability of this process, these experiments have been performed overnight to check whether the conversion remains constant. For residence times of 74 and 148 minutes, corresponding to \( CO_2 \) flowrates of 1 mL min\(^{-1}\) and 0.5 mL min\(^{-1}\), respectively, the conversion remains constant. However, at a residence time of 37 minutes, corresponding to a \( CO_2 \) flowrate of 2 mL min\(^{-1}\), a slight decrease in conversion is observed. At this higher \( CO_2 \) flowrate, the continuous outflow of \( CO_2 \) and products causes freezing of the pressure relief valve used for controlling the pressure. Normally the pressure is controlled within 0.2 MPa but in these experiments the pressure varied between 10 and 12 MPa. This variation in pressure can have a negative effect on the total conversion but it is also possible that the higher \( CO_2 \) flow is extracting too much water from the reaction medium.

![Figure 4.7 Stability of conversion overnight.](image)

**Figure 4.7 Stability of conversion overnight.** (■): \( t_{\text{res}} = 148 \) min (♦): \( t_{\text{res}} = 74 \) min (▲): \( t_{\text{res}} = 37 \) min.

**4.3.3 Process optimization**

At higher residence times of approximately 300 minutes, the calculations indicate that water will accumulate (Figure 4.6). This has to be resolved because it affects the
enzyme activity. Several ways to remove the water produced during the esterification reaction to obtain a higher productivity are available. However, completely integrated solutions as hetero-azeotropic distillation\textsuperscript{26} and reactive distillation\textsuperscript{27-28} are not possible in high pressure systems. Three possible options for process optimization in supercritical systems will be discussed. Also some general suggestions for improvement will be given.

First, the set-up can be extended with a water adsorbing column (Figure 4.8 a). The dried reactants and products can be lead to a second reactor to obtain a high conversion. Another way is to add an internal or external recycling loop through a water adsorbing column (Figure 4.8 b). Preferably, the system consists of two parallel columns in order to maintain a continuous system when one of the two columns is saturated.

![Figure 4.8 Process design a) Continuous setup including a water adsorbing column and a second reactor. b) Continuous setup including an external loop with a water adsorbing column.](image-url)
Second, a different solution will be the use of a membrane reactor where the enzyme is immobilized on the surface of the membrane\textsuperscript{29-30}. Several configurations are possible\textsuperscript{31}, and one of the options could be a water-selective membrane allowing high conversions and high productivities when the water selectivity is high enough. Third, a packed-bed reactor with CLEAs can be a feasible option as well. A few examples of using a packed bed for enzymes in supercritical CO\textsubscript{2} have been reported. Hampson\textsuperscript{32} showed that when dry CO\textsubscript{2} is lead through a fixed bed, water is stripped from the enzyme with a rate close to the maximum solubility. On average the water removal at 333 K and 27.5 MPa is 0.27 wt\%, while the expected removal related to the maximum solubility is 0.30 wt\%. This means that it is possible to remove the required amount of water by flowing CO\textsubscript{2} through the packed bed. The optimal conditions can be found by varying the CO\textsubscript{2} flow in combination with the amount of enzyme in the packed bed or the length of the column. Dumont\textsuperscript{33} has found a comparable behavior in organic solvent and scCO\textsubscript{2} using a packed bed for the enzymatic synthesis of ethyl myristate and therefore concludes that the main advantage of using supercritical CO\textsubscript{2} is the easier separation of products, which has been confirmed in other studies\textsuperscript{34}. On the other hand, Goddard\textsuperscript{35} reported increased catalytic activity in supercritical CO\textsubscript{2} as compared to organic solvents. Another improvement that can be made is avoiding the use of a catalyst basket. Under the applied conditions the catalyst basket prevents the loss of enzyme in the dead volume of the reactor, however, it also decreases the stirring efficiency and therefore the initial reaction velocity decreases with a factor 6 to 7. The activity of the CLEA-Calb is extrapolated to the ideal situation in which no catalyst basket is used and a high activity is reached (dashed line in Figure 4.9). The equilibrium conversion is 84 \% at 313 K but with a water removal step conversions close to 100 \% should be possible. The use of the catalyst basket can be avoided by using larger CLEA particles.
Another important issue is the choice of reactants. In this paper, model reactants are chosen for simplicity. However, acetic acid is not the reactant giving the highest activities since it is inhibiting to the enzyme\textsuperscript{36}. The use of other substrates can give even better results concerning reaction rates.

### 4.4. Conclusions

Results are presented for an enzymatic esterification reaction in supercritical carbon dioxide using CLEA-Calb. The initial activity of the CLEA-Calb shows a decreasing behavior with an increase in pressure which is comparable with the behavior of the ChiroCLEC\textsuperscript{TM}-CAB. Moreover, these CLEAs appear to be highly stable in supercritical carbon dioxide and maintain their activity also during continuous reactions. The performance has been tested in a continuously stirred tank reactor with a catalyst basket. Since this is not the most optimal configuration, suggestions for improved process designs have been given. The final choice of the process design depends on the reaction kinetics and type of substrates used.
References

Chapter 4
Chapter 5

Formation of Carbamic Acid in Organic Solvents and in Supercritical Carbon Dioxide

Abstract
The behavior of amines in various CO$_2$-rich solvents is described. $^1$H and $^{13}$C NMR spectra in DMSO, chloroform and benzene show that the amines are completely converted into carbamic acid by just bubbling atmospheric CO$_2$ through the solution. However, the chemical shifts of several protons and the carbonyl $^{13}$C in the newly formed compounds strongly depend on the organic solvent used. FT-IR measurements confirm the observation that carbamic acid formation occurs. Changing the solvent to supercritical CO$_2$ shows a temperature- and pressure-dependent equilibrium between the amine and the carbamic acid; higher pressures shift the equilibrium towards the carbamic acid, whereas higher temperatures favor the free amine.

5.1. Introduction

Current research is providing insight in the advantages and possibilities of using supercritical carbon dioxide (scCO$_2$) in (bio) chemical processes. The main advantages of scCO$_2$ include increased catalytic activities as a result of improved mass transfer, higher selectivities and strongly reduced organic waste streams$^{1-2}$. Not only the phase behavior but also the chemical properties of scCO$_2$ can be advantageously used. In this perspective, it is generally known that a primary or secondary amine is able to form a carbamate or carbamic acid with CO$_2$ (Figure 5.1).

![Figure 5.1 Schematic view of the spontaneous reaction between an amine and CO$_2$.](image)

Mechanistic studies of amine-CO$_2$ reactions are mostly performed in aqueous systems; in organic solvents this kind of information is scarcely available. However, a few amine-CO$_2$ reactions in organic solvents have been investigated with spectroscopic techniques including 1D and 2D NMR and IR$^{3-4}$. Essentially, this chemistry is an acid-base equilibrium in which one molecule of an amine and one molecule of CO$_2$ form a carbamic acid, whereas two amine molecules with one molecule of CO$_2$ form a carbamic salt$^{5-7}$. The formation of carbamic acid and/or ammonium carbamate is reversible as CO$_2$ can be released by heating or purging with a gas, which can be an attractive property for several purposes.

The binding between CO$_2$ and amines can be used in several areas. Polymer-bound amines are used as reusable CO$_2$-scrubbers to remove CO$_2$ from gaseous streams. Aqueous amine-CO$_2$ chemistry is important for the understanding of the interactions between enzymes, proteins, CO$_2$ and CO$_3^{2-}$ in body fluids, for which several groups have developed modeling, measuring and analytical methods$^{6, 8-11}$. Part of these studies includes the calculation of equilibrium constants in aqueous media.
Moreover, the formation of carbamic acid can also be advantageously used in several catalytic processes, e.g. for the replacement of highly toxic phosgene in alternative routes to produce isocyanates\textsuperscript{12} and urethanes\textsuperscript{13-15}. Other work shows facilitated formation of alkyl carbamates in \textit{CO}_2-rich environment\textsuperscript{16-17} and it has been reported that \textit{CO}_2 can serve as a temporary protecting group in ring closing metathesis\textsuperscript{18} as well as in rhodium-catalyzed hydroamino methylations\textsuperscript{19}, leading to reduced side-product formation and increased yields. From the foregoing it will be obvious that the effect of \textit{CO}_2 in reactions involving amines can be used advantageously, however, the basics in non-aqueous solvents are still rather unclear\textsuperscript{19}, this in contrast with the aqueous environment.

Several studies report the use of high pressure NMR for on-line investigation of the phenomena occurring in \textit{scCO}_2\textsuperscript{20-22}. Some of these studies include amines\textsuperscript{23-25} and show the presence of carbamic acid in \textit{CO}_2 at elevated pressures. However, the equilibrium of amine/carbamic acid at different temperatures and pressures has not been studied before. This work describes the formation of carbamic acids at low \textit{CO}_2 pressure in pure amines, in organic solvents as well as in \textit{scCO}_2 at various temperatures and pressures. The analysis techniques used are TGA-DSC, FT-IR, and low and high-pressure NMR.

5.2. Experimental

5.2.1 General

All used reagents and solvents were obtained from Sigma Aldrich and were used as received. \textit{\textsuperscript{1}H} and \textit{\textsuperscript{13}C} NMR spectra were recorded at room temperature on a 300 MHz Varian NMR spectrometer. Chemical shifts were measured relative to residual non-deuterated solvent resonances. The used deuterated solvents are DMSO-d6, chloroform and benzene. Spectra of amine in deuterated solvent with and without \textit{CO}_2-bubbling were measured. \textit{\textsuperscript{1}H} NMR spectra of isopropyl amine were measured in all solvents mentioned. \textit{\textsuperscript{1}H} and \textit{\textsuperscript{13}C} NMR spectra of hexyl amine, N-ethyl-N-butyl amine and aniline were only measured in DMSO-d6. The concentration used in these experiments was 0.1 M. FT-IR spectra were measured with a Remspec probe and analysed with Omnic software obtained from ThermoNicolet. IR spectra of
isopropyl amine (0.1 M) diluted in methanol and dioxane with and without CO\(_2\) bubbling were taken.

Carbamic acid formation was also measured with a Netzsch TGA-DSC apparatus coupled with a Pfeiffer mass spectrometer. Solutions of pure isopropyl amine, hexyl amine, N-ethyl-N-butyl amine and phenylethyl amine were prepared by CO\(_2\) bubbling. These samples were heated in the TGA-DSC equipment and CO\(_2\) release was measured with a mass spectrometer.

![Schematic picture of a high pressure NMR tube](image)

**Figure 5.2** Schematic picture of a high pressure NMR-tube A: titanium valve assembly, B: titanium pressure sensor, C: needle valve, D: sapphire tube, E: copper wire.

### 5.2.2 High pressure NMR

A specially designed high-pressure tube (Figure 5.2) was used to measure NMR high-pressure NMR spectra in scCO\(_2\) [22]. This tube is suitable for use in normal NMR equipment; the high pressure NMR spectra were obtained on Varian INOVA 300 and 500 MHz spectrometers at the University of Amsterdam. The cell consisted of a 10 mm outer diameter and an 8 mm inner diameter sapphire tube. A titanium high-pressure sensor enables the continuous measurement of the pressure in the cell during the NMR experiment. The pressure working range of this cell is 0-200 bar and it can be used between –60 and +150 °C. \(^1\)H NMR and \(^{13}\)C NMR chemical shifts
in CO₂ were obtained in the presence of chloroform (5 µl) as reference, ¹⁴N NMR chemical shifts were referenced to nitromethane. Every experiment was performed with an aliquot of 5 µl of the relevant amine (usually isopropyl amine).

5.3. Results and Discussion

5.3.1 Atmospheric pressure

Primary and secondary amines immediately react with CO₂ in a highly exothermic reaction. Mainly depending on the size of the amine, the carbamic acid is present in liquid form or forms a white waxy solid. The formation of the carbamic acid is also dependent on the basicity of the amine. Aniline, which is an aryl amine, is not able to react spontaneously with CO₂ to form the carbamic acid.

![Figure 5.3](image)

**Figure 5.3** ¹H NMR spectra of isopropyl amine before (a) and after (b) CO2-bubbling.

The presence of carbamic acid has been measured by ¹H and ¹³C NMR analysis of isopropyl amine solutions in DMSO. After bubbling of CO₂ into a 0.1 M solution of isopropyl amine in DMSO, δ (N-H) of the primary amine at 1.4 ppm completely disappears while δ (C-H) of the amine at 3.0 ppm shifts towards 3.5 ppm, which is indicative for δ (C-H) of the carbamic acid. The newly formed δ (O-H) appears at 6.2 ppm and δ (N-H) at 3.7 ppm (Figure 5.3).
Figure 5.4 $^{13}$C NMR spectra of isopropyl amine before (a) and after (b) CO$_2$-bubbling.

The formation of carbamic acid has been confirmed by recording the $^{13}$C NMR spectrum, which shows the formation of a new C=O functionality at $\delta$ (C=O) = 158 ppm (Figure 5.4). Hexyl amine and 1-phenylethyl amine were similarly evaluated and also show similar shifts in their $^1$H NMR spectra, indicating the transformation of the amine into the respective carbamic acid. These compounds also exhibited new $\delta$ (C=O) peaks in their $^{13}$C NMR spectra.

Since no water is present in the system, it is assumed that only carbamic acid is formed and not the ammonium carbamate. When both carbamic acid and carbamic salt would have been present, the spectra would have shown more peaks, since protonated acids will give different shifts. Moreover, no precipitation has been observed, what is also a strong indication for the absence of the carbamate salt. For the conditions applied here, it can be concluded that full conversion to carbamic acid is reached merely by bubbling atmospheric CO$_2$ through an amine solution in DMSO. However, DMSO is an organic solvent with properties that are substantially different from those of CO$_2$. For example, DMSO has a dielectric constant of 47, whereas this value for CO$_2$ is between 2 and 4, depending on the temperature and pressure. Also the polarity, measured as $E_T(30)$ and log P are quite different (Table 5.1).
Table 5.1 Solvent properties.

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</tbody>
</table>

Therefore, the ¹H NMR spectra of isopropyl amine diluted in chloroform and benzene, solvents which are more comparable to CO₂, are obtained. However, after bubbling CO₂ into this chloroform solution, it is not clear whether the chemical shift δ (N-H) of the primary amine at 1.2 ppm has completely disappeared or that it is covered by the major CH₃ peak at 1.1 ppm. It is interesting to see the appearance of the newly formed δ (O-H) at 9.2 ppm and δ (N-H) at 6.6 ppm. In DMSO, these signals appear at 6.2 and 3.7 ppm, respectively. The chemical shifts in benzene appear at yet different positions, at 7.9 and 3.9 for δ (O-H) and δ (N-H), respectively (Table 5.2).

Table 5.2 Chemical shifts of isopropyl amine in benzene, chloroform and dimethylsulfoxide.
The left number is before CO₂-bubbling, the right number is after CO₂-bubbling.

<table>
<thead>
<tr>
<th></th>
<th>δ (C-H)</th>
<th>δ (NH₂)</th>
<th>δ (N-H)</th>
<th>δ (O-H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene</td>
<td>2.8</td>
<td>3.0</td>
<td>0.6</td>
<td>3.9</td>
</tr>
<tr>
<td>chloroform</td>
<td>3.1</td>
<td>3.2</td>
<td>1.2</td>
<td>3.7</td>
</tr>
<tr>
<td>dimethylsulfoxide</td>
<td>3.0</td>
<td>3.5</td>
<td>1.4</td>
<td>6.6</td>
</tr>
</tbody>
</table>

The results given in Table 5.2 show indeed a very large solvent effect in the shift of these functionalities. The properties given in Table 5.1 like density, dielectric constant, acidity and basicity constant, E_T(30), log P and the solubility of CO₂ in the solvent do not give a clear trend to predict the placement of the shift. Another possibility is that not only the carbamic acid is formed but also the ammonium carbamate depending on the solvent as described by Masuda⁴. In this paper, it is
shown that in DMSO the carbamic acid is preferably formed, while in benzene and chloroform the ammonium carbamate is preferably formed. It is anticipated that the main reason for this effect is the influence of the solvent on the acid-base equilibrium.

FT-IR measurements have been performed with isopropyl amine diluted in two different organic solvents (dioxane and methanol). Figure 5.5 shows the resulting spectra before and after CO$_2$-bubbling and the two new peaks are obtained at 1650 cm$^{-1}$ and 1305 cm$^{-1}$. However, these peaks are not the expected carbamic acid or the ammonium carbamate peaks, but peaks from the carbonate which is formed in methanol by bubbling with CO$_2$.

![Figure 5.5 Isopropylamine in methanol before and after CO$_2$ bubbling. Appearing peaks after bubbling at 2300 cm$^{-1}$ (CO$_2$), 1650 cm$^{-1}$ (COOH), and 1305 cm$^{-1}$ (C-O).](image)

In Figure 5.6, the results of dioxane are shown. Here, it is shown that the carbon dioxide is partially dissolved in dioxane at 2300 cm$^{-1}$. Also new peaks appear at 1575 cm$^{-1}$ indicating the formation of the carbamate anion and at 1650 cm$^{-1}$ indicating the formation of the carbamic acid. In dioxane, both carbamic acid and ammonium carbamate are present but in DMSO, also dipolar and aprotic, mainly the carbamic acid is present. In dipolar and aprotic solvents it is expected that carbamic acid is predominantly formed, since the formation of salts is unfavorable. However, according to the dielectric constant, dioxane is relatively non-polar (Table 5.1). This
in combination with the higher CO₂-solubility in dioxane could lead to a significant amount of ammonium carbamate present in the solution.

![Figure 5.6 Isopropylamine in dioxane before and after CO₂ bubbling. Appearing peaks after bubbling at 2300 cm⁻¹ (CO₂), 1575 cm⁻¹ (COO⁻), and 1650 cm⁻¹ (COOH).](image)

TGA-DSC measurements combined with mass spectroscopy have provided data on the stability of the carbamic acid. For the amines tested, however, the mass is already decreasing from the beginning of the measurement (at 22°C) when the solution is only purged with argon. This indicates that the bonding between CO₂ and amine is not very strong. Still, with increasing temperature there is an explicit CO₂ release shown, at different temperatures for the different amines (Table 5.3).

<table>
<thead>
<tr>
<th>TGA-DSC</th>
<th>Mₘ</th>
<th>Tᵇ</th>
<th>Tᶠ</th>
<th>MS44</th>
<th>TGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropylamine</td>
<td>59</td>
<td>33</td>
<td>-37</td>
<td>76</td>
<td>80</td>
</tr>
<tr>
<td>Hexylamine</td>
<td>101</td>
<td>130</td>
<td>27</td>
<td>78</td>
<td>102</td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>121</td>
<td>188</td>
<td>69</td>
<td>79/102</td>
<td>130</td>
</tr>
<tr>
<td>Ethyl-N-butylamine</td>
<td>101</td>
<td>108</td>
<td>18</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 5.3 Overview of data from TGA-DSC measurements. Mₘ: molecular weight, Tᵇ: boiling temperature, Tᶠ: flash temperature, MS44: the temperature at which CO₂ is removed from the substance, TGA: the temperature at which all substance has disappeared.
A higher molecular weight of the amine requires a higher temperature for removing the CO$_2$. The aromatic amine, and to a lesser extent also the secondary amine shows a two-step CO$_2$ release indicating that possibly more than one single CO$_2$ molecule is bonded to this amine with different binding energies.

### 5.3.2 High pressure

Spectra of isopropyl amine have been recorded at higher pressures. Isopropyl amine was chosen since it is a primary amine with a relatively simple spectrum allowing for a more straightforward interpretation as compared to the other amines used in the experiments at atmospheric pressure.

![Figure 5.7](image)

Figure 5.7 $^{14}$N NMR (left) and $^{13}$C NMR (right) spectra of isopropyl amine at 152 bar and 50ºC.

Since lower concentrations are used in supercritical CO$_2$, analyzing spectra can be complicated as the signal is much weaker compared to the spectra obtained in organic solvents. Moreover, predicting chemical shifts is not trivial, which makes it difficult to assign the peaks. At 50 ºC and 152 bar, $^{14}$N and $^{13}$C NMR spectra have been recorded (Figure 5.7).

As can be seen from these spectra, the signals are indeed rather weak. The $^{14}$N NMR spectrum clearly shows two separate peaks indicating that both amine and carbamic
acid are present. Wittmann has reported high pressure $^{14}$N NMR of amines in the presence of CO$_2$ and in scCO$_2$ as well. Since the shifts in our spectra are comparable to theirs (Table 5.4), the same method of peak assignment has been applied. The shift $\delta$ (HN-C) at 279 ppm in the $^{14}$N NMR is assigned to carbamic acid, whereas the shift $\delta$ (HN-C) at 336 ppm denotes the presence of the amine.

Table 5.4 Comparison of the chemical shift at high pressure $^{14}$N NMR.

<table>
<thead>
<tr>
<th></th>
<th>Chemical shift ($\delta$) Wittmann, 2001</th>
<th>Chemical shift ($\delta$) This paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamic acid</td>
<td>285</td>
<td>279</td>
</tr>
<tr>
<td>Amine/Ammonium</td>
<td>336</td>
<td>336</td>
</tr>
</tbody>
</table>

Judging from the chemical shifts alone, this peak might also be assigned to the ammonium ion since it is not possible to distinguish between these two forms. However, since the substance remains soluble it is assumed that the amine is present and not the ammonium ion. Applying this assignment, peak areas could be estimated and the ratio between carbamic acid and amine was calculated to be 0.66. The same procedure was followed for the $^{13}$C NMR spectra; also here two different peaks are present, as well as a clear carbonyl peak at higher shifts. Peak assignment for the $^{13}$C NMR spectra was successfully performed according to the Varian predictor program, which predicts that the carbonyl function of the amine appears at 26 ppm, left from the carbonyl function of the carbamic acid which appears at 23 ppm. Although the peaks are shifted to 24 ppm and 21 ppm, respectively, it is assumed that the left peak is from the carbonyl function of the amine. The calculated ratio of carbamic acid and amine from the $^{13}$C NMR spectrum is 0.61, a value comparable to the ratio obtained from the $^{14}$N NMR spectrum.

However, when the same analysis was performed by using $^1$H NMR at the same pressure and temperature, the carbamic acid/amine ratio appears to be 0.92. This value differs substantially from those found by $^{13}$C and $^{14}$N NMR. Probably, relaxation problems have played a role and the integrals obtained in the $^{13}$C and $^{14}$N measurements will have a large error since the areas are very small. Therefore the $^{13}$C and $^{14}$N NMR data are only used for qualitative purposes. Since $^1$H NMR gives...
better signals and the analysis time is short, $^1$H NMR is the most straightforward technique to use for the calculations. A priori, the carbamic acid/amine ratio is expected to be dependent on temperature and/or pressure; so two series of measurements have been performed where the amount of isopropyl amine was kept constant. The starting pressures used and the pressure applied during the experiment were different as a consequence of gradually increasing the temperature. A point of interest is the movement of one peak, probably $\delta$(OH), from 1 to 4.6 ppm, expressed as a function of temperature as shown in Figure 5.8.

![Figure 5.8 Moving peak shifts in the $^1$H spectra as a result of increasing temperature and pressure.](image)

With increasing temperature and/or pressure, this peak moves from a chemical shift of 4.5 ppm towards 1.0 ppm. This effect is reversible since this peak moves back to 4.5 ppm with decreasing temperature / pressure. This seems more a pressure-related effect then a temperature effect since in the series started at 157 bars, this peak is already close to 1 ppm and the change in chemical shift is not very drastic here. As shown above, changing the organic solvent can induce a change in chemical shift, an effect that also occurs in scCO$_2$. Previous work also shows clear shifts of $^{17}$O from CO$_2$, especially in the transition of CO$_2$ from the liquid to the supercritical phase$^{23}$. The effect of changing temperature/pressure on the carbamic acid/amine ratio is shown in Figure 5.9. Obviously, the spectra where the moving peak is overlapping
with the used carbamic acid and amine peaks are not used. The upper line is the experiment that has been started at 35°C, 157 bar and ended at 75°C, 200 bar (limit of the high-pressure cell). The lower line is the experiment that has been started at 35°C, 105 bar and ended at 95°C, 185 bar. In this figure no correction is made for the larger amount of CO₂ present in the upper line as a result of the higher starting pressure, since CO₂ is present in excess in both series of experiments.

![Graph showing carbamic acid/amine ratio vs. temperature](image)

**Figure 5.9 Carbamic acid/amine ratio as derived from the ¹H NMR spectra of isopropyl amine. Two series are performed at different starting pressures.**

When looking at the trends towards higher temperatures, both lines show a clear decline in carbamic acid/amine ratio. At higher temperatures, the carbamic acid becomes less stable and the equilibrium shifts towards the ‘free’ amine and CO₂. This effect was also shown earlier in this paper where TGA-DSC measurements combined with mass spectroscopy show a release of CO₂ between 70-80 °C for all amines. Another effect, which can be derived from this figure, is the dependence on the pressure. At 35 °C, two measurements have been made at various pressures. The measurement performed at 105 bar gives an acid/amine ratio of 0.35, whereas the measurement at 157 bar yields a ratio of 2.0. So, at higher pressures the carbamic acid clearly prevails. However, since the peak areas are rather small, the error in the data is relatively large and the accuracy of these numbers is estimated to be in the order of 20%.
5.4. Conclusions

In this paper, we have shown that the spontaneous reaction between an amine and $\text{CO}_2$ occurs in organic solvents as well as in supercritical solvents, but not to the same extent. In dilute DMSO solution, the formation of carbamic acid is complete, but changing the solvent to supercritical $\text{CO}_2$ leads to the establishment of an equilibrium, which depends on pressure and temperature. Future studies should be aimed at improving the accuracy in order to obtain more precise estimates of the carbamic acid/amine ratio as a function of temperature and pressure. The obtained knowledge can be useful in utilizing the properties of $\text{CO}_2$ to best avail, especially when employing the protective function of carbamic acids for preventing the formation of undesired side-products.

References

Chapter 6

Production of Polyurethanes using Carbon Dioxide

Abstract
Two different routes for the production of polyurethanes without phosgene have been explored. The first route describes the use of carbon dioxide as a building block, making use of the spontaneous reaction of carbon dioxide with diamine. The second route describes the use of a cyclic carbonate as a building block to produce pre-polyurethane groups. The preliminary results obtained for these two routes will be presented, from which it can be concluded that both routes have resulted in the production of polyurethanes. Also, several options to improve the polyurethane formation have been given.

This chapter is based on: Z.J.Dijkstra, M. Aerts, J.M.M. Simons, J.T.F. Keurentjes, Phosgene-free production of polyurethanes using carbon dioxide, in preparation
6.1 Introduction

Polyurethanes belong to a commercially important class of polymers because of their wide variety of possible applications in different industries. Some of the applications of this versatile polymer include adhesives, foams, flexible wires, isolation and paints. A polyurethane has a significant number of urethane groups with the following characteristic configuration (Figure 6.1).

![Figure 6.1 A urethane group.](image)

The most widely used production processes of polyurethanes are based on isocyanates, produced from a primary amine and phosgene (Figure 6.2).

![Figure 6.2 Reaction scheme of the conventional route for the production of polyurethanes.](image)

The drawbacks of this process are clear: the use of the highly toxic and corrosive phosgene and the liberation of hydrogen chloride as a co-product. Also, working with diisocyanates demands special care since they are toxic and represent an environmental hazard. Significant efforts have been made to replace the use of phosgene in this process. Here, the possibilities to incorporate CO₂ or a cyclic carbonate into the polyurethane have been investigated. Three possible routes will be discussed in more detail. Two routes are based on the incorporation of CO₂ and one is based on the incorporation of cyclic carbonate (Figure 6.3).

The background of these three routes is first explained in the literature section. In the remaining of this chapter, the possibilities to incorporate CO₂ into the polymer through the diisocyanate route will be explored (Route 1a). The possibility of using enzymes to couple a carbamate, originating from a diamine and CO₂, with a diol or acid has been explored as well (Route 1b). The next part describes the use of cyclic
carbonates in the production of polyurethanes through an enzymatic route (Route 2). In this reaction, two different methods of water withdrawal have been applied, including the use of a vacuum and a molecular sieve. The experiments have been performed in a solvent-free medium and in CO$_2$. For both routes, the influence of the presence of CO$_2$ to reduce the viscosity and to increase the mass transfer has been investigated. Finally, the obtained products have been characterized using several analytical techniques.

**Route 1a**

\[
\text{Diamine} \xrightarrow{\text{CO}_2} \text{Carbamate} \xrightarrow{-\text{H}_2\text{O}} \text{Diisocyanate} \xrightarrow{\text{Diol}} \text{Polyurethane}
\]

**Route 1b**

\[
\text{Diamine} \xrightarrow{\text{CO}_2} \text{Carbamate} \xrightarrow{\text{Diol / Diacid Enzyme}} \text{Polyurethane}
\]

**Route 2**

\[
\text{Diamine} \xrightarrow{\text{Cyclic carbonate}} \text{Bis-carbamate} \xrightarrow{\text{Diol / Diacid Enzyme}} \text{Polyurethane}
\]

Figure 6.3 Alternative routes for the production of polyurethane.

### 6.2. Literature

#### 6.2.1 Route 1a

A possible alternative for producing polyurethanes is based on the use of carbon dioxide. Using carbon dioxide, it will be possible to produce isocyanates without phosgene (Route 1a). Carbon dioxide reacts in a spontaneous reaction with a primary or secondary diamine to a biscarbamate (Figure 6.4). Several articles in literature describe the activation of the carbamate anion by the addition of an organic base$^{2-3}$. The added base drives the equilibrium of the reaction towards the carbamate formation. Complete conversion of the amine is necessary to prevent unwanted side reactions in the next steps of the synthesis$^4$. 

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A few methods to convert the carbamates into diisocyanates are available, including the reaction of bis-carbamates and alkyl halides in the presence of potassium carbonate and a catalytic onium salt\(^3\), thermal decomposition of carbamate esters\(^5\), and dehydration of the carbamates\(^5-8\) (Figure 6.5).

Several dehydrating agents promote the highly selective synthesis of isocyanates from carbamate anions avoiding the use of phosgene. Chemicals that are able to withdraw the water from the carbamate are the toxic and corrosive POCl\(_3\), the solid o-sulfobenzoic acid anhydride and montmorillonite clay\(^5\). The o-sulfobenzoic acid anhydride is a versatile reagent; it gives rise to high yields and selectivities of diisocyanates under mild conditions\(^7\). Moreover, this reagent provides an opportunity to generate diisocyanate in situ followed by immediate conversion into polyurethane upon addition of a diol and simultaneously increasing the temperature. Ideally, the reaction will proceed as shown in Figure 6.6.
6.2.2. Route 1b

The previous paragraph explained how the biscarbamates can be used in the formation to polyurethanes. However, the bis-carbamates can also be transformed into polyurethanes without the use of diisocyanates. McGhee et al. found that, in a palladium diolefin system, the carbamate anion can react with another nucleophile to form low-molecular weight polyurethanes (Figure 6.7).

\[
RR_1NCO_2^- (+\text{HBase}) + \text{nucleophile} \xrightarrow{\text{PdL}_n} O_2CNRR_1
\]

**Figure 6.7** Reaction scheme of the palladium-catalyzed polyurethane formation by using the carbamate anion as a nucleophile.

Abla et al. investigated the conversion of carbon dioxide into urethanes by homogeneous catalysis. A tin-catalyst can catalyze the reaction of dense CO\textsubscript{2} with amines and alcohols into polyurethanes. The water produced is trapped by an acetal to regenerate an alcohol. The proposed mechanism is shown in Figure 6.8. Since it seems chemically possible to couple the carbamate anion to another nucleophile, coupling these molecules with an enzyme might be a versatile option as well.

**Figure 6.8** Reaction scheme of the urethane synthesis by homogeneous catalysis.
6.2.3 Route 2

Another alternative for producing polyurethanes is the use of cyclic carbonates. In this reaction the urethane function is produced from the spontaneous reaction of a diamine with a cyclic carbonate (Figure 6.9).

\[
\text{cyclic carbonate} + \text{diamine} \rightarrow \text{bis-carbamate}
\]

Figure 6.9 Reaction scheme of the reaction of a carbonate with a diamine.

This bis-carbamate contains two alcohol groups and can therefore be used for polymerization through enzymatic polycondensation, as recently described by McCabe\textsuperscript{10}. In a second step, Novozyme 435 is used to catalyze the esterification reaction of the hydroxide groups from the bis-carbamate and a dicarboxylic acid (Figure 6.10).

\[
m \{ \text{bis-carbamate} \} + n \text{1,4-butandiol} + (m+n) \text{adipic acid} \rightarrow \text{polyurethane} + 2(m+n)-1 \text{H}_2\text{O}
\]

Figure 6.10 Reaction scheme for the enzymatic synthesis of polyurethane.

The polymer synthesis is a step growth reaction in which each step is an esterification reaction of an acid and an alcohol end-group. The reaction mechanism of the enzymatic polyesterification between diacids and diols was studied by Binns\textsuperscript{11}.
and is given in Figure 6.11. This mechanism shows that it is the acid group and not the alcohol group that attacks the enzyme. After one cycle, the alcohol groups terminate the polymer on both ends. The acid groups occur only on the acid and alcohol-acid monomers A and AB in Figure 6.11.

![Figure 6.11 The reaction cycle for enzymatic polyurethane synthesis.](image)

The reaction as described above is a condensation polymerization reaction in which water is produced. With water in the system, spontaneous hydrolysis of the polymer chain occurs causing the production of low molecular weight polymers. Also an increase in the amount of water will decrease the activity of the enzyme. Methods to circumvent the production of water are the use of activated diacids. Several studies concerning condensation polymerization reactions between diols and activated diacids have been performed\textsuperscript{12}. Chloroesters have been used to produce a side product with a low boiling point which can be easily removed\textsuperscript{13-14}, divinyl esters have been used since the side product tautomerizes to acetaldehyde and in this way the reaction is driven to the product side\textsuperscript{15-17}, and finally ring opening reactions have been performed as well. In the ring opening reaction no side products are involved and no water is produced\textsuperscript{18}. 

\[ A = \text{Acid} \quad B = \text{Diol} \quad E = \text{Enzyme} \]
6.3 Experimental

6.3.1. CO₂ as building block (Route 1)

**Carbamate formation**

In an Erlenmeyer flask isophoronediamine or 1,6-hexamethylene diamine was dissolved in N,N-dimethylformamide (DMF) with a concentration range between 0.024 mol L⁻¹ and 0.24 mol L⁻¹.

![Figure 6.12 Schematic setup for the carbamate formation reaction.](image)

The reaction between the amine and carbon dioxide can be performed in a variety of solvents but it is preferred to perform such a reaction in a polar aprotic solvent to avoid isolation of the carbamate salt. Alternative solvents which can be used are dimethylsulfoxide (DMSO) or acetonitrile, although the diisocyanate is less soluble in these solvents as compared to DMF. Triethylamine (TEA) was added to the diamine solution in a molar ratio of 5:1 (TEA : diamine). The Erlenmeyer was placed in a bath with cold water since the reaction is exothermic. Gaseous carbon dioxide was led through the solution for 1 to 3 hours by means of an end filter with 1 µm pores (Figure 6.12).

**Diisocyanate and polyurethane formation**

All synthesis steps involving isocyanate groups were performed under a continuous flow of nitrogen to prevent contact with moisture from the air. Two (molar) equivalents of dehydrating agent per reactive nitrogen were slowly introduced into the carbamate solution over a time period of 30 minutes while continuously stirring with a magnetic stirrer and cooling in a water bath. The dehydrating agents were o-
sulfobenzoic acid anhydride powder, montmorillonite K12 clay, molecular sieve (Zeolite 4A) and acetic acid anhydride. Diisocyanate was produced in situ and subsequently 1,4-butanediol was added to generate the polyurethane. The polymerization reaction was performed at 353 K for at least 2 hours. To investigate the influence of CO$_2$-pressure, the reactions were also performed in a stainless steel reactor at 20 bar. In this way CO$_2$ was used both as a solvent and reactant. The reactor containing the hexanediamine and triethylamine mixture, in DMF or solvent-free, was filled with CO$_2$ at room temperature. After 30 minutes o-sulfobenzoic acid anhydride dissolved in DMF was added using a high pressure liquid pump and after another 30 minutes the butanediol was added to the autoclave. At this moment the temperature was changed to 353 K and the mixture was stirred for 2.5 or 24 hours, respectively.

**Enzymatic polyurethane formation**

In the atmospheric experiment, the bis-carbamate solution was prepared before adding butanediol, adipic acid and enzyme to this bis-carbamate solution. In the high pressure experiment, the diamine was dissolved in DMF, with and without TEA, subsequently 1,4-butanediol, adipic acid and the enzyme Novozyme 435 were added. In both cases, the amount of the reactants was calculated such that the molar amount of butanediol is 10 times that of the bis-carbamate. The molar quantity of adipic acid was equal to the sum of the molar amount of butanediol and the carbamate anion. Finally, 2 wt% Novozyme 435 relative to the amount of added adipic acid was added and this mixture was stirred for several days at a temperature between 333 and 343 K.

**6.3.2. Cyclic carbonate as building block (Route 2)**

**Bis-carbamate formation**

The reactions were performed in a closed 100 mL Erlenmeyer flask. The reaction mixture contained 50 mL 1,4-butanediol with ethylene carbonate (2.95 g) and 1,6-hexane diamine (1.95 g) in stoichiometric ratio. The amounts were calculated such that the final bis-carbamate would be about 5 to 10 mol % as compared to the diol. The reactions were carried out between 333 and 343 K for 20 hours.
**Enzymatic polyurethane formation in 1,4-butanediol**

A stoichiometric amount of adipic acid, as compared to the amount of alcohol group present in the bis-carbamate solution and 2.5 wt% of Novozyme 435, as compared to the adipic acid added, were added to the bis-carbamate solution. In the first procedure, vacuum was applied (approximately 6.5 mPa) for 72 hours, while the reaction temperature was kept between 333 and 343 K. In the second procedure, molecular sieve was added instead of applying vacuum. The molecular sieve used, Zeolite 4A, can adsorb up to 20 wt% of water. 1.23 g molecular sieve was used per gram of adipic acid, this amount of molecular sieve should be able to completely withdraw the water formed during the reaction.

**Enzymatic polyurethane formation in CO₂**

A scheme of the pressurized reaction setup is shown in Figure 6.13. The main part of the setup is a stainless steel reactor with a volume of 650 mL and maximum operating pressure of 60 bar. A temperature and a pressure sensor are connected to the top of the reactor as well as a CO₂ and N₂ inlet.

The reagent amounts were chosen such that the reaction mixture would cover the stirrer completely. First, the bis-carbamate was synthesized in a closed 200 mL Erlenmeyer. 32.8 g butanediol, 4.23 g 1,6-hexanediamine and 6.42 g ethylene carbonate were added to the Erlenmeyer and the reaction mixture was left overnight at 343 K. After the synthesis of the bis-carbamate, the content of the Erlenmeyer was added to the reactor, together with 58.6 g adipic acid, 1.46 g Novozyme 435 and 36.0 g molecular sieve. The reactor was then firmly tightened and pressurized with carbon dioxide. At the end of the reaction, the pressure was released from the reactor with the top valve. This was carried out slowly with small intervals to prevent excessive foaming. The reactor was opened and samples were taken.
6.3.3 Characterization

**GPC**

To examine the molecular weight and molecular weight distribution, the synthesised polymers were analysed with GPC. The apparatus consisted of a WATERS Model 510 pump, Model 486 UV detector (at 254 nm), and Model 410 refractive index detector (at 313 K). Injections were done by a WATERS Model WISP 712 autoinjector, the injection volume used was 50 µL. The columns used were a PLgel guard (5 µm particles) 50*7.5 mm guard column, followed by 2 PLgel mixed-C 300*7.5 mm columns (313 K) in series. Tetrahydrofuran (Biosolve, stabilised with BHT) was used as eluent at a flow rate of 1.0 mL/min. Calibration has been done using polystyrene standards (Polymer Laboratories, M = 580 to M = 7.1*10^6). Data acquisition and processing were performed using WATERS Millennium32 (v 4.0) software. Samples are filtered over a 13 mm * 0.2 µm PTFE filter (PP housing, Alltech).


NMR

$^1$H-NMR spectra were recorded from all synthesized compounds with a Varian Oxford 200 MHz NMR.

GC

Gas chromatography was used to measure the conversion of the biscarbamate during reaction. The chromatograms were recorded with a GC8000 with an RTX-5 column from Interscience.

FT-IR

FT-IR was used to measure the elongation of the polyurethanes. The spectra were recorded with a Nicolet Smart Golden Gate$^\text{TM}$, a Nexus model 670/870.

TGA-DSC-MS

The thermal stability of the synthesized bis-carbamates was analyzed with a Netzsch TGA-DSC apparatus coupled with a Pfeiffer mass spectrometer. With TGA (thermo-gravimetric analysis) the weight loss of a sample as a function of temperature is measured. The sample is placed in an inert sample holder that is coupled to a micro-gram balance during the experiment. The decomposition products that evaporate from the sample are directly analyzed with the coupled mass spectrometer.

MALDI-TOF-MS

MALDI-TOF-MS was used to characterize the co-polymer that was formed during polymerization. A scheme of the setup is shown in Figure 6.14$^{19}$. The analysis consists of three steps. In the first step is matrix assisted laser desorption/ionization is used. In particular, a nitrogen laser pulse with a wavelength of 337 nm is used to generate plumes of ejected particles. The pulses follow each other with a frequency of 20 Hz. In the second step, called Time of flight (TOF), the positive ions are separated from the ejected particle plume by means of electric fields. The ions are accelerated into a TOF-tube, which is a high vacuum enclosure with an electrical potential of 25 kV relative to the sample probe. Faster ions with the same mass
penetrate the electrical field further. This causes a delay and minimizes the difference in flight time between fast and slow ions of the same mass. The final step is the detection of the released ions. The detector functions as a very precise stopwatch. The molecular masses of the ions that arrive at the detector can be calculated from the flight time.

![Figure 6.14 Experimental setup for MALDI–TOF–MS analysis.](image)

6.4 Results and Discussion

6.4.1 CO$_2$ as building block

**Non-enzymatic route (Route 1a)**

First the influence of bubbling carbon dioxide through a solution of hexanediamine or isophoronediamine has been investigated. The resulting $^1$H NMR spectrum of the isophoronediamine and the carbamate in dimethyl(d6)sulfoxide are presented in Figure 6.15. These results show the change in spectrum after bubbling with carbon dioxide through the sample. The proton peak of the amine group, peak A and B at 2.2 ppm, has disappeared in the presence of CO$_2$. In the second spectrum (Figure 6.15 b), the protons of NH in the carbamate function are measured at 6.5 and 6.8 ppm (peaks C and D). In the additional step a dehydrating agent is added to generate diisocyanate. GC-MS spectra are used to confirm the presence of the diisocyanates. The addition of Montmorillonite clay K10 and acetic acid anhydride has not resulted...
in the formation of diisocyanate, but the addition of the molecular sieve and o-sulfobenzoic acid anhydride has produced diisocyanates. O-sulfobenzoic acid anhydride gives much higher conversions as compared to the molecular sieve and is therefore used in the polyurethane formation experiments.

![Diisocyanate Molecule]

**Figure 6.15** $^1$H NMR spectra of isophorone diamine before (a) and after (b) CO$_2$-bubbling.

In the literature, the polymerization reaction of diisocyanate and an alcohol is described as very exothermic$^{20}$. Nevertheless, it is not a spontaneous reaction. The temperature is kept constant between 343 and 373 K to overcome an energy barrier. The production of the polyurethane under constant N$_2$-flow at 353 K is followed with IR. Figure 6.16 shows the formation and elongation of the isophorone-polyurethane. Samples are taken after 2, 3 and 4 hours of reaction.
Molecular weights are determined by viscometry with the Mark-Houwink equation\textsuperscript{21}. A disadvantage of this analysis method is that no information about polydispersity can be obtained. The measured molecular weights are given as a function of time (Figure 6.17). An exponential function is fit through the data points as a guide to the eye. The increase in molecular weight as a function of time typically proceeds in the shape of an S-curve\textsuperscript{22-23}. There is a clear difference in the reaction rate between isophorone diisocyanate and hexane diisocyanate which is probably due to steric differences.

DSC measurements have been performed to measure the glass transition temperature of the two polymers. The glass transition temperature of the hexane-polyurethane is found at 241 K, and the $T_g$ of the isophorone-polyurethane is found at 263 K. The existence of only one $T_g$ suggests that these polyurethanes are linear, amorphous and monophasic.

However, in a CO\textsubscript{2} rich environment at 50 bar, there is no formation of polymers. During the reaction, the reaction mixture in CO\textsubscript{2} is present in one liquid phase with on top a gaseous CO\textsubscript{2}-phase. This has been checked with a mixture of the diamine, DMF and 1,4-butanediol, with and without TEA up to 50 bar. After opening the reactor, it seemed that the o-sulfobenzoic acid anhydride precipitated out of the
solution, and as a result no diisocyanate is formed, which explains the absence of polyurethanes.

![Graphs showing molecular weight over time for a) isophoronepolyurethane and b) hexane-polyurethane.](image)

**Figure 6.17** The size of the polyurethane chain expressed as molecular weight as a function of time for a) isophoronepolyurethane and b) hexane-polyurethane.

**Enzymatic route in subcritical CO₂ (Route 1b)**

The use of CO₂ in the enzymatic catalysis of polyurethanes gives poor results. During the first stage the isophorone-bis-carbamate anion, stabilized by TEA, is used as starting material. The reaction of the carbamate with butanediol and with a butanediol-adipic acid mixture results in a two-phase product after depressurization. A turbid solution is obtained in which solid particles are present. During the reaction, the reaction mixture in CO₂ is present in one phase. This is confirmed by pressurizing the reactants with CO₂ in a view cell. All reactions are performed at 50
bar and 333 K with a reaction time of 1 week. After this, the reaction products are analyzed by means of GPC. The reaction between the butanediol and the isophoronediamine results in an oligomer of 3,600 g mol$^{-1}$, while the reaction with the hexanediamine gives a molecular weight of 540 g mol$^{-1}$. However, for both diamines the conversion is low. This is partly due to the low activity of the enzyme in DMF. Although the carbamate and diisocyanate are soluble in DMF, it is clearly not the best solvent for the enzyme. It is, therefore, anticipated that higher conversions can be obtained by using other solvents.

**Perspectives**

The option of using CO$_2$ as a building block in the production of polyurethanes with the intermediate carbamate and isocyanate is very promising. Carbamate formation occurs spontaneously and o-sulfobenzoic acid anhydride can be used as a dehydrating agent. Although o-sulfobenzoic acid anhydride can be regenerated and re-used, it is worthwhile to investigate other options for the dehydration method. One can think of a strong water adsorbing column to lead the carbamate through. When the adsorbing capacity of the column is high enough diisocyanate is formed, and in the second step a diol can be added to form the polyurethane (Figure 6.18). So far, the experiments where CO$_2$ has been applied have failed to produce the polyurethane. Using another dehydrating method, as suggested above, is possibly a successful alternative. Another unclarity in this procedure is the solubility of the polyurethane in the reaction medium when CO$_2$ is applied. The progress of the polymerization will depend on the solubility in the CO$_2$-rich medium.

The enzymatic route where the carbamate should be converted into the polyurethane without isocyanate as intermediate is clearly a more complex route. The carbamate is probably partly present as acid and as carbamate anion. Treating this carbamate as an acid is therefore not clear-cut. However, since literature showed that this conversion is possible with a palladium catalyst, it might be possible to perform the reaction with enzymes as well.
6.4.2 Cyclic carbonate as building block (Route 2).

The reaction of 1,6-hexanediocamine with propylene carbonate has led to the formation of a yellowish liquid, which on cooling formed yellow crystals. The conversion has been followed by measuring the ethylene carbonate and 1,6-hexanediocamine concentration with GC (Figure 6.19).

The Fourier transformed NMR spectrum of 1,4-butanediol and the synthesized bis-carbamate from 1,6-hexanediocamine and ethylene carbonate in CDCl$_3$ are shown in Figure 6.20a. The diamine and ethylene carbonate have disappeared completely. None of the peaks from the diamine or ethylene carbonate appear in the bis-carbamate spectrum. In Figure 6.20b the origin of the different peaks is indicated.
Figure 6.20 a) NMR spectrum for di(hydroxyethyl) hexamethylene bis-carbamate in 1,4-butanediol and b) peak assignment of 1,4 butanediol and di(hydroxyl)hexamethylene bis-carbamate.

Figure 6.21 Decomposition of di(hydroxyethyl) hexamethylene bis-carbamate as a function of temperature. The left y-axis shows the decrease in mass as measured with TGA. The right y-axis shows the release of CO$_2$ as measured with a mass spectrometer.
The results from the TGA-MS measurements are shown in Figure 6.21. Almost no decrease in mass occurs until a temperature of 433 K is reached. The small decrease that does occur originates from the water adsorbed on the sample. When the sample is heated above 433 K, the product decomposes and carbon dioxide ($M_w = 44$ g mol$^{-1}$) is released as shown in Figure 6.21. At 773 K, the decomposition process is complete. This analysis shows that di(hydroxyethyl) hexamethylene bis-carbamate is stable up to a temperature of 433 K.

**Polyurethane formation**

The molecular weights of the synthesized polymers have been analyzed with gel permeation chromatography (GPC). To evaluate the effect of removing water from the system, the experiment has first been carried out without removing water from the reaction mixture. Samples have been taken from this system at 24, 48 and 72 hours. In these experiments, oligomers with molecular weights around 1100 g mol$^{-1}$ have been produced, indicating the importance of water removal during the polyesterification process.

<table>
<thead>
<tr>
<th></th>
<th>$M_{\text{peak}}$</th>
<th>$M_w$</th>
<th>$M_n$</th>
<th>$M_w/M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-Butanediol polyester</td>
<td>9566</td>
<td>10739</td>
<td>5253</td>
<td>2.044</td>
</tr>
<tr>
<td>Polyurethane from</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>di(hydroxyethyl)hexamethylene bis-carbamate</td>
<td>13931</td>
<td>14064</td>
<td>7001</td>
<td>2.009</td>
</tr>
<tr>
<td>Polyurethane from</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>di(hydroxyethyl)isophorone bis-carbamate</td>
<td>4346</td>
<td>4819</td>
<td>2587</td>
<td>1.863</td>
</tr>
<tr>
<td>Polyurethane from</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>di(hydroxyethyl)hexamethylene bis-carbamate (318-323 K)</td>
<td>9956</td>
<td>9585</td>
<td>5576</td>
<td>1.719</td>
</tr>
</tbody>
</table>

The first polymerization reactions have been performed by applying vacuum (Table 6.1). The polymer in which isophoronediamine is used shows a lower molecular weight and this can be explained by the more complex structure of isophorone
diamine as compared to hexane diamine. Also, there is only a small effect of the temperature on the reaction rate. This effect has also been observed in previous research\textsuperscript{22-24}.

Another method applied to withdraw water from the reaction mixture has been the addition of a molecular sieve. After 72 hours of reaction between 338 and 343 K, the molecular weight of the polymer is about 65,200 g mol\textsuperscript{-1}. The experiments in the high pressure reactor are carried out at 1 and 50 bar. The molecular weight of the polymer obtained in the experiment performed at 1 bar is $M_w$ 7,600 g mol\textsuperscript{-1}, while the experiment performed at 50 bar results in a polymer with a molecular weight of $M_w$ 30,000 g mol\textsuperscript{-1}. These results show that the CO\textsubscript{2}-expanded reaction mixture indeed has a positive effect on the molecular weight of the polymer which is most probably due to increased overall reaction rates. The change from the glass setup to the high pressure setup has decreased the molecular weight of the polymer tremendously; it decreased from 65,200 g mol\textsuperscript{-1} down to 7,600 g mol\textsuperscript{-1}. This effect can be explained by the difference in mixing. In the glass setup, the reaction mixture is more ideally mixed as compared to the high pressure setup.

In the high pressure setup only a few experiments results in the formation of a polymer. An explanation has been found in the adsorbing capacity of the molecular sieves. The molecular sieves are dried overnight at 393 K which might not be enough to activate the molecular sieves so that less water than expected is adsorbed. Another reason for the failure of the molecular sieve is the adsorption of CO\textsubscript{2} instead of water. Also here, less water than expected is adsorbed, resulting in a low molecular weight of the polymer.

**Co-polymer characterization with MALDI – TOF – MS**

To characterize the type of co-polymer that has been formed, the polymer is analyzed with MALDI–TOF–MS. The NMR spectra show the formation of a polymer, but do not provide information to what extent the urethane groups are included in the polymer. For the MALDI analysis, a polymer has been synthesized at atmospheric pressure and at 50 bar with an equal molar amount of 1,4-butanediol and di(hydroxyethyl)hexamethylene bis-carbamate. This gives insight in the relative reaction rates of the diol and the bis-carbamate.
The polyurethane segments in the polymer, as illustrated in Figure 6.9, have a molecular weight of 402 g mol\(^{-1}\). The polyester segments have a molecular weight of 200 g mol\(^{-1}\). By taking linear combinations of the molecular weights from these two segments and adding the molecular weight of an end group, the molecular weights of the polymer measured with MALDI can be matched. The results from these calculations are shown in Figure 6.22a.

![Surface plot of co-polymer type analyzed with MALDI – TOF - MS.](image)

The surface plot shows that the polymer, synthesized at atmospheric pressure, consists mainly of polyester groups. The calculations show that the polymer consists for 82 % of polyester, and the polymer contains only alcohol end groups and no acid end groups, as was expected from the enzyme kinetics. Basically, this means that for every urethane group, six or seven diol groups are included. Indeed diol groups are more reactive and are therefore present in a larger fraction in the polyurethane. The main reason is that the diol group is much smaller and will react faster as compared to the urethane group\(^{20}\). The experiment performed at 50 bar seems to give a
different result. A problem with the analysis of this experiment is the low molecular weight. The polymer formed is actually an oligomer. Figure 6.22b shows that for every urethane group also one diol group is included. This could lead to the conclusion that working in CO$_2$ promotes the inclusion of urethane groups into the polyurethane. However, to confirm this, a polyurethane with a similar molecular weight as shown in Figure 6.22a should be synthesized and analyzed.

**Perspectives**

The option of using a cyclic carbonate for use in polyurethanes has been explored. In the experiments where a vacuum is applied molecular weights are reasonably high and show good reproducibility. However, the experiments performed with the molecular sieves give a different result at atmospheric pressure as compared to the results obtained at CO$_2$-pressure. First, the optimization of the practical procedure should be considered to obtain reproducible results. This will include a search for more appropriate water adsorbers since it is likely that the use of molecular sieves is one of the main reasons for the failed experiments. After this step, the experiments performed at different CO$_2$-pressures can be repeated and analyzed by their molecular weight, including a MALDI-TOF study to check how many urethane groups are build in.

**6.5 Conclusions**

Two applied routes, route 1a and 2, have resulted in the production of polyurethanes and also for both routes problems came up when transferring the reaction to a CO$_2$-rich environment. The main issue is the presence of water and the absence of a proper method to remove the water from the system. In route 1a, the removal of water is needed to form the diisocyanate, and in route 2, the removal of water is needed to avoid hydrolysis of the polymer and to keep the enzyme in an active state. The main advantage of route 1a as compared to route 2 is that in route 1a no catalyst has to be used. Almost all primary and secondary diamines can be used for the formation of carbamates and the synthesis of the diisocyanates, which is a rather toxic intermediate. However, working in a closed system, as suggested in Figure
6.18, limits the problems related to this toxicity. Route 2 has no harmful intermediates and can therefore be seen as a much cleaner method. Here, the enzymes should be recovered and re-used to prevent high enzyme costs.

References

Future Perspectives

Abstract
In this thesis, the catalytic activity of *Candida antarctica* lipase B, Calb, in supercritical carbon dioxide has been studied. Several reactions and immobilization preparations have been investigated. In this work, only the catalytic activity of Calb has been described. However, other types of enzymes might be interesting for use in scCO\textsubscript{2} as well. In this chapter, the potential and challenges of a number of industrially relevant enzymes will be evaluated. In particular, oxygenases, which are representative for types of enzymes that need a cofactor, lipoxygenase, epoxide hydrolases, aldolases, and decarboxylases will be discussed. The importance of choosing the proper reaction conditions is shown, taking into account the immobilization method, the addition of extra compounds, and the overall process design.
7.1 Introduction

In this thesis, the focus has been on the activity of *Candida antarctica* lipase B immobilized in different formulations. The two main reactions studied in this work are the enantioselective esterification of (R/S)-phenyl ethanol with vinyl acetate and the esterification of amyl alcohol with acetic acid. The first reaction is performed using ChiroCLEC™-CAB and Novozyme 435 for both batch and continuous conditions. The second reaction is performed using CLEA-Calb during continuous operation. Another type of esterification reaction is the condensation polymerization, which has been studied in chapter 6.

In potential, all enzymes that are active in organic solvents can be used for enzymatic catalysis in supercritical fluids. The potential and limitations of different types of enzymes with the emphasis on the possible use in scCO$_2$ will be evaluated. The most important parameters for optimizing enzymatic reactions and the progress of research in these areas are discussed with the focus on process design.

7.2 Potentially interesting enzymes

7.2.1 Cofactor dependent enzymes

A wide variety of enzymes need cofactors in order to catalyze a reaction. Table 7.1 gives an overview of the type of reactions that can be performed using cofactor dependent enzymes. This list is clearly not complete and should only be used as an indication of the functionality of several cofactor dependent enzymes. Epoxide carboxylase and dehydrogenase are of particular interest because CO$_2$ is used as one of the reactants. In the case of epoxide carboxylase$^1$, beta-keto acids are produced which can be useful as building blocks for fine chemicals. A combination of three different dehydrogenases makes it possible to produce methanol from CO$_2$ with a yield up to 92%$^2$. The monooxygenases as listed in Table 7.1 use molecular oxygen for the oxidation reactions. The high solubility of oxygen in scCO$_2$ makes it advantageous to use scCO$_2$ as the solvent. The main products produced from this type of oxidation reactions are alcohols and epoxides, which can be used as intermediates for more valuable products in the fine chemical industry. A special
type of enzyme is the cytochrome P450. This is a very general term for a wide variety of hemoproteins found in most organisms. The catalytic center contains an iron atom which is reduced to the ferrous state making it possible for molecular oxygen to bind to the complex. Cytochrome P450 monooxygenases are able to catalyze a wide variety of substrates of which the hydroxylation of fatty acids is one of the main research subjects.

The two most important issues of the enzymatic reactions listed in Table 7.1 are related to the stability and the regeneration of the cofactors. The stability of the enzymes and the cofactors needs to be improved before the cofactor dependent enzymes can be used in scCO$_2$.

Table 7.1 A selection of enzymes with interesting possibilities$^{3,5}$

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Product</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexanon monooxygenase</td>
<td>Cyclic ketones</td>
<td>Optically pure lactones</td>
<td>Building blocks for chemical industry</td>
</tr>
<tr>
<td>Styrene monooxygenase</td>
<td>Aryl-substituted vinyl groups</td>
<td>Epoxides</td>
<td>Intermediate for HIV blockers</td>
</tr>
<tr>
<td>Xylene monooxygenase</td>
<td>Toluene derivates</td>
<td>Alcohols, aldehydes</td>
<td>Flavorings and Fragrances</td>
</tr>
<tr>
<td>Methane monooxygenase</td>
<td>Alkanes</td>
<td>Alcohol</td>
<td>Functional groups</td>
</tr>
<tr>
<td>Alkene monooxygenase</td>
<td>Alkenes</td>
<td>Epoxides</td>
<td>Building blocks for e.g. pharmaceuticals</td>
</tr>
<tr>
<td>Cytochrome P450 monooxygenase</td>
<td>Fatty acids</td>
<td>Diol derivatives</td>
<td>Fine chemical intermediates</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>CO$_2$</td>
<td>Methanol</td>
<td>Renewable source</td>
</tr>
<tr>
<td>Epoxide carboxylase</td>
<td>Epoxide + CO$_2$</td>
<td>Beta-keto acid</td>
<td>Fine chemical intermediates</td>
</tr>
</tbody>
</table>
Regeneration of cofactors is preferably performed within whole cells, since this is the most stable environment to protect the cofactor. However, in whole cells several components are present, leading to impurities. Ex vivo, there are three main possibilities for regeneration\textsuperscript{6-7}. The first and most widely used method is the addition of an extra substrate. The obvious disadvantage is that an additional component is added to the system which needs to be separated afterwards. The second option is to use H\textsubscript{2} as H-donor, avoiding the presence of extra components in the medium, although the enzymes needed for these reactions are difficult to obtain and are unstable. Another problem with using H\textsubscript{2} is that in oxidation reactions with oxygen very explosive mixtures can be created. The third option, which is the best option for use in scCO\textsubscript{2}, is the use of electrodes to regenerate a cofactor. An H-donor is still needed but the reaction progress can be followed with the electrode. Normally, a mediator is used to improve the transfer of electrons between the cofactor and the electrode. An important cofactor for many enzymes is NADH. During a reaction NADH is oxidized to NAD\textsuperscript{+}. This is reduced to NADH in order to be available for a second reaction (Figure 7.1)

![Figure 7.1 Schematic view of a regeneration method coupled with an electrode.](image)

There is already some experience in aqueous systems, and the possibilities in organic solvents look also promising\textsuperscript{8}. However, the conductivity in scCO\textsubscript{2} might be too low\textsuperscript{9} but this can be increased by the addition of polar additives. The presence of water and a conducting additive increases for example the diffusion coefficient of ferrocene in CO\textsubscript{2} by a factor of ten as compared to acetonitrile\textsuperscript{10}. Supercritical fluids
like trifluoromethane and several other hydrofluorocarbons, HFCs, have been tested for their conductivity and different salts have been tested as well\textsuperscript{11-13}.

### 7.2.2 Lipoxygenase

Lipoxygenases are a group of non-heme iron containing dioxygenases able to catalyze the addition of molecular oxygen to fatty acids in a stereospecific as well as a regiospecific way.

![Lipoxygenase reaction](image)

**Figure 7.2** Reaction of linoleic acid with oxygen catalyzed by lipoxygenase.

![Conversion graph](image)

**Figure 7.3** Conversion of linoleic acid with O\textsubscript{2} in time. \textcolor{red}{$\blacktriangle$}: without bubbling of O\textsubscript{2}; \textcolor{blue}{$\blacksquare$}: with bubbling of O\textsubscript{2}. Lipoxygenase is immobilized on Eupergit C. The experiments were performed in phosphate buffer pH 7 with and without bubbling oxygen.

For this enzyme the natural substrate is linoleic acid (Figure 7.2), and the reactive part of the acid is the 1,4-diene unit. Other fatty acids can also function as substrates
leading to the production of several natural products\textsuperscript{14}. These reactions have been performed in organic solvents\textsuperscript{15}, ternary micellar systems\textsuperscript{16} and in supercritical CO\textsubscript{2}\textsuperscript{17}. The advantage of performing this reaction in scCO\textsubscript{2} is the compatibility of oxygen with CO\textsubscript{2}. High oxygen concentrations and therefore high reaction rates can be reached as illustrated in Figure 7.3. Furthermore the substrate inhibition is decreased in the presence of a higher initial oxygen concentration\textsuperscript{18}.

### 7.2.3 Epoxide hydrolase

Epoxide hydrolases belong to a class of ‘newly discovered’ enzymes which seem to be relevant for the synthesis of fine chemicals. Epoxide hydrolases are enzymes that catalyze the addition of water to epoxides resulting in the formation of the corresponding diols in a highly selective way (Figure 7.4).

![Figure 7.4 Enantioselective hydrolysis of a racemic epoxide catalyzed by epoxide hydrolase.](image)

Epoxide hydrolases do not require cofactors, and are available from different sources. They can exhibit high enantioselectivity, and can therefore be used to produce in a cheap and simple way enantiopure epoxides\textsuperscript{19-20}. The synthesis of these enantiopure epoxides is of great interest since they can be used as building blocks for the preparation of bioactive compounds such as active pharmaceutical ingredients (APIs) and agrochemicals.

### 7.2.4 Aldolase

Aldolases are enzymes that can couple aldehydes or aldehydes and ketons to produce new chiral components\textsuperscript{21}. The most promising enzyme of this group is deoxyribose-5-phosphate aldolase, DERA, which can catalyze sequential three to
four substrate aldol reactions (Figure 7.5)\textsuperscript{22}. In this way, sugar-like components can be made. Another option is to use threonine aldolase\textsuperscript{23}. This enzyme uses glycine as a cosubstrate to form amino acid-like components (Figure 7.6).

\begin{equation}
\text{R} - \text{O} \quad + \quad \text{O} \quad + \quad \text{O} \quad \xrightarrow{\text{DERA}} \quad \text{OH} \quad \text{OH} \quad \text{O} \quad \text{R} - \text{O} \quad \text{OH} \quad \text{OH} \quad \text{O} \quad \text{OH}
\end{equation}

**Figure 7.5** Reaction of two acetaldehydes and another aldehyde catalyzed by DERA.

\begin{equation}
\text{H}_2\text{N} - \text{CH}_2 - \text{CO} \quad + \quad \text{O} \quad \xrightarrow{\text{L-threonine aldolase}} \quad \text{OH} \quad \text{O} \quad \text{OH} \quad \text{NH}_2 \quad \text{OH} \quad \text{NH}_2 \quad \text{OH} \quad \text{OH} \quad \text{O} \quad \text{OH} \quad \text{7%} \quad \text{93%}
\end{equation}

**Figure 7.6** Reaction of glycine with acetaldehyde catalyzed by L-threonine aldolase.

Both aldolases are commercially available but not much is known about the stability and activity of these enzymes in scCO\textsubscript{2}. However, the CLECs of aldolase give 10 to 100 times higher reaction rates as compared to the lyophilized enzyme\textsuperscript{24-25}. Since good results are obtained with the CLECs of Calb in scCO\textsubscript{2}, it is possible that these aldolase-CLECs maintain their activity in scCO\textsubscript{2}. For this specific reaction type, there are a number of reasons why performing this reaction in scCO\textsubscript{2} could be advantageous. It is known that performing enantioselective or stereoselective reactions in scCO\textsubscript{2} can enhance selectivities which often depends on the CO\textsubscript{2} pressure. Furthermore, the small reactants have a high solubility in scCO\textsubscript{2} while the higher molecular weight product has not. Most likely, the product will precipitate from the reaction mixture, and therefore the equilibrium of these reactions is pulled to the product side leading to an increased yield.

### 7.2.5 Decarboxylases

Pyrrole-2-carboxylate decarboxylase is known to decarboxylate pyrrole. After purification from whole cells, this enzyme seems also capable of performing the reverse reaction using an organic acid as cofactor (Figure 7.7)\textsuperscript{26}. When this reaction...
Chapter 7

is performed with whole cells in scCO\textsubscript{2} the yield is about 12 times higher\textsuperscript{27}. In this way, CO\textsubscript{2} can be used both as solvent and substrate. Clearly, more research is required to explore the possibilities of this type of enzymatic reactions in scCO\textsubscript{2}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure77.png}
\caption{Carboxylation of pyrrole catalyzed by pyrrole-2-carboxylate decarboxylase.}
\end{figure}

7.3 Optimization of enzymatic catalysis in scCO\textsubscript{2}

7.3.1 Enzyme preparation

Several ways to immobilize enzymes are available as mentioned in chapter 1. However, it is difficult to predict which immobilization method will yield the highest catalytic activity and selectivity because this depends on the type of reaction and reaction environment. A clear illustration for this is given in Figure 7.8. The activity of a cross-linked enzyme crystal, ChiroCLEC\textsuperscript{TM}-CAB, and two different cross-linked enzyme aggregates, CLEA-O and CLEA-W, has been compared in hexane and in scCO\textsubscript{2}. CLEA-O, as used in chapter 4, is optimized for use in organic solvents, while CLEA-W is optimized for use in aqueous solvents. In both hexane and scCO\textsubscript{2}, a different activity is obtained for all three immobilization procedures while the same enzyme is used.

The difference in activity observed for the two CLEA preparations reveals the importance of the immobilization procedure. Changes in immobilization procedures (solvent, time, temperature, pH, additives) can have a major effect on the catalytic activity of the enzyme. The addition of crown-ethers\textsuperscript{28-29}, methyl-\(\beta\)-cycloextrin\textsuperscript{30-31}, and lyoprotectants\textsuperscript{32} in the immobilization procedure increases the activity of the enzyme. Therefore, it can be concluded that the state of the enzyme during the immobilization procedure is an important factor.
Enzyme immobilization is also important when looking at mass transfer limitations. Immobilization on a support could lead to mass transfer limitations and inaccessibility of the active centre for the substrates, as explained in chapter 1 and 2. This can have an influence on the catalytic activity of the enzyme. For practical reasons it might not be the best choice to make the enzyme particles as small as possible, since this can lead to other problems. In high-pressure equipment, small particles can accumulate in dead volumes of the reactor, and are therefore not used.
during the reaction. Another problem with small particles is the agglomeration of the particles with solids substances in the reaction medium. This agglomeration has been observed during the reactions with CLEA after the addition of salt hydrates (chapter 4).

### 7.3.2 Additives

The catalytic activity of enzymes can also be improved by the addition of solutes to the reaction mixture. Figure 7.9 shows the effect of the addition of triethylamine, TEA, on a transesterification reaction performed in hexane and scCO$_2$. In the presence of TEA a considerable increase in activity is observed for both systems. This is probably due to the removal of acid residues by TEA$^{33-34}$. However, it is also possible that TEA has an effect on the ionization state of the enzyme and increases the catalytic activity in that way. Previous research has shown that the addition of salt hydrates, added to fix the water activity, also has an effect on the catalytic activity$^{35-37}$. Salt hydrates have been found to affect the ionization state of the enzymes which can be prevented by adding solid-state acid-base buffers$^{38-40}$. This approach can also be used in supercritical fluids$^{40}$ although more research into this subject is needed.

![Figure 7.9 Conversion of hexyl alcohol with isopropenyl acetate as a function of time.](image)

- (■): in hexane without TEA
- (□): in hexane with TEA
- (▲): in scCO$_2$ without TEA (11 MPa)
- (▲): in scCO$_2$ with TEA (11 MPa)
7.3.3 Process Design

A good process design takes several issues into account. An improvement in a specific part of the process does not necessarily result in an overall improved process design. For example, the size of the cross-linked enzyme particles should be as small as possible due to possible mass transfer limitations while particles which are too small will accumulate in the dead volumes of the reactor. Also, the addition of extra components to the reaction medium can increase the catalytic activity as shown in the previous paragraph. However, from the point of view of a process design, it is a disadvantage to add additional compounds since these also need to be removed. These kinds of considerations need to be taken into account when applying improvements. Several reactor and process configurations in supercritical fluids have been investigated\textsuperscript{41}. In chapter 4 also some possible reactor designs have been discussed with the main conclusion that the optimal process design is largely determined by the reaction kinetics and the used substrates.

7.4 Future outlook

The practical use of enzymatic catalysis in supercritical carbon dioxide has to be found in producing high added value products since the high pressure equipment is expensive and the compression of CO\textsubscript{2} is energy consuming. Lipases have proven to be rather stable and active during long-term reactions in scCO\textsubscript{2}. However, it can not be stated that performing reactions in scCO\textsubscript{2} will always result in a higher activity. This is highly dependent on the type of reaction, the enzyme and the applied conditions. The main advantage of CO\textsubscript{2} is, therefore, the possibility of easier separation steps with complete removal of the solvent. More research into integrated reaction and separation steps is necessary with a focus on the separation and recovery of the products. One can think of membrane processes to separate substrates and products or use the difference in solubilities to precipitate the products without the need for depressurizing the whole system. Using enzyme crystals in a chromatography column could combine the reaction section with the separation section resulting in a very efficient process\textsuperscript{42-43}. Furthermore, the exploration of other enzymes in scCO\textsubscript{2} is valuable as well. Clearly, enzymatic
catalysis in supercritical fluids has an interesting future when biochemists and process technologists work together. The biochemist should focus on the development of stable enzymes, while the process technologist can use the improved enzymes to produce high added value products.

References

The interest in industrial biocatalysis is growing due to a rapidly expanding range of possibilities. The number of biotransformation processes performed on an industrial scale is gradually increasing, and about 50% of these processes are applied in the pharmaceutical industry. Many of these biotransformations are performed in organic solvents. However, increased concern for the environment has lead to awareness for environmentally friendly production methods. A general thought is that supercritical fluids can be used to reduce the amount of organic waste. An additional advantage of supercritical fluids is that separation after reaction is relatively simple which offers several process advantages. The aim of this thesis is to explore the potential of enzymatic catalysis in supercritical carbon dioxide.

The immobilization method of *Candida antarctica* lipase B has shown to be important for the catalytic activity in hexane as well as in supercritical carbon dioxide. This has been measured with a transesterification reaction. Two types of preparations have been used: Novozyme 435, immobilized onto a carrier, and ChiroCLEC™-CAB, a cross-linked enzyme crystal existing entirely of enzyme. For Novozyme 435 the initial activity obtained in supercritical carbon dioxide is always lower than the activity obtained in hexane. For the ChiroCLEC™-CAB the highest initial activity is observed for the reaction in supercritical carbon dioxide. Furthermore, Novozyme 435 exhibits a maximum in the catalytic activity whereas the initial activity of the ChiroCLEC™-CAB decreases with an increase in pressure. In continuous operation the ChiroCLEC™-CAB maintains its activity as well. However, at a high carbon dioxide flow rate water is stripped from the enzyme, resulting in a reduction in catalytic activity which can be reversed by the addition of water.

Another preparation of *Candida antarctica* lipase B, CLEA-Calb, has been used in an esterification reaction. CLEA-Calb is a cross-linked enzyme aggregate which has shown to give a good activity and which remains stable when used in supercritical carbon dioxide. Since water is produced, it is important to control the amount of
water in the system, especially in a continuous system. Suggestions for improved process designs have been given.

Amine groups can form carbamates with carbon dioxide. Therefore, the behavior of amines in various carbon dioxide-rich solvents has been evaluated. \(^{1}\)H and \(^{13}\)C NMR spectra in DMSO, chloroform and benzene show that the amines are completely converted into carbamic acid by just bubbling atmospheric carbon dioxide through the solution. Changing the solvent to supercritical carbon dioxide shows a temperature- and pressure-dependent equilibrium between the amine and the carbamic acid; higher pressures shift the equilibrium towards the carbamic acid, whereas higher temperatures favor the free amine.

Based on the spontaneous reaction of amines with carbon dioxide, the possibilities of producing polyurethanes in carbon dioxide without phosgene are explored. The first route describes the use of carbon dioxide as a building block employing the reaction of carbon dioxide with a diamine. The formed carbamic acid is dehydrated to a diisocyanate and upon addition of a diol, polyurethane is formed. The major bottleneck and possible solutions for producing this polyurethane in carbon dioxide are given. Another route describes the use of a cyclic carbonate which reacts spontaneously with a diamine. The formed biscarbamate is a linear molecule containing two urethane groups and has two alcohol groups at the end. These alcohol groups react with di-acids in an enzymatic condensation polymerization reaction.

Other types of enzymes are interesting for use in scCO\(_2\) as well. The potential of oxygenases, epoxide hydrolases, aldolases and decarboxylases have been indicated. Furthermore, the most important parameters to take into account for the process design of an enzymatic reaction in supercritical carbon dioxide are the immobilization method, the ionization state of the enzyme and the specific reaction requirements which define the way of processing. The future perspectives for this technology can be found in the combination of improving the stability and activity of the potentially interesting enzymes and an improved integration of the reaction and separation sections.
Samenvatting

De interesse en daarmee ook het aantal toepassingen op industriële schaal op het gebied van witte biotechnologie neemt toe, waarbij de farmaceutische industrie een belangrijke plaats inneemt. Door een toegenomen milieubewustzijn is de vraag naar milieuvriendelijke productiemethodes gestegen. Een algemene gedachte is dat superkritische fluïda gebruikt kunnen worden om de hoeveelheid organisch afval te verminderen. Een extra voordeel van superkritische fluïda is een eenvoudige scheiding van de componenten na de reactie wat een aantal procestechnologische voordelen geeft. Het doel van dit proefschrift is om de toepassingsmogelijkheden van enzymen in superkritisch koolstofdioxide te onderzoeken.

De immobilisatiemethode van Candida antarctica lipase B (Calb) blijkt een belangrijke parameter voor de katalytische activiteit in zowel hexaan als superkritische koolstofdioxide. Calb is gebruikt in twee verschillende immobilisatievormen: Novozyme 435, geïmmobiliseerd op een drager, en ChiroCLEC™-CAB, een gekristalliseerd en gecrosslinked enzym. Voor Novozyme 435 geldt dat de katalytische activiteit in superkritisch koolstofdioxide onder alle geteste condities lager is dan de katalytische activiteit in hexaan. Voor ChiroCLEC™-CAB geldt juist dat de katalytische activiteit in superkritisch koolstofdioxide altijd hoger is dan de activiteit in hexaan. Verder blijkt dat er een verschil is in de drukafhankelijkheid. De activiteit van Novozyme 435 geeft een optimum als functie van de druk, terwijl de activiteit van ChiroCLEC™-CAB afneemt met toenemende druk. In een continu proces behoudt ChiroCLEC™-CAB de katalytische activiteit, welke een afhankelijkheid vertoont met de waterconcentratie. Bij hoge CO₂ doorstroomsnelheden wordt er water onttrokken aan het enzym waardoor de activiteit daalt. Deze daling kan worden hersteld door extra water toe te voegen.

Een gecrosslinked aggregaat van Calb, CLEA-Calb, is gebruikt in een veresteringsreactie in superkritisch koolstofdioxide. De stabiliteit van CLEA-Calb is goed, aangezien de activiteit na verschillende reacties uitgevoerd te hebben nog steeds even hoog is. Omdat water geproduceerd wordt in dit type reactie is het
belangrijk dat de totale hoeveelheid water in het systeem gecontroleerd wordt. Dit is vooral van belang in een continu proces. Verschillende suggesties om de procesvoering te optimaliseren zijn besproken.

Vrije aminogroepen kunnen carbamaten vormen met koolstofdioxide. Daarom is gekozen naar het gedrag van amines in verschillende koolstofdioxide-rijke oplosmiddelen. $^1$H and $^{13}$C NMR spectra in DMSO, chloroform en benzeen laten zien dat het amine volledig omgezet wordt tot een carbamaat wanneer er koolstofdioxide door de oplossing geborneeld wordt. Wanneer het amine in superkritisch koolstofdioxide gebracht wordt, blijkt er een temperatuur- en drukafbhiindelijk evenwicht tussen het amine en het gevormde carbamaat te bestaan. Hierbij leidt een hogere druk tot een verschuiving naar het carbamaat, terwijl een hogere temperatuur leidt tot een verschuiving naar het amine.

Een aantal mogelijkheden om polyurethanes te maken zonder hierbij fosgeen te gebruiken zijn onderzocht. De eerste route betreft het gebruik van koolstofdioxide, gebruik makend van de spontane reactie van amines met koolstofdioxide. Het gevormde carbamaat wordt gedehydrateerd tot een diisocyanaat en na de toevoeging van een diol wordt een polyurethaan gevormd. Het belangrijkste probleem bij uitvoering van deze route in koolstofdioxide is de dehydratatiestap. De tweede route beschrijft het gebruik van een cyclisch carboonat welke spontaan reageert met een diamine. Het gevormde bis-carbamaat is een lineair molecuul waarin twee urethaangroepen zitten met twee alcoholgroepen aan het einde. Deze alcoholgroepen kunnen met dizuren reageren in een enzymatische condensatiepolymerisatie.

Alle beschreven resultaten zijn verkregen met een lipase, maar ook andere type enzymen kunnen interessant zijn voor gebruik in superkritisch koolstofdioxide. De potentie en huidige limiteringen van oxygenases, epoxide hydrolases, aldolases en decarboxylases voor gebruik in superkritisch koolstofdioxide is beschreven. Verder blijkt uit de resultaten dat de belangrijke parameters voor enzymatische katalyse de immobilisatiemethode en de ‘ionisatiestaat’ van het enzym zijn. Ook zijn de optimale condities waaronder de reactie uitgevoerd wordt voor verschillende typereacties verschillend. De toekomst van het gebruik van enzymatische katalyse ligt vooral in de combinatie van het verbeteren van de stabiliteit en activiteit van
potentieel interessante enzymen en een verbeterde integratie van de reactie en scheidingsstappen nodig om het gewenste product zuiver te verkrijgen.
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