Chemo-enzymatic epoxidation of rapeseed methyl esters:
Parameters influencing the reaction and enzyme stability

Ana Filipa Jacinto Severiano

Dissertação para a obtenção do grau de Mestre em
Engenharia Biológica

Júri:
Presidente: Prof. Luis Joaquim Pina da Fonseca
Orientador: Prof. Maria Manuela Regalo da Fonseca
Prof. Rajni Hatti-Kaul
Vogais: Prof. Suzana Ferreira-Dias Vicente

Setembro de 2008
Chemo-enzymatic epoxidation of rapeseed methyl esters:

Parameters influencing the reaction and enzyme stability

Ana Filipa Jacinto Severiano

Supervisor at Lund University
Department of Biotechnology (Lund, Sweden):
Anna Hagström

 Examiner at Lund University
Department of Biotechnology (Lund, Sweden):
Professor Rajni Hatti-Kaul

Supervisor at Instituto Superior Técnico (Lisbon, Portugal):
Professor Maria Manuela da Fonseca
Acknowledgements

I would like to thank all the people that helped me in this project.

Thank you to Professor Rajni Hatti-Kaul for giving me the opportunity to work in this project.

To Anna Hagström, for the guidance at the lab, for correcting my thesis, for picking me up in the first day at the train station, for getting me an appointment when I was sick. Thank you Mathias for helping me with the GC and Pår for translating the conversations. Thank you to the Bioorganic group for Fika.

To all the Portugueses in Lund, Bruno, Medronho, Ana Tomás, Luís, Salomé, Custódio, Jorge, Telmo, Tiago, Zé, Miguel, thanks for sharing the best and the worst of Sweden with me.

To my international friends, Stefano, Zsolti, Alba, Thomas, Keiichi, Agniezka, Charris, Çelem, İrem, Ivana, Reno. It was really a pleasure to meet you! To my corridor mates, Bruno, Stefano, Stephan and Rodrigo for being so nice and for keeping everything organized.

To Gui and Inês, my nephews, for talking with me on the phone and for giving me so much love. To my father, my mother and my sisters for always care about me.

To Paulo who always supported me and made me feel more optimistic.
Abstract

The chemo-enzymatic epoxidation of rapeseed methyl ester (RME) in a solvent-free medium has been studied using the immobilized *Candida antarctica* lipase B (Novozym® 435) as a catalyst in the peracid formation. Hydrogen peroxide was used as the oxygen donor. After the peracid formation, spontaneous transfer of the oxygen to the double bond occurs, and the epoxidized product is formed.

The enzyme cost is an important factor determining the economics of the process. High stability and the possibility to recycle the enzyme are therefore highly desirable. The parameters affecting the reaction performance were optimized in order to minimize the deactivation of the enzymatic preparation, allowing its recycling and maximizing the productivity in a shorter reaction time.

The hydrogen peroxide concentration was found to have a significant effect both on the epoxidation and the enzyme deactivation rates. The stability of the enzyme was studied in a 30 ml scale for initial hydrogen peroxide concentration in the water phase of 5, 10 and 20% (w/w). The epoxidation rate increased with increasing hydrogen peroxide concentration, however at the expense of enzyme inactivation. A temperature increase has a similar effect: the reaction rate increases but the residual enzymatic activity is reduced.

The epoxidation of RME was also performed in a 300 ml scale, in a batch reactor with continuous addition of hydrogen peroxide solution. Maintaining an approximately constant concentration of hydrogen peroxide in the water phase of 15 M, at 40°C and with an enzyme loading of 3%, a conversion of 83% was obtained after 14 hours and a residual enzymatic activity of 61%.

*Keywords:* Rapeseed methyl ester; epoxidation; Novozym® 435, operational stability.
Resumo

A epoxidação quimo-enzimática do éster metílico de colza (RME – *Rapeseed Methyl Ester*) foi estudada em meio sem solvente e utilizando como catalisador uma preparação comercial de lipase B de *Candida antarctica* (Novozym® 435). O peróxido de hidrogénio foi utilizado como dador de oxigénio. Após formação do perácido o átomo de oxigénio é espontaneamente transferido para a ligação dupla formando-se o produto epoxidado.

O custo da enzima é um dos importantes factores que determinam a viabilidade económica do processo. Elevada estabilidade e a possibilidade de reciclar a enzima são assim desejáveis. Procedeu-se à optimização dos principais parâmetros que afectam a reacção, de modo a maximizar a produtividade e diminuir o tempo de reacção.

A concentração de peróxido de hidrogénio mostrou ter influência significativa quer na velocidade da reacção quer na taxa de desactivação enzimática. Estudou-se a estabilidade operacional numa escala de 30 ml para concentrações iniciais de peróxido de hidrogénio na fase aquosa de 5, 10 e 20% (w/w). A velocidade da reacção aumentou com o aumento da concentração de peróxido de hidrogénio à custa, no entanto, de uma maior desactivação enzimática. O aumento da temperatura tem um efeito semelhante: a velocidade da reacção aumenta mas a actividade enzimática residual diminui.

A epoxidação do RME foi estudada ainda numa escala de 300 ml, num reactor *batch* com adição contínua de peróxido de hidrogénio. Mantendo-se uma concentração de peróxido de hidrogénio na fase aquosa de aproximadamente 15 M, a 40°C e com uma carga enzimática de 3%, foi obtida uma conversão de 83% após 14 horas e uma actividade enzimática residual de 61%.

**Palavras-chave:** Éster metílico de colza; epoxidação; Novozym® 435, estabilidade operacional.
Table of Contents

1 Introduction ................................................................................................................... 1
  1.1 Rapeseed methyl ester ............................................................................................. 2
  1.2 Epoxides .................................................................................................................... 2
  1.3 Chemo-enzymatic epoxidation .................................................................................. 3
  1.4 Lipases ....................................................................................................................... 4
    1.4.1 Reactions catalyzed........................................................................................... 5
    1.4.2 Candida antarctica lipase B ............................................................................... 6
    1.4.3 Stability of Candida antarctica lipase .............................................................. 7
      1.4.3.1 Operational stability ............................................................................. 8
  1.5 Parameters affecting the chemo-enzymatic epoxidation .............................................. 9
    1.5.1 Temperature ..................................................................................................... 9
    1.5.2 Enzyme loading ................................................................................................. 9
  1.6 Operation modes ......................................................................................................... 10
    1.6.1 Batch reactor ................................................................................................... 10
    1.6.2 Hilker reactor ................................................................................................... 10
    1.6.3 Membrane reactor .......................................................................................... 11

2 Aim of the study ........................................................................................................... 12

3 Materials and Methods ................................................................................................. 13
  3.1 Materials ................................................................................................................... 13
  3.2 Methods ..................................................................................................................... 13
    3.2.1 RME epoxidation in 30 ml scale ...................................................................... 13
    3.2.2 RME epoxidation in 300 ml scale .................................................................... 14
    3.2.3 Oxirane number titration ................................................................................ 14
    3.2.4 Acid number titration ...................................................................................... 15
    3.2.5 Hydrogen peroxide measurement .................................................................. 16
    3.2.6 Enzyme activity assay by esterification ........................................................... 16

4 Results and discussion .................................................................................................. 18
  4.1 Chemo-enzymatic epoxidation of RME in 30 ml scale ................................................. 18
    4.1.1 Hydrolysis ........................................................................................................ 19
    4.1.2 Hydrogen peroxide concentration .................................................................. 21
    4.1.3 Stability of Novozym® 435 during chemo-enzymatic epoxidation of RME in 30 ml scale ........................................................................................................... 23
    4.1.4 Optimal temperature for the chemo-enzymatic epoxidation of RME .......... 26
  4.2 Chemo-enzymatic epoxidation of RME in 300 ml scale ............................................... 28
    4.2.1 Comparison with the results obtained in the 30 ml scale ................................ 28
    4.2.2 Continuous additions of hydrogen peroxide ................................................... 30
List of tables

Table 1. Fatty acid composition of rapeseed methyl ester. ............................................... 2
Table 2. Initial hydrogen peroxide concentration in the water phase, weight of RME, volume of water and hydrogen peroxide 35% added, total system volume and percentage of the organic phase in the system volume. ................................................................. 13
Table 3. Comparison between the reaction performed with 10% initial H₂O₂ concentration in the water phase added at once in the beginning of the reaction and a reaction with continuous additions. ........................................................................................................ 33
Table 4. Comparison between reactions II and III. .......................................................... 35

List of figures

Figure 1. Chemo-enzymatic epoxidation of methyl esters .............................................. 3
Figure 2. Chemo-enzymatic epoxidation of methyl esters. ............................................. 4
Figure 3. Hilker or enzyme-recycle reactor ...................................................................... 11
Figure 4. Oxirane number during chemo-enzymatic epoxidation of RME in 30 ml scale, at 40°C, with an enzyme loading of 3% (w/w) (Novozym® 435) and an initial concentration of hydrogen peroxide in the water phase of 5%, 10% and 20%.. 18
Figure 5. Oxirane number during chemo-enzymatic epoxidation of RME at 40°C, with an enzyme loading (Novozym® 435) of 3% (w/w) and an initial concentration of hydrogen peroxide in the water phase of 20% and 35%. 19
Figure 6. Percentage of hydrolysis during chemo-enzymatic epoxidation of RME in the 30 ml scale, at 40°C, with an enzyme loading of 3% (w/w) and an initial concentration of hydrogen peroxide in the water phase of 5%, 10% and 20%................. 20
Figure 7. Percentage of hydrolysis after 24 hours versus percentage of organic phase in the total volume (v/v) in chemo-enzymatic epoxidation of RME at 40°C and with an enzyme loading (Novozym® 435) of 3% (w/w) ..........................................................20

Figure 8. Hydrogen peroxide concentration in the water phase during chemo-enzymatic epoxidation of RME in a 30 ml scale, at 40°C, with an enzyme loading of 3% (w/w) and an initial concentration of hydrogen peroxide in the water phase of 5%, 10% and 20% ......................................................................................................................21

Figure 9. Hydrogen peroxide concentration in the organic phase during chemo-enzymatic epoxidation of RME in a 30 ml scale, at 40°C, with an enzyme loading (Novozym® 435) of 3% (w/w) and an initial concentration of hydrogen peroxide in the water phase of 10% and 20% ...........................................................................................................................................22

Figure 10. Epoxidation of RME in a 30 ml scale with different initial hydrogen peroxide concentration in the water phase. Volumetric initial percentage of the organic phase in the system volume, final productivity per grams of catalyst, final conversion and hydrolysis after 24 hours of reaction and initial productivity ..........23

Figure 11. Remaining enzyme activity of Novozym® 435 during the chemo-enzymatic epoxidation of RME in a 30 ml scale ..............................................................................................................................................24

Figure 12. Oxirane number during the chemo-enzymatic epoxidation of RME. All reactions were performed in a 30 ml scale, with 5% initial hydrogen peroxide concentration in the water phase, at 40°C and 3% (w/w) enzyme loading. Series 1 represents 6 of the reactions performed; series 2 represents the 3 last reactions performed, in which a higher conversion is observed. ...........................................................................................................25

Figure 13. Oxirane number during the chemo-enzymatic epoxidation of RME. All reactions were performed in a 30 ml scale, with 10% initial hydrogen peroxide concentration in the water phase, at 40°C and 3% (w/w) enzyme loading. Series 1 represents 7 of the reactions performed; series 2 represents the 3 last reactions performed, in which a higher conversion is observed; series 3 represents 4 of the reactions performed, in which a lower conversion is observed ..............................................26

Figure 14. Oxirane number during the chemo-epoxidation of 12 g of RME, at 40°C and 50°C with 3% (w/w) enzyme loading and an initial hydrogen peroxide concentration in the water phase of 10% ...........................................................................................................................................27
**Figure 15.** Percentage of hydrolysis during the chemo-epoxidation of 12 g of RME, at 40°C and 50°C with 3% (w/w) enzyme loading and an initial hydrogen peroxide concentration in the water phase of 10%.

**Figure 16.** Oxirane number during the chemo-enzymatic epoxidation of RME in 30 ml scale and in 300 ml scale with an initial hydrogen peroxide concentration in the water phase of 5%, 10% and 20%.

**Figure 17.** Percentage of hydrolysis during the chemo-enzymatic epoxidation of RME in 30 ml scale and 300 ml scale with an initial hydrogen peroxide concentration in the water phase of 5%, 10% and 20%.

**Figure 18.** Oxirane number and hydrogen peroxide addition rate during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction I 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H₂O₂ solution. 54 ml H₂O₂ (35% solution) was added continuously with decreasing addition rate.

**Figure 19.** Hydrogen peroxide concentration in the water phase and organic phase during the chemo-enzymatic epoxidation of RME with continuous addition of hydrogen peroxide (Reaction I).

**Figure 20.** Oxirane number and H₂O₂ addition rate during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction II 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H₂O₂ solution. 75.6 ml H₂O₂ (35% solution) was added continuously with decreasing addition rate.

**Figure 21.** Hydrogen peroxide concentration in the water phase organic phase during the chemo-enzymatic epoxidation of RME with continuous addition of H₂O₂ (Reaction II).

**Figure 22.** Percentage of hydrolysis during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction II 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H₂O₂ solution. 75.6 ml H₂O₂ (35% solution) was added continuously; For Reaction III 6.0 g of Novozym® 435 was used with 200 g of RME and 20 ml of 5% concentration H₂O₂ solution. 57.8 ml H₂O₂ (60% solution) was added continuously.
Figure 23. Oxirane number during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction II 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H₂O₂ solution. 75.6 ml H₂O₂ (35% solution) was added continuously; For Reaction III 6.0 g of Novozym® 435 was used with 200 g of RME and 20 ml of 5% concentration H₂O₂ solution. 57.8 ml H₂O₂ (60% solution) was added continuously.

Figure 24. Hydrogen peroxide concentration in the water phase organic phase during the chemo-enzymatic epoxidation of RME with continuous addition of H₂O₂ for 12 hours (Reaction III).
1 Introduction

Implementing sustainable production has been a key issue for the chemical industry (1). On the way to sustainable production it is important to shift from fossil to renewable feedstocks. The increasing cost of petrochemicals and the high rate of depletion of this natural resource drives the search for new raw materials that can be synthesized into more environmentally friendly products.

Vegetable oils and their component fatty acids are important raw materials, offering a great number of possibilities for applications of interest for the chemical industry. They are replacing petroleum in the production of environmentally friendly products for coatings, paints, lubricants and surfactants, and even as fuel (1; 2). The increasing broad of applications for vegetable oils and specially the utilization as biodiesel makes the competition for land an important issue and may result in a higher price of food crops. Many of the applications require the modification of the oils or fatty acids by chemical or biotechnology processes. Epoxidation of the C-C double bond is one of the most important modification reactions, giving vegetable oils and fatty acids a more reactive group than the unsaturation (1). These epoxides can be produced by biocatalytic synthesis, using enzymes as a catalyst. The use of enzymes to catalyze reactions has recognized importance in several industries, including cosmetic, textile, paper, food and pharmaceutical.

The forces stabilizing an enzyme in its native state are not very strong (hydrogen bonds, Van der Waal bonds and hydrophobic interactions). Therefore, it is often easy to influence the enzyme conformation in such way that the enzyme activity is lost. The limited storage and specially the extremely limited operational stability of many enzymes is the major reason for their limited success as catalysts in a larger scale. Much effort has been put into various attempts to find rational ways to stabilize enzymes. Immobilization provides enzymes with new properties and often means improved stability due to increased molecular rigidity and locally high protein concentration (3). Besides that, immobilized preparations are easier to handle and thus easier to reuse. Therefore they are suitable for continuous processes, which make them very attractive in many industrial applications.
1.1 Rapeseed methyl ester

Rapeseed is an oil crop cultivated worldwide not only for food, but also to meet the demands of the chemical and energy sectors (1). Currently the main application is the production of rapeseed methyl ester (RME) for use as biodiesel. The increasing demand for biodiesel has increased the worldwide production of rapeseed to 46.4 million metric tonnes in 2005 (1).

The fatty acid composition of RME is given in Table 1.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>4.4</td>
</tr>
<tr>
<td>Stearic</td>
<td>1.6</td>
</tr>
<tr>
<td>Oleic</td>
<td>59.2</td>
</tr>
<tr>
<td>Linoleic</td>
<td>21.4</td>
</tr>
<tr>
<td>Linolenic</td>
<td>10.7</td>
</tr>
<tr>
<td>Arachidic</td>
<td>0.6</td>
</tr>
<tr>
<td>Gadoleic</td>
<td>1.2</td>
</tr>
<tr>
<td>Erusic</td>
<td>0.2</td>
</tr>
<tr>
<td>Others</td>
<td>0.7</td>
</tr>
</tbody>
</table>

It has been previously shown that rapeseed methyl ester can be used for production of fatty epoxides by an atom-efficient solvent-free lipase mediated process (1).

1.2 Epoxides

The epoxy group is a highly reactive moiety, which makes epoxides an important group of industrial organic intermediates. Polarity and ring strain make the oxirane ring highly reactive. Thus epoxides participate in numerous reactions, which make these compounds useful building blocks in organic synthesis (4).

Epoxidized plant oils constitute a valuable alternative to petroleum-based epoxides, due to their low toxicity and their availability from renewable resources. Epoxides derived from plant oils are used as PVC stabilizers and plasticizers and in polyurethane production. They can also be useful as reactive diluents for paints, production of surfactants, corrosion protection agents, additives to lubricants and in various types of composite materials.
Epoxidized oils, fatty acids and fatty acid esters are produced on an industrial scale via the Prileshajev epoxidation reaction (2). In this reaction, a peracid is used for the oxygen transfer to the double bonds in the fatty acid chains. The peracid is usually formed \textit{in situ} from acetic or formic acid, hydrogen peroxide and a strong mineral acid catalyst (1). The presence of the strong acid causes the formation of by-products.

### 1.3 Chemo-enzymatic epoxidation

The chemo-enzymatic epoxidation of vegetable oils is an alternative to chemical epoxidation and has been reported for the first time in 1995 by Warwel and Klaas (5). With the help of a biocatalyst for the generation of the peracid, epoxidation can be performed in high yields under mild and selective conditions, i.e. moderate temperatures and neutral pH, without the need of acetic acid or formic acid (1).

The mechanism of chemo-enzymatic epoxidation of methyl esters involves a two-step reaction (Figure 1). In the first step, the methyl ester reacts with hydrogen peroxide to form the peracid. This is a catalyzed step. In the second step the peracid donates the oxygen to a double bond, via an intermolecular process to form the epoxide. Both unsaturated fatty acid and fatty acid ester molecules can undergo the epoxidation.

![Figure 1](image)

\textbf{Figure 1.} Chemo-enzymatic epoxidation of methyl esters. First the lipase catalyses the peracid formation. Then the peracid spontaneously donates the oxygen to a double bond, to form the epoxide.

As an alternative to the mechanism in Figure 1, hydrolysis of the ester bond can take place. In this case the peracid is formed from the acid (Figure 2).
In the first studies, the chemo-enzymatic epoxidation has been carried out in an organic solvent but more recently the reaction has been performed in solvent-free medium, with the advantages of a more environmentally friendly process, a more volume efficient reactor and a simplified separation process (2).

As another environmental advantage, the epoxide product from the chemo-enzymatic epoxidation of RME is biodegradable and non-toxic up to high concentrations. The epoxidized RME showed no toxicity in a fast screening test using activated sludge flora between the concentrations tested by Törnvall et al., from 50 to 1000 ppm. The biodegradability test showed the RME to have a BOD5/COD value of 0.541 (1).

### 1.4 Lipases

Enzymes can increase the reaction rate by factors of at least one million by lowering the transition state energy. Changes in the enzymatic structure are often destructive in terms of enzyme activity. In fact, the mutation of one amino acid essential for the catalytic reaction is enough to significantly reduce the activity (6). The advantage of enzymes as catalysts is their high selectivity. Products of high purity can be produced by enzymatic synthesis at lower

---

1 Compounds with a BOD/COD ratio between 0.4-0.8 are regarded as biodegradable (1).
temperatures and with a minimum of chemicals when compared with the traditional synthetic chemistry (7).

Lipases (Triacylglycerol acylhydrolase, E.C. 3.1.1.3) are ubiquitous enzymes found in microorganisms, plants and animals. Their natural reaction is the hydrolysis of triglycerides into fatty acids, diglycerides, monoglycerides and glycerol.

The active site of lipases consists of a catalytic triad of serine, histidine and aspartame/glutamate residues. The reaction mechanism involves two steps with the formation of a stable acyl-enzyme complex.

In the first step the enzyme serine residue in the active site makes a nucleophilic attack on the carbonyl carbon of the acyl donor to form a tetrahedral intermediate, characterized by a negatively charged carbonyl oxygen atom. A proton is donated to the oxygen in an ester bond, which is thus cleaved. The product leaves the active site and the acyl-enzyme is formed. In the second step the acyl-enzyme is subjected to the nucleophilic attack resulting in a regenerated free enzyme and a final product molecule.

The number of electrophiles capable of forming acyl-enzyme complexes and the number of nucleophiles capable of deacylation of the complex is very large. Examples of acyl donors are carboxylic acids, carboxylic esters, thio-esters, and carbonates. Water, alcohols, hydrogen peroxide, amines and ammonia can serve as nucleophiles (4).

1.4.1 Reactions catalyzed

Lipases are versatile catalysts and therefore they have been used to catalyze a variety of reactions including hydrolysis, esterification, acidolysis, alcoholysis, transesterification, aminolysis and perhydrolysis. All these reactions are reversible and the amount of water present in the reaction mixture determines the direction of the reaction (4).

Hydrolysis is the reaction of ester with water producing acids and alcohols. Lipases catalyze for example the hydrolysis of triglycerides (4). Esterification is the reverse of ester hydrolysis and is performed by reaction of an acid and an alcohol in an environment with low water activity, to drive the equilibrium towards the ester formation. It is a very useful reaction for synthesis of a wide variety of products that can be used as surfactants, emulsifiers and personal care additives. The reaction of acid or ester with hydrogen peroxide giving peroxide acids is called
**perhydrolysis.** The ability of lipase to catalyze the formation of peracids has been used for epoxidation of fatty acids and fatty acid esters.

**Transesterification** is the process of exchanging an acyl group of an ester with another acyl group (8). If the acyl donor is a free fatty acid, the reaction is called **acidolysis**, which has successfully been applied for synthesis of phospholipids with defined fatty acid composition. If the acyl donor is an ester, the reaction is called **interesterification**. A good example of this type of reaction is the preparation of substitutes of cocoa butter from palm oil. If the acyl donor is an alcohol the reaction is called **alcoholysis** (4).

**Aminolysis** is a reaction of an ester with an amine to produce an amide.

Regarding its versatility and the number of reactions catalyzed, lipases may be used to produce fatty acids, biosurfactants, aroma and flavor compounds, lubricants and solvent esters, polyesters and bimodified fats, synthesis of biopolymers, biodiesel, and production of enantiopure pharmaceuticals, agrochemicals and flavor compounds (4).

### 1.4.2 Candida antarctica lipase B

"*Candida antarctica,* one of 154 species of the genus *Candida,* belongs to the Phylum Ascomycota and to the Class Ascomycetes. It is an alkali-tolerant yeast found in the sediment of Lake Vanda, Antarctica."

Two lipase variants from *Candida antarctica*, lipase A (CALA) and B (CALB), present extreme properties and have proven to be of particular interest to researchers (4). CALB is stable at a relatively higher pH (pH 7 – 10) as compared with CALA (pH 6 – 9). CALA is extremely thermostable with an optimum temperature of over 90°C. CALB is not as stable in solution, however, in an immobilized form it can retain its activity at higher temperatures for long periods. The structure of CALB was determined in 1994. It is constituted by 317 amino-acids and its core is a β-sheet formed by seven parallel β-strands (4).

One useful immobilized preparation of *Candida antarctica* lipase B is Novozym® 435. The lipase is produced by submerged fermentation of a genetically modified fungus, *Aspergillus* sp., the enzyme is isolated, concentrated and thereafter absorbed on a macroporous resin (4).

Novozym® 435 is probably the most commonly used lipase in biocatalysis, with activity towards a great variety of different substrates. Even though Novozym® 435 is used as a
catalyst in the synthesis of carbohydrate monoesters, it is primarily used as a high enantioselective catalyst in the synthesis of optically active alcohols, amines and carboxylic acids (4). The immobilized enzyme is a very thermostable and robust catalyst with activity in a number of organic solvents and also in solvent-free media. It can be used both in batch and column reaction operations, but is particularly well suited for fixed-bed reactors (4). Based on The advantages and the broad industrial applications, it would be of great interest to develop a cost-effective industrial process using Novozym® 435.

Among the various lipases studied so far, Novozym® 435 has been shown to be the most effective to convert unsaturated compounds to the corresponding epoxide.

1.4.3 Stability of *Candida antarctica* lipase

Deactivation of enzymes can be caused by several reaction parameters, including temperature, reactant ratio as well as substrate and co-substrate concentrations. Loss of water, essential for enzyme activity, can also decrease the lipase activity. Almost all enzymes become denatured if they are heated much above the physiological temperature. The denaturation can be reversible or irreversible. During irreversible denaturation, the conformation of the enzyme is altered such that the protein is not able to refold to its native conformation, which leads to a permanent loss of catalytic activity. Other parameters can also affect the enzymatic activity. Some of these parameters result in chemical modifications in the polypeptide chain, e.g. by oxidation, amidation, etc.

Hydrogen peroxide is a strong oxidizing agent. Oxidation of surface amino acids on a protein may not affect the enzyme activity, but side-chain to side-chain transfer reactions have been reported, transferring the initial site of oxidation to readily oxidized amino acids. Oxidation of amino acids buried within the hydrophobic interior of the protein molecule and those participating in the catalytic mechanism can have drastic effects on the enzyme conformation and activity (6). The amino acids that are sensitive to oxidation are methionine, histidine, cysteine, tryptophan and tyrosine (6).

Some authors reported loss of catalytic activity caused by impurities in the oils, and therefore they recommend the degumming of oils to remove phospholipids prior to lipase-catalyzed modification (8). Peroxides can also deactivate lipases. Activity loss of a pure, free lipase was caused by polymerization of the lipase by peroxidation products in the reaction mixture.
Pretreatment of oils with small amounts of base can reduce lipase deactivation. When interesterification of rapeseed oil and palm oil was carried out in the presence of a basic solution, the half-life of the enzyme activity was six times longer than without pre-treatment (8).

1.4.3.1 Operational stability
Operational stability is a particular term which indicates the useful lifetime or productivity of a given catalyst preparation. It does not necessarily reflect the specific activity of an enzyme (8). This is because the preparation may consist of an excess of enzyme so that several layers of catalysts are formed (8).

The enzyme cost strongly determines the economic feasibility of most of the enzymatic processes. Therefore, high enzyme stability and the possibility to recycle the enzyme are highly desirable (5). Immobilization preparations can increase dramatically the enzyme stability and make the downstream processing easier (8).

Warwel and Klaas developed an epoxidation process in toluene, where they found that the enzyme (Novozym® 435) was highly stable, with 75% of the activity being left after 15 reaction cycles (5). When Orellana-Coca and colleagues performed the chemo-enzymatic epoxidation in a solvent-free process and under conditions optimized for achieving high reaction rates and product yields, the enzyme was found to suffer loss in activity, hence limiting its recycling (2).

It has earlier been suggested that the very reactive peracid is the main cause of deactivation in the chemo-enzymatic epoxidation (9). Also the epoxidized fatty acid could be suspected to have an adverse effect on the enzyme activity due to its reactive nature. Törnvall et al. have investigated the deactivation of Novozym® 435 in contact with the epoxidation substrates and products at various temperatures (9). No enzyme deactivation could be detected after incubation with oleic acid. With epoxystearic acid, the lipase activity decreased to 70% of the original level (9). The same group did not observe any deactivation effect by the very reactive peracid.

Hydrogen peroxide is the only reactant besides RME, added for the chemo-enzymatic epoxidation and it has been proven to have a significant influence on the stability of immobilized Candida Antarctica lipase B during epoxidation of fatty acids (9).
It is obvious that a careful choice of the reaction conditions is critical for the Novozym® 435 stability, and to the development of an industrial cost-effective process for chemo-epoxidation of RME using this enzyme.

1.5 Parameters affecting the chemo-enzymatic epoxidation

1.5.1 Temperature

The effect of reaction temperature on the chemo-enzymatic epoxidation of linoleic acid (0.5 M) was evaluated by Orellana-Coca et al. (2). The reaction was performed at 20-60°C, using 10 mg of enzyme per mmol of linoleic acid. 2 mmol of 30% (w/w) H₂O₂ was added per mmol of fatty acid. They observed that at 40 and 50°C the reaction was complete and only the diepoxide was present, but at 60°C the presence of monoepoxides was observed, suggesting that the reaction was not complete, perhaps due to H₂O₂ decomposition and/or enzyme inactivation. The highest reaction rate was observed at 50°C (2).

In another study, Törnvall et al. investigated the parameters affecting the lipase activity in chemo-enzymatic epoxidation of fatty acids (9). The Novozym® 435 was incubated in the presence of various reaction components at temperatures between 20 and 60°C. To perform this study the enzyme preparation (100 mg Novozym® 435) was incubated with 2 ml toluene with a water-H₂O₂ phase volume of 200 µm. The residual activity was determined by an esterification reaction. They observed that the Novozym® 435 was stable even at 60°C when there was no H₂O₂ in the preparation. However, at 60°C and in the presence of 6 to 12 M H₂O₂, the enzyme lost activity rapidly. They also observed that the rate of deactivation increased with increasing hydrogen peroxide concentration (9). The temperature by itself does not have an effect on the deactivation within the temperature range studied, i.e. up to 60°C, since they found that the enzyme was stable at 60°C, when hydrogen peroxide was not present.

1.5.2 Enzyme loading

The amount of enzyme required to keep in pace with hydrogen peroxide addition is an important factor.
Orellana-Coca et al. performed a recycling study in which they used an excess of enzyme, to overcome the inactivation problem (10). They were able to do five cycles of reaction before the conversion dropped dramatically in the sixth cycle. This probably indicates that the enzyme loading was much higher than would be needed if no enzymatic deactivation occurs.

In subsequent studies, the chemo-enzymatic epoxidation of RME with much lower amounts of enzyme was studied (Hagström, non-published data). The studies have been performed with an enzyme loading between 0.5 and 10% (w/w) based on the RME amount.

1.6 Operation modes

1.6.1 Batch reactor

The chemo-enzymatic epoxidation of vegetable oils has been studied in a small scale, and therefore the preferential operation mode is the Batch. The batch operation mode has several advantages, including his simple operation and good mass transfer between the two phases. However, when the deactivation of the enzyme is pointed as cause of H2O2 contact, it can be beneficial to separate the water from the organic phase, and thus reduce the contact between the water phase with higher concentration of hydrogen peroxide and the enzymatic preparation. High yields can be obtained in this operation mode but the reusability of the enzyme might not be possible due to activity loss.

1.6.2 Hilker reactor

The Hilker or enzyme-recycle reactor consists on a semi-batch recycle reactor constructed to separate the aqueous hydrogen peroxide solution from the enzyme which could be oxidized and deactivated (Figure 3).

The enzyme particles are dispersed within the organic phase in a stirred tank reactor. In the outlet, a membrane is installed to hold back the particles. To prevent the membrane from blocking, it is flushed quickly at regular time intervals by turning the direction of the pump. In order to achieve saturation with hydrogen peroxide, the organic phase is passed through a bubble column filled with aqueous H2O2 (11).
This reactor was used to perform the chemo-enzymatic epoxidation in an organic solvent. It would be useful to perform studies in solvent free media and analyze the effect on enzyme deactivation of this type of reactor.

### 1.6.3 Membrane reactor

Another approach to separate the aqueous hydrogen peroxide solution from the enzyme would be the use of a selective membrane separating the organic phase with the enzyme preparation from the aqueous phase. The diffusion of hydrogen peroxide across the membrane would allow the peroxidation to take place in the organic phase.
2 Aim of the study

The aim of this study is to optimize the chemo-enzymatic epoxidation of RME in a solvent-free medium and identify the parameters that most significantly have an effect on the enzyme (Novozym® 345) inactivation. Various reaction variables with a possible impact on the enzyme activity and its operational stability have been studied.

A deeper knowledge of the factors causing enzyme deactivation will be very useful in both the development of lipase preparations with improved stability and for the process design providing optimal productivity. It is necessary to minimize the enzyme deactivation to create a method that allows enzyme recycling. Therefore, it is important to study the stability of Novozym® 435 in the presence of oxidants, i.e., in the presence of the H₂O₂, which takes part in the epoxidation reaction.

The operational stability of the enzyme as function of the hydrogen peroxide concentration was analyzed. These experiments have been carried out to better understand and define conditions that would lead to higher reaction rates with a minimal concurrent inactivation of the biocatalyst.
3 Materials and Methods

3.1 Materials

Rapeseed methyl ester (industrial grade) was a kind gift from Aarhus Karlshamns AB (Karlshamn, Sweden). Novozym® 435 (immobilized *Candida antarctica* lipase B) was generously donated by Novozymes A/S (Bagsvaerd, Denmark). Hydrogen peroxide (35% (w/w) in water) was purchased from Fischer Scientific (Gothenburg, Sweden). Glacial acetic acid, ethyl acetate, potassium hydroxide and phenolphthalein were purchased from Merck (Darmstadt, Germany). Ethanol (96% (v/v)) and potassium iodide were obtained from VWR, BDH Prolabo (Stockholm, Sweden). Tetraethyl ammonium bromide and potassium phthalate were purchased from Sigma Aldrich.

3.2 Methods

3.2.1 RME epoxidation in 30 ml scale

The reactions were performed in a 50 ml round-bottomed flask. To maintain the total volume and the enzyme loading constant (approximately 30 ml and 3% (w/w) of Novozym® 435, based on RME amount), the quantity of RME and thus the quantity of enzyme and H$_2$O$_2$ were changed in the different reactions. A 10% stoichiometric excess of H$_2$O$_2$ was used, to epoxidize all the RME. The ratio between the organic phase and the water phase was significantly changed when the hydrogen peroxide concentration in the water phase was changed, see Table 2. The reactions were stirred at 450 rpm using a magnetic bar and the reaction temperature was held at 40°C with an oil bath, unless stated otherwise.

In Table 2 the properties of the initial reaction mixtures are shown.

<table>
<thead>
<tr>
<th>H$_2$O$_2$ concentration (%)</th>
<th>RME (g)</th>
<th>Novozym® 435 (g)</th>
<th>H$_2$O$_2$ 35% (ml)</th>
<th>H$_2$O (ml)</th>
<th>Total system volume (ml)</th>
<th>% OP (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>6.58</td>
<td>18</td>
<td>0.54</td>
<td>7.8</td>
<td>34</td>
<td>60%</td>
</tr>
<tr>
<td>10%</td>
<td>3.29</td>
<td>12</td>
<td>0.36</td>
<td>5.2</td>
<td>13.2</td>
<td>42%</td>
</tr>
<tr>
<td>5%</td>
<td>1.65</td>
<td>8</td>
<td>0.24</td>
<td>3.5</td>
<td>21.0</td>
<td>27%</td>
</tr>
</tbody>
</table>
3.2.2 RME epoxidation in 300 ml scale

The reactions were performed in a 500 ml jacketed glass reactor, kept at 40°C by recirculation of water pumped from a water bath. The stirring was performed with an over-head stirrer using a four blade propeller at 350 rpm. The continuous addition of hydrogen peroxide was performed with a peristaltic pump. The total system volume was about 300 ml. The enzyme loading was maintained constant at 3% based on the RME amount.

3.2.3 Oxirane number titration

The reaction was followed by measuring the oxirane number in a similar procedure to what is described by Jay (12). One ml of sample was withdrawn from the reactor and centrifuged for 3 minutes at 13 400 rpm in an appendorf centrifuge (Minispin plus, Eppendorf). About 0.5 g of the organic phase was diluted in 10 ml ethyl acetate, and then 10 ml of 20% tetraethyl ammonium bromide in acetic acid was added. The mixture was titrated with 0.1 M perchloric acid in acetic acid using 702 SM Titrino and Metrhom TiNet 2.5 software (Metrohm, Herisau, Switzerland).

The solution of perchloric acid in acetic acid was prepared by diluting 8.6 ml of HClO₄ (70%) with glacial acetic acid to a total volume of one liter. For the standardization of this solution approximately 0.20 g of dried potassium phthalate was dissolved in 30 ml glacial acetic acid and titrated with the HClO₄ solution. The concentration of the perchloric acid in the acetic acid solution was obtained from the equation 1:

\[
C_{\text{HClO}_4} = \frac{m \times 1000}{M \times V} \tag{1}
\]

with \(m\) being the potassium phthalate weight (in g), \(V\) the consumed volume of the titration solution (in ml) and \(M\) the molecular weight of potassium phthalate (204.24 g mol\(^{-1}\)).

The oxirane number can be calculated according to equation 2 and is expressed in % (w/w), where \(V_{\text{titrand}}\) and \(V_{\text{blanc}}\) is the consumed volume of the HClO₄ solution for the sample titration and the blanc (ml) respectively, \(C_{\text{titrand}}\) is the HClO₄ solution concentration (M), \(M_{\text{oxygen}}\) is the oxygen atomic weight (16 g mol\(^{-1}\)) and \(m_{\text{sample}}\) is the sample weight (g).

\[
\text{Oxirane number} = \frac{(V_{\text{titrand}} - V_{\text{blanc}}) \times C_{\text{titrand}} \times M_{\text{oxygen}}}{m_{\text{sample}} \times 10} \tag{2}
\]
Inserting the concentration of the perchloric acid solution and the weight of the sample, the software will give the oxygen content in mol/g sample after the titration. Therefore, this value has to be multiplied by 1.6 to obtain the oxirane number in % (w/w).

The oxirane number at 100% conversion can be predicted according to equation 3, where $n$ is the number of C-C double bonds per molecule and $M$ is the molecular weight of the substrate. For RME, the maximum oxirane number was found to be 6.8.

$$\text{Max. ox. no.} = \frac{n \times M_{\text{oxygen}}}{M + n \times M_{\text{oxygen}}}$$  \hspace{1cm} (3)

3.2.4 Acid number titration

Approximately 0.1 g of the centrifuged organic phase (3 minutes, at 13 400 rpm in an eppendorf centrifuge) was dissolved in 10 ml ethyl acetate. About 20 drops of phenolphthalein (1% in ethanol) were added. The solution was titrated with a 0.25 M KOH solution in ethanol until a shift of color was observed. The acid number was calculated from the amount of KOH consumed to neutralize the acid in the sample according to equation 4, where $V$ (ml) is the volume of titrand used to reach the color shift, $C_{\text{KOH}}$ (M) is the concentration of the titrand, $M$ is the potassium hydroxide molecular weight (56.11 g mol$^{-1}$) and $m$ (g) is the sample size.

$$\text{Acid no.} = \frac{V \times C_{\text{KOH}} \times M}{m}$$ \hspace{1cm} (4)

For standardization of the KOH solution, approximately 0.03 g of dried benzoic acid was dissolved in 10 ml ethanol and 1 ml water. A few drops of phenolphthalein (1% in ethanol) were added and then titrated with the KOH solution. The concentration of the KOH solution in ethanol was obtained from equation 5, where $m$ is the benzoic acid weight (g), $M$ is the benzoic acid molecular weight (122.12 g mol$^{-1}$) and $V$ the consumed volume of the KOH solution (ml).

$$C_{\text{KOH}} = \frac{m \times 1000}{M \times V}$$ \hspace{1cm} (5)
3.2.5 Hydrogen peroxide measurement

One ml of reaction medium was withdrawn from the reaction and added to an eppendorf tube. After about 15 minutes the two phases were separated spontaneously and from the water phase 10 µl was withdrawn and diluted with 990 µl water for analysis in the spectrophotometer.

150 µl was withdrawn from the organic phase and 150 µl of water was added. This sample was then vigorously mixed for 1 minute in a vortex and then centrifuged for 3 minutes at 13 400 rpm (Minispin plus, Eppendorf). After centrifugation, 100 µl was withdrawn from the water phase to be analyzed later spectrophotometrically.

In a quartz cuvette 100 µl of acetic acid, 10 µl of sample, 690 µl of water and 200 µl of 6% potassium iodide was added. The absorbance at 360 nm was then measured for 90 seconds in a UV-1650PC Shimadzu spectrophotometer. The slope of the absorbance vs time from 30 to 60 seconds was calculated and according to the calibration curve established for the potassium iodide solution (Appendix A) the hydrogen peroxide concentration was calculated.

3.2.6 Enzyme activity assay by esterification

The residual activity of Novozym® 435 was determined by esterification reaction between ethanol and caprylic acid (octanoic acid):

\[
\text{Caprylic Acid} + \text{Ethanol} \rightarrow \text{Ethyl Caprylate} + \text{Water}
\]

\[
C_7H_{14}COOH + C_2H_5OH \rightarrow CH_3(CH_2)_7COOC2H_5 + H_2O
\]

Ethanol is also the solvent and the initial reaction rate is measured by analyzing the samples by gas chromatography (GC).

Ethanol (27 ml in the 30 ml scale, 269 ml in the 300 ml scale) and water (2 ml in the 30 ml scale, 21 ml in the 300 ml scale) were added to the enzyme in the reaction flask. The stirring was initiated and after a few minutes (to let the temperature adjust to 40°C) caprylic acid (200 mM; 975 µl in the 30 ml scale and 9.7 ml in the 300 ml scale) was added and the clock started. During 10 to 15 min, 100 µl samples were taken at intervals and diluted with 900 µl ethanol in a GC vial. The reaction samples were analyzed using a Shimadzu gas chromatograph (GC-14A, Shimadzu Corp., Kyoto, Japan). 1 µl of sample was injected into the GC. Separation was
performed on a Nukol™ column (15 m length, 0.53 mm i.d., 0.5 μm dₗ) from Supelco (Sigma-Aldrich, Steinheim, Germany). Both the ester, alcohol as well as the acid could be detected. However the ethanol was used as the solvent and this peak could not be integrated. Caprylic acid and ethyl caprylate had retention times of 5.5 and 2.3 minutes. The conversion to the ester was calculated according to equation 8, where \( A \) is the peak area and \( RF \) is the response factor for caprylic acid vs. ethyl caprylate.

\[
Conversion = \frac{A_{\text{Ethyl Caprylate}}}{A_{\text{Ethyl Caprylate}} + A_{\text{Caprylic Acid}} \times RF}
\]  \hspace{1cm} (6)

The peak area integration was calculated using HSM on-line software. The response factor for caprylic acid vs. ethyl caprylate is 0.67.
4 Results and discussion

4.1 Chemo-enzymatic epoxidation of RME in 30 ml scale

Based on data from Hagström, A. (non-published data), the chemo-enzymatic epoxidation of rapeseed methyl ester was performed in a 30 ml scale, at 40°C and with a 3% (w/w) enzyme loading. The hydrogen peroxide (10% excess) was added in the beginning of the reaction. Initial experiments were performed with 5%, 10% and 20% of H₂O₂ initial concentration in the water phase. The reactions were followed for 24 hours by oxirane number analysis.

Dividing the oxirane number obtained for a specific time by the maximum oxirane number, the conversion obtained is calculated, and thus the number of moles of epoxidized RME that have been formed can be calculated. It is also useful to compare the initial productivity in mass of product formed per unit of time and per initial volume of RME or gram of enzyme.

As can be seen in Figure 4 the oxirane number in the reaction with 5% initial concentration of H₂O₂ in the water phase, showed a low initial productivity rate (20 g l⁻¹ h⁻¹) and a low oxirane number at 24 hours (2.6). This corresponds to a final conversion of about 38%. When the initial concentration of H₂O₂ in the water phase was increased to 10%, the initial epoxidation rate increased almost five times (to 113 g l⁻¹ h⁻¹). The conversion after 24 hours was about 80%. With an initial concentration of 20% H₂O₂ in the water phase, the initial epoxidation rate increased to 160 g l⁻¹ h⁻¹ and the conversion after 24 hours is about 86%.

For more details see Table 2.
conversion is already higher than in the 24 hours of reaction with an initial 10% $\text{H}_2\text{O}_2$ concentration in the water phase.

It was observed in a previous work at a larger scale (Hagström, A.; non-published data) that when a 35% $\text{H}_2\text{O}_2$ initial concentration in the water phase is used, with the same enzyme loading and the same reaction parameters, the conversion after 12 and 24 hours were very similar to the conversion observed for 20% $\text{H}_2\text{O}_2$ initial concentration in the water phase (Figure 5). This might happen because although the initial production rate is higher in the first case, the enzyme deactivation is also higher, and then the reaction rate decreases faster. Considering these results, the following experiments were performed with concentrations of hydrogen peroxide lower than 20%.

![Figure 5](image)

**Figure 5.** Oxirane number during chemo-enzymatic epoxidation of RME at 40°C, with an enzyme loading (Novozym® 435) of 3% (w/w) and an initial concentration of hydrogen peroxide in the water phase of 20% ( ) and 35% ( ). The reaction with 35% of hydrogen peroxide in the water phase was performed in a 300 ml scale (Hagström, A., non-published data).

### 4.1.1 Hydrolysis

The hydrolysis of the ester bond is important to produce free carboxylic acids which can be oxidized to form peracid (see Figure 2). However, hydrolysis can also occur as a side reaction in the chemo-enzymatic epoxidation of RME. The final product of the chemo-enzymatic epoxidation of RME will therefore be a mixture of epoxidized fatty acids and fatty acid esters. The hydrolysis reaction is catalyzed by the lipase and will take place in the presence of water. The extension of the hydrolysis reaction can be calculated based on the acid number and is represented, in percentage, in Figure 6.
Figure 6. Percentage of hydrolysis during chemo-enzymatic epoxidation of RME in the 30 ml scale, at 40°C, with an enzyme loading (Novozym® 435) of 3% (w/w) and an initial concentration of hydrogen peroxide in the water phase of 5% ( ), 10% ( ) and 20% ( ). For details see Table 2.

It was observed that the percentage of hydrolysis is inversely proportional to the ratio between the phases. This can be seen in Figure 7, at a higher initial concentration of H₂O₂ solution, the percentage of the organic phase volume in the total system is higher and the hydrolysis is lower. For example, considering an initial H₂O₂ concentration in the water phase of 20%, if the aim is to decrease the hydrolysis by 20%, the organic phase percentage in the total volume should be increased by 20%. This means that a 34% H₂O₂ initial concentration in the water phase should be used.

Figure 7. Percentage of hydrolysis after 24 hours versus percentage of organic phase in the total volume (v/v) in chemo-enzymatic epoxidation of RME at 40°C and with an enzyme loading (Novozym® 435) of 3% (w/w). Squares: reactions performed in a 30 ml scale; circles: reactions performed in a 300 ml scale; open symbols: data obtained from Hagström, A. (non-published data).
4.1.2 Hydrogen peroxide concentration

Regarding the hydrogen peroxide concentration in the water phase, it can be seen in Figure 8 that when using H$_2$O$_2$ at initial concentration of 10% or 20% in the water phase, its concentration decreases with the reaction time. This means that the H$_2$O$_2$ is being transferred to the organic phase and consumed. The H$_2$O$_2$ can also be decomposed during the reaction time, although the reactor has been protected from light to minimize this effect. When 5% H$_2$O$_2$ initial concentration in the water phase is used, the concentration in the water phase is approximately constant, although decreasing slightly from the initial value. This possibly indicates that there is no efficient mass transfer between the two phases due to a very low difference between the hydrogen peroxide concentrations in the water and organic phase. Unfortunately the concentration of H$_2$O$_2$ in the organic phase was not measured because the percentage of organic phase was very low under these conditions. Therefore the sample volume needed to measure the concentration in the organic phase would be too high and the total system volume would substantially decrease with time. Moreover the measurement of the H$_2$O$_2$ concentration in the organic phase has a great associated error. When withdrawing the samples, the two phases can not be properly separated and thus some water phase can be withdrawn as well and then a higher amount of H$_2$O$_2$ in the organic phase will be detected. There could also be an error in the assumption that the H$_2$O$_2$ is expected to be transferred from the organic to the water phase when the sample is vigorously shaken in the vortex.

![Figure 8](image_url)

**Figure 8.** Hydrogen peroxide concentration in the water phase during chemo-enzymatic epoxidation of RME in a 30 ml scale, at 40°C, with an enzyme loading (Novozym® 435) of 3% (w/w) and an initial concentration of hydrogen peroxide in the water phase of 5% (---), 10% (---) and 20% (---).
It is interesting to notice that the final concentration in the three experiments (with 5, 10 and 20% \( \text{H}_2\text{O}_2 \) initial concentration in the water phase) is quite similar. Calculating the number of moles consumed, the final \( \text{H}_2\text{O}_2 \) concentration considering the total system volume can be calculated. This value is 0.78, 0.50 and 0.59 M for the reactions with 5%, 10% and 20% initial \( \text{H}_2\text{O}_2 \) concentration in the water phase, respectively. However as the ratio between the organic and water phases is different between the reactions (see Table 2), a comparison of these results is difficult.

![Figure 9](image)

**Figure 9.** Hydrogen peroxide concentration in the organic phase during chemo-enzymatic epoxidation of RME in a 30 ml scale, at 40°C, with an enzyme loading (Novozym® 435) of 3% (w/w) and an initial concentration of hydrogen peroxide in the water phase of 10% ( ) and 20% ( ).

It can be seen in **Figure 9** that after 10 hours, the \( \text{H}_2\text{O}_2 \) concentration in the organic phase is significantly different between the reactions performed with 10 and 20% initial \( \text{H}_2\text{O}_2 \) concentration in the water phase. The increasing concentration after 10 hours of the reaction with 10% is not easily explained. Identical behaviour was expected for both reactions, although the difference in the first hours could be explained by a lower mass transfer rate, as a consequence of a lower driving force for the mass transfer (i.e. a lower difference in the concentration between water and organic phase). As mentioned before, the measurement of the \( \text{H}_2\text{O}_2 \) concentration in the organic phase is sensitive to sample handling and withdrawal and so to be able to draw any conclusions from these results the measurements should be repeated.

**Figure 10** summarizes the results obtained in the 30 ml scale, when the hydrogen peroxide is added in the beginning of the reaction with a concentration in the water phase of 5%, 10% and
20%. Here we can observe that the hydrolysis is inversely proportional to percentage by volume of organic phase. Also, the initial epoxidation rate, or initial productivity depends on the hydrogen peroxide concentration. The final conversion and the product per gram of enzyme after 24 hours is not substantially higher when a 20% H₂O₂ initial concentration is used, compared with the 10%. This is probably the result of enzyme deactivation.

![Graph](image)

**Figure 10.** Epoxidation of RME in a 30 ml scale with different initial hydrogen peroxide concentration in the water phase. Volumetric initial percentage of the organic phase in the system volume, final productivity per grams of catalyst, final conversion and hydrolysis after 24 hours of reaction and initial productivity.

### 4.1.3 Stability of Novozym® 435 during chemo-enzymatic epoxidation of RME in 30 ml scale

In the 30 ml scale during 24 hours reaction, taking 7 samples of 2 ml each (1 ml for the oxirane value and acid number titrations and 1 ml for the determination of the H₂O₂ concentration in both phases), as much as half of the volume is lost, and thus, if the system is well mixed, also as much as half of the enzyme. Therefore, to perform the stability studies, it was decided to take only two samples during the reaction time and perform the oxirane number determination to make sure the reaction follows the same conversion profile as was previously obtained. Thus, when the reaction is finished and the esterification assay is performed, the amount of enzyme that has been lost is significantly lower.

To determine the initial enzymatic activity an assay was performed. The enzyme preparation (Novozym®435) was used to catalyze the esterification between ethanol and caprylic acid. No previous contact between the enzyme and the epoxidation reaction medium was performed. It
was observed however that if the samples were stored in the refrigerator for 4 days, the value of the enzymatic activity would be 2.6 times higher. Since Novozym® 435 is an immobilized lipase preparation absorbed to an organic carrier, these results could be explained by desorption of the active enzyme. The active enzyme present in the GC vial, after the residual enzymatic activity had been determined, continues the esterification reaction, and therefore a higher enzyme activity can be observed. Törnvall et al. (2007) had previously observed this phenomenon (9). They did not observe any activity in the filtrate if \( \text{H}_2\text{O}_2 \) was present and thus we might expect that for longer epoxidation times, the desorbed free enzyme was deactivated, probably by the presence of \( \text{H}_2\text{O}_2 \). Nevertheless, the samples should be analyzed on the GC as soon as possible.

To know the enzymatic activity after a certain time, under specific conditions, several epoxidation reactions were performed. Each reaction was stopped at a specific time, the reaction media was withdrawn and the enzyme was used for measuring the esterification activity. The enzyme preparation was not washed before the esterification. The residual enzyme activity was measured after reactions with 5%, 10% and 20% hydrogen peroxide initial concentration in the water phase.

![Figure 11. Remaining enzyme activity of Novozym® 435 during the chemo-enzymatic epoxidation of RME in a 30 ml scale. The reactions were performed with an initial concentration of hydrogen peroxide in the water phase of 5% ( ), 10% ( ) and 20% ( ).](image)

**Figure 11** summarizes the results obtained in the study of the remaining enzymatic activity. As the initial \( \text{H}_2\text{O}_2 \) concentration increases in the water phase, the enzyme deactivation occurs faster. The deactivation curve presents an exponential decay. When a 20% \( \text{H}_2\text{O}_2 \) initial concentration was used, the enzyme activity was more than 50% lower after the first hour.
However, when the 5% H₂O₂ initial concentration in the water phase was used, the remaining enzymatic activity after one hour is higher than 80%. The conversion figure between the 10% and the 20% H₂O₂ initial concentration is similar and the conversion after 24 hours is almost the same. However when a 10% H₂O₂ initial concentration is used about 15% of enzymatic activity is spared compared with a 20% concentration.

Unfortunately, in all the cases, the enzyme deactivation is quite fast and it continuously decreases to a residual value. This residual value is higher for lower concentrations of hydrogen peroxide.

In Figure 11 the results for 12, 14 and 20 hours with 5% initial H₂O₂ concentration in the water phase and for 20, 22.6 and 24 hours with 10% are higher than expected. Those reactions were the last ones to be performed and as shown in Figure 12 and Figure 13 higher epoxidation conversions were obtained when compared with previous reactions under the same conditions. A new RME and Novozym® 435 from a new batch were used to perform the last reactions. A higher initial enzymatic activity could be a plausible explanation for the higher conversion observed. However, the esterification results don’t confirm this theory, since the initial enzymatic activity was found to be approximately the same. Therefore, the results obtained from those reactions are not conclusive.

![Figure 12. Oxirane number during the chemo-enzymatic epoxidation of RME. All reactions were performed in a 30 ml scale, with 5% initial hydrogen peroxide concentration in the water phase, at 40°C and 3% (w/w) enzyme loading. Series 1 (●) represents 6 of the reactions performed; series 2 (■) represents the 3 last reactions performed, in which a higher conversion is observed.](image-url)
Figure 13. Oxirane number during the chemo-enzymatic epoxidation of RME. All reactions were performed in a 30 ml scale, with 10% initial hydrogen peroxide concentration in the water phase, at 40°C and 3% (w/w) enzyme loading. Series 1 (•) represents 7 of the reactions performed; series 2 (■) represents the 3 last reactions performed, in which a higher conversion is observed; series 3 (▲) represents 4 of the reactions performed, in which a lower conversion is observed.

When the experiments with 10% initial concentration of H_2O_2 in the water phase were performed, it was observed that some experiments showed a lower initial reaction rate. For all the parameters influencing the reaction rate the stirring in this scale seems to be the most sensitive. The stirring speed was maintained at 450 rpm on the stirring plate, but sometimes small changes in the mixing could be observed. A reasonable explanation to the lower oxirane number values would be a mass transfer limitation. If the stirring is enough a higher concentration of H_2O_2 in the organic phase and thus a higher reaction rate can be observed. The remaining enzymatic activity for these reactions were not included in Figure 10.

4.1.4 Optimal temperature for the chemo-enzymatic epoxidation of RME

The chemo-enzymatic epoxidation of oils has been performed at 40°C in most of the previous studies. The reason for this is that although this enzymatic preparation is stable at elevated temperatures, the effects of enzyme deactivation by action of the hydrogen peroxide are increased with an increase in temperature. This can be explained by the fact that when the temperature increases, enzymes acquire a more open conformation and thus, some amino acids that were protected can become exposed and suffer oxidation by the hydrogen peroxide, resulting in loss in enzymatic activity.
In order to observe the influence of temperature, the chemo-enzymatic epoxidation of RME was performed at 40°C and at 50°C in an oil bath, with an enzyme loading of 3% and an initial concentration of H₂O₂ in the water phase of 10%.

![Figure 14](image1.png)

**Figure 14.** Oxirane number during the chemo-epoxidation of 12 g of RME, at 40°C ( ) and 50°C ( ) with 0.36 g of Novozym® 435 (3% (w/w) enzyme loading) and an initial hydrogen peroxide concentration in the water phase of 10%.

No significant influence of temperature on the amount of hydrolysis was observed (**Figure 15**).

![Figure 15](image2.png)

**Figure 15.** Percentage of hydrolysis during the chemo-epoxidation of 12 g of RME, at 40°C ( ) and 50°C ( ) with 0.36 g of Novozym® 435 (3% (w/w) enzyme loading) and an initial hydrogen peroxide concentration in the water phase of 10%.

The remaining enzymatic activity after 24 hours was about 16% for the reaction at 40°C and 7% for the reaction at 50°C. It would be interesting to measure the remaining enzymatic
activity, for example after 10 hours. By that time, approximately 79 and 87% of conversion were obtained at 40 and 50°C, respectively.

The initial epoxidation rate increases with increasing temperature (Figure 14). If the hydrogen peroxide concentration is maintained constant, resulting in a constant epoxidation rate, higher conversions in a shorter period of time could be obtained. Therefore, the reaction can be stopped earlier and more remaining enzymatic activity could be expected.

More studies have to be performed, with continuous additions, to realize if there is some advantage in performing the reaction at higher temperatures. Higher temperatures involve higher epoxidation rates and shorter reaction times and thus shorter exposition time between the enzymatic preparation and the hydrogen peroxide, but also higher deactivation rate of the enzymatic preparation and higher operational costs. However, the operational costs might be lower if the reaction time is considerably shorter.

For future studies, it would be interesting to perform the reactions at different temperatures in a larger scale, with continuous addition of hydrogen peroxide, and measure the residual enzyme activity after a relatively high conversion is obtained.

4.2 Chemo-enzymatic epoxidation of RME in 300 ml scale

4.2.1 Comparison with the results obtained in the 30 ml scale

To confirm that the results obtained in the 30 ml scale are valid in the 300 ml scale, a new reaction, with 10% initial concentration hydrogen peroxide was performed in this larger scale with the same conditions (3% enzyme loading and 40°C). The results are impressively concurrent (Figure 16). Also comparing the results from the 30 ml scale performed in this work and the results from a previous study in 300 ml (Hagström, A., non-published data), both with 20% initial H₂O₂ concentration in the water phase, it is noticed that there is no difference between the conversion profiles. Therefore, there is no difference between both scales on the mass transfer limitations when a 10 and 20% H₂O₂ in the water phase is used. However it’s not possible to conclude that there are no mass transfer limitations.

Significant differences were observed between the results of the epoxidation in the larger and smaller scale for a 5% initial concentration of hydrogen peroxide in the water phase. The
reaction performed in the 30 ml scale shows a significantly lower epoxidation rate and thus, the conversions are considerably lower. These results might be explained by mass transfer limitations in the smaller scale. With the improved mixing in the larger scale, the mass transfer of hydrogen peroxide to the organic phase is more efficient and thus the concentration in this phase is higher and consequently the epoxidation rate is also higher.

Figure 16. Oxirane number during the chemo-enzymatic epoxidation of RME in 30 ml scale (filled symbols) and in 300 ml scale (empty symbols) with an initial hydrogen peroxide concentration in the water phase of 5%, 10% and 20%.

Figure 17. Percentage of hydrolysis during the chemo-enzymatic epoxidation of RME in 30 ml scale (filled symbols) and 300 ml scale (empty symbols) with an initial hydrogen peroxide concentration in the water phase of 5%, 10% and 20%.
4.2.2 Continuous additions of hydrogen peroxide

It has been shown previously in this study that the enzyme preparation, Novozym® 435, is inactivated in the presence of hydrogen peroxide. This deactivation is faster when the concentration of hydrogen peroxide is higher. Also, in Figure 16, it has been observed that the stirring in a 30 ml scale is not enough for an efficient mass transfer of the H₂O₂ between the two phases when lower concentrations are used. Therefore it is necessary to continue this study in a larger scale, where the mixing is improved and it is possible to add hydrogen peroxide continuously.

The studies in the 300 ml scale were performed with the aim to maintain the concentration of hydrogen peroxide low enough to keep a low but constant epoxidation rate and obtain a higher remaining enzymatic activity than in previous studies.

It was decided to start the reaction with a system of two phases. The water phase started with a concentration of hydrogen peroxide of 5%. The addition rate was estimated through the initial product formation in the 10% initial concentration of hydrogen peroxide in the water phase.

An addition rate program was chosen with decreasing addition rate with time, to compensate for the loss in enzymatic activity and the decreasing reaction rate. The oxirane number profile is similar to the one where all the hydrogen peroxide was added at once in the beginning of the reaction, with a 10% initial concentration in the water phase (Figure 18).

After 24 hours of reaction, the remaining enzymatic activity was extremely low (about 3%). Worth to mention is that when all the hydrogen peroxide was added at once, with 10% concentration in the water phase, the remaining enzymatic activity after 24 hours was about 9%. The loss of enzymatic activity can be explained when studying the hydrogen peroxide concentration with time in the reaction mixture (Figure 19). A high hydrogen peroxide concentration in the water phase for a long period of time is observed (compared with Figure 8). The addition rate of hydrogen peroxide has to be lower to keep a lower and constant hydrogen peroxide concentration in the water phase.
Figure 18. Oxirane number and hydrogen peroxide addition rate during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction I ( ), 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H$_2$O$_2$ solution. 54 ml H$_2$O$_2$ (35% solution) was added continuously with decreasing addition rate ( ). Previous results are shown for comparison: reactions in the 30 ml scale with 20% initial H$_2$O$_2$ concentration in the water phase ( ), and in the 300 ml scale with 10% ( ) and 5% ( ) initial H$_2$O$_2$ concentration in the water phase.

Figure 19. Hydrogen peroxide concentration in the water phase ( ) and the organic phase ( ) during the chemo-enzymatic epoxidation of RME with continuous addition of hydrogen peroxide (Reaction I).

A new reaction with lower addition rates was performed. To estimate the remaining enzymatic activity that has been saved by adding the hydrogen peroxide continuously, the reaction was stopped after 14 hours, when a good conversion was obtained and the remaining enzymatic activity was measured by esterification between ethanol and caprylic acid.
Figure 20. Oxirane number and H$_2$O$_2$ addition rate during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction II (---), 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H$_2$O$_2$ solution. 75.6 ml H$_2$O$_2$ (35% solution) was added continuously with decreasing addition rate (----). Previous results are shown for comparison: reactions in the 30 ml scale with 20% initial H$_2$O$_2$ concentration in the water phase (---), and in the 300 ml scale with 10% (---) and 5% (---) initial H$_2$O$_2$ concentration in the water phase.

At the end of the reaction (after 14 hours) an enzymatic activity of 61% was obtained. This value was quite good compared with previous results (see Table 3). Nevertheless, the enzyme reutilization in a larger scale process will not be economical feasible. The productivity and conversion after 12 hours was higher than when all the hydrogen peroxide is added at once with a 10% initial concentration in the water phase.

Figure 21. Hydrogen peroxide concentration in the water phase (---) and the organic phase (---) during the chemo-enzymatic epoxidation of RME with continuous addition of H$_2$O$_2$ (Reaction II).
Table 3. Comparison between the reaction performed with 10% initial \( \text{H}_2\text{O}_2 \) concentration in the water phase added at once in the beginning of the reaction and a reaction with continuous additions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reaction 10%</th>
<th>Reaction II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial epox. rate (ox. no. h(^{-1}))</td>
<td>0.76</td>
<td>0.49</td>
</tr>
<tr>
<td>Prod/cat after 12 h (g g(^{-1}))</td>
<td>25.8</td>
<td>27.3</td>
</tr>
<tr>
<td>Conversion after 12 h (%)</td>
<td>72%</td>
<td>76%</td>
</tr>
<tr>
<td>Productivity after 12 h (g l(^{-1}) h(^{-1}))</td>
<td>57.0</td>
<td>59.5</td>
</tr>
<tr>
<td>Total reaction time (h)</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Conversion (%)</td>
<td>80%</td>
<td>83%</td>
</tr>
<tr>
<td>% Organic phase (v/v)</td>
<td>47%</td>
<td>53% *</td>
</tr>
<tr>
<td>Residual enzyme activity (%)</td>
<td>9%</td>
<td>61%</td>
</tr>
<tr>
<td>Hydrolysis (%)</td>
<td>49%</td>
<td>39%</td>
</tr>
</tbody>
</table>

*calculated considering the hydrogen peroxide solution volume added during the reaction.

4.2.3 Reduction of the water content

In the end of Reaction II about 47% of the system volume was water phase. The reduction of water content in the system has several advantages. First, it reduces the total system volume, the liquid waste from the process and therefore the cost is reduced. Secondly, it has been shown that the percentage of hydrolysis is inversely proportional to the water content and thus, reducing the water content the extension of this side reaction is also reduced.

To perform the reduction of water content in the system, a higher concentration of hydrogen peroxide was used to perform the continuous additions, and thus the total volume added was decreased. The initial water content was also decreased starting with a lower number of moles of hydrogen peroxide. The addition rate was chosen to add the same number of moles of \( \text{H}_2\text{O}_2 \) per minute and per amount of RME as in reaction II.

Reaction II had a final 53% organic phase while reaction III had 74%. The hydrolysis was reduced from 39% to 24% (Figure 22).
Figure 22. Percentage of hydrolysis during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction II (---) 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H₂O₂ solution. 75.6 ml H₂O₂ (35% solution) was added continuously; For Reaction III (---) 6.0 g of Novozym® 435 was used with 200 g of RME and 20 ml of 5% concentration H₂O₂ solution. 57.8 ml H₂O₂ (60% solution) was added continuously.

As can be seen in Figure 23 a lower reaction rate was observed when the water content in the system was reduced.

Figure 23. Oxirane number during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction II (---) 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H₂O₂ solution. 75.6 ml H₂O₂ (35% solution) was added continuously; For Reaction III (---) 6.0 g of Novozym® 435 was used with 200 g of RME and 20 ml of 5% concentration H₂O₂ solution. 57.8 ml H₂O₂ (60% solution) was added continuously.
Figure 24. Hydrogen peroxide concentration in the water phase (●) and the organic phase (○) during the chemo-enzymatic epoxidation of RME with continuous addition of H$_2$O$_2$ for 12 hours (Reaction III).

Table 4. Comparison between reactions II and III.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reaction II</th>
<th>Reaction III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial epox. rate (ox. no. h$^{-1}$)</td>
<td>0.46</td>
<td>0.36</td>
</tr>
<tr>
<td>Prod/cat after 14 h (g g$^{-1}$)</td>
<td>29.0</td>
<td>23.5</td>
</tr>
<tr>
<td>Conversion after 14 h (%)</td>
<td>83%</td>
<td>66%</td>
</tr>
<tr>
<td>Productivity after 14 h (g l$^{-1}$ h$^{-1}$)</td>
<td>56.3</td>
<td>44.6</td>
</tr>
<tr>
<td>% Organic phase (v/v)</td>
<td>53%$^1$</td>
<td>74%$^1$</td>
</tr>
<tr>
<td>Residual enzyme activity (%)</td>
<td>61%$^2$</td>
<td>63%$^3$</td>
</tr>
<tr>
<td>Hydrolysis (%)</td>
<td>39%</td>
<td>24%</td>
</tr>
</tbody>
</table>

$^1$ calculated considering the hydrogen peroxide solution volume added during the reaction; $^2$ measured after 14 hours of reaction; $^3$ measured after 16 hours of reaction.
5 Conclusions

The chemo-enzymatic epoxidation of RME, using the enzyme preparation Novozym® 435 was performed in a 30 ml and a 300 ml scale. The enzyme stability during the chemo-enzymatic reaction is highly dependent on hydrogen peroxide concentration. When a 5% (w/w) initial hydrogen peroxide concentration was used in a 30 ml scale, adding all the hydrogen peroxide in the beginning of reaction, more than 50% of enzyme activity was lost in the first eight hours of reaction. The enzyme deactivation was even faster for higher hydrogen peroxide concentrations. At lower initial concentrations of hydrogen peroxide, differences between the two scales in terms of hydrogen peroxide mass transfer to the organic phase were observed. Therefore, better mixing is necessary to perform the chemo-enzymatic epoxidation at lower hydrogen peroxide concentrations to obtain high conversions. No difference in terms of conversion figures was observed between the two scales when higher initial hydrogen peroxide concentrations in the water phase were used.

It was shown that a significant amount of enzyme activity can be spared and high conversions can be obtained in a shorter reaction time adding the hydrogen peroxide continuously. However, the significant loss of enzyme activity suggests that this process can not be done on an industrial scale. More studies with different operations modes, temperatures and hydrogen peroxide concentration have to be performed in order to reduce the enzyme inactivation and transform the chemo-enzymatic epoxidation with Novozym® 435 in an economically viable process.

Along with the improvement of the process design, the development of a more stable preparation, less sensitive to oxidative damage, would be an alternative strategy.

Another important fact to consider is the characteristics of the epoxidized product and its applications that can make it a competitive product. It has been shown that other vegetable oils than RME, such as tall oil, are more competitive for the production of epoxidized products (1). The big advantage is that the tall oil is a by-product from pulp and paper industry, and is treated as waste. Also, when producing RME to obtain epoxidized products, there is also the question of competing land use option, since the land could be free if other sources were used. Moreover, earlier life cycle assessments have shown that using renewable feedstocks such as
an oil crop is not necessarily favorable in all situations, partly due to high environmental impact during cultivation (1).
References

1. Törnvall, Ulrika, et al. Fatty epoxides from rapeseed and tail oil derivatives using a solvent-free chemo-enzymatic process. non-published data.


Appendix

Appendix A – Spectrophotometer calibration curve

Figure A1. Calibration curve for the spectrophotometer with the first potassium iodide solution.

The linear regression gave the correlation

\[ C_{H_2O_2} \text{ (mM)} = \frac{mAbs/s - 3.65 \times 10^{-2}}{4.91 \times 10^{-2}} \]

\[ R^2 = 0.994 \]

Figure A2. Calibration curve for the spectrophotometer with the second potassium iodide solution.

\[ C_{H_2O_2} \text{ (mM)} = \frac{mAbs/s - 2.55 \times 10^{-2}}{3.51 \times 10^{-2}} \]

\[ R^2 = 0.975. \]