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

Ana Severiano, Anna Hagström, Rajni Hatti-Kaul, M. Manuela R. da Fonseca

The chemo-enzymatic epoxidation of rapeseed methyl ester (RME) in a solvent-free medium was studied using the immobilized *Candida antarctica* lipase B (Novozym® 435) as catalyst. Hydrogen peroxide (H_2O_2) was used as the oxygen donor. The enzyme cost is an important factor determining the economics of the process. High enzyme stability and the possibility to recycle the enzyme are therefore highly desirable. The stability of the enzyme was studied for initial H_2O_2 concentration in the water phase of 5, 10 and 20% (w/w). The epoxidation rate increased with increasing H_2O_2 concentration, however at the expense of enzyme inactivation. A temperature increase has a similar effect: the reaction rate increases but the remaining enzymatic activity is reduced. The epoxidation of RME was also performed with continuous addition of H_2O_2 solution. Maintaining an approximately constant concentration of H_2O_2 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 with a remaining enzymatic activity of 61%.

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

1 Introduction

Implementing sustainable production has been a key issue for the chemical industry (1). Vegetable oils and their component fatty acids are important raw materials. They are replacing petroleum in the production of environmentally friendly products for coatings, paints, lubricants and surfactants, and even as fuel (1; 2). Many of those 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 participate in numerous reactions, which make these compounds useful building blocks in organic synthesis (3). 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 *in situ* from acetic or formic acid, H_2O_2 and a strong mineral acid as catalyst (1). The presence of the strong acid causes the formation of by-products.

Epoxides can also be produced by biocatalytic synthesis. The use of enzymes as catalysts has recognized importance in several industries, including cosmetic, textile, paper, food and pharmaceutical.

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 (4). Besides that, immobilized preparations are easier to handle and thus easier to reuse.

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

1.1 Chemo-enzymatic epoxidation

The chemo-enzymatic epoxidation of vegetable oils 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 (1).

The mechanism of chemo-enzymatic epoxidation of methyl esters involves a two-step reaction (**Figure 1**). First the methyl ester reacts with H_2O_2 to form the peracid in a catalyzed step. Secondly 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. 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).

1.2 Lipases

Lipases (Triacylglyrerol acylhydrolase, E.C. 3.1.1.3) natural reaction is the hydrolysis of triglycerides into fatty acids, diglycerides, monoglycerides and glycerol. 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 (3).

Lipases A and B from *Candida antarctica*, present extreme properties and have proven to be of particular interest to researchers (3). Novozym® 435, a commercial immobilized lipase B from *Candida antarctica*, is a very thermostable and robust catalyst, with activity in a number of organic solvents and also in solvent-free media (3).

1.3 Enzyme stability

Changes in the enzymatic structure are often destructive in terms of enzyme activity (6). Deactivation can be caused by several reaction parameters, including temperature, reactant ratio as well as water, substrate and co-substrate concentrations. Other parameters can result in chemical modifications in the polypeptide chain, e.g. by oxidation, amidation, affecting the enzymatic activity. H_2O_2 is a strong oxidizing agent. Oxidation of amino acids buried within the hydrophobic interior of the protein molecule and the ones participating in the catalytic mechanism can have drastic effects on the enzyme conformation and activity (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 (7). 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 can reduce lipase deactivation: when intersterification 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 (7).

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

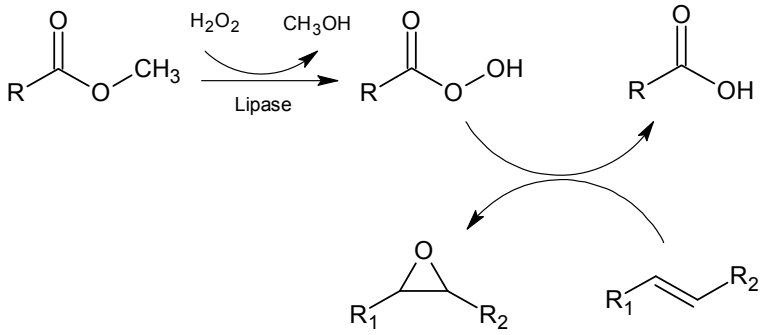

Figure 1. Chemo-enzymatic epoxidation of methyl esters. First the lipase catalyses the peracid formation. Then the peracid spontaneously donates the oxygen to a double bond, to form the epoxide.

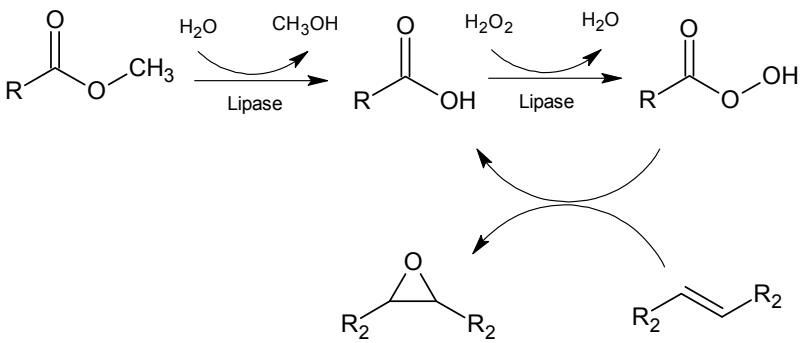

Figure 2. Chemo-enzymatic epoxidation of methyl esters. First the lipase catalyses the hydrolysis of the ester into the respective acid. Secondly the lipase catalyses the formation of the peracid from the acid. The peracid then spontaneously donates the oxygen to a double bond, to form the epoxide.

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).

Törnvall *et al.* have investigated the deactivation of Novozym® 435 in contact with the epoxidation substrates and products at various temperatures (8). No enzyme deactivation could be detected after incubation with oleic acid. With epoxystearic acid, the lipase activity decreased to 70% of the original level (8). The same group did not observe any deactivation effect by the very reactive peracid.

H_2O_2 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 Novozym® 435 during epoxidation of fatty acids (8).

1.4 Parameters affecting the chemo-enzymatic epoxidation

The effect of reaction temperature on the chemo-enzymatic epoxidation of linoleic acid (0.5 M) was previously 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) H_2O_2 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 H_2O_2 decomposition and/or enzyme inactivation. The highest reaction rate was observed at 50°C (2).

In another study, Novozym® 435 was incubated in the presence of various reaction components at temperatures between 20 and 60°C (8). They observed that the enzyme was stable even at 60°C when there was no H_2O_2 in the preparation. However, at 60°C and in the presence of 6 to 12 M H_2O_2, the enzyme lost activity rapidly. The rate of deactivation increased with increasing H_2O_2 concentration (8). 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 H_2O_2 was not present.

The amount of enzyme required to keep in pace with H_2O_2 addition is another important parameter. Orellana-Coca *et al*. performed a recycling study in which they used an excess of enzyme, to overcome the inactivation problem (9). They were able to do five cycles of reaction before the conversion dropped dramatically in the sixth cycle. In subsequent studies, the chemo-enzymatic epoxidation of RME was performed with much lower amounts of enzyme (0.5 and 10% (w/w) (Hagström, non-published data).

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 Novozym® 345 inactivation. 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.

2 Materials and Methods

2.1 Materials

RME (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). H_2O_2 (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.

2.2 Methods

2.2.1 RME epoxidation in 30 ml scale

The reactions were performed in a 50 ml round-bottomed flask. The total volume and the enzyme loading were maintained constant (approximately 30 ml and 3% (w/w) of Novozym® 435, based on RME amount). A 10% stoichiometric excess of H_2O_2 was used. 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. The properties of the initial reaction mixtures are shown in **Table 1**.

Table 1. Initial H_2O_2 concentration in the water phase, weight of RME, volume of H_2O_2 35% and water added and percentage of the organic phase in the system volume.

H_2O_2 concentration		RME	Novozym® 435	H_2O_2 35%	H_2O	% OP
% (w/w)	(M)	(g)	(g)	(ml)	(ml)	(v/v)
20%	6.58	18	0.54	7.8	6.0	60%
10%	3.29	12	0.36	5.2	13.2	42%
5%	1.65	8	0.24	3.5	21.0	27%

2.2.2 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 H_2O_2 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.

2.2.3 Oxirane number titration

The reaction was followed by measuring the oxirane number in a similar procedure to what is described by Jay (10). One ml of sample was withdrawn from the reactor and centrifuged for 3 minutes at 13 400 rpm in an eppendorf 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 oxirane number, expressed in % (w/w), can be calculated according to equation 1, where $V_{titrand}$ and V_{blanc} is the consumed volume of the $HClO_4$ solution for the sample titration and the blanc (ml) respectively, $C_{titrand}$ is the $HClO_4$ solution concentration (M), M_{oxigen} is the oxygen atomic weight (16 g mol^{-1}) and m_{sample} is the sample weight (g).

$$Oxirane\ number = \frac{(V_{titrand} - V_{blank}) \times C_{titrand} \times M_{oxygen}}{m_{sample} \times 10} \quad \textbf{(1)}$$

The maximum oxirane number for RME, i.e. the oxirane number at 100% conversion was found to be 6.8.

2.2.4 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.

2.2.5 H_2O_2 measurement

One ml of reaction medium was withdrawn 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 the H_2O_2 concentration was calculated.

2.2.6 Enzyme activity assay by esterification

The residual activity of Novozym® 435 was determined by esterification reaction between ethanol and caprylic acid. 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. Samples were taken at intervals and diluted with ethanol in a GC vial. The 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 d_f) from Supelco (Sigma-Aldrich, Steinheim, Germany). Both the ester, alcohol as well as the acid could be detected. 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 2, where A is the peak area and RF is the response factor for caprylic acid vs. ethyl caprylate.

$$Convertion = \frac{A_{Ethyl\ Caprylate}}{A_{Ethyl\ Caprylate} + A_{Caprylic\ Acid} \times RF} \quad \textbf{(2)}$$

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

3 Results and discussion

3.1 Chemo-enzymatic epoxidation of RME in 30 ml scale

Initial experiments were performed with 5%, 10% and 20% of H_2O_2 initial concentration in the water phase. The H_2O_2 was added in the beginning of the reaction. The oxirane number in the reaction with 5% initial

concentration of H_2O_2 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) (**Figure 3**). This corresponds to a final conversion of about 38%. When the initial concentration of H_2O_2 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% H_2O_2 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%. After 12 hours, the conversion is already higher than in the 24 hours of reaction with an initial 10% H_2O_2 concentration in the water phase.

3.1.1 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.

It was observed that the percentage of hydrolysis is inversely proportional to the ratio between the phases (**Figure 4**). At a higher initial concentration of H_2O_2 solution, the percentage of the organic phase volume in the total system is higher and the hydrolysis is lower.

3.1.2 H_2O_2 concentration

When using H_2O_2 at initial concentration of 10% or 20% in the water phase, its concentration decreases with the reaction time (**Figure 5**). This means that the H_2O_2 is being transferred to the organic phase and consumed. The H_2O_2 can also be decomposed during the reaction time, although the reactor has been protected from light to minimize this effect. When 5% H_2O_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 H_2O_2 concentrations in the water and organic phase. It is interesting to notice that the final concentration in the three experiments (with 5, 10 and 20% H_2O_2 initial concentration in the water phase) is quite similar.

It can be seen in **Figure 6** that after 10 hours, the H_2O_2 concentration in the organic phase is significantly different between the reactions performed with 10 and 20% initial H_2O_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). The measurement

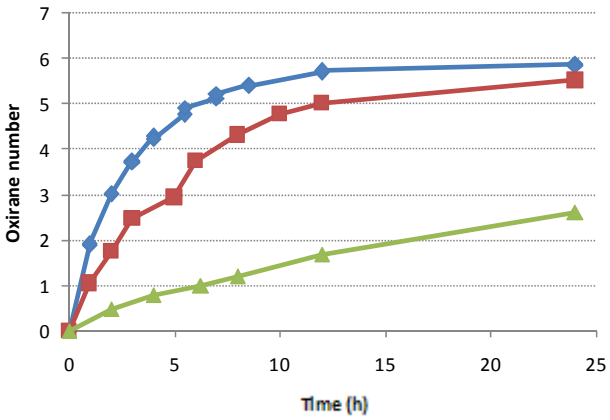

Figure 3: Oxirane number during chemo-enzymatic epoxidation of RME in 30 ml scale, at 40°C, with an enzyme loading of 3% (w/w) (Novozym® 435) and an initial concentration of H_2O_2 in the water phase of 5% ( ), 10% ( ) and 20% ( ). For more details see **Table 1**.

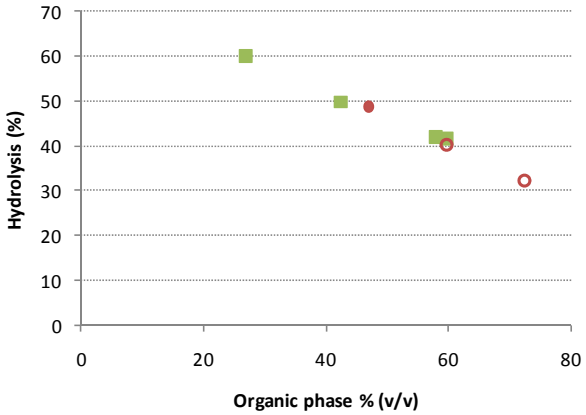

Figure 4. Percentage of hydrolysis after 24 hours versus percentage of organic phase in the total volume (v/v) in chemo-enzymatic epoxidation of RME at 40°C and with an enzyme loading (Novozym® 435) of 3% (w/w). Squares: reactions performed in a 30 ml scale; circles: reactions performed in a 300 ml scale; open symbols: data obtained from Hagström, A. (non-published data).

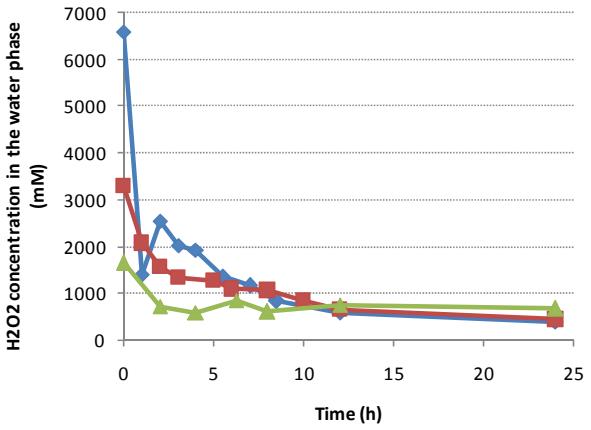

Figure 5. H_2O_2 concentration in the water phase during chemo-enzymatic epoxidation of RME in a 30 ml scale, at 40°C, with an enzyme loading (Novozym® 435) of 3% (w/w) and an initial concentration of H_2O_2 in the water phase of 5% (▲), 10% (■) and 20% (◆).

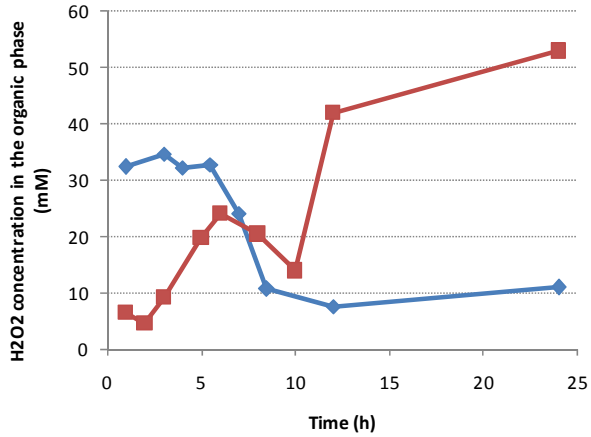

Figure 6. H_2O_2 concentration in the organic phase during chemo-enzymatic epoxidation of RME in a 30 ml scale, at 40°C, with an enzyme loading (Novozym® 435) of 3% (w/w) and an initial concentration of H_2O_2 in the water phase of 10% (■) and 20% (◆).

of the H_2O_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.

The hydrolysis is inversely proportional to percentage by volume of organic phase and the initial epoxidation rate, or initial productivity, depends on the H_2O_2 concentration (**Figure 7**). The final conversion and the product per gram of enzyme after 24 hours is not substantially higher when a 20% H_2O_2 initial concentration is used, compared with the 10%. This is probably the result of enzyme deactivation.

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

During the stability studies, only two samples were taken during the reaction time in order to minimize the lost of enzyme. The oxirane number determination was performed to make sure the reaction follows the same conversion profile as was previously obtained.

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.

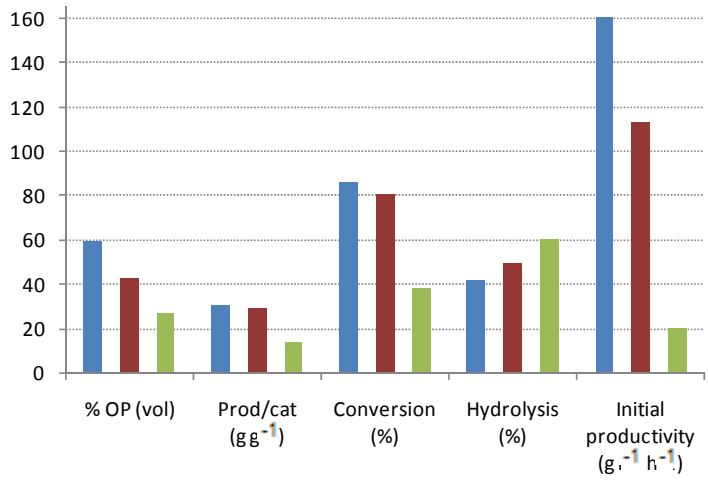

Figure 7. Epoxidation of RME in a 30 ml scale with 5 (■), 10 (■) and 20% (■) initial H_2O_2 concentration in the water phase. Volumetric initial percentage of the organic phase in the system volume, final productivity per grams of catalyst, final conversion and hydrolysis after 24 hours of reaction and initial productivity.

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% H_2O_2 initial concentration in the water phase.

As the initial H_2O_2 concentration increases in the water phase, the enzyme deactivation occurs faster (**Figure 8**). The deactivation curve presents an exponential decay. When a 20% H_2O_2 initial concentration was used, the enzyme activity was more than 50% lower after the first hour. However, when the 5% H_2O_2 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% H_2O_2 initial concentration is similar and the conversion after 24 hours is almost the same. However when a 10% H_2O_2 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 H_2O_2.

The results for 12, 14 and 20 hours with 5% initial H_2O_2 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 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.

3.1.4 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 Novozym® 435 is stable at elevated temperatures, the effects of enzyme deactivation by action of the H_2O_2 are increased with an increase in temperature (8). 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 H_2O_2, resulting in loss in enzymatic activity (8).

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 H_2O_2 in the water phase of 10%.

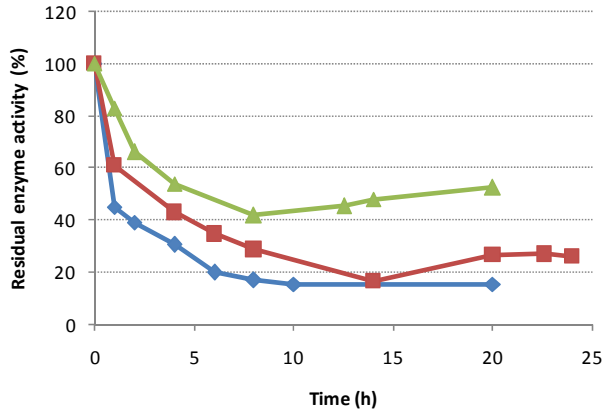

Figure 8: Residual enzyme activity of Novozym® 435 during the chemo-enzymatic epoxidation of RME in a 30 ml scale. The reactions were performed with an initial concentration of H_2O_2 in the water phase of 5% (—▲—), 10% (—■—) and 20% (—◆—).

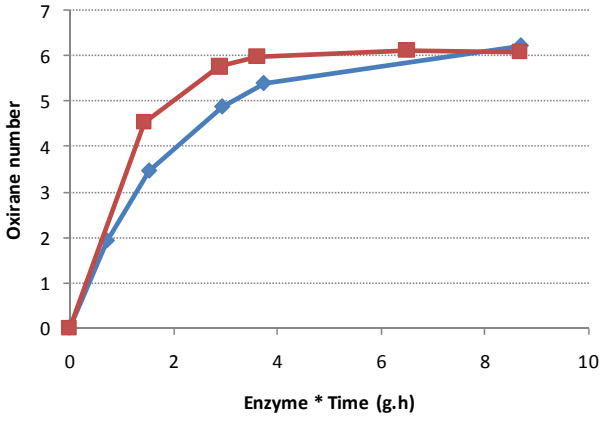

Figure 9. Oxirane number during the chemo-epoxidation of 12 g of RME, at 40°C (—◆—) and 50°C (—■—) with 0.36 g of Novozym® 435 (3% (w/w) enzyme loading) and an initial H_2O_2 concentration in the water phase of 10%.

No significant influence of temperature on the amount of hydrolysis was observed. 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 9**). If the H_2O_2 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 H_2O_2, 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.

3.2 Chemo-enzymatic epoxidation of RME in 300 ml scale

3.2.1 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 H_2O_2 was performed in this larger scale with the same conditions (3% enzyme loading and 40°C). The results are impressively concurrent (**Figure 10**). 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 H_2O_2 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% H_2O_2 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 H_2O_2 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 H_2O_2 to the organic phase is more efficient and thus the concentration in this phase is higher and consequently the epoxidation rate is also higher.

3.2.2 Continuous additions of H_2O_2

The reaction was started with a 5% H_2O_2 concentration 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 H_2O_2 was added at once in the beginning of the reaction, with a 10% initial concentration in the water phase (**Figure 11**).

After 24 hours of reaction, the remaining enzymatic activity was extremely low (about 3%). Worth to mention is that when all the H_2O_2 was added at once, with 10% concentration in the water phase, the remaining enzymatic activity after 24 hours was about 9%. A high H_2O_2 concentration in the water phase for a long period of time was observed. This can explain the low remaining enzymatic activity. The addition rate of H_2O_2 has to be lower to keep a lower and constant H_2O_2 concentration in the water phase.

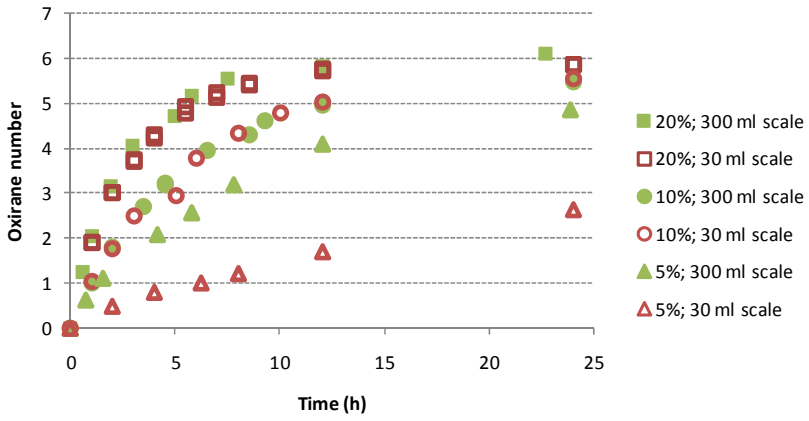

Figure 10. Oxirane number during the chemo-enzymatic epoxidation of RME in 30 ml scale (filled symbols) and in 300 ml scale (empty symbols) with an initial H_2O_2 concentration in the water phase of 5%, 10% and 20%.

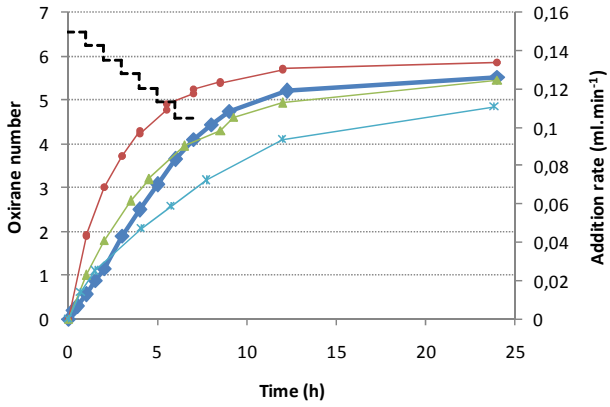

Figure 11: Oxirane number and H_2O_2 addition rate during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction I (—◆—) 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H_2O_2 solution. 54 ml H_2O_2 (35% solution) was added continuously with decreasing addition rate (- - - - -). Previous results are shown for comparison: reactions in the 30 ml scale with 20% initial H_2O_2 concentration in the water phase (—●—), and in the 300 ml scale with 10% (—▲—) and 5% (—✕—) initial H_2O_2 concentration in the water phase.

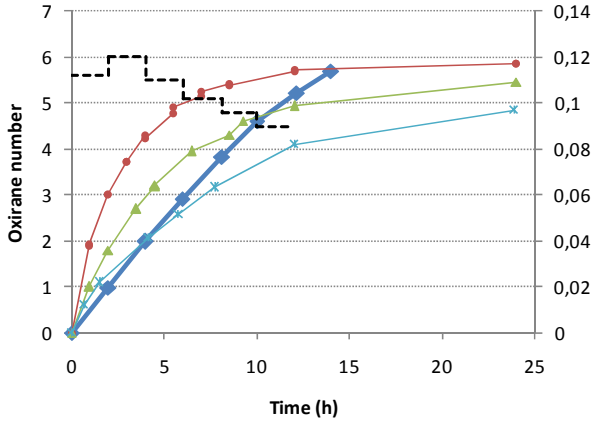

Figure 12. Oxirane number and H_2O_2 addition rate during chemo-enzymatic epoxidation of RME at 40°C and 3% (w/w) enzyme loading. For Reaction II (—◆—) 4.5 g of Novozym® 435 was used with 150 g of RME and 78 ml of 5% concentration H_2O_2 solution. 75.6 ml H_2O_2 (35% solution) was added continuously with decreasing addition rate (- - - - -). Previous results are shown for comparison: reactions in the 30 ml scale with 20% initial H_2O_2 concentration in the water phase (—●—), and in the 300 ml scale with 10% (—▲—) and 5% (—✕—) initial H_2O_2 concentration in the water phase.

A new reaction with lower addition rates was performed. To estimate the remaining enzymatic activity that has been saved by adding the H_2O_2 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. 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. The productivity and conversion after 12 hours was higher than when all the H_2O_2 is added at once with a 10% initial concentration in the water phase. Nevertheless, the enzyme reutilization in a larger scale process is still not economical feasible.

3.2.3 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.

Table 2. Comparison between the reaction performed with 10% initial H_2O_2 concentration in the water phase added at once in the beginning of the reaction and a reaction with continuous additions. *calculated considering the H_2O_2 solution volume added during the reaction.

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

To reduce the water in the system, a higher concentration of H_2O_2 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 H_2O_2. The addition rate was chosen to add the same number of moles of H_2O_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% and a lower reaction rate was observed (**Table 3**).

Table 3. Comparison between reactions II and III. [1] calculated considering the H_2O_2 solution volume added during the reaction; [2] mesured after 14 hours of reaction; [3] mesured after 16 hours of reaction.

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

4 Conclusions

The enzyme stability during the chemo-enzymatic reaction is highly dependent on H_2O_2 concentration. When a 5% (w/w) initial H_2O_2 concentration was used in a 30 ml scale, adding all the H_2O_2 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 H_2O_2 concentrations. To lower concentration, differences between the two scales were observed in terms of H_2O_2 mass transfer to the organic phase. Therefore, better mixing is necessary to perform the reaction with lower H_2O_2 concentrations and high conversions. No difference in terms of conversion figures was observed between the two scales when higher initial H_2O_2 concentrations in the water phase were used.

A significant amount of enzyme activity can be spared and high conversions can be obtained in a shorter reaction time adding continuously the H_2O_2. More studies have to be performed in order to reduce the enzyme inactivation and transform the chemo-enzymatic epoxidation with Novozym® 435 in an economically possible 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.

References

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

2. **Orellana-Coca, Cecilia, *et al.*** Chemo-enzymatic epoxidation of linoleic acid: Parameters influencing the reaction. *J. Lipid Sci. Technol.* 2005, Vol. 107, pp. 864-870.

3. **Orellana-Coca, Cecilia.** *Chemo-enzymatic Epoxidation of Unsaturated Fatty Acids.* Thesis, Department of Biotechnology, Lund University: s.n., 2006.

4. **Adlercreutz, Patrick, *et al*.** *Enzyme Technology.* Lund University : s.n., 2000.

5. **Warwel, Siegfried and Klaas, Mark Rüsch gen.** Chemo-enzymatic epoxidation of unsaturated carboxylic acids. *Journal of molecular catalysis B: Enzymatic.* 1995, Vol. 1, pp. 29-35.

6. **Davies, Michael J.** The oxidative environment and protein damage. *Biochimica et Biophysica Acta 1703.* 2005, pp. 93-109.

7. **Bloomer, Scott.** *Lipase-catalyzed Lipid Modifications in non-aqueous Media.* Thesis, Department of Biotechnology, Lund University : s.n., 1992.

8. **Törnvall, Ulrika, *et al*.** Stability of immobilized Candida antarctica lipase B during chemo-enzymatic epoxidation of fatty acids. *Enzyme and Micribial Thechnology.* 2007, Vol. 40, pp. 447-451.

9. **Orellana-Coca, Cecilia, *et al*.** Chemo-enzymatic epoxidation of oleic acid and methyl oleate in solvent-free medium. *Biocatalysis and Biotransformation.* November - December 2005, Vol. 23 (6), pp. 431-437.

10. **Jay, R. R.** Direct Titration of Epoxy compounds and Aziridines. *Analytical Chemistry.* 1964, Vol. 36 (3), pp. 667-668.

11. **Wu, Xiao Yan, Jääskeläinen, Sanna and Linko, Yu-Yen.** An investigation of crude lipases for hydrolysis, esterification, and transterification. *Enzyme and Microbial Technology.* 1996, Vol. 19, pp. 226-231.

12. **Orellana-Coca, Cecilia, *et al*.** Lipase mediated simultaneous esterification and epoxidation of oleic acid for the production of alkylepoxysterates. *Journal of Molecular Catalysis B: Enzymatic.* 2007, Vol. 44, pp. 133-137.

13. **Gan, L. H., *et al*.** Epoxidized esters of palm olein as plasticizers for poly(vinyl chloride). *Eur. Polym. J.* 1995, Vol. 31(8), pp. 719-724.

14. **Chen, Bo, *et al.*** Candida antartica lipase B chemically immobilized on epoxy-activated micro- and nanobeads: catalysts for polyester synthesis. *biomacromolecules.* 2008, Vol. 9, pp. 463-471.

15. **Turner, Nigel A. and Vulfson, Evgeny N.** At what temperature can enzymes maintain their catalytic activity? *Enzyme and Microbial Technology.* 2000, Vol. 27, pp. 108-113.

16. **Baldascini, Helen, *et al.*** Effect of mass transfer limitations on the enzymatic kinetic resolution of epoxides in a two-liquid-phase system. *Biotechnology and bioengineering.* 2001, Vol. 73 (1), pp. 44-54.

17. **Halling, Peter J.** Biocatalysis in multi-phase reaction mixtures containing organic liquids. *biotech. Adv.* 1987, Vol. 5, pp. 47-84.

18. **Borné, Johanna.** *Lipid Self-Assembly and Lipase Action.* Thesis, Physical chemistry 1, University of Lund : s.n., 2002.

19. **Persson, Mattias.** *Lipases as Biocatalyst in Organic Media - Influence enzyme preparation and reaction Conditions on Activity and selectivity.* Thesis, Department of Biotechnology, Lund University : s.n., 2002 .

20. **Hilker, I., *et al.*** Chemo-enzymatic epoxidation of unsaturated plant oils. *Chemical Engineering Science.* 56, 2001, pp. 427-432.