



LUND UNIVERSITY

Lipase catalysed synthesis of speciality chemicals: technical, economical & environmental aspects

Tufvesson, Pär

2008

[Link to publication](#)

Citation for published version (APA):

Tufvesson, P. (2008). *Lipase catalysed synthesis of speciality chemicals: technical, economical & environmental aspects*. [Doctoral Thesis (compilation), Biotechnology].

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00



LUND
UNIVERSITY

LIPASE CATALYSED SYNTHESIS OF SPECIALITY CHEMICALS

TECHNICAL, ECONOMICAL & ENVIRONMENTAL ASPECTS

Pär Tufvesson

Doctoral Thesis
Department of Biotechnology
December 2008

Akademisk avhandling för avläggande av teknologie doktorsexamen vid tekniska fakulteten vid Lunds universitet. Avhandlingen kommer att försvaras på engelska vid en offentlig disputation på Kemacentrum, Getingevägen 60, Lund, hörsal B, fredagen den 12 december 2008 kl 10.30. Opponent är professor Pierre Monsan från INSA, Toulouse, Frankrike.

Academic thesis which, by due permission of the Faculty of Engineering of Lund University will be publicly defended on Friday December 12 at 10.30 a.m. in Lecture Hall B, at the Center for Chemistry and Chemical Engineering, Getingevägen 60, Lund, for the degree of Doctor of Philosophy in Engineering. The faculty opponent is Professor Pierre Monsan from INSA, Toulouse, France.

Doctoral Thesis
Department of Biotechnology
Lund University
Sweden

© Pär Tufvesson
ISRN LUTKDH/TKBT--08/1111--SE
ISBN 978-91-89627-58-1
Printed by Media-Tryck, Lund, Sweden, 2008

ABSTRACT

Most people agree that the total ecological impact of society has become more than this planet can endure. This is because we consume our resources (such as oil and water) faster than they can regenerate and because we release more substances into the environment than can be assimilated (e.g. CO₂). In other words: our society is not sustainable on a long term basis.

Biocatalysis is being promoted as a clean, environmentally friendly technology because it is natural, inherently works at very mild conditions, and predominantly utilises raw material that comes from renewable resources. Biocatalysis is the use of whole cells or enzymes for the catalysis of chemical reactions. In this work two different model reactions have been studied: (1) production of an epoxide coating component (used e.g. in the painting of cars), through chemo-enzymatic epoxidation of allyl ethers, and (2) the production of alkanolamide surfactants through the amidation of fatty acid with ethanolamine.

For the synthesis of the epoxide (glycidyl ether) the aim was to find an alternative route to avoid the use of the toxic reagent epichlorohydrin, conventionally used in its manufacturing. Lipase B from *Candida antarctica* (CALB) was used to catalyse the formation of peracid from a fatty acid and hydrogen peroxide, which was utilised *in situ* for the epoxidation of the terminal unsaturated carbon-carbon bond of an allyl ether to form the desired epoxide. The reaction was found to be feasible in small scale and through optimisation a 75% yield of the product could be obtained from a reaction mixture containing up to one molar of the starting material. However, the conditions under which the epoxidation reaction was optimal was found to be too harsh for the enzyme. The need for a cheaper or more stable enzyme was identified as a major hurdle for industrial application of the technology.

The synthesis of alkanolamide surfactant was also catalysed by CALB; the reaction between dodecanoic acid and ethanolamine was used as a starting point. The reaction was

found to work very well under solvent free conditions if the amine was added in a step wise manner to avoid the formation of the viscous amine-acid salt. The yield of the reaction was pushed near to completion by removal of water, and only a minor amount of the amide-ester by-product was detected. The stability of the preparation was fairly good even at 90°C when the system was kept dry.

For large scale production using immobilised enzymes, the preferred reactor set-up is the packed-bed. This is due to easier handling of the catalyst as well as to avoid degradation of the preparation from prolonged stirring and repeated filtrations etc. For this reason a reactor system where the packed bed was connected to a reservoir holding the reactants via a loop was developed and characterised.

As the economics of the process was evaluated, it was found that both the stability and the cost of the biocatalyst had a great impact on the final cost of the product and at the current cost and deactivation rate of the enzyme, the enzymatic process would be approximately 30-40% more costly than a process catalysed by sodium methanolate.

In order to decrease the cost of the biocatalyst, the liquid lipase was absorbed onto a macroporous polypropylene carrier. A relatively low loading of the lipase was used to minimise the cost of the preparation per unit catalytic activity. The preparation obtained was found to be around 1/4 to 1/2 of the price of the commercial preparation. When studying the reaction of a fatty acid mixture (olein fatty acid) with ethanolamine at 70-90°C it was found that also the stability of the in-house preparation was superior. During repeated batches using a PBR-loop setup at 70°C, no deactivation of the in-house preparation could be seen after five batches. By this approach the added cost of the enzymatic process could probably be cut to an acceptable 0.1-0.2 euro per kilogram product. The added quality of the product, reduced risks of manufacturing, improved working environment and decreased energy requirement in the process might well motivate this cost. Further decreases in biocatalyst cost are possible if the catalyst finds a major application, such as in biodiesel production, then a cut in cost by a factor of ten is not unthinkable. This would undoubtedly open the door for many other applications.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Till dig som undrat vad jag egentligen har sysslat med de senaste fem åren:

I din kropp sker just nu tusentals kemiska reaktioner; korvmackan du åt till frukost bryts ner till sina minsta beståndsdelar för att sen antingen användas för att bygga upp dina muskler eller brytas ner ytterligare för att utvinna energi så att du ska orka läsa denna text. Enzymer är naturens katalysatorer, det vill säga, de har förmågan att göra kemiska reaktioner mycket snabbare utan att själv förbrukas.

Utan enzymer hade livet inte fungerat; korvmackan bryts ju inte ner av sig själv. Enzymerna tillverkas av levande organismer för att kontrollera de olika reaktioner som sker i cellerna. ”Korvmacka!? – jaha, då behöver vi stärkelsenedbrytaren, proteinnedbrytaren och fettnedbrytaren!” I kroppen och överallt i naturen finns enzymerna, till och med i så ogästvänliga miljöer som heta källor och saltsjöar.

Det dröjde ända till 1900-talets början innan man upptäckte att det fanns enzymer och man förstod hur de fungerade. Snart började man också fundera över hur dessa skulle kunna användas i människans tjänst. Nuförtiden används enzymer i en mängd produkter till exempel vid tillverkning av en billigare ersättare till kakaofett eller i tvättmedel för att bryta ner fläckar vid låg temperatur. Det är just enzyms förmåga att vara effektiva vid låg temperatur som gjort dem intressanta för användning vid produktion av kemikalier. Temperaturen i processen skulle då kunna sänkas (och därmed energiåtgången) och man skulle dessutom kunna använda naturliga råvaror i istället för sådana baserade på råolja. Enzymer är dessutom väldigt specifika, det vill säga de tillverkar bara det som ska tillverkas och inte en massa biprodukter. På så vis sparas också dyrbara råvaror – och därmed också miljön.

I mitt arbete har jag undersökt möjligheten att använda enzymer för att tillverka två olika kemikalier. Dels ett bindemedel i färg, en epoxid som idag tillverkas av bland annat epiklorhydrin, som är en potentiellt cancerframkallande kemikalie. Dels för tillverkning av en ingrediens i rengöringsmedel, en surfaktant. Klassiskt tillverkas surfaktanten vid hög

temperatur vilket ger upphov till oönskade biprodukter, dålig lukt och missfärgning. Detta har man löst genom att tillsätta en natriumbaserad katalysator som gör att processen kan köras vid lägre temperatur. Då får man inte så mycket föroreningar, men å andra sidan är katalysatorn farlig eftersom den kan ge upphov till explosioner, och dessutom måste man använda en annan råvara i tillverkningen som bildar metanol (träsprit) som biprodukt. Det hade helt enkelt varit mycket bättre om man hade kunnat använda enzymer för att katalysera reaktionen i stället!

Jag har använt ett enzym, upptäckt i en saltsjö på Antarktis, som i normala fall bryter ner fetter. Enzymet fungerar genom att fettmolekylen binder in i en liten ficka på enzymets yta där molekylen passar in – precis som en pusselbit. Därefter kommer en vattenmolekyl och reagerar med fettet som då spjälkas i två bitar. I mina processer har jag utnyttjat att enzymet också kan fungera ”baklänges” – om det inte finns något vatten i närheten kan enzymet också sätta ihop de två delarna till en. Man kan även sätta samman andra beståndsdelar, till exempel en amin och en fettsyra som då bildar surfaktanten. Mina försök visade också att detta fungerade precis som vi trott – vi fick de produkter vi hade hoppats på.

Vad är problemet då? Jo, enzymerna är väldigt dyra att tillverka. För att få ekonomi i tillverkningen måste man återanvända enzymet minst hundra gånger. Detta även om man bara behöver en hundradel så mycket enzym som vikten av det som tillverkas. För att kunna återanvända enzymerna tillräckligt antal gånger gäller det att hitta en balans mellan produktivitet i tillverkningsprocessen och stabilitet hos enzymet. I epoxidprocessen kunde vi tyvärr inte hitta någon sådan balans, men i surfaktantprocessen upptäckte vi att även om processen fungerade bäst vid hög temperatur, ledde en något lägre temperatur till att enzymet klarade sig längre. Med den slutliga processen skulle surfaktanten kunna tillverkas bara lite dyrare än idag och den extra kostnaden skulle säkert kompenseras av att processen blir säkrare och att produkten blir renare än idag.

LIST OF PAPERS

This thesis summarises the following papers, referred to in the text by their Roman numeral designations as I-V.

- I. Production of glycidyl ethers by chemo-enzymatic epoxidation of allyl ethers.
Tufvesson P, Adlercreutz D, Lundmark S, Manea M, Hatti-Kaul R.
Journal of Molecular Catalysis B: Enzymatic 54: 1–6 (2008)
- II. Solvent-free enzymatic synthesis of fatty alkanolamides.
Tufvesson P, Annerling A, Hatti-Kaul R, Adlercreutz D.
Biotechnology & Bioengineering 97: 447-453 (2007)
- III. Enzymatic synthesis of fatty alkanolamides in a recycle packed-bed reactor.
Tufvesson P, Karlsson A, Adlercreutz D, Hatti-Kaul R.
Manuscript.
- IV. Cost effective immobilization of Lipase B from *Candida antarctica* on Accurel MP1000 for synthesis of specialty chemicals
Tufvesson P, Carvalho J, Karlsson A, Hatti-Kaul R.
Manuscript.
- V. Technical, Economic and Environmental Evaluation of Enzymatic Alkanolamide Production.
Adlercreutz D, Tufvesson P, Karlsson A, Hatti-Kaul R.
Manuscript

MY CONTRIBUTION TO THE PAPERS

Paper I The project idea was from Stefan Lundmark and Nicola Rehnberg of Perstorp AB. The project was planned by me in collaboration with Rajni Hatti-Kaul (RHK) and Dietlind Adlercreutz (DIA). I did all the practical work. The first version of the manuscript was written by me and was finalised in collaboration with RHK and DIA.

Paper II The project idea was from Annika Annerling (later Karlsson) of AkzoNobel Surfactants. The project was planned by me in collaboration with DIA and RHK. I did most of the practical work; some lab work was performed by a Master student, Ana Monica Correia under my supervision. The first version of the manuscript was written by me and was finalised in collaboration with DIA and RHK.

Paper III The project idea was from me. The project was planned by me in collaboration with DIA and RHK. I did all the practical work. The first version of the manuscript was written by me and was finalised in collaboration with DIA and RHK.

Paper IV The project idea was from me and the project was planned by me. The practical work was mainly done by a Master student, Jorge Carvalho under my supervision; some laboratory work was done by me. The first version of the manuscript was written by me and was finalised in collaboration with RHK.

Paper V The project idea was from DIA and me. The project was planned by DIA in collaboration with me and RHK. I did the part of environmental analysis, some data collection for the simulation and a sensitivity analysis. The first version of the manuscript was written by DIA and was finalised in collaboration with me and RHK.

ABBREVIATIONS

LCA	Life cycle assessment
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
VOC	Volatile organic compound; causes smog and degrades ozone
CALB	<i>Candida antarctica</i> lipase B
N435	Novozym®435; CALB immobilised on Lewatit OC 1600
SEM	Scanning electron microscope
TMPME	Trimethylolpropane monoallyl ether
TMPTG	Trimethylolpropane triglycidyl ether
LA	Lauric acid; dodecanoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
TG	Triglyceride; i.e. oil or fat
MEA	Monoethanolamine
DEA	Diethanolamine
PBR	Packed-bed reactor
BSTR	Batch stirred tank reactor

ACKNOWLEDGEMENTS

I would like to acknowledge the following persons for their contributions to this thesis:

First and foremost I would like to thank my supervisor Rajni Hatti-Kaul. Thank you for accepting me as a student, thanks for your patience, for your never ending linguistic comments and for all the effort you have put into the projects and not least Greenchem. I am most sincerely grateful for all of this.

Dietlind – we finally managed to pull in the same direction and I think we both learnt very much getting there – joint custody of “babies” is never easy! Thank you for all your help – especially on how to write an academic paper.

I would also like to thank all my fellows within the industry: Thanks to all the people at AkzoNobel: Annika Karlsson and Ingegärd Johansson for all the input to the project and for keeping things realistic; many thanks to Annika for your encouragement and positive spirit. Special thanks for the possibility to work in the labs in Stenungsund and Arnhem. We had a really good time in Arnhem and were so well cared for by Pablo and Robert (Pablo: I owe you a cidra for the articles you sent me.) Thanks also to Margareta Strandbergh for sending me the fatty acids that I worked with.

Stefan Lundmark and Mircea Manea of Perstorp AB, thank you for your help in setting up the epoxidation project, for your enthusiasm and millions of ideas. Thanks also to Rebecka Lidholm who did GC-MS of some of my first epoxidised samples.

My portuguese project workers Ana Correia and Jorge Carvalho: agradecimentos tanto para o seu árduo trabalho!

I would also like to thank the many persons at the department of biotechnology who have contributed to a nice and quite unique atmosphere during these years; seniors and “juniors”.

Special thanks to Martin Hedström for your help with the MS and with this thesis and for having such a great positive attitude.

Thanks also to Harald Kirsebom for the help in taking the SEM-pictures, that was really fun.

Thanks to all our secretaries: Erik, Josse, Anna & Siv-å-Kersti for all your help with administrative matters.

I would like to thank the members of the Greenchem board, with the Green Knight, Harald Skogman, leading the way. Thanks for your commitment and especially for the funds to go to Biotrans in Oviedo, Spain, which was very rewarding.

Till Greenchem gänget: David, Mathias, Ulrika, Anna och Pernilla. Livet hade inte varit det samma utan er...och jag kommer aldrig att glömma er även om ni försvinner långt bort i världen ;-)

Linda, Freja och Axel: mina små älsklingar! Tack för att ni gett mig något viktigare att tänka på än enzymer och tack för att ni stått ut med mitt enzymsnack och mina kvällar och nätter i labbet.

Till mina kära syskon och föräldrar vill jag säga: tack för ert stöd i vått och torrt! Jag hoppas nu verkligen att ni läser avhandlingen så att ni äntligen förstår vad jag gjort!!

CONTENTS

1	INTRODUCTION	1
2	SUSTAINABLE CHEMISTRY	5
2.1	The dawn of environmentalism	5
	<i>Sustainable development</i>	6
2.2	Green chemistry	7
2.3	Governmental initiatives	10
	<i>Sweden's environmental quality objectives</i>	10
	<i>REACH</i>	10
2.4	Environmental management tools	11
	<i>Life cycle assessment</i>	11
	<i>Eco-efficiency analysis</i>	12
	<i>Responsible care ®</i>	13
3	BIOCATALYSIS FOR GREEN CHEMISTRY	15
3.1	Industrial biotechnology	15
3.2	Biocatalysis	16
	<i>Enzymes in organic media</i>	16
	<i>Enzyme stability</i>	19

3.3 Lipases	21
<i>General</i>	21
<i>Substrate specificity</i>	23
<i>Structure and mechanism</i>	24
<i>Candida antarctica lipase B</i>	24
3.4 Enzyme process engineering	29
<i>Immobilisation of enzymes</i>	29
<i>Water removal</i>	32
<i>Choice of reactor system</i>	33
4 LIPASE CATALYSED SYNTHESIS OF SPECIALITY CHEMICALS	39
4.1 Coatings	39
<i>Epoxides</i>	40
<i>Chemo-enzymatic epoxidation</i>	42
4.2 Biosurfactants	48
<i>Alkanolamides</i>	49
<i>Enzymatic synthesis of alkanolamides</i>	50
5 FUTURE PERSPECTIVES & CONCLUDING REMARKS	57
6 REFERENCES	61

1 INTRODUCTION

“A technological society has two choices: first it can wait until catastrophic failures expose systemic deficiencies, distortion and self-deceptions. Secondly, a culture can provide social checks and balances to correct systemic distortion prior to catastrophic failures”

Mahatma Gandhi

We are facing perhaps the greatest challenge in the history of mankind – the shift from a depleting to a sustainable society. Politicians, industry, the scientific community and last but not least the people, together have a responsibility in making this happen. It will not be easy, but our future may depend on our success. On the positive side there has been a surge of interest from the media concerning the effects of greenhouse gas emissions and the risks of global climate change. As people are recognising the problem they have the power to put pressure on companies and government to provide environmentally friendly products and policies. The question remains however if people are ready to pay the price of more environmentally friendly products or if they are willing to change their way of life in terms of a lower consumption and different travelling habits.

Other threats to the environment are the release of hazardous chemicals or heavy metals into the environment and chemicals that cause eutrophication or other effects on the

ecosystems where they are released. The 12 principles of Green Chemistry (see chapter 2) suggest a concept for a systematic approach to reduce or eliminate the generation or use of such hazardous substances and to minimise waste generation and energy consumption. This concept was adopted by the research programme *Greenchem – Speciality Chemicals from Renewable Resources* aiming towards the use of the tools of modern biotechnology to develop more environmentally friendly processes for production of chemicals.

Greenchem is a multidisciplinary programme based at Lund University involving researchers and students from different knowledge fields as well as representatives from Swedish companies involved in chemicals manufacturing and prospective users of new “green” products. The programme has been funded by MISTRA, the Swedish Institute for Strategic Environmental Research, since July 2003.

MISTRA promotes research that will have a positive impact on Sweden's future competitiveness and that plays a significant role in solving major environmental problems and contributes to the development of a sustainable society. One of the main goals is that a product is put on the market – thus really realising the environmental benefits of the research.

In *Greenchem* biocatalysis has been used with the aim of developing economically competitive, environmentally friendly processes. Biocatalysis is the use of whole cells or part of cells, e.g. enzymes, to carry out a chemical reaction. While enzymes carry out most of the thousands of reactions in the living cell, in recent years they have also been extensively explored for their capabilities to catalyse chemical reactions for which they were not intended in nature. The use of biocatalysis for industrial synthetic chemistry offers several potential advantages such as more selective processes, i.e. processes that yield more product per amount of starting material in fewer steps, and lower energy demand as process temperatures are normally lower than the corresponding chemical processes. However, generally enzymes are expensive and have a limited stability under process conditions. Thus the challenge is to find the optimal compromise between productivity and stability and to find those processes where biocatalysis can provide a significant added value compared to conventional chemistries.

This thesis deals with the biocatalytic production of two important classes of speciality chemicals: epoxides (**Paper I**) and fatty amides (**Paper II-V**). Epoxides are a group of reactive compounds that are used in coating applications. The present investigation has focused on finding alternative routes for the production of a special class of epoxides, glycidyl ethers, through a reaction combining an enzymatic peracid formation step with a chemical epoxidation step.

In the other part of the study, a solvent-free biocatalytic process for the production of fatty amides was developed (**Paper II**). Fatty amides are used as mild detergents and additives in cleaning products. To minimise the process cost, two different strategies were investigated: use of the enzyme in a packed bed format (**Paper III**) to maximise the process stability and immobilising the enzyme on a cheaper carrier material while maintaining the catalytic performance and operational stability (**Paper IV**). Finally the enzymatic process was benchmarked to existing production strategies with regards to economic and environmental performance (**Paper V**).

2 SUSTAINABLE CHEMISTRY

2.1 The dawn of environmentalism

Over the past century chemical science and technological inventions have made an incredible impact on our everyday lives. Major improvements in our quality of life have arisen thanks to pharmaceuticals, plentiful food and safe drinking water as well as comforts such as easy transportations and the wide range of consumer goods made from plastic materials. One effect of this development is that human life expectancy in the western world has increased from about 50 years at the beginning of the 20th century to almost 80 today. However, it can not have escaped many people that this development has also come at a price: climate change, pollution, extinction of species and depletion of non-renewable resources.

It seems that in the beginning of the industrial evolution few people understood the negative effect chemicals could have on the environment. Ironically the discovery of the insecticide DDT, today perhaps the world's most infamous chemical, was even awarded the Nobel Prize in 1948 for its success in battling typhus and malaria. In 1962 the book *Silent Spring* written by Rachel Carson was published, wherein she claimed that the use of insecticides such as DDT not only killed mosquitoes but also birds and that it was the cause of cancer in humans. Although the book has been criticised for being based more on fears than fact (Ronald Bailey, *Silent Spring* at 40, www.reason.com), it came to mark the beginning of the modern environmentalism movement and a growing broad public

concern for environmental issues which ultimately also led to action by governments and industry.

Sustainable development

With time, other environmental issues such as global warming, ozone depletion, desertification and the release of toxic chemicals came on the agenda. In response to these threats the United Nations General Assembly assigned Gro Harlem Brundtland chair of a commission that should “propose long-term environmental strategies for achieving sustainable development to the year 2000 and beyond”. The result was published in 1987 in *Our Common Future*¹, also known as the Brundtland-report. This is the origin of the most widely used definition² of sustainable development:

“Sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs”

The report stresses that the environment can not be seen as isolated from human actions, ambitions and needs. The problems have to be addressed in a technological, social and economical context. The report also proposes that the role of the scientific community as well as the co-operation with industry has to increase.

It appears that the Brundtland report actually had a large impact on the community, because in the years to come several different initiatives and ideas were launched, among them the concepts of *Industrial ecology*, *Cleaner production* and *Green Chemistry*. A

¹ Living standards that go beyond the basic minimum are sustainable only if consumption standards everywhere have regard for long-term sustainability. Yet many of us live beyond the world's ecological means, for instance in our patterns of energy use. Perceived needs are socially and culturally determined, and sustainable development requires the promotion of values that encourage consumption standards that are within the bounds of the ecological possible and to which all can reasonably aspire. – Our Common Future

² Angela Merkel: “use resources no faster than they can regenerate themselves and release pollutants to no greater extent than natural resources can assimilate them”

common feature is the idea of *waste minimisation*; it is better to avoid the formation of waste rather than cleaning up or treating it at the end of the process.

Waste minimisation hierarchy:

- (1) Eliminate or prevent source
- (2) Reduce source
- (3) Recycle in process
- (4) Reuse or recover
- (5) Convert to less harmful materials / Energy recovery
- (6) Dispose safely of waste which cannot be efficiently recycled or treated

Apart from this and the common goal of bringing about a change towards an environmentally sustainable industry, the three concepts differ somewhat in their approach of how this can be accomplished.

The *Industrial ecology* idea is that industry should be seen as an integral part of the ecosystem and that material flows should mimic nature in creating closed loops where the waste streams of one process is the raw material for the next process. This idea was not new in the end of the 80's but was made generally popular through an article by Robert Frosch in the journal *Scientific American* in 1989. Inherently from its concept, industrial ecology emphasises that a life cycle perspective is used in the development of processes and products.

The related concept of *Cleaner production* intends to minimise waste and emissions by analysing the flow of materials and energy in a company. Technological improvements but also improvements in organisation, education, documentation and process control are seen as important tools.

2.2 Green chemistry

In parallel, the concept of Green Chemistry was launched in the beginning of the 90's by the American Environmental Protection Agency (EPA), with the organic chemists Paul Anastas and John Warner being its founding fathers and its strongest advocates. Green Chemistry aims at helping chemists design chemical products and processes that reduce or

eliminate the use of hazardous chemicals. This is done by applying to a set of 12 principles (Anastas and Warner 1998):

- (1) Prevent waste: Design chemical syntheses to prevent waste, leaving no waste to treat or clean up
- (2) Design safer chemicals and products: Design chemical products to be fully effective, yet have little or no toxicity
- (3) Design less hazardous chemical syntheses: Design syntheses to use and generate substances with little or no toxicity to humans and the environment
- (4) Use renewable feedstock: Use raw materials and feedstocks that are renewable rather than depleting. Renewable feedstocks are often made from agricultural products or are the by-products of other processes; depleting feedstocks are made from fossil fuels (petroleum, natural gas, or coal) or are mined
- (5) Use catalysts, not stoichiometric reagents: Minimise waste by using catalytic reactions. Catalysts are used in small amounts and can carry out a single reaction many times. They are preferable to stoichiometric reagents, which are used in excess and work only once
- (6) Avoid chemical derivatives: Avoid using blocking or protecting groups or any temporary modifications if possible. Derivatives use additional reagents and generate waste
- (7) Maximise atom economy: Design syntheses so that the final product contains the maximum proportion of the starting materials. There should be few, if any, wasted atoms
- (8) Use safer solvents and reaction conditions: Avoid using solvents, separation agents, or other auxiliary chemicals. If these chemicals are necessary, use innocuous chemicals. If a solvent is necessary, water is a good medium as well as certain eco-friendly solvents that do not contribute to smog formation or destroy the ozone layer
- (9) Increase energy efficiency: Run chemical reactions at ambient temperature and pressure whenever possible
- (10) Design chemicals and products to degrade after use: Design chemical products to break down to innocuous substances after use so that they do not accumulate in the environment

(11) Analyse in real time to prevent pollution: Include in-process real-time monitoring and control during syntheses to minimise or eliminate the formation of by-products

(12) Minimise the potential for accidents: Design chemicals and their forms (solid, liquid, or gas) to minimise the potential for chemical accidents including explosions, fires, and releases to the environment.

One example of where Green chemistry is being promoted is a UK governmental program, Envirowise, which offers free advice to companies to help them implement green chemistry. It is argued that implementing the 12 principles will help companies comply with legislation, reduce waste and get a better public perception. One example of how manufacturers can benefit from environmentally friendly technology is an enzymatic polyester synthesis which is run at lower temperatures and avoiding the use of metal catalysts saving energy and giving a competitive edge through higher product quality (Forum-for-the-Future 2004).

Green Chemistry has indeed received a lot of interest in recent years, not least from academia. However, the green chemistry principles have also been criticised for being too narrow, One attempt to improve the green chemistry concept and to help compare the greenness of different processes and to assess the potential for waste minimisation was suggested by Neil Winterton through the “12 more principles of Green Chemistry” (Winterton 2001). These principles are more or less directed at getting a thorough understanding of the process that is being investigated, by analysing the process in terms of conversions, by-products, thermodynamics and use of utilities. It is also recommended to develop and apply measures of the sustainability of the process.

The Green chemistry concept has also been criticised for not taking the whole life-cycle of a product into account: “...failing to capture many environmental aspects of chemical manufacture, use and disposition” (Graedel 1999). The importance of a life cycle perspective has also been acknowledged by Paul Anastas in the article “Life cycle assessment and green chemistry: the yin and yang of industrial ecology” (Anastas and Lankey 2000) where it is argued that this is in fact inherent to the green chemistry principles where the term ‘chemistry’ is understood in its broadest sense, taking the hazards of e.g. global warming into account when considering energy generation and use.

2.3 Governmental initiatives

Sweden's environmental quality objectives

The Swedish Parliament has adopted 16 environmental quality objectives, which define the aim of the environmental work of the Swedish Chemicals Agency (<http://www.kemi.se/> 2008). Some of these are the same as the impact categories mentioned above, relating to: climate change, acidification and eutrophication. Special emphasis is given to the environmental goal: *A non-toxic environment*. The vision is that the environment will be free from man-made or extracted compounds and metals that represent a threat to human health or biological diversity and that, one generation from now, the major environmental problems currently facing us will have been solved. Milestones in this work are among others the phasing-out of particularly hazardous substances and the minimisation of risks for health and environment associated with the manufacture and use of chemical substances.

To accomplish this the Swedish government seeks to gain a better knowledge of the impact of chemical substances on the environment and health, a better information on the chemical substances we use, to get away from the use of particularly hazardous substances and to handle chemical substances more safely. For this to be a reality the chemical manufacturers need effective environmental management systems (EMS), but also a powerful legislation will be needed (Swedish Chemicals Inspectorate 2007).

REACH

A piece of legislation that is currently being implemented in the European Union is REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), which is the new European Chemicals Policy and was entered into force on June 1st 2007 (European Chemicals Agency 2008). REACH places greater responsibility on industry to manage the risks that chemicals may pose to the health and the environment. The industrial actors are the ones that must identify and manage the risks linked to the substances they manufacture and provide appropriate safety information to their users. All chemicals manufactured or imported into Europe over one tonne per year will also need

to be registered by the manufacturers and/or importers. If a substance is not registered it will be unlawful to manufacture or import it into the European Union.

The aims of REACH are not only to improve the protection of human health and the environment from the risks that are posed by chemicals, but also to enhance the competitiveness of the European Union's chemicals industry, to promote alternative methods for the assessment of hazards of substances and to ensure the free circulation of substances within its internal market (European Chemicals Agency 2008).

2.4 Environmental management tools

In order to assess the actual impact of a chemical or product on the environment different methods have been suggested such as Life Cycle Assessment (LCA) and the eco-efficiency method. These methods as well as the chemical industry's Responsible care ® initiative, will be discussed below.

Life cycle assessment

Life Cycle Assessment is a part of the ISO 14000 standardised methodology (ISO 1998) and is used for measuring the impact of a product including the full life cycle from cradle (raw materials production) to grave (waste disposal) as well as the impact during its use-phase (see Figure 2.1). One important lesson from LCA work is that it is crucial to identify the step in a product's life cycle that has the highest impact on the environment so that the efforts for improvements can be focused there and to avoid shifting the environmental burden of one phase into another, e.g. from the production to the use phase or vice versa or from one environmental impact to another (e.g. from greenhouse gas emissions to eutrophication). It was, for example, found in **Paper V** that the main contribution to energy consumption in the production of alkanolamide surfactant is not in the actual process step but rather in the production of the raw materials. This means that the benefit of biocatalysis does not come from the lower temperature in the process (as is generally stated) but from the improved utilisation of the raw materials, which can be achieved with a high process yield.

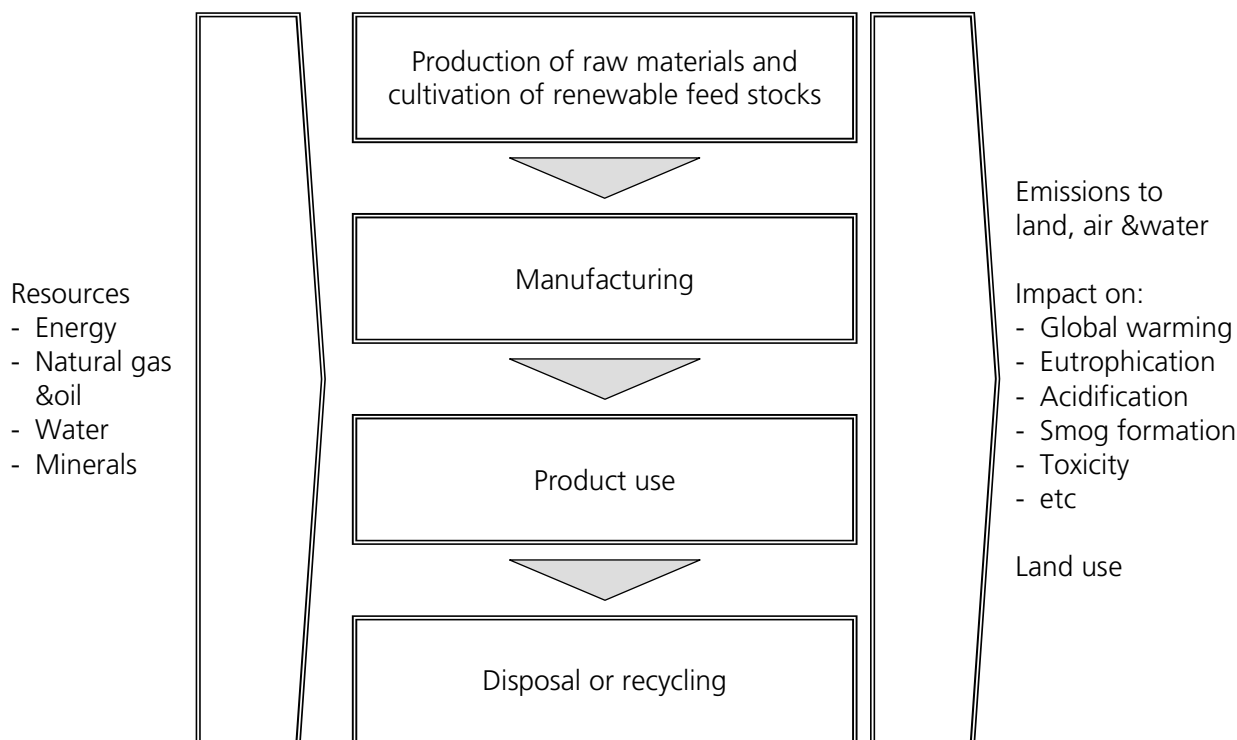


Figure 2.1 Overview of steps included in a Life Cycle Assessment of a chemical product from cradle-to-grave. The emissions from transportation are also included.

Some of the commonly used environmental effects or impact categories studied in an LCA are: global warming potential (CO_2 equivalents), eutrophication (PO_4^{3-} equivalents), acidification (SO_2 equivalents), photochemical smog generation, ecotoxicity human toxicity and raw materials and energy use.

In **Paper V** only the energies used in the production of the raw materials and the processing energies were calculated, but the impact on working environment also was discussed, however in more qualitative terms.

Eco-efficiency analysis

Eco-efficiency analysis is a strategic sustainability tool used by many companies. The method was developed to consider both the economic and life cycle environmental effects of a product or process, giving these equal weighting; the goal is to create more value with less environmental impact (Saling et al. 2002). The major elements are basically the same

as in LCA. National environmental goals and economic data are used to assess the relevance of the environmental and economic impacts (Shonnard et al. 2003).

Responsible care ®

The Responsible Care program is a voluntary environmental initiative launched by the chemical industry itself. Its main objectives are to improve the chemical industry's environmental performance and to improve the relationship with government and public. This is done by working together with other industries in national associations (<http://www.responsiblecare.org/> 2008). The program has also promoted the members' progress towards taking a responsibility for the environmental performance of products through a life-cycle perspective and also advocates a shift in attitude towards environmental issues that go "beyond compliance".

3 BIOCATALYSIS FOR GREEN CHEMISTRY

3.1 Industrial biotechnology

Industrial or 'white' biotechnology is the application of microorganisms and biological catalysts (enzymes) for the production of chemicals, materials and energy (EuropaBio). Historically microorganisms and enzymes were used without knowledge of their function. For instance, cheese, wine and bread have been prepared for many hundreds of years even without the knowledge that microorganisms or enzymes were even present; but with the advancement of science and biotechnology their unique properties are exploited in entirely new ways.

Hopes are that white biotechnology will transform the chemical industry; from petrochemically based oil refineries using harsh reaction conditions to so called bio-refineries where today's commodity chemicals can be produced from renewable feedstocks using mild bioprocesses and thereby contributing to a more sustainable chemical industry.

There are many examples of how biotechnology can be used in industrial sectors such as chemistry, food and feed, paper and pulp, textiles and energy. Some are the use of enzymes in detergents (which facilitates stain removal at lower temperatures), the production of amino acids and vitamins by fermentation, using recombinant microbial strains (Frazzetto 2003) and the use of immobilised glucose isomerase enzyme for the production of high fructose corn syrup (Liese et al. 2006).

3.2 Biocatalysis

Biocatalysis involves the use of enzymes or whole cells (containing the desired enzyme or enzyme system) as catalysts for chemical reactions. Indeed biocatalysis fits very well into the Green Chemistry and sustainable chemistry concepts (Jaeger 2004): the processes are inherently very benign as they are run at low or moderate temperatures, preferably natural substrates and no toxic chemicals are used in the process. Further enzyme processes are normally very selective and do not need protecting deprotecting steps, all of which leads to a high atom economy (Schmid et al. 2001). In a life-cycle assessment of emollient esters, a cosmetic ingredient, at Evonik GmbH comparing a biocatalytic and a conventional process it was shown that the bioprocess consumed 60% less energy and the emission of unwanted pollutants was reduced by up to 90% (Thum and Oxenbøll 2008).

In an overview by Straathof et al (2002) it is reported that the use of biocatalysis in industry has increased exponentially since the 1960's; although the number of processes was still limited to 134. The study showed that the majority of the processes have chiral compounds as products and that about half of the processes are for the production of pharmaceuticals; the rest are divided between food, agro, cosmetics, polymers and feeds sectors. Enzymes used are lyases, transferases, isomerases, oxidoreductases, oxidising or reducing whole cells and not least hydrolases, which make up almost 50% of the processes.

Clearly enzymes have found use in industry especially in the pharmaceutical industry (Liese et al. 2006). Some of the frontrunners are companies like DSM (the Netherlands), Lonza (Switzerland) and BASF (Germany). However, the number and diversity of processes is still low and the production of speciality chemicals using biocatalysis is restricted to a few examples, for instance the emollient ester synthesis at Evonik GmbH, Germany (Hills 2003). Sometimes availability of suitable enzymes is a limitation, but often low stability (Schoemaker et al. 2003) and high costs for the enzyme are the major barriers for use.

Enzymes in organic media

Nature is typically seen as a water based system. Therefore it is not surprising that enzymes were long seen as something that can only be used in water. This view was

radically changed in the late 70's and early 80's by reports of enzymes used in organic media (Klibanov et al. 1977; Tarquis et al. 1980) and particularly by the publications by Zaks and Klibanov (Zaks and Klibanov 1985; Zaks 1984) who showed that lipases were highly active and stable in different organic solvents at low water concentrations.

There are several advantages of conducting enzymatic conversions in organic solvents as opposed to water: most organic compounds are more soluble in non aqueous media than in an aqueous one which means that new reactions can be carried out and that the biocatalytic step is more easily integrated into a chemical process. Further, unwanted side reactions caused by water can be reduced and the equilibrium in hydrolytic reactions can be shifted towards synthesis (Kvittingen 1994; Zaks and Klibanov 1985).

If organic solvents are to be used it is generally recommendable to use hydrophobic solvents ($\log P > 4$) in order to keep the enzyme active (Laane et al. 1985; Reslow et al. 1987). To dissolve hydrophilic substrates, hydrophilic organic solvents such as acetonitrile or tert-butanol may be needed. However, these solvents are often seen to deactivate the enzyme more rapidly. A suggested explanation to this phenomenon is that hydrophilic solvents tend to strip the enzyme of essential water, thereby deactivating it (Gorman and Dordick 1992). It has also been observed that different organic solvents affect the flexibility of certain amino acids on the enzyme (Trodler and Pleiss 2008).

It could be seen as surprising that enzymes have the ability to work in organic solvents at all. Most enzymes have a hydrophilic outside and a relatively hydrophobic inside and thus it would be more thermodynamically favourable for the enzyme to literally turn inside out, displaying its hydrophobic interior to the solvent. It seems, however, that the enzyme is unable to unfold because of high kinetic barriers (Zaks and Klibanov 1988), i.e. it is trapped in its original state. On the other hand, there are also many examples showing that the enzymatic activity in organic solvents is orders of magnitude lower than in aqueous systems because of diffusional limitations, changes in protein flexibility, or destabilisation of the enzyme (Clark 2004). Some organic solvents could also disrupt many of the noncovalent interactions (*e.g.*, hydrogen bonding, salt-bridges, van der Waals contacts) that stabilise the folded enzyme (Toba and Merz 1997).

For environmental reasons the use of organic solvents can also be disadvantageous since they are a source of volatile organic compounds, causing low-level ozone and smog. Moreover many solvents are banned from use in food and personal care products (Lancaster 2002). If the use of solvents is inevitable, solvents with a low environmental impact should be selected. Doble and Kruthiventi (2007) suggest the use e.g. of isoamyl alcohol and other alcohols, ethyl lactate and dimethylpropylene urea, others promote supercritical carbon dioxide (Beckman 2004) or ionic liquids (Holbrey and Seddon 1999). However, the question is how to determine how green a solvent is. Life cycle assessment (LCA) methodology was used by Zhang et al. (2008) to compare the use of ionic liquids with conventional chemistry and found that ionic liquids were not the best choice due to low stability and recyclability. Another study combined LCA and an EHS (Environment, Health, Safety) tool to rank 26 different solvents and found that simple alcohols (methanol, ethanol; however not suitable for enzymatic synthesis) or alkanes (heptane, hexane) were environmentally preferable. In **Paper I** different solvents were tried, however toluene was seen to be by far the most effective solvent.

In many cases the reaction can be run in the absence of any solvents, i.e. solvent-free. For the work reported in **Paper II-V** it was a prerequisite, that no solvent was to be used in the process. This is obviously the best choice both from an environmental as well as from an economical perspective, as there will be no need for costly post treatment in the form of solvent evaporation and recycling or disposal of the solvents. Furthermore, dilution of the reactants generally results in lower reaction rates as well as the need for larger and more expensive reactors and auxiliary equipment.

pH effects in organic media

The pH and ionisation state of the enzyme will affect the activity and stability of the enzyme (Halling 1994). In organic media, organic salts can be added to buffer the reaction solution, however many times it is enough to immobilise or dry the enzyme at the correct pH using ordinary buffers. The enzyme will keep its ionisation state from the aqueous buffer. Further, the use of pH-additives would be impractical and un-economical. In the amidation reaction in **Paper II-V**, pH is likely to have shifted through the course

of reaction: from acidic to slightly basic. It does not seem however, that this affected the reaction significantly.

Enzyme stability

The operational stability of a biocatalyst is usually critical for its applicability in industry as it will directly influence the added cost of the enzyme per kilogram of product (Fernandes et al. 2005; Hills 2003). Enzymes lose their activity by different adverse influences. The loss in activity is either caused by unfolding of the enzyme and/or by direct chemical inactivation of the enzyme (Fagain 1995). When the enzyme is unfolded, it can theoretically fold back into the correct shape but often it is denatured. The unfolded state is generally also more susceptible to inactivating chemical effects like oxidation, deamidation, cleavage of sulphur bonds or hydrolysis of peptide bonds (Jaenicke 2000).

Temperature is obviously one factor that will influence the stability of enzymes very strongly. Not only does higher temperatures make unfolding of the enzyme more likely, but it will also increase the speed of the inactivating reactions. The Arrhenius equation states that the reaction rate of a chemical reaction will approximately double with an increase of ten degrees Kelvin. Since a higher temperature probably will also lead to faster rates of the desired reaction, a compromise in temperature will be needed. In **Paper IV** the stability of the enzyme preparation was investigated in the range of 70 to 90 °C and it was clear that the deactivation increases significantly with each increase in temperature by 10°. For the total economy of an enzymatic process a good stability of the enzyme is more important than high reaction rates (**Paper V**).

Solvents could also, as discussed above, affect the stability of the enzyme by destabilising the weak forces holding the enzyme together. This is especially true for protic hydrophilic solvents such as methanol, but also water is a very important factor for inactivation. Water is a part of many of the deleterious covalent reactions causing deactivation, and higher water activities also facilitate rearrangements, since an unfolded enzyme require more water molecules for solvation (Fagain 1995). Turner (1995) demonstrated that the temperature at which a protein undergoes thermal denaturation is highly dependent on

the water activity of the media. The denaturation temperature was found to be 30-50°C higher in anhydrous environments than in water. In another study, Turner et al also demonstrated that lipase from *Candida antarctica* can catalyse a transesterification reaction at 130°C for a significant amount of time, if the system was kept dry (Turner and Vulfson 2000). These findings are also supported by the observation in **Paper II**. When the reaction was carried out under conditions where the water could be evaporated the half-life of the enzyme was doubled.

An oxidative environment is often damaging to proteins. Oxidation of proteins can lead to a number of reactions such as hydroxylation of aromatic groups, sulfoxidation of methionine or cysteine residues, and also cleavage of the polypeptide chain (Stadtman and Levine 2003). Methionine, tryptophan, tyrosine, histidine, cysteine and cystine are amino acids especially sensitive to oxidation. Surface-exposed residues are particularly sensitive however oxidation can also occur at the protein backbone (Davies 2005). In **Paper I** the enzyme was exposed to high concentrations of hydrogen peroxide (20-50%), resulting in rapid loss of activity. The effects of hydrogen peroxide on lipase activity and stability during chemo-enzymatic epoxidation of fatty acids was investigated by Törnvall et al. (2007) by incubating the enzyme at different concentrations of H₂O₂ and at different temperatures. The enzyme was found to be reasonably stable at 20°C even at high concentrations of H₂O₂ (12M) whereas at a temperature of 60°C the inactivation was rapid with the rate of deactivation increasing with increasing hydrogen peroxide concentration.

There are different available options for obtaining a biocatalyst with improved stability: selection of enzymes from microorganisms that grow optimally at extreme temperatures pH, ionic strength, etcetera (Hough and Danson 1999; Lagarde et al. 2002) as these enzymes are inherently more stable. For example Ikeda and Clark (1998) characterised an esterase from *Pyrococcus furiosus* (a hyperthermophilic archeon) that showed an optimum temperature at 100°C and a half-life of 50 minutes at 126°C in aqueous buffer. Screening for thermostability might also be generally beneficial for the stability of the enzyme since there seems to be a correlation between thermostability and stability towards other factors (Owusu and Cowan 1989).

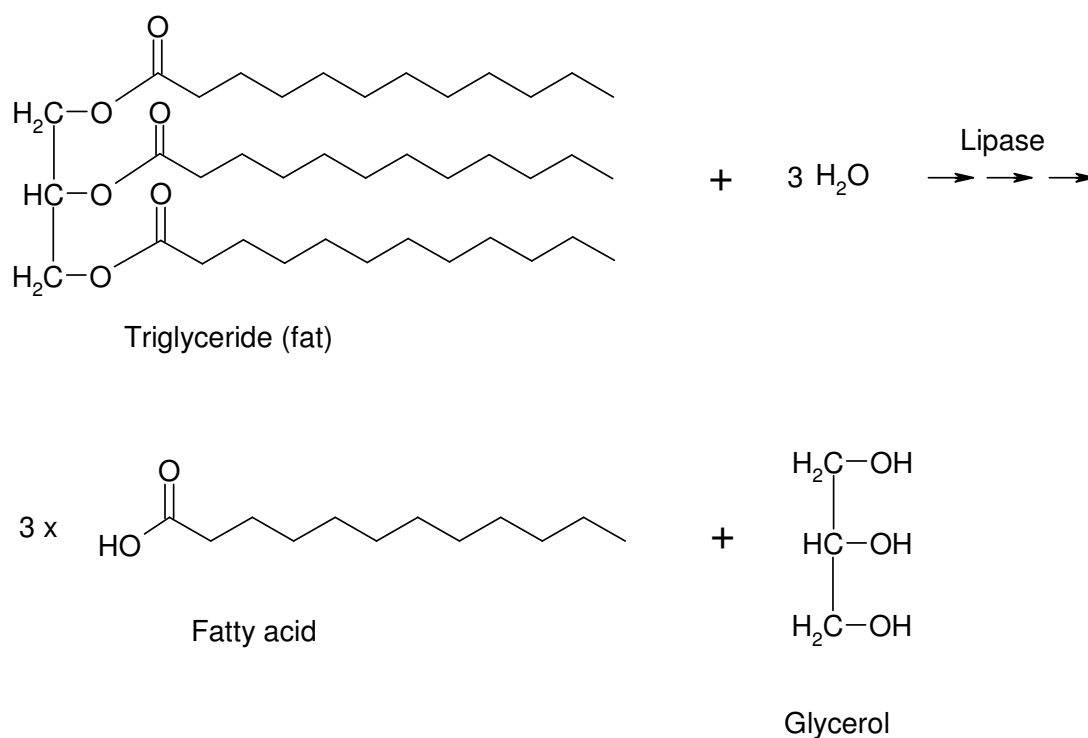


Figure 3.1 Enzymatic hydrolysis of a triglyceride into fatty acids and glycerol.

Another approach is the use of genetic engineering tools such as directed evolution (Fox et al. 2007; Kaur and Sharma 2006; Zhang et al. 2003) or rational design (Hult and Berglund 2003) to improve properties of the enzyme. This technique was for example successfully used to increase the melting temperature of an esterase from *B. subtilis* from 52-66°C without affecting the catalytic activity negatively (Giver et al. 1998).

3.3 Lipases

General

Enzymes are classified according to the type of reaction they catalyse, and lipases (3.1.1.3) are a sub-class of enzymes within the esterase family whose natural function is to hydrolyse long chain triacylglycerols (i.e. oils or fats) as depicted in Figure 3.1 (Schomburg and Salzman 1991). Lipases are found widely in nature in microorganisms, animals and plants where their function is to digest lipids in order to make these available as an energy source for the cells (Krishna and Karanth 2002). Fungi and bacteria secrete

lipases to their surroundings to facilitate nutrient absorption from the external medium. There are many potential applications of lipases as can be seen in Table 3.1, as in leather processing, animal feed, pulp and paper processing, etc (Pandey et al. 1999), however the most significant industrial applications of lipases have mainly been found in the pharmaceutical sector, in food and as already mentioned in detergents (Houde et al. 2004). One very useful trait of lipases is their enantioselectivity; this is exploited in the pharmaceutical industry where lipases are used for the preparation of single-isomer chiral drugs, either by kinetic resolution of racemic alcohols, acids, esters or amines, or by the desymmetrisation of prochiral compounds (Gotor-Fernández et al. 2006). The scope of the current thesis however, is in the preparation of non-chiral chemicals.

Table 3.1 Industrial applications of lipases

Industry	Product	Function	Reference
Food	Cocoa butter equivalent	Trans esterification	(Coleman and Macrae 1977)
	Human milk fat substitute		(Yang et al. 2003)
	Mono/diacyl glycerol	Emulsifier etc	(Bornscheuer 1995; Fureby 1995)
	Cheese	Flavour development	(Kirk et al. 2002)
	Flavour ester	Esterification	(Langrand et al. 1990)
	Bread	Dough stability and conditioning	(Kirk et al. 2002)
Cosmetics	Fatty acid ester	Emollient	(Hills 2003)
Detergents	Stain removal	Lipid degradation	(Kirk et al. 2002)
Pulp and paper	Pitch control, contaminant control	Lipid degradation	(Kirk et al. 2002)
Leather	Degreasing	Lipid degradation	(Kirk et al. 2002)
Organic synthesis		Resolution of chiral alcohols and amide	(Kirk et al. 2002)

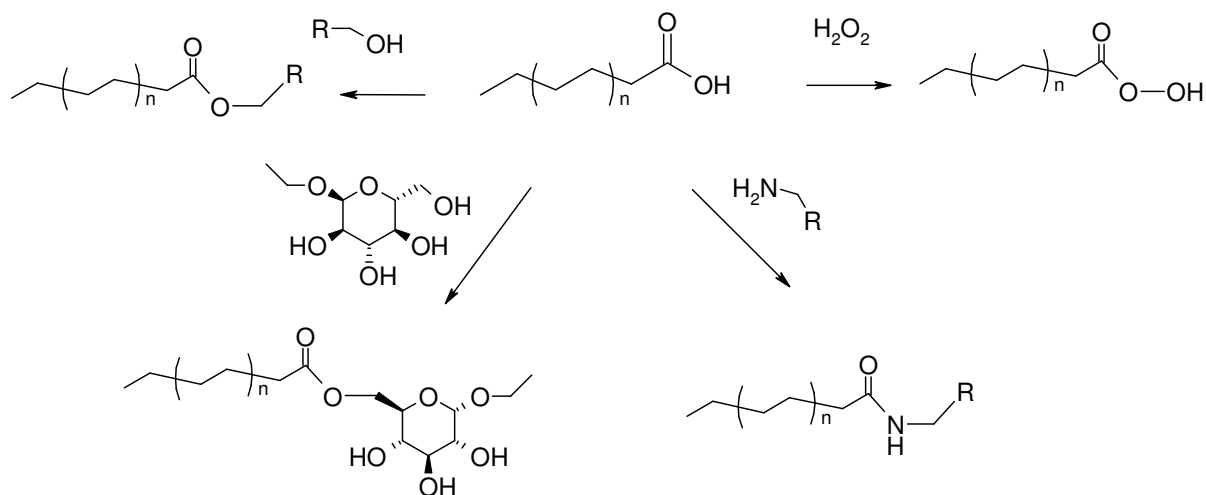


Figure 3.2 The lipase can catalyse many different synthetic reactions. Clockwise from top right: perhydrolysis, amidation, glucose ester and ester formation.

Substrate specificity

To understand the molecular basis of substrate specificity, Schmid and co workers analysed and compared six lipases with different substrate specificities for the acyl group (Pleiss et al. 1998). It was found that the lipases have a large, hydrophobic fatty acid binding site but that the lipases differ in the geometry of their binding sites. The lipases were subdivided into three sub-groups: (1) lipases with a hydrophobic, crevice-like binding site located near the protein surface (lipases from *Rhizomucor* and *Rhizopus*); (2) lipases with a tunnel-like binding site (lipase from *Candida rugosa*) (3) lipases with a funnel-like binding site (lipases from *Candida antarctica*, *Pseudomonas* and mammalian pancreas and cutinase).

The shape and micro-environment of the binding site determine the substrate specificity of the lipase. The broad synthetic potential of lipases is largely due to the fact that they accept a wide range of substrates (see Figure 3.2 for some examples). All lipases accept esters of medium (C4) to long chain (C16) fatty acids, some of them efficiently hydrolyse fatty acid esters as long as C22. Others have esterase activity, thus accepting short acetyl groups (Pleiss et al. 1998). Apart from aliphatic acids, lipases can also accept a wide range of unnatural substrates such as cyclic, and aromatic esters (Schmid and Verger 1998). In

the same way the properties of the alcohol binding site determines the selectivity for the nucleophile.

Structure and mechanism

Common to all lipases is the α/β -hydrolase fold structure, which means that the enzyme is composed of a core of predominantly parallel β strands surrounded by α helices. The folding pattern arranges the residues of the catalytic triad of serine, histidine and aspartic or glutamic acid (Brady et al. 1990) so that the nucleophilic serine rests at a hairpin turn between a β -strand and an α -helix (Cygler et al. 1994). The catalytic mechanism involving the typical catalytic triad, His-Asp-Ser (see Figure 3.3), is also strongly conserved (Pleiss et al. 1998). Many lipases display an increased activity at an oil-water surface. This has been termed interfacial activation and has been attributed to a 'lid' function of the lipase, which opens or closes the access to the catalytic site (Verger 1997).

***Candida antarctica* lipase B**

In all of the work covered in this thesis the lipase B from the yeast *Candida antarctica* was used (**Paper I-V**). *C. antarctica* was, as the name implies, originally found on Antarctica, isolated from a hypersaline lake called Vanda (Goto et al. 1969). The yeast expresses two lipases: lipase A (CALA) and lipase B (CALB) (de Maria et al. 2005; Nielsen and Kirk 1999). CALB consists of 317 amino acid residues which are folded into a globular α/β type protein with the dimensions of 30Å x 40Å x 50Å (Uppenberg et al. 1994) (see Figure 3.4). CALB shares the common catalytic triad Ser-Asp-His of other lipases, however it differs from the typical lipase in that it is not activated by interfaces. The optimal pH of CALB is 7, however the enzyme is stable in the range of pH 3.5-9.5 (Anderson et al. 1998) and it shows an unusual pH profile with a broad isoelectric region ranging from pH 4 to 8 (Trodler et al. 2008).

X-ray crystallography has revealed that the active site of CALB is composed of two channels, one hosting the acyl group, and the other hosting the nucleophile (Uppenberg et al. 1995). The channels are described as deep and narrow. This has implications for the

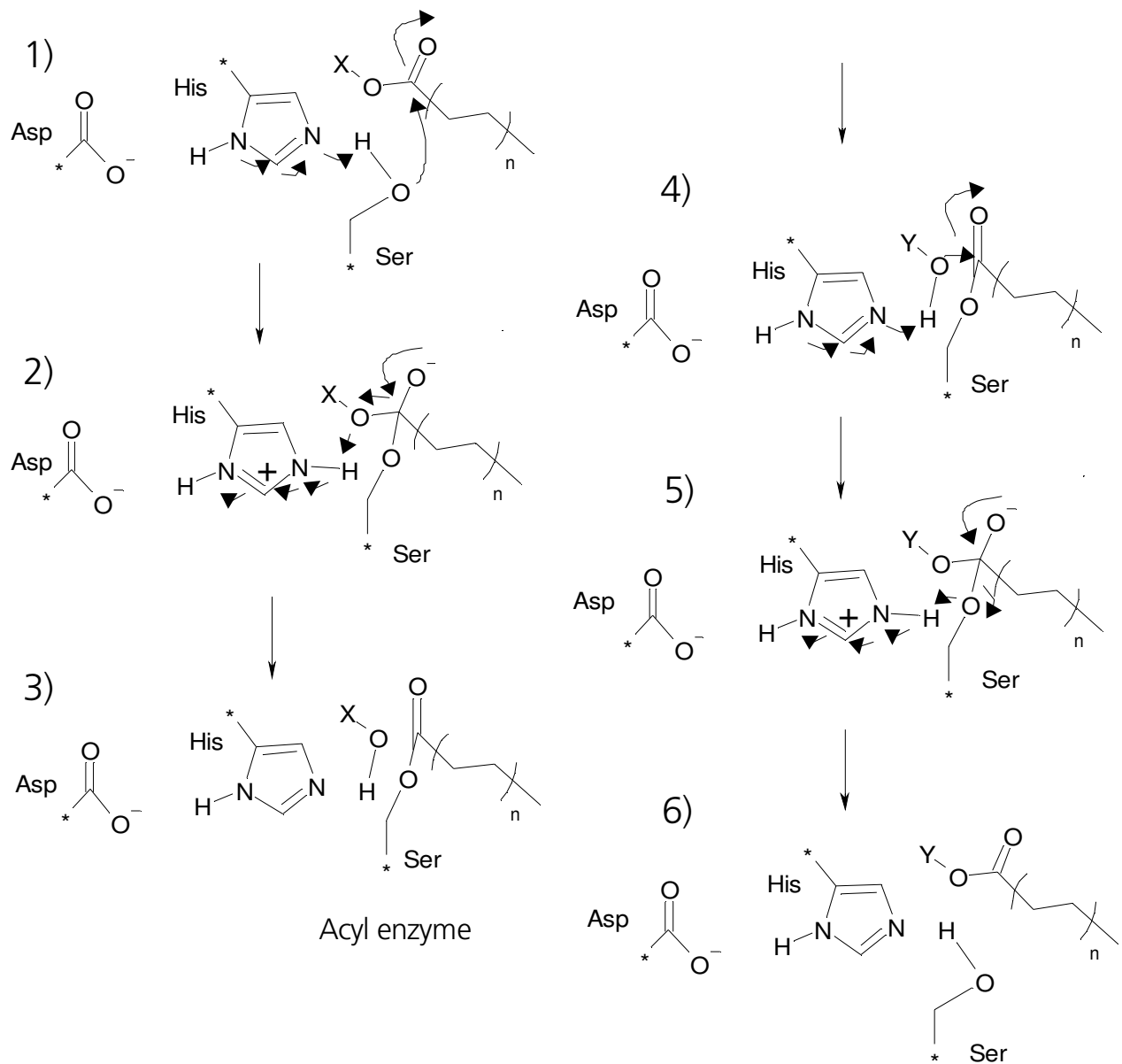


Figure 3.3 Catalytic mechanism of lipase: after binding of the ester substrate, a tetrahedral intermediate is formed by nucleophilic attack of the catalytic serine, which is facilitated by the catalytic histidine and aspartic acid (1); the negatively charged oxygen formed is stabilised by basic residues in the so-called oxyanion hole. Thereafter the leaving nucleophile is released (2) forming the acyl-enzyme (3). In the second half of the reaction (4-6), the acyl enzyme is hydrolysed by a second nucleophile yielding the final product.



Figure 3.4 Model of *Candida antarctica* lipase B that belongs to the α/β -hydrolase fold family of enzymes (Ollis et al. 1992). The α -helixes are shown in red and the β -sheet in yellow.

selectivity and specificity of the enzyme, which is known to be highly enantioselective and only accepting rather non-bulky substrates, especially for the nucleophilic substrate (Nielsen and Kirk 1999). Substrates are however not only restricted to the natural substrates fatty acids, water and glycerol; straight-chain primary alcohols are very good substrates for CALB and some secondary alcohols like 2-propanol are also accepted as substrates while tertiary alcohols are not accepted (Anderson et al. 1998). Along with alcohols, also hydrogen peroxide and amines works as nucleophiles in the synthesis of esters, peracids and amides, respectively. These possibilities have been exploited in the work of **Paper I-V**. Perhydrolysis was used in **Paper I** and the ability to do aminolysis and esterification was utilised in **Papers II – V**.

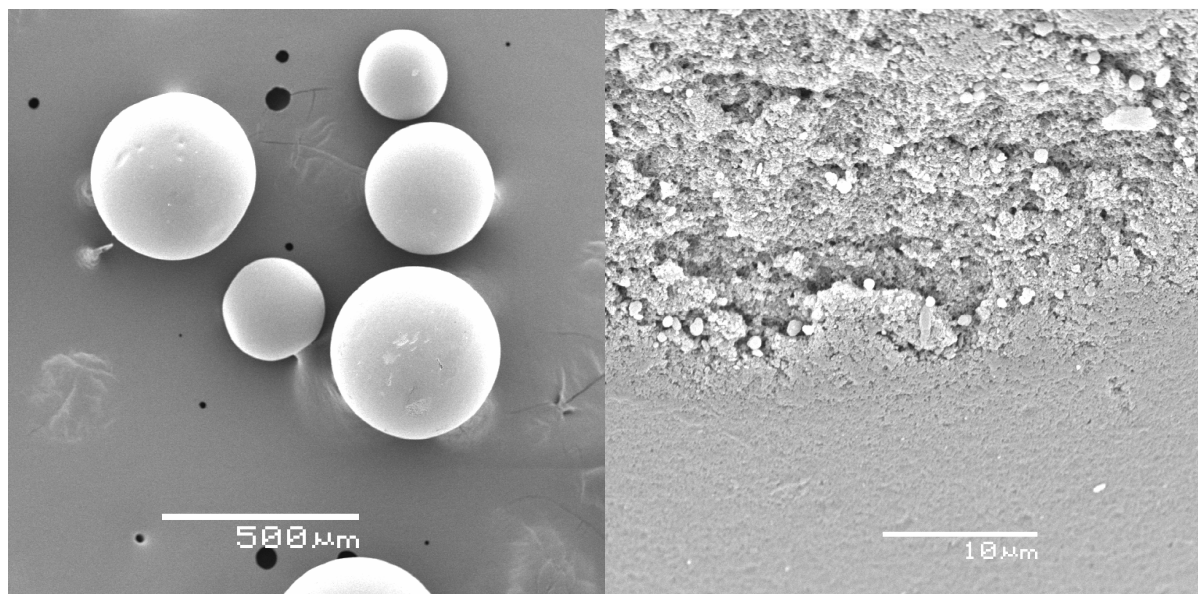


Figure 3.5 SEM pictures of Novozym®435 beads, the commercially available immobilised form of CALB, at different magnification. The resin (Lewatit OC 1600) is a macroporous, divinylbenzene-crosslinked polymer based on methacrylic ester. According to literature the enzyme loading is between 8.5 and 20%(w/w) (Chen et al. 2008; Nakaoki et al. 2005) The lipase is loaded in a 50-100 µm wide front in the outer part of the beads (Mei et al. 2003; Nakaoki et al. 2005).

Novozym®435

To improve the industrial production efficiency, CALB is expressed in *Aspergillus oryzae* by submerged fermentation (<http://www.novozymes.com/> 2008). Usually CALB is used in its immobilised form on a macroporous acrylic resin (Lewatit VP OC 1600). In this shape it is marketed by Novozymes A/S as Novozym®435 (see Figure 3.5) and by Roche Molecular Biochemicals as Chirazyme L-2. The temperature dependence of CALB/Novozyme®435 is very much determined by the conditions studied. There are reports stating that CALB denature already at 40°C in aqueous solutions (Homann et al. 2001), whereas when immobilised and kept dry it can withstand temperatures of beyond 100°C for an extended period of time. When used in an industrial application, the optimum temperature will be where there is a balance between productivity and stability of the enzyme. Novozym®435, has been suggested for the production of a number of

different speciality chemicals as can be seen from Table 3.2. The ideal temperature will probably be as low as 30°C or lower for processes involving substrates that have a clear adverse effect on the enzyme, as methanol in biodiesel production. For processes that only involve hydrophobic reactants, as is the case in the synthesis of wax esters, the process can probably be run at 70-80°C without severe inactivation of the enzyme, especially if the system is kept relatively dry (particularly avoiding the formation of a separate water phase). These preparations were mainly developed for the resolution of chiral intermediates and other high priced speciality chemicals. The physical characteristics of Novozym®435 (CALB/Lewatit OC 1600) are given in Table 3.3.

Table 3.2 Reaction rates and reaction conditions for some suggested applications of Novozym®435 for the synthesis of speciality chemicals

Product	Reaction	Temp. (°C)	Reaction rate (µmol/min/g)	Reference
Emollient ester	C14 acid + C14 alcohol	60	7000	(Hills 2003; Thum 2004; Veit 2004)
Wax ester	C16 acid + C16 alcohol	65	10.000	(Pettersson et al. 2005)
Ester	Decanoic acid + propane-2-ol	60	600	(Hills et al. 1990)
Ester	C12 acid + C10 alcohol	90	10.000	Paper II
Alkanolamide	Lauric acid + ethanolamine	90	1000	Paper II
Decyl lauryl amide	Lauric acid + decylamine	90	100	Paper II
Biodiesel	TG + methanol	30	50	(Shimada et al. 2002)
Sugar ester	Myristic acid + glucose	60	20	(Degn and Zimmermann 2001)
Peracid formation	Caprylic acid + H ₂ O ₂ (30%, aq.)	40	1100	Paper I
Polymer	ε-Caprolactone ring opening polymerisation	70	130	(Deng and Gross 1999)
Acrylation	Acrylic acid + octanol	50	100	(Nordblad and Adlercreutz 2008a)
t-acrylation	Ethyl acrylate + octanol	50	700	(Nordblad and Adlercreutz 2008b)
Acyl glycerols	Sunflower oil + glycerol	40	3000	(Damstrup et al. 2007)
Ceramides	2-hydroxy-propyl amine + octadeca-9,12-cis-cis-dienoic acid	65	>200	(Lassalle and Yvergnaux 2006)

3.4 Enzyme process engineering

The performance of a biocatalytic reaction system is not only determined by the choice of the enzyme. The reaction conditions used, the immobilisation method as well as the process set-up will strongly influence the productivity and process stability and ultimately the economic sustainability of the process (Burton et al. 2002). In the following section some of the most important aspects that need to be considered are discussed.

Immobilisation of enzymes

Even when an enzyme is identified as being useful for a given reaction, its application is often restricted by its lack of long-term stability under process conditions, and also by difficulties in recovery and recycling. Immobilisation is therefore often the key to improve the operational performance of an enzyme (Sheldon 2007). Especially for use in a dry media it would be very difficult to use enzymes without immobilisation or cross-linking since the enzyme molecules would be prone to aggregation and hence suffer from severe mass-transfer limitations.

The main advantage of immobilisation is that the enzyme becomes more stable and that it can be easily separated from the reaction mixture and thereby facilitating re-use of the preparation. Further it can be used in a packed-bed reactor or in a continuous reactor system. Immobilisation also prevents enzyme from contaminating the product (Bloomer et al. 1992; Bornscheuer 2003; Mateo et al. 2007; Monsan and Combes 1988); however leaking of the enzyme could be a problem also from immobilised preparations.

A wide range of ways to immobilise enzymes has been reported (Bloomer et al. 1992; Bornscheuer 2003; Mateo et al. 2007; Monsan and Combes 1988). These methods can be roughly divided into different categories: non-covalent adsorption, deposition, covalent attachment, entrapment in a polymeric gel, membrane, or capsule and cross-linking of an enzyme (such as CLEA). Of these methods, physical (non-covalent) adsorption is one of the more simple and cheap immobilisation procedures and was the procedure used in **Paper IV** to prepare a less costly biocatalyst, Accurel MP1000 (see Table 3.3 and Figure 3.6).

Table 3.3 Carriers evaluated for immobilisation of CALB in **Paper IV**.

The product information was supplied by the manufacturers.

Name	Manufacturer	Matrix	Type	SA (m ² /g)	Pores (Å)	Mean diam. (µm)	Density (kg/L)	Cost €/L
Amberlite XAD4	Rohm&Haas	Polystyrene divinylbenzene	Hydro- phobic	750	100	640	0.68	27
Amberlite XAD7HP	Rohm&Haas	Aliphatic acryl polymer	Hydro- philic	500	450	560	0.66	31
Duolite A568	Rohm&Haas	Phenol- formaldehyde condensate	Hydro- philic	NA	NA	NA	0.31	28
Accurel MP1000	Membrana	Polypropylene	Hydro- phobic	70	2000	~750	0.12	4.8
Lewatit OC 1600	Lanxess	DVB-crosslinked methacrylate		130	150	400	0.73	27

The choice of carrier for the enzyme is dependent on many characteristics. If the preparation is to be used in a batch reactor the carrier needs to withstand operations such as stirring and filtration and if it is to be used in a packed bed, the back-pressure needs to be controlled (Malcata et al. 1990). In this respect a carrier that does not get compressed and that consists of relatively larger particles is probably preferable. Also the thermal and chemical stability is important if the carrier is to be used under, for instance, acidic conditions and if organic solvents are used.

If the enzyme is prone to leaching from the carrier, it can be covalently bound to the carrier by some sort of chemical functionality e.g. epoxide, amino or aldehyde groups. How this affects the residues in the active site and/or the tertiary structure of the enzyme must then be considered. It is likely that chemical bonding will affect the activity of the enzyme negatively, but sometimes this strategy is inevitable.

The hydrophobic/hydrophilic character of the carrier will influence both the micro-environment (especially water) of the enzyme as well as the partitioning of substrates between the bulk phase and the carrier.

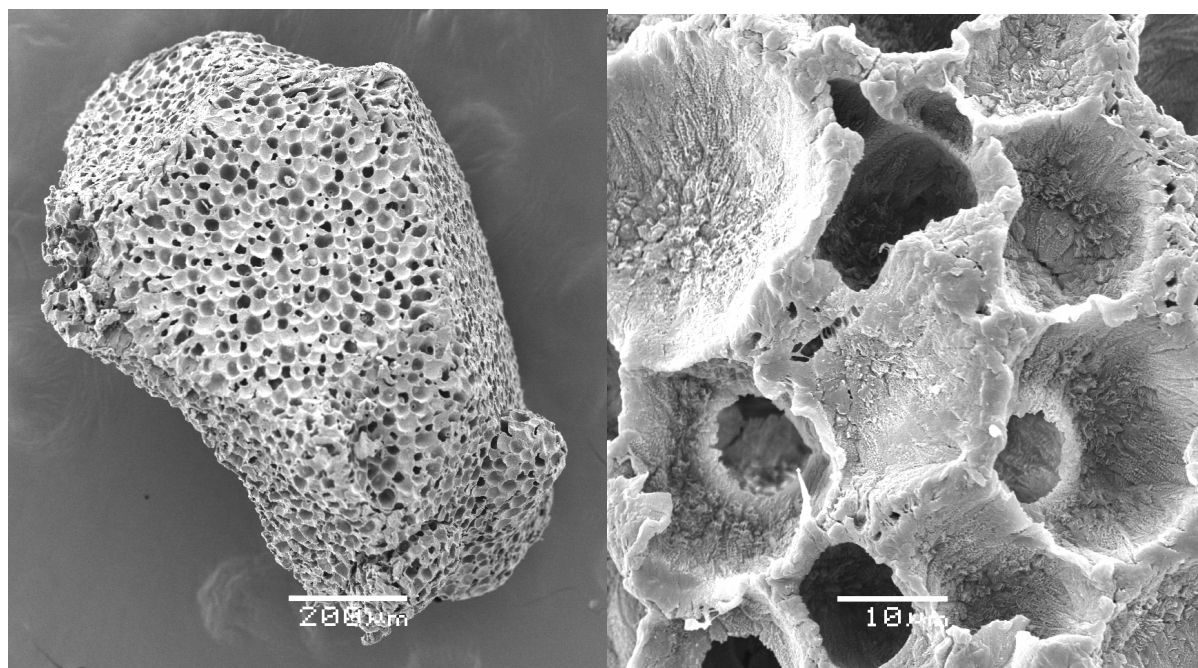


Figure 3.6 SEM pictures of the carrier material Accurel MP1000. The particles were sieved using a 500µm mesh. The micron marker reads 200µm (left) and 10µm (right).

The loading capacity determines the amount of activity per gram of preparation that can be achieved. Normally, a very high activity per gram is pursued and this value is often benchmarked against other preparations. However, this value does not have a very high impact on the applicability unless very low and thus limiting the total activity that can be added to the reactor.

Finally the cost of the carrier material is also of some importance. Most carriers for enzyme immobilisation are quite expensive (see Table 3.3); however a rough estimation shows that they only contribute about 5% of the total selling price of the preparation

By selecting the appropriate carrier some savings can be made in the added cost of the biocatalyst to the production costs of chemicals. Another key factor in the development of a cost-effective biocatalyst is the selection of an appropriate enzyme loading to the resins (Bosley and Peilow 1997). An optimal cost effectiveness will depend on the cost of both enzyme and immobilisation matrix and the proportions in which they are mixed.

The large-scale production, i.e. the immobilisation procedure, of the biocatalyst needs also to be considered: the procedures should be quick, robust and re-producible and the enzyme should be stable during each step. Also working environment issues such as the handling of crosslinking chemicals and dust-producing materials should be considered (Kirk and Christensen 2002).

Water removal

Water (or alcohol) is formed as a co-product in the condensation reactions mentioned above, e.g. esterification and amidation. Thus there will be an equilibrium between the reactants and the products, resulting in incomplete conversion. To shift the equilibrium in favour of the desired product the water needs to be removed. Although enzymes work well in organic solvents, they require some water for their function (sometimes referred to as greasing the enzyme). The thermodynamic water activity governs the activity of the enzyme (Halling 1994), an aspect to be considered so that the water removal does not inactivate the enzyme. The optimum water activity is different for different enzymes (Wehtje and Adlercreutz 1997). CALB used in **Paper I-V** is unusually effective at very low water activities which means that in most cases there will not be any risk of running the reactions too dry (Petersson et al. 2007).

There are many possible methods to remove water from the reaction system (Xu 2003). Vacuum evaporation or bubbling (sparging) of dry air or nitrogen through the reactor are probably the simplest ways to remove water. A system where either moist or dry air was bubbled through the reaction media was developed by (Won and Lee 2001) and was shown to be able to control the water activity in the system by monitoring the relative humidity in the headspace of the reactor and using this information for feed back control. This method could be used both industrially and in small-scale experiments and has the benefits of a high water transfer rate and that the water activity can be changed according to different optima at different stages of the reaction (Petersson et al. 2007). In an interesting article by Napier et al, a combination of vacuum evaporation and air sparging is explored (Napier et al. 1996). By allowing a controlled leak of air into the headspace of the reactor, water removal is achieved at a weaker vacuum than would be required in an ideal evacuated reactor. Napier also argues that uncontrolled air leaks explain the success

of some experiments that would not theoretically work using the weak vacuum reported. In the studies in **Paper II-IV** water was removed in small scale (4 ml vials) simply by running the reactions without lids, and letting the water evaporate, which seems like a fairly effective method at least at 90°C. In preparative scale (40 ml – 3 L) water was removed by applying a vacuum to the headspace of the reactor. It would be reasonable to believe that these set-ups could have been influenced also by air leaks. However, the vacuum applied would lower the boiling point of water below the temperature at which the reactions were run.

Vacuum evaporation or sparging techniques are most effective when the reactants or products are non-volatile; otherwise losses of the volatile compound may be significant. In that case, distillation or membrane pervaporation can be employed (Kwon et al. 1995; Wynn 1996). In the latter case a membrane helps to separate the water from other compounds.

For laboratory experiments a saturated salt solution with certain water activity can be used to maintain the water activity of the system (Bell et al. 2001). The vapour phases in the reaction system are circulated with the vapour phase of the saturated salt solution. Water content or activity in the reaction system can thus be regulated. A similar effect can be achieved by adding solid salt hydrates (Halling 1992). Drying agents such as molecular sieves (Bloomer et al. 1992) or silica gel, added to the reaction system (internal or external) can also be used to remove water. However, molecular sieves have been suspected to influence the pH of the system, and should therefore be used with some care.

Choice of reactor system

There are many reactor types available for enzymatic reactions, all of which offer specific benefits and drawbacks (Balcão et al. 1996; Straathof et al. 2002). There are many aspects that need to be considered: cost, space, mass-transfer, heating, ease of operation, etc, and due to the relatively high cost of immobilised enzymes the operational stability and reusability of the catalyst is of utmost importance (Fernandes et al. 2005).

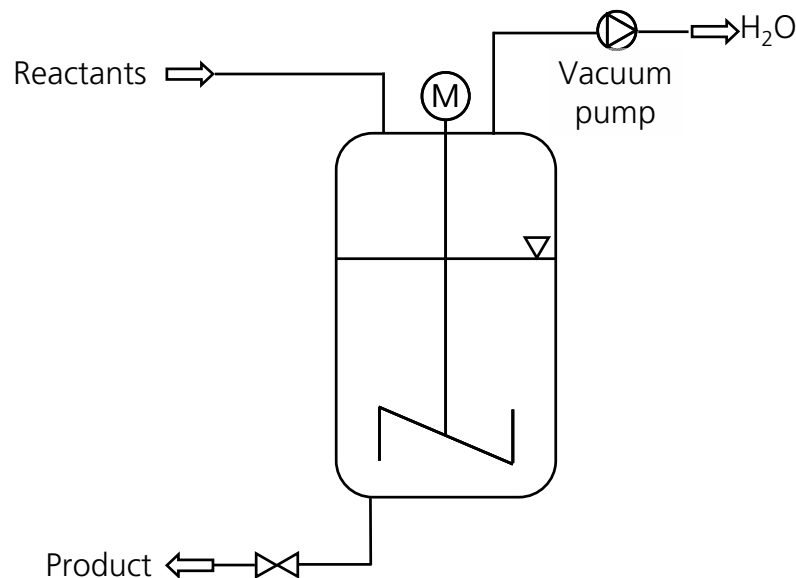


Figure 3.7 Batch stirred tank reactor connected to vacuum for water removal.

Batch Stirred Tank Reactor

The batch process is, due to the simple setup and the ease of operation, the most commonly used reactor set-up in the laboratory (Figure 3.7). It is probably also the best set-up to get basic data about the process, such as K_m and V_{max} , without worrying about mass transfer limitations or temperature gradients. Hence, this setup was used as a reference point for the work in **Paper III and IV** when working on alternative reaction set-ups.

A drawback of this setup in large scale is the low volumetric productivity and that immobilised enzyme will be exposed to mechanical stress from the stirring and multiple filtrations which could lead to the physical loss of enzyme preparation and thereby contamination of the product and significantly decreased catalytic activity (Hilterhaus et al. 2008; Shimada et al. 2002; Watanabe et al. 2005). Another difficulty is how to deal with the inevitable gradual decline in enzyme activity as the number of re-uses increases (Nielsen et al. 2008); either the reaction time has to be prolonged or more enzyme must be added to the reactor as the activity decreases. Both of these strategies will naturally only be feasible to a limited extent.

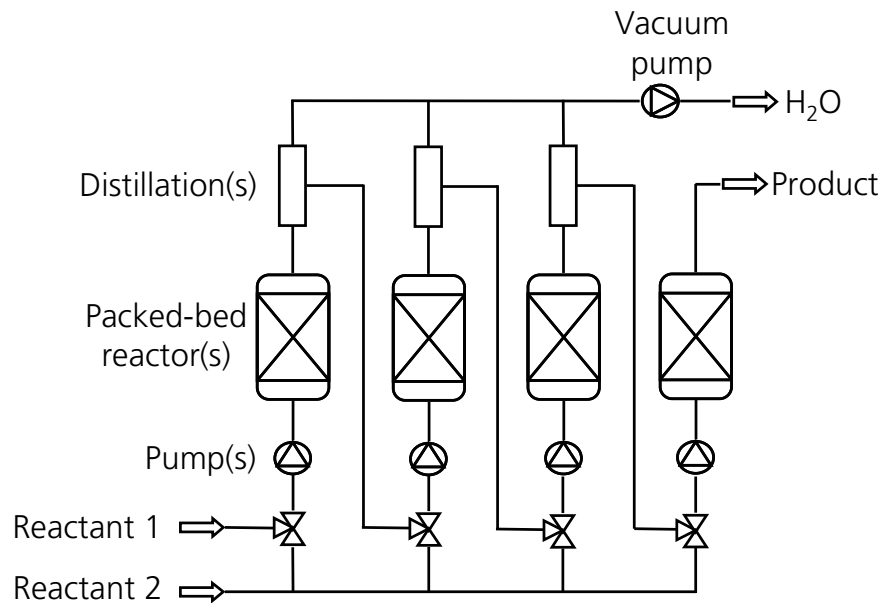


Figure 3.8 Sequential packed-beds with intermittent water removal and addition of reactants. Water removal is required to shift the equilibrium of a water generating reaction; step-wise addition of one reactant can be done if e.g. the reactant is inhibitory to the enzyme.

Packed bed reactor

The packed bed reactor is the industrially preferred set-up for running enzymatic conversions using immobilised enzymes (Hills 2003). The benefits over the BSTR are generally the low investment cost and its higher volumetric productivity and the fact that it can be run in a continuous mode. More importantly the problems of mechanical shear forces are minimised (Hilterhaus et al. 2008; Xu 2003), and separation of the enzyme from the product is simplified. The shorter reaction time can also lead to less side reactions.

On the other hand, drawbacks of using a PBR could be internal and external mass transfer limitations, channelling over the bed and high back pressures. A specific problem experienced with water generating reactions such as amidation or esterification is the water removal; this is difficult to do *in situ*, i.e. on the packed bed, although the use of co-

solvents (Colombié et al. 1998) or semi-permeable tubes penetrating the bed to remove the water have been suggested (Wehtje et al. 1997).

A simpler way to deal with this problem is probably by running several PBRs sequentially with intermittent water removal as shown in Figure 3.8. This set-up also has the added benefit that reactants can be added step-wise. This could be very useful when one reactant causes deactivation of the enzyme, such as in methanolysis of triglycerides (Nielsen et al. 2008), or as in amidation where the amine forms a highly viscous salt with the acid (**Paper II-IV**).

Bubble reactor

A bubble column or air lift reactor is a reactor in which the reaction medium is kept mixed and aerated by introduction of air into the bottom. This reactor type is mainly applied to facilitate the contact and/or reaction of a liquid and a gaseous phase. Recently this set-up was demonstrated by Liese and co-workers for use in enzymatic reactions where high viscosity of the reactants made the use of a PBR impractical (Hilterhaus et al. 2008). The air serves both as a non-abrasive mixer as well as a medium for removing the water formed in the reaction. This reactor type was also considered for the amidation reaction and the results showed similar reaction rates as in batch reaction (un-published results), however problems arose with foam formation and losses of reactants and enzyme through the outlet vents and this set-up was therefore judged to be unsuitable for our application.

PBR loop

Yet another alternative is where the PBR is connected to a vessel via a loop through which the reaction mixture is repeatedly pumped from a stirred tank (Figure 3.9) (Hills 2003; Veit 2004). This configuration combines the features of both the batch and packed-bed mode – allowing mixing of non-homogeneous reactants by stirring without disturbing the enzyme preparation and a simple water removal system – a vacuum or air flow is applied to the stirred tank to remove the water formed. This set-up was used in **Paper III and IV** and compared with a normal BSTR.

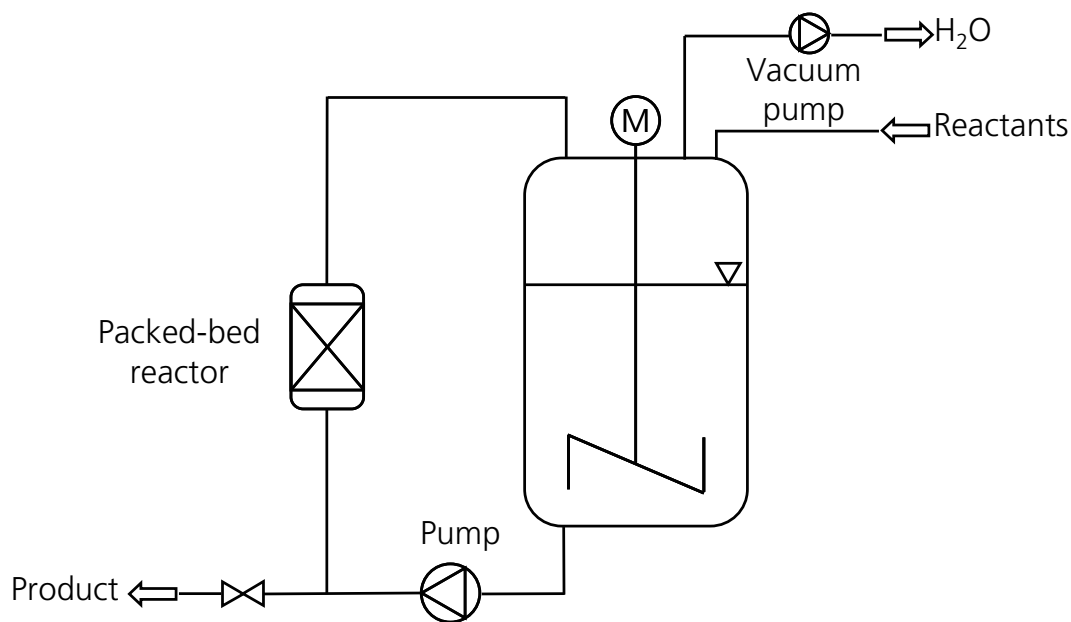


Figure 3.9 Reactor set-up where the packed-bed is attached to a stirred tank via a loop. In **Paper III** and **IV** such system was compared to a BSTR set-up.

Generally the reactions proceeded marginally slower using the PBR-loop, which was mainly attributed to the more difficult water removal mechanism, i.e. water needs to be carried by the reaction mixture to the BSTR vessel where it is evaporated. When using CALB immobilised on 500 μm particles of macroporous polypropylene (MP1000) no such effects were seen (**Paper IV**). This could be because of the lower hydrophilicity of polypropylene compared to the acrylic resin used in Novozym[®]435 or possibly because MP1000 has larger pores and therefore mass-transport is faster.

4 LIPASE CATALYSED SYNTHESIS OF SPECIALITY CHEMICALS

Specialty and fine chemicals are high value added products where innovation is the driver behind better competitiveness by developing new products, applications or process technologies to produce products for both traditional and niche markets. Fine chemicals are typically high purity pharmaceutical or pesticide ingredients and enzymes have already been widely adopted in the synthesis of chemicals in this segment. Speciality chemicals are chemicals such as surfactants, coatings and adhesives, pigments, additives, flavours and fragrances. These chemicals are typically produced in small volumes (from a few tons to a few hundred tons) but still represent an industry worth hundreds of billion euros (Ash and Ash 2003), often sold based on performance rather than price per kg. As a rule of thumb the price of specialty chemicals is around 2 €/kg and above as compared to base chemicals which are sold at prices around 0.1-0.4 €/kg (IChemE October 2008).

In the following chapter two classes of speciality chemicals will be covered; coatings and surfactants and the application of biocatalysis to their production.

4.1 Coatings

A coating can generally be described as a material that is applied to a surface for decoration or protection of the particular surface. It was estimated that each person in the

industrialised world uses more than 25 kg of coating each year. Consequently the market value of coatings is large and it keeps growing: in 1988 the market value was \$ 36 billion (Stoye et al. 2006) and by the year 2000 this value had almost doubled (Wicks 2002).

A typical coating or paint formulation contains several components. Depending on the properties of the surface that are desired different formulations are used; aspects such as application method, and economic and environmental considerations will affect the choice (Stoye et al. 2006). The most important component is the binder, which will basically determine the properties of the coating. Solvents are also commonly used in the formulation to control the application viscosity (Van De Mark et al. 2005). In latex-type coatings (i.e. acrylics, vinyls and styrene-based resins) water is used as the solvent (40-65% solvent) whereas in alkyd and oil-based resins usually alcohols or aromatic solvents like xylene or toluene are used; sometimes up to 70%. Organic solvents are however undesirable due to the emissions of VOC to the atmosphere. To avoid solvents one option is to use powder coatings that are solid powders which are melted on the surface to form the coating. Powder coatings have also been shown to have other environmental benefits as much less material need to be applied to the surface than in solvent based coatings (Gustafsson and Börjesson 2007).

Pigments and different additives such as dispersants, wetting aids, defoamers, biocides, anti-corrosives, and other components are also added to the formulation (usually <3%) (Van De Mark et al. 2005).

A direction within the industry is the development of more environmentally friendly coatings for instance by excluding organic solvents and other hazardous compounds, as well as using raw materials from renewable resources. Progress is also made by developing more effective application technologies in order to reduce losses in material and energy (Goldschmidt and Streitberger 2003).

Epoxides

Among the most common binders are the epoxy resins. Epoxides are characterised by the highly reactive epoxy or oxirane group which is a cyclic three-membered ether group. The polarity and the ring strain make the epoxy group very reactive and thus, epoxides are also

useful for a wide range of other applications. Often epoxides formed in an initial step react further to provide industrially important products, such as surfactants or plastics.

Epoxy resins have wide use in coatings due to the high mechanical strength and resistance of the formed polymers (Pham and Marks 2004). Epoxides also have good thermal and adhesive properties, no volatiles are emitted when curing and they have low shrinkage upon cure. The largest use of epoxy resins is in protective coatings (> 50 %). Other applications are found in electrical devices, for tooling, molding, and casting, in flooring and as adhesives. In 1995 ca. 700 000 tonnes of epoxy resins was used worldwide by the coating industry.

Epichlorohydrin substituted polyols

Glycidylation is the most common way of introducing epoxy functionality into resins. This is done by reacting a hydroxyl group with epichlorohydrin and then treating with a base to form the glycidyl ether.

Glycidyl ethers are widely used in protective coatings where high chemical resistance and adhesion are important properties. By far the most commonly used glycidyl ethers are the ones derived from 2,2-bis(4-hydroxyphenyl) propane commonly called bisphenol A or BPA, and aliphatic polyols (see figure 4.1), respectively (Pham and Marks 2004). The polyol based products are often used to improve the properties, such as viscosity, of the BPA based glycidyl ether resins (Park et al. 2000).

Epoxides are not only extremely active in chemical synthesis; they are also equally active in the human body. Low molecular weight epoxides can enter the cell and react with, for instance, DNA causing mutations. They are thus classified as alkylating agents. More specifically epichlorohydrin is a mutagenic substance (Giri 1997), and persons working with this compound require continuous medical supervision (Sienel et al. 2000). Recently a process for the production of epichlorohydrin from glycerol, a by-product from biodiesel production has been developed (Bell et al. 2008). This possibly improves its environmental profile when it comes to CO₂ emissions etc; however the main reason for avoiding the use of epichlorohydrin is its toxicity. This, however, requires that an alternative synthetic route for glycidyl ether production is established. The aim of **Paper I**

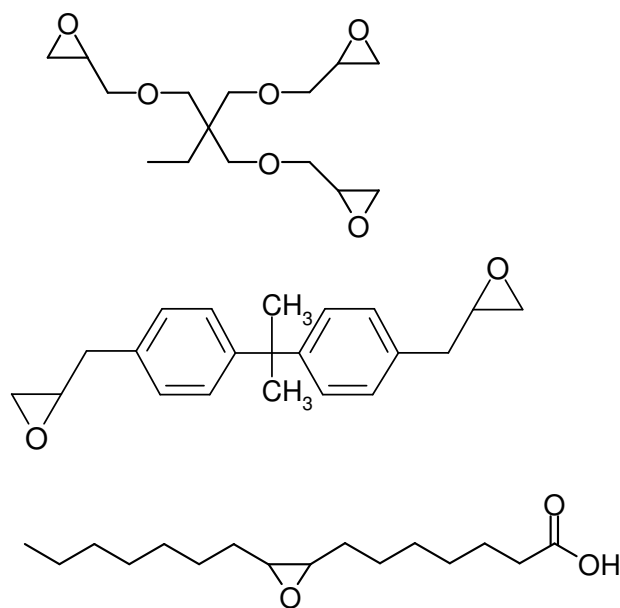


Figure 4.1 Examples of epoxides used in coatings: a glycidylated polyol, TMPTG (top) BPA (middle) and an epoxidised fatty acid (bottom).

was therefore to develop an alternative route for glycidyl ether production which would exclude the use of epichlorohydrin.

Chemo-enzymatic epoxidation

One possible alternative for the production of glycidyl ethers would be through the epoxidation of the corresponding allyl ether by a Prileshajev epoxidation process, in which a peracid is used to epoxidise a double bond (Swern 1947). The oxygen atom is transferred from the peracid to the alkene in a cyclic single-step mechanism with the formation of an epoxide and a carboxylic acid as seen in Figure 4.2 (Solomons 1996). Epoxidation with peracid is the most commonly used method for converting alkenes to epoxides (Sienel et al. 2000). Peroxy acids can be synthesised by the acid or base catalysed reaction of a carboxylic acid with hydrogen peroxide. Three peroxyacids are produced commercially: peroxyacetic acid as a 40 wt % solution in acetic acid, m-chloroperoxybenzoic acid (MCPB), and magnesium monoperoxyphthalate hexahydrate (MMPP).

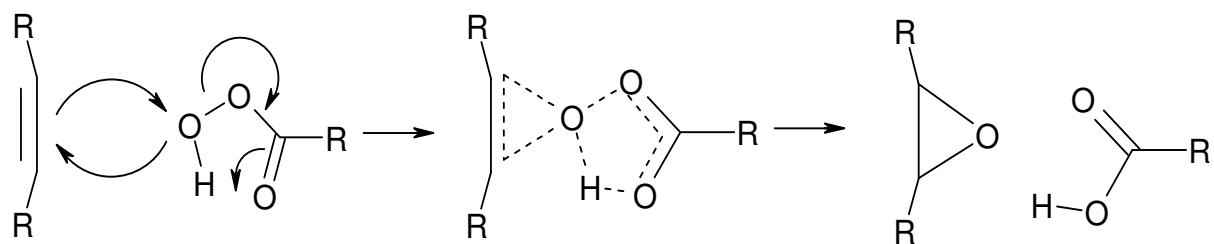


Figure 4.2 Reaction mechanism for the epoxidation of a double bond with a peracid (Prileshajev epoxidation).

The peroxy acid can also be produced *in situ* from a carboxylic acid and hydrogen peroxide using a strong acid or ion-exchange resin as catalyst, as is common for e.g. peroxyformic acid (Musante et al. 2000). A biocatalytic route for the production of peracids has also been presented (Björkling et al. 1990). It was shown that lipases such as CALB catalyse the formation of peroxy carboxylic acids from hydrogen peroxide and the corresponding carboxylic acid. It was also shown that the formed peroxyacid could be used to epoxidise the double bond of an unsaturated compound. Since its discovery the method has been used for epoxidation of many different alkenes (see Table 4.1) and also to perform Baeyer-Villiger reactions (Carboni-Oerlemans et al. 2006). For the study in **Paper I** TMPME (Trimethylolpropane monoallyl ether; see Figure 4.3) a terminally unsaturated polyol derivative was used as a model compound.

Epoxidation with peracid is an exothermic, second-order reaction; in a second order reaction the reaction rate is determined by the following equation:

$$\text{Reaction rate} = k \times C[\text{peracid}] \times C[\text{alkene}] \quad (4.1)$$

The properties of the alkene and peracid greatly influence the reaction rate constant (k). Electron-donating groups in the vicinity of the double bond increase the reaction rate while electron-withdrawing groups, such as allylic oxygen, have the opposite effect (Sienel et al. 2000). As an example, cyclohexene reacts 600 times faster than allyl acetate with peracetic acid at 25°C (Höllriegelskreuth 1981). Most of the reported studies on chemo-enzymatic epoxidation have investigated alkenes that are easily epoxidised (e.g. cyclooctene) (Sandersson 2000).

Table 4.1 Chemo-enzymatic epoxidation using N435

Acyl compound	C acid (M)	Alkene	C alkene (M)	T (°C)	Enzyme (mg/ml)	Epoxidation rate (μmol/min/ml)	Reference
C14 acid	1-sf	1,2 decene	5	25	67	7.5	(Björkling et al. 1992)
Oleic acid	0.5	Oleic acid	0.5	RT	10	0.4	(Warwel and Klaas 1995)
C14-acid	0.5	1,2 octene	0.5	40	10	0.4	(Klaas and Warwel 1997)
Ethyl acetate	sf	1,2 octene	0.1	40	20	0.1	(Klaas and Warwel 1999)
Stearic acid	0.1	Linseed oil	0.2	40	8	1.2	(Hilker et al. 2001)
C12 acid	0.6	Styrene	1	55	6	11	(Yadav and Borkar 2006)
Octanoic acid	2	α-pinene	2	RT	24	12.5	(Skouridou et al. 2003)
Linoleic acid	0.5	Linoleic acid	1	30	10	3.1	(Orellana-Coca et al. 2005a)
Oleic acid	3	Oleic acid	3	50	60	11.5	(Orellana-Coca et al. 2005b)
Octanoic acid	3	TMPME	1	40	25	3	Paper I

Abbreviations: sf - solvent-free; RT- room temperature

This is unfortunate because industrial interest would be for epoxidation of compounds on the other end of the reactivity spectra such as terminal olefins and allylic compounds. In **Paper I**, the potential of the chemo-enzymatic approach to epoxidise such a terminal unsaturated bond in the proximity of an electron withdrawing oxygen, namely trimethylolpropane monoallyl ether (TMPME), was evaluated (see Figure 4.3).

Similarly, the reactivity of the percarboxylic acid is also subject to electronic effects. Electron-withdrawing groups increase the reaction rate of epoxidation by enhancing its electrophilicity while electron-donating groups reduce the reaction rate.

For the study, caprylic acid (octanoic acid) was used for peracid formation because it is a good substrate for the lipase and easy to work with because of its high solubility in toluene (which was used as a solvent) allowing it to be used at a wider range of concentrations and temperatures as compared to longer chain acids which are insoluble at higher

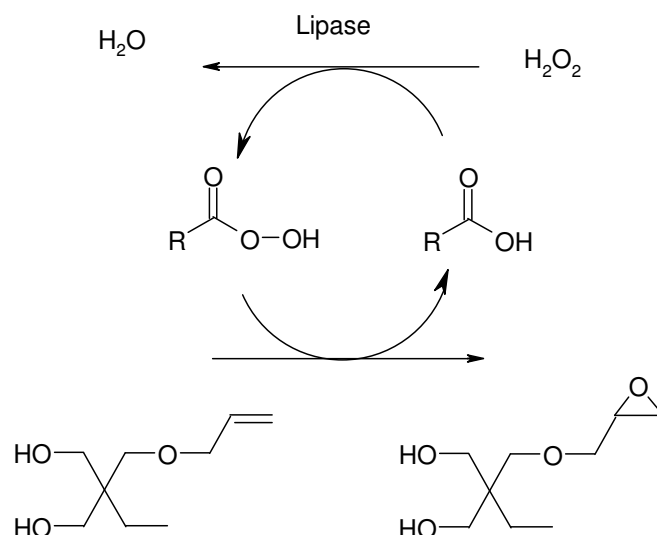


Figure 4.3 Chemo-enzymatic epoxidation of trimethylolpropane monoallyl ether (TMPME). Lipase catalyses the formation of peracid from carboxylic acid and hydrogen peroxide. The peracid then reacts with the alkene to yield the epoxide and the carboxylic acid.

concentrations ($\sim 0.3M$) at room temperature. Also ethyl acetate and dimethyl carbonate were investigated as acyl donors with the idea of facilitating purification of the product downstream of the synthesis as well as minimising the acid related epoxide degradation (Klaas and Warwel 1999). However, ethyl acetate resulted in slow epoxidation rates, while no conversion using dimethyl carbonate could be seen. The choice of solvent is also known to have a marked effect on the reaction rate (Sienel et al. 2000). Hydrophilic solvents retard the rate while chlorinated and aromatic solvents like toluene are more suitable for faster reaction rates.

The reaction conditions employed do not only influence the epoxidation rate but also determine the rate of side reactions. Carboxylic acids and water will attack epoxides to produce mono esters and vicinal diols. Strong acids have a marked catalytic effect on ring cleavage. Loss of epoxide in the presence of acid is generally inevitable, although careful choice of the percarboxylic acid and reaction conditions can minimise loss (Sienel et al. 2000).

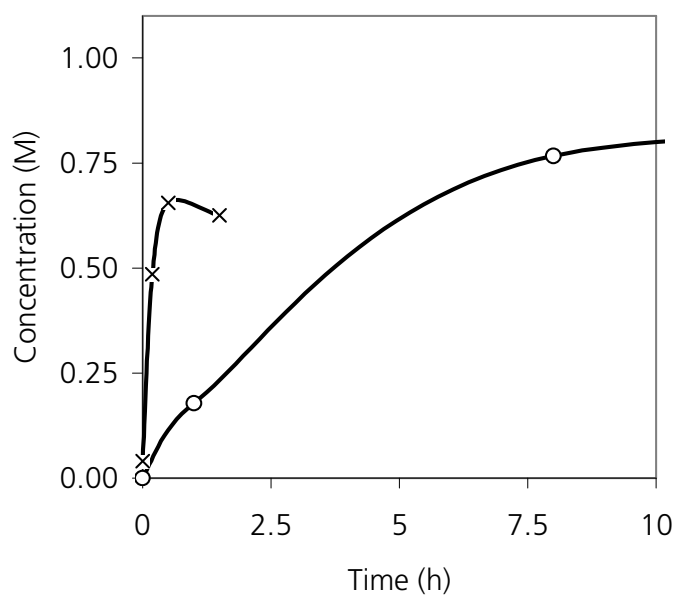
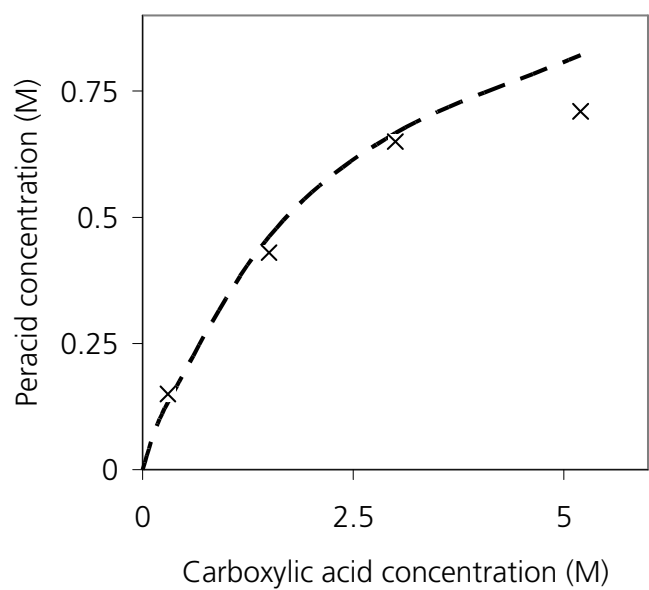


Figure 4.4 The final concentration of peracid is plotted (top) as function of carboxylic acid concentration in the presence of 50% H_2O_2 (1.2M based on total volume). The lower figure shows the formation of peracid (x) and epoxide (o) in the chemo-enzymatic epoxidation of 1M TMPME.

In fact, in chemo-enzymatic epoxidation there are four parallel reactions taking place: (1) peracid formation, (2) epoxidation, (3) degradation of the epoxide and (4) inactivation of the enzyme. To obtain the optimal productivity these processes need to be balanced against each other to find an appropriate window of operation.

The first reaction, peracid formation, is favoured by high concentration of hydrogen peroxide and high concentration of carboxylic acid as there is an equilibrium formed determined by the equation:

$$C[\textit{Peracid}] = K \frac{C[\textit{Acid}] \times C[\textit{H}_2\textit{O}_2]}{C[\textit{water}]} \quad (4.2)$$

In **Paper I** this relationship was confirmed (see figure 4.4), but it was seen that at the highest concentrations of acid the degradation of the epoxide also increased due to the increased amount of aqueous hydrogen peroxide (and thus water) in the system.

The rate of the epoxidation step is according to equation 1 determined by the peracid concentration. This was seen to be true in **Paper I**, where it was also observed that temperature had a strong influence on the reaction rate. In line with earlier findings the epoxidation reaction was rate determining for the conversion of the alkene (Björkling et al. 1992). The peracid was thus quickly accumulated in the system as can be seen from Figure 4.4; this was also seen to be essential to obtain high yields of the epoxide.

Trials to match the formation of peracid with the epoxidation by lowering the amount of enzyme failed because the enzyme became inactivated before the conversion of alkene was finished.

In summary: a maximal epoxide yield of about 75% from 1 M TMPME could be achieved using 3M of the carboxylic acid and 50% H₂O₂ at 40°C. However to obtain a process which would be industrially attractive, peroxidation and epoxide productivity in the system would need to be balanced against epoxide and enzymatic stability. However, these seem to be excluding each other (see table 4.2); the conditions under which the enzyme would be reasonably stable (for instance 25°C and 10% H₂O₂) make the epoxidation too slow and the conditions favouring the epoxidation are much too harsh for the enzyme.

Table 4.2 Factors influencing the epoxidation rate, epoxide stability and enzyme stability in chemo-enzymatic epoxidation

		Epoxidation rate	Epoxide stability	Enzyme stability
Temperature	High	High	Low	Low
	Low	Low	Moderate	Moderate
Acid conc.	High	High	Moderate	(unaffected)
	Low	Low	High	(unaffected)
H ₂ O ₂ conc.	High	High	Moderate	Very low
	Low	Low	Low	Moderate

4.2 Biosurfactants

Surfactants constitute another important class of commodity chemicals used in several everyday applications. Surfactants are amphipathic molecules, i.e. the molecules consist of one hydrophilic part and one hydrophobic part. This is what gives them their special characteristics, e.g. dissolving fatty stains or emulsifying water in oil (creams) such as, cleaning and washing, in cosmetics and pharmaceuticals, in food and in a number of other products (Tadros 2006).

The world production of surfactants is about 15 million tons (Hauthal 2004). Soaps (sodium salt of fatty acid) account for about half of this and thereafter the alkylbenzenesulfonates are the most important class of surfactants with an annual production of ca 2 million tons per year (Kosswig 2000). Because of the large production volumes and the exposure to humans and aquatic eco-systems the human and aquatic toxicity and biodegradability are main environmental concerns. Already, the most skin irritating and persistent surfactants have been excluded from use; however efforts are made

to improve this further by developing natural or bio-based surfactants (Clapés and Infante 2002). These so called biosurfactants use natural raw materials such as fatty acids or alcohols, sugars, amino acids, peptides and other amines to make (typically with the help of enzymes) for instance sugar esters, alkylglucosides, glucamides, peptide based surfactants and alkanolamides (Holmberg 2001).

Alkanolamides

Alkanolamides from fatty acids are a minor class of surfactants, produced in quantities of about 100 000 tons per year world wide. Alkanolamides are considered to be environmentally benign and are used in a wide range of applications including personal care products, hard surface cleaners, emulsifiers in metal working, rust inhibitors, lubricants and dye dispersants (Johansson 2003).

Chemically, alkanolamides are condensation products of fats and alkanolamines. In conventional alkanolamide manufacturing a fatty acid, a fatty acid methyl ester, or a triglyceride is reacted with an alkanolamine such as monoethanolamine or diethanolamine using either elevated temperatures (usually 180 °C), or in the case of methyl esters a metal oxide catalyst at about 100°C (Maag 1984). Yield can usually be improved by using an excess of amine and by the removal of the by-products, water and methanol, respectively. Depending on the starting material and the production strategy, products with an alkanolamide content ranging from 60 to >90 % are obtained (see Table 4.3).

Table 4.3 Conventional manufacturing methods for fatty alkanolamides

	Acyl donor	Reaction conditions	Temp. (°C)	Yield (%)	Properties
Type 1	FA	1:1	140-180	<50	Low purity Water Soluble
Type 2	FA	2:1	100-300	60-80	Medium purity Water Soluble
Type 3	FAME	1:1	100 NaOMe	>90	High purity
Type 4	TG	6:1	75 NaOMe	80-85	Medium purity; contains glycerol

Abbreviations: FA - fatty acid; FAME - Fatty acid methyl ester; TG - Triglyceride; EA - Ethanolamine

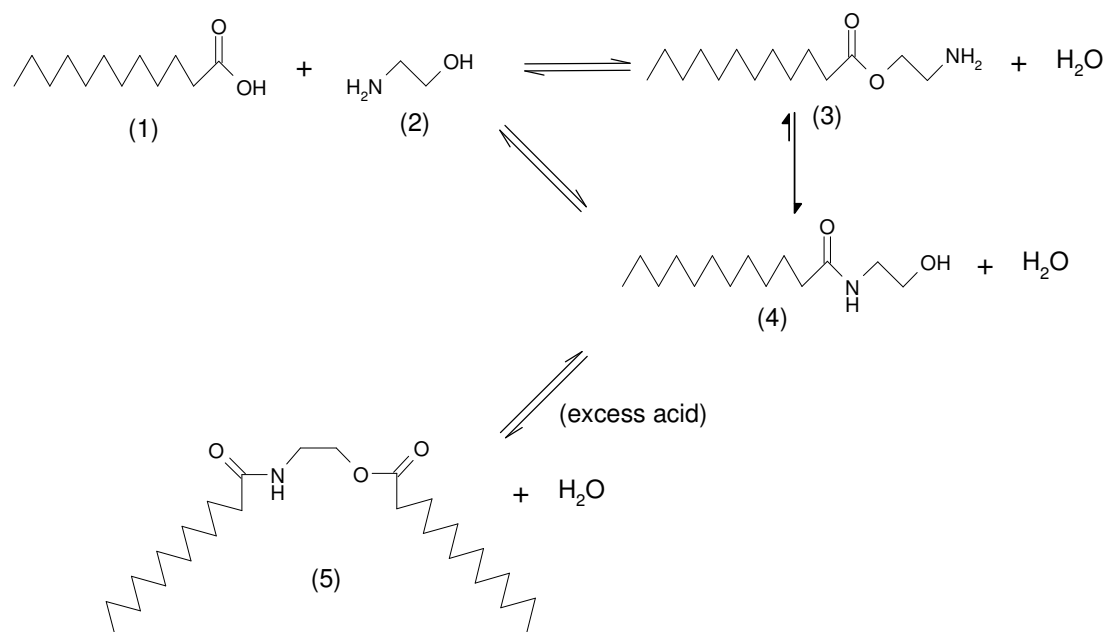


Figure 4.5 The reaction scheme of the reaction between a fatty acid and ethanolamine catalysed by CALB.

As can be seen from Table 4.3 high yields can be achieved with the type 3 process using sodium methoxide as catalyst and fatty methyl esters as starting material. However, this catalyst is highly explosive and it can be a serious health hazard to humans upon contact (Kotrba 2005). Further, its use requires raw materials free from water and free fatty acids as this otherwise will cause problems in the process.

Enzymatic synthesis of alkanolamides

Many different groups have studied different lipase catalysed amidation reactions of fatty acids or their esters (including triglycerids). Some of these have been compiled in Table 4.4. Lipases have also been investigated for their ability to catalyse the amidation of alkanolamines. An important issue in early investigations was chemo-selectivity, since alkanolamines are susceptible for acylation both at the amine and alcoholic group (Furutani 1996; Kanerva 1992). It was found that most lipases catalyse both the amidation and the esterification of alkanolamines, however in either case the predominant final product is the amide, formed not only via amidation but also via esterification and subsequent acyl migration from the alcohol to the amine, the thermodynamically more

Table 4.4 Examples of lipase catalysed amidation reactions

Substrate	Conc (M)	Solvent	Enzyme	T (°C)	Yield (%)	Prod. (U/g enz)	Reference
FFA + C ₈ NH ₂	0.02	Hexane	PPL	40	50	low	(Tuccio et al. 1991)
FAME + Primary amine	0.25	Hexane	Lipozyme IM (<i>R miehei</i>)	20	80	90	(Bistline et al. 1991)
FA-hexanoate + aminoalcohol C>3	0.1	t-amyl alcohol	Lipase PS	40	50	0.02	(Kanerva 1992)
Oleic acid + taurine salt	0.12	2M2B hexane	SP435 (CALB)	55	25	<0.5	(Maugard et al. 1997b)
FFA/FAME C18 + N-me-glucamine	0.35	2M2B hexane	SP435 (CALB)	90	90	70	(Maugard et al. 1997a)
TG + NH ₃	0.1	t-butyl alcohol	N435 (CALB)	60	94	5	(De Zoete 1996a)
FFA C8 via ester + NH ₃	0.7	t-amyl alcohol	CALB on MP1000	40-50	97	400	(De Zoete 1996b)
FFA C4-Oleic + NH ₄ -carbamate	0.05	MIBK	N435 (CALB)	35	95	8	(Litjens 1999)
FFA(C8) + NH ₃	0.3	Ionic liquid	N435 (CALB)	40	100	2	(Lau et al. 2000)
FFA Oleic + NH ₄ -carbamate	1	2M2B	N435 (CALB)	60	85	30	(Slotema 2003)
FFA + NH ₄ carbamate	0.1	2M2B	N435 (CALB)	55	95	30	(Levinson 2005)
FFA /FAEE C8 + NH ₂ OH	2	Dioxane hexane,	CALB on MP1000	40	93	300	(Hacking et al. 2000)
FFA via ester C6 + n-propylamine	0.6	BuOH/ IPA	N435 (CALB)	30	100	1	(Baldessari and Mangone 2001)
FFA C12 + 1-phenylEt-amine	Solvent-free		N435 (CALB)	55	50	15	(Irimescu 2004)
FFA C4-C8 + 2-Et-hexyl-amine	Solvent-free		N435 (CALB)	90	85	10	(Prasad 2005)
FAME N-Me-EtOH amine	0.005	ACN	Chriazyme (CALB)	50	97	12	(Sharma et al. 2005)
FFA N-Me-EtOH amine	0.05	2M2B	N435 (CALB)	50	95	35	(Khare et al. 2008)

Abbreviations: U – units (μmol/min); PPL – porcine pancreatic lipase; 2M2B – 2-methyl-2-butyl alcohol; MIBK – methyl isobutyl ketone; IPA – 2-propanol; FFA – free fatty acid; FAME – fatty acid methyl ester; FAEE – fatty acid ethyl ester; TG – triglyceride;

favoured position (see reaction scheme in Figure 4.5). Acyl migration was observed to be faster in the shorter chain alkanolamines (C3) than the longer (C6) ones. Acyl migration in ethanolamine was not studied as the rearrangement was thought to be too fast to be observed.

The impact of various reaction parameters on the lipase-catalysed production of alkanolamides from mono- and diethanolamine has been studied by Otero and co-workers, using Novozym®435 in various organic solvents at concentrations of the reactants up to 1 mol/L of free fatty acid (Fernández-Pérez 2001; Fernández-Pérez 2003). The Novozym®435-catalysed reaction yielded either mono-acylated ethanolamide and/or the di-acylated amide ester depending on the choice of reaction conditions. If amidation occurred directly or via esterification and acyl migration was not investigated. One of their major findings was that the kinetics for the amidation using free acids was controlled by the solubility of the ion pair formed by the reactants. Moreover it was found that the selectivity of the reaction depended on the solubility of the product in the solvent used and that the choice of solvent was critical in obtaining an efficient process.

Table 4.5 Examples of lipase catalysed synthesis of alkanolamides

Substrate	Conc. (M)	Solvent	Enzyme	T (°C)	Yield (%)	Prod. (U/g enz)	Reference
FFA +1/2 MEA	1	CF	Lipozyme IM (<i>R. miehei</i>)	60	NR	40	(Chandrasekharan and Basu 1996)
FFA C10-18 +MEA	0.5	Hexane	N435 (CALB)	37	<20	5	(Rashidah et al. 2001)
FFA C6-18 +DEA	0.1 0.35	ACN	N435 (CALB)	10-60	53	0.2	(Liu et al. 2001)
FFA C12 +MEA	1	Dioxane	N435 (CALB)	40	95	40	(Fernández-Pérez and Otero 2001)
FAME C12 +MEA	2	ACN	N435 (CALB)	40	92	40	(Fernández-Pérez and Otero 2001)
FFA C12 + MEA	Solvent-free (ca 5M)		N435 (CALB)	90	95	1000	Paper II

Abbreviations: U – units (µmol/min); ACN – acetonitrile; FFA – free fatty acid; FAME – fatty acid methyl ester; MEA - monoethanolamine

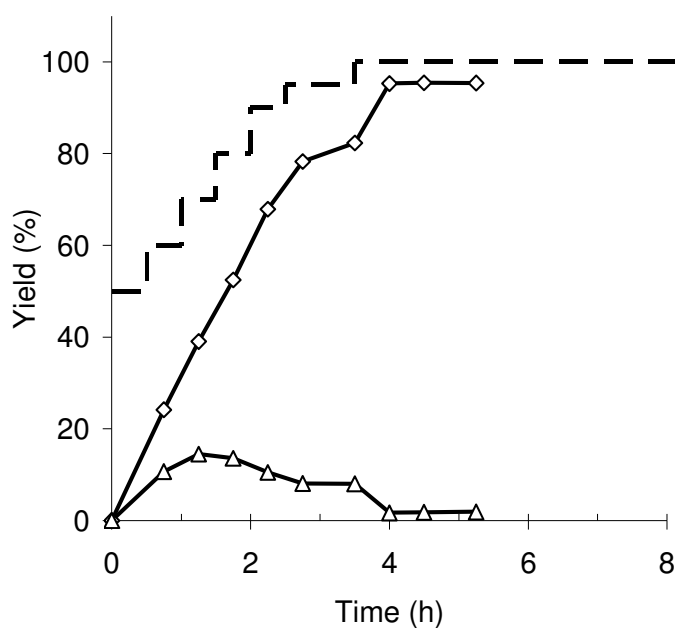


Figure 4.6 Amide (◇) and amide-ester (△) formation in the reaction between MEA and lauric acid catalysed by N435. Broken line indicates the addition of MEA (**Paper II**)

Our aim was to develop an economically competitive, solvent-free method for the production of alkanolamide. The goal was to obtain at least 90% yield, in less than 8h. Starting with the model reaction of lauric acid (dodecanoic acid) and monoethanolamine (MEA) we concluded early on that the reaction temperature needed to be above the melting point of the product. A process temperature of 90°C was selected, but even at this temperature the mixture of the amine and acid formed a very viscous liquid due to the salt formation, when mixed in equimolar amounts. This problem was remedied by adding only half of the amine initially; a free flowing liquid was obtained. By removing the water formed in the reaction by vacuum evaporation the conversion of the fatty acid could be pushed almost to completion. The excess of acid in the beginning of the reaction favours the formation of amide ester (diacylated ethanolamine). It was found that adding the rest of amine as quickly as possible and in small steps helped suppress its formation (see Figure 4.6). This also kept losses of amine due to co-distillation with the water to a minimum, since the added amine could ion-pair with free fatty acids present.

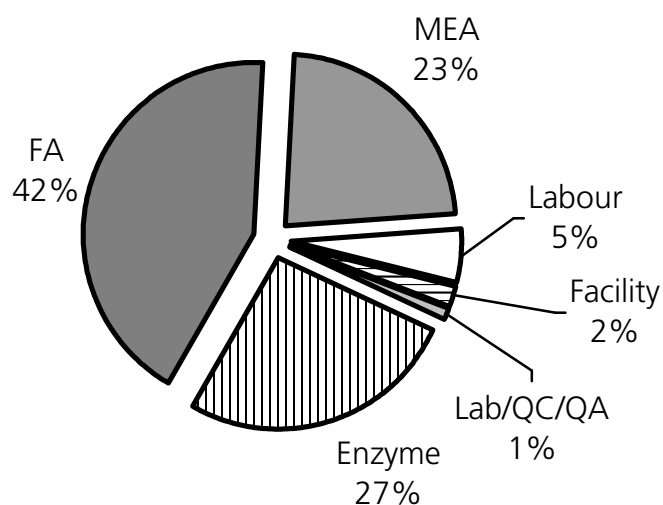


Figure 4.7 Representation of the itemised cost of enzymatic alkanolamide production assuming a cost of the enzyme of 900 €/kg and 80 re-uses (**Paper V**)

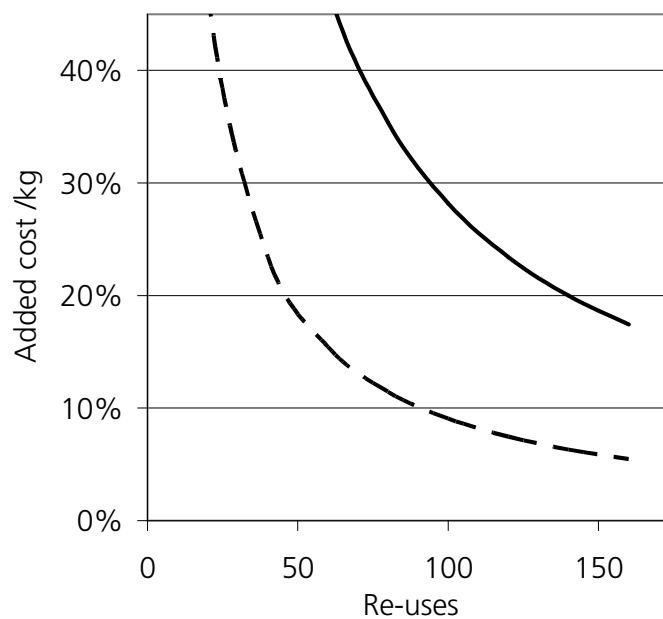


Figure 4.8 Figure shows the percentage of added cost of enzyme to the total alkanolamide production cost of ca 1.4 €/kg. The solid line assumes for a price of 900 €/kg and the broken line a price of 300 €/kg.

It was also observed that the amide ester could be converted back into amide. In **Paper II** a final yield of 95% could be achieved in 4 hours using 5% Novozym®435. The final experiment was scaled from 100 ml to a 3L batch reactor and the same results were obtained (unpublished data). As can be seen from Table 4.5 the productivity is significantly higher than the previously reported synthesis alkanolamides using solvents.

For industrial scale production pure substrates are seldom used, therefore three different technical grade fatty acids were evaluated: coco (from coconut oil), olein (from rapeseed oil) and tall (from tall oil) fatty acids. When using immobilised enzymes, the preferred reactor set-up is, as already mentioned, the packed bed. In the development of a packed-bed reactor system, the olein fatty acid was selected as the most appropriate substrate because of its lower melting point, which would facilitate pumping, etc. A reactor system where the packed bed was connected to a reservoir holding the reactants via a loop was then developed and characterised (**Paper III**).

As the economics of the process was evaluated (**Paper V**), it was found that both the stability and the cost of the biocatalyst had a great impact of the final cost of the product (see Figure 4.7 and Figure 4.8) and at the current cost and enzyme deactivation rate found in **Paper I**, the enzymatic process would be approximately 30-40% more costly than a process catalysed by sodium methoxide.

In order to decrease the cost of the biocatalyst, CALB (liquid) was absorbed onto different carriers (see Table 3.3). A macroporous polypropylene carrier, Accurel MP1000, was seen to give the most cost effective preparation (**Paper IV**). A relatively low loading of the lipase was used in order to minimise the cost of the preparation (see Figure 4.8). The preparation obtained was found to be around 1/4 - 1/2 of the price of the commercial preparation, when processing costs were not considered. Further more it was found that the stability of the in-house preparation was superior to Novozym®435.

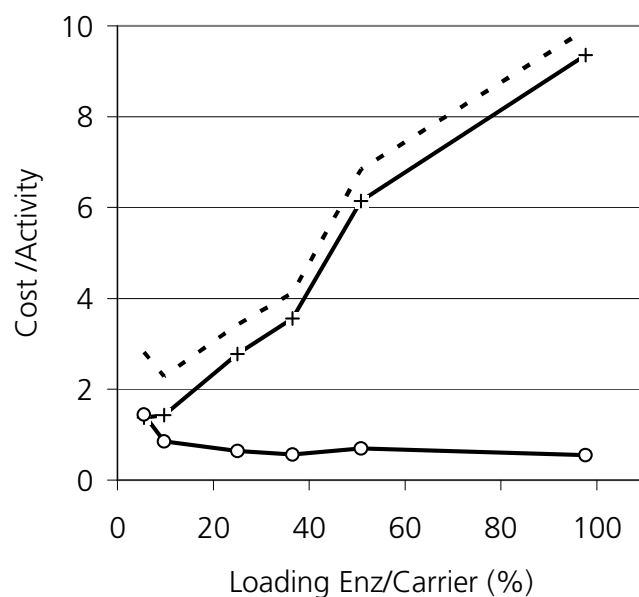


Figure 4.9 The figure shows the cost of the immobilisation of CALB on MP 1000 (particles > 500µm) using different loadings of enzyme. The solid thick line indicates the sum of the costs for lipase (+) and carrier (o). An optimum in the cost per activity profile was found at around 10mg/g.

During repeated batches using the PBR-loop setup at 70°C, no deactivation of the in-house preparation could be seen after five 10-hour batches. By this approach the added cost of the enzymatic process could probably be cut to 0.1-0.2 € per kilogram product, which seems quite acceptable from an economic point of view compared to the total production cost of 1.4 €/kg for the chemically catalysed product.

From an environmental standpoint, it was found that the energy gains in reducing the process temperature from 160°C to 90°C were negligible compared to the energy put into the raw materials during cultivation of the oil crop and synthesis of the ethanolamine from natural gas. On the other hand, this means that the yield of the process will be very important – a process yielding 75% product requires 33% more raw material input than a 100%-yield process. Thus, what makes enzymes superior catalyst for the environment is the ability to work selectively rather than the ability to work under mild conditions.

5 FUTURE PERSPECTIVES & CONCLUDING REMARKS

It is clear that our impact on the environment needs to be reduced. It is encouraging that so many efforts are made within the chemical industries to improve the environmental profile of the companies, and there really seems to be a shift in mentality from trying to get away with as much as possible to a “beyond compliance” way of thinking. Being green is hot and no company can afford not to think about their environmental profile.

It is naturally compelling to hope for a “quick fix” technological solution. Applying to the principles of Green Chemistry thus seems like a straightforward way to make chemical processes greener. However, there is a risk that without a life cycle perspective and a thorough knowledge of the processes only sub-optimisations will be achieved - the environmental load might just be shifted from one phase to another or from one environmental impact to another. There is also a risk that applying to one or a few principles of Green Chemistry will be used to “Green wash” products, i.e. give environmental justification for something that might not be environmentally favourable from a broader perspective³. It is also important to keep in mind that no technology can

³ One example is the ionic liquids; often marketed as ‘green’ solvents although their greenness is highly questionable

totally eliminate the environmental impact of a product since the raw materials themselves contribute significantly; this holds true whether the raw material comes from renewable sources or not (although renewable resources generally contribute slightly less).

It was our goal from the outset to develop more environmentally friendly chemical processes by using enzyme catalyst. We developed two quite different processes for the production of epoxides and alkanolamides, respectively. While the epoxidation process was seen to be uneconomical, the alkanolamide process is technically feasible using a packed-bed setup and is probably within range of being also economically sustainable. It was seen that if temperatures was kept below 70°C etc, the added cost of the enzymatic amidation process could probably be cut to an acceptable 0.1-0.2 euro per kilogram product. The added quality of the product, improved working environment, decreased risks, exclusion of decolorisation and deodorisation as well as slightly lower energy requirements in the processes might well motivate this cost. From the processes we have worked with and from observing the progress of other projects within Greenchem it can still be concluded that the limiting factor for using enzymes in the production of speciality chemicals is seldom the availability of a suitable enzyme but the cost and stability of the enzyme. I find it hampering to the development of enzyme technology that these issues seldom are addressed in academic papers – what gets measured gets managed!

An opportunity for the decrease in biocatalyst cost would be if the enzyme finds a major application, such as in biodiesel production. In that case a cut in cost by a factor of ten is not unthinkable (Nielsen 2008). In fact Novozymes A/S has an ongoing project to develop enzymatic biodiesel production, so this is not an unlikely scenario. For the process economics to be sustainable a significantly cheaper enzyme preparation will be required. If this became a reality it would undoubtedly open the door for many other applications.

Although reduction in the price of enzyme is likely and modern biotechnology offers possibilities to improve enzyme properties –enzymes will still have a hard time competing with chemical catalysts only on terms of cost. True benefits of enzymes are only realised if the selectivity can be used to the benefit of the product – for instance if the product can not be made chemically at all or without major by-product formation. Therefore it is

essential to select the appropriate product candidate for enzymatic synthesis, where these benefits will give an added value to the process or product. Also there should be a potential for high stability of the enzyme, i.e. the process should avoid the use of extremely harsh conditions.

Choosing the right products also applies to the environmental issues. Some companies have started talking about Eco-effectiveness – to not only reduce the emissions while using the same products but reviewing which materials that should not be used at all and replacing them with better alternatives. – a systematic and ongoing procedure to find where it is most effective to put the money and efforts. Indeed sustainability covers both economic and environmental aspects and both are equally important!

Certainly enzymes are incredible catalysts and their role in the chemical industry is likely to grow over the years as new products from natural sources are being developed. Probably enzymes will become standard catalysts for certain reactions and applications, a tool among many others for the modern chemist.

6 REFERENCES

- Anastas, PT, Lankey, RL. 2000. Life cycle assessment and green chemistry: the yin and yang of industrial ecology. *Green Chemistry* 2(6):289-295.
- Anastas, PT, Warner, JC. 1998. *Green Chemistry: Theory and Practice*. Oxford, UK: Oxford University Press.
- Anderson, E, Larsson, K, Kirk, O. 1998. One biocatalyst - Many applications: The use of *Candida antarctica* B-lipase in organic synthesis. *Biocatalysis and Biotransformation* 16(3):181 - 204.
- Ash, M, Ash, I. 2003. *Specialty Chemicals Source Book - Third Edition*: Synapse Information Resources.
- Balcão, VM, Paiva, AL, Malcata, FX. 1996. Bioreactors with immobilized lipases: State of the art. *Enzyme & Microbial Technology* 18:392-416.
- Baldessari, A, Mangone, CP. 2001. One-pot biocatalyzed preparation of substituted amides as intermediates of pharmaceuticals. *Journal of Molecular Catalysis B-Enzymatic* 11(4-6):335-341.
- Beckman, EJ. 2004. Supercritical and near-critical CO₂ in green chemical synthesis and processing. *Journal of Supercritical Fluids* 28(2-3):121-191.
- Bell, BM, Briggs, JR, Campbell, RM, Chambers, SM, Garenstroom, PD, Hippler, JG, Hook, BD, Kearns, K, Kenney, JM, Kruper, WJ and others. 2008. Glycerin as a renewable feedstock for epichlorohydrin production. The GTE process. *Clean* 36(8):657-661.
- Bell, G, Halling, P, May, L, Moore, B, Robb, D, Ulijn, R, Valivety, R. 2001. Methods for measurement and control of water in nonaqueous biocatalysis. In: Vulfson E,

- Halling P, Holland H, editors. *Methods in Biotechnology*. Totowa, NJ: Humana Press. p 105–126.
- Bistline, R, Bilyk, A, Fearheller, S. 1991. Lipase catalyzed formation of fatty amides. *Journal of the American Oil Chemists' Society* 68(2):95-98.
- Björkling, F, Frykman, H, Godtfredsen, SE, Kirk, O. 1992. Lipase catalyzed synthesis of peroxycarboxylic acids and lipase mediated oxidations. *Tetrahedron* 48(22):4587-4592.
- Björkling, F, Godtfredsen, SE, Kirk, O. 1990. Lipase-mediated formation of peroxycarboxylic acids used in catalytic epoxidation of alkenes. *Journal of the Chemical Society - Chemical Communications*(19):1301-1303.
- Bloomer, S, Adlercreutz, P, Mattiasson, B. 1992. Facile synthesis of fatty acid esters in high yields. *Enzyme & Microbial Technology* 14(7):546-552.
- Bornscheuer, UT. 1995. Lipase-catalyzed syntheses of monoacylglycerols. *Enzyme & Microbial Technology* 17(7):578-586.
- Bornscheuer, UT. 2003. Immobilizing enzymes: How to create more suitable biocatalysts. *Angewandte Chemie-International Edition* 42(29):3336-3337.
- Bosley, JA, Peilow, AD. 1997. Immobilization of lipases on porous polypropylene: reduction in esterification efficiency at low loading. *Journal of the American Oil Chemists' Society* 74:107-111.
- Brady, L, Brzozowski, A, Derewenda, ZS, Dodson, E, Dodson, G, Tolley, S, Turkenburg, JP, Christiansen, L, Huge-Jensen, B, Norskov, L and others. 1990. A serin protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature* 343:767-770.
- Burton, SG, Cowan, DA, Woodley, JM. 2002. The search for the ideal biocatalyst. *Nature Biotechnology* 20(1):37-45.
- Carboni-Oerlemans, C, Dominguez de Maria, P, Tuin, B, Bargeman, G, van der Meer, A, van Gemert, R. 2006. Hydrolase-catalysed synthesis of peroxycarboxylic acids: Biocatalytic promiscuity for practical applications. *Journal of Biotechnology* 126(2):140-151.
- Chandrasekharan, CV, Basu, AK. 1996. Synthesis, characterisation and surfactant behaviours of acylaminoethyl esters of fatty acids. *Journal of the Indian Chemical Society* 73(2-3):123-128.
- Chen, B, Hu, J, Miller, EM, Xie, W, Cai, M, Gross, RA. 2008. *Candida antarctica* Lipase B chemically immobilized on epoxy-activated micro- and nanobeads: catalysts for polyester synthesis. *Biomacromolecules* 9(2):463-471.

- Clapés, P, Infante, MR. 2002. Amino acid-based surfactants: enzymatic synthesis, properties and potential applications. *Biocatalysis and Biotransformation* 20(4):215-233.
- Clark, D. 2004. Characteristics of nearly dry enzymes in organic solvents: implications for biocatalysis in the absence of water. *Philosophical Transactions of the Royal Society B: Biological Sciences* 359:1299–1307.
- Coleman, MH, Macrae, A; 1977. Vervarhen zur umlagerung von fettsaeureresten in fettreationsteilnehmern. Germany patent Geman patent DE 2705608
- Colombié, S, Tweddell, RJ, Condoret, J-S, Marty, A. 1998. Water activity control: A way to improve the efficiency of continuous lipase esterification. *Biotechnology and Bioengineering* 60(3):362-368.
- Cygler, M, Grochulski, P, Kazlauskas, RJ, Schrag, JD, Bouthillier, F, Rubin, B, Serreqi, AN, Gupta, AK. 1994. A structural basis for the chiral preferences of lipases. *Journal of the American Chemical Society* 116:3180-3186.
- Damstrup, ML, Kiil, S, Jensen, AD, Sparso, FV, Xu, XB. 2007. Process development of continuous glycerolysis in an immobilized enzyme-packed reactor for industrial monoacylglycerol production. *Journal of Agricultural and Food Chemistry* 55(19):7786-7792.
- Davies, MJ. 2005. The oxidative environment and protein damage. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics* 1703(2):93-109.
- de Maria, PD, Carboni-Oerlemans, C, Tuin, B, Bargeman, G, van der Meer, A, van Gemert, R. 2005. Biotechnological applications of *Candida antarctica* lipase A: State-of-the-art. *Journal of Molecular Catalysis B: Enzymatic* 37(1-6):36-46.
- De Zoete, MC, Kock-van Dalen, A C, van Rantwijk, F, Sheldon, R A. 1996a. Lipase-catalysed ammoniolysis of lipids. A facile synthesis of fatty acid amides. *Journal of Molecular Catalysis B: Enzymatic* 2:141-145.
- De Zoete, MC, Kock-van Dalen, A C, van Rantwijk, F, Sheldon, R A. 1996b. A new enzymatic one-pot procedure for the synthesis of carboxylic amides from carboxylic acids. *Journal of Molecular Catalysis B: Enzymatic* 2:19-25.
- Degn, P, Zimmermann, W. 2001. Optimization of carbohydrate fatty acid ester synthesis in organic media by a lipase from *Candida antarctica*. *Biotechnology and Bioengineering* 74(6):483-491.
- Deng, F, Gross, RA. 1999. Ring-opening bulk polymerization of ϵ -caprolactone and trimethylene carbonate catalyzed by lipase Novozym 435. *International Journal of Biological Macromolecules* 25(1-3):153-159.

- Doble, M, Kruthiventi, AK. 2007. Green Chemistry & Engineering: Elsevier Science & Technology Books.
- EuropaBio. Industrial or White Biotechnology: A driver of sustainable growth in Europe.
- European Chemicals Agency. 2008. <http://www.echa.europa.eu>.
- Fagain, CO. 1995. Understanding and increasing protein stability. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* 1252(1):1-14.
- Fernandes, JFA, McAlpine, M, Halling, PJ. 2005. Operational stability of subtilisin CLECs in organic solvents in repeated batch and in continuous operation. *Biochemical Engineering Journal* 24:11-15.
- Fernández-Pérez, M, Otero, C. 2001. Enzymatic synthesis of amide surfactants from ethanolamine. *Enzyme & Microbial Technology* 28:527-536.
- Fernández-Pérez, M, Otero, C. 2001. Enzymatic synthesis of amide surfactants from ethanolamine. *Enzyme & Microbial Technology* 28:527-536.
- Fernández-Pérez, M, Otero, C. 2003. Selective enzymatic synthesis of amide surfactants from diethanolamine. *Enzyme & Microbial Technology* 33:650-660.
- Forum for the Future. 2004. A Vision for the Sustainable Production & Use of Chemicals. <http://www.forumforthefuture.org.uk>.
- Fox, RJ, Davis, SC, Mundorff, EC, Newman, LM, Gavrilovic, V, Ma, SK, Chung, LM, Ching, C, Tam, S, Muley, S and others. 2007. Improving catalytic function by ProSAR-driven enzyme evolution. *Nature Biotechnology* 25(3):338-344.
- Frazzetto, G. 2003. White biotechnology. *EMBO reports* 4(9):835-837.
- Frosch, RA, Gallopoulos, NE. 1989. Strategies for manufacturing. *Scientific American*(September):94.
- Fureby, AM. 1995. Aspects on lipase-catalysed preparation of partial glycerids [Doctoral thesis]. Lund: Lund University, Sweden.
- Furutani, T, Furui, M, Ooshima, H, Kato, J. 1996. N-Acylation of β -amino alcohol by acyl migration following enzyme-catalysed esterification. *Enzyme & Microbial Technology* 19:578-584.
- Giri, AK. 1997. Genetic toxicology of epichlorohydrin: A review. *Mutation Research* 386(1):25-38.

- Giver, L, Gershenson, A, Freskgard, PO, Arnold, FH. 1998. Directed evolution of a thermostable esterase. *Proceedings of the National Academy of Sciences of the United States of America* 95(22):12809-12813.
- Goldschmidt, A, Streitberger, H-J. 2003. *BASF Handbook on basics of coating technology*. Norwich, NY: William Andrew Publishing.
- Gorman, LAS, Dordick, JS. 1992. Organic-solvents strip water off enzymes. *Biotechnology and Bioengineering* 39(4):392-397.
- Goto, S, Sugiyama, J, Iizuka, H. 1969. A taxonomic study of antarctic yeasts. *Mycologia* 61(4):748-774.
- Gotor-Fernández, V, Brieva, R, Gotor, V. 2006. Lipases: Useful biocatalysts for the preparation of pharmaceuticals. *Journal of Molecular Catalysis B: Enzymatic* 40:111-120.
- Graedel, T. 1999. Green chemistry in an industrial ecology context. *Green Chemistry* 1(5):G126-G128.
- Gustafsson, LM, Börjesson, P. 2007. Life cycle assessment in green chemistry - A comparison of various industrial wood surface coatings. *International Journal of Life Cycle Assessment* 12(3):151-159.
- Hacking, MAPJ, Akkus, H, van Rantwijk, F, Sheldon, RA. 2000. Lipase and esterase-catalysed acylation of hetero-substituted nitrogen nucleophiles in water and organic solvents. *Biotechnology and Bioengineering* 68:84-91.
- Halling, P. 1992. Salt hydrates for water activity control with biocatalysts in organic media. *Biotechnology Techniques* 6(3):271-276.
- Halling, PJ. 1994. Thermodynamic predictions for biocatalysis in nonconventional media: Theory, tests, and recommendations for experimental design and analysis. *Enzyme and Microbial Technology* 16(3):178-206.
- Hauthal, HG. 2004. CESIO 2004 - Dynamic surfactants and nanostructured surfaces for an innovative industry. *SÖFW-Journal* 10:3-17.
- Hilker, I, Bothe, D, Pruss, J, Warnecke, H-J. 2001. Chemo-enzymatic epoxidation of unsaturated plant oils. *Chemical Engineering Science* 56(2):427-432.
- Hills, G. 2003. Industrial use of lipases to produce fatty acid ester. *European Journal of Lipid Science and Technology* 105:601-607.
- Hills, G, Macrae, A, Poulina, R; Unichema chemie, Unilever, assignee. 1990. Ester preparation patent EP0383405

- Hilterhaus, L, Thum, O, Liese, A. 2008. Reactor concept for lipase-catalyzed solvent-free conversion of highly viscous reactants forming two-phase systems. *Organic Process Research & Development* 12(4):618-625.
- Holbrey, JD, Seddon, KR. 1999. Ionic Liquids. *Clean Technologies and Environmental Policy* 1(4):223-236.
- Holmberg, K. 2001. Natural surfactants. *Current Opinion in Colloid and Interface Science* 6(2):148-159.
- Homann, MJ, Vail, R, Morgan, B, Sabesan, V, Levy, C, Dodds, DR, Zaks, A. 2001. Enzymatic Hydrolysis of a Prochiral 3-Substituted Glutarate Ester, an Intermediate in the Synthesis of an NK1/NK2 Dual Antagonist. *Advanced Synthesis & Catalysis* 343(6-7):744-749.
- Houde, A, Kademi, A, Leblanc, D. 2004. Lipases and their industrial applications: An overview. *Applied Biochemistry and Biotechnology* 118:155-170.
- Hough, DW, Danson, MJ. 1999. Extremozymes. *Current Opinion in Chemical Biology* 3(1):39-46.
- <http://www.kemi.se/>. 2008. Website of the Swedish Chemicals Inspectorate.
- <http://www.novozymes.com/>. 2008. Webpage of Novozymes A/S.
- <http://www.responsiblecare.org/>. 2008. Webpage of the Responsible care organisation.
- Hult, K, Berglund, P. 2003. Engineered enzymes for improved organic synthesis. *Current Opinion in Biotechnology* 14(4):395-400.
- Höllriegelskreuth. 1981. Peroxide-Chemie: A 0.1.2 Peroxygen compounds in organic synthesis, Epoxidation, Chapter 6. p 11-17.
- IChemE. October 2008. The Institution of Chemical Engineers website.
- Ikeda, M, Clark, DS. 1998. Molecular cloning of extremely thermostable esterase gene from hyperthermophilic archaeon *Pyrococcus furiosus* in *Escherichia coli*. *Biotechnology and Bioengineering* 57(5):624-629.
- Irimescu, R, Kato, K. 2004. Lipase-catalysed enantioselective reaction of amines with carboxylic acids under reduced pressure in non-solvent system and in ionic liquids. *Tetrahedron Letters* 45:523-525.
- ISO. 1998. ISO 14040-43, Life cycle assessment. Geneva: International Organization for Standardization.
- Jaeger, K-E. 2004. Protein technologies and commercial enzymes: White is the hype - biocatalysts on the move. *Current Opinion in Biotechnology* 15:269-271.

- Jaenicke, R. 2000. Stability and stabilization of globular proteins in solution. *Journal of Biotechnology* 79(3):193-203.
- Johansson, I, AkzoNobel Surface Chemistry AB. 2003. Amides, Fatty acid. *Kirk-Othmer Encyclopedia of Chemical Technology*. New York: John Wiley & Sons, Inc.
- Kanerva, LT, Kosonen, M, Vanttinen, E, Huuhtanen, T T, Dahlqvist, M. 1992. Studies on the chemo- and enantio-selectivity of the enzymatic monoacylations of amino alcohols. *Acta Chemica Scandinavica* 46:1101-1105.
- Kaur, J, Sharma, R. 2006. Directed evolution: An approach to engineer enzymes. *Critical Reviews in Biotechnology* 26(3):165-199.
- Khare, SK, Kumar, A, Kuo, TM. 2008. Lipase-catalyzed production of a bioactive fatty amide derivative of 7,10-dihydroxy-8(E)-octadecenoic acid. *Bioresource Technology* In Press, Corrected Proof.
- Kirk, O, Borchert, TV, Fuglsang, CC. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology* 13(4):345-351.
- Kirk, O, Christensen, MW. 2002. Lipases from *Candida antarctica*: unique biocatalysts from a unique origin. *Organic Process Research & Development* 6:446-451.
- Klaas, MRG, Warwel, S. 1997. Lipase-catalyzed preparation of peroxy acids and their use for epoxidation. *Journal of Molecular Catalysis A: Chemical* 117(1-3):311-319.
- Klaas, MRG, Warwel, S. 1999. Chemoenzymatic epoxidation of alkenes by dimethyl carbonate and hydrogen peroxide. *Organic Letters* 1(7):1025-1026.
- Klibanov, AM, Samokhin, GP, Martinek, K, Berezin, IV. 1977. A new approach to preparative enzymatic synthesis. *Biotechnology and Bioengineering* 19(9):1351-1361.
- Koswig, K. 2000. Surfactants. *Ullmann's Encyclopedia of Industrial Chemistry*. Weinheim, Germany: Wiley-VCH.
- Kotrba, R. 2005. A catalyst for change. *Biodiesel Magazine* August/September.
- Krishna, SH, Karanth, NG. 2002. Lipases and lipase-catalysed esterification reactions in nonaqueous media. *Catalysis Reviews* 44:499-591.
- Kvittingen, L. 1994. Some aspects of biocatalysis in organic solvents. *Tetrahedron* 50(28):8253-8274.
- Kwon, SJ, Song, KM, Hong, WH, Rhee, JS. 1995. Removal of water produced from lipase-catalyzed esterification in organic-solvent by pervaporation. *Biotechnology and Bioengineering* 46(4):393-395.

- Laane, C, Boeren, S, Vos, K. 1985. On optimizing organic solvents in multi-liquid-phase biocatalysis. *Trends in Biotechnology* 3(10):251-252.
- Lagarde, D, Nguyen, HK, Ravot, G, Wahler, D, Reymond, JL, Hills, G, Veit, T, Lefevre, F. 2002. High-throughput screening of thermostable esterases for industrial bioconversions. *Organic Process Research & Development* 6(4):441-445.
- Lancaster, M. 2002. *Green Chemistry: An Introductory Text*. Cambridge: Royal Society of Chemistry.
- Langrand, G, Rondot, N, Triantaphylides, C, Baratti, J. 1990. Short chain flavour esters synthesis by microbial lipases. *Biotechnology Letters* 12(8):581-586.
- Lassalle, L, Yvergnaux, F; 2006. New method for synthesizing ceramide-type compounds. US Patent application.10/559,713
- Lau, RM, van Rantwijk, F, Seddon, KR, Sheldon, RA. 2000. Lipase-catalyzed reactions in ionic liquids. *Organic Letters* 2(26):4189-4192.
- Levinson, WE, Kuo, T M, Kurtzman, C P. 2005. Lipase-catalysed production of novel hydroxylated fatty amides in organic solvent. *Enzyme & Microbial Technology* 37:126-130.
- Liese, A, Seelbach, K, Wandrey, C. 2006. *Industrial Biotransformations*. Weinheim, Germany: Wiley-VCH.
- Litjens, MJJ, Sha, M, Straathof, A J J, Jongejan, J A, Heijnen, J J. 1999. Competitive lipase-catalysed ester hydrolysis and ammoniolysis in organic solvents; equilibrium model of a solid-liquid-vapour system. *Biotechnology and Bioengineering* 65:347-356.
- Liu, KJ, Nag, A, Shaw, JF. 2001. Lipase-catalyzed synthesis of fatty acid diethanolamides. *Journal of Agricultural and Food Chemistry* 49(12):5761-5764.
- Maag, H. 1984. Fatty acid derivatives: important surfactants for household and industrial purposes. *Journal of the American Oil Chemists Society* 61:259-66.
- Malcata, FX, Reyes, HR, Garcia, HS, Hill, CG, Amundson, CH. 1990. Immobilized lipase reactors for modification of fats and oils - a review. *Journal of the American Oil Chemists Society* 67(12):890-910.
- Mateo, C, Palomo, JM, Fernandez-Lorente, G, Guisan, JM, Fernandez-Lafuente, R. 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme & Microbial Technology* 40(6):1451-1463.
- Maugard, T, Remaud-Simeon, M, Petre, D, Monsan, P. 1997a. Lipase-catalysed chemoselective N-acylation of amino-sugar derivatives in hydrophobic solvent: acid-amine ion-pair effects. *Tetrahedron* 53:7587-7594.

- Maugard, T, Rемаud-Simeon, M, Petre, D, Monsan, P. 1997b. Lipase-catalysed production of N-oleoyl-aurine sodium salt in non-aqueous medium. *Biotechnology Letters* 19(8):751-753.
- Mei, Y, Miller, L, Gao, W, Gross, RA. 2003. Imaging the distribution and secondary structure of immobilized enzymes using infrared microspectroscopy. *Biomacromolecules* 4(1):70-74.
- Monsan, P, Combes, D. 1988. *Enzyme Stabilization by Immobilization*. Methods in enzymology. London: Academic Press, Inc.
- Musante, RL, Grau, RJ, Baltanas, MA. 2000. Kinetic of liquid-phase reactions catalyzed by acidic resins: the formation of peracetic acid for vegetable oil epoxidation. *Applied Catalysis, A* 197(1):165-173.
- Nakaoki, T, Mei, Y, Miller, LM, Kumar, A, Kalra, B, Miller, ME, Kirk, O, Christensen, M, Gross, RA. 2005. *Candida antarctica* lipase B catalyzed polymerization of lactones: Effects of immobilization matrices on polymerization kinetics & molecular weight. *Industrial Biotechnology* 1(2):126-134.
- Napier, PE, Lacerda, HM, Rosell, CM, Valivety, RH, Vaidya, AM, Halling, PJ. 1996. Enhanced organic-phase enzymatic esterification with continuous water removal in a controlled air-bleed evacuated-headspace reactor. *Biotechnology Progress* 12(1):47-50.
- Nielsen, PM. 2008. Conversation at Greenchem symposium in Malmö.
- Nielsen, PM, Brask, J, Fjerbaek, L. 2008. Enzymatic biodiesel production: Technical and economical considerations. *European Journal of Lipid Science and Technology* 110(8):692-700.
- Nielsen, TB, Kirk, O. 1999. Lipase A and B from the yeast *Candida antarctica*. In: Margesin R, Schinner F, editors. *Biotechnological applications of cold-adapted organisms*. New York: Springer.
- Nordblad, M, Adlercreutz, P. 2008a. Effects of acid concentration and solvent choice on enzymatic acrylation by *Candida antarctica* lipase B. *Journal of Biotechnology* 133(1):127-134.
- Nordblad, M, Adlercreutz, P. 2008b. Efficient enzymatic acrylation through transesterification at controlled water activity. *Biotechnology and Bioengineering* 99(6):1518-1524.
- Ollis, DL, Cheah, E, Cygler, M, Dijkstra, B, Frolow, F, Franken, SM, Harel, M, Remington, SJ, Silman, I, Schrag, J and others. 1992. The α/β hydrolase fold. *Protein Engineering* 5(3):197-211.

- Orellana-Coca, C, Camocho, S, Adlercreutz, D, Mattiasson, B, Hatti-Kaul, R. 2005a. Chemo-enzymatic epoxidation of linoleic acid: Parameters influencing the reaction. *European Journal of Lipid Science and Technology* 107(12):864-870.
- Orellana-Coca, C, Törnvall, U, Adlercreutz, D, Mattiasson, B, Hatti-Kaul, R. 2005b. Chemo-enzymatic epoxidation of oleic acid and methyl oleate in solvent-free medium. *Biocatalysis and Biotransformation* 23(6):431-437.
- Owusu, RK, Cowan, DA. 1989. Correlation between Microbial Protein Thermostability and Resistance to Denaturation in Aqueous-Organic Solvent 2-Phase Systems. *Enzyme and Microbial Technology* 11(9):568-574.
- Pandey, A, Benjamin, S, Soccol, CR, Nigam, P, N, K, Soccol, VT. 1999. The realm of microbial lipases in biotechnology. *Biotechnology and Applied Biochemistry* 29:119-131.
- Park, S-J, Kim, T-J, Lee, J-R. 2000. Cure behavior of diglycidylether of bisphenol A/trimethylolpropane triglycidylether epoxy blends initiated by thermal latent catalyst. *Journal of Polymer Science Part B: Polymer Physics* 38(16):2114-2123.
- Petersson, AEV, Adlercreutz, P, Mattiasson, B. 2007. A water activity control system for enzymatic reactions in organic media. *Biotechnology and Bioengineering* 97(2):235-241.
- Petersson, AEV, Gustafsson, LM, Nordblad, M, Borjesson, P, Mattiasson, B, Adlercreutz, P. 2005. Wax esters produced by solvent-free energy-efficient enzymatic synthesis and their applicability as wood coatings. *Green Chemistry* 7(12):837-843.
- Pham, HQ, Marks, MJ. 2004. Epoxy Resins. *Kirk-Othmer Encyclopedia of Chemical Technology*. Hoboken, NJ: John Wiley.
- Pleiss, J, Fischer, M, Schmid, RD. 1998. Anatomy of lipase binding sites: the scissile fatty acid binding site. *Chemistry and Physics of Lipids* 93:67-80.
- Prasad, AK, Husain, M, Singh, B K, Gupta, R K, Manchanda, V K, Olsen, C E, Parmar, V S. 2005. Solvent-free biocatalytic amidation of carboxylic acids. *Tetrahedron Letters* 46:4511-4514.
- Rashidah, A, Abu Bakar, S, Mahiran, B, Che Nyonya, A. 2001. Enzymatic synthesis of palm based fatty monoethanolamides: product analysis and comparative study. *Malaysian Journal of Biochemistry and Molecular Biology* 6:1-6.
- Reslow, M, Adlercreutz, P, Mattiasson, B. 1987. Organic solvents for bioorganic synthesis. *Applied Microbiology and Biotechnology* 26:1-8.
- Saling, P, Kicherer, A, Dittrich-Kramer, B, Wittlinger, R, Zombik, W, Schmidt, I, Schrott, W, Schmidt, S. 2002. Eco-efficiency analysis by BASF: The method. *International Journal of Life Cycle Assessment* 7(4):203-218.

- Sandersson, W. 2000. Cleaner industrial processes using hydrogen peroxide. *Pure and Applied Chemistry* 72(7):1289-1304.
- Schmid, A, Dordick, JS, Hauer, B, Kiener, A, Wubbolts, M, Witholt, B. 2001. Industrial biocatalysis today and tomorrow. *Nature* 409:258-268.
- Schmid, RD, Verger, R. 1998. Lipases: Interfacial enzymes with attractive applications. *Angewandte Chemie International Edition* 37:1608-1633.
- Schoemaker, HE, Mink, D, Wubbolts, MG. 2003. Dispelling the myths-biocatalysis in industrial synthesis. *Science* 299:1694-1697.
- Schomburg, D, Salzmann, M. 1991. *Enzyme Handbook 3*. Berlin: Springer-Verlag.
- Sharma, J, Batovska, D, Kuwamori, Y, Asano, Y. 2005. Enzymatic chemoselective synthesis of secondary-amide surfactant from N-methylethanol amine. *Journal of Bioscience and Bioengineering* 100(6):662-666.
- Sheldon, RA. 2007. Enzyme immobilization: The quest for optimum performance. *Advanced Synthesis & Catalysis* 349(8-9):1289-1307.
- Shimada, Y, Watanabe, Y, Sugihara, A, Tominaga, Y. 2002. Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. *Journal of Molecular Catalysis B: Enzymatic* 17(3-5):133-142.
- Shonnard, DR, Kicherer, A, Saling, P. 2003. Industrial Applications Using BASF Eco-Efficiency Analysis: Perspectives on Green Engineering Principles. *Environmental science & technology* 37(23):5340-5348.
- Sienel, G, Rieth, R, Rowbottom, KT. 2000. Epoxides. *Ullmann's Encyclopedia of Industrial Chemistry*. Weinheim, Germany: Wiley-VCH. p 531-545.
- Skouridou, V, Stamatis, H, Kolisis, FN. 2003. A study on the process of lipase-catalyzed synthesis of α -pinene oxide in organic solvents. *Biocatalysis and Biotransformation* 21(6):285-290.
- Slotema, WF, Sandoval, G, Guieysse, D, Straathof, A J J, Marty, A. 2003. Economically pertinent continuous amide formation by direct lipase-catalyzed amidation with ammonia. *Biotechnology and Bioengineering* 82:664-669.
- Solomons, TWG. 1996. *Organic Chemistry*. Hoboken, NJ: John Wiley.
- Stadtman, ER, Levine, RL. 2003. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 25(3-4):207-218.
- Stoye, D, Funke, W, Hoppe, L, Hasselkus, J, Curtis, LG, Hoehne, K, Zech, H-J, Heiling, P, Yamabe, M, Dören, K and others. 2006. *Paints and Coatings*. Ullmann's Encyclopedia of Industrial Chemistry. Weinheim, Germany: Wiley-VCH.

- Straathof, AJJ, Panke, S, Schmid, A. 2002. The production of fine chemicals by biotransformations. *Current Opinion in Biotechnology* 13:548-556.
- Swedish Chemicals Inspectorate. 2007. Report Nr 4/7.
- Swern, D. 1947. Electronic interpretation of the reaction of olefins with organic per acids. *JACS* 69:1692-8.
- Tadros, T. 2006. Surfactants. *Kirk-Othmer Encyclopedia of Chemical Technology*. Hoboken, NJ: John Wiley&Sons.
- Tarquis, D, Monsan, P, Durand, G. 1980. Amino-acids esters synthesis by enzymic catalysis using an organic-solvent. *Bulletin De La Societe Chimique De France Partie Ii-Chimie Moleculaire Organique Et Biologique(1-2)*:76-79.
- Thum, O. 2004. Enzymatic production of care specialties based on fatty acid esters. *Tenside Surfactants Detergents* 41(6):287-290.
- Thum, O, Oxenbøll, KM. 2008. Biocatalysis – A Sustainable Method for the Production of Emollient Esters. *SÖFW-Journal* 134:44-47.
- Toba, S, Merz, KM. 1997. The Concept of Solvent Compatibility and Its Impact on Protein Stability and Activity Enhancement in Nonaqueous Solvents. *Journal of the American Chemical Society* 119(42):9939-9948.
- Trodler, P, Nieveler, J, Rusnak, M. 2008. Rational design of a new one-step purification strategy for *Candida antarctica* lipase B by ion-exchange chromatography. *Journal of Chromatography A* 1179(2):161-168.
- Trodler, P, Pleiss, J. 2008. Modeling structure and flexibility of *Candida antarctica* lipase B in organic solvents. *BMC Structural Biology* 8(1):9.
- Tuccio, B, Ferre, E, Comeau, L. 1991. Lipase-catalyzed syntheses of N-octyl-alkylamides in organic media. *Tetrahedron Letters* 32(24):2763-2764.
- Turner, NA, Duchateau, DB, Vulfson, EN. 1995. Effect of hydration on thermostability of serine esterases. *Biotechnology Letters* 17(4):371-376.
- Turner, NA, Vulfson, EN. 2000. At what temperature can enzymes maintain their catalytic activity? *Enzyme & Microbial Technology* 27(1-2):108-113.
- Törnvall, U, Orellana-Coca, C, Hatti-Kaul, R, Adlercreutz, D. 2007. Stability of immobilized *Candida antarctica* lipase B during chemo-enzymatic epoxidation of fatty acids. *Enzyme & Microbial Technology* 40(3):447-451.
- Uppenberg, J, Hansen, MT, Patkar, S, Jones, TA. 1994. The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure* 2(4):293-308.

- Uppenberg, J, Oehrner, N, Norin, M, Hult, K, Kleywegt, GJ, Patkar, S, Waagen, V, Anthonsen, T, Alwyn Jones, T. 1995. Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecificity pocket for secondary alcohols. *Biochemistry* 34(51):16838-16851.
- Van De Mark, MR, Sandefur, KD, Durham, KA. 2005. Paints. Kirk-Othmer Encyclopedia of Chemical Technology. Hoboken, NJ: John Wiley.
- Warwel, S, Klaas, MRG. 1995. Chemo-enzymatic epoxidation of unsaturated carboxylic acids. *Journal of Molecular Catalysis B: Enzymatic* 1(1):29-35.
- Watanabe, T, Sugiura, M, Sato, M, Yamada, N, Nakanishi, K. 2005. Diacylglycerol production in packed bed bioreactor. *Process Biochemistry* 40:637-643.
- Wehtje, E, Adlercreutz, P. 1997. Water activity and substrate concentration effects on lipase activity. *Biotechnology and Bioengineering* 55(5):798-806.
- Wehtje, E, Kaur, J, Adlercreutz, P, Chand, S, Mattiasson, B. 1997. Water activity control in enzymatic esterification processes. *Enzyme & Microbial Technology* 21(7):502-510.
- Veit, T. 2004. Biocatalysis for the production of cosmetic ingredients. *Engineering life science* 4(6):508-511.
- Verger, R. 1997. 'Interfacial activation' of lipases: facts and artifacts. *Trends in Biotechnology* 15(1):32-38.
- Wicks, ZWJ. 2002. Coatings. Kirk-Othmer Encyclopedia of Chemical Technology: John Wiley & Sons.
- Winterton, N. 2001. Twelve more green chemistry principles. *Green Chemistry* 3(6):G73-G75.
- Won, K, Lee, SB. 2001. Computer-aided control of water activity for lipase-catalyzed esterification in solvent-free systems. *Biotechnology Progress* 17(2):258-264.
- Wynn, NP. 1996. Pervaporation. Kirk-Othmer Encyclopedia of Chemical Technology. Hoboken, NJ: John Wiley & Sons, Inc.
- Xu, X. 2003. Engineering of enzymatic reactions and reactors for lipid modification and synthesis. *European Journal of Lipid Science and Technology* 105:289-304.
- Yadav, GD, Borkar, IV. 2006. Kinetic modeling of microwave-assisted chemoenzymatic epoxidation of styrene. *American Institute of Chemical Engineers Journal* 52(3):1235-1247.
- Yang, TK, Xu, XB, He, C, Li, LT. 2003. Lipase-catalyzed modification of lard to produce human milk fat substitutes. *Food Chemistry* 80(4):473-481.

- Zaks, A, Klibanov, A. 1988. Enzymatic catalysis in nonaqueous solvents. *The Journal of Biological Chemistry* 263(7):3194-3201.
- Zaks, A, Klibanov, AM. 1985. Enzyme-catalyzed processes in organic solvents. *Proceedings of the National Academy of Sciences of the United States of America* 82(10):3192-3196.
- Zaks, A, Klibanov, A M. 1984. Enzymatic catalysis in organic media at 100 degrees C. *Science* 224:1249-1251.
- Zhang, N, Suen, W-C, Windsor, W, Xiao, L, Madison, V, Zaks, A. 2003. Improving tolerance of *Candida antarctica* lipase B towards irreversible thermal inactivation through directed evolution. *Protein Engineering* 16(8):599-605.
- Zhang, Y, Bakshi, BR, Demessie, ES. 2008. Life Cycle Assessment of an ionic liquid versus molecular solvents and their applications. *Environmental Science and Technology* 42(5):1724-1730.