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2013

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Citation for published version (APA):

Chávez, G. (2013). *Oxidation of Ketones: A (Chemo-) Enzymatic Approach Using Oxygenases and Hydrolases*. [Doctoral Thesis (compilation), Biotechnology]. Lund University (Media-Tryck).

Total number of authors:

1

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Oxidation of Ketones:

A (Chemo-) Enzymatic Approach Using Oxygenases and Hydrolases

Georgina Chávez



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Department of Biotechnology
Doctoral thesis
May 2013

Academic thesis, which by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Thursday, May 30 at 10:30 a.m. in Lecture Hall B, at the Center of Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent: Dr. Mats Clarsund, Executive Vice President for Research and Development, Enzyrnatica AB, Lund, Sweden.

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ISBN 978-91-89627-93-2

ISRN LUTKDH/TKBT--13/1147--SE

Cover Design

Front: a plate showing the colorimetric assay described in Paper I

Back: the chemo-enzymatic reaction for the oxidation of cyclohexanone in Paper III.

Printed in Sweden by Media-Tryck, Lund University
Lund 2013



Organization LUND UNIVERSITY Department of Biotechnology P.O. Box 124, SE-22100 Lund, Sweden	Document name DOCTORAL DISSERTATION	
	Date of issue April 18, 2013	
Author(s) Georgina Chávez	Sponsoring Organization Sida/SAREC; Marie Curie actions	
Title and subtitle: Oxidation of Ketones: A (Chemo-) Enzymatic Approach Using Hydrolases and Oxygenases		
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Keywords Baeyer-Villiger, monooxygenase, Lipase B from <i>Candida antarctica</i> , perhydrolyase, caprolactone.		
Classification system and /or index terms (if any)		
Supplementary bibliographical information	Language English	
ISSN and key title	ISBN 978-91-89627-93-2	
Recipient's notes	Number of pages 120	Price
	Security classification	

Distribution by Georgina Chávez, Department of Biotechnology, Lund University, P.O. Box 124 SE-221 00 Lund, Sweden.

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In loving memory of Jorge Chávez

Abstract

Oxidation reactions are important in organic chemistry as well as in nature. In industry, oxidations are commonly used for the synthesis of chemicals and pharmaceuticals, however such processes have a number of limitations, e.g. they use chlorinated solvents, stoichiometric oxidation reagents, and in some cases the reagents that have risks of explosion during transportation and storage. This has called for more environment-friendly alternative technologies for oxidation reactions. Baeyer-Villiger oxidation is a reaction in which a ketone is oxidized to an ester or a cyclic ketone to a lactone by treatment with peroxyacids. Lactones constitute an important group of chemicals used in flavors, fragrances, pharmaceutical intermediates and polymer building blocks. The work presented in this thesis concerns enzymes, including Baeyer-Villiger monooxygenases (BVMOs) that catalyse the Baeyer-Villiger oxidation using molecular oxygen as an oxidant, and perhydrolytic enzymes that can be used for *in situ* generation of peracid for oxidation of cyclic ketones. A simple colorimetric method was developed for detection of BVMO activity and was based on the formation of a purple colored product between an enolizable ketone and 3,5-dinitrobenzoic acid in an alkaline solution. The method was shown to have potential for screening of both wild type and recombinant microbial cells as well as for quantitative measurement of BVMO activity. Further, a recombinant BVMO from a strain of *Dietzia* was characterized. The sequence of the enzyme suggested that it is related to Ethionamide monooxygenases. The recombinant enzyme was active in whole cells and crude lysate but lost activity on purification. The enzyme was shown to have high activity towards several linear alkenes, and was also moderately active towards cyclobutanone, phenylacetone and thioanisole.

Two perhydrolytic enzymes able to produce peracids from a carboxylic ester and hydrogen peroxide were studied for oxidation of cyclohexanone to ϵ -caprolactone, a chemical of immense importance. The enzymes were immobilized as cross-linked enzyme aggregates (CLEAs). The well-studied lipase B from *Candida antarctica* (CaLB) gave a maximal caprolactone yield of 80% with ethyl acetate as acyl donor. The perhydrolase was able to produce peracids in an aqueous medium with ethylene glycol diacetate and hydrogen peroxide, and gave ϵ -caprolactone yield of 70%. In both cases the formation as acetic acid as a coproduct showed to be an important factor for the deactivation of the enzyme.

The use of monooxygenases, lipases and perhydrolases for the Baeyer-Villiger reaction constitutes a greener alternative to traditional chemical processes but the problem of enzyme stability remains to be solved.

Keywords: Lipase B from *Candida antarctica*, perhydrolase, caprolactone, cross linked enzyme aggregates.

Popular summary

The thesis that you are reading at this moment was printed using climate compensated paper, a sign that consciously or not you are contributing somehow to control the current environmental issues in our planet. It would not be nice if every single article used in your daily life contains information about how environmentally friendly that product is? We have to think “big” and start to work for a real solution. It is of common knowledge that the chemical industry has changed the world by providing us with innumerable products but at the same time has been one of the major contributors to pollution on our planet. Investments on research to allow us to make a green process along with policies regulating the production of chemicals can be a real solution to several of our environmental problems.

Oxidations are important reactions in chemistry. Oxidations in industry are done generally using chlorinated solvents, large amounts of chemicals some of which pose risk of explosion during transportation and storage. A greener alternative is the use of enzymes which are able to oxidise compounds with molecular oxygen (as monooxygenases) or enzymes able to produce oxidants during the process (lipases and perhydrolases). Enzymes usually work in mild conditions, making the process not just greener but also safer as avoid the risk of explosion. In addition, green reactions should also consider changing traditional solvents to greener alternatives, especially considering that solvents *per se* constitute around 80% of the generated organic waste.

This thesis concerns work with the above-mentioned enzymes with a target to produce lactones, the products used in flavors, fragrances, pharmaceuticals and polymers. To contribute to the efforts of green chemistry, the work covers the development of a new colorimetric method for the search of oxidizing enzymes from bacteria, the description of one such enzyme from a bacteria isolated from a soda lake, and development of an enzymatic process to produce caprolactone. Who knows, maybe in the future if you decide to read this thesis again, in the corner of your glasses in very tiny letters you can read something like: this product was made using materials according to green chemistry regulations.

List of Publications

This thesis is based on the following papers and/or manuscripts, referred to in the text by their Roman numerals.

- I. Javier Linares, Georgina Chávez, Rodrigo Villagomez, Gashaw Mamo and Rajni Hatti-Kaul (2012) A method for rapid screening of ketone biotransformations: Detection of whole cell Baeyer–Villiger monooxygenase activity. *Enzyme and Microbial Technology* **50**, 101-106.
- II. Serena Bisagni, Justyna Pertek, Georgina Chávez and Gashaw Mamo (2013) Cloning, expression and characterization of a Baeyer-Villiger monooxygenase from *Dietzia* sp D5. Manuscript
- III. Georgina Chávez, Rajni Hatti-Kaul, Roger Sheldon and Gashaw Mamo (2013) Baeyer-Villiger oxidations with peracids generated *in situ* by CaLB-CLEA catalyzed perhydrolysis. *Journal of Molecular Catalysis B: Enzymatic* **89**, 67-72.
- IV. Georgina Chávez, Jo-Anne Rasmussen, Michiel Janssen, Gashaw Mamo and Roger Sheldon (2013) Baeyer-Villiger oxidation of cyclohexanone with *in situ* generation of peracid with a perhydrolase CLEA. Manuscript.

My contribution to the papers

- I. Javier Linares Pastén provided the project idea and experimental design. I participated in the assay development and performed the cell assays. The first version of the manuscript was written by Javier Linares and later finalized with assistance of all the authors.
- II. The idea came from Serena Bisagni. Planning and experimental parts were done mainly by Serena Bisagni and Justina Pertek. I performed some of the biotransformations and contributed with writing of the manuscript.
- III. I developed an idea from Gashaw Mamo. I planned and performed the experiments. I wrote the first version of the manuscript, and finalised it in collaboration with the other authors.
- IV. The project idea came from Michiel Janssen. I planned the project in close collaboration with Jo-Anne Rasmussen and Michiel Janssen. I did all the experimental part, wrote the first draft of the manuscript and finalized it with assistance of the other authors.

Abbreviations

ATP	Adenosine Triphosphate
BVMO	Baeyer-Villiger Monooxygenase
C	Cytosine
CaLB	<i>Candida antarctica</i> lipase B
CHMO	Cyclohexanone Monooxygenase
CL	Caprolactone
CLEA	Cross Linked Enzyme Aggregates
DKCMO	Diketocamphane monooxygenase
EC	Enzyme Commission
EthA	Ethionamide
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FMO	Flavin containing monooxygenase
G	Guanine
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NCIMB	National Collection of Industrial, Marine and Food Bacteria

Contents

Abstract

Popular Summary

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1. Introduction

The current environmental situation of our planet is critical and efforts in different fields are being made in order to preserve our natural resources. One of the major contributors to pollution has been the petro-chemical industry. To diminish the negative environmental impact of chemicals a new branch of chemistry was born in the 90's: Green Chemistry, based on 12 principles with a goal to eliminate or reduce the use of non-renewable resources, minimize energy requirement, -build up and release of hazardous wastes and emissions.

Biocatalysis, based on enzymes as catalysts, has become a central part of green chemistry. The use of enzymes for production of chemicals confers many benefits to the process, which often include mild reaction conditions, and also fewer reaction steps and less waste thanks to their high regio-, stereo and enantioselectivity. The enzymes have the added advantage of being biodegradable and renewable catalysts.

The enzyme catalyzed reactions being run or developed in chemical and pharmaceutical industry include (trans-)esterification, amidation, glycosylation, carbon-carbon formation and cleavage, hydrolysis and redox reactions (Wohlgemuth, 2010). Among the redox reactions involving introduction of one oxygen atom, the Baeyer-Villiger oxidation is of industrial interest as the use of enzymes makes the process not just cleaner but also safer by avoiding the use of large amounts of oxidants that pose risk of explosion during transportation and storage.

Several microorganisms found in different ecosystems possess enzymes, *oxygenases*, that transfer oxygen from molecular oxygen to a substrate, and depending on the insertion of one or two atoms of oxygen the enzymes can be divided into monooxygenases and dioxygenases respectively. Monooxygenases that carry out the Baeyer Villiger oxidation are cofactor dependent enzymes. Although being able to add oxygen to the substrate in one single step, the labile nature of the monooxygenases, along with the need to add external cofactor, limits their use in whole cell systems. An alternative to the use of monooxygenases for the Baeyer-Villiger oxidations is the use of enzymes with perhydrolytic activity to generate peracid from which the oxygen is spontaneously transferred to the substrate. These enzymes are usually more resistant and the cell-free enzyme can be immobilized for use in the reaction.

1.1. Scope of the thesis

The aim of the work presented in this thesis was to investigate biocatalytic routes as greener alternatives to current processes for production of specialty chemicals through Baeyer-Villiger oxidation. The main focus was the oxidation of ketones, with emphasis on the oxidation of cyclohexanone to produce ϵ -caprolactone.

The thesis is based on four papers that describe the search and the use of whole cells with monooxygenase activity in the oxidation of cyclohexanone and other (cyclo-)ketones and the use of an immobilized perhydrolytic enzyme for chemo-enzymatic oxidation of cyclohexanone. The work covers the development of a colorimetric method that can be applied for screening of wild and recombinant strains with monooxygenase activity towards ketones (**Paper I**). A monooxygenase with the ability to oxidize linear ketones, from a *Dietzia* species isolated from an alkaline soda lake, is described in **Paper II**. The chemo-enzymatic production of caprolactone and optimization of different parameters of the reaction in green solvents using lipase and perhydrolase, respectively, is the subject of Paper **III** and **IV**.

The following chapters give a background of the areas covered in the thesis. **Chapter 2** introduces the concepts of sustainable chemistry. **Chapter 3** covers the description of Baeyer-Villiger oxidation, the microorganisms and also their enzymes involved in the reaction. In **Chapter 4**, a comparison between the traditional chemical way to produce caprolactone and the biocatalytic systems are described. Finally, in **Chapter 5** the conclusions and future perspectives of this work are included.

2. Sustainable Chemistry

The concept of sustainability is in general the capacity to support, maintain or endure. It was not until the “Limits to growth” (Meadows et al., 1972) published at the beginning of the 70’s, when the world started to consider sustainability as the fundamental base to save our resources. The book was based on computational models that predict that by the year 2100 all our non-renewable resources will disappear if we do not immediately take a drastic action. Unfortunately 40 years later, the model seems to be correct.

The growth of petrochemical industry during the 20th century had a tremendous impact on global economy and transformed the world forever. Fossil based products – energy, chemicals and materials - have become an integral part of our lives. About 90% of the chemicals are produced from fossil resources. The world market of chemicals was estimated at €1736 billion in 2004 (SusChem, 2005). During the past two decades, however, there is an increasing concern that fossil oil and gas will become a scarce commodity in the future. Already the systematic increases in the price of mineral oil and gas have reduced the profit margins for the chemical industry. Moreover, fossil based production has also been linked to several of the environmental problems being faced today such as greenhouse effect, ground- and water pollution, and climate change. The recent United Nations meeting in Rio 2012 recognised that management of chemicals and waste throughout their life cycle is crucial for protection of human health and environment (UN, 2012). More and more chemical companies are beginning to realise that sustainable development is an effective way to improve their businesses and profitability, and are considering both resource and environmental aspects of their operations. The European chemical industry has called for an urgent need for enhancement of innovation efforts to provide the technology base for more sustainable chemicals production (CEFIC, 2004).

Sustainable Chemistry also known as Green Chemistry, is the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substance (E.P.A.). The term “green chemistry” was first introduced by Paul Anastas and John Warner (Anastas and Warner, 1998) and was summarized later by Roger Sheldon as: “efficiently using raw materials

and solvents in the manufacture and application of chemical products” (Sheldon, 2000).

Green Chemistry is based on the following principles (Anastas and Warner, 1998)

1. It is better to prevent waste than to treat or clean up waste after it is formed.
2. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity.
4. Chemical products should be designed to preserve efficacy of function while reducing toxicity.
5. The use of auxiliary substances should be made unnecessary whenever possible, and innocuous when used.
6. Energy requirements should be recognized for their environmental and economic impact and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.
7. A raw material should be renewable whenever technically and economically practical.
8. Unnecessary derivatization should be avoidable whenever possible.
9. Catalytic reagents are superior to stoichiometric reagents.
10. Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products.
11. Analytical methodologies need to be further developed to allow for real-time in process monitoring and control prior to the formation of hazardous substances.
12. Substances and the form of a substance used in a chemical process should be chosen so as to minimize the potential for chemical accidents, including explosions, fires and releases to the environment.

A lot of efforts are ongoing to develop new reaction concepts or modify the existing ones in agreement with the green chemistry principles. Among the technologies expected to hold promise for providing clean processes is industrial biotechnology.

2.1. Industrial Biotechnology

Industrial biotechnology, known as “white biotechnology” has been recognized as an important technology area for the chemical industry in providing clean processes, especially based on renewable resources as raw materials. According to the European Association for Bioindustries (EuropaBio) “White Biotechnology is the application of nature’s toolset to industrial applications” (EuropaBio, 2003). This toolset includes living cells and enzymes that are used in fermentation and biocatalytic processes, respectively, and the industries include sectors as diverse as chemicals, food and feed, healthcare, paper and pulp, detergents, textiles and biofuels.

The concept of industrial biotechnology, although having been around for some decades has gotten revived during the late 1990s, the encouragement coming from modern tools of genetic engineering, genomics, proteomics, etc. Biotechnology is being used today to convert agricultural feedstocks into bulk chemicals, fine chemicals, bioplastics, biofuels, etc. by means of fermentation and biocatalysis (Hatti-Kaul R. et al., 2007; OECD, 2001) Some examples of current industrial biotechnology processes in the market are listed in Table 1.

Table 1 White Biotechnology processes in industry

Product or process	Company	Biotechnological approach	Reference
Vitamin B2	BASF	Fermentation	(Saling, 2005)
Cephalexin	DSM	Fermentation and 2 enzymatic steps	(Schroën et al., 2002)
Scouring of cotton	Novozymes	Use of scouring enzyme in water	(Nielsen et al., 2009)
Bio-based polymers	Cargill Dow	Lactic acid production by fermentation	(Lunt, 1998)
Acrylamide	Nitto Chemical Ltda.	Nitrilase catalyzed production of acrylamide	(Crich et al., 1993)
Poly-4-hydroxybutyrate	Tepha	Fermentation	(Martin and Williams 2003)

The aim of white biotechnology is to create a sustainable society (Gupta and Raghava, 2007). According to an estimate by McKinsey & Company, the generated added value by industrial biotechnology in the chemical industry alone is estimated to amount up to EUR 11-22 billion per annum (www.europabio.org). The greatest impact may be on the fine chemicals segment where up to 60 % of products may be based on biotechnology.

2.2. Biocatalysis

Biocatalysis refers to the use of biological agents such as microorganisms or enzymes as catalysts that activate or speed up a chemical reaction. The biocatalysts catalyse reactions under mild conditions of pH, temperature and pressure, and are non-toxic and biodegradable. The use of biocatalysts in chemical synthesis is in line with the principles of green chemistry.

Enzymes are increasingly penetrating the chemical industry as catalysts in a number of reactions. Biocatalysis is often an attractive alternative in cases where reactions cannot easily be conducted by classical organic chemistry, or replacing processes that requires several steps. Hence, biocatalysis is having an increased impact on organic synthesis, especially on the synthesis of enantiopure compounds (Kazlauskas, 2004). The synthesis of polyester (Habeycha et al., 2011; Tilman et al., 2007) interesterification of triglycerides and fatty acids by lipase (Freitas et al., 2009), peptide synthesis by proteases (Sun et al., 2011), are some of the examples used at industrial scale. Currently, processes like asymmetric reductions, stereospecific oxidations, C-C, C-O and C-N bonds formation, etc. are being developed using biocatalysis (Goldberg et al., 2007; Nuijensa et al., 2012; Resch et al., 2011).

Very few enzymes, commercially available today, catalyse the reactions under conditions that are industrially and economically convenient. This is due to the low stability of the enzymes under extreme conditions. The impetus in applied biocatalysis has put a demand on discovery of new biocatalysts. Novel biocatalysts can be discovered or developed by two different routes: by exploring nature for novel variants, or by genetic *in vitro* development of available enzymes, or a combination of both. Microorganisms still constitute an important source of enzymes. Biocatalysts are being discovered from novel microorganisms isolated from e.g. extreme environments, and also from the genetic material from uncultured microorganisms present in environmental samples (metagenomics). Evolution of the enzymes has further been possible

in vitro at a significantly higher pace than achieved in nature. The more traditional has been the rational design approach, where knowledge of existing structures is used to predict and design modified enzymes. Currently, the increasing availability of genome sequences in databases provide easy access to the gene sequences encoding different enzymes, and further allows comparison of the sequences to facilitate the choice or design of a suitable sequence that can be synthesized.

The mild conditions required for a reaction while using enzymes and the fact that organic compounds have poor solubility in water or polar solvents, led to the use of biocatalysts in water-organic phase biphasic systems or preferably in a water-poor medium. Developments in non-aqueous enzymology for the past 20 years have brought to light immense possibilities of synthesizing chemicals with enzymes (Dordick, 1989; Klivanov, 2001; Koeller and Wong, 2001; Schmid et al., 2001)

In this thesis, the interest was to discover and develop biocatalysts and biocatalytic processes for oxidation of ketones and cycloketones.

2.3. Oxidation reactions

Oxidation reactions are powerful tools to convert a position that is protected in a lower oxidation state to the desired functionality and for the functionalization of otherwise unfunctionalized positions (Caron et al., 2006). Oxidations are important reactions used for the synthesis of bulk- and fine chemicals, and pharmaceuticals. These include transformations that either remove hydrogen from the molecule (for example, alcohol to aldehyde), or insert an oxygen into a C–C (Baeyer–Villiger, epoxidation) or C–H bond (aldehyde to acid) (Hudlicky, 1990; Marko, 1998). A lot of progress has been made on the development of environment-friendly oxidation reagents, however there are still several limitations among existing methods that need to be addressed. For example, majority of oxidation reactions are still conducted in inert, nonflammable chlorinated solvents. Oxidants such as dichromate/sulfuric acid, chromium oxides, permanganates, periodates, chlorine, etc., used in stoichiometric amounts, resulting in high salt and heavy metal containing wastes, are still used (Constable et al. 2007). Also transportation and storage of organic peracids, commonly used for epoxidation, incur significant costs.

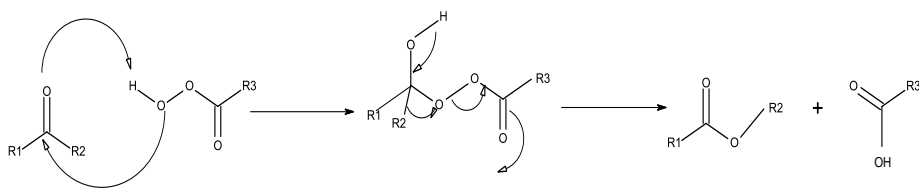
The use of enzymes as catalysts further contributes to the greenness of the process by allowing selective oxidations and hence less steps and waste. The use of enzymes allows the synthesis of chiral intermediates that are impossible

or difficult to obtain in high yields by the use of conventional catalysts. Molecular oxygen or air is the ideal oxidant; however, aerating flammable solvents is a significant safety concern that can only be fully addressed if the reaction can be conducted efficiently in water. Hydrogen peroxide is the second best choice with respect to atom economy, but utilization, efficiency and narrow range of scope limit its application (Constable et al., 2007). In this work, molecular oxygen is used by monooxygenases (**Papers I** and **II**), and hydrogen peroxide is used for production of peracids for the Baeyer-Villiger oxidation (**Paper III** and **IV**)

3. Biocatalytic Baeyer-Villiger oxidation

The Baeyer-Villiger oxidation reaction was described for the first time by Adolph von Baeyer and Victor Villiger in 1899 (Baeyer and Villiger, 1899). The reaction involves the oxidative cleavage of a carbon-carbon bond adjacent to a carbonyl, which converts ketones to esters and cyclic ketones to lactones. These reactions are of great importance in synthetic organic chemistry. The oxidants typically used to carry out the Baeyer-Villiger reaction are m-chloroperoxybenzoic acid, peroxyacetic acid or peroxytrifluoroacetic acid.

The mechanism of the reaction is known to involve two main steps (Scheme 1). The first is the carbonyl addition of a peroxyacid to the carbonyl, producing a tetrahedral adduct known as the Criegee intermediate (Criegee, 1948). The second step is the migration of the alkyl or aryl group adjacent to the carbonyl group to the nearest peracid oxygen atom, with the simultaneous dissociation of the O–O bond. If the migrating carbon is chiral, stereochemistry is retained. The mechanism was first proposed by Doering and Dorfman in 1953 based on isotope labeling experiments (Doering and Dorfman, 1953).



Scheme 1 Reaction mechanism of the Baeyer-Villiger reaction using peracids

Stereospecificity and regiochemistry are the typical features of Baeyer-Villiger oxidations. The regioselectivity of the reaction depends on the relative migratory ability of the substituents attached to the carbonyl. Substituents which are able to stabilize a positive charge migrate more readily, so that the order of preference is: tertiary alkyl > cyclohexyl > secondary alkyl > phenyl > primary alkyl > CH₃ (Friess and Pinson, 1952), (Hawthorne et al., 1958), (Winnik and Stoute, 1973). A hydrogen as –R group almost never migrates. In

some cases, stereoelectronic or ring strain factors also affect the regiochemical outcome.

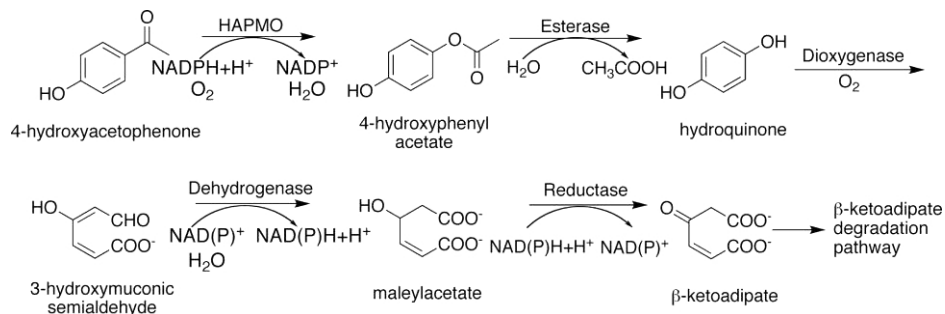
In **Paper I**, there is a negative relation of ring size/yield, the bigger the ring the lower the conversion. In **Paper III** there is an apparent steric effect as larger molecules showed lower yields; the conversion of cyclopentanone reached 88% while the conversion of cyclooctanone was in the range 0-2%. Similarly, the position of the ring substituent has an influence on the conversion as location of the methyl group in position *ortho* led to a conversion of 76-84%, in meta position to a conversion between 57-62%, and in *para* a conversion of 77-80%.

3.1. The Baeyer-Villiger oxidation in nature

Oxidation reactions are extremely important for maintenance of life, being involved in one fourth of all processes present in living cells. For example oxidation is vital in energy generating release pathways and formation of a vast group of essential compounds such as Adenosine Triphosphate (ATP), acetyl coenzyme A, oxaloacetate, etc. In living organisms, oxidation reactions are catalyzed by enzymes such as dehydrogenases that oxidize substrates by transferring one or more (H-) to an electron acceptor, peroxidases that requires H₂O₂, and oxidases, monooxygenases and dioxygenases, that use molecular oxygen as oxidants (de Gonzalo et al., 2010). There are enzymes that catalyse the Baeyer-Villiger reaction which are known as Baeyer-Villiger monooxygenases (BVMOs).

In living cells, the first evidence for this type of reaction was discovered in 1948, when Turtiff and coworkers observed that several fungi were able to convert steroids via Baeyer-Villiger oxidation (Turfitt, 1948). Years later, it was proposed that Baeyer-Villiger oxidations might be involved during the conversion of progesterone and testosterone to Δ^1 -testololactone (Peterson et al., 1953). BVMO involved in the degradation of aliphatic linear ketones were observed in various *Pseudomonas* strains (Forney and Markovetz, 1953),(Shum et al., 1974). The Baeyer-Villiger reaction is commonly followed by an esterase catalyzed hydrolysis. Moreover, genetic analysis has shown that the location of the gene of BVMO is close to the genes encoding a esterase and an alcohol dehydrogenase (Geitner et al., 2007; Rehdorf et al., 2007; Rehdorf et al., 2009; Rodríguez et al., 2007). As an example the degradation pathway of 4-hydroxyacetophenone in *Pseudomona putida* JD1 includes an oxidation

for a monooxygenase to form 4-hydroxyphenyl acetate, followed by the action of an esterase to form the correspondent hydroquinone which is further degraded by a dehydrogenase and a reductase to form β -ketoadipate (Scheme 2).



Scheme 2 Proposed mechanism for the degradation of 4-hydroxyacetophenone catalyzed by the enzymes of *P. putida* JD1 (Rehendorf et al., 2007).

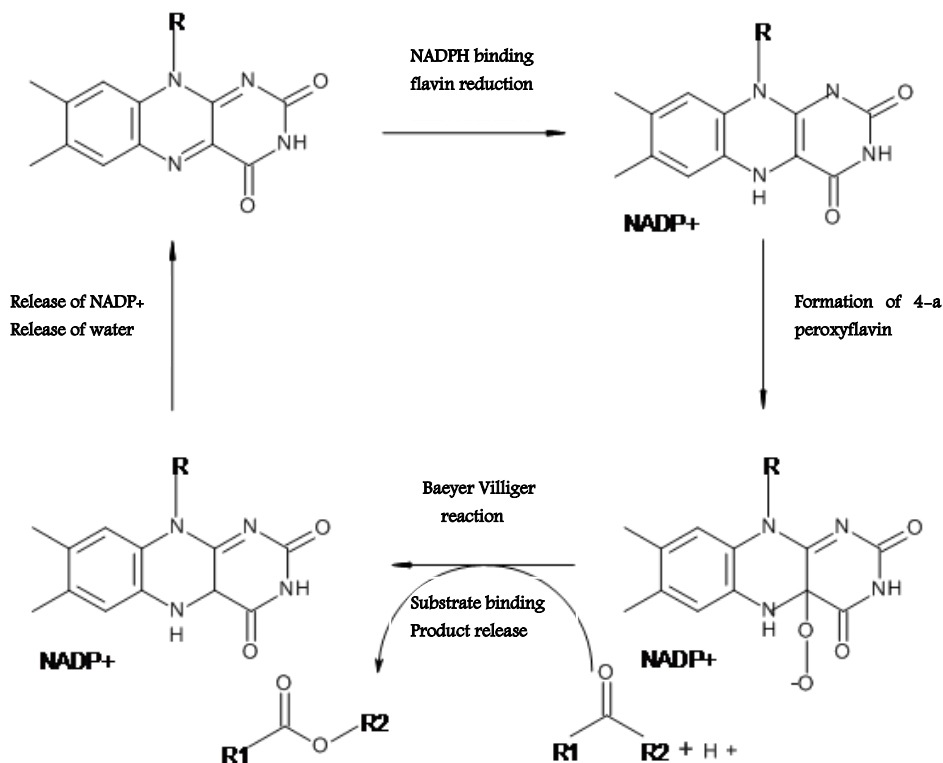
Baeyer-Villiger monooxygenases (BVMOs) are used to produce important substances, for example toxins and steroids, metabolize various carbon sources, such as aliphatic and acyclic ketones (Kamerbeek et al., 2003). Although a dedicated BVMO has evolved for each metabolic step, biocatalytic studies have shown that individual BVMOs can accept different substrates.

Distribution of BVMOs is quite interesting, they are abundant in some specific microbes e.g. actinomycetes and some filamentous fungi, while they are hardly represented in some bacteria and absent in higher eukaryotes and archaea. Microorganisms using various hydrocarbons as carbon source have been found to use BVMOs. By using BVMO-specific protein sequence motif, it has been possible to identify that at least one BVMO gene is present in 15% of all sequenced microbial genomes (Fraijee et al., 2002).

Cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB is the most studied BVMO and has shown an impressive substrate scope and exquisite chemo-regio and enantioselectivity (Opperman and Reetz, 2010). This enzyme was discovered by Donogue in 1976 (Donoghue et al., 1976) and later overexpressed in *Escherichia coli* and *Saccharomyces cerevisiae*. Other cycloketone monooxygenases have been reported from *Comamonas* sp., *Xanthobacter* sp., *Rhodococcus* sp. (Van Beilen, 2003), *Nocardia* sp. (Donoghue et al., 1976), *Brevibacterium* sp. (Brzostowicz et al.,

2002), *Arthrobacter* sp. (Kim et al., 2008) and *Pseudomonas* sp. (Rehdorf et al., 2009)

The proposed mechanism for the enzymatic Baeyer-Villiger oxidation was based on data obtained from cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* (Scheme 3). Kinetic studies revealed that BVMOs catalysis is achieved by the formation of a peroxyflavin (Ryerson et al., 1982; Sheng et al., 2001). The enzymatic process is initiated by the tightly bound Flavin Adenine Dinucleotide (FAD) by (Nicotinamide Adenine Dinucleotide Phosphate Hydrogen) NADPH followed by rapid oxidation by molecular oxygen to produce flavin 4-a peroxide anion, which acts as the oxygenation specie. Nucleophilic attack of the substrate carbonyl group by the flavin 4-a peroxide anion results in the "Criegge" intermediate, which undergo rearrangement to form the product lactone and 4-a hydroxy flavin. The catalyst cycle is terminated by elimination of water to form FAD and the release of the product and co-factor.



Scheme 3 Scheme of the catalytic mechanism of type I BVMO (de Gonzalo et al., 2010).

3.2. *Dietzia* species

This taxon was proposed in 1995 by Raynei (Rainey et al., 1995). *Dietzia* species have been isolated from a diverse range of aquatic and terrestrial habitats (Goodfellow and Maldonado, 2006). *Dietzia maris* has been isolated from halibut (Harrison, 1929) and later from soil, skin and the intestinal tract of carp (Nesterenko et al., 1982) deepest sea mud and sediments (Colquhoun, 1998; Takami et al., 1997). *Dietzia aerolata* was isolated from a bioaerosol allocated in a duck barn (Kampfer et al., 2010). *Dietzia cercidiphylli* and *Dietzia schimae* were isolated from surface sterilized plant material (Li and 2008) (Li and 2008). *Dietzia cinnamea* (Yassin et al., 2006) and *Dietzia papillomatosis* are species of human origin (Jones et al., 2008). *Dietzia lutea* (Li, 2010), *Dietzia kunjamensis* (Mayilraj et al., 2010), and *Dietzia timorensis* (Yamamura, 2010) were obtained from soil while *Dietzia natronlimnae* was recovered from a soda lake (Duckworth et al., 1998).

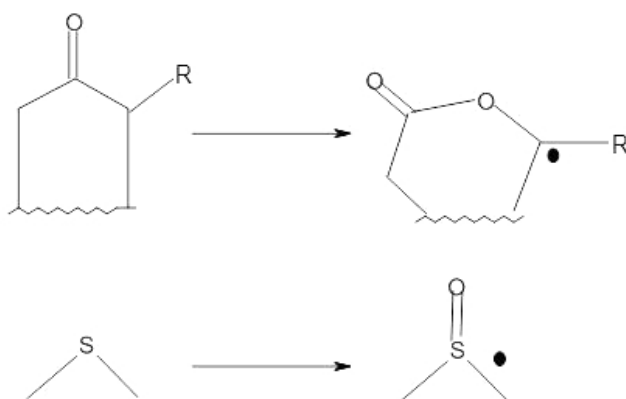
Dietzia is an aerobic Gram-positive alkaliphilic bacterium, non-acid alcohol, non-spore forming cocci that grow into short rods and catalase positive. On agar plates the bacteria form circular, raised or convex, glistening, orange to coral red colonies with entire edges (Koerner et al., 2009). The strains are chemoorganotrophic and have oxidative metabolism. The major glycan in the cell wall of the organism is N-acetyl muramic acid, and the main sugars are arabinose and galactose. Short chain mycolic acids, long chain fatty acids and tuberculostearic acid are present.

Based on 16S rRNA gene sequence the species of *Dietzia* are from a distinct family level lineage within the order Corynebacter of the class Actinobacteria. Another important characteristic of genus *Dietzia* is its high mol% G + C content (66-73% in their genome).

Dietzia species are able to grow on a large number of hydrocarbons including hexadecane, cyclohexanone, n-hexane, cyclohexanol and catechol, as the sole source of carbon and energy (Iwaki et al., 2008). They are found in many environmental niches and have a remarkable ability to metabolize a wide variety of xenobiotic compounds (von der Weid, 2007; Yumoto, 2002). The *Dietzia* strain studied in this thesis was isolated from an Ethiopian soda lake sample, and was cultivated initially in a medium containing cyclohexanol.

3.3. Baeyer-Villiger monooxygenases

Baeyer Villiger-monooxygenases (BVMOs E.C. 1.14.13.x) belong to the class of oxidoreductases. They incorporate one atom of molecular oxygen (O_2) into the substrate and the other atom is reduced to water. BVMOs catalyse the nucleophilic oxygenation of ketones and boron and the electrophilic oxygenation of sulphur, selenium, nitrogen and phosphorus (Scheme 4). Two types of BVMOs are known: Type I and Type II, the former containing flavin adenine dinucleotide (FAD) as cofactor use NADPH as a source of electrons and consist of identical subunits, while Type II BVMOs contain flavin mononucleotide (FMN) as cofactor and use Nicotinamide Adenin Dinucleotide Hydrogen (NADH) as electron donor (Kamerbeek et al., 2003; Rehdorf et al., 2009) So far all BVMOs investigated are Type I enzymes.



Scheme 4 General scheme of BVMO catalysed oxidation of cyclic ketones and organic sulfides (Alphand et al., 2003)

Classification of BVMOs in the family of flavoprotein (flavin-containing) monooxygenase (FMO's), places the Type I BVMOs in the Class B FMOs and the type II in the class C FMO's, while type O BVMOs belongs to class A FMO (van Berkel et al., 2006).

Type I monooxygenases consist of one polypeptide chain, with two Rossmann sequence motifs, which allow the binding of the two cofactors, FAD and NADPH (Wierenga et al., 1986). Among bacteria, Type I BVMOs are

prevalent in actinomycetes as in **Paper II**, rendering these bacteria an interesting source of novel BVMOs, while fungal genomes are also rich in BVMO sequences but are relatively unexplored. Type I BVMOs are not present in humans, plants or animal genome (Torres Pazmiño, 2010).

Type II BVMOs are composed of two different subunits. Sequence data suggest a sequence relationship with the flavin dependent luciferases (van Berkel et al., 2006). There are only few examples of Type II BVMOs, two diketocamphocamphane monooxygenases (DKMO), the enzymes involved in camphor metabolism from *P. putida* (Kadow et al., 2012), two FMN and NADH dependent luciferases from *Photobacterium phosphoreum* and *Vibrio fischeri* (Villa and Willetts, 1997), and a BVMO from *Rhodococcus erythropolis* involved in the metabolism of limonene. (van der Werf et al., 1999).

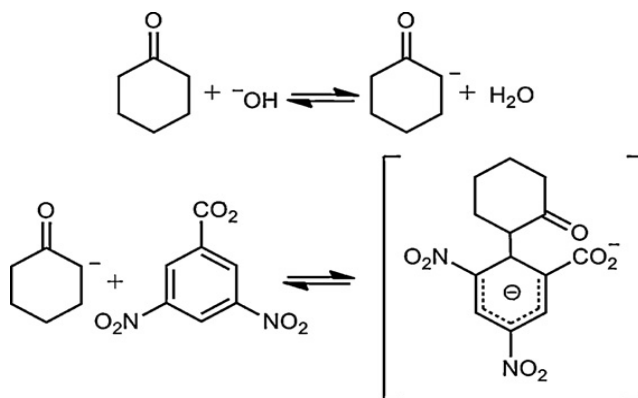
Type O BVMO is represented by MtmOIV from *Streptomyces argillaceus*, a key enzyme in the biosynthetic pathway of mithramycin (Torres Pazmiño and Fraaije, 2007). The enzyme contains FAD bound to the surface and is structurally related to FAD dependent hydroxylases (Beam et al., 2009; Gibson et al., 2005). The finding of novel BVMO types indicates that during evolution several different enzymes have evolved into BVMOs and it is possible that more BVMO types may be discovered in the coming years (Torres Pazmiño and Fraaije, 2007).

3.3.1. Screening of BVMOs

Being important catalysts and also having varying substrate preference and molecular properties, there is a need to find new BVMOs, in different habitats and to screen recombinant libraries with mutants with varied activity, selectivity and stability features. Considering that BVMOs are cofactor dependent many screening methods are based on the decrease in absorbance at 340 nm due to consumption of NADPH (Ryson et al., 1982). Alternatives to depletion of NADPH are assays to measure product formation based on pH change after hydrolysis of the product (Gutierrez et al., 2003), determination of lactones formed by gas chromatography (Goncalves et al., 2004), measurement of fluorescence of umbelliferone release from 2-coumaroyloxy ketone substrates (Sicard et al., 2005), or a modification of an adrenaline assay based on the back titration with iodate (Wahler and Reymond, 2002). Although these methods are efficient, they are not easy to perform when a library of mutants must be screened. For this purpose, spectrophotometric methods are the most convenient choice. One example is the method proposed

by SaB (SaB, 2012) which is a two step method based on the oxidation p-nitroacetophenone by a BVMO as a first step to form the corresponding acetate, which is later hydrolysed by an esterase to give p-nitrophenylate which is measured at 410 nm.

In **Paper I**, a colorimetric method based on the Janovsky reaction was developed for screening of BVMOs, based on the reaction of cyclohexanone (or other cyclic ketone) with 3,5-dinitrobenzoic acid in an alkaline solution (Scheme 5).



Scheme 5 Proposed reaction of cyclohexanone monooxygenase with 3,5-dinitrobenzoic acid in an alkaline solution to give purple colored Sigma (Janovsky) complex. Reproduced from **Paper I**

This method was used to detect Baeyer-Villiger monooxygenase activity in whole cells. It is a simple and fast method and is appropriate for both qualitative screening and quantitative monitoring consumption of cyclohexanone and other cycloketones. Figure 1 shows the consumption pattern of cyclohexanone by different bacteria using this method by determining a decrease in color of the Janovsky complex (**Paper I**). *Dietzia sp.* showed maximum consumption (84%) of 50 mM cyclohexanone. The decrease in the purple color seen visually on consumption of cycloketone can be seen in Figure 2.

In **Paper II** evaluation of the monooxygenase activity was done by determining conversion of substrates using a gas chromatography method. In this work, a new ethionamide monooxygenase like Baeyer-Villiger monooxygenase recombinantly expressed in *E. coli* showed activity to transform a number of linear alkenes and also some other substrates.

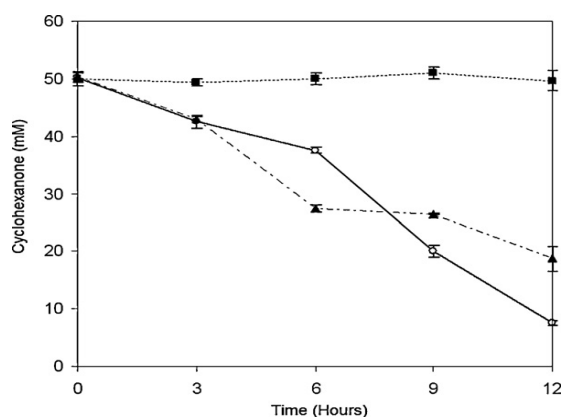


Figure 1 Cyclohexanone consumption by *Dietzia* sp. (○), *Brevibacterium* sp. (▲) and *E. coli* BL21 (DE3) (■) at pH 6.8 and 20 °C (**Paper I**).

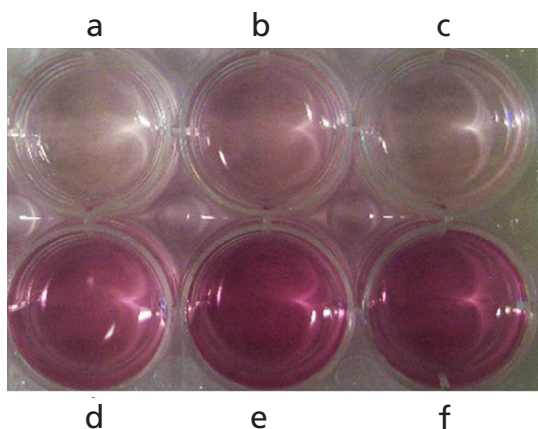


Figure 2 Qualitative assay of cyclohexanone consumption using (a) *Dietzia* sp., (b) *Brevibacterium* sp., (c) *Methylobacterium extorquens*, (d) *Xanthobacter flavus*, (e) *E. coli* BL21 (DE3), and (f) blank (**Paper I**).

3.3.2. Properties of Type I BVMOs

Type I BVMO members have been extensively studied, and currently they are the most important biocatalysts used for Baeyer-Villiger oxidation. Typically, they exhibit an optimum activity at slightly basic pH. Type I BVMOs are sequence related to two other flavoprotein monooxygenase classes: heteroatom flavin-containing monooxygenases and N-hydroxylating flavin-

containing monooxygenases (van Berkel et al., 2006). This reflects a shared reactivity as all three flavoprotein classes employ a FAD cofactor to perform oxygenation reactions.

Type I BVMOs consist of only one polypeptide chain. Three-dimensional structure reveals the presence of two domains resembling disulfide oxidoreductases, one that tightly binds FAD and one for NADPH binding (Pazmiño and Fraaije, 2007). In each of these domains a Rossmann fold (GXGXXG) that is typical for the interaction with the cofactor is present. Another characteristic feature of all Type I BVMOs is the presence of a protein sequence motif ((FxGxxxHxxxW(P/D) that can be used to identify novel Baeyer-Villiger monooxygenases. This motif is also important for catalytic activity of the enzymes (Fraaije and Janssen, 2004).

The active site of BVMO contains one arginine residue highly conserved throughout the entire group, which plays an extremely important role in biocatalysis. This residue has the ability to stabilize the peroxyflavin intermediate that is a highly oxidizing species necessary for the completion of the reaction (Scheme 3).

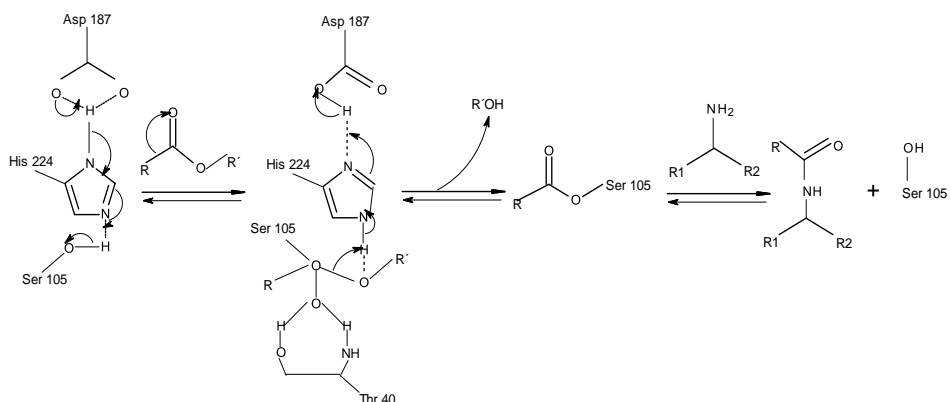
3.4. Perhydrolysis and Baeyer-Villiger oxidation

Generation of peracid by transfer of oxygen from hydrogen peroxide to a carboxylic acid or an ester followed by spontaneous transfer of the oxygen from the peracid to the ketone substrate has been used for Baeyer-Villiger oxidation reaction (**Papers III and IV**). The enzymes catalyzing perhydrolytic reaction belong to a subgroup of serine hydrolases with catalytic site containing the catalytic triad Ser-His-Asp (Yin D.L. et al., 2010). Perhydrolytic activity has been found in lipases (Björkling et al., 1990), aryl esterases (Yin D.L. et al., 2010), (Lee, 2010), xylan esterases (Park, 2011) and haloperoxidases (Picard et al., 1997).

Lipases (E.C. 3.1.1.3) belong to the family of α/β hydrolases (Brzozowski, 1991). The α/β hydrolase fold consists of a central hydrophobic eight stranded β -sheet packed between two layers of amphilic α -helices, providing a stable scaffold for the active site. Undoubtedly, one of the most studied enzymes is *Candida antarctica* lipase B (CaLB). The enzyme is a small protein of 317 amino acids and is known for its promiscuity as it is able to catalyze a number of reactions including aminolysis, alcoholysis, (trans)esterification, amidation

and perhydrolysis (Kapoor and Gupta, 2012). CaLB was used in **Paper III** to generate peracids through the perhydrolysis reaction.

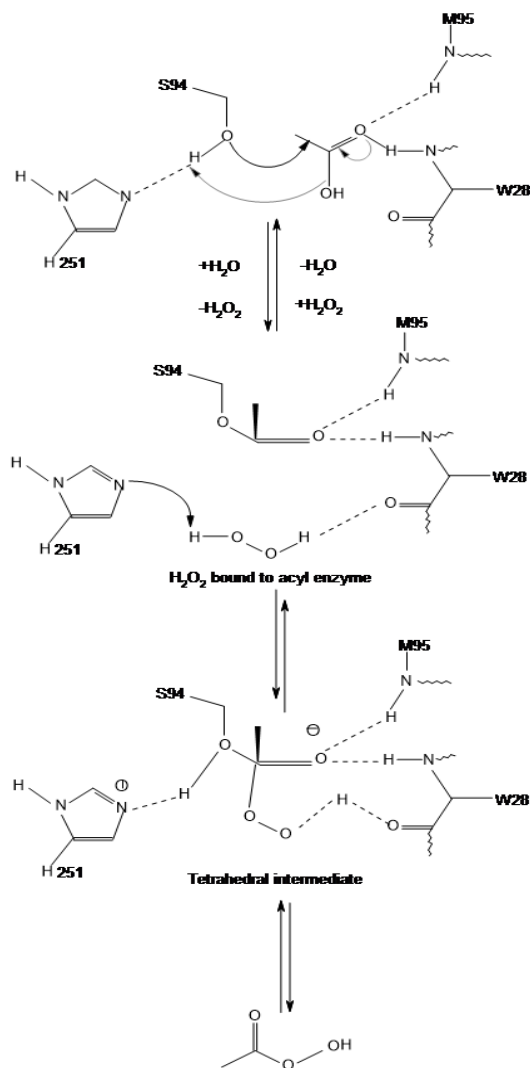
The reaction using lipase CaLB begins with the nucleophilic attack of the hydroxyl group of the Ser residue on an ester moiety, a tetrahedral intermediate is formed, which is assisted by the His and Asp groups. As a result, the negatively charged carbonyl oxygen is stabilized by hydrogen bonding with the peptide backbone NHS of the oxyanion hole. Next, the release of the R'OH group enables formation of the acyl enzyme intermediate. Finally, formation of the product and regeneration of the enzyme is achieved by reaction of the acyl intermediate with the nucleophile (Scheme 6). In case of hydrolysis the nucleophile is water, but in absence of water any nucleophile can react with the acyl intermediate, hence allowing a number of transformations (de Zoete, 1995; Faber, 1997).



Scheme 6 Mechanism of CaLB catalyzed acyl transfer within the active site (Kotowska-Miernowska, 2010).

In the reaction with carboxylic acid, the amount of peracid generated is thermodynamically controlled ($K_{eq} = 3$), while the reaction with an ester is kinetically controlled, hence a higher concentration of peracid can be generated (Yin, 2011).

In other esterases, perhydrolysis takes place most likely by a ping-pong bi-bi mechanism. The catalytic serine attacks the carbonyl carbon of the carboxylic acid and displaces water to form an acyl-enzyme intermediate. Then, hydrogen peroxide reacts with the acyl enzyme to form the peracid and to regenerate the catalyst. Hydrogen bonds from two main chain amides, called the oxyanion hole, activate the carbonyl group for attack and stabilize the tetrahedral intermediate (Yin D.L. et al., 2010) (Scheme 7).



Scheme 7 Proposed ping-pong bi-bi mechanism for perhydrolysis of acetic acid for an aryl esterase. The first diagram shows the enzyme-acetic acid complex. The γ -hydroxyl group of active site serine 94 attacks the carbonyl group of acetic acid to form a tetrahedral intermediate. Next, this intermediate collapses to form an acetyl-enzyme intermediate. Finally hydrogen peroxide binds to yield the complex shown in the second diagram. The N-H's of M95 and W28, called the oxyanion hole, donate hydrogen bonds to the carbonyl oxygen. Nucleophilic attack of hydrogen peroxide on the acyl enzyme forms a second tetrahedral intermediate (Yin D.L. et al., 2010).

3.5. Immobilized biocatalysts for Baeyer-Villiger oxidations

Immobilization of enzymes or whole cells is a strategy employed to allow recovery and recycling of the biocatalyst and also render it more stable (Hanefeld et al., 2009). The use of whole cells is preferred if the enzyme in an isolated form is inactive or unstable, or if there is need for cofactors and cofactor regeneration, or for multistep enzyme reactions. Immobilization is traditionally performed by binding the biocatalyst to an insoluble matrix by different mechanisms e.g. adsorption, covalent coupling, crosslinking and entrapment. For use in organic syntheses under non-aqueous conditions, the enzymes are invariably adsorbed to the matrix. Novozym®435 is one such commercial preparation of immobilized *Candida antarctica* lipase B that is the most reported biocatalyst in literature.

Other forms of immobilizations have also been developed such as coupling to soluble polymers and cross-linked enzyme aggregates (CLEA). CLEAs preparation involves the precipitation of the enzyme from aqueous buffer followed by cross-linking of the resulting physical aggregates of enzyme molecules, the enzyme does not need to be in a pure form as CLEAs preparation is somehow a combination of purification and immobilization (Sheldon et al., 2005) (Figure 3). One can even produce the immobilized enzyme in a single operation directly from a fermentation broth. Advantages of CLEAs over other immobilized preparations includes high catalyst productivities and low cost as avoids the use of carriers (Sheldon, 2011).

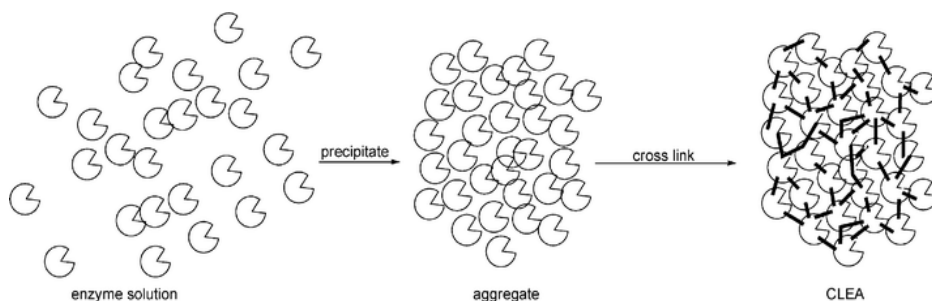


Figure 3 CLEA preparation

Preparation of CLEAs can also be done by performing the cross-linking in the presence of a monomer that undergoes polymerization under these conditions. This results in the formation of CLEA-polymer composites with tunable physical properties. For example, cross-linking in the presence of a siloxane

and subsequent polymerization can provide a CLEA-silica composite (Kim, 2007).

To date there is no monooxygenase immobilized as a pure enzyme. There is however one report of cyclohexanone monooxygenase immobilized along with a glucose-6-phosphate dehydrogenase immobilized on polyethyleneimine-glyoxyl-agarose showed to be an efficient system as the use of the second enzyme allows cofactor recycling (Atia, 2005). The poor stability, need of a cofactor, and the possible steric hindrances for the substrate and product with immobilized monooxygenases, led us to use alternative mode of Baeyer-Villiger oxidation. In this case immobilized forms of CaLB and a perhydrolase in the form of Cross Linked Enzyme Aggregates (CLEAs) were used in **Papers III** and **IV**. In **Paper III**, CALB-CLEAs were also compared with Novozym®435. Both the biocatalyst preparations exhibited similar activity with different acyl donors and cyclic ketones in the reaction. In **Paper IV**, the perhydrolase-CLEA could be used in an aqueous medium for generation of peracid using ethylene glycol diacetate and H_2O_2 . In both cases, generation of high concentrations of acetic acid once the oxygen has been transferred from the peracid to the substrate, led to deactivation of the biocatalyst with time.

4. Synthesis of ϵ -caprolactone

Lactones are cyclic esters with two or more carbon atoms and a single endocyclic oxygen coupled with an adjacent ketone. Lactones are widely used for production of biodegradable polymers by ring-opening polymerization. One of the most well known lactones is ϵ -caprolactone (CL) having a seven-membered ring with the formula $(\text{CH}_2)_5\text{CO}_2$. It is a colorless liquid miscible with most organic solvents. The major producers of CL are the European companies, BASF and Perstorp, Daicel in Japan, and several other companies around the world. CL is the largest commercially available lactone; other lactones with smaller ring sizes such as β -propio-, γ -butyro-, and δ -valerolactone, are also available and serve as building blocks for polyesters for different applications (Nobes et al., 1996).

The annual global consumption of CL is estimated at around 50 thousand tons (2008). The most well known applications of CL are its use as precursor for caprolactam which is used for producing synthetic nylon, and poly(ϵ -caprolactone) (PCL), a biocompatible and biodegradable aliphatic polyester that is bioresorbable and non-toxic for living organisms. PCL is easily biodegraded under physiological conditions (such as in the human body) and as a result, has received a great deal of attention as an implantable biomaterial with good mechanical properties. The polymer has been widely applied in medicine as artificial skin, artificial bone, and containers for sustained drug release.

Other important uses of caprolactone include components for urethane coatings, elastomers, solvent diluent for epoxy resins, synthetic fibers, organic synthesis (Ash and Ash, 2004). Therapeutic uses of caprolactone include their use as matrix for drug delivery systems (Pérez de la Ossa D et al., 2012) and bone regenerative therapy (Im et al., 2003).

Industrially caprolactone is produced by oxidation of cyclohexanone with peracetic acid at 50 °C and atmospheric pressure; the usual selectivities are 90% based on cyclohexanone and about 85-90% based on peracetic acid (; Wittcoff et al., 2004). Other oxidants used include m-chloroperoxybenzoic acid, hydrogen peroxide, peroxyacetic acid and trifluoroperoxyacetic acid (Krow, 1993), however they also oxidise other functional groups including alkenes, thioethers, and amines, which constrains synthetic strategies and

require protection and deprotection steps. Because common peroxyacids are achiral, they afford racemic esters. The desire for a safer Baeyer-Villiger oxidant with higher chemo- and enantioselectivity has stimulated a worldwide search for other methods. These methods include chemical reagents for racemic Baeyer Villiger oxidations such as magnesium monopero-phthalate (Mino et al., 1997), metal-mediated O₂/aldehyde reagents (Kaneda et al., 1995; Kaneda et al., 1994; Mino et al., 1997; Yamada et al., 1991), and a heterogeneous catalysis method that uses hydrogen peroxide (Corma et al., 2001). Metal-based oxidants that provide good and in some cases, excellent enantioselectivities have been introduced by Bolm (Bolm et al., 1997; Bolm et al., 1994) Strukul (Strukul, 1997), and Lopp (Lopp et al., 1996).

Although there is an important progress made in developing “chemical” oxidation methods, even the development of selective, environmentally benign methods based on molecular oxygen (monooxygenases) or alternatives that allow the production of oxidants *in situ*, avoiding handling of reactive peracids directly (chemo-enzymatic methods) is ongoing (**Papers III** and **IV**).

4.1 Whole cell synthesis of caprolactone

Due to the low stability of BVMO enzymes outside the cells and also due to the need for the cofactors, reactions catalyzed by the monooxygenases are often performed using whole microbial cells. However, wild type microorganisms are not used due to low level of activity and consumption of the product in subsequent reactions in the metabolic pathway (as seen in Scheme 2). Recombinant microorganisms are instead used for the synthesis of caprolactone. The preferred hosts for carrying the cyclohexanone monooxygenase gene are bakers yeast (Cheesman, 2001; Stewart et al., 1996) and *Escherichia coli* (Chen et al., 1999; Chen et al., 1988).

Attempts to produce caprolactone, have however not left the laboratory scale, as the use of living cells suffer from 3 main disadvantages: Oxygen supply, which is difficult to control once the cells reach high optical density, or the demand is too high to afford the survival and proper metabolism of the cells, product removal since the product (caprolactone) above a certain concentration inhibits the reaction, and operational stability of the biocatalyst since in large scale the cells would need to be refreshed or a feed maintained constantly to maintain the cells in a growing state. Moreover, the presence of caprolactone could be damaging for the cells (Law et al., 2006).

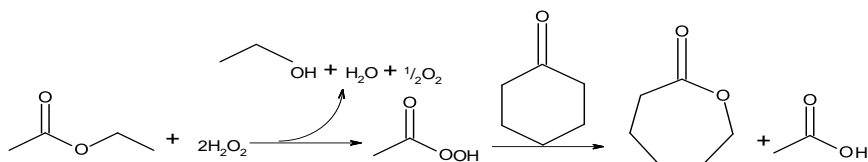
In comparison, the use of resting cells has been shown to be an efficient alternative to the use of living microorganisms; the yields obtained with this

strategy reach up to 88% (Walton and Stewart, 2002). Although the oxidation of methyl substituted cyclohexanones, was investigated using a recombinant *E. coli* expressing two monooxygenases from *Brevibacterium*, a modest yield of 60-65% was obtained (Mihovilovic et al., 2003).

4.2 Chemo-enzymatic synthesis of caprolactone

The first report on the use of a chemo-enzymatic system to produce lactones dates from 1995 (Lemoult et al., 1995); the oxidation of different cyclic ketones was performed using the lipase B from *Candida antarctica* (CaLB) as catalyst and myristic acid as acyl donor in toluene giving product yields of 20-73%. Later the reaction was tried without an acyl donor since the caprolactone product is hydrolysed further in the lipase catalyzed reaction to dihydroxyhexanoic acid and can serve as an acyl donor (Pchelka et al., 1998). However, the yields obtained in this system were quite modest and not higher than 55%. To avoid ring opening and to get better yields an approach using ethyl acetate as both acyl donor and solvent was developed (Rios et al., 2007); the product yield obtained on oxidation of cyclohexanone was 80%, however the reaction took 6 days.

To fill the gaps in the state-of-the-art regarding chemo-enzymatic production of caprolactone, ethyl acetate was used along with the CaLB immobilized as CLEAs and/or the commercial preparation Novozym®435, and hydrogen peroxide as oxidant for the oxidation of 0.5 mmol cyclohexanone (**Paper III**) (Scheme 8). The yield of caprolactone obtained was 69-70%, but the time required was reduced from 6 to 2 days by a slight increase in the temperature (Figure 4). Mass spectrometry analysis showed no ring opening of the product. But the formation of large amounts of acetic acid as by product was responsible for the loss of enzyme activity (Figure 5)



Scheme 8 Perhydrolysis reaction used in **Paper III** for the production of peracid using ethyl acetate as acyl donor and hydrogen peroxide as oxidant. This reaction was coupled to the chemical Baeyer-Villiger oxidation of cyclohexanone for the production of caprolactone.

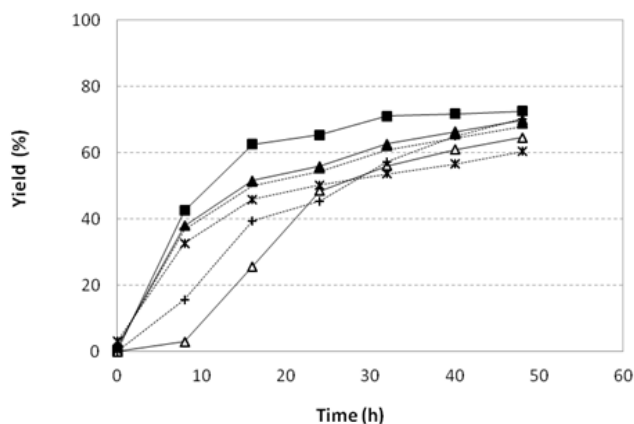


Figure 4 Time course of the production of ϵ -caprolactone by CaLB CLEA OM4 (dotted line) using 0.6 mmol (+), 1 mmol hydrogen peroxide (*) or 1 mmol urea-hydrogen peroxide (-), and by Novozym[®] 435 (line) with 0.6 mmol hydrogen peroxide (Δ) or 1 mmol hydrogen peroxide (\blacktriangle) and 1 mmol urea-hydrogen peroxide (\blacksquare), respectively. The reactions were carried out with 25 mg of enzyme, 0.5 mmol cyclohexanone and 911 μL or 866 μL of ethyl acetate for reactions using 0.6 and 1 mmol of oxidant respectively, incubated at 40 $^{\circ}\text{C}$, 400 rpm during 48 hours (**Paper III**).

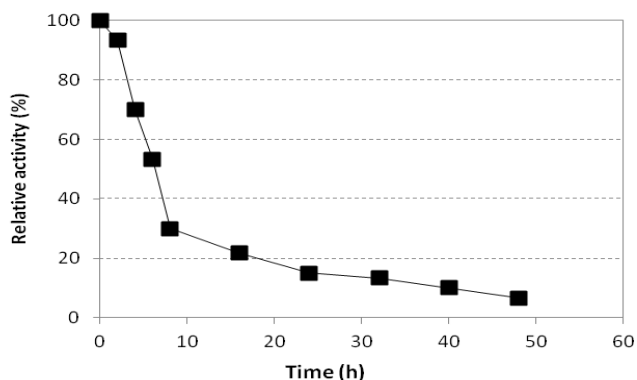
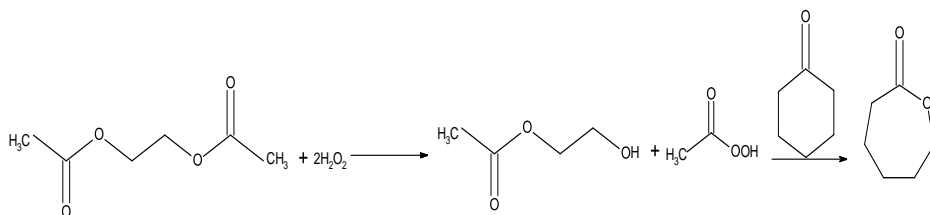


Figure 5 Relative activity of CaLB CLEA OM4 during the course of the reaction. Conditions: 0.5 mmol of cyclohexanone with 0.6 mmol of hydrogen peroxide using 25 mg of CaLB CLEA in ethyl acetate, incubated during 48 h at 40 °C and 400 rpm (**Paper III**).

In order to avoid the excessive generation of acetic acid, another perhydrolase enzyme immobilized as CLEAs was used with ethylene glycol diacetate as acyl donor. The added advantage of using this enzyme was that the reaction could be done in water, although to avoid drastic changes in pH a buffer system was used (Scheme 9) (**Paper IV**). In this system a 63% yield of caprolactone was reached in 72 hours. Further optimization is required to improve the yields.



Scheme 9 Perhydrolytic reactions for *in situ* generation of peracid and coupling to the chemical oxidation of cyclohexanone, using ethylene glycol diacetate as acyl donor (**Paper IV**).

5. Concluding remarks and future perspectives

Monooxygenases are the enzymes of choice for the Baeyer-Villiger oxidation of alkenes and so far only a limited number of such enzymes have been discovered. Screening for enzymes from the microbial diversity in the environment and also from the libraries of mutants generated in the laboratory, requires a simple, rapid and reliable assay. The screening method developed in **Paper I** could be used to detect monooxygenase activity by depletion in color of the Janovsky complex formed between a ketone and dinitrobenzoic acid. The method is applicable for most of the cyclic ketones, and showed to be more sensitive for smaller cyclic substrates such as cyclobutanone. It is cheap, reliable and fast method, especially applicable when sophisticated equipment is not available, and opens the opportunity to discover more strains with monooxygenase activity towards cycloketones.

Microorganisms that are able to consume and metabolise hydrocarbons are normally a rich source of oxygenases. The organisms belonging to the genus *Dietzia* are among such and several of them have been isolated from areas with oil spills. *Dietzia sp.* D5, was found to grow in media with different hydrocarbons and cyclohexanol. Recently, genome of the organism has been sequenced in our laboratory, which has revealed a large number of oxygenases (unpublished data). Since *Dietzia sp.* D5 grows slowly and reaches low cell densities, it becomes essential to clone and express the genes encoding the monooxygenases in heterologous microbial hosts that can grow easily in the laboratory. One of the *Dietzia sp.* D5 monooxygenases whose gene sequence was closely related to ethionamide (EthA) like BVMOs was expressed successfully in *E. coli* strain that could accept genes with high GC content and even rare codons. The recombinant whole cells or the crude cell lysate was able to oxidise a number of linear ketones; the best substrate for the enzyme was 2-nonanone. The enzyme did not however show detectable activity towards ethionamide. Attempts for purifying this enzyme were not successful as the enzyme was deactivated. More studies will be necessary in order to understand the structure of the *Dietzia sp.* BVMO, how it differs from other EthA like monooxygenases, and to get a more stable enzyme.

Although monooxygenases are able to carry out the Baeyer-Villiger oxidations of different substrates their low stability and the need for a cofactor makes difficult their use in industry. So other alternatives for carrying out the Baeyer-Villiger oxidation using *in situ* generated peracid, which are not selective like the BVMOs *In situ* production of peracids using enzymes with perhydrolytic activity makes possible the Baeyer-Villiger oxidation of alkenes. These reactions are environment-friendly alternative to the current chemical process used in industry, as they use green solvents as ethyl acetate or even water. As a model reaction the oxidation of cyclohexanone for the formation of ϵ -caprolactone was used, as this product is of high importance in industry. The yields obtained with this process varied between 62-80% depending on the enzyme and conditions used. This approach uses mild conditions, e.g. room temperature or 40 °C, and atmospheric pressure. The amount of acetic acid formed as by product however has detrimental consequences for the enzyme, limiting the recyclability. Current studies in our department are being carried out to remove acetic acid from the reaction system without affecting the amounts of substrate or product. A successful solution will be of great use especially in chemo enzymatic systems like the ones developed in this work, and will allow the improvement of the yields and process economy in large scale. A more expensive approach will be the genetic modification of enzymes in order to be able to tolerate acidic conditions without losing activity.

In a nearer future the use of chemo-enzymatic processes seems to be more realistic than the use of monooxygenases for the production of caprolactone. In a longer-term perspective, BVMOs and monooxygenases in general need to be studied further, and designed to become robust biocatalysts for the chemical and pharmaceutical industry.

Acknowledgements

The period of PhD studies has been a great adventure, a period of growth in many senses. I will certainly never forget this time of my life. Therefore, I would like to express my gratitude to some special people who, in one way or the other, made this thesis possible along with me.

First of all I would like to thank my supervisor Prof. Rajni Hatti-Kaul for allowing me to be a part of the DSP group, for sharing your scientific knowledge, ideas, suggesting experiments and correcting my manuscripts. For teaching me how to do research and pushing me to be a better scientist.

Prof. Bo Mattiasson, thanks for starting and keeping the collaboration with Bolivia in the ASDI-SAREC project and for believing in me in that first presentation in La Paz. I am very grateful for the opportunity you gave me to do my studies in Sweden.

Prof. Roger Sheldon, it has been an honor to work with you, thank you for accepting me in the Biotrains project. The experience I gained at CLEA Technologies B.V. is priceless.

My co-supervisor Dr. Gashaw Mamo for all your scientific advices, but mainly for transmitting me that confidence in the hard times. My co-authors, Javier Linares, I learnt a lot from you and about you. Dr. Michiel Janssen, thanks for making always time for answering my questions and solving my doubts. Dr. Jo Anne- Rasmussen, for all your enthusiasm and energy during the perhydrolase days. Serena for your spirit of collaboration, I know you have a great future in science.

Many thanks to Natalia and Paula for all the help with the printing matters. In fact, no one will be reading this document if both of them do not help me.

Thanks to the people from UMSA, the authorities that made it possible for me to come to Lund, the different directors that passed for DIPGIS and all the administrative staff.

To the IIFB for being my cradle in research. Thanks to Dr. Alberto Gimenez for opening the door of the institute for me, to Dr. Maria Teresa Alvarez for being my supervisor during my master years and my friend during my PhD studies. To Kike (R.I.P) for all the good advices, you are really missed.

I was very lucky to be part of the DSP group, being in such an international environment opened my eyes. I have shared with people from all around the world and some of you have really left a print on my heart. I would specially like to write some lines to my favorite ones: Marlene, for being my best friend, and my confidante, Thuy, I feel great respect and admiration for you, because you are one of the few ones I consider a “real doctor”, Rawanilla, for sharing the “history of your name” with me, along with many other things, the trips I had with you were epic in every sense, Victoriano da Silva for all the nice chats and advices, Deepti for all the fun that we had in our coffee breaks. Tarek and Roya for trying to keep the group united and organized.

Thanks to Siv Holmqvist and Frans Peder for all the help with administrative and computer matters and to my friends in other Biotechnology groups specially Marika, Maryam, Maria, Christina, Zulma, Marta, Lesedi, Kosin, you all created a very nice environment for working.

To my bolivian friends at KC, especially the ones who were with me when I lost my father. To Hector for being the first “cochala” that I really appreciated; you Cecilia were the second, and now the third one is Claudia, oh no!!! Neida and Karen for all the little-big favors during the last part of my stage.

Dhanyavaadaalu Ravi Kiran. You helped me so much with my computer and documents and never got paid for that. You were not officially my supervisor but you taught me many things. You do not appear as a co-author in my papers but you certainly did a lot in some manuscripts. You are not my mother but you took care of me when I was sick in the hospital. Please, just remember that Kazakhstan is the best country in the world. See you there.

To my non-scientific friends, because you all help me to remember I am still normal.

Big thanks to Michael Jackson and Ellen DeGeneres for being the best company when I was alone. Michael, hearing one of your songs changed my mood so radically that it would be unfair not to say thanks. Ellen, when I was really stressed I just watched your show and came back to the battlefield.

A mi familia, en especial gracias a mis primos Vanessa y Pablo por estar ahí todo este tiempo, es tan lindo saber que puedo contar con ustedes siempre en los buenos y malos momentos y para los favores chicos y grandes.

Mamá: Perdóname por todo este tiempo que te deje sola, aunque sabemos que mi papá se quedo siempre contigo, así que de alguna forma estabas en buenas manos. Gracias por todas las oraciones que has hecho por mí. Tú eres mi razón para volver a Bolivia. Por favor acompáñame mucho tiempo más. Por el momento me voy a olvidar de esto por un rato y te voy a compensar el tiempo perdido.

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Paper I



A method for rapid screening of ketone biotransformations: Detection of whole cell Baeyer–Villiger monooxygenase activity

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ARTICLE INFO

Article history:

Received 3 June 2011

Received in revised form 19 October 2011

Accepted 26 October 2011

Keywords:

Baeyer–Villiger monooxygenase

Screening assay

Cycloketone

Lactone

3,5-Dinitrobenzoic acid

ABSTRACT

A method for screening of ketone biotransformations was developed and applied to the identification of Baeyer–Villiger monooxygenase (BVMO) activity. The method was based on the formation of a purple coloured product on reaction between an enolizable ketone and 3,5-dinitrobenzoic acid in an alkaline solution. Absorbance of the colour decreased with the size of the cycloketone ring. Stoichiometric ratio between cycloketone and 3,5-dinitrobenzoic acid was 1:1 at maximum absorbance. The method was applied for monitoring the consumption of cyclohexanone by bacteria under aerobic conditions, and was found to be potentially useful for both screening assays and quantitative measurements of BVMO activity. Compared to other existing methods, this method is faster, cheaper and amenable for whole cell assays.

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1. Introduction

Reactions involving the insertion of oxygen atom into a ketone are known as Baeyer–Villiger reactions after the work of Baeyer and Villiger in 1888 [1], and are of enormous value in synthetic organic chemistry. Many chemical methodologies have been developed to carry out these reactions [2–4], but most of them are characterized by uncontrolled stereochemistry and result in a racemic product mixture. In contrast, the reactions catalysed by enzymes known as Baeyer–Villiger monooxygenases (BVMOs), often produce single stereoisomers [5] and exhibit a superior degree of enantio- and regioselectivity [6].

A number of BVMOs, acting on small cyclic ketones, larger cyclic ketones, aromatic ketone derivatives and steroids have been described, however the most well-studied among them is cyclohexanone monooxygenase [7]. Cyclohexanone is an intermediate product found both in aerobic and anaerobic degradation pathways of many cyclic compounds in a number of microorganisms [8,9] (Fig. 1). Cyclohexanone monooxygenase catalyses the insertion of oxygen into the alicyclic ring resulting in the formation of caprolactone.

The best-studied cyclohexanone monooxygenase is from *Acinetobacter calcoaceticus* NCIMB 9871 [10–14]. Transformation of more

than 100 different substrates by this bacterial monooxygenase have been reported over the years, and the enzyme is serving as a prototype BVMO to show that these enzymes are not only capable of performing Baeyer–Villiger oxidations but also can oxygenate sulfides, selenides, amines, phosphines, olefins, and iodide- and boron-containing compounds [15]. So far, cycloketone-converting monooxygenases have been reported from *Comamonas* sp., *Xanthobacter* sp., *Rhodococcus* sp. [16], *Nocardia* sp. [10], *Brevibacterium* sp. [17], *Arthrobacter* sp. [18] and *Pseudomonas* sp. [19]. As the application potential of these monooxygenases is enormous, there is a need for expanding the biocatalytic diversity and hence the search for monooxygenase producing microorganisms has continued.

The availability of a rapid, simple and straightforward method that is applicable to whole cell systems is critical for the success of a screening process. The current screening systems for BVMO activity include monitoring oxidation of the nicotinamide cofactor [20], detection of lactones by gas chromatography [21], measuring fluorescence of umbelliferone released from 2-coumariloxyl ketone substrates (made by alkylation of umbelliferone with chloroketones) [22], and determination of the products formed by a coupled reaction using an esterase that hydrolyzes the lactone, resulting in acids or diols. Acid formation is detected using pH indicators in a weakly buffered or non buffered system [23], whereas the diol formed is measured by reacting it with NaIO₄ and determining the unconsumed periodate by back-titration with adrenaline that leads to the formation of the chromophore adrenochrome [24]. However, most of these methods are time-consuming, expensive and/or

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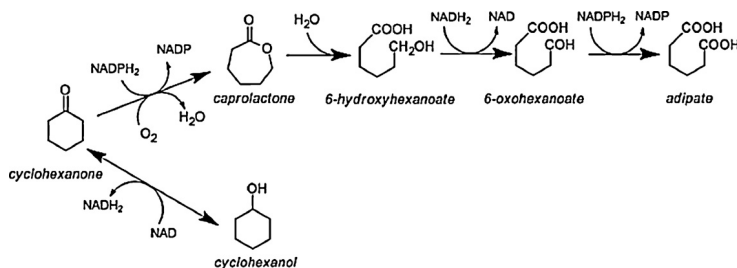


Fig. 1. Microbial oxidation and reduction of cyclohexanone. The product of the Baeyer–Villiger oxidation is caprolactone that is later converted to adipic acid, while the product of the reduction reaction is cyclohexanol.

are not readily suitable for screening BVMO activity directly in the whole cells.

In this work we have developed a fast assay for screening of BVMO activity in the microorganisms, based on the Janovsky reaction, i.e. the reaction of an enolizable ketone with polynitroaromatic compounds in alkaline solution leading to the formation of coloured complexes [25] (Fig. 2).

2. Materials and methods

2.1. Chemicals

3,5-Dinitrobenzoic acid (DNB), ϵ -caprolactone, δ -valerolactone, cyclobutanol, cyclopentanol, cyclohexanol, cyclobutanone, cyclopentanone, cycloheptanone, propanone, 2-butanone, 2-pentanone, 2-hexanone and 2-heptanone were purchased from Sigma–Aldrich (Taufkirchen, Germany), while cyclohexanone was procured from four different vendors (Acros, Sigma, VWR and AppliChem). Nutrient broth was from Difco and other chemicals of analytical quality were obtained from VWR (Darmstadt, Germany).

2.2. Bacterial strains

Escherichia coli BL21 (DE3) was purchased from Novagen (Darmstadt, Germany), *Methylobacterium extorquens* CCUG 2084 was from the Culture Collection of the University of Göteborg (CCUG, Göteborg, Sweden), *Xanthobacter flavus* DSM 338 and *Micrococcus luteus* DSM 20030 were procured from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany), while other strains used in this work were from the culture collection at Department of Biotechnology, Lund University.

2.3. Assay development

The reaction between cyclohexanone and DNB was performed as follows: to 1 ml solution of 20 mM cyclohexanone in 20 mM phosphate buffer, pH 6.8 was added 500 μ l of 50 mM freshly prepared DNB (in ethanol) and the solution was agitated for 3 min at 20 °C. Then 500 μ l of fresh 200 mM KOH (in ethanol) was added, and the

reaction mixture was left mixing on a rocking table at room temperature for about 15 min until the colour became stable. The reaction conditions were optimized using different concentrations of DNB (0–50 mM) and base (0.02–200 mM KOH) according to a factorial experimental design [24]. The reaction was also evaluated with other cyclic and linear ketones including cyclobutanone, cyclopentanone, cycloheptanone, propanone, 2-butanone, 2-pentanone, 2-hexanone and 2-heptanone, lactones such as ϵ -caprolactone and δ -valerolactone, and cyclic alcohols such as cyclohexanol, cyclopentanol and cyclobutanol.

2.4. Stoichiometry of the reaction

The molar ratio of cyclohexanone and DNB as well as cyclopentanone and DNB were determined by the continuous variation method (Job's method) [17]. The following stock solutions were prepared: 80 mM cyclohexanone, 50 mM cyclopentanone, 50 mM and 80 mM DNB respectively, and 200 mM KOH. Fourteen mixtures for each cycloketone were prepared by mixing 0–3 ml of the ketone stock solution with 3–0 ml of DNB (with the same concentration of the ketone) and 1 ml of KOH to a total volume of 4 ml. Absorbance spectra (400–800 nm) were then obtained for each reaction. The experiments were carried out in duplicates and the average curve was adjusted by polynomial regression. Finally, the maximum of the polynomial function was determined by the first derivative.

2.5. Standard curve for cyclohexanone determination using the colorimetric assay, GC and HPLC analyses

Different concentrations of cyclohexanone (from 4 different vendors) ranging from 0 to 50 mM were prepared in 20 mM phosphate buffer and reacted with DNB as described above. From the absorbance of the solution, a standard curve was plotted. The results were analyzed in terms of linearity range, reproducibility, detection, and quantification limits. Linearity is defined as the concentration interval with a response factor (concentration/absorbance) within $\pm 10\%$ of the average response factor for all samples analyzed. Reproducibility was determined as the variation coefficient of absorbance at three levels (5, 20 and 50 mM). Detection and quantification limits were calculated as the concentrations that gave signals 3- and 10-times the noise signal, respectively.

Analysis of cyclohexanone was also performed using gas chromatography (GC) and HPLC, respectively. For GC analysis, the instrument (Varian 430-GC, Varian, USA) equipped with FactorFour Capillary column, VF-1ms (Varian, 15 m \times 0.25 mm) and a flame ionization detector was used. The initial column oven temperature was increased from 50 to 250 °C at a rate of 20 °C/min. The samples, diluted with equal volume of ethyl acetate, were injected in a volume of 2 μ l at 275 °C. The retention time for cyclohexanone was 1.46 min.

HPLC analysis was done on the instrument (Jasco) equipped with an autosampler, a refractive index detector (ERC-7515A) and an oven (Shimadzu CTO-6A). Aminex HPLX-87H (Biorad) was used as the analytical column, and separation and quantification was performed by isocratic elution with 5 mM H₂SO₄ as mobile phase at a flow rate of 0.6 ml/min at 55 °C. The injection volume was 50 μ l. The retention time for cyclohexanone was 24 min. The calibration curves were made in the same range of cyclohexanone concentration as for the colorimetric assay (0–50 mM).

For comparison of the methods, the absorbance registered by the colorimetric assay was plotted versus the peak area obtained by GC and HPLC, respectively.

2.6. Whole cell assays

Different bacterial cultures were grown in nutrient broth at 30 °C for 1–5 days (depending on the growth rate) and the cells recovered after centrifugation were washed twice with 20 mM phosphate buffer pH 6.8 and finally re-suspended in the buffer to give an OD₆₀₀ of about 10. About 250 μ l of the cell suspension was added

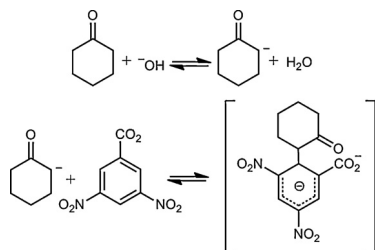


Fig. 2. Proposed reaction of cyclohexanone with dinitrobenzoic acid in an alkaline solution to give purple coloured sigma (Janovsky) complex.

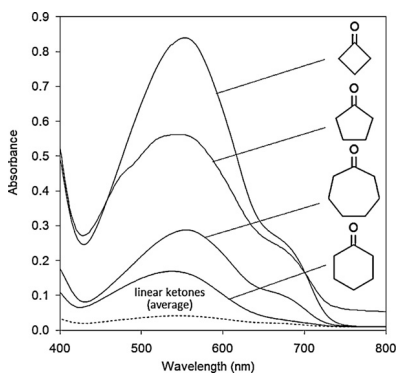


Fig. 3. Absorbance spectra for the products of different ketones (20 mM) reacted with 3,5-dinitrobenzoic acid (50 mM) in 200 mM KOH at 20 °C. The absorbance spectra of the linear ketones analyzed (2-propanone, 2-butanone, 2-pentanone, 2-hexanone and 2-heptanone) were very similar, and are hence shown in the figure as an average with dotted line.

to 750 μ l solution of the phosphate buffer containing 50 mM cyclohexanone. The solutions without any bacterial cells or heat-treated cells were used as blanks. To provide ample time for the cells to transform the cyclohexanone, the mixture was incubated for 12 h at 20 °C, after which 500 μ l of 50 mM DNB solution was added and incubated for 3 min prior to addition of 500 μ l of 200 mM KOH solution. The reaction was continued for 15 min at the same temperature until the colour formation was stable. A decrease in the intensity of the purple colour observed visually is attributed to the consumption of cyclohexanone, which in turn is a qualitative indication of the presence of cyclohexanone monooxygenase activity. After removing the cells from the solution by centrifugation or filtration, the change in the colour intensity of the solution was measured spectrophotometrically at 550 nm.

For quantitatively following the cyclohexanone consumption by different bacteria, fresh cell pellet obtained from 5 ml bacterial suspension was washed twice with 20 mM phosphate buffer pH 6.8 and re-suspended in the buffer containing 50 mM cyclohexanone to an OD₆₀₀ of 2.5. The suspension was incubated at 20 °C, and 1 ml samples were withdrawn every 3 h and analyzed with DNB as described above. The absorbance at 550 nm was used to calculate the concentration of cyclohexanone using standard curve prepared with known concentrations of cyclohexanone. The dry weight of the cells was measured both at the start and the end of the incubation period by centrifuging the suspension at 2500 \times g for 5 min and drying the cell pellet at 80 °C for 12 h. Any loss in dry weight was assumed to be due to cell lysis.

3. Results and discussion

3.1. Reaction

The reaction between cycloketones (cyclobutanone, cyclopentanone, cyclohexanone and cycloheptanone) and DNB in a basic medium resulted in purple coloured products with maximum absorbance at 550 nm while the reactions between linear ketones and DNB gave a much lower intensity of coloured products (Fig. 3). Absorbance spectra of the reaction mixtures showed a considerable bathochromic change in the maximum absorbance of DNB to the visible electromagnetic region (from 430 nm to 520–550 nm). The formation of purple coloured products has been reported earlier when enolizable ketones react with nitroaromatic compounds in alkaline medium – well known as “Janovsky reaction” [25]. Any ketone with a methylene group at a position α with respect to the carbonyl forms a carbanion under alkaline conditions [26]. The coloured species is suggested to be an anionic σ -complex (Janovsky complex) formed by the attack of the conjugate base of ketone on the 4-position of 1,3-dinitrobenzene and its derivatives [26–28] (Fig. 2). A similar reaction between picric acid and creatinine or cyclohexanone, called as Jaffé reaction, has been studied earlier and

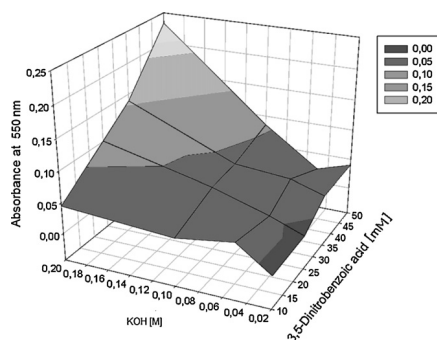


Fig. 4. Surface response of reaction of 50 mM cyclohexanone with 10–50 mM 3,5-dinitrobenzoic acid and 20–200 mM KOH.

the coloured product formed is suggested to be a Janovsky complex [26].

As seen in Fig. 3, the absorbance of the product appears to be dependent on the ring size of the substrate, the highest value being obtained for cyclobutanone and the lowest for cyclohexanone and cycloheptanone. This behavior may be due to steric hindrance of the more flexible, larger cycloketones to form a complex with DNB. The reaction was also tested with saturated lactones such as ϵ -caprolactone and δ -valerolactone (which are the oxidation products of cyclohexanone and cyclopentanone, respectively) at concentrations up to 1 M, and 50 mM cyclic alcohols e.g. cyclohexanol, cyclopentanol and cyclobutanol (which are the reduction products of the corresponding cycloketones). These compounds were unable to form detectable coloured-complexes. On the other hand, the α,β -unsaturated lactones, as represented by cardiac glycosides, cardenolides and other secondary metabolites of animals and plants are known to give coloured product with DNB through Kedde reaction [30–32]. Hence, from an analytical perspective, the assay is suitable for monitoring the depletion of cycloketones as in reactions catalysed by Baeyer–Villiger monooxygenases or alcohol dehydrogenases.

The concentrations of both DNB and KOH were optimized for reaction with cyclohexanone using a factorial design, and as shown in Fig. 4 the maximum absorbance was obtained when 50 mM DNB and 200 mM KOH were used in the assay. It was not possible to test the effect of higher concentration (>200 mM) of KOH due to limited solubility of the alkali in 95% ethanol. Additionally, the stoichiometric ratio between DNB and cycloketones (cyclopentanone and cyclohexanone) in the reaction were analyzed by Job's method of continuous variation [29]. In both cases, the Job's plot reached a maximum value at a molar fraction of 0.5 (shown for cyclohexanone in Fig. 5), which confirmed that the molar ratio between the components in the complex is 1:1.

3.2. Quantitative assay and validation

The assay was further tested under optimal conditions with different concentrations of cyclohexanone from four different vendors. Cyclohexanone was used in spite of the lower absorbance of its complex with DNB as compared to cyclobutanone and cyclopentanone because it is the most commonly used cycloketone used with respect to the Baeyer–Villiger reaction. A variance analysis was done giving no significant difference between the cyclohexanone stocks and a standard curve was plotted, which showed

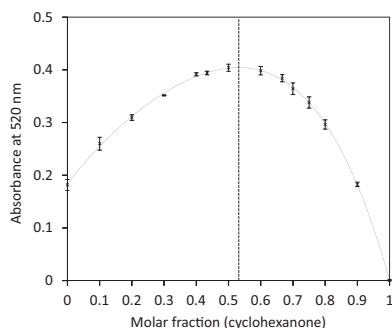


Fig. 5. Continuous variation plots for absorbance at 520 nm of the coloured product formed in the reaction between cyclohexanone and 3,5-dinitrobenzoic acid. The adjusted polynomial curve is shown. The equation of the curve is $-0.956x^4 + 0.915x^3 - 0.943x^2 + 0.802x + 0.183$ with a correlation, $R^2 = 0.999$.

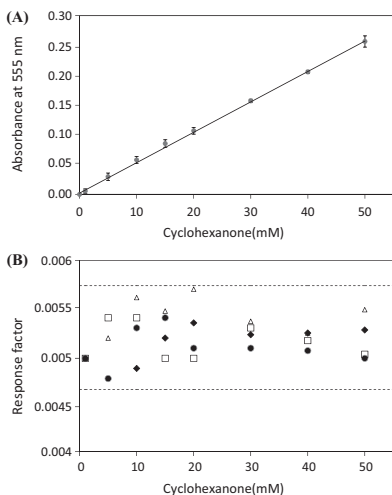


Fig. 6. Linearity of the assay: (A) standard curve ($y = 0.0052x + 0.0021$, $R^2 = 0.999$) and (B) linearity of the response factor using cyclohexanone from different vendors: (●) Acros, (□) Sigma, (▲) VWR and (△) AppliChem as analyte in the assay developed with 1% (w/v) 3,5-dinitrobenzoic acid, and 200 mM KOH at 20 °C. The linearity is within $\pm 10\%$ limits of the average response factor for all samples analyzed.

a linearity range of 0–50 mM cyclohexanone (Fig. 6). Detection and quantification limits were determined to be 0.5 and 5 mM, respectively. Reproducibility of the assay was acceptable at all concentrations, with variations coefficients of $<5\%$ (Table 1).

Table 1

Reproducibility of the colorimetric assay, expressed as the variation coefficients from six determinations of each sample, for three concentrations of cyclohexanone.

Cyclohexanone	Average of absorbance	Variation coefficient (%)
20 mM	0.129	1.79
50 mM	0.334	2.48

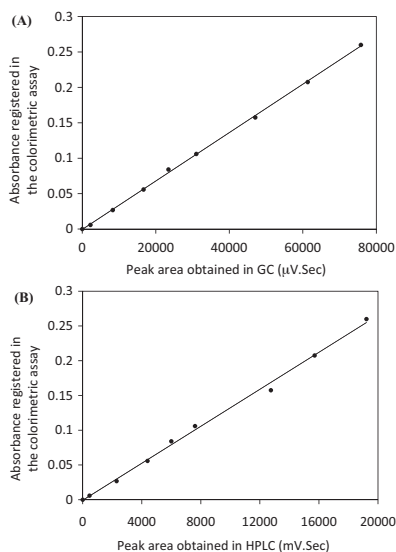


Fig. 7. Correlation curves between the absorbance registered in the colorimetric assay for cyclohexanone versus peak areas obtained in (A) GC ($y = 3 \times 10^{-5}x - 0.0006$, $R^2 = 0.999$) and (B) HPLC (Eq. $y = 1 \times 10^{-5}x - 0.0008$, $R^2 = 0.999$) analyses, respectively.

Linearity of the assay was very similar to that of GC as well as HPLC analysis, as seen by the linear correlation of the plot of the absorbance versus the GC/HPLC peak areas (coefficient of 0.999 and 0.997, respectively) (Fig. 7). The limits of detection and quantification for the colorimetric assay were very similar to that of the HPLC method, however the GC is more sensitive with the quantification limit lower than 0.1 mM (Table 2).

3.3. Whole cell assays for Baeyer–Villiger monooxygenase activity

Different bacteria from our culture collection and from standard sources were screened for the consumption of cyclohexanone (Table 2 and Fig. 8). The bacterial pellets were incubated overnight with 50 mM cyclohexanone after which the residual cyclic ketone was evaluated qualitatively by reacting with 3,5-dinitrobenzoic acid. *Dietzia* sp. D5, a bacterial isolate from a soda lake sample, showed a major depletion of cyclohexanone, reflected in the least colour development. The boiled cell suspension of *Dietzia* sp. D5

Table 2

Qualitative analysis of bacterial monooxygenase activity based on depletion of cyclohexanone; (+++) represent high activity, (++) medium activity, (+) low activity, and (–) no detectable activity, respectively.

Bacteria	Cyclohexanone consumption
<i>Bacillus cereus</i>	–
<i>Brevibacterium</i> sp.	+
<i>Dietzia</i> sp.	+++
<i>Escherichia coli</i> BL21 (DE3)	–
<i>Halomonas</i> sp.	++
<i>Methylobacterium extorquens</i> CCUG 2084	++
<i>Micrococcus lysodeikticus</i>	+
<i>Xanthobacter flavus</i>	+

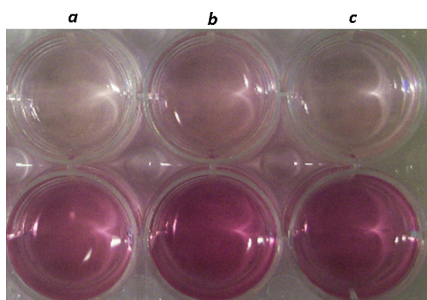


Fig. 8. Qualitative assay of cyclohexanone monooxygenase activity after 12 h of cyclohexanone addition using (a) *Dietzia* sp. D5, (b) *Brevibacterium* sp., (c) *Methylobacterium extorquens*, (d) *Xanthobacter flavus*, (e) *E. coli* BL21 (DE3), and (f) blank.

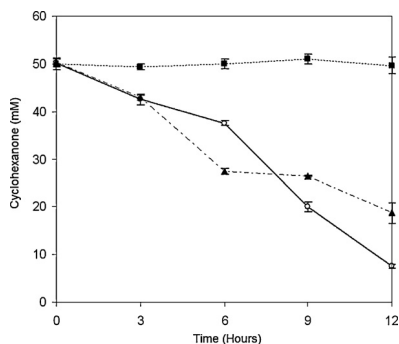


Fig. 9. Cyclohexanone consumption by *Dietzia* sp. D5 (○), *Brevibacterium* sp. Strain 5 (▲) and *E. coli* BL21 (DE3) (■) at pH 6.8 and 20 °C.

(OD=2.5) was tested under similar conditions and <5% depletion of cyclohexanone was noted, which could be attributed to the lysis of cells on heating and release of denatured macromolecules (proteins) with exposed hydrophobic moieties that could bind cyclohexanone by hydrophobic interactions.

As seen in Table 2, most of the other organisms showed a marginal reduction in the colour intensity of the complex. *Bacillus cereus* and *E. coli* BL21 (DE3) strains did not show any colour change and were similar to blank samples (cyclohexanone solution in buffer without bacteria). There is in fact no report so far on consumption of cyclohexanone or production of cyclohexanone monooxygenase by *B. cereus* and *E. coli* BL21 (DE3).

Based on the results shown in Table 2, *Brevibacterium* sp. Strain 5, *Dietzia* sp. D5 and *E. coli* BL21 (DE3) were selected for the quantitative assay of cyclohexanone oxidation, where the latter two organisms served as positive and negative controls, respectively. Aliquots of the reaction mixtures were taken at different time intervals and assayed for measuring the cyclohexanone content using the DNB assay. Fig. 9 shows that there is a significant decrease in the cyclohexanone concentration over a period of 12 h with *Brevibacterium* sp. and *Dietzia* sp., while no change was observed in case of *E. coli* BL21 (DE3). The cell dry weight measurements showed the

biomass to be constant during the whole process, indicating that no cell lysis occurred during the 12 h duration of the reaction.

4. Conclusion

Reaction between a cyclic ketone and 3,5-dinitrobenzoic acid was used to develop a method for screening of BVMO activity. The method was found to be useful for both qualitative screening assays and quantitative monitoring of cyclohexanone consumption by microbial cells. It may also be appropriate for detecting other BVMOs such as cyclopentanone monooxygenases. This method has a number of advantages over the currently used ones – it is simple and rapid, and can be used as an initial screen for activity detection directly in whole cells, and is also readily amenable for high-throughput screening of mutant and metagenome libraries of BVMOs. Since the method is based on the consumption of cycloketones, it may also be used for screening of enzymes (alcohol dehydrogenases) catalysing their reduction. The enzymes can subsequently be characterized by another technique for monitoring the product formed and determining the enantioselectivity.

Acknowledgements

This work was financially supported by the Research Department of the Swedish International Development Cooperation Agency (SIDA-SAREC) and the Swedish Foundation for Strategic Environmental Research (Mistra).

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Paper II

Cloning, expression and characterization of a Baeyer-Villiger monooxygenase from *Dietzia* sp. D5

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Abstract

A novel Baeyer-Villiger monooxygenase from *Dietzia* sp. D5 has been cloned and functionally expressed. Sequence similarity search revealed that the enzyme belongs to a group of BVMOs that are closely related to ethionamide monooxygenase. Better expression of the soluble enzyme was achieved using *E. coli* CodonPlus(DE3)-RP and ArcticExpress(DE3)-RP. The best expression of the enzyme has been obtained when the gene encoding the enzyme was expressed in *E. coli* CodonPlus(DE3)-RP, induced with 0.1 mM of IPTG, at 15 °C and using TB medium. Although crude enzyme is active, the purified enzyme did not show any measurable activity. Thus, the substrate scope of the enzyme has been determined using whole-cell and crude extract systems. The enzyme was most active towards aliphatic acyclic substrates. However, it has shown good degree of conversion of cyclobutanone, 2-methylcyclohexanone, Bicyclo[3.2.0]hept-2-en-6-one, phenylacetone and thioanisole. There was no measurable conversion when ethionamide, cyclohexanone and acetophenone were used as substrates.

Keywords: *Dietzia*, Baeyer-Villiger monooxygenase, aliphatic ketones, ethionamide

Introduction

Baeyer-Villiger monooxygenases catalyze the oxidation of ketones and heteroatoms (i.e. sulphur, nitrogen, phosphorous, boron and selenium) to lactones and oxides, respectively. Three types of BVMOs have been described so far of which Type I BVMOs that use FAD as a prosthetic group and are dependent on NADPH, are the most studied (E. Torres Pazmiño and W. Fraaije 2007). Based on the sequence similarity, Type I BVMOs have been grouped into clusters (Bisagni, Linares-Pastèn et al. 2012). One of these BVMO groups is represented by an enzyme known as ethionamide monooxygenase (EthA) which is isolated from *Mycobacterium tuberculosis* and identified to be responsible for the activation of the pro-drug ethionamide into the bio-active sulfoxide intermediate (Vannelli, Dykman et al. 2002; Fraaije, Kamerbeek et al. 2004). Apart from ethionamide, the enzyme is active towards aliphatic ketones such as 2-octanone and 2-decanone and ketones containing an aromatic group like phenylacetone (Fraaije, Kamerbeek et al. 2004). *M. tuberculosis* has two more EthA monooxygenase like enzymes. These two BVMOs are known to catalyze the oxidation of thioanisole to the corresponding sulfoxide and the oxidation of 2-octanone and bicyclohept-2-en-6-one (Bonsor, Butz et al. 2006). Among the 23 BVMOs from *Rhodococcus jostii* RHA1 only two of them, MO13 and MO16 share significant similarity with EthA monooxygenase. MO16 oxidizes different substrates (cyclobutanone, 2-methylcyclopentanone, bicyclohept-2-en-6-one and 2-octanone) but the substrate scope of MO13 is not reported because of its poor expression. A BVMO from *Pseudomonas putida* KT2440 is the other enzyme that shows sequence similarity to EthA monooxygenase and reveals highest activity with 4-decanone as the substrate (Rehendorf, Kirschner et al. 2007).

Recently, we have sequenced the genome of a *Dietzia* strain which is found to be rich in different oxygenases. Four BVMOs have been identified from the draft genome sequence and BVMO3 is the only BVMO that is closely related to EthA monooxygenase (Bisagni et al. 2013). In this paper, we report the cloning, expression and substrate scope of this BVMO.

Materials and methods

Organisms and Plasmid

E. coli CodonPlus(DE3)-RP and ArcticExpress(DE3)-RP were purchased from Agilent Technologies (Santa Clara, USA). *E. coli* NovaBlue and the plasmid pET-22(b)+ were purchased from Novagen (Darmstadt, Germany). QIAGEN Plasmid Mini Kit and QIAEX II Gel Extraction Kit (Qiagen, Sollentuna, Sweden) were used to extract plasmids from cells and DNA from agarose gel, respectively. Genomic DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, USA).

Chemicals for biocatalysis studies

All chemicals used in the study are of the highest available purity obtained from standard sources.

Gene Cloning

The gene encoding BVMO3 was amplified from the genome of *Dietzia* sp. D5 using a pair of primers, BVMO3-F: ATTACCATGGCTGGTAGCACCCACCTC and BVMO3-R: ATTACTCGAGTGATCGGGCCACCTCGTC, which were designed based on the identified gene sequence in the draft genome sequence of *Dietzia* sp. D5. The forward and reverse primers had *Nco*I and *Xho*I (underlined and italic) restriction sites, respectively. A High Fidelity PCR enzyme mix (Fermentas) was used to amplify the gene following the manufacturer instructions. DMSO (5%) was added to the PCR mix to improve the amplification. After cleaning with Qiagen PCR cleaning kit, the PCR product was digested with *Nco*I and *Xho*I and ligated to the expression vector pET-22b(+) digested with the same restriction enzymes. The ligation product was transformed into competent *E. coli* NovaBlue cells and spread on LB agar plates containing ampicillin. Colonies were picked from the agar plates and recombinant plasmids were extracted and sequenced. The plasmid containing the correct sequence was transformed to expression host *E. coli* CodonPlus(DE3)-RP or ArcticExpress(DE3)-RP.

Protein expression and purification

The recombinant *E. coli* cells were cultivated using low salt Luria Bertani medium (LB), terrific broth (TB, composed of tryptone 12g, yeast extract 24g, glycerol 4ml, filtered solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 100 ml per liter) and M9 medium (1 M MgSO_4 2ml, 20% glucose 20 ml, 1 M CaCl_2 0.1ml, M9 salts 200 ml per liter). The M9 salts comprised 164 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 15 g KH_2PO_4 , 2.5 g NaCl and 5 g NH_4Cl in water to a final volume of one liter.

Cultivation of *E. coli* for the recombinant protein expression was initiated by inoculating the culture media with a pre-inocula corresponding to 5% of the final culture volume. After three hours of cultivation at 30 °C, protein expression was induced by addition of appropriate concentration of IPTG or lactose. During the expression phase the cells were incubated at 15 °C for 16 h with shaking at 150 rpm. Cells were spun down by centrifuging for 10 minutes at 8000 rpm in Sorvall RC5C centrifuge at 4 °C and then resuspended in 100 mM potassium phosphate buffer pH 7.5 containing 10% glycerol. Then cell suspension was placed on ice and sonicated in 3 cycles of 45 sec (Hierscher UP400S Ultrasonicator; amplitude 50%, cycle 0.5) with 1 min break between the cycles. The sonicated cells were centrifuged for 15 minutes at 10 000 rpm in Sorvall RC5C centrifuge at 4 °C to remove cell debris. The clarified lysate was used as the enzyme source.

The BVMO was purified from the above solution by immobilized metal ion affinity chromatography on a Cu^{2+} bound column and eluted with imidazole

The activity of the recombinant enzyme was followed by conversion of 10 mM 2-nonanone and measuring the product by gas chromatography analysis.

Biotransformation

Whole-cell biotransformations were performed using both growing and resting cells. For growing cells, a flask with TB medium was inoculated with a pre-culture of *E. coli* CodonPlus(DE3)-RP carrying the plasmid for the expression of the recombinant BVMO. The cells were grown for 3 hours at 30 °C and 150 rpm in a shaker incubator. Immediately after adding IPTG (final concentration 100 μM) to induce the expression of the recombinant enzyme, the biotransformation reaction was initiated by adding the substrate to a final concentration of 5 mM. The biotransformation reaction was done by cultivating the cells with shaking at 150 rpm for 16 h at 15 °C. The

biotransformation activity was measured by determining the product formed using gas chromatography.

For resting cell biotransformation study the cells were grown as described above. After overnight induction with IPTG, the cells were harvested by centrifugation and resuspended in an equal volume of 20 mM sodium phosphate buffer pH 7.4 containing 5 mM substrate and 25 mM glucose and incubated further for 16 h at 15 °C.

Biocatalysis with crude extract

The soluble cell extract was prepared as described above. To this solution was added 25 mM glucose, 0.4 U of glucose dehydrogenase (GDH), 133 μ M NADP⁺ and 25 mM substrate from a 1 M stock solution in ethanol. The reaction mixture was incubated with shaking at 150 rpm for 24 hours at 15 °C. Product formation was followed using gas chromatography as described below.

GC analysis

A sample of the biotransformation reaction (400 μ l) was extracted twice with ethyl acetate (500 μ l) and analysed using Varian 430-GC gas chromatograph (Agilent Technologies, Santa Clara, USA). The column used for analyte separation was FactorFour VF-1ms 15 m x 0.25 mm (0.25) column (Varian, Strathaven, UK) with 1 min hold at 50°C; heating ramp from 50°C to 225°C at a rate of 25°C/min; and 2 min hold at 225°C (the injector and the detector temperature was maintained at 275°C).

Results

Sequence analysis

The gene encoding for BVMO3 is located between dehydrogenase and transcription regulator as shown in Fig. 1. Part of the gene encoding the mature polypeptide was identified and primers were designed for PCR amplification. The translated sequence was used for database searching. Although the similarity was less than 43%, the sequence similarity search for

the deduced BVMO3 amino acid sequence has shown that the BVMO belongs to a group of BVMOs related to EthA monooxygenase. Based on the alignment of these related BVMO sequences a phylogenetic tree was constructed (Fig.2), which shows that the enzyme is branched out from the rest of the related BVMOs that have been functionally characterized. The secondary structure of the enzyme shown in Fig. 3 contains several beta strands and helices connected by loops and is comparable to what has been reported for other BVMOs. The alignment study revealed that some residues that are highly conserved in all the other BVMOs except BVMO3 (Fig. 4).

Cloning, expression and purification

The gene encoding the BVMO could not be expressed in the common *E.coli* expression strains such as BL21 (DE3). This is mainly due to its high GC content (>70%) and the presence of several rare codons. Thus, *E. coli* strains designed to overcome codon bias and high GC content expression problems were chosen to express BVMO3. *E. coli* CodonPlus(DE3)-RP and ArcticExpress(DE3)-RP were tested and resulted in functional expression of BVMO3. The ArcticExpress system in addition to having rare tRNA plasmid also carries cold active chaperon and hence needs induction to be done at 10-12 °C, which led to reduced growth and concomitant low expression of the soluble protein. Therefore, *E. coli* CodonPlus(DE3)-RP which gave a better expression profile was chosen as production host.

Optimization of some induction parameters including induction temperature, inducer type and concentration, and cultivation media, has been tried to increase the yield of soluble and active BVMO3. The highest amount of soluble enzyme has been obtained when the enzyme was expressed in *E. coli* CodonPlus(DE3)-RP, induced with 0.1 mM IPTG, at 15 °C and in TB medium. These conditions were used in all the following experiments.

Initially the cells were resuspended and lysed in 50 mM sodium phosphate buffer pH 7.5 but the enzyme activity was completely lost. It has been suggested that this type of BVMOs could be membrane associated *in vivo* (Britton and Markovetz 1977, Fraijee et al 2004), hence different additives such as glycerol and Triton X-100 were included in the resuspension buffer. As shown in Fig.4, the addition of glycerol resulted in a slight improvement in the amount of soluble protein. On the other hand, when the enzymatic activity was measured, all the soluble extracts converted 10 mM 2-nonanone

completely. Therefore, potassium phosphate buffer containing 10% glycerol was chosen as the cell lysis medium for further experiments. The recombinant was purified from the active crude extract using metal ion affinity chromatography, but the purified enzyme did not show any activity.

Whole-cell biotransformation

A range of substrates containing different chemical functions (ketones with aliphatic, alicyclic, aromatic moieties and sulphides) that are known to be transformed by BVMO were used as substrates (Table 1). The best conversion was achieved for the linear aliphatic ketones, with the highest degree of conversion measured for 2-nonanone and 3-decanone. Interestingly, when the ketone is on the second carbon there is only one oxidation product but when the carbon is in position three two products are revealed by gas chromatography. The ratios for among the two products vary for the 3-keto substrates (Table 1).

Biotransformation using resting cells and soluble cell extract

Bioconversion of aliphatic ketones was also investigated using resting cells expressing the BVMO and soluble cell extract (Table 1). Resting cells were prepared by resuspension in sodium phosphate buffer of *E. coli* cells after the overnight expression of BVMO3. This system led to poor substrate conversion. The reactions with crude extract exhibited complete conversion of the 5 mM substrate rapidly and hence a higher substrate concentration (25 mM) was used in the conversion studies. The cofactor NADPH was enzymatically regenerated by glucose dehydrogenase and the co-substrate glucose was added in stoichiometric amount to the substrate. As shown in Table 1, high conversion has been achieved for most of the substrates tested.

Discussion

This work presents the cloning, expression and characterization of a Baeyer-Villiger monooxygenase from a strain of *Dietzia*. Despite the similarity in structure (and cofactor/electron donor dependence is common amongst Type I BVMOs), there are sequence features that justify further division of Type I Baeyer-Villiger monooxygenases. Sequence analysis of this monooxygenase

revealed that this enzyme falls into a group of monooxygenases that contain EthA monooxygenase and two other BVMOs from *Mycobacterium tuberculosis* H37Rv (Fraaije, Kamerbeek et al. 2004; Bonsor, Butz et al. 2006), BVMO from *Pseudomonas putida* KT2440 (Rehdorf, Kirschner et al. 2007) and two BVMOs from *Rhodococcus jostii* RHA1 (Szolkowy, Eltis et al. 2009). The EthA monooxygenase has been interesting because of its activity for converting the pro-drug ethionamide to a biologically active form. Mutations of this gene and its regulator EthR trigger resistance mechanisms towards this drug (Brossier, Veziris et al. 2011).

Dietzia sp. D5 BVMO3 gene is 1556 nucleotides long. Similar to EthA monooxygenase, close to the BVMO3 gene there is a transcriptional regulator coding sequence in the opposite strand (Fig. 1), which is analogous to ethR. However, unlike the EthA encoding gene, BVMO3 is followed by a dehydrogenase as in the case of *R. jostii* (RHA1_ro02919) BVMO encoding gene. The sequence of BVMO3 differs at sites that are highly conserved in other EthA monooxygenase like BVMOs, which suggests that this enzyme is novel from sequence point of view. However, since there is no determined 3D structure for any of these BVMOs and due to significant sequence difference between these EthA monooxygenase like BVMOs and other BVMOs for which there are determined structures, it is not easy to make structural analysis and predict the possible role of the observed sequence differences.

Although members of this BVMO group are expected to have a similar substrate scope, the characterization study results do not reflect this expectation. BVMO3 exhibited strong preference towards linear aliphatic ketones such as 2-nonanone and 3-decanone, which is comparable to that of *P. putida* BVMO and EthA monooxygenase of *M. tuberculosis* but not with the other enzymes of the group. The fact that some of the other enzymes did not convert this type of substrates might be related to their poor expression such as the *Rhodococcus* BVMOs or it could be due to intrinsic properties differences as can be seen in the case of the other two BVMOs from *Mycobacterium* which are reported to oxidize sulphides but not 2-octanone. A comparison of the conversion yields with BVMO previously characterized is shown in Table 2. The most interesting feature is that BVMO3 cannot oxidize ethionamide but oxidizes thioanisole. Among the linear substrates, the conversion increases with increase in chain length.

In whole- cell biotransformation, higher conversion yields were obtained when growing cells were used. Although the use of resting cells is time consuming it has been reported that biotransformation with the resting cells instead of growing cells results in higher conversion (Redhorf et al., 2007). However, the results in this study show that the conversion achieved by the resting cells was lower. This can be partly due to the difference in the *E.coli* strains used in the

studies. Moreover, the extra long incubation after induction might have affected the metabolic efficiency of the cells.

In case of the crude extract, the amount of substrate used was five-times higher compared to that with the whole cells. wherein the cell growth was negatively affected by substrate concentration higher than 5 mM. Using the crude extract, 5 mM linear ketones were completely converted within a short time due to easy access of the substrate to the enzyme since there is no need for its transportation across the cell envelope. Besides the aliphatic ketones, 77% conversion of thioanisole was also achieved by the crude extract; the low conversion of 32% with whole cells could be due to the toxicity of thioanisole to the cells. In general, the trend of conversion of aliphatic ketones by whole cells or soluble extract was quite comparable, although some differences are clearly visible, both in the degree of conversion and in the nature of the products. Further studies are needed to characterize the systems in detail.

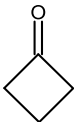
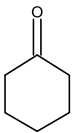
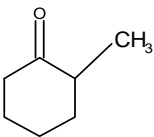
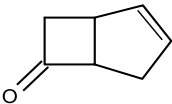
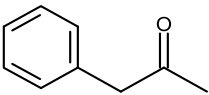
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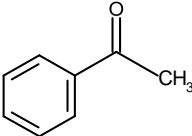
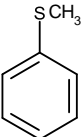
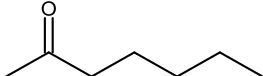
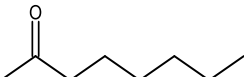
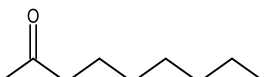
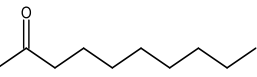
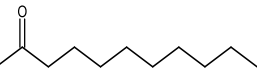
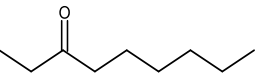
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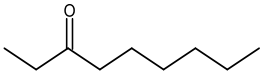
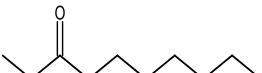
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Table 1 Substrate conversion by whole-cell and cell extract systems.

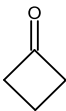
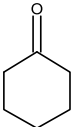
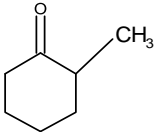
Substrate	Whole cell Conversion (%) /Ratio	Resting cells Conversion (%)/Ratio	Soluble extract Conversion (%)/Ratio
 Cyclobutanone	34.2		
 Cyclohexanone	NC		
 2-methylcyclohexanone	21.0		
 bicyclo[3.2.0]hept-2-en-6-one	13.5		
 Phenylacetone	35.2		

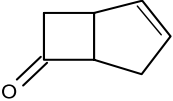
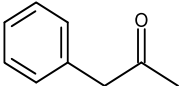
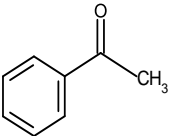
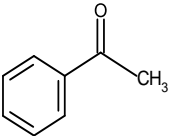
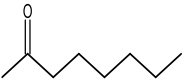
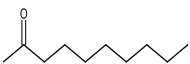
 <p>Acetophenone</p>	NC		
 <p>Thioanisole</p>	32.0		77.6
 <p>2-heptanone</p>	41.1	Traces	73.6
 <p>2-octanone</p>	60.8	6.6	78.3
 <p>2-nonanone</p>	89.8	3.2	82.4
 <p>2-decanone</p>	80.8%	10.5	83.7
 <p>2-undecanone</p>	63.9		57.9
 <p>3-octanone</p>	44.3 /(1:1)	5.9/(2:3)	43.6/(1:1)

 3-nonanone	64.2/ (1:1)	1.6/(2:3)	45.9/(1:2)
 3-decanone	85.9 (2:1)	Traces/ ND	59.0/(1:1)

Whole cell biotransformations with growing and resting cells were performed with 5 mM substrate, while biotransformations with crude extract contained 25 mM substrate and glucose/glucose dehydrogenase for cofactor regeneration. The reaction mixtures were incubated at 15 °C with shaking at 150 rpm for 16 hours (whole cells and resting cells) or 24 hours (soluble cell extract).

Table 2 Comparison of substrate conversion by resting cells of *Dietzia* sp. with previous reports of EthA monooxygenase like group enzymes. Colours represent the conversion rate: green >55%, orange between 55% and 10%, blue less than 10%, and pink no conversion. 1) BVMO3, 2) MO3083, 3) MO0565C, 4) MO13, 5)MO16, 6)EthA, 7)BVMP.

Substrate	1	2	3	4	5	6	7
 Cyclobutanone	Orange				Orange		
 Cyclohexanone	Pink				Blue		Blue
 2-methylcyclohexanone	Orange						

 bicyclohept-2-en-6-one							
 Phenylacetone							
 Acetophenone							
 Thioanisole							
 2- octanone							
 2-decanone							

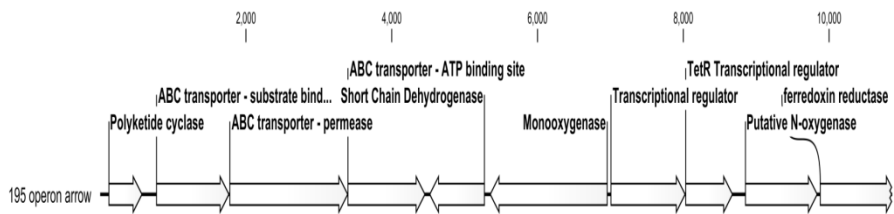


Figure 1 ORFs surrounding BVMO3 gene obtained from *Dietzia* sp. D5 sequencing (Bisagni 2012).

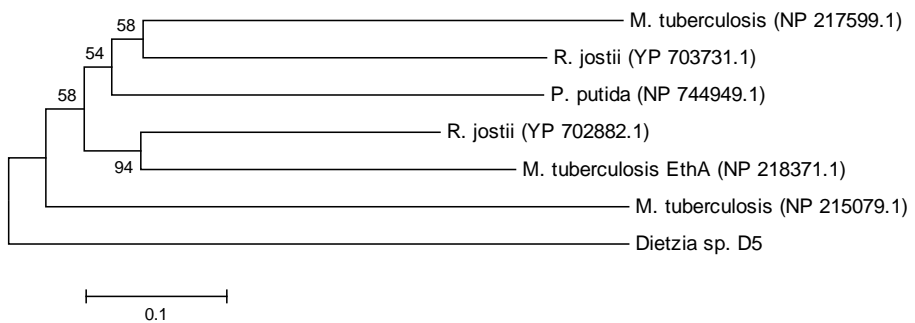


Figure 2 The phylogenetic tree constructed from sequence alignment to show the position of BVMO3 from *Dietzia* sp. D5.

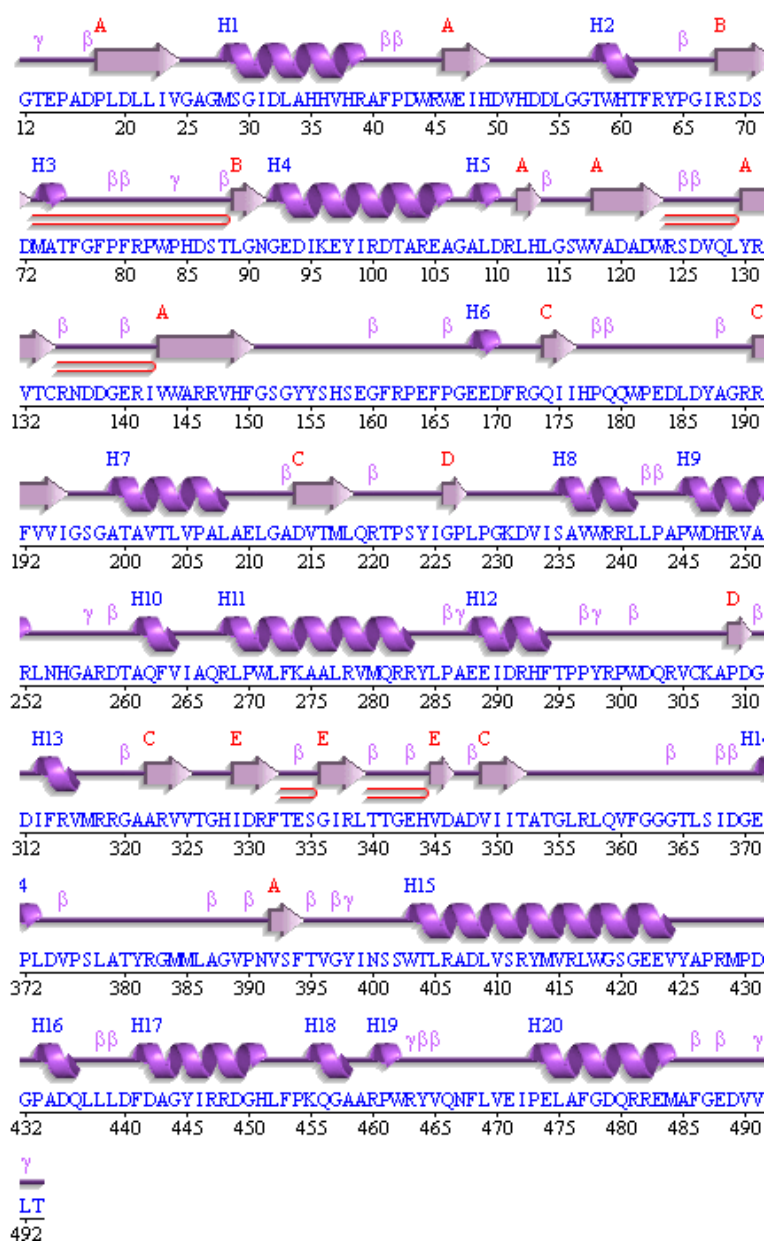


Figure 3 Predicted secondary structure of BVMO3.

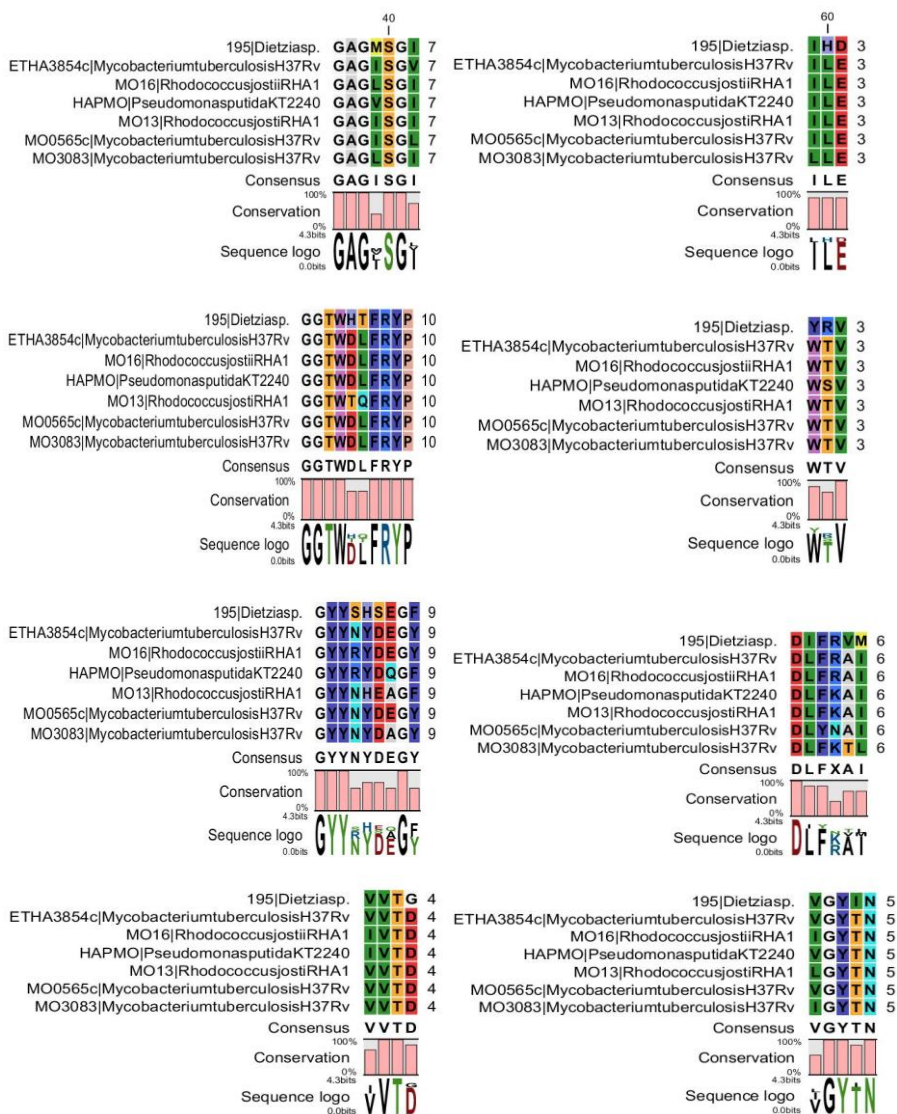


Figure 4 Alignment of sequence fragments of the BVMOs related to BVMO3 (BVMO 195).

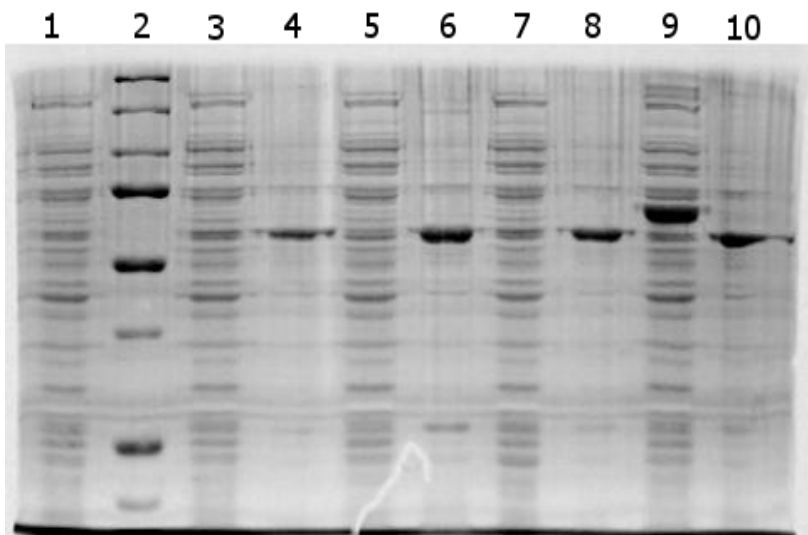


Figure 5 SDS-PAGE showing the influence of resuspension buffers on BVMO3 solubility. Lane 1: Soluble fraction in potassium phosphate buffer; Lane 2: AllBlue Marker, the two thick bands in the center of the gel with molecular weight 75 and 50 kDa, respectively; Lane 3 and 4: soluble and insoluble fraction in potassium phosphate buffer with 10% glycerol; Lane 5 and 6: soluble and insoluble fraction in potassium phosphate buffer with 0.1% Triton X-100; Lane 7 and 8: soluble and insoluble fraction in EthA buffer; soluble and insoluble fraction in potassium phosphate buffer with 1 g/L BSA.

Paper III



Baeyer–Villiger oxidation with peracid generated *in situ* by CalB–CLEA catalyzed perhydrolysis

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ARTICLE INFO

Article history:

Received 13 July 2012

Received in revised form 4 December 2012

Accepted 19 December 2012

Available online 29 December 2012

Keywords:

Lipase

CLEAs

Baeyer–Villiger oxidation

ϵ -Caprolactone

ABSTRACT

Candida antarctica lipase B, immobilized as cross linked enzyme aggregates (CLEAs) was used to mediate the Baeyer–Villiger oxidation of cyclohexanone to ϵ -caprolactone, and the reaction was compared with the one using Novozym® 435 as catalyst. The conversion was dependent on the initial concentration of cyclohexanone, and was about 90% after 48 h at concentrations of up to 0.25 M but was decreased at higher concentrations. Caprolactone concentrations up to 0.6 M had no effect on the reaction efficiency. Among the cyclic ketones tested, the highest degree of conversion was achieved for cyclopentanone (88%) and the lowest for cyclooctanone (about 2%). The effect of methyl substitution and position of substitution on the cycloketone was studied using methylcyclohexanone and it has shown to influence the conversion efficiency. Both hydrogen peroxide and the reaction by-product acetic acid had a deleterious effect on the stability of the biocatalyst.

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1. Introduction

The global consumption of ϵ -caprolactone is about 30 kton annually for various applications such as production of synthetic fibers, plastics, films, coatings, and plasticizers. The current industrial production of caprolactone is based on the oxidation of cyclohexanone with peracetic acid at 50 °C and atmospheric pressure [1]. However, the high risk of explosion associated with the transport and storage of peracetic acid makes the *in situ* generation of peracids an attractive and necessary alternative.

Peracids can be generated *in situ* by reaction of carboxylic acids or esters with hydrogen peroxide (perhydrolysis), catalyzed by certain hydrolases. In 1990, Björklund et al. [2] reported the generation of peracids mediated by lipases for the first time. Subsequently, enzymatically generated peracids have been used for epoxidation of alkenes [3–7] and for the Baeyer–Villiger reaction [8–11] (Fig. 1). In nature, monooxygenases catalyze the Baeyer–Villiger oxidation of cyclic ketones with molecular oxygen [12–17]. However, the need for NADPH as a stoichiometric cofactor, FAD supplementation, poor enzyme stability, low product yield, and lack of commercially available enzymes represent serious obstacles to their use on industrial scale. Therefore, a chemo-enzymatic approach using *in situ* generation of peracid catalyzed by a lipase could be an interesting choice as the reaction does not require

any co-factor and the enzyme is relatively stable and commercially available.

Candida antarctica lipase B (CalB) has been the enzyme of choice for catalyzing many processes including aminolysis, alcoholysis, (trans)esterification, amidation and perhydrolysis [18,19]. The commercially available immobilized form of CalB, Novozym® 435 in which the enzyme is adsorbed on a polymethylmethacrylate carrier, has been shown to be a highly efficient biocatalyst, mainly due to its tolerance to organic solvents and reasonable thermal stability [20–22]. However, its operational stability is often limited due to enzyme leaching [23] and its sensitivity to high concentration of oxidants [24] and pH changes [25]. These limitations make the biocatalyst the predominant cost-determining factor in such a process. Other carriers have been reported for immobilization of CalB, such as polystyrene, polypropylene and polysiloxane [26,27], and different immobilization techniques such as entrapment and crosslinking [28,29] have been used to improve the stability of the catalyst and to decrease the cost.

Cross linked enzyme aggregates (CLEAs) have been developed as alternative carrier-free immobilized preparations, in which the enzyme is precipitated from an aqueous solution by adding a salt, organic solvent or a polymer, and the resulting aggregates of protein molecules are subsequently cross linked with a bifunctional agent [30]. CalB has been immobilized as CLEAs, and different preparations have been optimized by fine-tuning the hydrophobicity to allow its use in organic media [31–33]. Unlike Novozym® 435, CalB–CLEA® does not leach under aqueous conditions and,

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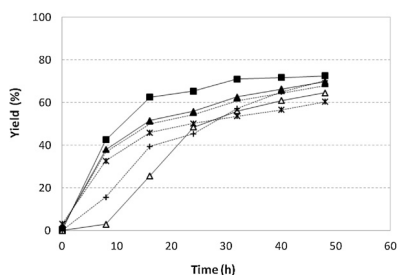


Fig. 1. Time course of the production of ϵ -caprolactone by CalB-CLEA OM4 (dotted line) using 0.6 mmol (+), 1 mmol hydrogen peroxide (*) or 1 mmol urea-hydrogen peroxide (–), and by Novozym® 435 (solid line) with 0.6 mmol hydrogen peroxide (Δ) or 1 mmol urea-hydrogen peroxide (\blacksquare), respectively. The reactions were composed of 25 mg of enzyme, 0.5 mmol cyclohexanone, and 911 μ L or 866 μ L of ethyl acetate for reactions containing 0.6 and 1 mmol of oxidant, respectively. The reaction mixture was incubated for 48 h at 40 °C with agitation at 400 rpm.

because of the smaller particle size, it is expected to have lower mass transfer limitation.

In this paper, the chemo-enzymatic Baeyer–Villiger oxidation of cyclohexanone to ϵ -caprolactone using hydrogen peroxide as the oxidant, immobilized CalB (CLEA OM4) as the catalyst and ethyl acetate as both acyl donor and solvent is reported (Scheme 1). Ethyl acetate is, from a green chemistry perspective, a preferred solvent for use in chemical reactions [34], and has an added advantage of having a low boiling point that allows easy downstream processing. The reaction catalyzed by Novozym® 435 was also run for comparison.

2. Materials and methods

2.1. Materials

Novozym® 435 (10.5 U/mg) was kindly donated by Novozymes A/S, Bagsvaerd, Denmark. CalB-CLEA OM4 (5.3 U/mg) was provided by CLEA Technologies BV, Delft, The Netherlands. Cyclohexanone (99%), ϵ -caprolactone (97%), methyl benzoate (99.5%), hydrogen peroxide (50%, w/w), urea-hydrogen peroxide (97%), bovine liver catalase (4966 U/mg), ethyl acetate (99.8%), acetic acid (99%), cyclopentanone (99%), cyclooctanone (98%), and 2-, 3- and 4-methyl cyclohexanone (98%, 97% and 99%, respectively) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents used were of the highest purity available.

2.2. ϵ -Caprolactone production

A typical reaction was carried out as follows: to a mixture containing 1 mL of 0.5 M cyclohexanone (0.5 mmol) in ethyl acetate and 37 μ L of 50% (w/w) hydrogen peroxide or 56 mg of urea

hydrogen peroxide (0.6 mmol, 1.2 molar equivalent) in 4 mL vials was added 25 mg of the immobilized lipase preparation. The vials were placed in a HTMR-131 thermomixer (HLC, Germany) at 400 rpm and 40 °C, and the formation of ϵ -caprolactone was monitored by GC as described below.

The effect of varying concentrations of reactants and products on the yield of the reaction was investigated. Reaction mixtures of 1 mL with cyclohexanone concentrations ranging from 0.05 to 2 M in ethyl acetate with 37 μ L of hydrogen peroxide (50%, w/w) and 25 mg of lipase CLEA OM4 were incubated under the conditions described above. The effect of ϵ -caprolactone concentration was investigated by adding at the start of the reaction 23, 34, 46, 57 and 70 μ L of ϵ -caprolactone to make the final concentrations of 0.2, 0.3, 0.4, 0.5 and 0.6 M, respectively, and incubating the vials under the conditions described above. The initial concentration of hydrogen peroxide in the reaction mixture was varied from 0.2 to 2 M while keeping the amount of cyclohexanone and biocatalyst constant.

2.3. Enzyme stability

Vials containing the reaction mixture comprising 1 mL of 0.5 M cyclohexanone in ethyl acetate, 37 μ L of hydrogen peroxide (50%, w/w) and 25 mg of immobilized lipase preparation were incubated at 40 °C with shaking at 400 rpm. Samples were taken after 2, 4, 6, 8, 16, 24, 32 and 48 h of incubation, after which the immobilized enzyme was filtered, washed twice with ethyl acetate, dried, weighed, and residual enzymatic activity was measured using the titration method described below.

The effect of acetic acid on the enzyme stability was studied by incubating 25 mg of CLEA OM4 in 1 mL of 1, 2, 5 and 10 M, respectively, of the acid dissolved in ethyl acetate. After 8 h of incubation at 40 °C and 400 rpm shaking, the immobilized enzyme was filtered and washed as described earlier and the residual activity was determined by the titrimetric method.

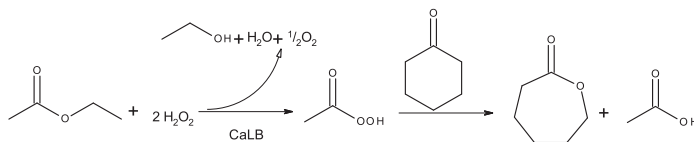
2.4. Analysis

2.4.1. Determination of lipase activity

Lipase activity was assayed using a modified titrimetric method described by Sémériva [35], using Metrohm 665 Dosimat automatic titrator. To 20 mL of 25 mM phosphate buffer pH 7.5 at 40 °C, were added 2 mL of tributyrin and 20 mg of the immobilized enzyme. The volume of the titrant (0.1 M KOH) consumed was monitored during 5 min and was used to calculate the amount of butyric acid produced. One unit of activity was defined as the amount of enzyme that produces 1 μ mol of butyric acid per minute under the assay conditions.

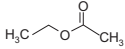
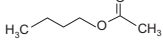
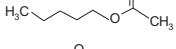
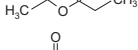

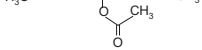
2.4.2. Peracid number determination

The peracid number of the reaction mixture was determined following the method described by Minning et al. [36]. One milliliter of the reaction mixture was incubated with 20 mg catalase (4966 U/mg) at room temperature (about 22 °C) for 5 min, and then 65 mL of water, 2 g of potassium iodide and 10 mL of 0.5 M sulfuric acid were added. This solution was titrated with 0.05 M



Scheme 1. Lipase mediated chemo-enzymatic conversion of cyclohexanone to ϵ -caprolactone.







Table 1Effect of acyl donors on yield and conversion of cyclohexanone to ϵ -caprolactone using CaLB-CLEA OM4 and Novozym® 435, respectively.

Acyl donor	Structure	Yield (%)		Conversion (%)	
		CLEA OM4	Novozym® 435	CLEA OM4	Novozym® 435
Ethyl acetate		70	69	79	80
Butyl acetate		64	69	75	79
Amyl acetate		2	2	10	7
Ethyl propionate		62	70	85	84
Methyl caproate		59	50	79	71
Triacetine		65	62	76	74

The reaction mixtures were composed of 25 mg of the immobilized enzyme preparation, 0.5 mmol of cyclohexanone and 0.6 mmol H₂O₂, and acyl donors to a final volume of 1 mL. The reaction was performed for 48 h at 40 °C with shaking at 400 rpm.

Table 2

Baeyer–Villiger oxidation of different cyclic ketones using CaLB-CLEA OM4 and Novozym® 435, respectively.

Substrate	Structure	Conversion (%)	
		CLEA OM4	Novozym® 435
Cyclopentanone		88	88
Cyclohexanone		79	80
Cyclooctanone		0	2
2-Methylcyclohexanone		84	76
3-Methylcyclohexanone		57	62
4-Methylcyclohexanone		77	80

The reaction mixtures were composed of 25 mg of the immobilized lipase preparation, 0.5 mmol cyclohexanone and 0.6 mmol H₂O₂ and ethyl acetate to a final volume of 1 mL. The reaction was performed for 48 h at 40 °C with shaking at 400 rpm.

sodium thiosulfate until the color of the mixture changed to a very light yellow, after which 2 mL of indicator (2% (w/v) starch solution) was added and addition of thiosulfate was continued until the mixture was colorless.

2.4.3. Analysis of cyclohexanone and ϵ -caprolactone

Cyclohexanone and ϵ -caprolactone were analyzed by gas chromatography using Shimadzu Chromatograph equipped with a flame ion detector and a ZB-Wax Plus column (Phenomenex). Hydrogen was used as a carrier gas and the inlet pressure was 33 psi. The temperature of the column was programmed from 50 °C to 240 °C with a ramp of 20 °C/min. The temperature of the injector was set at 250 °C. Ten microliter samples were taken from the reaction mixtures and diluted 100 times in ethyl acetate containing 1 g/L methyl benzoate as internal standard. The solutions were dried with magnesium sulfate and filtered prior to analysis. The retention times for cyclohexanone, acetic acid, methylbenzoate, and ϵ -caprolactone were 6.1, 7.5, 8.5 and 10 min, respectively. The total analysis time was 18 min.

Reaction rates were calculated as the molar change in concentration per hour using 25 mg of immobilized CaLB and 1 mL reaction volume.

2.4.4. Mass spectrometry

The presence of ϵ -caprolactone in the product was confirmed by mass spectrometry. Prior to, and in sequence with MS, the sample components were separated by injecting 50 μ L of 500 \times diluted samples in acetonitrile containing 1% formic acid on a reversed phase C18 column (5 μ m, 100 Å, 150 mm \times 4.6 mm; Eka Chemicals, Bohus, Sweden) connected to a Perkin Elmer HPLC-system (Boston, USA) equipped with a UV-detector Spectra Series UV100, Thermo Separation Products, Riviera Beach, USA. Then the samples entered the turbo ion spray source on a Θ STAP[®] pulsar-*i*-Q-TOF mass spectrometer (PE Sciex, Toronto, Canada). The MS was set to positive ion mode with a source voltage of +5500 V. The quadrupole system was adjusted to scan between m/z 50 and 500 in TOF-MS.

3. Results and discussion

3.1. Screening of acyl donors

Table 1 shows the oxidation of cyclohexanone with different acyl donors using CLEA OM4 and Novozym[®] 435, respectively, to catalyze the perhydrolysis reaction. With the exception of amyl acetate, all the acyl donors tested showed reasonably good conversion of cyclohexanone (71–85%) and ϵ -caprolactone yield (59–70%) in a relatively shorter time than what has been previously reported [8–10]. Ethyl propionate gave the highest conversion (84–85%) while ethyl acetate gave the highest yield (69–70%). Due to the better conversion and yield achieved, and that it is a green solvent [34], ethyl acetate was selected as acyl donor for further studies. Both lipase preparations used gave fairly similar conversion and product yield (Table 1), even though their specific activities differed by a factor of almost two (5.3 and 10.5 U/mg for CLEA OM4 and Novozym[®] 435, respectively). The higher conversion efficiency achieved by the CLEA OM4 preparation could be ascribed to reduced mass transfer limitation due to smaller particle size of the biocatalyst.

3.2. Oxidation of cyclic ketones

Oxidation of 0.5 mmol cyclohexanone was studied using CLEA OM4 and Novozym[®] 435 in the presence of H₂O₂ (0.6 or 1 mmol) or urea-hydrogen peroxide (1 mmol) (0.6 and 1 M final concentration of oxidant, 1.2 and 2 molar equivalent). CLEA OM4 exhibited a higher initial reaction rate with 0.6 mmol H₂O₂, while at higher

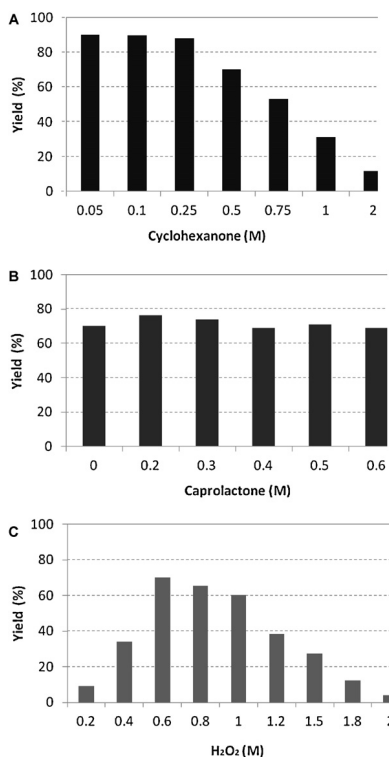


Fig. 2. Effect of (A) cyclohexanone, (B) ϵ -caprolactone and (C) hydrogen peroxide concentration on the production of ϵ -caprolactone by chemo-enzymatic oxidation reaction catalyzed by CaLB-CLEA OM4 (25 mg) in ethyl acetate. Experimental details of A and B are described in Section 2.1, and C is described in Section 2.2.

peroxide concentration or with urea-H₂O₂ the initial reaction rates were similar for the two preparations but over time Novozym[®] 435 retained relatively higher reaction rate possibly due to higher content of active enzyme (Fig. 1). The higher amount of peracid accumulated during the initial phase of reaction with 1 mmol H₂O₂ leads to a higher degree of deactivation of the enzyme. As a result, there was no significant difference in the final product yield and the maximum production of ϵ -caprolactone achieved after 48 h of reaction was in the range of 60–73% (Fig. 1), which was similar to what has been reported earlier [10].

The oxidation of a range of ketones by CaLB has been studied [8–10], and chemo-enzymatic production of ϵ -caprolactone was shown to be a very slow process that could take up to 6 days when the reaction was run at lower temperature (25 °C) and relatively low shaking rate (250 rpm) [10]. On the other hand a faster reaction and higher yield (75% within 20 h) have been achieved using 200 times higher amount of enzyme than that used in this study [9]. Recently, Baeyer–Villiger oxidation in ionic liquids has also been reported yielding 62% of ϵ -caprolactone in 5 h using octanoic acid as acyl donor at 50 °C [37].

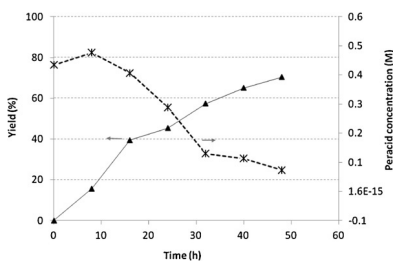


Fig. 3. Time course of ϵ -caprolactone (▲) and peracid (●) production in the reaction mediated by CalB-CLEA OM4. The reaction mixture containing 25 mg biocatalyst, 0.5 mmol cyclohexanone, 0.6 mmol hydrogen peroxide (1.2 molar equivalent) and 911 μ L of ethyl acetate, was incubated for 48 h at 40 °C with agitation at 400 rpm.

The reaction temperature was maintained at 40 °C in the present study; increase in temperature would increase the initial reaction rate but also compromises the enzyme stability, and moreover it increases the risk of explosion due to peracetic acid. In chemo-enzymatic oxidation, the rate limiting step is not the generation of the peracid but the subsequent transfer of oxygen for oxidation of the substrate [22], which can be promoted by increase in temperature.

CLEA OM4 and Novozym® 435 were used to catalyze the oxidation of different cycloketones, and both the preparations gave similar degrees of conversion (Table 2). The oxidation efficiency of the cycloketones was correlated to the ring size, the larger the ring the slower was the oxidation. The highest conversion was obtained with cyclopentanone while there was very low or no conversion of cyclooctanone. About 88% of cyclopentanone was converted in 48 h (Table 2), resulting in 51% yield of δ -valerolactone (data not shown), as compared to 68% yield in 72 h reported earlier when using Novozym® 435 [11]. The methyl substitution and position of substitution on the cycloketone was also shown to influence the conversion efficiency; 2-methyl cyclohexanone was a better substrate than cyclohexanone while 3-methyl cyclohexanone was a significantly poorer substrate (Table 2).

3.3. Effect of cyclohexanone, ϵ -caprolactone and peroxide concentration on product yield

In order to find the optimum conditions for performing the reaction, the effect of the different reactants and products on the activity and stability of CLEA OM4 was investigated. Performing the reaction at initial cyclohexanone concentrations in the range of 0.05–0.25 M resulted in a similar ϵ -caprolactone yield (88–90%); however, at concentrations of 0.5 M and above the product yield started to decrease significantly and was only 10% at 2 M cyclohexanone (Fig. 2A). Variation of cyclohexanone concentration alters the cyclohexanone:H₂O₂ ratio, which is a critical factor in determining the rate of chemo-enzymatic reactions as reported earlier [38,39].

The effect of ϵ -caprolactone was studied by adding different amounts (to reach initial concentration of 0.2–0.6 M) at the start of the reaction. No substantial effect was observed on the final product yield, which varied in the range of 69–77% (Fig. 2B). Mass spectrometry analysis of the product revealed no ring opening of ϵ -caprolactone by the water generated in the reaction.

Fig. 2C shows the conversion of cyclohexanone with respect to the hydrogen peroxide concentration used in the reaction. Maximum ϵ -caprolactone yield was obtained when using 0.6 mmol (1.2 equivalent) H₂O₂, a concentration that is 4 times lower than that reported previously [8], and 2 times lower than the urea-hydrogen peroxide used by Rios et al. [10]. Higher peroxide concentrations result in enzyme deactivation due to oxidation of sensitive amino acids [28]; loss of enzyme activity is not immediate but occurs with time and eventually the oxidation process results in disruption of disulfide bridges and loss of structure [40,41]. The loss of lipase activity occurs at a lower peroxide concentration if the reaction temperature is increased to 60 °C [41]. Urea-hydrogen peroxide is claimed to be a milder oxidant and also decreases the water formation in the reaction [10,11]; however, no significant improvement in the product yield was observed in this study and recycling of the biocatalyst was not possible. Hence, considering the availability, lower cost and easier handling in liquid form, H₂O₂ was chosen as the oxygen donor for further studies.

H₂O₂ concentration used determines the rate and amount of peracid formed which in turn influences the efficiency of the chemo-enzymatic reaction. In order to have a constant production of ϵ -caprolactone, peracid concentration should be kept constant. As shown in Fig. 3 the peracid level was maintained at a high level

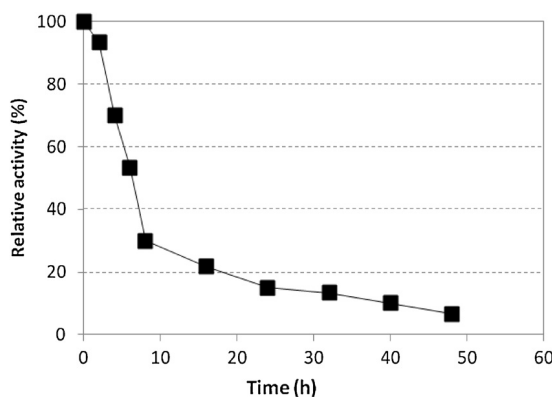


Fig. 4. Residual activity of CalB-CLEA OM4 during the course of the chemo-enzymatic oxidation reaction. Experimental details are given in Section 2.2.

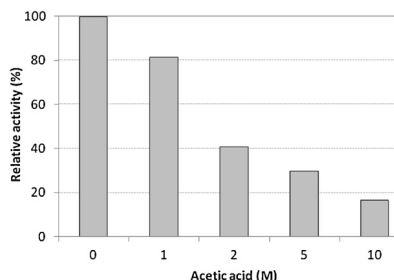


Fig. 5. Effect of acetic acid on the activity of CalB-CLEA OM4. The reaction mixture consisted of 25 mg of the biocatalyst with 0.1, 2, 5 or 10 M of acetic acid and ethyl acetate to a final volume of 1 mL. The mixture was incubated at 40 °C for 8 h with agitation at 400 rpm.

(0.48 M) up to 8 h but decreased thereafter as it is consumed in cyclohexanone oxidation and is not replenished. The peracid level declined to 0.13 M after 30 h and then dropped below 0.1 M after 48 h of reaction. In fact, if the rate of production in the first 16 h (0.0106 M h⁻¹) had continued, the maximal product yield should have been reached already at 31 h.

3.4. Influence of acetic acid by-product on the biocatalyst activity

Determination of the residual enzyme activity of CLEA OM4 during the course of chemo-enzymatic oxidation revealed loss of activity from 5.3 U/mg to 0.69 U/mg at the end of the reaction at 48 h (Fig. 4), which makes its recycling/longer use impossible. As the low hydrogen peroxide concentration used in our experiments was not expected to result in enzyme deactivation, the loss of CalB activity observed in our system was suspected to be due to the acetic acid co-product generated in the reaction. The acetic acid reaches a concentration of 5.2 M in the initial 8 h of the reaction. Fig. 5 shows the loss of CalB activity with increase in acetic acid concentration. The acidic environment affects the protein conformation and also results in the cleavage of disulfide bridges [42]. As a result, the enzyme loses its ability to form more peracid and hence the rate of formation of ϵ -caprolactone also decreases. A way to overcome this problem could be the use of buffering salts in the system.

The stability of the enzyme is also influenced by the size of the acyl donor used [24]; the larger the acyl donors produce longer chain peracids which are less aggressive to the enzyme but are less reactive than the shorter chain peracids. The steric effect of the aliphatic chain of the acyl donor could be an important factor to be considered as bulky chains might reduce the rate of nucleophilic attack by H₂O₂ which consequently reduces the reaction rate.

4. Conclusions

Chemo-enzymatic oxidation is a challenging reaction for a biocatalyst owing to the use as well as generation of molecules with a severely denaturing effect. In spite of that, the immobilized CalB appears to be a promising biocatalyst for the conversion of cyclohexanone to ϵ -caprolactone. This study showed that the reaction catalyzed by CLEA OM4 was comparable with that using equal amount of Novozym® 435 which has two times higher specific activity. A further step would be to design a suitable process to maintain the denaturants at sufficiently low concentrations in the reactor so as to extend the operational lifetime of the biocatalyst. Protein engineering of the enzyme could also be a complementary

approach to provide a biocatalyst with higher stability against the effect of the oxidizing and/or low pH conditions.

Acknowledgments

The authors are grateful to the Swedish Agency for Research Cooperation with Developing Countries (Sida-SAREC) and Marie Curie Actions for the financial support of this project, and to Dr. Martin Hedström for mass spectrometry analysis.

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Paper IV

Baeyer-Villiger oxidation of cyclohexanone with *in situ* generation of peracid with a perhydrolase CLEA

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Abstract

A perhydrolase, immobilized as a cross linked enzyme aggregate (CLEA), was used to perform the *in situ* formation of peracids using ethylene glycol diacetate as acyl donor. The perhydrolase CLEA proved to be a very efficient enzyme with values of K_m 118 mM and V_{max} 2815 mM * min⁻¹. The produced peracid was used for the Baeyer Villiger oxidation of cyclohexanone, yielding 63% of caprolactone after 72 hours at room temperature. Interestingly, the enzyme was able to perform better in aqueous solution than the well studied lipase B from *Candida antarctica*. Parameters such as the type and amount of acyl donor, solvent, pH, temperature and ratio of cyclohexanone to oxidant were also studied. The best conditions found involved the use of 100 mM of ethylene glycol diacetate in phosphate buffer (pH 6), stirring at room temperature and a ratio of cyclohexanone to hydrogen peroxide of 0.25. The *in situ* generated peracid could also be of used in other oxidations.

Keywords

Perhydrolase CLEA, Baeyer Villiger oxidation, aqueous system, caprolactone

Introduction

Over the last few years, the concern for environmental issues has increased dramatically. The recent United Nations Conference of Sustainable Development in Río de Janeiro, Brazil¹ pointed out that the management of chemicals is crucial and extra effort must be made to enhance the technology for environmentally sound chemicals. Diminishing the use of chemicals is one key point in achieving this goal, and considering that the solvent contribution to waste produced in a process can be around 80% in some cases², to make a process greener the use of organic solvents should be diminished or avoided. In this sense, the use of water as a solvent is an environmentally friendly alternative to some traditional chemical processes³, with the added advantages of its low impact on health issues, and being an economical and readily available resource. On the other hand, the use of enzymes is another way to contribute to the greenness of an organic synthesis as enzymes generally work under mild conditions such as physiological pH, room temperature and atmospheric pressure. Safety issues can also be solved by the use of enzymes for the *in situ* generation of chemicals avoiding the direct manipulation of dangerous reagents. As an example, the current industrial production of caprolactone is based on the oxidation of cyclohexanone with peracetic acid at 50 °C and atmospheric pressure⁴. However, the high risk of explosion associated with the transport and storage of peracetic acid makes *in situ* generation an interesting alternative.

Perhydrolases are enzymes that are able to catalyze the formation of peracids from a carboxylic acid and hydrogen peroxide. They belong to a subgroup of serine hydrolases in which the catalytic site contains the catalytic triad Ser-His-Asp⁵. Perhydrolytic activity has been found in lipases⁶, aryl esterases^{5,7}, xylan esterases⁸ and haloperoxidases⁹.

Peracids are widely used in industry, mainly in bleaching¹⁰, waste water treatment¹¹, disinfecting agents¹² and for the removal of lignin for biomass¹³. Peracids can also be used in organic synthesis, *e.g.* as an oxidant for the Baeyer Villiger reaction^{14,15} and for the epoxidation of ketones^{16,17}. In the latter cases, it is particularly important that perhydrolases shows good resistance to changes in pH and oxidizing conditions. As the formation of the peracetic acid is a faster reaction than the subsequent oxidation of ketones¹⁸, it is possible for the accumulation of peracid in the systems to occur.

For practical applications, it is well known that immobilization of enzymes is a good strategy to increase the stability of the enzyme¹⁹. Cross-linked enzyme aggregates (CLEAs) are immobilized preparations, in which the enzyme is precipitated from an aqueous solution by adding a salt, water miscible organic solvent or a polymer, and cross linking of the resulting protein aggregates with a bifunctional agent²⁰. We previously reported the use of CLEAs from lipase

CalB for the *in situ* formation of peracids in ionic liquids²¹ and organic solvents²². In the present study we used a perhydrolase CLEA to generate peracids *in situ* in a chemo-enzymatic Baeyer-Villiger oxidation of cyclohexanone to ϵ -caprolactone in an aqueous environment, using hydrogen peroxide as the oxidant and ethylene glycol diacetate as the acyl donor (Scheme 1). The proposed system is an environmentally friendly alternative to conventional syntheses of lactones and can be extended to other oxidations with proper optimization. To our knowledge, this is the first report using CLEAs of perhydrolase for production of caprolactone in aqueous system.

Results and discussion

Effect of the pH on the reaction

In a chemo-enzymatic process the optimum pH for the enzymatic step may be very different to that of the chemical step. Finding a pH that favors both is critical for the successful development of a chemo-enzymatic process. Enzymes able to catalyze the perhydrolysis reaction work optimally in the pH range 5-9^{5,6}. On the other hand, the chemical Baeyer-Villiger reaction is preferably done under acidic conditions as a basic medium favors the decomposition of peracids²³. Figure 1 shows that pH 6 favors the production of caprolactone which indicates the possibility of carrying out the reaction in tap water (pH 6.5-7) which is advantageous from an economical point of view. Moreover, this pH is between the optimum range for the enzyme activity and favors the chemical oxidation without affecting the product hydrolysis.

Effect of the ratio of cyclohexanone to oxidant

The ratio of substrate to oxidant is an important factor in chemo-enzymatic oxidations^{24,25}. As shown in Figure 2, we observed that a ratio of 0.25 is ideal for the production of caprolactone. This ratio is similar to the one used by Lemoult¹⁴ who obtained yields of 57-69% of caprolactone. In the initial experiments a lower yield (51%) was obtained. Figure 3 shows the effect of increasing the amount of acyl donor. A decrease in the amount of ethylene glycol diacetate from 200 mM to 100 mM lead to a yield of 63% without altering the ratio of cyclohexanone to hydrogen peroxide (Fig. 4). Oxidants can have detrimental effects on the enzyme. In this sense, previous works have tried to keep the amount of oxidant as lower possible, for example a ratio of 0.5 was used by Ríos²⁶, for a maximum production of caprolactone of 88% in 6 days; we used half of the ratio to reach 63% in 3 days. Higher production of caprolactone can be obtained in a system were the ratio cyclohexanone to oxidant is kept lower than 0.5.

Enzymatic production of peracid

Peracid formation is a very fast reaction¹⁸. As can be seen for the kinetics values obtained, (Table 1) the enzyme is highly efficient and catalyzes rapidly the production of peracid when using immobilized perhydrolase. The formation rate of peracid at different concentrations of ethylene glycol diacetate can be seen in Figure 3, as observed at concentrations of substrate above 200 mM there is no considerable increase in the rate of peracid formation, this value is also higher than the K_m (118 mM), so we kept the ethylene glycol concentration high enough to ensure the perhydrolysis reaction keeping a low rate of reaction, so we used 100 mM of ethylene glycol for future experiments.

Formation of ethylene glycol monoacetate was monitored during the reaction as a way to indirectly control the performance of the enzyme and the formation of peracid. As observed in Figure 4, ethylene glycol monoacetate is formed at a very high rate during the first 8 hours. This is an indirect indication of the formation of peracetic acid and is in agreement with the fast formation of caprolactone during the first hours. However, the rate of formation of caprolactone is maintained up to 12 hours, when the concentration of ethylene glycol monoacetate has already dropped, this can be explained because of the difference of velocity between the enzymatic and the chemical reaction. The apparent accumulation of ethylene glycol monoacetate during the last part of the reaction could be due that the enzyme is unable to use ethylene glycol monoacetate as acyl donor, accumulating this in the system (Fig.4).

The difference in the rates between the production of peracid (enzymatic reaction) and the oxidation of cyclohexanone (chemical reaction) makes the enzyme susceptible to exposure for a longer time to higher concentrations of peracetic acid and consequently to a lower pH, affecting not just the enzyme, but also the product. Although the use of buffers could contribute to diminishing the impact of drastic changes in the pH, other ways to keep the enzyme stability in the reaction media must also be kept in mind.

Effect of the enzyme loading

Varying the immobilized enzyme loading (0.2-5% vol/vol) showed that the maximum amount of caprolactone was produced when using 1% of the enzyme preparation in the reaction (Fig. 5). Lower amount of enzyme loads were not able to produce enough peracid and hence there was no detectable Baeyer-Villiger oxidation. Higher amounts of enzyme will produce peracid so efficiently (Table 1) which accumulated in the system leading to deactivation of the enzyme and/or caprolactone ring opening.

Choice of acyl donor

As can be observed in Table 2, the best conversions and yield were obtained with an acyl donor having a glycol moiety in its structure, this can be attributed to the stabilizing effect that glycols can have on the enzyme²⁷. The conversion achieved using diacetin and triacetin as acyl donors was good with modest yields. Interestingly, tributyrin did not show good conversions and this is probably due to steric effect²⁸. the bulky acyl donor possibly blocked the active center of the enzyme and hydrogen peroxide was not able to reach the active site for the generation peracid. Surprisingly, ethyl acetate, being a small, short acyl donor, was not able to act as an acyl donor contrary to reports in other studies²⁶. As ethylene glycol diacetate showed higher yield; is highly soluble in water and has an apparent stabilizing effect on the enzyme, it was chosen as the acyl donor for further studies.

Effect of the temperature

The course of the reaction was followed at different temperatures. As observed in Figure 6, the best production of caprolactone (73%) was reached after 12 hours at 40 °C. During the first few hours of the reaction, an increase in caprolactone production has been achieved with an increase in the reaction temperature. However, a rapid loss of the product due to ring opening is also observed with an increase in reaction temperature. This effect has also been observed in other chemo-enzymatic reactions²⁹ where side reactions lead to product degradation. At room temperature the yield of caprolactone formation reached 62% in 48 hours, which is considerably less than what is obtained in other works using chemo-enzymatic processes^{14,15,26}.

Production of caprolactone in different solvents

As the production of caprolactone with *in situ* generated peracid has been commonly studied using organic solvents^{14,26} and due to the ability of organic solvents enhancing enzymatic activities³⁰, we tried the reaction using different organic solvents and buffer phosphate. As shown in Figure 7, maximum yields were found after 72 hours of reaction. In phosphate buffer, the highest caprolactone yield was 63% while using ethyl acetate, toluene and MTBE resulted in 30%, 17% and 10% yields, respectively. There was no detectable product when the reaction was performed in dimethyl carbonate. A comparison with lipase CalB immobilized as CLEAs and the commercial Novozyme 435 revealed that the perhydrolase and lipases reached similar

yields of caprolactone formation (28-30%) in ethyl acetate (Figure 7). However, among the three catalysts tested, the perhydrolase CLEA was the only preparation capable of performing the chemo-enzymatic oxidation of cyclohexanone in phosphate buffer, reaching a 63% yield of caprolactone in 72 hours.

Caprolactone degradation

Stability of caprolactone can be affected by several different factors during the course of the reaction. In Table 3, the effect of hydrogen peroxide, peracetic acid and acyl donor concentrations on caprolactone after 72 hours is shown. It is clear that the main component affecting this system is the peracetic acid, with 52% caprolactone hydrolysis in phosphate buffer alone and 59% when the enzyme is added. This slight increase in hydrolysis in the presence of the enzyme is possible due to an autocatalytic effect of the enzyme¹⁵. The autocatalytic effect of perhydrolase is also confirmed by the decrease in the caprolactone concentration when using hydrogen peroxide in the presence of the enzyme, it can be seen that hydrogen peroxide is not degrading the product by itself, however when the enzyme is present, perhydrolysis of caprolactone lead to the formation of hydroxyacids, perhydrolase is able to use these hydroxyacids as an acyl donor and produce peracetic acid. However, the concentration of hydrogen peroxide and peracetic acid used in our experiments (100 mM) would be used for the Baeyer-Villiger reaction if cyclohexanone was present, diminishing in this way the negative effect on the product.

Epoxidation of oleic acid and methyl oleate with *in situ* generated peracid

It has been previously demonstrated that peracids produced using lipases can be used for epoxidation of alkenes^{31,32}. In Table 4, the yields obtained after 72 hours of incubation with oleic acid and the methyl ester of oleic acid are shown. Although the yields obtained are modest (44% for oleic acid and 26% for methyl oleate), we suggest that the system could be optimized further, particularly the reaction temperature. As the solubility of fatty acids in aqueous solutions is limited, is possible that elevated temperatures favor the reaction to get good epoxidation yields. However; separation of the epoxides would be facilitated while using a room temperature system. A possible solution could be the generation of peracids in one container and the chemical epoxidation in a second system. Although more research is necessary for using

in situ generated peracids with other substrates, the results with alkene epoxidation showed promising.

Experimental

Materials

Cyclohexanone, caprolactone and ethylene glycol diacetate were purchased from Merck (Hohenbrunn, Germany). Dodecane and methyl oleate were purchased from Acros (Geel, Belgium). Hydrogen peroxide 30% (w/w), oleic acid and methanol were purchased from Carl Roth GmbH (Karlsruhe, Germany). *p*-Nitrophenyl acetate and catalase were purchased from Sigma-Aldrich. All other chemicals were of analytical grade. Ethylene glycol monoacetate and CLEA from perhydrolase were from CLEA Technologies B.V.

General procedure for the production of caprolactone

The reactions were performed in 10 mL vials containing 0.25 or 0.5 mmol of ethylene glycol diacetate with 13 μ L of cyclohexanone (0.125 mmol), 52 μ L of hydrogen peroxide (0.5 mmol) and 25 μ L of CLEA suspension (31 MU/mL) in phosphate buffer (50 mM pH 6). The final volume of the reaction was 2.5 mL. Dodecane (1mM) was present as internal standard. The samples were placed on a magnetic stirrer at 750 rpm at room temperature (aprox. 20 $^{\circ}$ C), for 72 hours. Samples were withdrawn at appropriate time intervals and analyzed by GC.

Effect of the pH

Influence of the pH in the production of caprolactone was assayed by carrying out the reaction using 2 mmol of cyclohexanone with 2 mmol of hydrogen peroxide and 25 μ L of CLEA suspension using citrate buffer (50 mM, pH 4-5.5) or phosphate buffer (50 mM, pH 6-8) containing dodecane (1 mM). The final volume of the reaction was 2.5 mL. The samples were stirred at room temperature at 750 rpm. After 48 hours the samples were withdrawn and analyzed by GC.

Effect of the ratio of cyclohexanone to hydrogen peroxide

To determine the best ratio of cyclohexanone: hydrogen peroxide, the reactions were carried out as follows: to a vial containing 100 mM of ethylene

glycol diacetate, cyclohexanone was varied while keeping the hydrogen peroxide constant. 6.25, 13, 26, 52, 104 or 156 μL of cyclohexanone was mixed with 52 μL of hydrogen peroxide to give final ratios of 0.125, 0.125, 0.375, 0.5, 1 and 1.5 respectively. The final volume of the reaction was 2.5 mL. The vials were stirred at room temperature at 750 rpm for 48 hours, after this the samples were analyzed by GC.

Enzymatic formation of peracid in the absence of cyclohexanone

Formation of peracid by CLEA perhydrolase was performed by adding 416 μL of hydrogen peroxide (0.5 mmol) in a vial containing 200 μL of CLEA suspension (31 MU/mL) in phosphate buffer (50 mM, pH 6), the concentration of ethylene glycol diacetate was varied from 100 to 1000 mM of . The final volume of the reaction was 20 mL. No cyclohexanone was added in the mixture. After leaving the reaction under stirring at room temperature during 1 hour the samples were withdrawn and treated as explained lines down. Kinetic data were calculated for perhydrolase CLEA.

Epoxidation of oleic acid and methyl oleate

The reactions were performed in 30 mL vials containing 0.25 mmol of ethylene glycol diacetate with 156 μL of cyclohexanone (0.125 mmol), 624 μL of hydrogen peroxide (0.5 mmol) and 300 μL of CLEA suspension (31 MU/mL) in phosphate buffer (50 mM pH 6). The final volume of the reaction was 30 mL. Dodecane (1mM) was present as internal standard. The samples were placed on a magnetic stirrer at 750 rpm at room temperature (aprox. 20 $^{\circ}\text{C}$), for 72 hours. After that the oxirane number was determined by titration.

GC analysis

Samples (200 μL) were taken from the reaction and extracted twice with 600 μL of ethyl acetate containing 1 mM dodecane as internal standard. The solutions were treated with MnO_2 and MgSO_4 , filtered, and analyzed by GC using a Shimadzu Chromatograph equipped with a flame ion detector and a ZB-Wax Plus column (Phenomenex). Hydrogen was used as a gas carrier and the inlet pressure was 33 psi. The temperature of the column was programmed from 50 $^{\circ}\text{C}$ to 240 $^{\circ}\text{C}$ with a ramp of 20 $^{\circ}\text{C}/\text{min}$. The temperature of the injector was set at 250 $^{\circ}\text{C}$. The retention times for dodecane, cyclohexanone, ethylene glycol diacetate, ethylene glycol monoacetate and caprolactone and

were 5.2, 6, 8, 8.5 and 10 min, respectively. The total analysis time was 18 min.

Titration

Oxirane number

The epoxidation yield of methyl oleate and oleic acid was determined by a titration according to a modified version of the method described by Jay³³. A sample of 0.1 g was dissolved in 10 ml of ethyl acetate. Subsequently 10 ml of tetramethyl ammonium bromide (20% (w/v) in glacial acetic acid) and 6 drops of crystal violet indicator were added. The solution was then titrated with perchloric acid (0.1 M in glacial acetic acid) until change of color to a light green.

Peracid number

A sample (1 mL) of the reaction mixture was incubated with 20 mg catalase (4966 U/mg according to specifications of the supplier) at room temperature (22 °C) for 5 min, and then 65 ml of water, 2 g of potassium iodide and 10 mL of 0.5 M sulfuric acid were added. This solution was titrated with 0.05 M sodium thiosulfate, until the color of the mixture changed to a very light yellow, then 2 mL of indicator (2% (w/v) starch solution) was added and addition of thiosulfate continued until the mixture was colorless.

Enzymatic activity

Enzymatic activity was determined by hydrolysis of p-nitrophenyl acetate (p-NPA). To a cuvette with 1970 μ L of phosphate buffer (25 mM pH 7.5), 20 μ L of p-NPA (1 mM solution in methanol) and 10 μ L of CLEA suspension were added and the absorbance was followed for 5 minutes at 348 nm in a spectrophotometer. The activity of the esterase is defined as the amount in μ mol of p-NPA liberated per minute per gram of enzyme.

To compare the performance of perhydrolase CLEA with CalB (in the form of CLEAs or in the commercial presentation of Novozyme 435) the hydrolysis of ethylene glycol diacetate was used by a titrimetric method using a 665 Dosimat (Metrohm). To 20 mL of phosphate buffer (25 mM, pH 7.5) at 40 °C, were added 2 ml of ethylene glycol diacetate, and 200 μ L of CLEA perhydrolase suspension or 20 mg of the immobilized lipase. The volume of the titrant (100 mM KOH) consumed was monitored during 5 minutes and was used to calculate the amount of acetic acid produced. One unit of activity was

defined as the amount of enzyme that produces 1 μmol of acetic acid per minute under the assay conditions.

Conclusions

Perhydrolase CLEA proved to be a suitable catalyst for *in situ* production of peracids in aqueous buffer and successfully used in Baeyer-Villiger oxidation of cyclohexanone to caprolactone. Perhydrolase CLEA performed better than immobilized lipases for this reaction under the aqueous condition. The process is economically viable and environmentally friendly. Moreover, the use of an aqueous system and the mild conditions is an interesting beginning for a green, “organic solvent-free” approach to the Baeyer-Villiger oxidation.

Acknowledgements

Financial support from the Swedish International Development Agency for Research Collaboration with Developing countries (Sida/SAREC) and FP7 Marie Curie ITN People Programme in the project BIOTRAINS are gratefully acknowledged.

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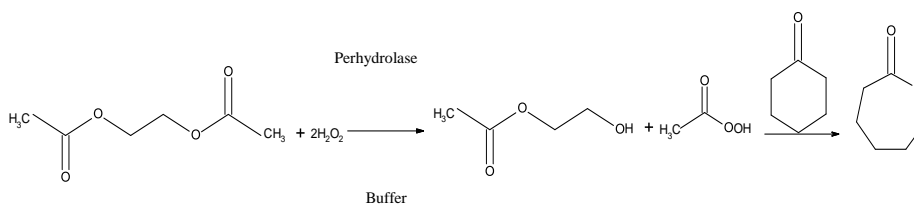
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Results

Scheme 1

Chemo-enzymatic production of caprolactone by *in situ* peracid generated by perhydrolase



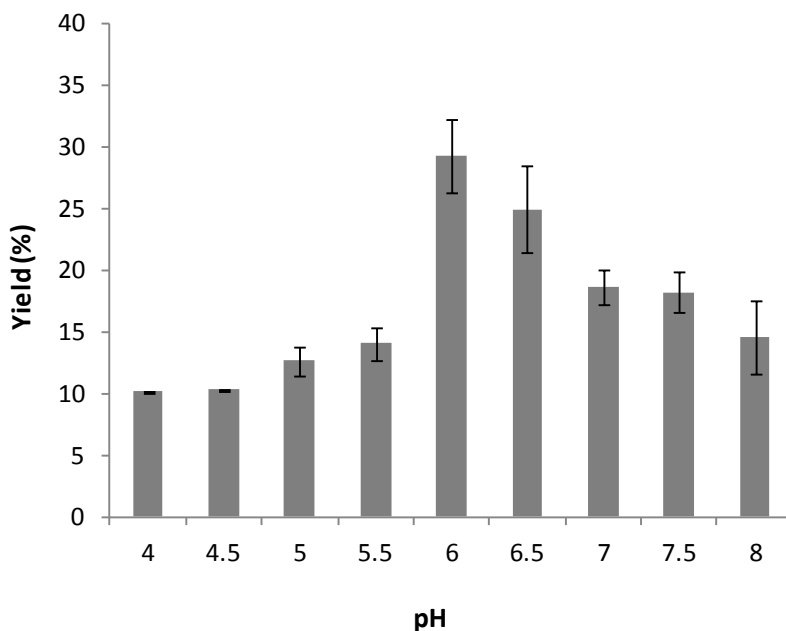


Fig 1. Influence of the pH in the production of caprolactone. Reaction conditions: ratio cyclohexanone:hydrogen peroxide =1, 0.5 mmol ethylene glycol diacetate and 1% of enzyme at different pHs of citrate buffer (50 mM, pH 4-5.5) or phosphate buffer (50 mM, pH 6-8). The samples were analyzed after stirring for 48 hours at room temperature (aprox. 20°C) at 750 rpm stirring.

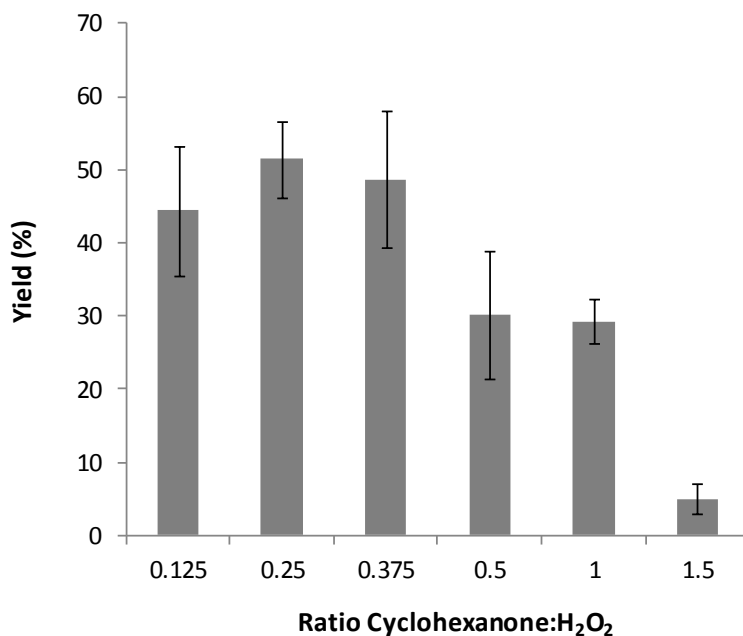


Fig 2. Influence of the molar ratio cyclohexanone:hydrogen peroxide (moles of hydrogen peroxide were kept constant) in the production of caprolactone. The reaction was carried out with 0.5 mmol of ethylene glycol diacetate as acyl donor and 1% of enzyme in phosphate buffer (50mM, pH 6). The samples were analyzed after 48 hours of stirring at room temperature (aprox. 20°C) at 750 rpm.

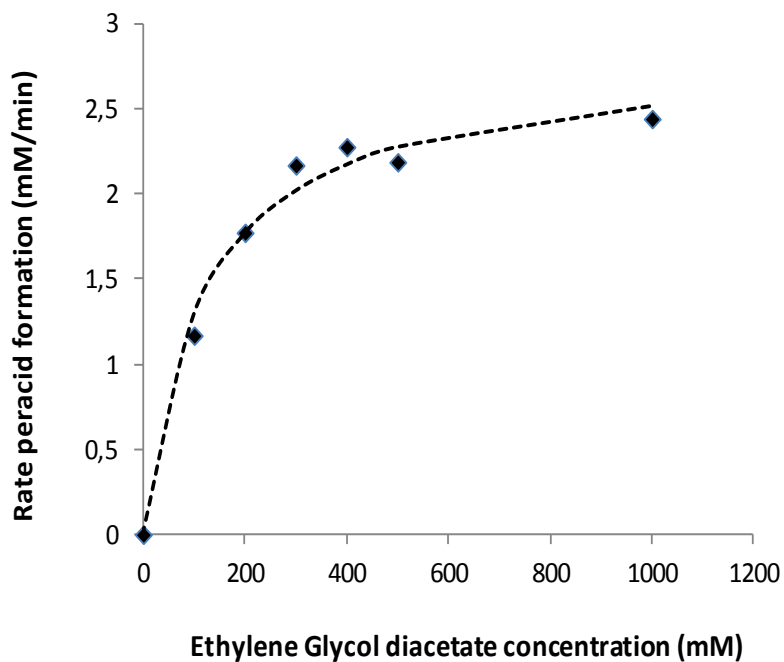


Fig 3. Initial rate of formation of peracid using ethylene glycol diacetate at different concentrations, using 416 μL of hydrogen peroxide 30% and 1% of enzyme in phosphate buffer (50 mM, pH 6). The final volume of the reaction was 20 ml. The dotted line represents the Michaelis-Menten equation.

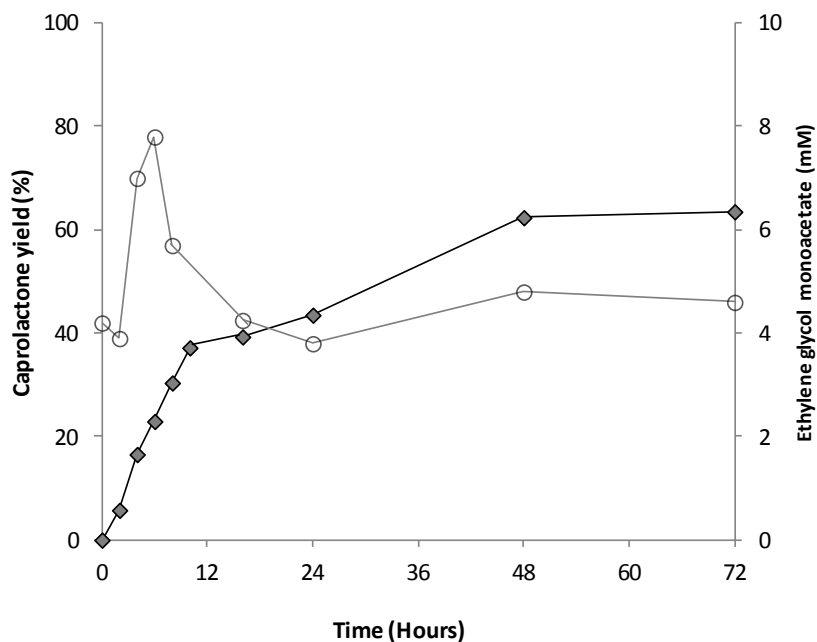


Fig 4. Time course of the chemo-enzymatic production of caprolactone and the formation of ethylene glycol monoacetate production of with perhydrolase CLEA at 20 °C. The reaction was carried out with 0.25 mmol of ethylene glycol diacetate as acyl donor, using a ratio cyclohexanone:hydrogen peroxide of 0.25 and 1% of enzyme in phosphate buffer (50 mM, pH 6) stirring at room temperature (aprox. 20°C) at 750 rpm.

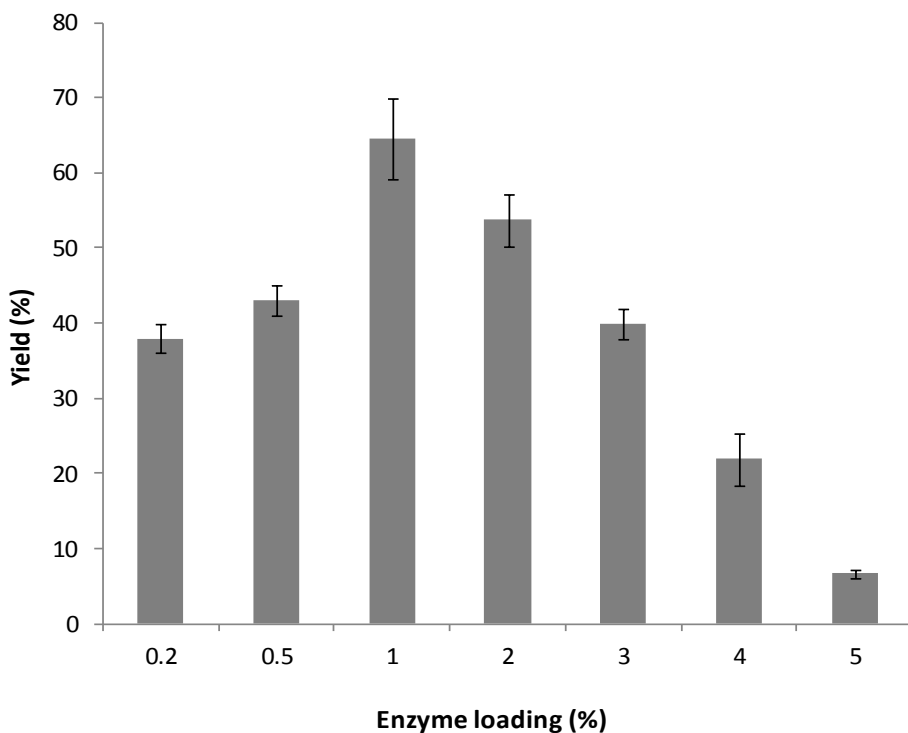


Fig 5. Effect of the enzyme loading in the production of caprolactone. The reaction was carried out with 0.25 mmol of ethylene glycol diacetate as acyl donor, ratio cyclohexanone: hydrogen peroxide 0.25 and 1% vol/vol of enzyme in phosphate buffer (50 mM, pH 6). The samples were analyzed after 48 hours at room temperature (aprox. 20°C) and 750 rpm stirring.

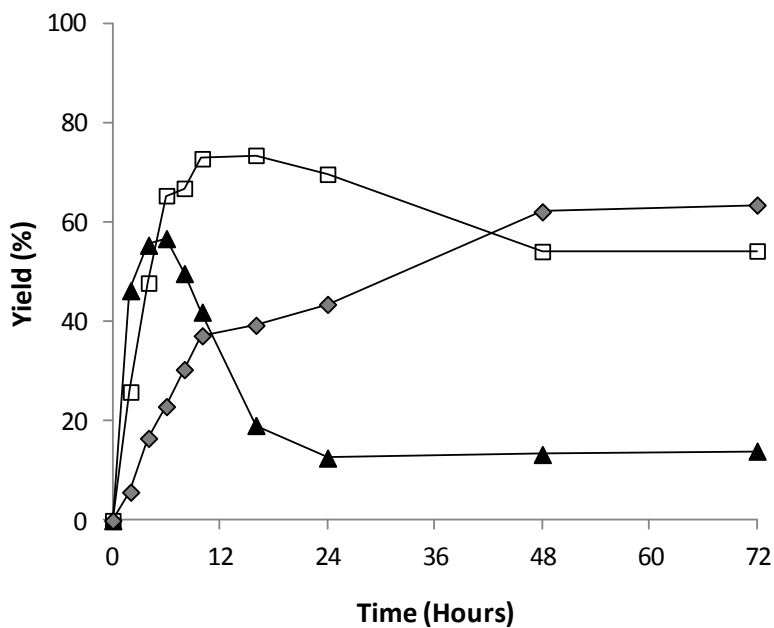


Fig 6. Time course of the chemoenzymatic production of caprolactone with perhydrolase CLEA at 20 °C (□), 40 °C (◆) and 60 °C (▲). The reaction was carried out with 0.25 mmol of ethylene glycol diacetate as acyl donor, using a ratio cyclohexanone:hydrogen peroxide of 0.25 and 1% of enzyme in phosphate buffer (50 mM, pH 6) with 750 rpm stirring.

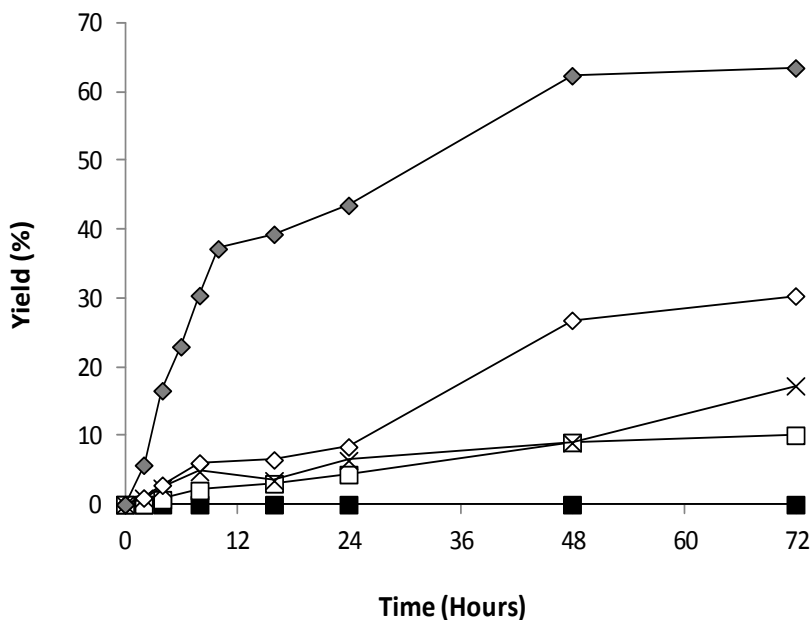


Fig 7. Time course of the chemoenzymatic production of caprolactone with perhydrolase CLEA in different solvents: phosphate buffer pH 6 (◆), ethyl acetate (◇), toluene (x), MTBE(□) and dimethylcarbonate (■). The reaction was carried out with 0.25 mmol of ethylene glycol diacetate as acyl donor, using a ratio cyclohexanone:hydrogen peroxide of 0.25 and 1% of enzyme suspension in phosphate buffer (50mM, pH 6) at room temperature (approx. 20°C) with 750 rpm stirring.

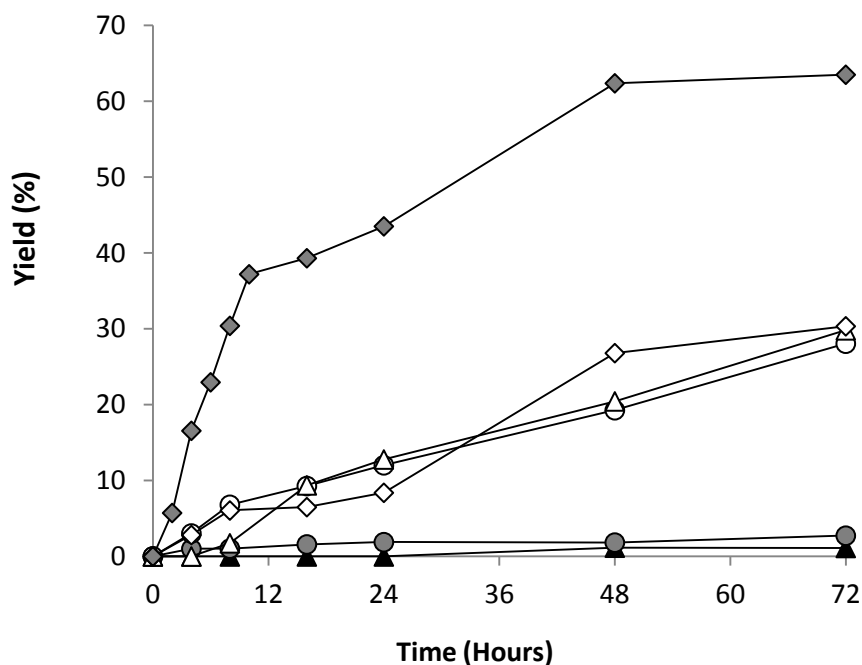
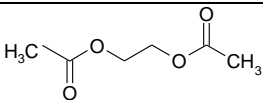
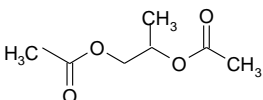
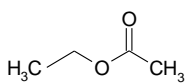
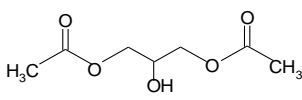


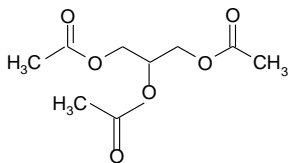
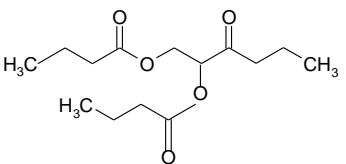
Fig 8. Time course of the chemo-enzymatic production of caprolactone in phosphate buffer (50 mM, pH 6) with perhydrolase CLEA (◆), CLEA CalB (▲), Novozyme 435 (●), and in ethyl acetate using Perhydrolase CLEA(◇), CLEA CalB(△)and Novozyme 435 (○).The reaction was carried out with 0.25 mmol of ethylene glycol diacetate as acyl donor, using a ratio cyclohexanone:hydrogen peroxide of 0.25 and 1% of CLEA perhydrolase enzyme suspension in phosphate buffer, 25 mg Novozyme 435 or 1.5 mg of CLEA CalB at 20 °C with 750 rpm stirring.

Table 1. Kinetic parameters for the production of peracid using CLEA perhydrolase

Parameter	Value
K_m	118 mM
V_{max}	2815 mM x min ⁻¹
K_m/V_{max}	23.85 min ⁻¹
K_{cat}	1.26 x 10 ⁷ min ⁻¹
K_{cat}/K_m	1.07 x 10 ⁵ mM ⁻¹ x min ⁻¹

Table 2. Conversion and yields of the chemo-enzymatic production of caprolactone using CLEA perhydrolase and different acyl donors.

Acyl donor	Structure	Conversion (%)	Yield (%)
Ethylene glycol diacetate		82	62
Propylene glycol diacetate		89	54
Ethyl acetate		23	1
Diacetin		83	52

Triacetin		86	45
Tributyryn		26	6

The reactions were carried out in phosphate buffer using 0.25 mmol of ethylene glycol, a ratio cyclohexanone:hydrogen peroxide of 0.25 and 1 % of enzyme suspension. The samples were stirred for 48 hours at room temperature with 750 rpm stirring.

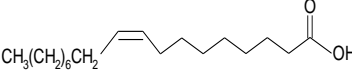
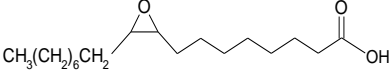
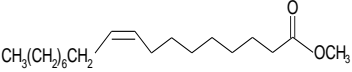
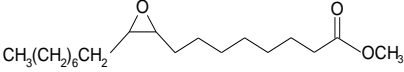
Table 3. Hydrolysis of caprolactone with different components and/or products of the reaction

Reaction component or product	Perhydrolysis (%)	
	Caprolactone	Caprolactone + CLEA perhydrolase
Buffer phosphate 50 mM pH 6*	0*	9*
Hydrogen Peroxide 100 mM	5	39
Peracetic acid 100 mM	52	59
Ethylene Glycol diacetate 100 mM+ Hydrogen Peroxide 100 mM	19	42
Ethylene Glycol monoacetate 100 mM + Hydrogen Peroxide 100 mM	52	59

*** Hydrolysis**

Caprolactone was stirred for 72 hours at room temperature at 750 rpm stirring containing 100 mM (final concentration) of the reaction or product component in a 10 ml vial. Reactions containing perhydrolase CLEA contained 1% of enzyme suspension.

Table 4. Epoxidation of oleic acid and its ester with peracid produced by CLEA perhydrolase.

Alkene	Epoxide	Yield (%)
 <p>Oleic acid</p>	 <p>Epoxy stearic acid</p>	44
 <p>Methyl oleate</p>	 <p>Epoxy stearic acid methyl ester</p>	26

The reactions were carried out in phosphate buffer using 0.25 mmol of ethylene glycol diacetate as acyl donor, a ratio cyclohexanone:hydrogen peroxide of 0.25 and 1 % of enzyme suspension.

