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The chemical industry has greatly contributed to the advancement of the human civilization. However, the side effects of this industry have become a real environmental burden. The message in this thesis is that enzymes have a great potential as catalysts for clean and sustainable processes. With three enzymes: an esterase, a lipase and a phytase, the thesis demonstrated how to rationally engineer the enzyme structure to improve its performance for a certain task, how to engineer an enzyme-based reaction to run solvent-free processes, the advantages of biocatalysts over conventional chemical catalysts, and the importance of green metrics in the evaluation of the developed processes. The integration of computer science, bioinformatics, molecular biology, and chemistry is traced throughout this thesis. Such an interdisciplinary approach is promising and necessary for the future development of biocatalysis and green chemistry.

Yasser Gaber is affiliated to Beni-Suef University, Egypt. He obtained his B.Sc. in pharmaceutical sciences and M.Sc. in microbiology from Cairo University. He started his Ph.D. studies in 2008 at the Biotechnology department, Lund University under the supervision of Professor Rajni Hatti-Kaul. His research is focused on enzymes, bioinformatics, biocatalysis, and green chemistry.



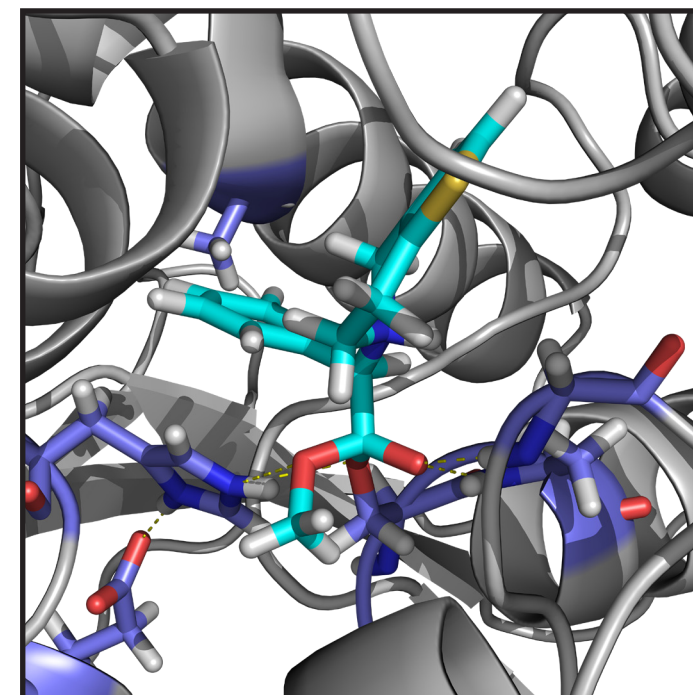
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Hydrolases as Catalysts for Green Chemistry and Industrial Applications

Esterase, Lipase and Phytase



Yasser Gaber

Doctoral Thesis





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Hydrolases as Catalysts for Green Chemistry and Industrial Applications

Esterase, Lipase and Phytase

Yasser Gaber*

Department of Biotechnology
Lund University

Doctoral Thesis
May 2012

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Academic thesis, which by due permission of the Faculty of Engineering of Lund University, will be publicly defended on Thursday May 24 at 10:30 a.m. in Lecture Hall A, at the Center for Chemistry and Chemical Engineering, Getingevägen 60, Lund, for the degree of Doctor of Philosophy in Engineering. The faculty opponent is Roger Sheldon, Professor Emeritus of Biocatalysis and Organic Chemistry at Delft University of Technology and Chief Executive Officer of CLEA Technologies B.V., Delft, The Netherlands.

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Cover picture:
Tetrahedral intermediate of clopidogrel R isomer and
a mutant esterase

Dedicated to
Soul of my father
My mother
My wife, Rasha
My children: Abdelrahman & Jody

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Key words Biocatalysis, rational design, PLE, esterase, clopidogrel, enantioselectivity, kinetic resolution, solvent-free, CalB, Novozym 435, green chemistry, metrics, biodegradable, b-propeller, phytate, metallo-enzyme, mutation, HPLC-EAT, sustainable environment			
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Abstract

The use of enzymes in industrial applications has been recognised for providing clean processes with minimal impact on the environment. This thesis presents studies on engineering of enzymes and enzyme-based processes in the light of green chemistry and environmental sustainability, and focuses on three hydrolases: esterase, lipase and phytase.

The use of esterase has been investigated to provide an alternative clean route for the synthesis of a chiral pharmaceutical compound, *S*-clopidogrel, by selective hydrolysis of the racemic precursor. Current production of the pure *S*-clopidogrel isomer involves the use of a resolving agent, *L*-camphorsulfonic acid, and organic solvents. Screening of different hydrolases revealed that crude pig liver esterase (PLE), a mixture of different isoenzymes, selectively acts on the *R* isomer, with $E = 8.3$. Two PLE isoenzymes, PLE-1 and PLE-3 were tested individually as catalysts for the reaction. Molecular modeling simulations indicated that a phenylalanine F407 residue is destabilizing for the *R*-isomer-PLE-1 tetrahedral intermediate. PLE-1 and its mutants were expressed in *Escherichia coli* with a chaperon system. Interestingly, mutations of F407 to alanine or leucine led to a dramatic increase in activity but with reversed selectivity ($E=3.3$ and $E>100$ respectively towards the *S* isomer). On the other hand, PLE-3 isoenzyme selectively hydrolysed the correct isomer, *R*, with $E=10$.

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The enzyme phytase hydrolyses phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate), an anti-nutrient compound present in cereals and grains, and increases the bioavailability of phosphorus and other nutrients. A recombinant thermostable *Bacillus* sp. MD2 alkaline phytase, a metallo-enzyme, was characterized and the effect of various divalent metal ions on its stability and catalytic properties was studied. The presence of calcium ions on both the enzyme and the substrate was required for optimal activity and stability of the enzyme. Furthermore, site-directed mutagenesis of the enzyme was done to improve its activity and stability in the acidic environment. Mutation of a glutamate residue in the enzyme active site to serine E227S led to a slight decrease of the optimum pH and higher stability at low pH.

List of papers

This thesis is based on the following research papers, which in the text are referred to by their Roman numerals:

- I. Gaber, Y.**, Ismail M., Takwa M., Amin, M. A., & Hatti-Kaul, R. (2012). Enzymatic asymmetric hydrolysis of racemic clopidogrel: rational design investigation (Manuscript).
- II. Gaber, Y.**, Törnvall, U., Orellana-Coca, C., Amin, M. A., & Hatti-Kaul, R. (2010). Enzymatic synthesis of N-alkanoyl-N-methylglucamide surfactants: solvent-free production and environmental assessment. *Green Chemistry* 12(10), 1817-1825.
- III. Åkerman, C.O., Gaber, Y.**, Ghani, N.A., Lämsä, M., Hatti-Kaul, R., (2011) Clean synthesis of biolubricants for low temperature applications using heterogeneous catalysts. *Journal of Molecular Catalysis B: Enzymatic* 72(3-4), 263-269.
- IV. Gaber, Y.**, Törnvall, U., Kumar, M.A., Ali Amin, M., Hatti-Kaul, R., (2011) HPLC-EAT (Environmental Assessment Tool): A tool for profiling safety, health and environmental impacts of liquid chromatography methods. *Green Chemistry* 13(8), 2021-2025.
- V. Tran, T. T., Hashim, S.O., Gaber, Y.**, Mamo, G., Mattiasson, B., & Hatti-Kaul, R. (2011). Thermostable alkaline phytase from *Bacillus* *sp.* MD2: Effect of divalent metals on activity and stability. *Journal of Inorganic Biochemistry* 105(7), 1000-1007.
- VI. Tran, T.T., Mamo, G., Búxo, L., Le, N.N., Gaber, Y.**, Mattiasson, B., Hatti-Kaul, R., (2011) Site-directed mutagenesis of an alkaline phytase: Influencing specificity, activity and stability in acidic milieu. *Enzyme and Microbial Technology* 49(2), 177-182.

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My contributions to the papers

Paper I. Prof. Rajni Hatti-Kaul initiated the project and I demonstrated the enantioselectivity of crude PLE under different co-solvent and temperature conditions. I prepared the homology model of PLE-1, and did the molecular modeling simulations. Dr. M. Takwa supervised the run and analysis of the simulations results, the selection of mutation sites and the experiments. I contributed partially to the other experiments that were mainly done by M. Ismail, and I wrote the draft of the manuscript.

Paper II. I performed the experiments under supervision of Dr. Cecilia Orellana-Åkerman, and wrote the manuscript with Dr. Ulrika Törnvall. Prof. Rajni Hatti-Kaul started the project and revised the manuscript.

Paper III. I was involved in the analysis of the results, writing and editing the manuscript.

Paper IV. I suggested the idea of the study, designed the first version of HPLC-EAT software and wrote the draft of the manuscript.

Paper V. I was involved in the interpretation of the metal-enzyme interaction and its effect on the enzyme stability and activity. I prepared the phytase structure and run the substrate-enzyme docking study.

Paper VI. I participated in interpretation of the mutation results, molecular modeling study and writing and editing the manuscript.

Abbreviations

EC	Enzyme Commission
3D	Three-dimensional
PDB	Protein Data Bank file format
PLE	Pig liver esterase
PLE-1	Gamma pig liver esterase
PLE-3	Isoenzyme of pig liver esterase
THI	Tetrahedral intermediate
E	Enantioselectivity; enantiomeric ratio; E-value
ee	Enantiomeric excess
CalB	<i>Candida antarctica</i> lipase B
N435	Novozym®435
E factor	Environmental factor (kg kg ⁻¹)
S ⁻¹	Mass index (kg kg ⁻¹)
Q	Unfriendliness quotient
EATOS	Environmental Assessment Tool for Organic Synthesis
EI _{in}	EATOS unit; Environmental Impact for input (PEI kg ⁻¹)
EI _{out}	EATOS unit; Environmental Impact for output (PEI kg ⁻¹)
HPLC-EAT	High performance liquid chromatography-Environmental Assessment Tool
MEG	Methyl glucamine
MEGA	N-alkanoyl-N-methyl glucamide
TMP	Trimethylolpropane
BPP	β-propeller phytase
HAP	Histidine acid phosphatase phytase
PAP	Purple acid phosphatase phytase
P _i	Inorganic phosphate

Amino Acid	Abbreviation		Amino Acid	Abbreviation	
	1 letter	3 letters		1 letter	3 letters
Alanine	A	Ala	Leucine	L	Leu
Arginine	R	Arg	Lysine	K	Lys
Asparagine	N	Asn	Methionine	M	Met
Aspartic Acid	D	Asp	Phenylalanine	F	Phe
Cysteine	C	Cys	Proline	P	Pro
Glutamic Acid	E	Glu	Serine	S	Ser
Glutamine	Q	Gln	Threonine	T	Thr
Glycine	G	Gly	Tryptophan	W	Trp
Histidine	H	His	Tyrosine	Y	Tyr
Isoleucine	I	Ile	Valine	V	Val

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1. Introduction

Environmental sustainability has become a global concern. The Earth's resources are continuously being depleted to meet the needs of the growing population and, on the other hand, the human activities have resulted in an enormous burden on the environment from toxic wastes and greenhouse gas emissions and the resulting climate change. The chemical industry is a keystone of human development that influences all aspects of modern society. The start of industrial revolution during the 19th century was dependent on coal and fossil oil as energy and carbon sources. Nowadays, fossil oil represents the main source of most of chemical products used by modern society, starting from plastics and rubber to perfumes and pharmaceuticals. Fossil oil is a finite resource as well as a source of environmental pollution. It is evident that we need to make efficient use of the available resources, reduce the dependence on fossil feedstocks and the environmental impact of the production methods and products [1-3].

In light of the problems of the fossil-based processes, the concept of green chemistry was formulated: "the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances." [4]; and a road plan for shaping the future of sustainable chemistry known as the twelve principles of green chemistry was presented by Paul Anastas and John Warner in 1991 [5]. These principles carry common-sense goals such as using safer chemicals, reducing the risk of the produced waste and replacing fossil resources with the renewable ones. Based on the volumes produced, chemicals are generally classified as bulk chemicals (produced in millions of tonnes), specialty chemicals such as surfactants, lubricants, adhesives, pigments and flavours (are produced in relatively smaller amounts), and fine chemicals and pharmaceuticals (produced in low volumes and to a very high purity) [6]. After two decades of having introduced the green chemistry concept, scientists have contributed with many novel solutions [7-9]. The use of catalysts, which are selective and recyclable, is one of the important principles of green chemistry [10].

Enzymes are powerful natural catalysts that have an enormous capacity to increase the speed of biochemical reactions. While un-catalyzed reactions might need several million years to take place, milliseconds are enough for enzyme-catalysed reactions [11]. Enzymes lower the activation energy required for the reaction to take place by forming a complex with the reactants and stabilizing the transition state of the reaction (Figure 1). Enzymes are present in all living organisms, catalysing the multitude of reactions that enable different functions

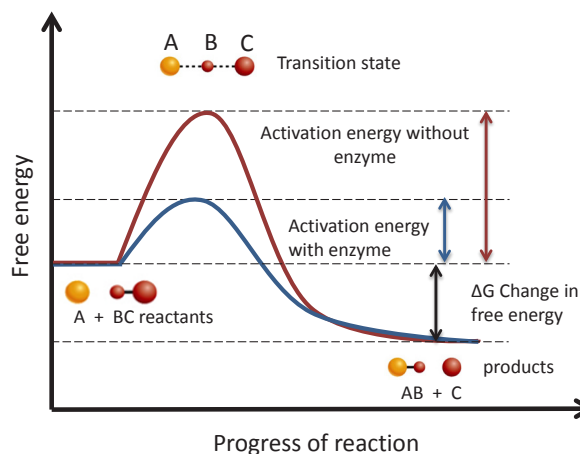


Figure 1 Illustration of the energy profile of an enzyme-catalyzed reaction (blue curve) and un-catalyzed reaction (red curve). The enzyme lowers the activation energy needed for the reaction to progress without affecting the overall free energy change (ΔG) [16].

and survival. The wonderful power of enzymes has been harnessed for applications in the food, chemical, pharmaceutical, and pulp and paper industries, detergent production, and preparation of animal feeds, among others. The market of the industrial enzymes is progressively growing; in the year 2000 it was estimated at \$1.5 billion and almost doubled in value by the year 2009 [12, 13]. This rapid development in the enzymes market is a results of the advances in the technology for discovering new enzymes from novel microorganisms isolated from different environments, the production of enzymes in heterologous microbial hosts, the development of enzyme variants by means of molecular biology, the advent of protein engineering, and also the improvement of the durability of enzymes by various techniques such as immobilization. Hydrolases are a group of enzymes that naturally catalyse the breakdown of molecules and account for the highest share of enzymes used for industrial purposes. Many industrial sectors such as the detergent, leather, textiles, pulp and paper, foods and feeds, dairy, and biofuels industries depend on hydrolases. In most of these applications, the use of enzymes results in reduced consumption of raw materials, energy, chemicals and water, and also reduced production of wastes and emission of greenhouse gases [14, 15].

Biocatalysis is the application of enzymes for the syntheses of organic compounds. The biocatalyst can be whole cells or the isolated enzyme. However, other biomolecules like antibodies, viruses and nucleic acids can also be used as

biocatalysts [17]. Enzymes as catalysts provide many benefits such as high regio-, stereo- and enantioselectivity, avoiding the use of toxic reagents and extreme reaction conditions. An example of green chemistry using biocatalysis is the clean synthesis of ethyl (*R*)-4-cyano-3-hydroxybutyrate (known as hydroxynitrile), a precursor for Lipitor®, the best-selling anti-lipidemic drug in the market. The conventional way for hydroxynitrile production involves the use of hazardous chemicals like hydrogen bromide and energy-demanding steps such as high-vacuum fractional distillation. An improved process involving three enzyme-catalyzed steps was developed by Codexis, Inc. for the production of this precursor compound [18]. The process runs under ambient pressure with increased productivity and is environmentally friendly and safer for the workers. In a more recent example, Merck developed an enzymatic method for the synthesis of the anti-diabetic agent sitagliptin. This method takes advantage of the selectivity of an engineered transaminase to catalyse the synthesis of a chiral amine from the prostereogenic ketone (non-chiral precursor). The traditional process for the production of sitagliptin involved a rhodium-catalyzed asymmetric enamine hydrogenation step run under high pressure, and a mass-intensive purification step [8, 10, 19-21].

1.1 Scope of the thesis

This thesis is based on six papers that include studies performed on three hydrolases, an esterase, a lipase and a phytase, respectively, with the objective of achieving efficient, selective and environment-friendly processes, and further highlight the use and development of user-friendly computational tools to determine the environmental impact of processes at the lab scale.

Paper I deals with the search for an enantioselective enzyme for resolution of racemic clopidogrel. Clopidogrel is among the best selling drugs in the world. In the traditional synthetic route, a large amount of resolving agent and organic solvents is used. Developing an enantioselective enzymatic process would be beneficial from an economic and environmental perspective. State-of-art tools were used to engineer the esterase from pig liver into an efficient enantioselective biocatalyst. The study is an application of the rational design strategy and employed molecular modeling simulations.

Successful examples of the use of enzymes for clean production of chemicals from renewable resources are presented in Papers II and III, using lipase B from the yeast *Candida antarctica*. The green chemistry perspective has been clearly highlighted in these two papers. The reactions were engineered to run the

synthesis under solvent-free conditions for synthesis of biosurfactants and biolubricants. Green metric computational tools were applied for lab scale evaluation of the processes. During the progression of these two studies, the need for a customized tool for the evaluation of the greenness of the liquid chromatography-based separation methods became evident and, thus, a free java-based software was developed for this purpose (Paper IV).

A thermostable phytase obtained from *Bacillus* sp. MD2 was studied and engineered in the context of its usability as a feed additive. In Paper V, a study aiming to reveal the effect of divalent metals ions bound to the enzyme and to the substrate phytate on the enzyme activity and stability was conducted. The phytase was also engineered to modulate its substrate specificity and optimum pH (Paper VI).

The following chapters will help give a background of the areas covered in the papers and will also summarise the outcome and potential of the investigations. Chapter 2 provides an introduction for hydrolase enzymes with focus on the three enzymes investigated in this thesis. The chapter presents briefly the important features of the catalytic mechanisms and the protein structures. Chapter 3 deals with the current status of protein engineering strategies including directed evolution and rational design. The tools available for rational design of enzymes such as molecular modeling, homology modelling, docking, and the new generation of protein databases are also presented. Furthermore, examples the methods used for the biocatalyst development and production, such as site-directed mutagenesis, heterologous expression in *E. coli*, and enzyme immobilization are concisely discussed. Chapter 4 presents the green chemistry principles, the concept of green chemistry metrics, biocatalysis in solvent-free media and the promising non-conventional media for green reactions. The case studies involving the three enzymes are summarised in Chapter 5. The thesis ends with concluding remarks and future perspectives in Chapter 6.

2. Hydrolases

Hydrolases are enzymes that catalyse the breakdown of chemical bonds in the presence of water. Hydrolases are classified as EC 3 according to the nomenclature system maintained by the *Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)*. In this thesis, three ester bond hydrolases (Table 1) were studied: the triacylglycerol lipase CalB (EC 3.1.1.3) and pig liver esterase (EC 3.1.1.1) for biocatalytic applications, and a phytase (EC 3.1.3.8) as a final enzyme product for use in animal feed industry [22]. Hydrolases in general have attractive properties for biocatalytic and industrial applications, e.g., they are co-factor independent, they accept unnatural molecules as substrates, possess high stereoselectivity, and the ability to carry out reversed reactions under water-free conditions [23, 24].

2.1 Esterase and lipase: α/β -hydrolase fold enzymes

One of the most common protein folds found in nature is that of α/β -hydrolases (Figure 2). This fold comprises many hydrolysing enzyme classes, for example lipases, esterases, dehalogenases and peroxidases [26]. Typically, this fold is composed of a central β -sheet enclosed by α helices. Catalytically, many of the enzymes in this group belong to serine hydrolases, which are characterized by a conserved catalytic triad formed by Ser, His and Asp or Glu. The nucleophile, e.g. Ser, is located in what is known as nucleophilic elbow which can be generally described by the conserved motif Sm-X-Nu-X-Sm (Sm = small residue, X = any residue and Nu = nucleophile). This motif restricts the mobility of the nucleophile residue and at the same time exposes it to the substrate and water

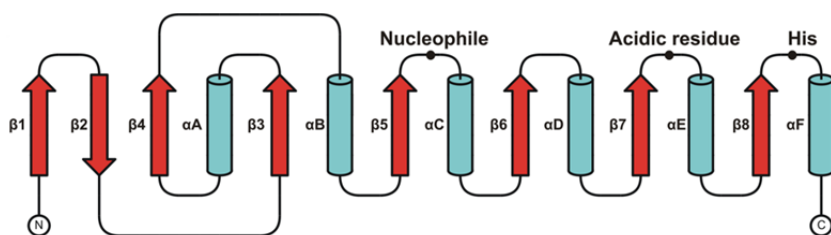


Figure 2 Illustration of the topology diagram of canonical α/β -hydrolase fold. The positions of the catalytic triad residues: His, acidic, and nucleophile (Ser in case of PLE and CalB) are indicated. Beta strands are shown in red and helices are shown in green cylinders. The figure is reprinted with kind permission from Springer [25].

during catalysis [27, 28].

The mechanism of serine hydrolases catalysis is illustrated in Figure 3 using the active site of pig liver esterase as a model [23, 27]. The enzyme catalysed reaction starts by the attack of the serine hydroxyl group on the carbonyl carbon atom of the acyl donor (e.g. carboxylic acid ester) to form the first tetrahedral intermediate, hereinafter, is referred to as THI. The generated oxyanion in the tetrahedral intermediate is oriented to what is known as the oxyanion hole, which is the GGGA(X)-motif in case of PLE. The oxyanion hole aids the stabilization of THI by formation of two hydrogen bonds; another hydrogen bond to the oxyanion is formed with Ala205, the residue following the nucleophile (Ser204). Subsequently, the leaving group (alcohol) is released and acyl enzyme complex is formed. The second substrate (water in the case of hydrolysis or an alcohol in the case of esterification/transesterification reactions) then attacks the carbonyl carbon atom to initiate the deacylation of the enzyme passing via the tetrahedral intermediate 2. Finally the free enzyme is regenerated and is ready for another catalytic round. Hult and Berglund reviewed the activation role of the oxyanion hole on the carbonyl double bond of the acyl donor before the serine attack. This

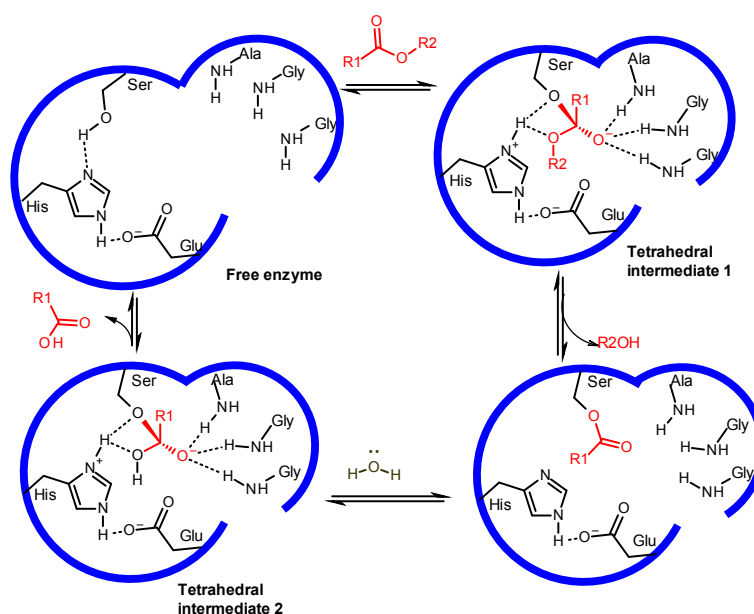


Figure 3 Reaction mechanism of ester hydrolysis by PLE (studied in Paper I) as a representative of α/β hydrolases and serine hydrolases. Catalysis occurs in two steps: acylation and deacylation. In each step, a tetrahedral intermediate is formed and stabilized by hydrogen bond formation from the catalytic His449, GGG(A)X-motif and Ala205.

activation mechanism was employed in a Michael addition reaction catalysed by a ser105 deleted CalB variant as an example of promiscuous lipase activity [29].

Pig liver esterase

Pig liver esterase (PLE) also known as hog or porcine liver esterase (EC 3.1.1.1), is an important enzyme among the few available esterases for biocatalytic applications [23, 31]. PLE is a carboxylesterase that acts as a scavenger of xenobiotics and the toxic esters passing the pig liver, and thus, it is characterized by broad substrate specificity, a desirable feature for organic synthesis. The first report of PLE application in organic synthesis was in 1903 by Dakin [32]. During 70s, biochemical characterization of the enzyme was done and three fractions of PLE (α , β , and γ) were described. The active form of PLE was described as a trimer mainly formed by γ and α fractions [33-35]. PLE was extensively exploited in chiral chemistry because of its ability to asymmetrically hydrolyse many



Figure 4 Homology model of PLE-1 used in Paper I, the two helices (blue and yellow) in the front view are located on the entrance to the active site [30]. The catalytic triad residues Ser204, His449 and Glu336 as well as their hydrogen bonds in the free state of the enzyme are shown.

racemic mixtures [36]. These applications were augmented thanks to the commercial availability of the enzyme. However, the fact that the preparation is a mixture of different isoenzymes extracted from the pig liver is a limitation for most of synthetic applications [37]. The mixture form of PLE is a limitation for most of synthetic applications. Different isoenzymes have different specificities and enantioselectivities towards the same substrate. Recently, by means of mRNA extraction from the pig liver, reverse-transcription, cDNA synthesis, and expression in *Pichia pastoris* or *Escherichia coli*, 7 isoenzymes belonging to PLE could be characterized: PLE-1 (γ -PLE), PLE 2-6 and PLE-7 (alternative PLE) [38]. The sequence differences in these isoenzymes were presented by Hasenpusch et al. [30]. Recently, the Bornscheuer and Schwab research groups have contributed towards the characterization of PLE isoenzymes [38-42] and a ton-scale application based on one of the PLE isoenzymes has been implemented by DSM Pharma chemicals [43]. PLE isoenzymes 1-6 are also commercially available from the Enzymicals Company based in Greifswald, Germany.

The crystal structures of the PLE isoenzymes have not yet been determined. However, the structures of homologous carboxylesterases from human and rabbit (PDB: 1MX9 and 1K4Y) were solved [44, 45]. In Paper I, the homology model of PLE-1 based on the human carboxylesterase was built and used for the molecular modeling studies (Figure 4). The structure is stabilized by two disulphide bonds. Two helices form the entrance of the active site of PLE-1 and their amino acid sequence varies in different PLE isoenzymes. These helices contribute to the PLE isoenzymes selectivities to different substrates [30, 46]

***Candida antarctica* lipase B, CalB**

Candida antarctica is an extremophile yeast isolated from a hypersaline lake in Antarctica. It produces two lipases, CalA and CalB [47]. CalB is a small protein of 317 amino acids (Figure 5). CalB is not a typical lipase, as it features a mix of esterase and lipase properties. In general, there are more similarities between lipases and esterases than differences as both act on the same set of substrates. However, esterases act on small molecules and short-chain triacylglycerols, whereas lipases act on long-chain triacylglycerols. One main difference between esterases and lipases is the interfacial activation phenomenon encountered in lipases. Consistently with their natural physiological role, lipases have the ability to work in water-oil emulsion systems. The contact of the lipase enzyme with the oil phase induces the opening of a lid that covers the enzyme active site allowing catalytic activity. Due to this lid-activation phenomenon, lipase kinetics cannot be described using the Michaelis-Menten model. The substrate should reach a critical

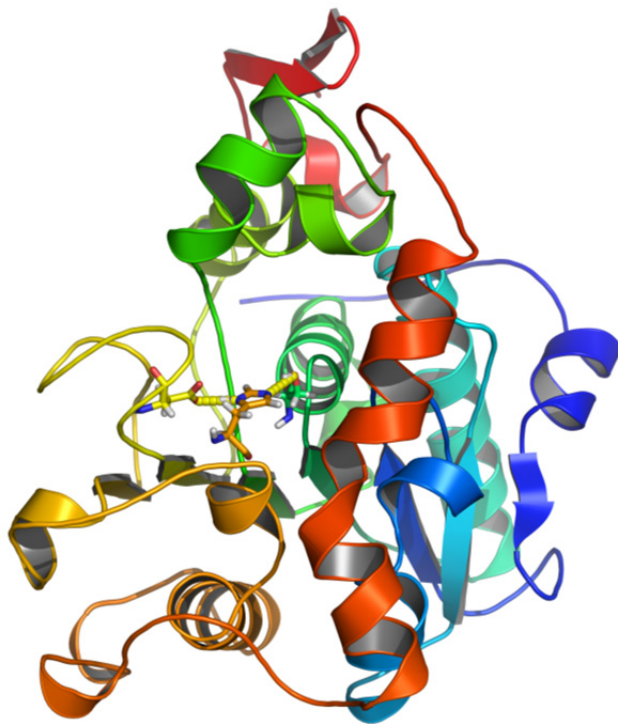


Figure 5 The 3D structure of CalB, the catalytic triad residues Ser105, His224 and Asp 187 and their hydrogen bonds in empty enzyme state are shown. The figure is prepared using PyMOL based on PDB: 1TCA.

concentration before the activation takes place. In this context, *C. antarctica* lipase B is an exception as it lacks the lid on the active site, and can work without the need of interfacial activation [48]. CalB is described as a promiscuous enzyme as it is able to catalyse different kinds of reactions such as esterification (Paper III) transesterification [49], epoxidation [50], amidation (Paper II, [51]), aldol addition [52], perhydrolysis [53] and Michael addition [54]. The catalytic mechanism of CalB is similar to the mechanism of PLE described previously. The catalytic triad is formed of Ser105, His224, Asp187 and the oxyanion hole is formed by Thr40 and Gln106. The crystal structure of CalB was reported by Uppenberg et al. [55, 56]. Klivanov et al. [57] demonstrated the ability of lipases to act in organic solvents, opening the door for organic synthesis and setting a milestone in biocatalysis.

2.2 Phytases

Phytate is the main storage form of phosphorous in plant seeds, representing 75-85 % of their total phosphorus content [58]. Its Chemical composition consists of 6 dihydrogen phosphate groups esterified to myo-inositol (myo-inositol hexakisphosphate). Phytases are hydrolases that dephosphorylate phytate into less phosphorylated myo-inositol and inorganic phosphorus. Phytases are thus important for the germination process of the plant as they restore phosphorus, which is required for ATP synthesis. Phytases are found in plants, microorganisms and some animals. Yeasts, bacteria and fungi represent an important source of phytases for biotechnological applications [59]. Phytases can be classified based on their mode of activity on phytate or on their catalytic features. According to NC-IUBMB phytases are classified into two groups: 3-phytases (EC 3.1.3.8), which start hydrolysis in the position number 3 of phytate and are mostly found in microorganisms; and 6-phytases (EC 3.1.3.26), which hydrolyse the phosphate at the position number 6 in phytate and are mostly found in plant tissues with some exceptions (*E. coli* phytase is a 6-phytase) [59]. Based on their catalytic features, phytases can be divided into four classes: histidine acid phosphatase (HAP) phytases, protein tyrosine phosphatase PTP phytase, purple acid phosphatases PAPs and β -propeller phytases (BPPs). HAPs

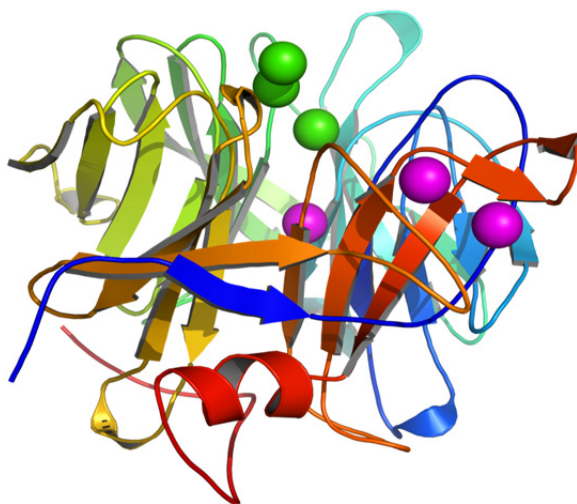


Figure 6 β -propeller phytase is illustrated based on PDB: 2POO, calcium atoms in the high-affinity binding site are shown as spheres in pink, and calcium atoms in the low-affinity binding site are shown as green spheres. The figure is prepared using PyMOL.

are the most important phytases from the commercial point of view and widely used in the animal feed industry [60]. HAP phytases have the conserved RHGXRRP motifs for performing the catalytic activity in two steps: the nucleophilic His residue attacks the phosphorus atom followed by hydrolysis of the phosphorus-histidine intermediate [61].

β -propeller phytases

Alkaline β -propeller phytases (BPPs) are metallo-enzymes widely distributed in the nature. Compared to HAPs, they are less studied. As opposed to HAPs, BPPs are active in neutral and alkaline conditions and possess high thermostability. These are attractive properties for developing phytases as animal-feed additives as they would withstand the high-temperature pelleting process during used in the formulation and preparation of the feeds. *Bacillus* species are the main source of phytase sequences deposited in the protein databanks and a number of structures have been solved during the last decade [62, 63]. The structure is composed mainly of β sheets in the shape of a propeller. Five four-stranded and one five-stranded sheets are the main components of BPB (Figure 6). One important feature of BPPs is their dependence on metal ions such as Ca^{2+} for their catalytic activity and stability. Two locations for the metals were found in the phytase structure from *Bacillus amyloliquefaciens*: high-affinity and low-affinity binding sites. The catalytic mechanism of BPB, Figure 7, was described by Oh et al. and Shin et al. [64, 65]. BPB has two sites: the cleavage site (P1) and the binding site (P2). The model for these two sites was suggested by Shin et al. to describe the phytate binding and catalytic action of BBP. A water molecule activated by two Ca^{2+} atoms in the cleavage site forms the nucleophilic moiety for attacking the

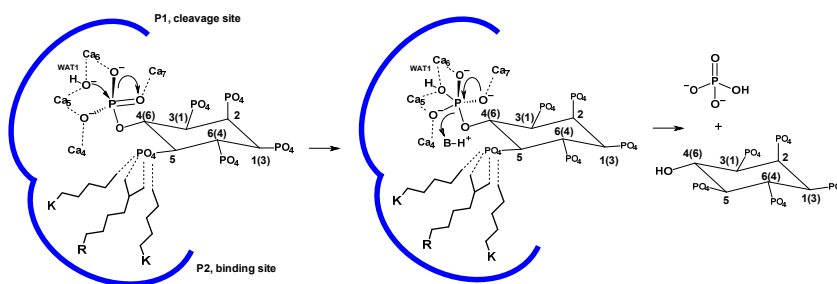


Figure 7 Proposed mechanism of BPP phytase. Two phosphates groups occupy the cleavage site and binding site simultaneously. Water molecule (WAT1) activated by two calcium atoms: Ca5 and Ca6 is acting as a nucleophile for attack the phosphorus atom of the phosphate group to initiate the pentavalent transient state and 4 Ca^{+2} ions in the cleavage site, stabilize the formed transient pentavalent phosphate.

2. Hydrolases

phosphorous atom. The binding site consists of basic residues coordinating to the negatively charged phosphate group adjacent to the phosphate group sitting in the cleavage site. A confirmation of this model was recently provided by Zeng et al. [62].

Table 1 Summary of the features of hydrolases used in this thesis

Feature	PLE-1	CalB	MD2 phytase
Class	EC 3.1.1.1	EC 3.1.1.3	EC 3.1.3.8
Synonyms	Gamma pig liver esterase,	Lipase B	beta-propeller phytase
Origin	<i>Sus scrofa</i> (Pig)	<i>Candida antarctica</i>	<i>Bacillus</i> sp. MD2
Uniprot code	Q29550	P41365	D2JYH3
No. of a.a	544	317	354
Metals and ions	None	None	Ca ²⁺
Isoenzymes	PLE 2-7	CalA	Non
Protein structure class	α/β hydrolase	α/β hydrolase	β -propeller
PDB	Non	1TCA, 1LBT	Non
Related PDB	1MX9, 1K4Y		2POO, 3AMR, 3AMS
Catalytic nucleophile	Ser204	Ser105	Ca-activated water
Transition state stabilizing residues	GGG(A)X-motif, Ala205	Thr40, Gln106	Calcium atoms: Ca4, Ca5, Ca6, Ca7
Used or studied as	Soluble & immobilized on His Mag Ni Sepharose TM beads	Novozym®435 (immobilized preparation)	Soluble form
Applications	Kinetic resolution of racemic clopidogrel	Solvent-free production of a biosurfactant and biolubricatns	As final product enzyme for food and feed industry
	Paper I	Papers II & III	Papers V & VI
References	[30, 33, 38, 41, 43]	[56, 66-68]	[62, 69-71]

3. Enzyme engineering: strategies and methods

Enzymes in their native form suffer several limitations for use as industrial catalysts, such as instability at high temperature, narrow substrate spectrum or insufficient enantioselectivity. These limitations may be overcome by engineering the enzyme at the genetic level or by modification of the enzyme without structural changes e.g., immobilization to a support. The former approach is commonly termed protein engineering, and involves introduction of changes in the gene coding for the enzyme, e.g., by rearrangement of the order of the amino acid sequence (circular permutations) or by introducing mutations either randomly or rationally. In general, protein engineering can be achieved via three main strategies: directed evolution, rational design and a hybrid of these two approaches known as semi-rational approach (Figure 8). The application of these tools has resulted in the successful engineering of biocatalysts from different enzyme families and has been reviewed elsewhere [8, 26, 80-84].

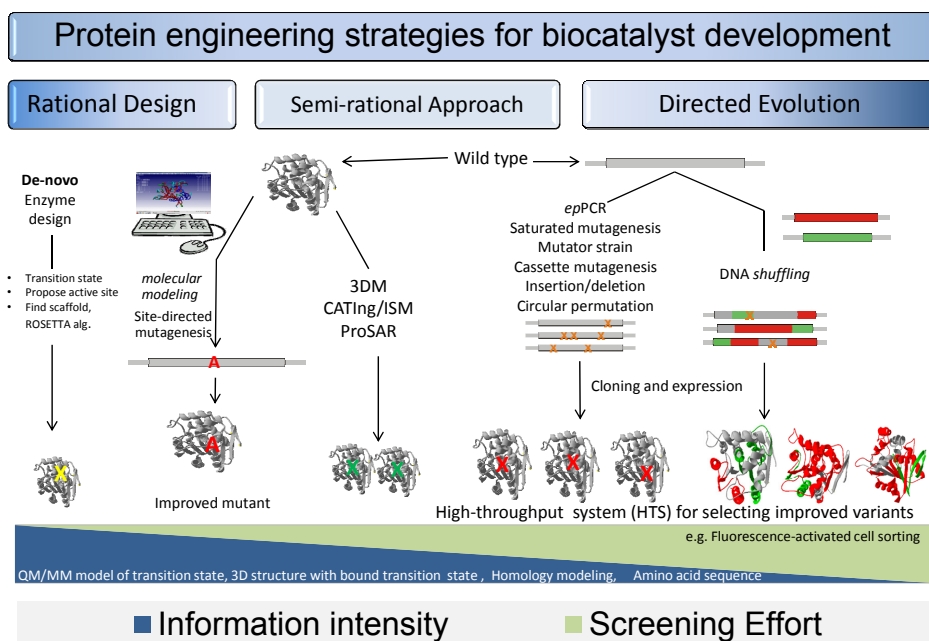


Figure 8 Illustration of the main strategies utilized for engineering proteins for the development of new biocatalysts. The acronyms are as follows: epPCR, error prone PCR; CASTing, Combinatorial Active Site Saturation Test; ISM, iterative saturation mutagenesis; ProSAR, Protein Sequence Activity Relationship [72-79].

3.1 Directed evolution

The directed-evolution approach is characterized by two main features: random generation of mutations resulting in a library of mutants, and a good screening method for the desired mutants. The screening could be done either by manually picking colonies from agar plates or by employing advanced methods like fluorescence-activated cell sorting, which allows screening of a large number of cells [85]. One way of creating a library is based on gene shuffling in which the starting genetic material comprises different genes that are related to each other but differ in some features like thermostability and substrate specificity. The library produced will contain various chimeras of these genes with new properties (Figure 8). Gene shuffling has led to the development of many successful biocatalysts with increased thermostability or improved activity [72]. Another method for library generation is to start with a single sequence which is mutated by, for example, error-prone PCR, mutator strain, circular permutation, or saturation mutagenesis [72, 86-88].

3.2 Rational design

Rational design in protein engineering science refers to the use of structural and mechanistic information to modify a protein. For the purpose of rational design, the three-dimensional (3D) structure should be known as it is essential in understanding at the molecular level the protein function and interactions with other protein or ligands (substrates). The protein structure is determined using either X-ray crystallography techniques or multidimensional NMR in the case of small proteins [89]. Many enzyme structures have been solved at high resolution, which has paved the way for using computer modeling for protein engineering. In addition, the advances of molecular modelling software, new generation of databases and computational tools allow non-experts to perform rational design for protein engineering. The approach of rational design was successfully applied in many cases to engineer enzyme selectivity and activity, [Paper I, [90]. Rational design has reached advanced levels with the help of quantum mechanical calculations and information about the reaction mechanism. For example, Baker and co-workers have designed “de-novo” enzymes for reactions without known natural enzymatic catalyst [79, 91, 92].

3.2.1 Homology modeling

More than 6 million protein sequences are deposited in public databases nowadays, of which only 72 000 sequences have solved structures. This urges the

computational biology scientists to develop algorithms to predict the 3D structure of proteins from their amino acid sequences. One effective approach is the prediction of the structure based on an already known structure (template) sharing similarity with the target sequence (40 % or higher). The process of homology modeling involves the following steps: secondary structure prediction, template identification, alignment of the target sequence over the template, model building, refinement and evaluation of the model. Bordoli et al. [93] have described in detail the use of the SWISS-MODEL web server for obtaining a homology model for a protein sequence. In Paper I, the Yasara software was used for building a homology model of PLE-1 using mainly human carboxyl esterase (PDB 1MX9) as a template [94].

3.2.2 Molecular Modeling

Molecular modeling is an *in-silico* simulation of the behaviour of the molecules, which requires devising a number of equations and algorithms describing inter- and intra-molecular interactions. Application of quantum mechanics equations is highly accurate in describing the atom behaviour in molecules, especially of small molecules. However this approach is expensive in terms of computational processor units. Instead, simplified algorithms based on the molecular mechanics approach have been successfully used in a number of softwares. For example, AMBER, Assisted Model Building with Energy Refinement, is a family of force fields, i.e. a set of parameters and equations that describes the behaviour of atoms during the simulations, widely used for proteins and DNA simulations studies.

What will a molecular modeler look for in a rational design experiment? For illustration, the case presented in Paper I was about improving enzymatic activity of PLE-1 selectively towards the *R*-isomer of clopidogrel. The molecular modeling experiments were performed to investigate the enzyme-substrate complex formed during the catalytic process (THI, Figure 3) using the molecular modeling software Yasara [94, 95]. The positioning of the catalytic residues relatively to the substrate in terms of proper angles and distances was checked to determine the compliance with the criteria required for obtaining a stabilized THI conformation [96]. The THI conformation that shows the typical hydrogen bond pattern according to Uppenberg et al. [55] is termed productive binding mode. Steric hindrance observations and/or improper hydrogen bond network in the THI are important criteria to be considered during the modeling experiments [90, 97]. The geometry of the THI was examined to identify sources of instability. As seen in Figure 9, a steric problem was identified due to the presence of the substrate chlorophenyl ring between F407 and His449 residues. The chlorophenyl

ring displaced the His449 imidazole ring downward and prevented the formation of the catalytically required hydrogen bonds (Figure 3).

In silico mutation of F407 to Ala followed by molecular modeling simulations of the THI indicated that His449 formed the two hydrogen bonds with the oxygen atoms of the leaving group and the Ser204. The imidazole ring of His449 was rotated to be activated by E452 instead of E336 (Figure 10). The results of simulations also indicated that the oxyanion was stabilized by G126 from the motif GGG(A)X and A205 (Figure 10). Experimentally, this mutation PLE-1-F407A resulted in a dramatic improvement in the enzymatic activity as described in Paper I.

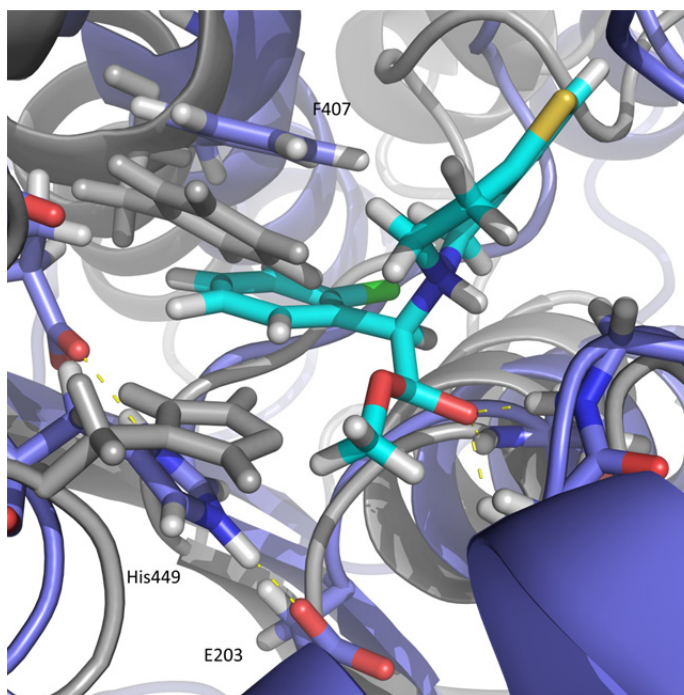


Figure 9 Molecular modeling simulation (Paper I). The PLE-1 homology model without substrate (grey) is superimposed on PLE-1 (blue) linked to the R-isomer of clopidogrel (cyan) forming the tetrahedral intermediate THI. The chlorophenyl ring of the substrate was situated between F407 and His449. Due to steric effect, the His449 imidazole ring was displaced by a value of 2.59 Å from its original position and did not form the hydrogen bonds with the oxygen atoms of the Ser204 residue and the leaving group (methanol) of the substrate which are needed of the THI stability.

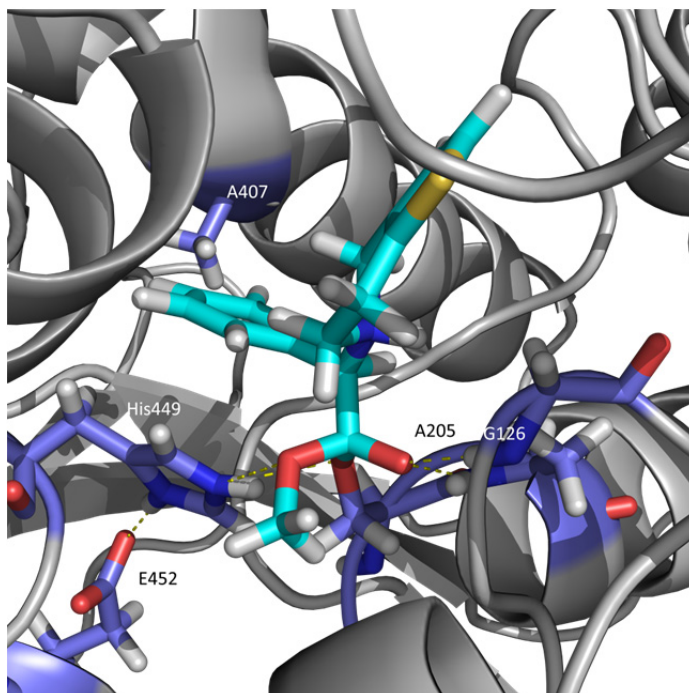


Figure 10 *In-silico* mutation of the F407 in PLE-1 to Ala showed correction of the His449 positioning in the active site and formation of two hydrogen bonds with the oxygen atoms of the leaving group (methanol) and Ser204. The THI oxyanion formed two hydrogen bonds with G126 and Ala205. His449 formed hydrogen bond with E452 instead of E336. The figure is reproduced from Paper I.

3.2.3 Docking

Docking refers to the prediction of the conformation that a small molecule (ligand or substrate) will acquire in the active site of a receptor macromolecule (enzyme, other types of protein or DNA), and estimation of the binding affinity [98]. The docking technique was initiated for purposes of drug discovery i.e. *in-silico* screening of a molecule library against certain receptors to find potential hits. AutoDock4 is one of the most commonly used docking softwares [98]. A more recent and improved version, AutoDock-Vina, has higher accuracy and shorter calculation time. Recently, AutoDock and AutoDock-Vina have been combined in a software package, PyRx, which allows easy running of the docking experiments [99-101]. For example, in Paper V, AutoDock 4 was used for studying the effect of the metals present in the active site of the *Bacillus* sp. MD2 phytase on the conformation acquired by the substrate phytate. In the case of phytase loaded with Ca ions, the docking results indicated that phytate fulfils the criteria required for catalysis (Figure 7): one phosphate group occupied the

cleavage site formed by Ca4, Ca5, Ca6 and Ca7 ions and an adjacent phosphate was accommodated by the binding site (formed by residues K76, K77, R122) [102]. These docking results are in line with the observations reported recently by Zeng et al. [62].

3.3 Semi-rational approach

Each of the aforementioned strategies for enzyme engineering has pros and cons. The directed-evolution approach is very powerful in finding the required enzyme variant. However, a good screening method is essential. In the case of rational design, such highthroughput screening methods are not necessary, but, a minimum level of information about the enzyme structure and mechanism of action is needed. Some approaches combine features from these two approaches in what is known as semi-rational or combined approach. Reetz et al. have developed methods such as combinatorial active site saturation mutagenesis (CASTing) and iterative saturation mutagenesis (ISM) for improving enzymatic activity or selectivity. These methods apply saturation mutagenesis on defined locations inside the active site of the enzyme [76, 77, 103-105].

New generation of protein databases

The last two decades have witnessed an explosion in the amount of biological data thanks to the advances in the DNA sequencing technologies, X-ray crystallography and computational biology. As a result, storing and retrieving data in a convenient and proper way has become a necessity. The protein data bank (www.rcsb.org), Uniprot, www.uniprot.org, PubMed, and other publicly available databases represent first-generation databases which depend on annotated submitted sequences or structures. Linking the information of related proteins together is hampered by the variation in numbering schemes of sequences and structures. 3DM is an example of a new generation of databases which extract new information based on the data already available in the current databases. The principal idea behind 3DM is to align structurally related proteins in a superfamily from which a consensus core is deduced. Related protein sequences are then collected and added to the superfamily. A 3DM numbering scheme is given to the core to facilitate the correlation between amino acids having similar 3D positioning in different structures. The Mutator software collects the mutation information in literature and links it to the protein sequences in the superfamily. The 3DM database of α/β hydrolase fold enzymes has been successfully applied in engineering an esterase for improving its thermostability [106].

3.4 Site-directed mutagenesis

There are many well-established techniques for introducing mutations in target genes, such as point mutation and saturation mutagenesis. Point-mutation mutagenesis refers to the introduction of a single amino acid change in the protein sequence by swapping the corresponding DNA codon while saturation mutagenesis involves replacing the amino acid by all the other 19 amino acids to generate all possible mutations at that position. The QuikChange Site-directed mutagenesis kit (Stratagene) provides an easy-to-use method to mutate a specific site in a gene (Figure 11). The site-directed mutagenesis methodology was applied in Paper I for preparing five single mutations and in Paper VI for preparing 7 single and one double mutation [107].

3.5 *Escherichia coli* as an heterologous protein-expression system

E. coli is the workhorse for lab-scale protein production. The organism has many advantages as a protein-expression system: it grows fast, requires inexpensive culture medium, and as it is a well-studied organism there is ample amount of information available [108]. On the other hand, it lacks some desirable features found in other systems like yeast or fungi, (e.g. the ability to perform post-translational modifications). The PLE-1 used in Paper I is an enzyme from an eukaryotic organism and it has two

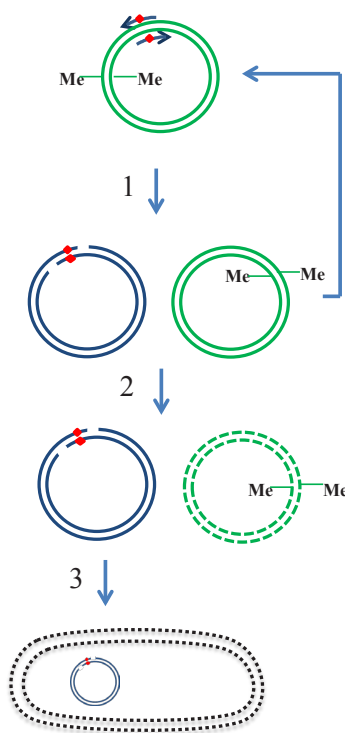


Figure 11 Illustration of QuikChange site-directed mutagenesis technique used in Papers I and VI. Parenteral methylated DNA is shown in green and the primers including mismatching base pairs in the middle are indicated in red. In step 1, PCR extends primers to daughter nicked plasmid. In step 2, Dpn I digests methylated parenteral DNA and other methylated DNA present in reaction mixture. In step 3, transformation of mutated DNA to XL10-Gold Ultracompetent Cells is done to repair the nick present in the daughter plasmid. Figure is modified from Stratagene 2009 [107].

Paper I is an enzyme from an eukaryotic organism and it has two disulphide bonds stabilizing the structure. For its cytoplasmic overexpression, the *E. coli* strain Origami™ 2DE(3) was utilized as it offers an oxidizing environment in the cytoplasm by deletion of two genes from its chromosome: thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) [109, 110]. PLE-1 was sub-cloned into the pET15b vector fused to a His-tag sequence at the N-terminus. In addition, simultaneous expression of the GroEL-GroES chaperon encoded in the pGro7 plasmid was used to aid in the proper folding of the overexpressed enzyme (Figure 12). The procedures published by Bornscheuer and co-workers for the expression of PLE-1 were followed [39, 41, 42] except that a codon-optimized synthetic gene was used. Codon optimization is an important factor for the heterologous expression of eukaryotic genes in lower organisms like *E. coli* as indicated by Welch et al. [111].

In Paper V and VI, a *Bacillus* MD2 phytase was overexpressed in *E. coli* BL21 (DE3). The gene was inserted in the multiple-cloning site of the pET22b(+) vector and fused to a *pelB* leader peptide sequence at the N-terminal end. The leader peptide enabled the expressed enzyme to be exported to the periplasmic space [112]. The enzyme was accumulated in the periplasmic space and stimulated loss of membrane integrity and subsequently leakage of the enzyme to the extracellular space, which is desirable for downstream processing [69, 113].

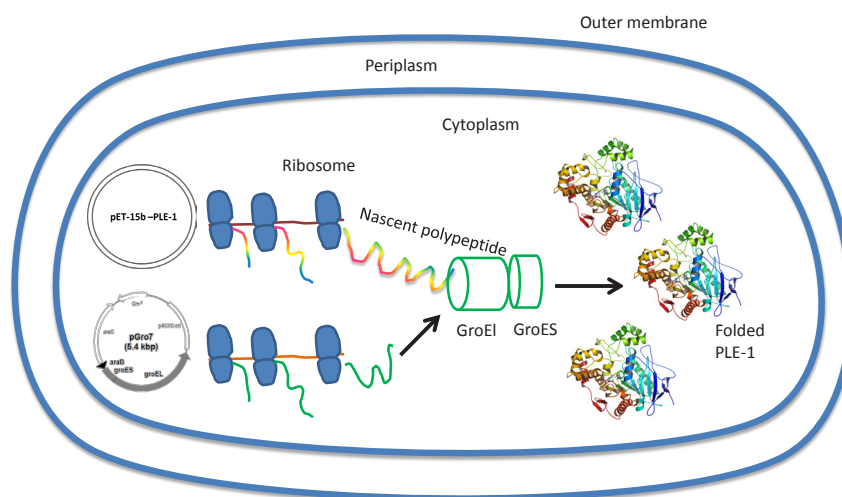


Figure 12 Illustration of expression of PLE-1 sub-cloned in the pET-15b plasmid in *E. coli*. The chaperone system GroEL-GroES is encoded by the pGro7 plasmid and helps the nascent polypeptide be folded correctly, Paper I.

3.6 Enzyme immobilization

Enzymes are preferably used in immobilized form in many applications to enable easy handling, recycling, improved stability, and, hence, process economy. Immobilized enzymes can be prepared by various techniques including non-covalent adsorption to solid matrices, covalent coupling to active groups on matrices, and cross-linked enzyme aggregates or crystals [114-116]. When choosing the immobilization technique, simplicity, stability of the enzymes and cost efficiency should be considered.

Novozym®435 is the commercially immobilized preparation of the *Candida antarctica* lipase B CalB. CalB immobilization is performed by hydrophobic adsorption on a macroporous acrylic resin. Novozym® 435 has shown very good operational stability under different experimental conditions (Papers II and III). The applications are restricted to non-conventional media such as organic solvents, ionic liquids and supercritical fluids as there is a risk of enzyme leaching in aqueous media. However, hydrolysis reactions can be run in organic solvents under controlled water activity. In Paper I, hydrolysis of racemic clopidogrel was achieved with Novozym 435 in an organic solvent (*t*-amyl alcohol) at controlled water activity of 0.3. Mora-Pale et al. [67] have reported enhanced hydrolysis of lutein diesters using Novozym®435 under very low water activities. Novozym®435 is stable up to 150 °C as reported by Lozano et al [117]. However, 50-60 °C is recommended by the manufacturer for long-term applications [118]. The cost of Novozym®435 represents a great limitation for scaling up lab-scale processes. However for fine chemicals and specialty chemicals with high degree of purity the cost factor is not significant.

In Paper I, His-tag linked to the overexpressed PLE-1 and its mutants, was used to achieve enzyme immobilization and purification all in one step. Commercially available cross-linked sepharose beads with magnetic properties and derivatized with Ni²⁺ ions were used for protein capture [119, 120]. Sampling during the reaction was done by the use of a magnet without the need for centrifugation. Nguyen et al. [121] have described the use of a similar preparation for setting up a small-scale high-throughput protein purification and screening system.

In the absence of a solid carrier, the enzymes can be insolubilized by crosslinking the enzyme aggregates obtained by addition of reagents that reduce protein solubility such as ammonium sulphate. Cross-linked enzyme aggregates (CLEAs) of several different enzymes have been prepared. The cross linking can

be achieved by using glutaraldehyde, or other milder cross-linkers like dextran polyaldehyde. Cross linking has also been done for enzyme crystals in the form of cross-linked enzyme crystals CLECs. CLECs have many useful applications and a good stability profile but not all enzymes can be crystallized easily and, in addition, the preparation procedures are not as easy as in the case of CLEAs [116, 122].

4. Green chemistry and biocatalysis

The initiative of Anastas and Warner for guiding the chemical industry to less hazardous practices has been welcomed by chemists, industry and policy makers. The increasing number of publications dealing with developing new routes for the clean, green, and sustainable synthesis of chemicals is a clear indication of this. The twelve principles set as a guide for Green chemistry as defined by its founders [5] are:

1. **Prevention**
It is recommended to prevent or minimize the waste generated from chemical synthesis.
2. **Atom Economy**
Design of the chemical reaction to incorporate all/most of the starting material into the desired product
3. **Less Hazardous Chemical Syntheses**
Design of chemical synthesis should consider use and generation of substances of low or no toxicity to human health and the environment.
4. **Designing Safer Chemicals**
The chemical product should be designed to obtain the effect with minimum toxicity.
5. **Safer Solvents and Auxiliaries**
Auxiliary substances commonly used in chemical synthesis such as solvents should be minimized or, whenever possible, omitted from the reactions, otherwise be innocuous when used.
6. **Design for Energy Efficiency**
If possible, chemical processes should be run at ambient temperature and pressure. Generally, the energy requirements of chemical processes should be defined for their environmental and economic impacts and should be minimized.
7. **Use of Renewable Feedstocks**
Use of renewable raw material or feed stock is preferred over the use of depleting resources.
8. **Reduce Derivatives**
It is common in chemical synthesis to use derivatization, protection/deprotection, and blocking agents. These practices should be avoided or minimized as much as possible.
9. **Catalysis**
Stoichiometric reagents are not preferred; however, it is better to use selective catalysts.
10. **Design for Degradation**

The fate of the produced chemical in the environment should be considered; hence design of biodegradable chemicals is of benefit to the environment.

11. **Real-time analysis for Pollution Prevention**

It would be beneficial to have in-line monitoring and real-time analysis of the processes to follow the formation of the hazardous substances to minimize or eliminate it.

12. **Inherently Safer Chemistry for Accident Prevention**

Design the process, chemicals and their forms (solid, liquid, gas) to minimize the risk for chemical accidents, like fire, explosions, and release to the environment.

4.1 Green chemistry metrics and software

The ultimate aim of green chemistry is the reduction of waste and risk of chemical processes, and enhancement of the process sustainability. The achievement of these goals needs a set of efficient green metrics to evaluate the processes. One early approach for quantitative description of chemical reaction greenness is the (E)nvironmental factor suggested by Sheldon in the late 1980s. The E factor is the ratio of waste mass generated per mass of product obtained. In the bulk chemicals industry, the E factor is 1-5, while in the pharmaceutical industry the E factor is in range of 25-100 due to the large amounts of solvents and other auxiliaries required in the purification processes to produce highly pure chemicals. The oil refining and bulk chemical industries have the lowest E factor, < 0.1 and 1-5 respectively. It is important to note, though, that the environmental impact of 1 kg of hexane as waste is not the same as that of 1 kg of water waste. Therefore, Sheldon recommended coupling the use of the E factor to an environmental quotient that describes how environmentally harmful the waste is [7, 123]. Atom economy, introduced by Trost in 1991 [124], is another metric for describing the efficiency of a chemical reaction and it is a measurement of how many of the reactant atoms are incorporated in the desired product. This metric is in agreement with Principle number 2 of the Green Chemistry principles. A reaction of 100 % atom economy will incorporate all the input substrates into the final product.

Developing green, environmentally-friendly processes or syntheses needs to be accompanied by a valid quantitative evaluation of the methods. Tools developed for such purpose can be divided into two broad categories: holistic and complex tools represented by life cycle assessment (LCA) and simpler tools that provide an easy-to-use guide about the greenness of a certain process like GREENSCOPE (Gauging Reaction Effectiveness for the Environmental Sustainability of Chemistries with a multi-Objective Process Evaluator), EHS

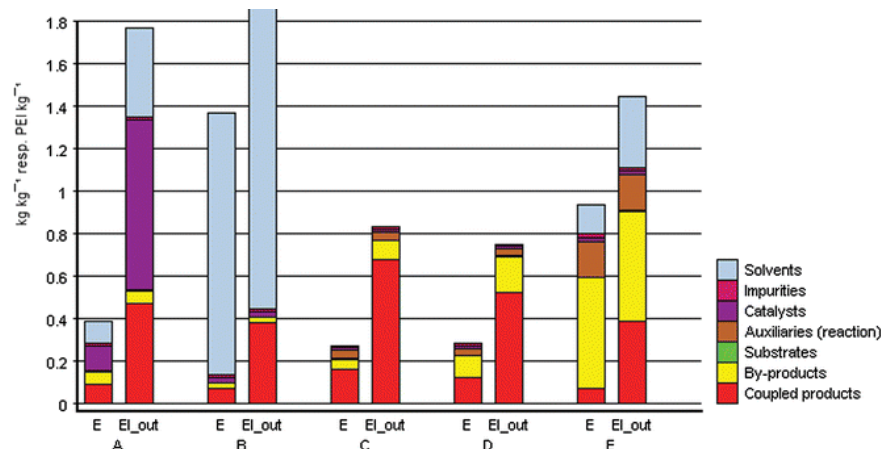


Figure 13 Illustration of EATOS output graph showing bars of E factor, and EI_out parameters. EATOS evaluated two reactions reported in literature: A [126] and B [127] and reactions (C-E), Table 4, developed in Paper II for production of a biosurfactant. The solvent-free processes showed lower E factor value and environmental impact compared to process B which used the same biocatalyst, Novozym®435. Data are reproduced from Paper II.

(Environmental, Health and Safety), EATOS (Environmental Assessment Tool for Organic Synthesis) and HPLC-EAT which we have developed in Paper IV. Leading companies such as GlaxoSmithKline (GSK) have developed their own tools to support their transition towards greener processes. GSK solvent selection guide is an example of such tools [125].

One freely available software for assessment of lab-scale chemical synthesis has been presented by Eissen and Metzger in 2002, known as EATOS [128]. EATOS calculates the input and output of certain chemical process in terms of mass used, determines the mass index (weight of input materials to unit mass of product produced) and the E factor of the process. EATOS uses information of the materials involved in the chemical process such as log*P* value, human toxicity, flammability and hazardousness which are found in the material safety datasheet. Based on this input, EATOS assigns an environmental-unfriendliness quotient (Q) for each substrate, by-product or auxiliary material utilized in the chemical synthesis. Based on the E factor and Q values, the risk of the Environmental impact of output waste (EI_out) can be evaluated and comparison of different synthetic routes is possible. EATOS has been effectively applied in Papers II and III for evaluation of lipase-catalysed synthesis of a biosurfactant (Figure 13) and biolubricants. The results highlighted the high environmental impact of organic solvents. For example, removing or decreasing *t*-amyl alcohol from the reaction

(Figure 13 and Table 4, entry B) led to a significant decrease in the E factor and EI_{out} of the process (entries C, D, and E, Figure 13 and Table 4).

In line with these studies, a similar green assessment tool customized for liquid chromatography methods was designed and is now available free of charge for users at www.biotech.lu.se/hplc-eat. The tool avoided one limitation of EATOS i.e., the need to manually enter material parameters regarding toxicity and hazardousness (Table 2). Manual entry of data in EATOS can result in significant variation of the assigned unfriendliness quotient Q, hence solid background about the way EATOS works is needed to have reliable results. In the case of HPLC-EAT, a database is already included for the most commonly used organic solvents in chromatography separation. HPLC-EAT has been used in the evaluation of study cases collected from the literature (Paper IV). This

Table 2 Comparison of the HPLC-EAT tool developed in the present thesis to other freely available tools for greenness evaluation

	EATOS	EHS	HPLC-EAT
Availability	Downloadable Java-archive file	Downloadable Excel file	Downloadable Java-archive file or Excel file
Purpose	Calculates the unfriendliness quotient Q and environmental quotient EQ values	Calculates what is equivalent to unfriendliness quotient Q values.	Analytical and preparative Liquid chromatographic separation methods
Advantages	In principle, evaluates any process. Informative and versatile outputs (S ⁻¹ , E, EI _{in} , EI _{out}).	No manual data entry Discriminating.	No manual data entry Discriminating
Limitations	Manual data entry Non- discriminating in some aspects A familiarity about the tool is a must before use.	Restricted to a small list of chemicals (mainly organic solvents).	Limited to liquid chromatographic applications.
Reference	[128]	[129]	Paper IV

simple tool could discriminate quantitatively and rank the different methods in order of greenness.

4.2 Medium of biocatalytic reactions

The conventional medium for enzymes is water, which prevails in the biological systems. In the 1980s Klibanov et al. [57, 130] described the use of enzymes in organic solvent as a non-conventional medium. Non-conventional media commonly used for biocatalytic reactions are: organic media, ionic liquids, supercritical fluids and gaseous media. Enzymatic reactions in non-conventional media allow the reversal of the hydrolytic reactions [57], increases the stability of some enzymes, and enables dissolution of hydrophobic substrates. Nowadays, many successful processes are based on enzymatic reactions in organic solvents [131]. Organic solvents have some attractive features e.g. they are able to solubilize apolar substrates and can be easily evaporated from the reaction at lower temperatures compared to water. Organic solvents were and still are indispensable components in chemical synthesis, and their complete displacement is almost impossible [132, 133]. Solvents participate in several steps in the chemical synthesis starting from dissolving the reactants and running the reaction to crystallization and purification of the products in addition to their role in the analytical procedures. Reduction in the use of organic solvents will result in a healthier and safer environment and reduced product cost. Hence many efforts are concentrated on reducing the use of organic solvents.

4.2.1 Solvent -free reactions

Organic media of biocatalytic reactions can be divided into two types: organic solvents and solvent-free media. In the latter, the substrates are dissolved in each other as in an esterification reaction of a fatty acid and a long chain alcohol. Running chemical reactions in solvent-free systems possess an obvious environmental benefit. Solvent-free synthesis decreases the production cost by reducing the cost of materials and waste treatment, and the volumetric productivity is increased. Moreover, the final product is free from solvent residues. Enzymatic reactions in heterogeneous systems where the substrates are present in a high mass-to-volume ratio are well reviewed [134]. These systems require a small amount of solvent (can either be water or an organic solvent) that acts as an adjuvant between the substrates. Solubility of the substrates and products in the adjuvant controls the overall product yield. The lower the solubility of substrates, the higher their activity and the reaction kinetics are driven forward. Also, if the products precipitate easily from the adjuvant the

reaction will be favoured in the direction of the products. Cao et al. [135] described a solid-phase reaction process in which a small amount (eq. 2 w/w solvent to substrates) of a solvent system (acetone and *t*-butanol) was used as adjuvant. In Paper II, a reduced-solvent system for the synthesis of a biosurfactant was presented. Concentration of the substrates to > 6 M (Entry E, Table 4) in *t*-amyl alcohol resulted in 56 % yield. Moreover, the reaction had a greener profile compared to reactions with high solvent content even if the product yield was higher (Entry B) (Figure 14). Grafting a reactive chemical group to substrates was used to aid in the progression of solvent-free reactions. Fregapane, et al. [136] reported an enzymatic solvent-free synthesis of sugar esters using activated sugar in the form of acetal. Another example is the use of an activated acyl moiety in the form of active gamma lactone for the synthesis of fatty acid glucamides, the surfactant class produced in Paper I. Activation of the acyl moiety by lactonization was initiated by acid catalyst [137]. However, this activation was not applicable to the synthesis of N-alkanoly-N-methylglucamide, case of Paper I, as the fatty acid substrate used is saturated.

The most influencing factors on enzymatic solvent-free reactions are the molar ratio, the optimal temperature, the water activity and the hydrophobic character of the immobilized enzyme. Adlercreutz reviewed these parameters for controlling enzymatic reaction in non-conventional media [138]. In Paper I, it was difficult to reach the optimal molar ratio of the reaction (1:1) between the

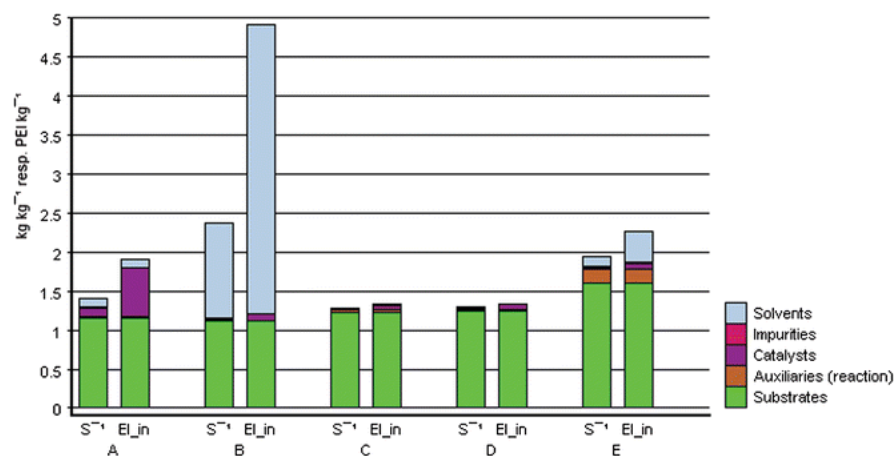


Figure 14 EATOS evaluation of reactions performed in solvents (A and B), solvent-free reactions (C and D) and a reduced-solvent reaction (E) for production of N-alkanoly-N-methylglucamide. S^{-1} is the mass index (mass of reaction input materials per unit mass of product), EI_in is environmental impact of process feedstock input, low values indicate better environmental impact. Data are reproduced from Paper II

substrates; therefore a 2:1 molar ratio (entry C Table 4) was used. To increase the yield of the process, a simple and hydrolytic step was applied for the by-product which resulted in 99 % yield (entry C_h). In another solvent-free contribution, Tufvesson et al. [51] made use of the ion-pair salt formed between the ethanol amine and the fatty acids to run an enzymatic solvent-free synthesis of alkanolamide surfactants. The study revealed the effect of the molar ratio on the reaction progression. An initial molar ratio of 1:1 between the substrates was not possible due the viscosity limitation. Gradual addition of one of the substrates (ethanol amine) resulted in an acceptable viscosity of the reaction medium and a high yield (> 90 %) was obtained.

Water control in solvent-free medium

Enzymatic reactions in solvent-free systems require fine control of the water available in the system. Some enzymes such as CalB show stability and function efficiently under very dry conditions, while others need water to act as a lubricant for the enzyme molecules. In reactions designed to be reversed by hydrolases like esterification, water should be removed from the system efficiently to push the reaction in the direction of the products. Water can be removed from solvent-free media in different ways; for example, by running the reaction under vacuum (Paper III), in an open reactor at high temperature (Paper II), or by sparging dry gas into the reactor. Water produced from the reaction can also be selectively removed from the organic solvent at azeotropic temperature (Paper III). The dried organic phase can be returned to the reactor. Hult et al. [139] have engineered CalB to decrease the hydrolysis rate compared to the synthesis pathway. The CalB structure is proposed to have a tunnel through which the water molecules pass during the catalysis. Blocking this tunnel by a Ser47Leu mutation resulted in enhancement of the esterification over hydrolysis.

4.2.2 Promising non-conventional media

One of the most important factors that determine the greenness of a chemical process is the type and amount of solvents used. In Europe, the annual consumption was estimated at 4 million tonnes at 2004 [140]. Organic solvents, also known as Volatile Organic Compounds (VOCs), impose a risk to the personnel in the chemical industry and are harmful to environment because of they are generally flammable, toxic or carcinogenic. Therefore, development of alternatives to organic solvents that are inert, do not have vapour pressure, are less toxic, and are preferably derived from renewable resources is encouraged. Ionic liquids (ILs) are a relatively new kind of solvents that might fulfil some of these criteria. ILs are a mixture of cations and anions present in liquid form at

room temperature, not volatile and with no substantial vapour pressure. An example is ethyl-ammonium nitrate $[\text{EtNH}_3][\text{NO}_3]$ that was first described in 1914 by Walden. Many applications have been reported showing the opportunities to use ionic liquids in green synthesis [141]. However many issues have to be addressed regarding ionic liquids, for example their toxicity to aquatic organisms and solubility in water which facilitates the entrance to environmental water bodies. In addition, organic solvents might eventually be needed to extract the product from the ILs solution. Another emerging solvents which share many properties with ILs are deep eutectic solvents (DES), which can be defined as low melting point liquids derived from an organic salt complexed with a hydrogen-bond donating species. A typical example of DES is the 1:2 molar ratio mixture of choline chloride (T_m 302 °C) with urea (T_m 133 °C) which forms a free-flowing fluid with T_m of 12 °C. Most DES systems studied are based on choline chloride that is produced by an efficient route with an E factor close to zero [142].

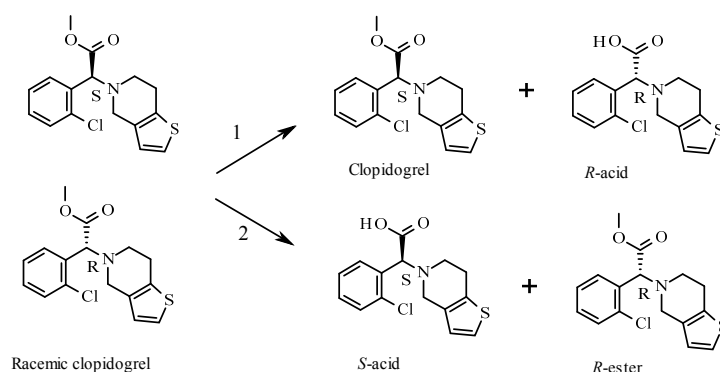
Supercritical fluids are also attractive and already applied green solvents. Carbon dioxide, water and ethane are examples of compounds used in the supercritical-fluid state for running biocatalytic reactions [123, 143, 144]. The supercritical state occurs when these compounds are present above the critical temperature and critical pressure but below the pressure required to condense them into solids. For example, CO_2 at 25 °C and 50 bar is found in both liquid and gaseous states and a distinct meniscus can be seen between these two phases. Increasing the temperature and the pressure will bring the densities of the two phases closer until a fusion occurs and the system becomes one phase which is the supercritical state. Beckman has reviewed the use of supercritical CO_2 in green chemistry applications [145]. CO_2 in the supercritical state is a very attractive green solvent candidate as it is cheap, non-inflammable, relatively non-toxic, and a typical renewable compound. Moreover, no residues will remain in the final products. However, the fact that this is a high-pressure process should be considered as a potential disadvantage because of its inherent risk and high energy demands.

5. Case studies with the hydrolases

This chapter presents the studies with esterase, lipase and phytase within the frame of this thesis.

5.1 Kinetic resolution of racemic clopidogrel

Clopidogrel is currently the most effective anti-platelet aggregation drug. It was originally marketed under the trade name Plavix®. It is the second best-selling drug in the world with a market estimated at US\$ 6 billion in 2005 [146]. Clopidogrel is used for the treatment of atherosclerotic events such as myocardial infarction, stroke, and vascular death and has fewer side effects compared to aspirin [147, 148]. The *S* isomer is the only active form of clopidogrel, while the *R* isomer is therapeutically inactive and induces convulsions at high doses in experimental animals [149, 150]. As a consequence, synthesis of pure *S*-clopidogrel has drawn the attention of several research groups [151, 152]. Two major routes for synthesis of racemic clopidogrel are reported in the literature. The first route starts from the pure isomer, for example methyl (*R*)-*o*-chloromandelate obtained via a biocatalytic route [153]. The second route, which is predominant in industry, consists of synthesizing the racemic clopidogrel and purifying the *S* isomer by a diastereomeric resolution process using *L*-camphor sulphonic acid, [151, 154] which forms physically different salts of *R* and *S* isomers that can be separated by crystallization from a suitable solvent system. This final resolution process consumes *L*-camphor sulphonic acid in stoichiometric amounts and large volumes of solvents. Moreover a purification process for removal of the residual chemicals is needed. The application of enzymatic route for selective resolution of racemic clopidogrel (scheme 1) is a



Scheme 1 Resolution of racemic clopidogrel via two enzymatic routes developed in Paper I: (1) PLE-3 and (2) PLE-1-F407L.

desired method since the process of the racemic synthesis is already established and the racemic clopidogrel as a raw material is available. A preliminary patent search has shown no processes on the enzymatic kinetic resolution approach.

Enzymes are able to discriminate between enantiomers based on the formation of different substrate-enzyme complexes in the transition state of the catalysis. In the case of an enantioselective enzyme, one enantiomer will be utilized faster than the other. Biocatalysis is applied for synthesis of chiral compounds via three routes [23]: a) asymmetric synthesis, where the starting substrate is a prochiral compound and a final yield up to 100 % can be achieved, b) kinetic resolution (Paper I), where one enantiomer will be utilized faster by the enzyme compared to the other one and a maximum yield of 50 % is possible, while the other isomer is recycled, c) dynamic kinetic resolution, where the kinetic resolution is accompanied by a racemization process to convert the slow-reacting compound into a fast-reacting one and, theoretically, a final yield of 100 % can be achieved. Enzyme enantioselectivity is described by the enantiomeric ratio or *E* value, which describes the discrimination of the enzyme towards the different isomers; an *E* value of 1 means no enantioselectivity. For industrial applications, an *E* value in the range of 20-50 can be used, while the optimum is *E* > 100. For an enantioselective reaction, three variables can be measured: enantiomeric excess of substrate (*ee_s*), enantiomeric excess of product (*ee_p*), and extent of conversion (*c*). Based on these variables, the *E* value can be determined based on one of the following equations [155]:

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]}; E = \frac{\ln[(1 - c)(1 - ee_s)]}{\ln[(1 - c)(1 + ee_s)]}; E = \frac{\left[\frac{1 - ee_s}{1 + (ee_s/ee_p)} \right]}{\left[\frac{1 + ee_s}{1 + (ee_s/ee_p)} \right]}$$

Screening of different hydrolases for enantioselective hydrolysis of racemic clopidogrel has resulted in identification of crude PLE as an enantioselective enzyme with *E* = 8.3. However, the utilization of PLE in its crude form is prohibited for pharmaceutical compounds. Therefore, pure isoenzymes of PLE were tested. Based on information in the literature, two isoenzymes among the seven reported were selected for further experiments: PLE-1 or gamma PLE, which is the main fraction of PLE and was thought to be responsible for the detected enantioselectivity of the crude enzyme, and PLE-3 which has significant sequence and structural differences with PLE-1. Design of mutations of the PLE-1 based on molecular modeling simulations was also performed. The simulations have revealed one potential site for mutation which is F407 (Figures 9 and 10).

Table 3 Enantioselectivities of PLE-1 and its variants and PLE-3 towards racemic clopidogrel

Enzyme	Time (h)	ee _s (%)	Conv. (%)	<i>E</i>	Preference
PLE-1	55	12	17	4.4	<i>R</i>
PLE-1-V76A	55	9	15	3.8	<i>R</i>
PLE-1-E203D	55	1	5	1.5	<i>R</i>
PLE-1-M344A	55	4	8	2.9	<i>R</i>
PLE-1-F407A	10	51	59	3.3	<i>S</i>
PLE-1-F407L	12	99	52	>100	<i>S</i>
PLE-3	30	69	51	10.0	<i>R</i>

^a Conditions: 4ml scale reaction, 50 mM phosphate buffer, DMSO 20 %, 0.1 mM racemic clopidogrel, 100 µl immobilized enzyme, 30 °C. *E* value was calculated using the equation $E = \frac{\ln[(1-c)(1-ees)]}{\ln[(1-c)(1+ees)]}$. Data are reproduced from Paper I.

Glu203, also, formed a catalytically unwanted hydrogen bond with His449 as noticed in the molecular modeling simulations (Figure 9).

Mutation of Phe with Ala resulted in a dramatic improvement in PLE-1 activity towards racemic clopidogrel: 59 % conversion after 10 hours was recorded compared to only 15 % after 55 hours in the case of wild type PLE-1 (Table 3). However, the enzyme enantioselectivity was reversed, i.e., *S* was the fast-reacting isomer (*E*=3.3). Interestingly, the F407L mutation caused a dramatic inversion of PLE-1 enantioselectivity: *E* >100 was recorded towards the *S* isomer compared to *E*=4.4 to the *R* isomer in the case of the wild-type enzyme. Single mutations in two residues (M344A and V76A) located in the helices at the entrance of the active site [30, 31] did not improve the enzyme activity or selectivity. Mutation of Glu203 to Asp resulted in minimal PLE-1 activity (5% conversion after 55 hours). Heinze et al. [156] mutated the same residue in an esterase from *Bacillus* sp, and increased the enantioselectivity towards esters of tertiary alcohols which has the chirality centre in the acyl acceptor moiety i.e. the alcohol.

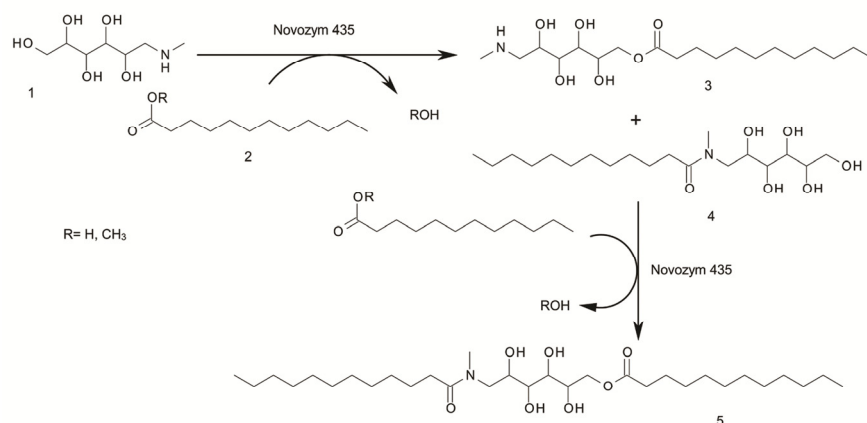
The PLE-3 isoenzyme, on the other hand, exhibited the desired enantioselectivity with *E* = 10 and > 99 ee% after 55 hours. Structurally, PLE-3 is different from PLE-1 by 20 amino acids. Investigation of the superimposed structures of THI of PLE-1 and PLE-3 revealed that PLE-3 has A236 and G237 residues in the active site while PLE-1 has V236 and A237 respectively. These small residues provide a bigger active site for the substrate and are probably the cause of PLE-3 enhanced activity and selectivity compared to PLE-1.

In conclusion, enzymatic resolution of racemic clopidogrel has been achieved for the first time with pig liver esterase isoenzyme 3. However, further improvement of the E value is needed. Based on the molecular modeling simulations, the F407A mutation in PLE-1 improves the enzymatic activity. Another mutation, F407L, has dramatically reversed the enantioselectivity of the enzyme with $E > 100$ (S is the fast-reacting isomer).

5.2 Lipase-catalyzed, solvent free synthesis of a biosurfactant

According to the green chemistry principles, solvents in a chemical process should be minimized or excluded [5, 10]. This is not an easy task, especially when the reactants have low solubility or miscibility, and the reaction system is viscous. Solvents are necessary in chemical reactions for efficient mass transfer and maintaining the system homogeneity. *N*-alkanoyl-*N*-methyl glucamide (MEGA) is a non-ionic biosurfactant composed of a hydrophilic part, *N*-methyl glucamine (MEG), linked via an amide bond to the hydrophobic lauric acid. Both moieties are derived from renewable resources [157, 158]. Chemically, this compound was synthesized with sodium methoxide as a catalyst in an organic solvent [126]. A lipase-catalyzed synthesis of MEGA was also reported in a solvent system [127]. In both cases methyl laurate was used as the acyl donor. A solvent-free synthesis of MEGA catalyzed by the immobilized lipase Novozym®435 (N435) was developed and its environmental impacts was compared to that of the methods reported in the literature.

To achieve a solvent-free synthesis of MEGA, a mixture of lauric acid, methyl laurate and MEG was employed. By the formation of the ion-pair salt between lauric acid and MEG, a homogenous mixture was formed and N435 could catalyse the amide bond formation. MEG contains two functionalities i.e., a primary alcohol hydroxyl group and a secondary amine, which leads to the formation of an amide product (4, Scheme 2) and an amide-ester, a major by-



Scheme 2 Amidation reaction of lauric acid and/or methyl laurate (2) with methyl glucamine MEG (1) catalyzed by Novozym®435 (Paper II). The surfactant is the amide product (4) and amide-ester (5) is the main by-product of the reaction.

5. Case studies with the hydrolases

Table 4 Solvent free synthesis of N-alkanoyl-N-methyl glucamide (entries C-E) in comparison to processes described in literature A [126] and B [127].

Entry	Lauric acid mmol	Methyl laurate mmol	MEG mmol	Molar ratio	Solvent	Yield %
A	0	500	500	1:1	Propane-1,2-diol	95
B	0	3.5	3.5	1:1	<i>t</i> -amyl alc. (8 g)	80
C	3.5	3.5	3.5	2:1	None	34
C _h ^a	3.5	3.5	3.5	2:1	None	99
D	0.87	1.34	1.75	1.25:1	None	58
E	0	1.4	1.4	1:1	<i>t</i> -amyl alc. (0.24 g)	56

Catalyst: Novozym®435 in entries B-E and sodium methoxide in entry A.

^a Reaction C after hydrolysis. Data are reproduced from Paper II.

product (5, scheme 2). Formation of the amide was enhanced by increasing the amine molar ratio. The amide yield was 58 % after 40 hours (entry D, Table 4) with a molar ratio close to the ideal reaction of 1:1 (Figure 15 A). When the molar ratio of methyl laurate to MEG was increased to reduce the reaction viscosity (entry C, Table 4), the yield decreased (34 %) and formation of amide-ester was enhanced (Figure 15 B). Taking advantage of the chemical properties of the amide and the by-product, a simple and efficient hydrolysis of the amide-ester was successfully applied to the final reaction mixture to increase the amide yield to 99 % (entry C_h and Figure 16).

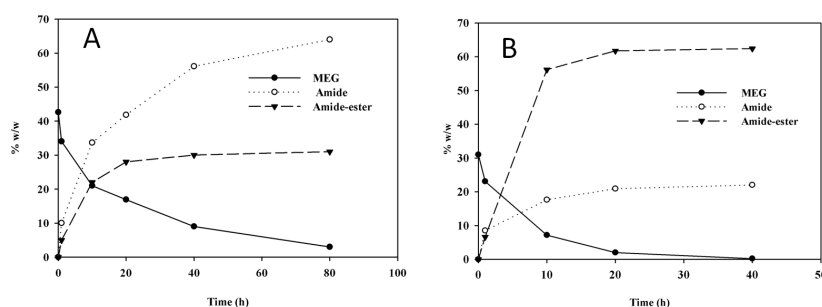


Figure 15 (A) Lipase-catalyzed amidation reaction with lauric acid, methyl laurate and methyl glucamine MEG at a molar ratio of 1.25:1 (see Entry D Table 4). Amide yield of 58 % was achieved after 40 hours. (B) Higher molar ratio (2:1) of acylating material, methyl laurate and lauric acid to MEG was used in the reaction. All the starting MEG substrate was converted into amide and amide-ester. This entry was followed by a simple hydrolytic step (Entry C_h) ending with a final yield of 99 %.

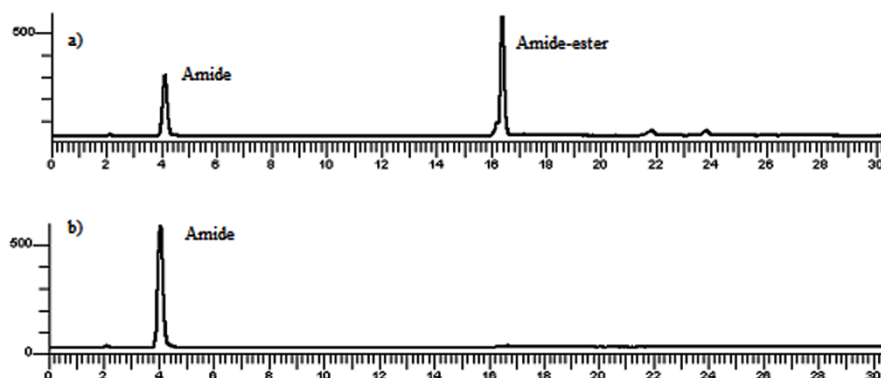
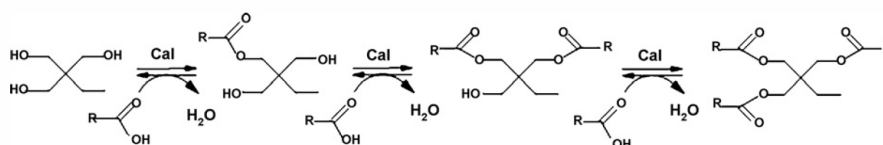


Figure 16 HPLC chromatograms of the reaction mixture obtained from entry C (a) and after applying the aqueous hydrolysis step (b) (entry C_b). Conversion of all amide-ester, the by-product, into amide was observed.

In conclusion, the surfactant obtained from the enzymatic solvent-free process developed in this study is based on renewable resources and clean in terms of solvent and catalyst residuals compared to the product of the chemical or the enzymatic reactions described earlier. The process can be performed without the risk of using hazardous sodium methoxide. In addition, running the reaction in a solvent-free medium resulted in a lower E factor and environmental impact. The preliminary environmental assessment shows that even if the developed method (entry C) gives a lower yield than the traditional methods, it provides benefits in terms of lower environmental impact (Figures 13 and 14). By-product conversion using a simple step hydrolysis into product greatly improved the entry C in terms of yield (entry C_b 99 %).

5.3 Lipase-catalysed synthesis of biolubricants

Lubricants are a group of specialty chemicals with an enormous market worldwide. The annual consumption was estimated at 37 million metric tonnes [159-162]. Most of these lubricants end up in the ecosystem by leakage, which represent a potential source of contamination to the soil and water. Europe consumes one third of the total lubricants produced world-wide. In Germany, for example, the approximate loss of lubricants into the environment is 520 000 tonnes per year [163]. Therefore, interest in synthesis of lubricants that are biodegradable and based on renewable resources is growing [159]. Vegetable oils are a very good candidate for synthesis of biodegradable lubricants, as they are easily degraded either aerobically or anaerobically in the environment and are derived from plant crops. However, the crude form of vegetable oils lacks stability and is sensitive to oxidation. In addition, their tribological¹ properties are poor. To improve the properties of plant oils for lubricant applications, chemical modifications such as epoxidation or esterification to polyol compounds are required. Synthetic esters of short- to long-chain (C5-C18) carboxylic acids with polyols such as neopentyl glycol, pentaerythritol and polymethylol propane have good properties for various lubricant applications. Trimethylolpropane (TMP) oleate is produced from esterification of TMP with oleic acid (Scheme 3), and is mainly used as a hydraulic fluid. Traditionally, TMP-oleate synthesis is catalyzed by an acidic catalysts such as p-toluenesulphonic acid. In Paper III, an immobilized CalB (N435) was used to catalyse the synthesis of TMP oleate as a biolubricant for sub-zero applications and compared to other two heterogeneous chemical catalysts: silica-sulphuric and Amberlyst-15.



Scheme 3 Illustration of the esterification reaction of Trimethylolpropane (TMP) with a carboxylic acid

¹ Related to the science studying the mechanism of friction, lubrication, and wear of interacting surfaces that are in relative motion

The esterification reaction of TMP with oleic acid catalysed by N435 and Amberlyst-15, respectively, was run in solvent-free conditions. The produced water from the reaction was removed by applying vacuum 20 mbar. In the case of silica-sulphuric acid, it was not possible to run the reaction under solvent-free conditions and toluene was used instead and the water produced from the reaction was removed by azeotropic distillation applying Dean stark apparatus. The results obtained showed high initial reaction rate in the case of silica-sulphuric (10 mmol h⁻¹g⁻¹) compared to Amberlyst-15 (3 mmol h⁻¹g⁻¹) and N435 (6 mmol h⁻¹g⁻¹) (Figure 17). Silica-sulphuric acid and N345 showed higher conversion after 24 hours (> 90 %) while Amberlyst-15 converted approximately 70 % after 24 hours (Paper III). About 96 % of the final mixture was triester in the N435 catalysed reaction, and 90 % with silica-sulphuric acid as the catalyst. The product colour produced in the reaction with silica-sulphuric acid was dark brown while in the case of N435 it was much lighter and had lower pour point (-42 °C), which is a desired property in lubricants used at sub-zero applications. The reason behind the dark colour was that the acid catalyst protonates the double bond present in oleic acid and forms a carbocation which reacts with another molecule of oleic acid to form a dimer and a trimer known as estolide. These by-products decrease the product quality in terms of colour and tribological properties such as pour point and viscosity. Moreover, dark-colored products need to go through a process of bleaching and purification before being released to the market. Therefore, the high conversion and fast reaction in silica-

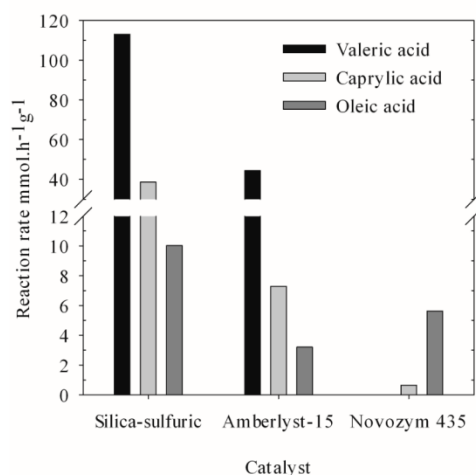


Figure 17 Initial reaction rate recorded in case of esterification of trimethylolpropane with valeric, caprylic and oleic acid, respectively, catalysed by silica-sulphuric acid, Amberlyst-15 and Novozym® 435. The figure is reproduced from Paper III.

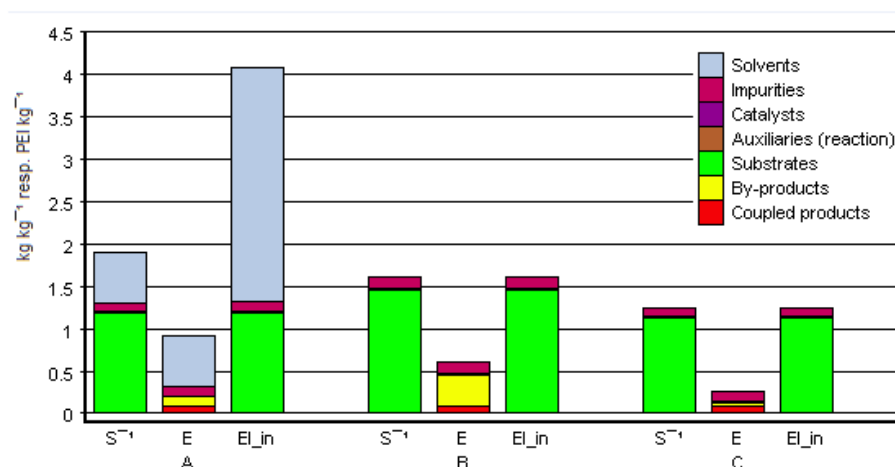


Figure 18 Environmental assessment of TMP-oleate synthesis using three catalysts: A, silica-sulphuric acid, B Amberlyst-15 and C Novozym® 435 (CalB). Toluene was used in case A to aid water removal. The figure is reproduced from Paper III.

sulphuric acid catalyzed reaction did not produce a high quality lubricant compared to the N435 reaction. Application of lab-scale green metric assessment by means of the EATOS software was performed for these three reactions (Figure 18). The mass index S^{-1} (mass of the chemicals entering the reaction per one kg of product) was 1.89 kg kg^{-1} in route A with silica-sulphuric acid compared to 1.23 kg kg^{-1} in the N435 route (C). The difference in S^{-1} between the two is attributed to the use of toluene during the reaction and ethyl acetate after termination of the reaction. Consequently, the potential environmental impact of the reaction input (EI_in) in route A was approximately four-fold higher compared to the route C. The E factor for the three routes was calculated with EATOS; the enzymatic route showed the lowest E factor compared to the silica-sulphuric acid and Amberlyst-15 routes.

In conclusion, lipase-catalysed synthesis of a biolubricant derived from renewable resources was presented to give a process with better environmental profile. The enzyme selectivity led to formation of higher-quality product compared to chemical catalysts.

5.4 *Bacillus* sp. MD2-Phytase: effect of metals and protein engineering

Phytases are widely used in the feed industry with a market estimated at US \$ 350 million per year and 10 % annual growth rate [164]. Phytases are used as feed supplements in the diets of monogastric animals, poultry and fish [165]. They hydrolyse phytate, an anti-nutritional factor commonly found in plant food materials like seeds, leading to the release of its phosphorous content. This eliminates the need to add external phosphorus (e.g., in the form of di-calcium phosphate) to the animal diet. Approximately, addition of 250 g of phytase would save the use of 10 kg of di-calcium phosphate [166]. Addition of phytases also has an environmental benefit by lowering the level of phytate and hence the inorganic phosphate (Pi) released in the livestock manure. Release of Pi in water has a deleterious environmental impact on rivers, streams and lakes known as eutrophication². Phytases are not yet used as supplement for human food, although there is promising ongoing research that shows the benefits from addition of phytase to foods such as treating iron-deficiency symptoms and increasing the nutritional value foods [167-169]. For successful industrial application, phytases are preferred to be thermostable to withstand the pelleting process during the feed formulation [170, 171]. In addition, resistance to inactivation by pepsin and low pH values found in the animal stomach is required for a successful industrial phytase [165].

In Papers V and VI, a thermostable phytase was obtained from *Bacillus* sp. MD2. The phytase was studied and engineered to increase its activity and stability at lower pH values. MD2-phytase has a beta propeller protein fold with high- and low-affinity metal binding sites. The high-affinity sites are located at the periphery of phytase molecule where the calcium atoms Ca1, Ca2 and Ca3 bind. The low-affinity sites are located in a shallow cleft on the top of the structure and binds three calcium atoms: Ca4, Ca5 and Ca6, which are involved in the catalytic activity (Figure 6) [63]. During the catalytic process, one more Ca atom binds to the low-affinity site which coordinates to one of the phosphate groups of the substrate (PDB: 1H6L) [65]. In Paper V, the role of Ca²⁺ ions and other divalent metals was investigated. Charging the metal-depleted phytase with Ca²⁺ or related divalent atoms, Ba²⁺, Sr²⁺, Mn²⁺ or Mg²⁺ resulted in recovery of 89-100 % of the enzyme activity. The recovery of the enzyme activity with these metals indicates that they bind to the same loci of Ca²⁺ ions in the enzyme structure and can

² Increase in the concentration of nutrients like phosphate and nitrates in water bodies promotes excessive growth of algae and consequently reduction of the water oxygen and death of aquatic organisms.

5. Case studies with the hydrolases

Table 5 Effect of divalent metal ions on the relative activity of metal depleted *Bacillus* MD2 phytase^a

Metal ions	Assay temperature	
	37 °C	70 °C
CaCl ₂	100.0 ± 1.6	100.0 ± 3.1
BaCl ₂	90.9 ± 3.7	98.5 ± 6.7
CoCl ₂	31.7 ± 0.5	19.1 ± 2.9
CuCl ₂	14.9 ± 0.2	16.8 ± 4.8
MnCl ₂	91.5 ± 3.1	99.5 ± 3.9
MgCl ₂	88.7 ± 1.2	101.0 ± 3.1
FeCl ₂	ND	ND
SnCl ₂	12.7 ± 0.6	ND
SrCl ₂	98.3 ± 1.5	98.8 ± 4.1
ZnCl ₂	ND	ND

^a Metal depleted enzyme (3.5 µM) was incubated with different metal ions (5 mM) overnight at 4 °C. The enzyme samples were then diluted 100 times in 10 mM Tris–HCl buffer pH 7 prior to determination of activity in 0.1 M Tris–HCl pH 7 containing 5 mM CaCl₂ and 1.5 mM sodium phytate at 37 °C and 70 °C, respectively. Activity of calcium activated enzyme (289.7 U/ml at 37 °C and 5 mM CaCl₂; 685.2 U/ml at 70 °C and 5 mM CaCl₂) was considered as 100%. Activity of the control sample (metal depleted enzyme without any treatment) showed (0.7±0.1) % relative activity at 37 °C and (2±0.9) % relative activity at 70 °C. Data are reproduced from Paper V. ND-not detected.

perform the catalytic role i.e., stabilization of the formed pentavalent phosphorous transition state (Figure 7). Some other metals such as Fe²⁺ and Zn²⁺ were not able to restore the enzymatic activity. Differential scanning calorimetry (DSC) showed an increase in the apparent denaturation temperature (T_m) of the enzyme charged with Ca²⁺ by 12 degrees relative to the Ca-free enzyme. In addition, the optimum enzyme activity was recorded at 70 °C in the presence of Ca²⁺ ions. Other divalent metals could also stabilize the structure but to a lower extent compared to Ca²⁺ ions.

MD2 phytase has shown good thermostability. However, it works optimally at neutral pH conditions. It would be industrially beneficial if the MD2 phytase could be engineered to be active at low pH values, i.e., at the physiologic pH of the stomach. Histidine acid phosphatases (HAP) have acidic pH optima, which enables these enzymes to be active in the acidic environment of the animal gastric condition. In Paper V, engineering of MD2 phytase was based on structural and sequence comparison with phytases active in acidic media: histidine acid phytase from *E. coli* (PDB 1DKQ) and the phytase sequence of *Bacillus licheniformis*. The

active site of HAP contains more positively charged residues, four arginine and one lysine residues, compared to MD2 phytase (three Lys and one Arg). Mutation of MD2 phytase was based on increasing the positive charge content of the catalytic surface and the active site of MD2 phytase or decreasing the negative charge by replacing glutamate residues with neutral residues. The selection was guided by sequence comparison with phytase from *Bacillus licheniformis*, which has higher activity at lower pH values.

Experimentally, two mutations at the catalytic surface E229V and S283R resulted in increase in the specific activity of the wild type enzyme by 13 and 19 %, respectively. The mutation in the active site catalytic residue, K77R resulted in significant reduction in the enzyme activity. By using docking and molecular modeling, the reason behind this low enzyme activity was revealed. The K77R mutation led to the formation of two extra hydrogen bonds with phytate compared to the wild-type Lys residue, which has an over-stabilization effect on the enzyme-substrate complex and retarding the subsequent steps of catalysis. Mutation E227S led to resistance to lower pH values by retaining 80 % of its initial activity after incubation in acidic medium, while the wild type retained only 40 % of its initial activity (Figure 18). In addition, the pH optimum of E227S mutant was shifted to the acidic region, and showed optimal activity at pH 5.5 compared to pH 6 of the wild type. However the E227S mutation showed much

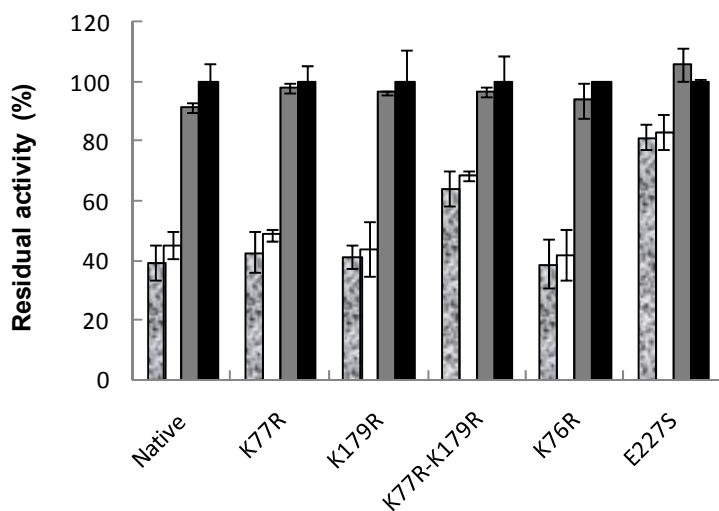


Figure 19 Effect of different pH values on the native *Bacillus sp.* MD2 phytase and some mutants presented in Paper VI. The enzymes were incubated in buffers of different pH values for 3 hours: pH 2.6, dotted bar; pH 3, open bar; pH 3.6 grey filled bar; black bar, control sample without treatment. Figure is reprinted from Paper VI.

lower specific activity in comparison to the wild type enzyme.

In conclusion, although the study in Paper V has revealed the role of many metal ions on the MD2 phytase activity and stability, still more research is needed. The inhibitory role of both Zn^{2+} and Fe^{2+} might be explored by X-ray structure determination of the phytase in presence of these metals. The engineering of MD2 phytase still needs more effort to achieve the desired shift in pH optimum without compromising the specific activity. For improving MD2 phytase stability in acidic medium the semi-rational approach could be applied by considering the availability of a robust screening method. In this approach, a library of mutants would have to be designed focused only on the catalytic surface and the active site and screened for improved mutants by a suitable screening method. Shivange et al. [172] have recently described a simple high-throughput screening method for improving the activity of a phytase derived from *Yersinia mollaretii*.

6. Concluding remarks and future perspectives

The message conveyed by this thesis is that enzymes have great potential as catalysts in clean and sustainable processes. With three different types of hydrolases (esterase, lipase and phytase) the thesis has demonstrated how to engineer rationally the enzyme structure to carry out a selective process (Paper I), how to engineer an enzyme-based reaction to run solvent-free reactions (Papers II and III) and the importance of application of green metrics for laboratory-scale process evaluation. The integration of computer science, bioinformatics, molecular biology, and chemistry is traced throughout this thesis. Such interdisciplinary approach has a very positive effect on development of biocatalysis and green chemistry.

Paper I is the first report on enzymatic resolution of racemic clopidogrel. Further studies are needed to improve the enantioselectivity via more rounds of rational design of PLE-1 and PLE-3, or applying semi rational strategy. The one-step method used for enzyme purification and immobilization will make the handling of large number of mutants easy. The pig liver esterase utilized in the current thesis has many advantages as biocatalyst for organic synthesis. The case study presented in this thesis shows that PLE can be subjected to rational design based on the already available information. Moreover the enzyme is easily expressed in *E. coli* with good stability. Research on developing a stable immobilized preparation of PLE is worth continuing. In addition, engineering PLE for running reactions in organic solvent could be interesting for increasing its scope of application. The approach used can be applied even to other PLE isoenzymes for different synthetic applications.

The immobilized preparation of CalB has been demonstrated to be a very practical and suitable choice for many organic synthesis applications. In the two cases presented in the thesis, the biosurfactant and biolubricant products obtained were of higher quality and had improved environmental profile. Lipase catalysis showed high selectivity in the esterification reaction while an alternative heterogeneous sulphuric acid-based catalyst led to the formation of unwanted by-products. The cost of immobilized preparation however could be the main obstacle for the large-scale production of specialty chemicals that have lower cost margin compared to the pharmaceuticals. Hence, development of cheaper and more stable biocatalyst preparations is definitely a priority for the future.

6. Concluding remarks and future perspectives

The thesis has also presented *Bacillus* sp. MD2 phytase as a promising thermostable enzyme for industrial application (Paper V and VI). The role of Ca^{2+} and other divalent metal ions on the phytase stability and activity has been deeply investigated. Further research is ongoing to investigate the inhibitory role observed in case of Zn^{2+} and Fe^{2+} metals. In addition, research on the potential application of this phytase in organic synthesis would be interesting. The thermostable *Bacillus* sp. MD2 phytase could provide an interesting biocatalyst for the production of myo-inositol related molecules for pharmaceutical applications. The semi-rational design approach can be a good choice in the future for developing a phytase variant active in acidic medium, especially if a 3DM database for β -propeller fold proteins is constructed. Such database could identify hotspot residues for activity in acidic medium, and then a focused library of MD2 phytase can be produced and screened for improved mutants.

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