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Engineering and Application of Enzymes for Lipid Modification, an Update

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Abstract
This review first provides a brief introduction into the most important tools and strategies for protein engineering (i.e. directed evolution and rational protein design combined with high-throughput screening methods) followed by examples from literature, in which enzymes have been optimized for biocatalytic applications. This covers engineered lipases with altered fatty acid chain length selectivity, fatty acid specificity and improved performance in esterification reactions. Furthermore, recent achievements reported for phospholipases, lipoxygenases, P450 monooxygenases, decarboxylating enzymes, fatty acid hydratases and the use of enzymes in cascade reactions are treated.

Keywords
Biocatalysis, lipase, lipid modification, phospholipase, P450 monooxygenase, protein engineering

1. Introduction
Development of sustainable energy and material production processes is one of the major challenges faced in the prosecution of a more eco-friendly chemical industry. In order to overcome these environmental concerns, the application of greener reaction technologies, as well as the use of renewable raw material sources, is needed to cover the increasing demand of fuels and chemicals as well as to provide compounds for healthy human nutrition. In this sense, the use of vegetable oils – and to a smaller extent of animal fats – together with the implementation of biotechnological methodologies, is an important alternative to traditional petrol derived processes.
Lipid biotechnology employs whole (engineered) microorganisms or isolated enzymes as biocatalysts to obtain a great variety of specialties, which are used for the production of healthy fats and oils, cosmetics, lubricants, coatings, surfactants, biofuels, and many other useful products [1-3]. Commonly used enzymes for the modification of lipids, fats and oils, include mostly lipases, but also phospholipases, P450-monooxygenases and lipoxygenases amongst others [4]. Enzymes can offer

numerous advantages as catalysts, such as high efficiency, chemo-, regio- and stereoselectivity, avoid the need for functional group activation and protection/deprotection steps and are environmentally friendly, since enzymes are biodegradable, use mostly water as a solvent system and work under mild reaction conditions [5]. Despite all these benefits, there are some limitations for the effective implementation of enzymes in industrial processes such as narrow substrate scope, insufficient stereoselectivity or intrinsic instability towards harsh reaction conditions like high temperature, extreme pH, presence of organic co-solvents or long-term process stability. To extend their applicability several strategies can be applied to obtain biocatalysts displaying the desired characteristics: (i) search for new strains and enzymatic activities, including classical enrichment cultivation, the metagenome approach or *in silico* screening for new enzymes; (ii) development of alternative reaction media and immobilization methodologies, and (iii) variation of the enzyme properties by protein engineering [6-9].

Several publications have extensively covered this subject of enzymatic lipid modification in the past few years [2, 4, 10, 11]. Therefore, the aim of this review is to summarize the most important tools and strategies for protein engineering and to illustrate the recent achievements in the field of enzymatic lipid modification.

2. Protein engineering

As pointed out above, protein engineering has emerged as an important tool to overcome the limitations of natural enzymes as biocatalysts. Two main strategies for protein engineering are used to optimize the enzyme properties towards the desired application: rational design or directed evolution [11-13]. Rational protein design requires structural and mechanistic information of the enzyme of interest (or a good homology model). Using this data and molecular modeling, usually a few interesting amino acids can be identified to alter or induce the desired properties. Then, the chosen variants are created in the laboratory and their features are tested experimentally. On the other hand, directed evolution involves either a random mutagenesis of the target gene encoding the catalyst (e.g. by error-prone PCR) or recombination of a set of related genes (e.g. by DNA shuffling). By means of these methods a mutant library of the protein is generated and subsequently screened to find the best mutants exhibiting the desired property. Variants showing promising results can be subjected to further rounds of evolution. Although both approaches have shown their usefulness, some limitations have to be taken into account. For instance, while rational protein design needs a deep and accurate understanding of the protein to make reliable predictions, directed evolution does not require structural data. Furthermore, the effect of simultaneous mutations is often extremely difficult to predict by rational methods. Luckily, nowadays there is an increasing availability of sequences, structures and biochemical data of proteins, combined with an amazing progress in computational technologies [14, 15]. On the other side, when using directed evolution huge mutant libraries are usually created (>10^4–10^7 variants), hence, a high-throughput screening or selection system must be available. Moreover, bias in the experimental

methods and degeneracy of the genetic code restricts the library design. These issues have been recently addressed in different articles [12, 16, 17]. Another alternative to overcome the restraints of each method is to use semi-rational design, a combination of these two strategies. This concept merges mechanistic and structural information, as well as computational predictive algorithms to preselect promising target sites. The focus on specific regions of the enzyme translates into dramatically reduced library sizes with yet higher hit rates, which is especially advantageous if no high-throughput assay system is available [12, 18].

In addition to the aforementioned strategies, *de novo* protein design has emerged as a promising tool for generating tailor-made biocatalysts. Currently, strong efforts are made to expand the set of reactions catalyzed, including non-biological reactions such as multistep retroaldol transformation [19], Diels–Alder cycloaddition [20], and Kemp elimination [21]. Typically these designed enzymes do not reach the activities displayed by natural enzymes, however they can be significantly improved by directed evolution [22-24].

3. Lipases

Lipases naturally catalyze the hydrolysis of triacylglycerols (TAG) into glycerol and non-esterified fatty acids (NEFA). Furthermore, lipases are able to perform synthetic reactions in organic solvents, mostly transesterification and esterification reactions. Thus, fatty acid alkyl esters can be formed, which are used as biodiesel [2]. Besides this, lipases can catalyze a range of other reactions. For instance, numerous reactions have been described for lipase B from *Candida antarctica* (reclassified as *Pseudozyma antarctica*) (CAL-B), such as epoxidation of α,β-unsatured fatty acids, amide hydrolysis, and – very slow – aldol addition [25].

There are numerous features that make lipases useful biocatalysts for industry such as (i) broad substrate spectrum, (ii) excellent chemo-, regio- and stereoselectivity, (iii) high stability towards harsh reaction conditions, (iv) independence of cofactors and, furthermore, (v) a wide variety of lipases is commercially available [26]. Due to all these advantages lipases are used in industry for the removal of fats and oils or for the production of NEFA, monoacylglycerols (MAG), and diacylglycerols (DAG) as raw material [27]. Large scale applications of lipases in industry can be found in the dairy, baking, and detergent industry [28-30], but also for the production of *trans*-fatty acid free margarines and biodiesel (fatty acid methyl esters). Each individual application of lipases requires certain selectivity or stability towards temperature, pH and/or organic solvents. Processes used to be designed around the features of the applied lipase. Nowadays, in order to expand the use of lipases in industry, protein engineering is applied to adapt them to the process requirements.

During the past decades lipases have been studied extensively and are well understood [1, 4, 10, 31-33]. In this section we are going to focus on the most recent progresses in the improvement of lipases by protein engineering.

3.1. *Candida antarctica* lipase A like group

The quite new lipase subclass of "*Candida antarctica* lipase A (CAL-A) like lipases" is assigned to the group of fungal lipases that contain a Y-type oxyanion hole [34]. Their similarity relies on a conserved pentapeptide GESAG in their active site and a unique cap domain.

Structurally, CAL-A possess two differentiated domains that play a major role in interfacial activation of the enzyme: the cap domain and the flap domain (Figure 1) [35]. The CAL-A cap domain involves 92 amino acid residues (amino acids 217-308), which are mainly hydrophobic, and create six α-helices. This domain participates in forming the acyl-binding tunnel and contributes to substrate-specificity and stability. Thus, single amino acid changes can induce differences in selectivity and specificity of the enzyme [36]. Regarding to the flap domain, it is formed by 10 amino acid residues that create two N-terminal β-sheets (amino acids 426-436) [35]. This structure flap lays on the entrance of the substrate-binding pocket, covering the active site. Therefore, when the flap moves, it leaves the active site pocket accessible for the substrate. Interestingly, by removing the flap domain, Wikmark et al. could demonstrate a preservation of the selectivity, specificity, and stability of CAL-A wild-type and, on the other hand, a Michaelis-Menten-like kinetic applicable to the truncated variant without the interfacial activation phenomenon known for typical lipases [37].

Figure 1

For a rational protein design of CAL-A two crystal structures are available (PDB entries: 2VEO and 3GUU). Both structures only show the inactive form of CAL-A with the cap domain closed, which makes it more difficult to perform docking studies with possible substrates. By a molecular dynamics simulation the closed form of CAL-A was opened. On the basis of this model, docking studies with (*R*)- and (*S*)-phenylethyl butyrate were performed to identify residues in close proximity to the tetrahedral intermediate [38]. These compounds are the possible products of enantioselective transacylation of a small *sec*-alcohol with vinyl butyrate, which are usually not converted enantioselectivly by CAL-A. Seven positions were identified and used for site-directed mutagenesis to change the special scope of the residues. Variant Y93L/L367I presented improved enantioselectivity towards a couple of tested *sec*-alcohols and displayed (*R*)-selectivity.

CAL-A was used in several protein engineering approaches to alter its selectivity. For instance, CAL-A variants had been generated with high specificity for medium-chain-length fatty acids (C_6-C_{12}) [39]. For this purpose, rational protein design was used to block the primary acyl-binding tunnel to force the binding of the acyl chain to the small alternative binding tunnel of CAL-A. As shown previously for *Candida rugosa* lipase 1, stepwise shortening of the acyl-binding tunnel by the introduction of bulky amino acid residues resulted in different specificities towards fatty acids with shorter chain-length [40]. As a hot spot, the residue G237 in CAL-A – located at the entrance of the acyl-binding tunnel – was identified. Mutants G237A/V/L/Y (Figure 2) were generated and their substrate spectrum was determined with *p*-nitrophenyl esters ranging from chain-

length C_2-C_{18} and with TAGs varying from C_4-C_{14} fatty acids. It was shown that mutant G237A possessed a reduced activity against long chain fatty acids, whereas the other three mutants led to variants unable to hydrolyze fatty acid chains longer than C_6, due to the complete blocking of the primary acyl-binding tunnel with their bulky residues. For variants G237V and G237Y an up to threefold higher activity towards *p*NP-hexanoate compared to CAL-A wt could be observed [41].

Figure 2

In another project CAL-A has been engineered to convert *trans*-fatty acids with higher reaction rates [36]. *Trans*-fatty acids are an undesired by-product in industrial hydrogenation of vegetable oils containing polyunsaturated fatty acids. Their content in food is restricted because they account as health risk factor for coronary heart disease. Naturally, CAL-A exhibits a 2.5 fold higher reaction rate for esterification of elaidic acid (Δ9-*trans* C_{18}-fatty acid) with *n*-butanol in comparison with oleic acid (Δ9-*cis* C_{18}-fatty acid). This unique feature was also described for the closely related lipase from *Ustilago maydis* [42]. By computer modelling experiments of the CAL-A crystal structure, several amino acids were identified as targets for site-directed saturation mutagenesis. The twelve chosen amino acids were located in the ~30 Å long fatty acid binding tunnel coordinating the Δ9 position or flanking the entrance or end of the tunnel. Libraries of mutants were expressed in *E. coli* and screened on microtiter plate (MTP) basis. For this purpose the hydrolytic activity of 5,000 clones was tested in separate wells in the presence of *p*NP-elaidic acid and *p*NP-oleic acid esters. *Trans*-over *cis*-selectivity (ToC) was calculated for each clone, identifying five amino acid positions that showed up to 15-fold higher ToC values than the CAL-A wt. The six best variants were expressed in *P. pastoris* and tested towards partially hydrogenated soybean oil containing 18% *trans*-fatty acids (Table 1). Variants T221H and I301H were the most selective ones only hydrolyzing the *trans*- and saturated fatty acids of the glyceride fraction. In these mutants, the introduced changes to the acyl-binding tunnel resulted in a linear geometry. Thus, linear saturated or *trans*-fatty acids were able to bind and non- linear acyl chains (*cis*-) were excluded.

Table 1

Another unique feature of CAL-A like lipases is their acyltransferase activity in the presence of water, as recently published by Müller et al. However, in CAL-A catalyzed transesterification reactions in the presence of water, still a small amount of NEFA are formed as an unwanted side product [43]. Using rational protein design four hydrophilic amino acid residues in close proximity to the active site were identified (T118, D122, T221, and E370), and changed by site-directed mutagenesis to more hydrophobic residues. Increased hydrophobicity in the active site should enhance the preference of binding alcohols over water. The obtained mutants were tested in the transesterification reaction of palm kernel oil with ethanol. When comparing the ethyllaurate synthesis of

the mutants and the wild type enzyme, three possible improved variants were identified (T118, D122, and T221). After longer incubation times these three variants and the CAL-A wt reached an equilibrium threshold of 80% conversion. Further investigations showed that only mutant D122L exhibited an increased transesterification rate, together with a decreased hydrolytic activity. Comparable pH stability, temperature-dependence, and thermo-stability profiles were also obtained for all improved variants. Only the temperature optimum was slightly decreased for the D122L mutant in comparison to CAL-A wt. By improving the reaction conditions, up to 95% ester formation – notably in the presence of water – after 24 h was achieved [44]. Another structural homolog of CAL-A, lipase 2 from *Candida parapsilosis* (CpLIP2), was investigated recently [45]. This enzyme can accept a higher percentage of water in acyltransfer reactions in comparison to CAL-A and preserves high activity at low temperatures. Although CpLIP2 and CAL-A share a low sequence identity (31%), a structural model of CpLIP2 on the basis of the structure of CAL-A was generated [46]. Accuracy of the structural model was determined by site-directed mutagenesis experiments on crucial amino acid residues for lipase activity and specificity. Afterwards, experiments of partial proteolysis were performed to distinguish regions with higher accessibility, lower stability and/or low interactions from the rest of the protein. Site-directed mutagenesis was also used to generate CpLIP2 mutants with changed activity and specificity. It is noteworthy to mention that the mutant Y197F showed decreased hydrolysis and improved transesterification ratio with methanol as acyl-donor compared to the wt, as previously described for the CAL-A D122L mutant [43]. Furthermore, this mutant showed increased specific activity of 40% towards the acyl-donor ethyl laurate by discriminating between saturated and unsaturated substrates.

3.2 *Improving lipase stability by protein engineering*

Protein surface properties, especially the charge distribution, have a great influence on the stability of the enzyme [28]. Introducing hydrogen bonds in flexible regions by site-directed mutagenesis is one approach to increase the rigidity and/or improve the hydrophobic packaging of the protein and, therefore, its stability. This has been shown for lipases such as CAL-B [47] or a lipase from *Pseudomonas* sp. [48].

Aldehydes and ketones are compounds, which affect lipase stability negatively, supposedly by their covalent reaction with nucleophilic amino acid residues such as lysine, histidine and cysteine. Unfortunately, these compounds are formed as secondary lipid peroxidation products of oils and fats with a high content of unsaturated fatty acids. In a protein engineering approach lysine and histidine residues, excluding the catalytic histidine and disulphide bond forming cysteines, of *Rhizopus oryzae* lipase were chosen for saturation mutagenesis [49]. Mutants were tested towards hydrolysis of *p*NP-butyrate in the presence of a variety of aldehydes. The improved variants H201S and H201S/K168I showed, respectively, 60% and 100% improved stability towards octanal, while preserving the specific activity of the wt enzyme.

3.3. *Understanding the phenomenon of interfacial activation*

As mentioned above for CAL-A, most lipases develop their full catalytic function on a lipid-water interface by interfacial activation [50, 51]. This also applies for the lipase from *Thermomyces lanuginosus* (TLL). Its lid region is composed of one α-helix flanked by an anterior and posterior hinge domain [52]. Supposedly, the opening of the lid is unfavoured under aqueous conditions, due to the exposure of hydrophobic areas to the aqueous solvent while the lid is opened [50]. In contrast, lipase activity increased more than 10-fold upon reaching the critical micelle concentration of the substrate [53]. This behaviour of the lid is supported by molecular dynamic studies [54]. To understand this fact, Skjold-Jørgensen et al. generated TLL variants with changed behaviour on lipid-water interfaces by altering the lid domain [55]. Owing to the structural similarity of TLL with a ferulic acid esterase from *Aspergillus niger* (ANFAE), this enzyme was used to generate TLL variants containing the lid region of ANFAE or hybrids of both of them. A study of activity and spectroscopic measurements was combined for the characterization of the variants and their activation mechanism. The results exhibited that, while most of the new variants displayed a decrease in their thermal stability, their structure remained intact. Furthermore, it was shown that some variants, especially the ones with only and exclusively the ANFAE lid domain, displayed esterase activity, while losing their lipase character. Other variants revealed combined features such as activity towards water-soluble and -insoluble substrates and fast activation at the lipid-water interface. It is noteworthy to mention here that the hybrid with an included PQ-motif in the posterior hinge domain, which is often found in hydrolases, showed this combined lipase and esterase character. Further investigations by site-directed fluorescence labelling were performed by modifying the C225, placed at the catalytic cavity, with bimane. The interaction with the C225-bimane with Tyr or Trp, present in the lid domain of the TLL variants, was monitored to show that the lid movement is dependent on the amino acid composition of the lid and the solvent polarity [56]. In high polarity environment only the TLL variant with the ANFAE lid domain displayed an open confirmation, whereas in low polarity solvents all variants, including the TLL wt and the variant with the lid hybrid, displayed an open conformation.

3.4. *Application of lipases in biodiesel production*

Biodiesel is a sustainable alternative to traditional fossil fuel [57]. However, biofuel production is currently not economically competitive to petroleum derived fuels. Nowadays the industrial production of biodiesel relies on a chemical process that uses alkali as catalyst to convert the TAG from high quality vegetable oils and methanol to FAME [58]. Lipase catalysed transesterification could overcome some of the major drawbacks of the chemical process [59-63]. For instance, lipases catalyze the transesterification of acylglycerols and, moreover, they catalyze even faster the esterification of NEFA contained in lower grade oils. However, the only industrial applications of lipase-catalyzed biofuel production have been reported in China and Brazil [28, 64].

To increase the economic viability of biodiesel production, recent research is focusing on process optimization. Among the enzymatic production of biodiesel, CAL-B is the most frequently applied lipase [65, 66]. Indeed, CAL-B was used in a recent study of Novozymes for pre-treatment of high-NEFA oils, to make them compatible with the chemical process [67]. Thus, they investigated immobilized Novozyme 435 on degummed rapeseed oil, which they spiked with oleic acid to vary the NEFA content. Different factors were considered in the optimization process like (i) NEFA content, (ii) methanol content, and (iii) processing time. Best conditions were determined, being 4% (v/v) methanol and 15% (w/w_{oil}) NEFA with a controlled reaction intensity, which is defined as the product of the amount of enzyme and time used to convert a given amount of oil (g_{enz} h L^{-1}). Additionally, in a recent publication of Novozymes, up-scaling of an enzymatic biodiesel production was described [68]. In this case, a liquid formulation of a modified *Thermomyces lanuginosus* lipase (NS-40116) was used to convert industrial waste oils as used cooking oil, with a NEFA content of <15%, and brown grease, with a NEFA content of >55%, into FAMEs. Furthermore, they were able to develop a continuous operation and a methodology to adapt conditions for fast batch optimization. Thus, they were able to perform the process in a 40 m^3 reactor in an affordable manner.

4. Phospholipases

Phospholipases are hydrolytic enzymes that act on phosphoglycerides and split them into a variety of products, like lysophospholipids, NEFA, DAG or phosphatidates, depending on the site of hydrolysis. Consequently, phospholipases can be classified into five groups (PLA1, PLA2, PLB, PLC, and PLD) based on their substrate cleavage site. This class of enzymes is commonly used in industrial applications such as the food industry and for oil degumming [28, 69].

The presence of phospholipids in crude vegetable oils negatively impacts the oil stability, color, and flavor. Traditional oil refining is performed by the addition of water to crude oil that causes hydration of the phosphate esters, bringing the phospholipids to the oil-water interface and allowing their separation by centrifugation. The main drawback in water degumming is the oil yield loss by oil entrainment. This fact is due to formation of an emulsion owed to the presence of intact phospholipids. To overcome this issue, several approaches employing phospholipases have been developed for oil refining. Early processes used a mammalian phospholipase from porcine pancreas specific for the ester bond at *sn*-2-position (PLA2) (Scheme 1). This enzyme was firstly extracted from pig pancreas as a side product from insulin production and, afterwards, it was expressed in other hosts than the native, leading to a safer enzyme to ensure enzyme supply, purity and a better performance in the process. Besides, another advantage of producing a porcine enzyme in a microorganism is that it can be regarded as kosher and halal. A later approach involved an enzyme obtained from *Fusarium oxysporum*, which exhibits *sn*-1-selectivity (PLA1) [70] (Scheme 1). Subsequent combination of the genes encoding the *Fusarium oxysporum* phospholipase and a lipase from *Thermomyces lanuginosus* resulted in a chimeric enzyme, which kept the

stability of the parental lipase and the PLA1-activity [71]. Both enzymes, PLA1 and PLA2, produce lysophospholipids that can be easily hydrated, allowing the reduction of the phospholipid content to below 10 ppm. A more recent alternative established by the company DSM (formerly Verenium/Diversa, San Diego, USA) implies the hydrolysis of the phosphorylated head-group present in phospholipids by a PLC (Scheme 1). This reaction produces *sn*-1,2(2,3)-DAG, which are oil-soluble and the phosphate residue as water-soluble fragment. Thereby, emulsion formation is reduced leading to less yield loss due to entrained oil. Furthermore, phosphate removal can be achieved as efficiently as with PLA1 or PLA2 [72]. Also our group discovered several PLCs after screening of different *Bacillus* sp. strains that could be functionally expressed in *Bacillus subtilis* for use in degumming [73, 74]. Concomitantly, an assay for the reliable determination of their activity was developed in which the phosphoester residue released by the PLC is cleaved with an alkaline phosphatase. Afterwards, the phosphate formed can be quantified as phosphomolybate complex previous extraction with *n*-butanol [75].

Scheme 1

Phospholipase D can be used for industrial phospholipid syntheses, because of their broad substrate specificity toward alcohol compounds [28, 69]. However, the synthesis of phosphatidylinositol (PI), a phospholipid that is attracting attention because its potential health benefits [76-78], could only be catalyzed by some PLDs from plant origin. To address this problem Iwasaki's group studied the wild-type *Streptomyces antibioticus* PLD to establish an efficient enzymatic system for PI production from phosphatidylcholine and myo-inositol as an alcohol acceptor. This goal was achieved by site-directed saturation mutagenesis to generate PI-synthesizing variants. Firstly, they targeted on positions involved in acceptor accommodation (W187, Y191, and Y385). Unfortunately, the obtained variants generated PI as a mixture of positional isomers [79-81]. Further studies faced the improvement of the positional specificity of the W187N/Y191Y/Y385R (NYR) variant (76/24 1-PI/3-PI ratio) by subjecting four residues (G186, K188, D189, and D190) of its acceptor-binding site to saturation mutagenesis. Subsequent screening showed that NYR-186T and NYR-186L were the most improved variants, producing an excellent ratio of 1-PI/3-PI of >93/7 at 20 ºC [82].

5. Lipoxygenases

Lipoxygenases (LOXs) are a family of non-heme iron- or manganese-containing oxidative enzymes that can be found ubiquitously in plants, animals, bacteria and fungi. They catalyze the dioxygenation of polyunsaturated fatty acids with a *cis*-1,4-pentadiene unit, leading to the formation of a conjugated hydroperoxydienoic acid [83].
Production of hydroperoxides mediated by LOXs can be exploited in different industrial applications, including bleaching of colored components in food, paper and textile processes; modification of lipids from different raw materials and production of oleochemicals and aroma compounds. Despite this great potential, the development of

industrial applications involving this class of enzymes is currently hampered by the poor stability, low catalytic activity and lack of efficient overexpression systems capable to produce sufficient amounts of enzymes [84]. So far, the main commercial application reported is the bleaching of wheat flour where the carotenoid pigments are oxidized by LOX enriched soybean flour [85]. Aiming to improve their usefulness, over the last years LOXs have been subjected to protein engineering to alter their regio- and stereospecificity [86], structure–function relationships [87], metal selectivity [88] and stability [89]. As a result, several critical amino acids have been identified at the active site of selected LOX isozymes. Regarding the stereoselectivity, a single conserved amino acid in the active site seems to be responsible for this feature in LOXs analyzed until now. This key amino acid residue corresponds to an alanine in (*S*)-specific LOXs and a glycine in (*R*)-LOXs. Mutational studies replacing the glycine to an alanine succeeded in partially switching the position of oxygenation and chirality of the product, thus converting a (*S*)- into a (*R*)-LOX enzyme and *vice versa* (Scheme 2) [90, 91].

Scheme 2

Production of hydroperoxides by LOX has been investigated on laboratory scale by Villaverde et al. They have described the synthesis of 13-hydroperoxy-(9*Z*,11*E*)-octodecadienoic acid from linoleic acid by means of two lipooxygenases from *Gaeumannomyces graminis* [92] and *Pseudomonas aeruginosa* [93]. Both biotransformations resulted in high yield (88% and 75%) and selectivity (74% and 61%) of the desired product. When comparing both reactions, it could be observed that maximum conversion with *P. aeruginosa* LOX required a substrate concentration 10 times lower (10 g L^{-1}) and longer reaction times (48 h). A scale-up attempt of the reaction was performed using *G. graminis* LOX in the presence of industrially relevant linoleic acid concentrations (100–300 g L^{-1}). The new conditions caused a yield decrease of 40% leading to a volumetric productivity of 3.6 g L^{-1} h^{-1}. The authors of this research suggested that the process could likely be improved by facilitating the solubility of the fatty acid as well as oxygen supply to the reaction medium [92].

6. P450 Monooxygenases

Cytochrome P450 monooxygenases (CYPs) are a diverse super-family of heme-containing enzymes that catalyze the regio- and stereospecific oxidation of a wide variety of substrates. Typically, CYPs require their association with a redox partner system that transfers electrons from NAD(P)H to the P450 heme center. This class of enzymes plays an essential role in xenobiotic metabolism and is involved in the biosynthesis of numerous natural products [94, 95]. However, in spite of their great potential in biotechnology, industrial application of CYPs is still very limited due to some hurdles such as low stability and poor activity. Often the enzymes are membrane bound or membrane-assoicated, they require pyridine cofactors such as NADPH in stoichiometric amounts and they need an efficient electron transfer system. To

circumvent these problems, several approaches rely on protein and metabolic engineering and on the development of electrochemical and enzymatic approaches for the replacement or regeneration of the cofactor. Recently, different reviews have addressed this topic [96-100].

6.1 Synthesis of hydroxyfatty acids

Long-chain fatty acids can undergo ω-oxidation to produce ω-hydroxy fatty acids, which can be subsequently converted to dicarboxylic acids. Both are multifunctional compounds useful for the production of polymers and fragrances. In this context, CYPs could be valuable tools to produce ω-oxyfunctionalized fatty acids.

When using CYPs as biocatalyst, one of the strategies to enhance the previously described electron transport while avoiding the use of several enzymes is by using a self-sufficient CYP. This type of CYPs has the advantage that their heme domain is fused with the reductase domain in a single polypeptide. As a result, this type of CYP usually exhibits the highest turnover frequency compared to separated P450 monooxygenase/reductase systems. For instance, one of the best studied self-sufficient P450s is the fatty acid hydroxylase CYP102A1 from *Bacillus megaterium* (P450 BM3). This enzyme catalyzes the subterminal hydroxylation of long chain fatty acids (C12-C22) in a regio- and stereoselective manner. Furthermore, their turnover frequencies towards fatty acids are the highest among those reported for P450 monooxygenases so far (several thousand per minute) [101]. Many successful efforts have been presented using protein engineering on this enzyme for the modification of its activity, stereo-, regio- and chemoselectivity, stability, co-factor reliance and substrate specificity [102]. The resulting data have provided a better understanding of the influence that certain amino acid substitutions might display on P450 BM3 performance. Indeed, position F87 was identified in several studies to be a hotspot, mediating substrate specificity and regioselectivity [103-105]. A recent example described the application of iterative cycles of random and targeted mutagenesis to create variants of P450 BM3 able to synthesize terminal hydroxyfatty acids. The best variants identified acquired up to 11 amino acid alterations, being only three of them (F87, I263, and A328) located relatively close to the substrate binding site. In fact, a mutant containing these three substitutions alone showed 41% terminal oxidation specificity whereas the yield observed for best variant was 74%. Thus, non-obvious residues showed surprisingly strong contributions to the increased selectivity for the terminal position (Scheme 3). Unfortunately, in this case the improved regioselectivity came along with a decoupling of the cofactor reduction activity [106]. In addition to P450 BM3, other self-sufficient CYPs belonging to the *Bacillus* and *Streptomyces* sp. have also been characterized [107-110].

Scheme 3

Alternatively, in other studies self-sufficient CYPs were taken as model for the design of new chimeric proteins containing both, monooxygenase and reductase domains. In

this sense, a fusion between the monooxygenase CYP153A from *Marinobacter aquaeloei* and the reductase domain of P450 BM3 from *Bacillus megaterium* was constructed for the production of ω-hydroxy dodecanoic acid from dodecanoic acid (lauric acid). I*n vivo* studies in recombinant *E. coli* strains resulted in 1.2 g L^{-1} from 10 g L^{-1} C12-FA with high regioselectivity (> 95%) for the terminal position. A second strategy increased the product formation up to 4 g L^{-1} by replacing the original substrate with the methyl ester derivative, utilizing a two phase system to avoid low substrate solubility and toxicity problems by continuous extraction and coexpressing a membrane transport system to enhance substrate transfer into the cell [111].
Utilization of a whole cell approach facilitated the application of separated CYP/reductase systems as this methodology enhanced enzyme stability, and cofactor regeneration is typically provided by the cell's own metabolic machinery. Synthesis of different medium chain-length ω-hydroxyacids has been achieved by overexpressing P450 monooxygenases from *Sorangium cellulosum* [112] and *Fusarium oxysporum* [113] in *E. coli* and *S. cerevisiae*, respectively.

6.2 *Alkene formation*
Terminal alkenes are valuable precursors to commodity chemicals such as lubricants, pesticides, polymers, and detergents. Their desirability relies on the many potential derivatizations that their alkene group can allow such as olefine metathesis to make valuable compounds from renewable resources. Furthermore, they are of particular interest as "drop-in" compatible hydrocarbon fuels due to their high energy content and their compatibility with the already existing engine platform and fuel distribution systems. Saturated fatty acids can be used as starting materials for the synthesis of 1-alkenes. Several chemical methods have been developed to achieve this transformation. Nevertheless, these methods require precious transition metal catalysts and high temperatures. Besides, the final yield of the reaction can be reduced due to the formation of internal olefins [114]. Enzymes could be an alternative to avoid these limitations in the olefin synthesis. Unfortunately, the great importance from an industrial point of view of terminal alkenes is not correlated with the insignificant role that they play in metabolic pathways. Interestingly, a few enzymes able to perform the synthesis of these compounds have been described recently.
Rude et al. reported in 2011 a new P450 fatty acid decarboxylase (OleT) from *Jeotgalicoccus* sp. (ATCC 8456). This enzyme belongs to the CYP152 peroxygenase family and is able to catalyze the decarboxylation of fatty acids (C16:0, C18:0, C20:0) into long-chain terminal alkenes with uneven chain length (such as 1-pentadecene, 1-heptadecene or 1- nonadecene) by using H_2O_2 as electron and oxygen donor [115] (Scheme 4A). Thereafter, Belcher et al. presented the crystal structure and a biochemical and kinetic characterization of OleT, broadening the substrate scope to C14:0 and C12:0 and obtained 1-tridecene and 1-undecene [116]. A possible drawback for large-scale alkene production using OleT could be the necessity of H_2O_2, since the utilization of large amounts of peroxide is expensive and a high concentration in the reaction medium can affect the activity of the enzyme [117]. In order to avoid this issue

two different approaches have been made: (i) the use of O_2 as oxidant by coupling the OleT with a biosynthetic redox cascade and (ii) H_2O_2 *in situ* generation. Liu et al. fused OleT to the P450 reductase domain RhFRED from *Rhodococcus* sp. Thus, the resulting fusion protein can transfer electrons from NADPH to the heme iron reactive center using O_2 as the oxidant. The same approach using non-fused *E. coli* flavodoxin reductase and flavodoxin was capable of supporting OleT activity as well [118]. An alternative system also involving OleT, O_2 as the oxidant, and NAD(P)H as the electron donor, was the construction of an efficient electron-transfer chain employing putidaredoxin CamAB in combination with NAD(P)H dehydrogenase-recycling at the expense of glucose, formate, or phosphite (Scheme 4B). Interestingly, in this study the FA chain length was varied from C4:0 to C22:0 demonstrating that short-chain FAs (<C9) could be decarboxylated [119]. With respect to H_2O_2 *in situ* generation, Zachos et al. described the combination of OleT with a light-driven peroxide-generation. In this system the flavin mononucleotide (FMN) serves as a photocatalyst, which in its excited state oxidizes a sacrificial electron donor (EDTA). Subsequently, the resulting reduced FMN is reoxidized by O_2 yielding H_2O_2 that is used by OleT for the oxidative decarboxylation of fatty acids to the corresponding terminal alkenes (Scheme 4C) [120].

Scheme 4

Another option for the biocatalytic synthesis of alkenes has been reported by Zhang's group. They discovered a non-heme iron oxidase (UndA) able to convert medium-chain fatty acids (C10–C14) into their corresponding terminal olefins using an oxygen-activating, non-heme iron-dependent mechanism. To find the responsible gene in *Pseudomonas* strains both, an *in silico* as well as a synthetic approach were performed. Unfortunately, no homologues of the known enzymatic systems for alkene formation were revealed in the *Pseudomonas* genome search, and comparative genomics analysis yielded thousands of gene candidates. Alternatively, a genome library of the 1-undecene-producing *Pseudomonas fluorescens* Pf-5 was created in *E. coli* followed by heterologous expression and phenotype screening of the alkene producing clones. Preliminary studies showed that *in vitro* experiments with purified UndA resulted in single-turnover reactions and, consequently, productivities remained low. This fact suggests that the conversion leaves the enzyme with an inactive oxidized iron species. Furthermore, this is supported by the partial recovery of the activity by the addition of Fe^{2+} or reducing agents such as ascorbic acid. The best total turnover number was achieved under continuous *in situ* generation of oxygen combined with the addition of reducing agents [121, 122].

7. Fatty acid double bond hydratases

Fatty acid double bond hydratases (EC 4.2.1.53) catalyze the stereo- and regio-selective hydration of isolated *cis* double bonds in NEFA to form hydroxy fatty acids by the addition of water. These distinct FAD-containing enzymes, which only share the

FAD binding motif with other proteins, act predominantly on the Δ9*cis* and Δ12*cis* double bonds of mono- and poly- unsaturated fatty acids with a chain length varying from C14 to C22, but C18 prevailing to form mainly the 10-hydroxy- and to a minor extent the 13-hydroxy derivatives of these fatty acids. Since no uniform designation of these enzymes exists in literature, most fatty acid double bond hydratases are also described as oleate hydratases, Δ9-hydratases or 10-hydroxy hydratases.

Despite the fact that the first description of a microbial hydration of oleate to form 10-hydroxystearic acid by *Pseudomonas* sp. date back to 1962 [123] and was succeeded by the description of several other microbial fatty acid hydrations by bacteria and fungi of various genera, the corresponding hydratase was first cloned and characterized by Bevers et al. in 2009 [124]. Next, Volkov and others were able to reattribute MCRAs (myosin-cross-reactive-antigens) from *Streptococcus pyogenes*, *Bifidobacterium breve* and *Lactobacilli* as Δ9 hydratases [125-127]. The first crystallization of a Δ9-hydratase from *Lactobacillus acidophilus* (LAH) in apo form with (pdb:4IA6) and without a ligand (pdb: 4IA5) followed promptly [128]. LAH is a structural homodimer and possesses a long substrate binding channel for fatty acids with an N-terminal lid domain in each protomer. In 2015, Engleder et al., were able to resolve the holo structure of the oleate hydratase from *Elizabethkingia meningoseptica* with its FAD cofactor (pdb: 4UIR) [129]. Together with a structure-based mutagenesis study, new insights were gained on the reaction mechanism of Δ9 hydratases, especially on the activation of water, the protonation of the double bond and the reductive state of the FAD cofactor.

Meanwhile several other Δ9 hydratases have been recombinantly expressed as for instance the oleate hydratases from *Lysinibacillus fusiformis* [130], *Stenotrophomonas maltophilia* [131] or from *Macrococcus caseolyticus* [132]. These oleate hydratases have in common, that they do not at all or only to a minor extent hydrate Δ12 double bonds in linoleic acid. Oh and coworkes, however, have identified a new Δ12-hydratase from *Lactobacillus acidophilus*, which predominantly forms the 13-hydroxy derivative of linoleic acid [133, 134]. In addition, Hirata and coworkers showed that the Δ12-hydratase from *Lactobacillus* not only converts C18 *cis* PUFAs, but also arachidonic acid (C20) and DHA (C22) into the corresponding 15-hydroxy fatty acid and the14-hydroxy fatty acids, respectively [135].

Moreover, recent reports on Δ9 hydratases processes with recombinant enzymes underline their potential applicability for the large-scale production of 10-hydroxy fatty acids derivatives. For a process employing the recombinant Δ9 hydratase from *Lactobacillus plantarum* in *E. coli* to produce different 10-hydroxy fatty acids substrate concentrations of 1 M were reported [136]. Jeon *et. al.* describe an industrially relevant *E. coli* whole-cell biotransformation utilizing a Δ9 hydratase from *Stenotrophomonas maltophilia* with a final product concentration of approximately 46 g/L and a product yield of 91% and a productivity of 8.2g/L*h in a 1 L-bioreactor scale [137].

What is more, the importance of regiospecific biocatalytic hydration as key step for the production of high-value-added products like flavors, resins, waxes, nylons, plastics, lubricants, polymers *etc*. from fatty acids can well be exemplified by multienzyme cascade reactions as described in three recent articles by Park and coworkers [134,

138, 139]. In these sequential enzymatic reactions, the hydration step of either oleic acid by a Δ9 hydratase [139] or linoleic acid by a novel Δ12 hydratase [138] forms the starting point for multi branched enzymatic cascades. However, the Δ9- and Δ12-hydratase cascade published 2013 and 2015 have the following sequence in common: First the formed hydroxy-group is oxidized by an alcohol dehydrogenase (ADH) to give the corresponding oxo-fatty acid. Next the sequence is split in an oxidation catalyzed by two different Baeyer-Villinger monooxygenase (BVMO) to access different regioisomeric esters: the BVMO from *P. putida* provides access to ω-hydroxyfatty acids whereas the BVMO from *P. fluorescens* leads to the formation of an α,ω-dicarboxylic acid ester after an esterase-catalyzed hydrolysis (Scheme 5). In a subsequent work, this sequence was extended further by employing both an alcohol hydrogenase to yield α,ϖ-dicarboxylic acids and a (*S*)-transaminase to gain access to ω-aminocarboxylic acids, which are industrially relevant building blocks [134]. When using linoleic acid instead of oleic acid as starting material, Oh *et al.* [138] were also able to form lactones by adding whole-cell biotransformations with yeast to the sequence. In sum, these new enhancements made the multi-cascade reactions with hydratases as starting point more versatile for new value-added products from fatty acids of vegetable oils. However, the applicability of these approaches have to be proven to be also relevant for large scale applications.

Scheme 5

8. Conclusion

Utilization of renewable raw materials as well as the application of green reaction technologies is emerging as a thriving alternative to traditional oleochemistry processes. This fact is due to the wide variety of specialties that these biotechnological strategies have the potential to achieve, while contributing to a more environmentally friendly industrial manufacturing. In this sense, this contribution demonstrates that the use of enzymes as catalyst may play a major role in industrial lipid modification for the obtaining of all kind of products, including healthy fats and oils, cosmetics, lubricants, coatings, surfactants, and many other useful products. Furthermore, the development of protein engineering is contributing to solve the previous drawbacks of the biocatalytical approaches, enhancing the stability, activity, selectivity and specificity of the enzymes, or allowing the design of new metabolic pathways in microorganisms, to yield a high level production of lipid-related products starting from simple compounds. To accomplish this goal, the design of new tools for computational protein analysis continues to make fascinating advances that allow the gaining of interesting structural data and the creation of smaller and smarter mutant libraries. The expansion of all these techniques will go along with the replacement of the already existing processes as well as with the discovery of biocatalytic approaches to new products, resulting in a “greener” chemical future.

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10. References

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Figure captions

Figure 1. Structural characteristics of CAL-A. a) The cap domain is shown in dark red. Helix αD2 – where position G237 is located – is highlighted in light orange. The movable flap domain is shown in green and the α/β-hydrolase fold is highlighted in blue/grey. b) Topology diagram of CAL-A with β-sheets (arrows), α-helices (cylinders) and active site residues (circles). αD1-D6 represent the cap domain.

Figure 2. *p*-Nitrophenyl hexanoate (blue) illustrates the two possible locations in the fatty acid binding tunnel of CAL-A shown as wireframe. The tunnel is mainly formed by the six consecutive helices of the cap domain shown in dark red. Position of G237 is shown as dots in helix αD2 (light orange). The active site residues are highlighted as green sticks.

Scheme 1. Hydrolytic reactions catalysed by PLA1, PLA2 and PLC for oil degumming. This results in hydratable phospholipids present in the aqueous phase for easy separation from the lipid phase thus resulting in low phosphate content in the process oil.

Scheme 2. Synthesis of hydroxyeicosatetraenoic acid (HPETE) derivatives from araquidonic acid using two (*S*)-lipoxygenases (mouse 8*S*-LOX and human 15-LOX-2) and two (*R*)-lipoxygenases (8*R*-LOX from *Plexaura homomalla* and human 12*R*-LOX). The table summarizes the products obtained by the wt LOXs and their Gly or Ala variants.

Scheme 3. Reactions catalysed by P450 BM3 and its variants. The table shows product profiles of the P450 BM3 wt and the best variants selected. [1)]Sum OPs = Sum of all oxidation products from palmitic acid; [2)] KETOPs = keto-palmitic acids.

Scheme 4. Alkene synthesis catalysed by OleT. (**A**) Decarboxylation of fatty acids using H_2O_2 via the peroxide shunt. (**B**) Example of enzymatic redox cascade process for the decarboxylation of fatty acids using O_2 with cofactor recycling (DH=dehydrogenase). (**C**) Decarboxylation of fatty acids with photocatalytic *in situ* generation of H_2O_2 using FMN.

Scheme 5. Enzyme cascade reaction to convert unsaturated fatty acids such as oleic acid into ω-hydroxycarboxylic acids or dicarboxylic acids. Cofactors are not shown for clarity [139].

Figure 1

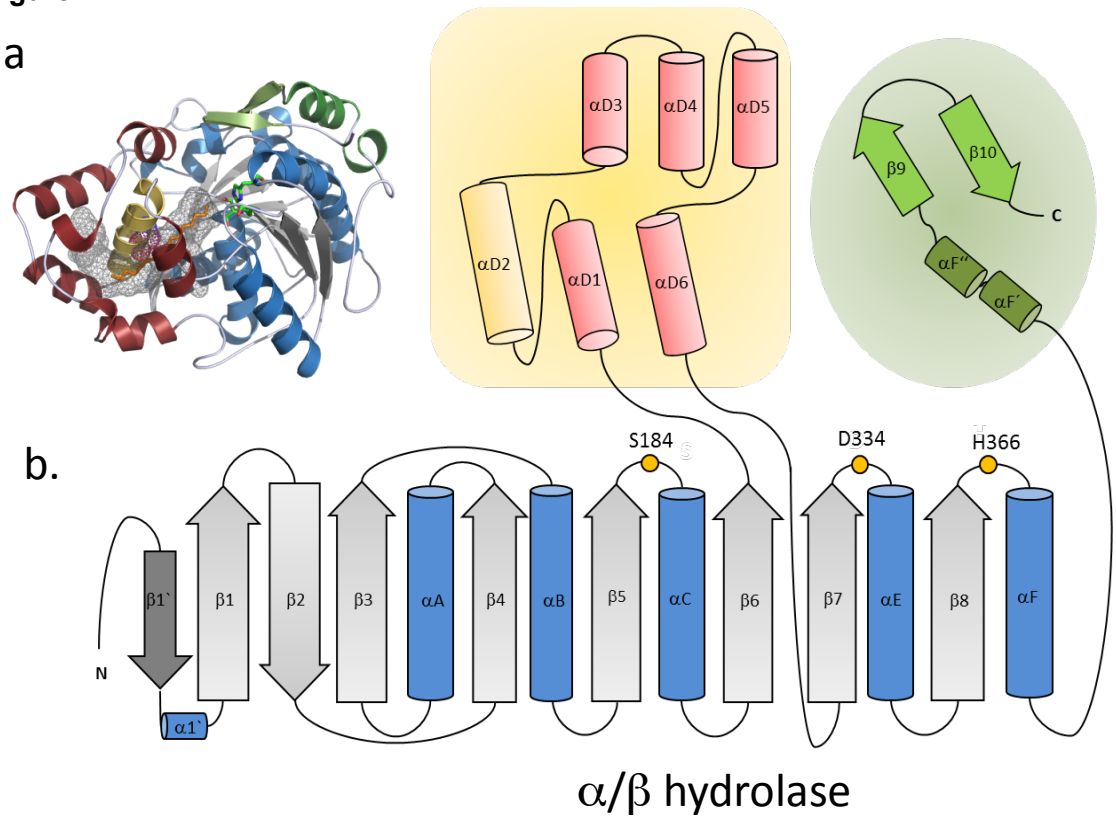
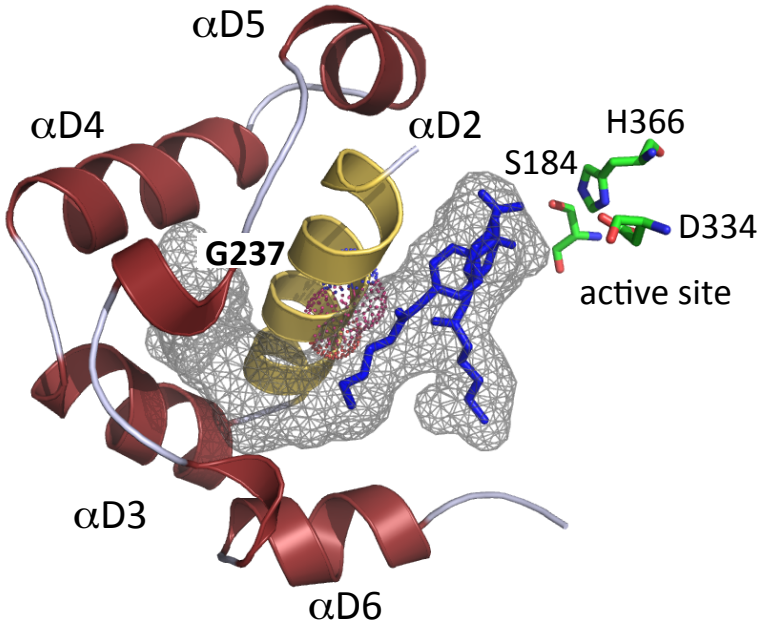
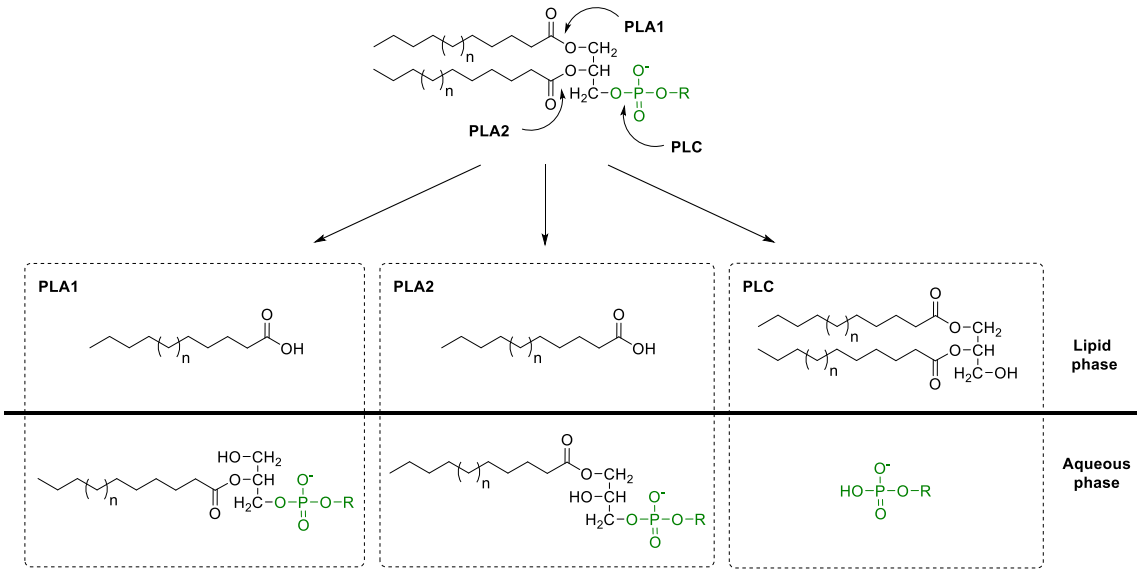

a
αD3
αD4
αD5
αD2
αD1
αD6
β9
β10
C
αF''
αF'
b.
S184
D334
H366
N
β1`
β1
β2
β3
αA
β4
αB
β5
αC
β6
β7
αE
β8
αF
α1`
α/β hydrolase

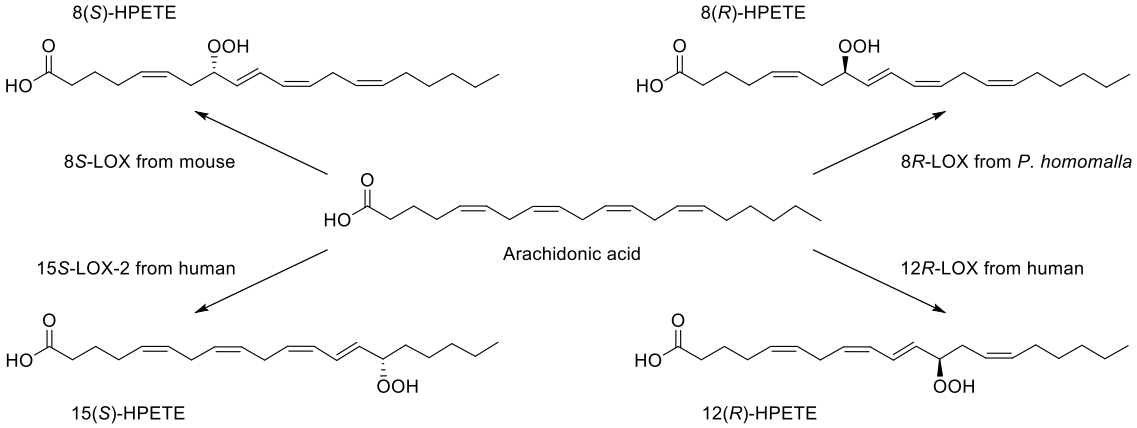
Figure 2



Scheme 1

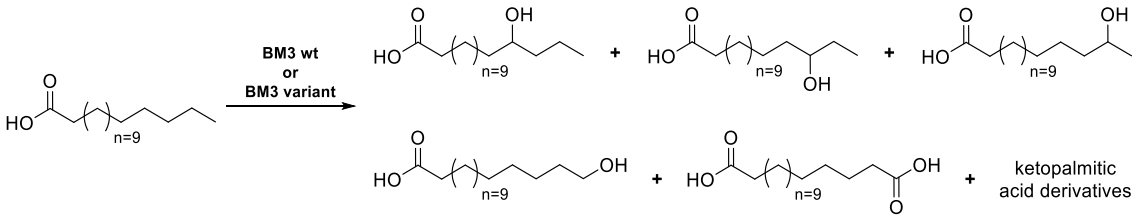


Scheme 2



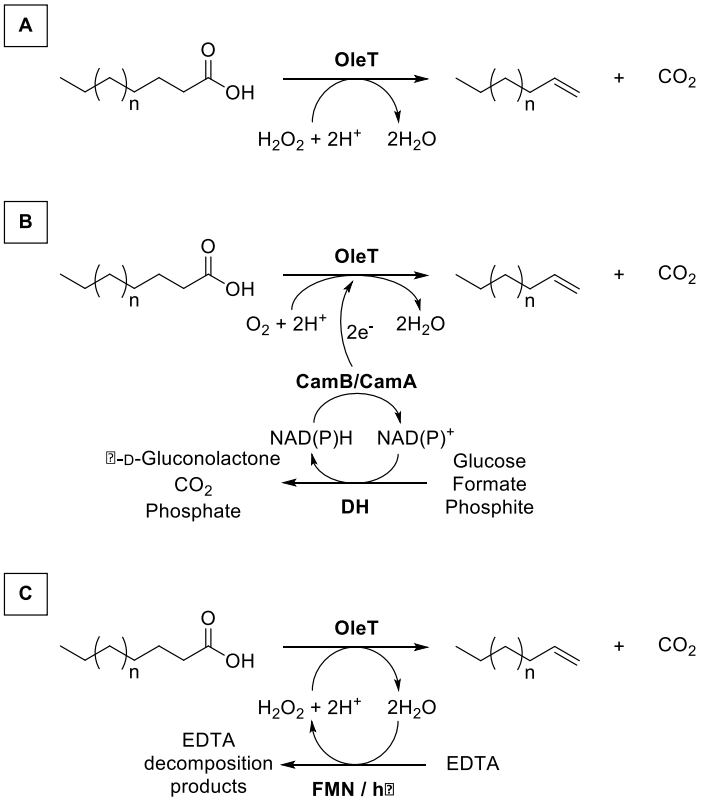
LOX	Variant	Products	Configuration	Ratio
8*S*-LOX from mouse	wt	8-HPETE	*S* (>99%)	—
	Ala417Gly	12-HPETE	*R* (>99%)	1.8:1
		8-HPETE	*S* (>95%)	
15*S*-LOX-2 from human	wt	15-HPETE	*S* (>99%)	—
	Ala416Gly	11-HPETE	*R* (>99%)	1.5:1
		15-HPETE	*S* (>94%)	
8*R*-LOX from *Plexaura homomalla*	wt	8-HPETE	*R* (>99%)	—
	Gly427Ala	12-HPETE	*S* (>98%)	—
12*R*-LOX from human	wt	12-HPETE	*R* (>99%)	—
	Gly441Ala	8-HPETE	*S* (>91%)	1.4:1
		12-HPETE	*R* (>92%)	

Scheme 3

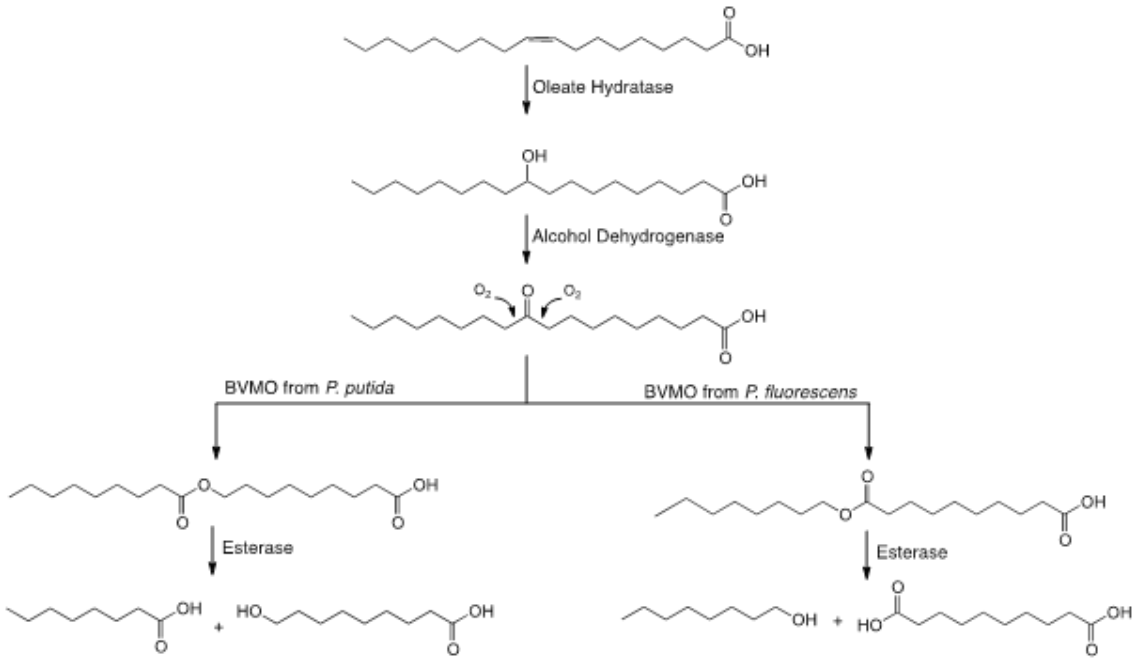


P450 BM3 variant	Product yield					
	Sum OPs[1)] (ppm)	Ω (%)	Ω-1 (%)	Ω-2 (%)	Ω-3 (%)	KETOPs[2)] (%)
wt	66	< 0.2	25	46.7	21.3	7.3
29E12 Q55P, N70S, F87I, M185T, A197V, K202R, V216A, M237L, N239T, I263F, A328V	3.0	73.5	12.3	4.0	2.0	8.4
44G7 N21S, N70S, F87I, L148F, K202R, D214G, N239T, I263F, A328V, D422V	1.2	72.6	17.1	5.1	2.6	2.6
36B7 N70S, F87I, M119I, L148F, K202R, D214G, N239T, I263F, A328V, D422V	2.5	72.2	12.4	4.8	2.4	8.1

Scheme 4


A
OleT
$H_2O_2 + 2H^+$
$2H_2O$
+ CO_2
B
OleT
$O_2 + 2H^+$
2e-
$2H_2O$
+ CO_2
CamB/CamA
NAD(P)H
$NAD(P)^+$
-D-Gluconolactone
CO_2
Phosphate
Glucose
Formate
Phosphite
DH
C
OleT
$H_2O_2 + 2H^+$
$2H_2O$
+ CO_2
EDTA
decomposition
products
EDTA
FMN / h

Scheme 5


Oleate Hydratase
Alcohol Dehydrogenase
BVMO from P. putida
BVMO from P. fluorescens
Esterase
Esterase

Table

Table 1. Hydrolysis of partially hydrogenated soybean oil using different CAL-A variants comparing the rates for release of *trans* and *cis* fatty acids (ToC) [36].

CAL-A variant	Conversion [%]	ToC
wt	22	1.36
L305N	14	2.50
F149D	11	27.77
F222S	16	7.02
F222C	20	3.53
T221H	5	>100
I301H	12	>100