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Review

Fatty Acid Hydratases: Versatile Catalysts to Access Hydroxy Fatty Acids in Efficient Syntheses of Industrial Interest

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Abstract: The utilization of hydroxy fatty acids has gained more and more attention due to its applicability in many industrial building blocks that require it, for example, polymers or fragrances. Furthermore, hydroxy fatty acids are accessible from biorenewables, thus contributing to a more sustainable raw material basis for industrial chemicals. Therefore, a range of investigations were done on fatty acid hydratases (FAHs), since these enzymes catalyze the addition of water to an unsaturated fatty acid, thus providing an elegant route towards hydroxy-substituted fatty acids. Besides the discovery and characterization of fatty acid hydratases (FAHs), the design and optimization of syntheses with these enzymes, the implementation in elaborate cascades, and the improvement of these biocatalysts, by way of mutation in terms of the substrate scope, has been investigated. This mini-review focuses on the research done on process development using fatty acid hydratases as a catalyst. It is notable that biotransformations, running at impressive substrate loadings of up to 280 g L^{-1}, have been realized. A further topic of this mini-review is the implementation of fatty acid hydratases in cascade reactions. In such cascades, fatty acid hydratases were, in particular, combined with alcohol dehydrogenases (ADH), Baeyer-Villiger monooxygenases (BVMO), transaminases (TA) and hydrolases, thus enabling access to a broad variety of molecules that are of industrial interest.

Keywords: biocatalysis; cascades; enzymes; fatty acids; hydratases; hydroxy fatty acids

1. Introduction

Hydroxy fatty acids are of the highest interest for industrial applications, since they serve as starting materials for polymers [1] or cyclic lactones [2], which are used in fragrances and antibiotics [3], as well as in plasticizers [4], surfactants [5], lubricants [6] and detergent formulations [7]. Besides being available in nature and accessible through plant oil hydrolysis (e.g., in the case of ricinoleic acid from castor oil [8]), chemical approaches towards hydroxy-functionalized fatty acids include oxygenation, epoxidation [9–11] and ozonolysis [12] as key reaction steps. Enzymatically, hydroxy fatty acids can be synthesized by means of oxidation reactions in the presence of 12-hydroxylases [13], cytochrome P450 monooxygenases, lipoxygenases, and peroxygenases [12]. An enzyme-class, which catalyzes the addition of water to non-activated C=C double bonds are hydratases. Representative examples for such enzymes are the linalool dehydratase-isomerase, which converts myrcene towards linool [14], or carotenoid 1,2-hydratase [15], which catalyzes the conversion of acyclic carotenes. However, one of the most common enzymes for the conversion of unsaturated, non-activated C=C-double bonds are fatty acid hydratases (FAHs) [16,17]. Hydratases can only be found in microorganisms [18]. After being reported for the first time by Wallen et al. in 1962 [19], they gained high interest since [20,21]. Until now, no detailed statements can be made about the physiological role of fatty acid hydratases. Some

possible functions are the detoxification of fatty acids [22] or changes in host–microbe interactions due to their capacity to enhance cell hydrophobicity [23]. FAHs are FAD-dependent enzymes [24], in which the FAD seems to stabilize the active conformation. The FAD, which is none-covalently bound to the conserved N-terminal nucleotide binding motif (GXGXXGX21E/D) [25], is, however, not involved in the reaction mechanism for the hydration of the unsaturated fatty acid [22,25]. Furthermore, there are a range of structural prerequisites for the substrates in order to be tolerated by this enzyme class. Accordingly, fatty acid hydratases require a carboxylic group in the substrate, a distance of nine carbons between the double bond and the acid group, a minimum chain length of C-14 and a *cis*-conformation of the double bond [26]. For these substrates, the hydration then proceeds by the simple addition of water, which makes such a transformation a highly attractive reaction, which is also the case for industrial purposes.

2. Application of Fatty Acid Hydratase in Synthesis and Process Development

The utilization of enzymes in the chemical industry has become indispensable, and today numerous large-scale applications, based on the use of enzymes, are already known [27–33]. Industrial processes that use enzymes as a catalyst often have excellent productivities. Well-known examples are the production of acrylamide [34] in the presence of a nitrile hydratase or the use of lipases for the hydrolysis of milk fat, oils and fat [27]. In addition, the industry also makes use of the naturally high stereoselectivity of enzymes, and representative examples in this field are the enzymatic production of L-amino acids by means of an aminoacylase [35] or the use of a penicillin acylase for the production of antibiotics [36].

A further enzyme class with broad potential applicability for industrial purpose are fatty acid hydratases, which are capable of hydrating unsaturated fatty acids, thus forming hydroxy-substituted fatty acids [37]. This type of reaction only requires water as a reagent, which also makes this transformation attractive from the point of view of sustainability. Since fatty acids are key components of fats and oils as renewable raw materials, this type of biotransformation is also of interest within the current research efforts to develop processes for industrial chemicals based on biorenewable feedstocks instead of petrochemical sources. In Figure 1, a representative example for such a transformation with a fatty acid hydratase is shown, exemplified by the synthesis of 10-hydroxyoctadecanoic acid starting from oleic acid.

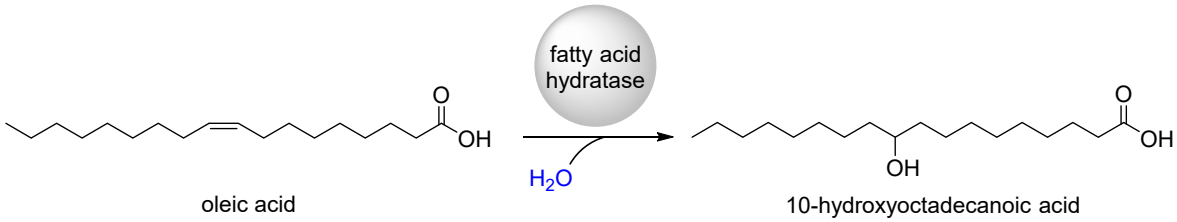


Figure 1. Reaction scheme for the synthesis of 10-hydroxyoctadecanoic acid starting from oleic acid as a selected example for the synthesis of hydrated fatty acids [37].

The characterization of fatty acid hydratases has been intensively studied, as well as the process development of hydroxy fatty acid production by means of this approach. One of the first applications of hydroxy fatty acid hydratases in the process development for the synthesis of hydroxy fatty acids is the use of the strain *Candida tropicalis* DSM 3152 and a mutant M 25 in the transformation of oleic acid into 3-hydroxy-Δ^9-cis-1,18-octadecenedioic acid, which gave the desired product with 19.4 g L^{-1} after 223 h in the fermentation process [38]. In addition, a range of other comprehensive studies, including other microbes, which possess the ability to convert unsaturated fatty acids into hydroxy fatty acids, were published, such as resting *Saccharomyces cerevisiae* cells [39], ruminal bacteria [40] or bacteria isolated from compost or soil [41,42], to name just a few, which converts oleic acid into 10-hydroxyoctadecanoic acid. In 2009, one of the most frequently used fatty acid hydratases was

described for the first time. In detail, the isolation and biochemical characterization of the oleate hydratase from *Elizabethkingia meningoseptica* (formerly known as *Pseudomonas* sp. strain 3266 and widely found in nature, e.g., in soil and water) [43], was reported by Hagen et al. [44]. In 2011, Oh et al. described a fatty acid hydratase of *Stenotrophomonas nitritireducens*, the genus *Stenotrophomonas* lives in close association with plants [45], and by means of the corresponding whole cells containing this enzyme, with 31.5 g of 10-hydroxystearic in 4 h, were formed when starting from 30 g of oleic acid [46]. Since 2012, the interest in the development of fatty acid hydratases and its usage in elevated gram-scale applications further increased.

In 2012, Oh et al. described a putative fatty acid hydratase, which was isolated from *Stenotrophomonas maltophilia* and was defined as an oleate hydratase. In the presence of this enzyme, 10-hydroxystearic acid was obtained with 49 g L^{-1} in 4 h, corresponding to a conversion of 98% [37]. The same group also reported a recombinant enzyme from *Lysinibacillus fusiformis* (organism isolated from soil) [47], which was isolated and defined as an oleate hydratase. Under optimized reaction conditions, 40 g L^{-1} (133 mM) of the desired product 10-hydroxystearic acid was isolated, which corresponds to a yield of 94% [48]. Park et al. used the oleate hydratase from *Stenotrophomonas maltophilia* and recombinantly expressed the ohyA gene in *E. coli*. This biocatalyst was able to produce 10-hydroxystearic acid with a final concentration of 46 g L^{-1} in the culture medium [49]. Subsequently, Oh et al. described a process with an oleate hydratase from *Lysinibacillus fusiformis*, which converted ricinoleic acid to 10,12-dihydroxystearic acid, which was obtained with 13.5 g L^{-1} [50]. *Lactobacillus* species are mostly found in dairy products, such as fermented milks and cheeses [51]. Additionally, Ogawa et al. classified genes that can be found in the gut bacterium *Lactobacillus plantarum*, encoding for enzymes, which are involved in the metabolism of polyunsaturated fatty acids [52]. Moreover, the LAH (*Lactobacillus acidophilus* hydratase) was described as a hydratase [53], whereas, in the same year, the crystal structure of this enzyme in the apo form was determined, showing a structural similarity to the 10,12-CLA-producing fatty acid double-bond isomerase from *Propionibacterium acnes* (PAI) and an amine oxidoreductase from *Calloselasma rhodostoma*. This crystallization gave an insight into the protein, which consist of two identical monomers, whereas an additional domain at the C-terminus acts as a lid, which covers the entrance of the substrate-binding channel. This lid probably opens through the recognition of the carboxylate residue on the surface of the enzyme. The opening of one channel led to the opening of the other protomer [54]. In 2014, Oh et al. described a fatty acid hydratase from *Stenotrophomonas nitritireducens*, which converted 5.3 g L^{-1} linoleic acid and 0.93 g L^{-1} oleic acid [55]. The same group also utilized a linoleate 13-hydratase from *Lactobacillus acidophilus* to convert linoleic acid to hydroxy fatty acid *via* the use of recombinant whole cells, which produced 79 g L^{-1} within 3 h [56]. In 2015, Ogawa et al. described a further gut bacterium (*Lactobacillus acidophilus* NTV001), which expresses a gene called FA-HY1 that is suitable to convert C18, as well as C20 and C22 unsaturated fatty acids [57]. Additionally, Oh et al. identified a double-bond hydratase that produces 13(*S*)-hydroxy-9(*Z*)-octadecenoic acid with high stereoselectivity [12]. Another milestone was the clarification of the crystal structure of *Elizabethkingia meningoseptica*, which also shows FAD as a bound cofactor. These findings gave a deep insight into the process of enzymatic water addition to C=C double bonds. The postulated mechanism starts with the protonation of the double bond by the means of a tyrosine. This positive charge is on the one hand stabilized by FAD and on the other hand by the means of an asparagine and a phenylalanine, which are localized at the C-terminus of a α-helix. A water-molecule, which is activated by a glutamate, then is able to attack on the partially charged double bond [25].

The production of a hydroxylated fatty acid at very high substrate loading could be achieved with the fatty acid hydratase of *Lactobacillus plantarum*, which was overexpressed in *E. coli* and used for the conversion of 280 g L^{-1} linoleic acid into (*S*)-10-hydoxy-*cis*-12-octadecenoic acid (HYA), leading to an enantiomeric excess (*ee*) of > 99% [58]. In addition, Oh et al. described an oleate hydratase from *Stenotrophomonas nitritireducens*, which converts plant oils to 10-hydroxy fatty acids. Hauer et al. summarized, in the Hydratase Engineering Database (HyED) 2046, putative oleate hydratases from

eleven homologous families [59]. Moreover, Oh et al. used a 10-hydratase from *Stenotrophomonas maltophilia* DSM 29579 and 13-hydratase from *Lactobacillus acidophilus* ATCC 4796 for the conversion of 31.7 and 15.6 g L^{-1} of unsaturated fatty acids in OA-rich oil [60]. All hydroxy fatty acids, which have been synthesized from fatty acids at substrate loadings that are on a multi gram per liter scale, are listed in Table 1, together with further information on experimental data. Additionally, all characterized oleate hydratases are categorized in two groups, namely OhyA1 or OhyA2. This categorization is based on the fourth conserved amino acid of the flavin adenine dinucleotide (FAD)-binding motif [61]. Moreover, it is possible to use a plant oil as a cheap and renewable [62] starting material in combination with a hydroxy fatty acid hydratase and a lipase.

Furthermore, several examples of the synthesis of hydroxy fatty acids, starting from the cleavage of the oils (Figure 2), have been demonstrated, and in the following, some selected examples are given. Kim et al. used a lipase from *Candida rugosa* to hydrolyze olive and soybean oil, and the resulting fatty acids were then further converted by means of the *Flavobacterium sp.* strain DS5, which was used to oxygenate fatty acids, thus leading to 10-ketostearic acid as major product and a minor amount of 10-hydroxystearic acid when starting from olive oil [63]. *Candida rugosa* can also be used for the hydrolysis of safflower oil [43], as well as perilla seed oil [55,64] and tree oils [65]. The lipase from *Thermomyces lanuginosus* is able to hydrolyze olive and soybean oil [66].

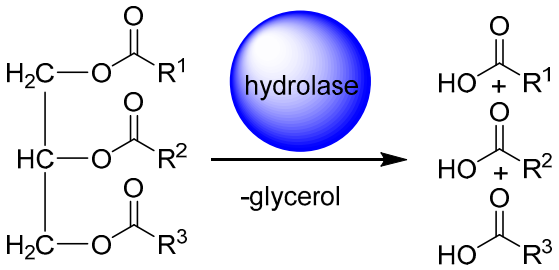
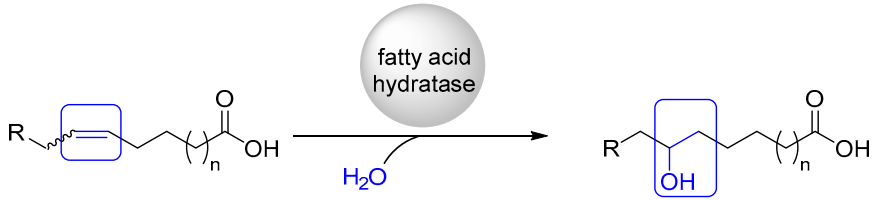


Figure 2. Enzymatic hydrolysis of oils, thus releasing fatty acids and glycerol.

Table 1. Summary of fatty acid hydratases and resulting products synthesized on multi gram-scale. Exp. in *E. coli*: expressed in *E. coli*; —: no information was given.



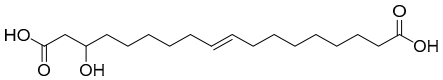
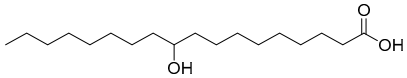
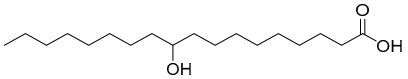
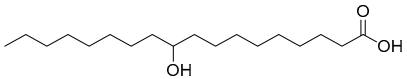
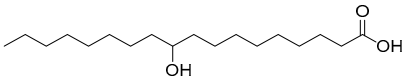
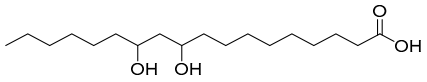
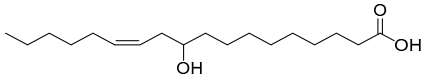
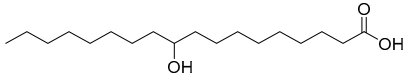
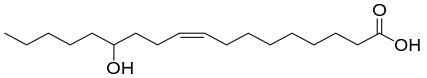
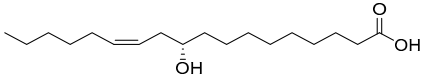
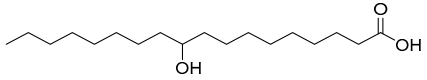
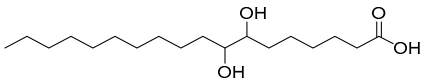
Strain (Source of Fatty Acid Hydratase)	Product	Conv./%	Product-Amount/ g L^{-1}	PRODUCTIVITY/ g L^{-1} h^{-1}	Substrate Loading
Candida tropicalis DSM 3152 (wild-type) [38]	 3-hydroxy-Δ^9-*cis*-1,18-octadecenedioic acid	-	19.4	0.8	70 mL d^{-1}
Stenotrophomonas nitritireducens (wild-type) [46]	 10-hydroxystearic acid	-	31.5	7.9	15 g/L
Stenotrophomonas maltophilia (exp. in *E. coli*) [37]	 10-hydroxystearic acid	98	49.0	12.3	50 g/L
Lysinibacillus fusiformis (exp. in *E. coli*) [48]	 10-hydroxystearic acid	94	40.0	384	40 g/L
Stenotrophomonas maltophilia (exp. in *E. coli*) [49]	 10-hydroxystearic acid	91	46.0	197	50 g/L

Table 1. *Cont.*

Strain (Source of Fatty Acid Hydratase)	Product	Conv./%	Product-Amount/ g L^{-1}	PRODUCTIVITY/ g L^{-1} h^{-1}	Substrate Loading
Lysinibacillus fusiformis (exp. in *E. coli*) [50]	 10,12-dihydroxystearic acid	90	13.5	108	15 g/L
Stenotrophomonas nitritireducens (exp. in *E. coli*) [55]	 10-hydroxy-12(*Z*)-octadecenoic acid	89; 88	5.0, 0.85	102, 22	7.5 g/L
	 10-hydroxysteric acid				
Lactobacillus acidophilus (exp. in *E. coli*) [56]	 13-hydroxy-9(*Z*)-octadecenoic acid	79	79.0	631	100 g/L
Lactobacillus plantarum (exp. in *E. coli*) [58]	 (*S*)-10-hydoxy-*cis*-12-octadecenoic acid	98	280.0	552	90 g/L
Stenotrophomonas maltophilia, *Lactobacillus acidophilus* (exp. in *E. coli*) [60]	 10-monohydroxy fatty acids	65, 81	21.7, 13.3	-	50 mL (reaction volume)
	 7,8-dihydroxy fatty acids				

3. Cascade Processes Involving Fatty Acid Hydratase

From the year 2013 on, a range of enzymatic cascades combining fatty acid hydratases with other enzymes have been reported in the literature. In such cascades, fatty acid hydratases, in combination with other enzymes, were used to build-up a broad range of industrially relevant products. Such cascades combining various enzymatic steps are of interest also from an industrial perspectives as work-steps of intermediates can be avoided, thus enabling a better process economy with decreased solvent consumption and solvent waste formation. A key prerequisite (and at the same time a challenge) for realizing such cascades is to achieve compatibility between the different enzymatic reaction steps.

Examples of the usage of fatty acid hydratases in biocatalytic cascade processes for the synthesis of long-chain α,ω-dicarboxylic acids and ω-hydroxycarboxylic acids are summarized in Figure 3 and are described in the following. Park et al. developed a cascade starting with a hydratase catalyzing the hydration of the fatty acid double bond. Afterwards, the alcohol is oxidized by means of an alcohol dehydrogenase (ADH) and a Baeyer-Villiger monooxygenase (BVMO). In the final step, the ester is then hydrolysed towards α,ω-dicarboxylic acids and ω-hydroxycarboxylic acids (Figure 4). All these enzymes were combined in one whole cell, and the process was conducted without addition of an external cofactor. Such products are of particular interest for applications in the field of polymers as these bifunctional molecules can serve as monomers for the synthesis of corresponding polyesters [67].

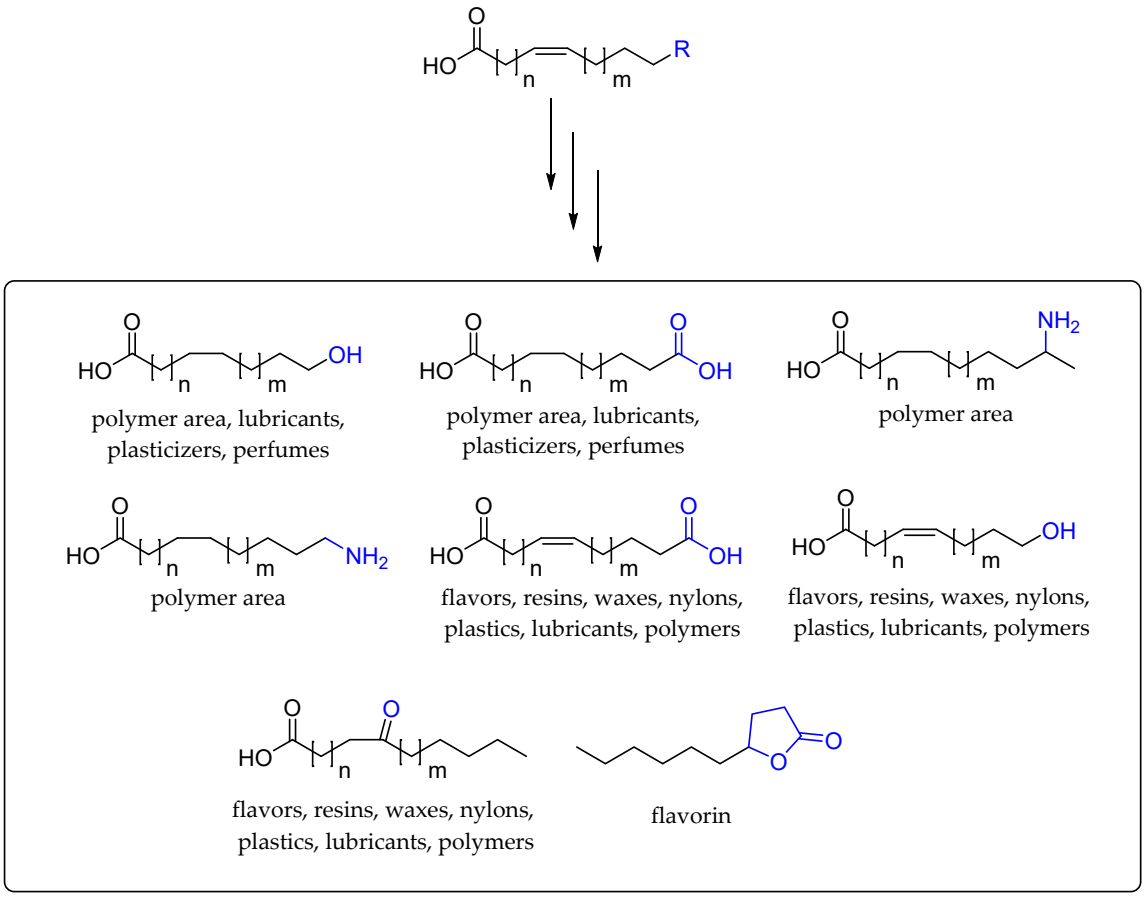


Figure 3. Collection of different molecules that were synthesized in a cascade that involve fatty acid hydratases. Listed are ω-hydroxycarboxylic acids [66] α,ω-dicarboxylic acids [68], aminocarboxylic acids [68,69], oxostearic acid [70] and lactones [2].

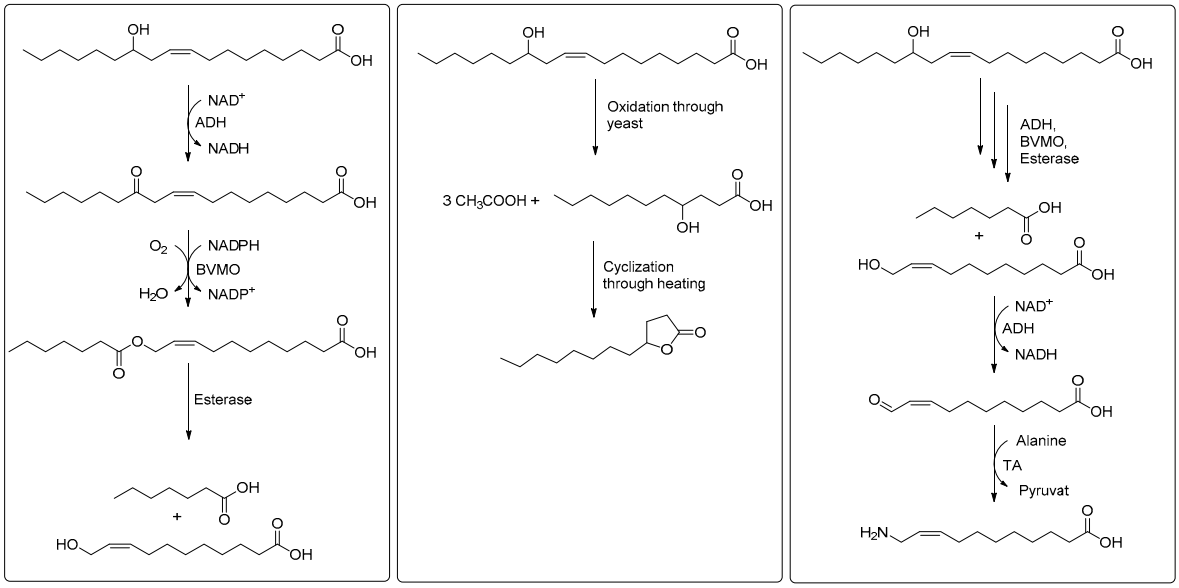


Figure 4. Chosen examples for the enzymatic cascade reactions involving fatty acid hydratases. Shown are the synthesis of ω-hydroxycarboxylic acid [67], ω-aminocarboxylic acids [68,69] and γ-lactone [2]. NAD/NADH (Nicotinamide adenine dinucleotide), ADH (Alcohol dehydrogenase), BVMO (Bayer-Villiger monooxygenase), TA (Transaminase).

Besides being interesting building blocks as starting materials in the polymer field, these products can be also utilized for the preparation of lubricants, plasticizers or perfumes [70]. This cascade process was then further expanded by coupling the cascade with an alcohol dehydrogenase, which oxidized the ω-hydroxycarboxylic acid towards the corresponding diacid or the aldehyde [67]. The latter one is subsequently transformed *via* a transaminase towards the primary amine (Figure 4) [67]. It is noteworthy that, by means of this concept, fatty acids can be converted into ω-substituted amino acids, which are of interest as monomers for polyamides.

Furthermore, this cascade was combined with a double bond hydratase to obtain unsaturated α,ω-dicarboxylic acids and ω-hydroxycarboxylic acids [71,72]. In addition, a related cascade system using a whole cell catalyst, which overexpresses a fatty acid double bond hydratase (OhyA) of *Stenotrophomonas maltophilia*, the ADH of *Micrococcus luteus*, and the engineered Baeyer-Villiger monooxygenase (E6BVMO) of *Pseudomonas putida* KT2440, was developed. Furthermore, the long chain fatty acid transporter FadL was overexpressed, which transports the fatty acids into the whole cell. This overexpression further contributes to the improvement of the whole-cell biotransformation. It should be added that this cascade starts and ends with a hydrolysis. In the initial step, a plant oil is hydrolyzed, and the final step consists of the hydrolysis of the ester by the means of a lipase from *Thermomyces lanuginosus*. The ω-hydroxycarboxylic acid is then further derivatized by means of chemical [64] or enzymatic oxidation, and the resulting product can then further be used as a monomer for the production of polyesters and polyamides [73]. This synthetic method was further extended towards a process based on the use of a whole cell catalyst, which converts long chain fatty acids like ricinoleic acid, in combination with an added aldehyde dehydrogenase, in order to obtain the corresponding ω-hydroxycarboxylic acid (Figure 4) [74].

In further studies by Xu et al., the synthesis of 10-oxostearic acid was reported, utilizing a novel oleate hydratase from *Paracoccus aminophilus* with a high specific activity of 5.21 U mg^{-1}. In the hydration step, 90 g L^{-1} of hydrated oleic acid was obtained. The formed 10-hydroxystearic was converted afterwards by an alcohol dehydrogenase from *Micrococcus luteus*, which was used in combination with a lactate dehydrogenase for co-factor recycling. After 10 h reaction time at a scale of 1 L, a conversion of 95% was reached [69]. This concept also shows the high suitability of

combining a lyase (exemplified by a hydratase) and redox enzymes (exemplified by alcohol and lactate dehydrogenases).

Another building block that is of interest for a different industrial product segment was synthesized by Oh et al. in 2014, which also utilize fatty acid hydratases. In detail, they produced γ-dodecalactone from safflower oil, which is widely used in perfume industry or enhancing aroma or taste [55]. The cascade starts with the hydrolysis of safflower oil by means of lipase from *Candida rugosa* producing free fatty oleic and linoleic acid. Afterwards, the hydration was catalyzed by the FAH from *Stenotrophomonas nitritireducens*, followed by lactonization in the presence of these *Candida boidinii* whole cells [57].

Furthermore, Park et al. very recently published a cascade for the production of aliphatic amines from renewable fatty acids, which are interesting for applications in the polymer area such as for the synthesis of carbamates and polyurethanes [67]. This concept is based on the combined use of three types of enzymes, namely a fatty acid hydratase, an alcohol dehydrogenase and a transaminases [68].

4. Conclusions

The importance of fatty acid hydratases in biotechnology has been demonstrated by the many achievements of different groups, including the development of suitable biocatalysts, microorganisms or isolated enzymes overexpressed in *E. coli*. The biocatalysts were further used for the process development of fatty acid hydration in huge scale applications, producing industrially relevant hydroxy-substituted fatty acids from renewable raw materials, such as, e.g., vegetable oils as raw material. Nevertheless, high cell or enzyme amounts were used, as well as no elaborate isolation methods being presented, which is a bottleneck for further utilization. Moreover, the combination of fatty acid hydration with other enzymes in complex cascade processes was intensively studied for the further production of industrially valuable molecules. However, up to now, only little is known about the catalytic mechanism, which is due to the fact that only a limited number of crystal structures are available [25,54,75,76]. The advanced knowledge could contribute to the future improvement of productivity and the design of novel biotransformation pathways. Other confines are the substrate scope, which is limited by the seven carbon atoms between carboxylic residue and double bond, as well as the length of the fatty acids and the carboxylic acid itself. These problems have already been addressed in some publications, like the hydration of unactivated olefins by using short chain acids as decoy molecules [77], the rational of engineering a fatty acid hydratase to enhance the substrate scope [78] and the hydration of oleic acid derivatives without a free carboxylate moiety [79]. Thus, the current status represents a highly promising basis for further studies in this field with the perspective of a range of industrial applications for a sustainable manufacture of industrial chemicals for various commercial segments, based on the utilization of biorenewable raw materials.

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Conflicts of Interest: The authors declare no conflict of interest.

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