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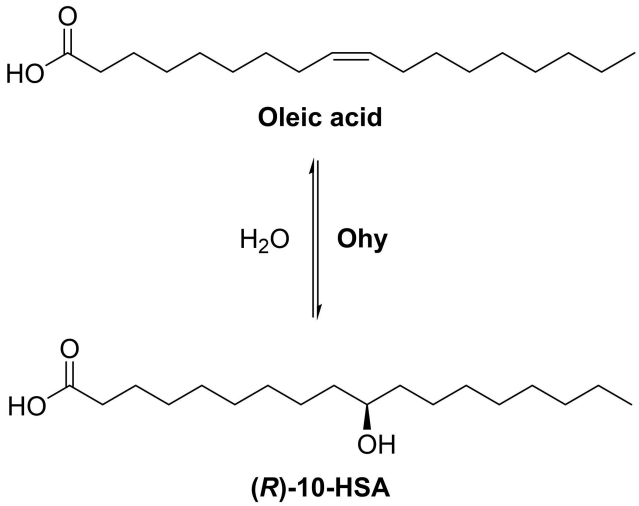
Immobilization of Oleate Hydratase on Solid Supports

Keiko Oike,[a] Rob Schoevaart,[b] Frank Hollmann,[a] Ulf Hanefeld,[a] and Peter-Leon Hagedoorn*[a]

Oleate hydratases open a biocatalytic access to hydroxy fatty acids by hydration of unsaturated fatty acids. Their practical applicability, however, is hampered by their low stability. In this study we report the immobilization of the oleate hydratase from *Rhodococcus erythropolis* PR4 on functionalized porous, spherical polymer beads. Different carrier materials promoting covalent, hydrophobic, ionic and his-tag affinity were screened and immobilization yields typically >95% were observed. The highest activity recovery of 32% was achieved by immobilization via ionic interaction with quaternary ammonium functionalized beads. Biochemical properties of the enzyme immobilized via ionic interaction remain unchanged upon immobilization. The immobilized enzyme was applied for synthesis of 10-hydroxystearic acid remaining stable under process conditions. Conversion of up to 100 mM oleic acid gave 10-hydroxystearic acid achieving a TON of up to 19,000. Successful recycling of the biocatalyst for up to ten cycles further demonstrate its potential for the synthesis of 10-hydroxystearic acid.

Introduction

Fatty acids play an important role as new biorenewable feedstocks for the chemical industry and alternative to fossil resources.[1] The introduction of hydroxy moieties is a key step in valorization of certain unsaturated fatty acids. A prominent example of a hydroxy fatty acid is 12-hydroxy-9-*cis*-octadecanoic acid (ricinoleic acid), the main product of castor oil hydrolysis (ca 70% w/w), a cheap biorenewable resource for products such as decalactone and 12-hydroxystearic acid (12-HSA). The latter has numerous applications in polymer chemistry and in the cosmetic industry. The established manufacturing process of 12-HSA features energy intensive processes such as hydrogenation using Raney-Ni and saponification starting from ricinoleic acid.[2] While chemical hydration is neither regio- nor stereoselective, enzymatic hydration is both.[3] The use of a single stereoisomer is beneficial for industrial processes such as preparation of polyester compared to the use of an isomer mixture.[4] An alternative chemical with similar properties derived from oleic acid is 10-hydroxystearic acid (10-HSA).[1–2] Oleate hydratases (Ohys, EC 4.2.1.53), are a class of flavoenzymes catalyzing water addition to oleic acid under mild conditions forming (*R*)-10-HSA (Scheme 1).[3a] A possible native function of this enzyme is proposed to be the protection of bacteria from antimicrobial unsaturated fatty acids secreted from mammalian skin as demonstrated for the Ohy from *Staphylococcus aureus* (*Sa*Ohy).[5] Beside the single-enzyme detoxification, a cascade featuring hydration by Ohy followed by dehydration to the *trans*-olefin and subsequent reduction to the saturated fatty acid has also been discussed.[6] Ohys require the cofactor flavin adenine dinucleotide (FAD) which is non-covalently bound to the enzyme.[7] Amongst the 11 specific oleate hydratase families (HFams), to date, crystal structures from five oleate hydratases of the families HFam 2 (Ohy from *Lactobacillus acidophilus* (*La*Ohy) and *Sa*Ohy), HFam 3 (Ohy from *Rhodococcus erythropolis* CCM2595: *Re*Ohy$_{CCM2595}$) and HFam 11 (Ohy from *Elizabethkingia meningoseptica*: *Em*Ohy and *Stenotrophomonas* sp. KCTC 12332: *St*Ohy) have been reported in literature.[8] The enzyme structure consists of four domains and contain a Rossmann fold or a Rossmann fold like structural motif binding the cofactor FAD non-covalently.[3b]



Scheme 1. Conversion of oleic acid to (*R*)-10-HSA catalyzed by Ohy.

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The current state of the art process for enzymatic 10-HSA production is a batch process employing Ohy containing cell lysates or purified protein. The reaction product is extracted using organic solvents, leading to complete inactivation of the enzyme.[2] Besides production of hydroxy fatty acids, coupling of Ohys in enzymatic cascade reactions gives access to fatty ketones, alkanes, fatty acid esters, etc[9] derived from plant-based resources. High enzyme loadings are required due to the very low stability of Ohys especially in the presence of high concentrations of the substrate, which could be attributed to the detergent properties of fatty acids.[10] Processes employing resting cells are limited by the incorporation of fatty acids in the cell membrane making downstream processing more challenging. Reuse of the enzyme catalyst was not described in any of the previously reported processes. Enzyme immobilization is a technique for improving enzyme performance by physically separating the enzyme from the reaction solution.[11] This can be achieved by different means such as covalent anchoring (typically via a reaction of epoxide-functionalized carrier with lysine residues on the surface),[12] non-covalent interactions, the formation of enzyme aggregates and entrapment.[13] The interaction between enzyme and carrier, especially if multi-point interactions are generated, can lead to increased enzyme stability.[14] There is only one report on the immobilization of an Ohy. *Em*Ohy covalently immobilized on magnetic chitosan beads showed an activity recovery of 23%. Other carrier materials led to inactivation of the enzyme. This report focused on the characterization of the immobilizate regarding its biochemical properties and storage stability, and data regarding the application of the immobilized enzyme for synthetic purposes were not reported.[15] In this study, we report the immobilization of an Ohy on spherical polymeric beads called Immobeads produced by ChiralVision.[16]

Results and Discussion

Production of *Re*Ohy

In our previous study, we developed a photometric assay for kinetic characterisation of oleate hydratases. In addition, we also identified an Ohy from *Rhodococcus erythropolis* PR4 (*Re*Ohy) exhibiting a broad substrate scope.[17] This Ohy has a sequence identity of >99% to the previously described *Re*Ohy$_{CCM2595}$.[8c] In *Re*Ohy, three amino acids are substituted (V89I_E174D_T432A) compared to *Re*Ohy$_{CCM2595}$. Both Ohys from *Rhodococcus erythropolis* are members of the specific oleate hydratase family HFam 3. *Re*Ohy$_{CCM2595}$ is the only structurally characterized member of HFam 3 and is to date the only monomeric Ohy.[8c] A model of *Re*Ohy based on the crystal structure of *Re*Ohy$_{CCM2595}$ shows that these enzymes are structurally nearly identical (Supporting information Figure S1). Both enzymes lack an α-helix in the *N*-terminal region which is suggested to mediate the dimer formation.[8c] All other structurally characterized Ohys from the families HFam 2 and HFam 11 form dimeric structures.

We expressed *Re*Ohy heterologously in *E. coli* and purified the enzyme via Ni^{2+}-NTA affinity chromatography. We investigated the biochemical properties of purified *Re*Ohy in a standard biotransformation of oleic acid forming 10-HSA (Supplementary information Figures S4-S8) observing similar properties to those of *Re*Ohy$_{CCM2595}$ (see details below).

Immobilization Carrier Screening

Next, we conducted a screening for a suitable carrier for immobilization using the commercially available screening kit Immobeads from ChiralVision based on functionalized porous spherical beads (Supporting information Tables S1 and S2). The functionalization enables different interaction types with the enzyme: a) covalent (COV), b) adsorption (ADS), c) cationic (CAT), d) anionic (ANI) and e) his-tag affinity (HIS). The screening kit offers the advantage that not only different interaction types and functional groups, but also different bead and pore sizes are included which increases the probability to find a suitable carrier material. For immobilization of *Re*Ohy onto the carriers, the enzyme was incubated overnight at 4 °C. A pretreatment of the carrier materials was not necessary. Enzyme immobilization on all carriers materials was performed using the same procedure as a first approach. Unbound enzyme was washed off from the support using buffer. Subsequently, the immobilizates were used in wet form. The immobilization efficiency was determined using the protein concentration in the supernatant before and after incubation (Figure 1, upper). In general, high immobilization efficiencies >95% were observed for most carriers. While the immobilization efficiency on the unpolar adsorption-based carriers (ADS1-3) was high, it was moderately lower for the polar ADS4 carrier. The anionic charged carrier CAT-1 showed a very low immobilization efficiency of ~20%.

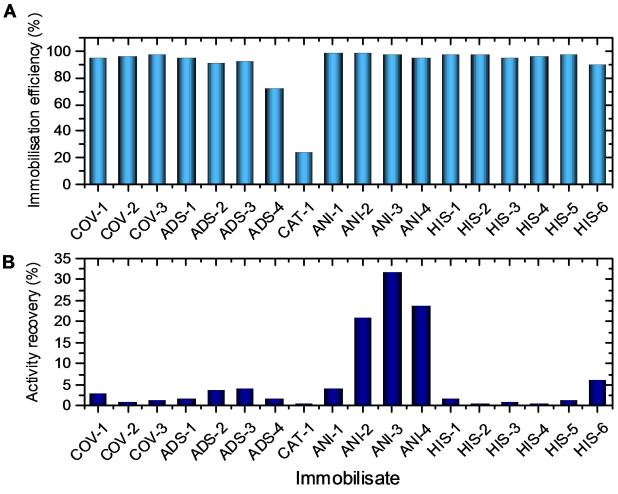

Figure 1. Immobilization efficiencies (upper) and activity recovery (lower) for *Re*Ohy on different solid supports. Activity recovery calculated by comparing the activity of the immobilized enzyme against the initially immobilized activity. Reaction conditions: V = 0.5 mL, 50 mM buffer (pH 4.0–8.0), 20 μM FAD, 5% DMSO, 0.5 μM oleic acid, 5.0 μM immobilized *Re*Ohy, 25 °C, 850 rpm orbital shaking, 45 min reaction time. Experiments were performed in duplicates.

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This is expectable taking into account that the CAT-1 carrier is functionalized with anionic sulphonate groups as *Re*Ohy is negatively charged at pH 7.0 (calculated pI = 5.4). *Re*Ohy is therefore more suitable for immobilization via anionic interaction.

Subsequently, the activity of the immobilized *Re*Ohy was tested in a standard biotransformation of oleic acid and compared to the activity of the free enzyme (Figure 1, lower). The conditions from Lorenzen et al. used for the characterization of $ReOhy_{CCM2595}$ were adapted.[8c] For most carriers, activity recoveries below 5% were observed. This suggests that the enzyme is deactivated by the immobilization process. Activity tests with the supernatant of the immobilization batches proved that the non-immobilized *Re*Ohy remains active (data not shown). (Partial) unfolding, immobilization in unfavorable orientation and diffusion limitation are possible factors causing the deactivation of *Re*Ohy upon immobilization. More specific procedures tailored for the different type of carrier materials, especially COV-1 to 3, may result in higher recoveries,[18] but were not explored here. While activity recoveries of up to 23% were reported for covalently immobilized *Em*Ohy,[15] immobilization of *Re*Ohy through covalent interaction was unsuccessful. In contrast, good activity recoveries > 20% were achieved for the carriers binding *Re*Ohy via anionic interaction with polar functionalized surfaces (ANI-2, ANI-3 and ANI-4). These three carrier materials are based on tertiary amine (ANI-2) or quaternary ammonium functionalized resins (ANI-3, ANI-4). Amongst these three, the highest activity was maintained with the largest particle (800 μm) and pore size (72% pore volume). *Re*Ohy immobilized on ANI-3 carrier showed an activity retention of 32%. This value is clearly the highest amongst all tested carriers and exceeds the reported activity recovery of 23% for *Em*Ohy covalently immobilized on magnetic chitosan beads.[15]

Characterization of the Immobilized *Re*Ohy

The treatment of the immobilizate can have a huge impact on the activity of the immobilized enzymes.[19] We tested different workup methods of the carrier (Table 1). Washing of the immobilizate with water instead of PIPES buffer (Entry 1) lead to an even higher activity recovery of 45% (Entry 2). At pH 7.0, PIPES is predominantly present in its monoanionic form which can also interact with the quaternary ammonium groups of the carrier materials. The washing step is possibly removing the bound buffer salt which could promote access of the hydrophobic substrate to the immobilized enzyme. A wet formulation of the immobilizate is necessary. Drying or acetone-treatment of the carrier leads to a sharp decrease of the activity recovery to below 5% (Entries 3, 4). All immobilized *Re*Ohy subsequently used in this study was washed with water before storage and usage in biotransformations.

Table 1. Impact of different treatments of *Re*Ohy immobilized on ANI-3 beads on the activity recovery.

Entry[a]	Treatment	Activity recovery [%][b,c]
1	PIPES buffer wash (wet)	32
2	Water wash (wet)	45
3	Air dried after water wash	3
4	Air dried after acetone wash	4

[a] Reaction conditions: V = 0.5 mL, 50 mM PIPES buffer (pH 7.0), 20 μM FAD, 5% DMSO, 0.5 mM oleic acid, 5.0 μM immobilized *Re*Ohy (ANI-3), 25 °C, 850 rpm orbital shaking, 45 min reaction time, experiments were performed in duplicates; [b] Calculated by comparing the activity of the immobilized enzyme against the initially immobilized activity; [c] Error range ± 3%.

With the immobilized enzyme in hand, we investigated common biochemical properties and compared them to purified *Re*Ohy (Figure 2). In detail, we determined the dependence of *Re*Ohy activity on the pH-value using different buffers, the thermostability and the impact of the presence of FAD for enzyme activation.

First, different buffers in the range between pH 4.0 and 9.0 were tested (Figure 2A, D). Both free *Re*Ohy in solution and the immobilized *Re*Ohy showed the highest activity in 50 mM PIPES buffer at pH 7.0. At the same pH, lower activities were observed in potassium phosphate or Tris-HCl buffer for both the free enzyme (ca. 80%) and the immobilized enzyme (ca 50%). The enzyme was significantly more active in a range between pH 5.0 and 7.5 depending on the buffer type. The activity gradually increased from pH 5.0 to 7.0 and sharply decreased at higher pH. Interestingly, immobilized *Re*Ohy showed a higher relative activity in sodium citrate buffer compared to the free enzyme in the pH range from 5.0 to 6.0. This observation is opposite for sodium phosphate and Tris-HCl buffer where higher relative activity was observed with free *Re*Ohy.

Next, the thermostability of the enzyme was tested (Figure 2B, E). The enzyme was incubated for two hours at a specific temperature in 50 mM PIPES buffer (pH 7.0) before starting the reaction under standard conditions. The free enzyme was stable at temperatures up to 35 °C above which the activity rapidly declined. The immobilized enzyme showed a similar pattern, but was only stable up to 30 °C. Thus, it can be assumed that the binding to the carrier causes a distortion due to multi-point anchoring by hydrophobic interaction with the porous carrier and ionic interactions with the functionalized carrier surface.[14] Furthermore, enzyme immobilized via ionic interactions are reported to be prone to deactivation under higher temperatures due to facilitated dissociation of the enzyme-carrier aggregate.[13]

The dependence of Ohys on FAD is well established in literature, the enzymes are inactive in absence of the cofactor.[3b] However, the precise role of the FAD in the catalytic cycle remains unknown. FAD binding is non-covalent, thus a more or less profoundly exhibited on-off binding mechanism of the cofactor[20] is expected.

Proposed functions of the FAD are the stabilization of the enzyme structure and an assisting role in the substrate binding.[8d] UV-vis measurements suggest that the cofactor is not present in purified *Re*Ohy (Supporting information Figure S2), thus FAD is typically supplemented to the reaction

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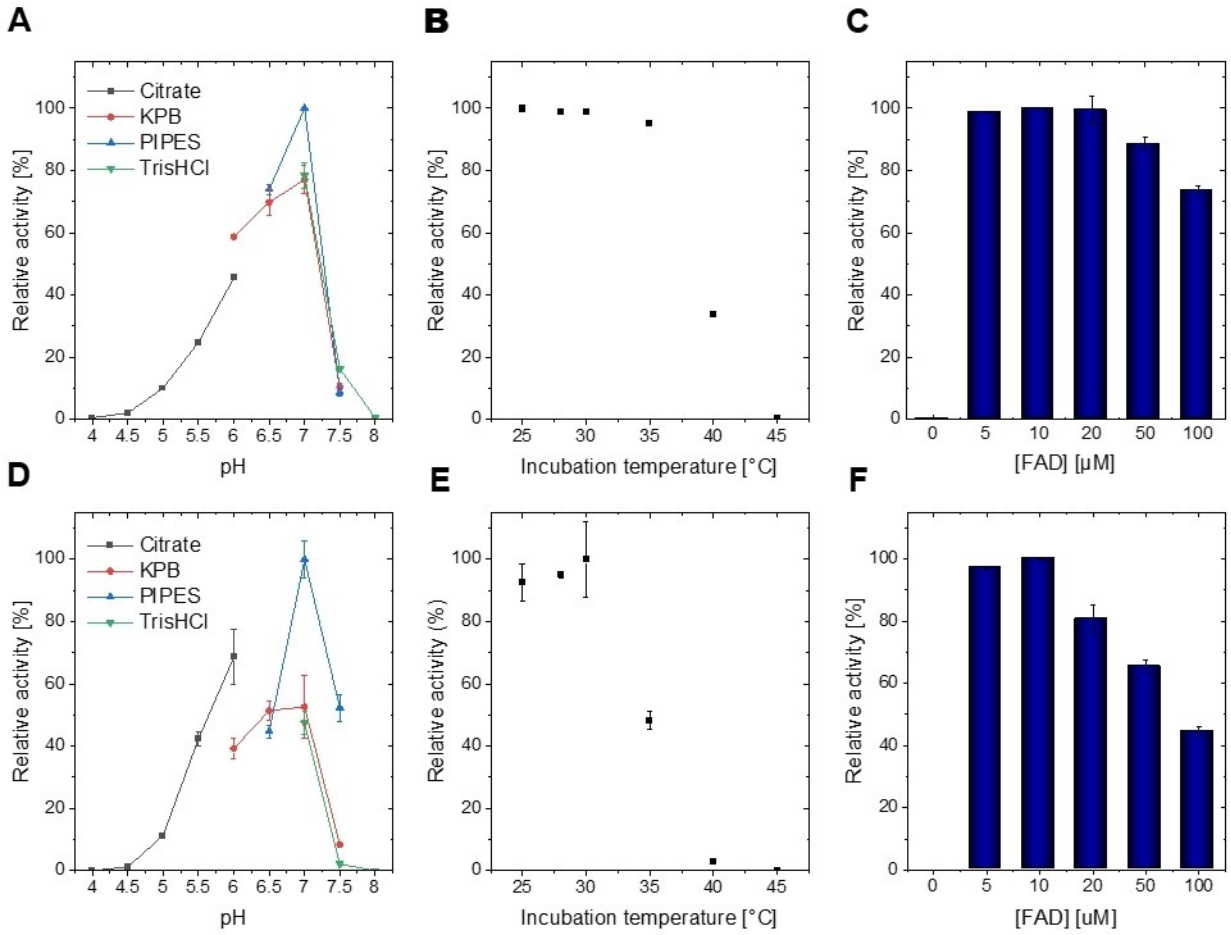

Figure 2. Biochemical characterization of *Re*Ohy in free (A–C) and immobilized form (ANI-3, D–F). Reaction conditions: V = 0.5 mL, 50 mM buffer (pH 4.0–8.0), 0–100 μM FAD, 5% DMSO, 0.5 mM oleic acid, 5.0 μM *Re*Ohy (immobilized on ANI-3), 800 rpm orbital shaking, reaction time 15 or 45 min. Experiments were performed in duplicates. 100% correspond to 40.1 mU mg^{-1} (purified *Re*Ohy) and 18.2 mU mg^{-1} (*Re*Ohy immobilized on ANI-3).

solution. The effect of different concentrations supplemented FAD on the activity of *Re*Ohy was tested by varying the concentrations between 0 and 100 μM (Figure 2C,F). As expected, no conversion of oleic acid was observed without external supply of the cofactor. The highest activity was observed at 10 μM FAD (two equivalents compared to the amount of enzyme) both for free and immobilized *Re*Ohy. At higher FAD concentrations, the activity decreased. This effect was more prominent for immobilized *Re*Ohy. The decrease might be due to inhibitory effects caused by unselective binding of the additional FAD in the active site. In previous reports on *Re*Ohy$_{CCM2595}$, 20 μM FAD equivalent to four equivalents compared to the enzyme were supplied in all experiments and the effect of the FAD concentration was not investigated.[8c] Also for other Ohys, especially these deficient of FAD after purification, supplementation of the cofactor is crucial for the enzymatic activity.[7,8c] Nevertheless, achieving optimal activity at low FAD concentrations suggests a high affinity of *Re*Ohy for the cofactor and is beneficial from an economic perspective considering the high price of FAD.

Performance of Immobilized *Re*Ohy in Biotransformations

Next, we aimed to perform preparative biotransformations employing immobilized *Re*Ohy. Therefore, we first tested the stability of both free and immobilized *Re*Ohy against a selection of water (im-)miscible organic solvents (Supporting information Table S3). Unfortunately, all tested organic cosolvents except DMSO completely deactivated the enzyme. DMSO was tolerated well in concentrations up to 5% (Figure 3). At higher co-solvent concentrations, the activity of *Re*Ohy was notably decreased. The immobilization of *Re*Ohy on the anionic carrier led to a moderately decreased tolerance against higher DMSO concentrations. Most biochemical properties of the immobilized enzyme did not undergo major changes upon immobilization.

In the following, we increased the substrate loading of oleic acid in analytical scale biotransformations (500 μL reaction solution). 5% of DMSO was added as co-solvent. Alternatively, the substrate oleic acid was added in pure form without co-solvent (Table 2). While for no reaction setup full conversion was achieved after one day reaction time using 5.0 μM enzyme, conversions of > 80% were observed for substrate loadings up to 25 mM after one week reaction time. No significant differ-

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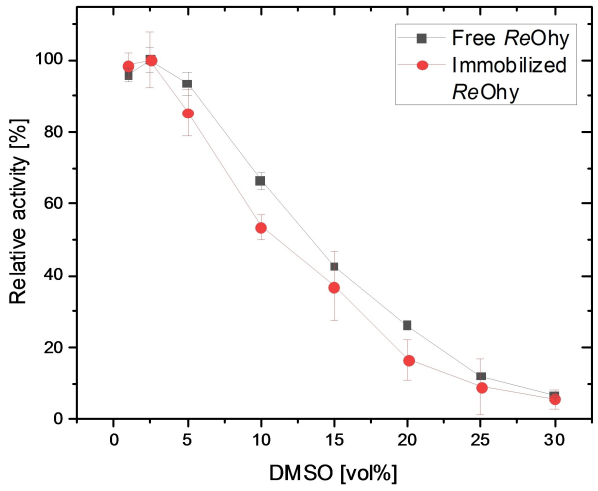

Figure 3. Tolerance of *Re*Ohy against different concentrations of DMSO. Black squares: free *Re*Ohy; red circles: immobilized *Re*Ohy (ANI-3). Reaction conditions: V = 0.5 mL 50 mM PIPES buffer (pH 7.0), 20 μM FAD, 2.5–30% DMSO, 500 μM oleic acid, 5.0 μM *Re*Ohy (immobilized, ANI-3), 25 °C, 800 rpm orbital shaking, 45 min reaction time. Experiments were performed in duplicates. 100% correspond to 41.9 mU mg^{-1} (purified *Re*Ohy) and 18.5 mU mg^{-1} (immobilized *Re*Ohy).

ence observed for the reactions carried out in the absence of DMSO. The absolute production rate of 10-HSA leveled off above 25 mM. This suggests apparent maximal reaction rates above 25 mM oleic acid, which could reflect the K_m value of the enzyme. However, it has to be considered that the reaction rates are also strongly dependent on the physical forms in which oleic acid exists at these concentrations and conditions (e.g. formation of micelles). No apparent loss in hydratase activity was observed over seven days in the biotransformations at higher substrate loadings suggesting a high stability in immobilized form.

Diffusion limitation is often a problem in biotransformations using immobilized enzymes and especially prominent when performing reactions on a very small scale.[21] Thus, it was investigated if the reaction proceeds better when carried out on larger scale. Therefore, reactions were performed in Erlenmeyer flasks (5 mL reaction volume in 50 mL flask volume) with shaking at 200 rpm (Figure 4). This led to a remarkable improvement of catalyst performance using the same catalyst loading. A possible reason is the improved mixing achieved by orbital shaking in the larger reaction vessel. In addition, the substrate can interact with the polypropylene reaction tube used for small scale reactions which impedes the diffusion from the solution to the catalyst. For a substrate loading up to 50 mM oleic acid, conversions of >90% were achieved after one day reaction. Even with 100 mM oleic acid, approximately 70% of the substrate was converted in one day. Conversions >95% for up to 100 mM oleic acid were observed after one week reaction time. At elevated concentrations (>50 mM), the reaction mixture appeared as two separate phases as previously indicated in literature. Interestingly, the product precipitates out of the reaction solution as an off-white powder. This effect probably has a beneficial effect on the reaction equilibrium shifting it to the product side. We could isolate 10-HSA from the biotransformation at 50 mM substrate loading in 80% yield by filtration of the precipitate. The immobilizate was removed by a further filtration step for which the product was dissolved in ethyl acetate. The purity was >95% according to ^{1}H NMR spectroscopy (Supporting information Figure S9). While at 200 mM substrate concentration, the initial conversion after one day was only 4%, close to 50% of the substrate were converted after one week reaction time. High viscosity of the oil

Table 2. Conversion of oleic acid to 10-HSA with increased substrate loading.

Entry[a]	[Oleic acid] [mM]	Reaction time [d]	Conv. (no cosolvent) [%]	Abs. conv. (no cosolvent) [mM]	Conv. (5% DMSO) [%]	Abs. conv. (5% DMSO) [mM]
1	5	1	–[b]	–[b]	68	3.4
2		7	–[b]	–[b]	95	4.8
3	10	1	–[b]	–[b]	32	3.2
4		7	–[b]	–[b]	95	9.5
5	25	1	14	3.5	25	6.3
6		7	91	22.8	87	21.7
7	50	1	9	4.5	5	2.5
8		7	50	25.0	31	15.5
9	100	1	2	2.0	2	2.0
10		7	22	22.0	22	22.0
11	200	1	1	2.0	1	2.0
12		7	7	14.0	10	20.0

[a] Reaction conditions: V = 500 μL, 50 mM PIPES buffer (pH 7.0, not adjusted after substrate addition), 10 μM FAD, optional 5% DMSO, 5–200 mM oleic acid, 5.0 μM immobilized *Re*Ohy (ANI-3), 25 °C, 800 rpm orbital shaking, one or seven days reaction time. Experiments were performed in duplicates; [b] Experiment not attempted due to technical difficulties adding small substrate volumes.

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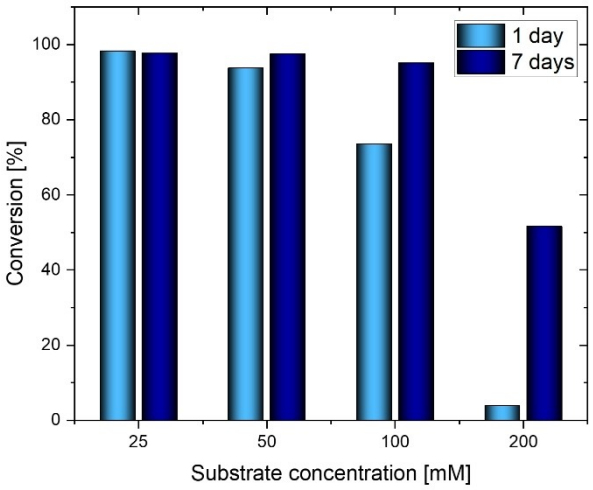

Figure 4. Conversion of oleic acid to 10-HSA dependent on the substrate concentration (5.0 mL scale). Light blue: One day reaction time, dark blue: Seven days reaction time. Reaction conditions: V = 5 mL (50 mL Erlenmeyer flask), 50 mM PIPES buffer (pH 7.0), 10 μM FAD, 5% DMSO, 25–200 mM oleic acid, 5 μM immobilized *Re*Ohy (ANI-3), 25 °C, 200 rpm orbital shaking, 1 or 7 days reaction time. Experiments were performed in duplicates.

phase is reported to limit the mass transfer into the aqueous phase.[22] Another factor contributing to the lower conversion might be a decrease of pH in presence of higher substrate concentration.

The product titre for the biotransformation at 100 mM substrate concentration was ca. 28 g L^{-1}. This value is in comparable range to other literature reported reactions between 15 and 60 g L^{-1} (Table 3).[1] The corresponding space time yield (STY) calculated based on the conversion after one day is ca. 0.8 g L^{-1} h^{-1} (Entry 1). This productivity is in the range of biotransformations with resting cells wild-type bacterial strains such *Nocardia paraffinae* induced with oleic acid.[23] Other reactions with whole-cells achieve 8 to 12 g L^{-1} h^{-1}. In general, our observed STY is approximately 15 to 30 times lower compared with other reaction systems employing cell lysate, purified enzyme or resting whole cells (Entries 2–5). The seemingly low STY obtained with immobilized *Re*Ohy is misleading, as this can be fully attributed to the low enzyme loading in our experiments. While we applied only 6 U L^{-1} immobilized enzyme, at least 300 times higher loadings (based on the enzymatic units) were applied in the experiments reaching higher STYs. It is very likely that an increased enzyme load in our biotransformations would further improve the STY. Furthermore, the STYs reported in literature are often calculated based on initial productivities and not when reaching higher conversions. Nevertheless, it can be concluded that the major advantage of immobilized *Re*Ohy is the high stability. While most Ohys are prone to deactivation, we observed conversion also after long reaction times of up to one week. In contrast, an engineered variant of the Ohy from *Paracoccus aminophilus* (*Pa*$Ohy_{T15N/F122L/F233L}$) has a half-life at 30 °C of 48 min compared to 14 min for the wild type.[10b] Other members of the Ohy HFam 3, namely from *Lactobacillus rhamnosus*, sharing ca. 55% sequence identity and the monomeric structure with *Re*Ohy were recently discovered to be dependent on the reduced flavin cofactor $FADH_2$.[24] These enzymes displayed lower stability in their free form. In contrast, our enzyme shows to be active with the oxidized form of the enzyme. However, the actual binding situation of the cofactor still needs to be elucidated. To this end, only detailed crystal structural analysis will shed further light on this behaviour. But so far, soaking and co-crystallization of *Re*$Ohy_{CCM2595}$ did not lead to cofactor binding in the crystal pointing towards a low affinity for this cofactor.[8c] The suggested FAD binding mechanism causing a structural change of a free loop in *Re*$Ohy_{CCM2595}$[8c] might be affected by the structural distortion assumed to occur upon immobilization. The increased inhibition at higher FAD concentrations in immobilized form supports the theory. Thus, we hypothesize that the FAD affinity is increased in immobilized form contributing to the high enzyme stability.

We observed a maximum total turnover number (TON) of up to 19,000. Until now, total turnover numbers have been scarcely reported for Ohy catalyzed biotransformations as their calculation in lysate or whole cell catalyzed reactions is very challenging.

Recycling of Immobilized *Re*Ohy

Another advantage of immobilized enzymes is their recyclability. While cell lysates or resting whole cells can hardly be recycled, immobilized enzyme can be removed by filtration or centrifugation.[26] Lastly, we investigated the recycling of the

Table 3. Comparison of STYs for selected biotransformations of oleic acid with Ohys.

Entry[a]	Enzyme and formulation	Enzyme loading [U·L^{-1}]	Substrate loading [mM]	STY [g·L^{-1} h^{-1}]	STY per enzyme unit [g·L^{-1} h^{-1} U^{-1}]	Ref.
1[b]	*Re*Ohy (purified, immobilized)	6	100	0.8	0.13	This study
2[c]	*Pa*OH (lysate, lyophilized)	4000	321	23.0	0.006	[10a]
3[c]	*Pa*$OH_{T15N/f122L/F233L}$ (crude extract)	1500	283	22.5	0.015	[10b]
4[c]	*Lf*Ohy (purified)	25 10^6	142	16.0	6 10^{-7}	[25]
5[c]	*Sm*Ohy (recombinant resting *E. coli* cells)	8900	179	12.3	0.001	[22]

[a] The activity of enzyme utilized have to be taken with care as they strongly depend on the assay. Reaction volumes and time differ in each setup. Therefore, the data may not be directly comparable and has to be taken with care; [b] Calculated based on the conversion after one day; [c] As given in the original work. Experiments were performed in duplicates.

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immobilized biocatalyst (Figure 5). *Re*Ohy does not contain bound FAD after purification and external FAD supplementation is crucial for the enzymatic activity (see also Figure 2C, F). UV-vis measurements suggested that FAD is quite tightly bound to the immobilized enzyme upon incubation (Supporting information Figure S3). We investigated if the FAD remained bound to the immobilized enzyme after subsequent reaction cycles. This can either be the case directly to the enzyme or via ionic interactions with the carrier.[27] Therefore, time course measurements were performed for two conditions: a) fresh supply of FAD every cycle, b) supply of FAD only in the first cycle. For the first six cycles, conversions of >95% after 24 hours were observed in both setups. The initial conversion rate decreased slightly when fresh FAD is supplied every cycle and moderately when no fresh FAD is supplied. While in the reactions with fresh FAD final conversions after 24 hours reaction times >90% are maintained even after ten cycles, a significant decrease of the final conversions is observed for the reactions without fresh FAD supply after seven cycles.

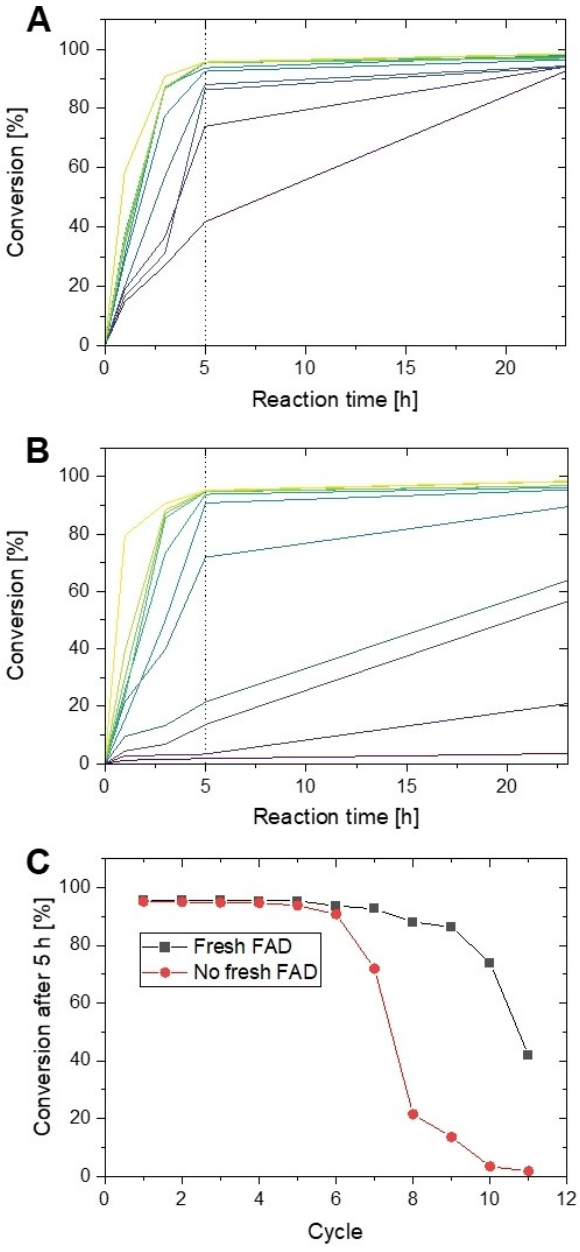

Figure 5. Recycling of immobilized *Re*Ohy (ANI-3) in biotransformation of 2.5 mM oleic acid in 10 mL reaction scale in Erlenmeyer flasks. A) Supply of 10 μM FAD every cycle; B) Supply of 10 μM FAD only in first cycle. The color gradient from yellow (first cycle) to purple (11th cycle) indicates the number of cycles the immobilizate was used. The dotted lines at t = 5 hours indicate the data points used for graph C; C) Conversions after 5 hours reaction time for each cycle. Reaction conditions: V = 10 mL (50 mL Erlenmeyer flask), 50 mM PIPES buffer (pH 7.0), 10 μM FAD, 5% DMSO, 2.5 mM oleic acid, 5.0 μM immobilized *Re*Ohy (ANI-3), 25 °C, 200 rpm orbital shaking.

These results prove that the immobilized *Re*Ohy can also be recycled many times. As purified enzymes are expensive catalysts in the chemical industry, recyclability is an important factor for industrial application.[28] It is likely advantageous to add FAD after a certain number of cycles to optimise the biocatalyst performance from an economical perspective. Furthermore, the FAD is assumed to stays bound to the enzyme more tightly as monitored by recyclability over several cycles. This also supports our hypothesis of prolonged stability when FAD stays bound to the enzyme.

In addition, these results suggest that the FAD stays bound to the enzyme after the reaction. Even after three weeks of recycling experiments (10 days reaction at 25 °C and at least ten days storage at 4 °C), notable activity can be observed. This is in contrast to the activity of cofactor free pure and immobilized enzyme. This decreases by >50% after one week storage at 4 °C (Supporting information Table S4). Thus, it can be assumed that the binding of the FAD cofactor in the active site increases the enzyme stability in contrast to the apoenzyme. The presence of the fatty acid substrate and product could also contribute to the stability, but this was not further investigated.

Conclusions

*Re*Ohy was successfully immobilized on anionic carriers and showing good activity recoveries. The best performance was achieved on quaternary ammonium functionalized porous spherical polyacrylic beads. The biochemical properties of the immobilized enzyme did not change significantly due to the immobilization. The presence of organic solvents deactivates the enzyme also in immobilized form. Preparative biotransformations on analytical scale did not lead to satisfactory results which could be significantly improved by changing the reaction vessel from Eppendorf Tubes to Erlenmeyer flasks and increasing the scale from 0.5 to 5 mL. High conversion of >90% of 10-HSA at 100 mM substrate loading corresponding to a TON of up to 19,000 were achieved in preparative biotransformations with immobilized *Re*Ohy. These results represent the highest STY per unit of enzyme used so far reported for immobilized oleate hydratases. We demonstrated the recyclability of an Ohy for the first time. Reuse of the biocatalyst for up to ten cycles was possible. In addition, we identified a beneficial impact of the immobilization on the FAD binding properties of *Re*Ohy which contributed to improved enzyme stability.

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Supporting Information

Additional experimental data, experimental procedures and spectroscopic data are found in the Supporting Information.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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