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Immobilization of Aldoxime Dehydratases and Their Use as Biocatalysts in Aqueous Reaction Media

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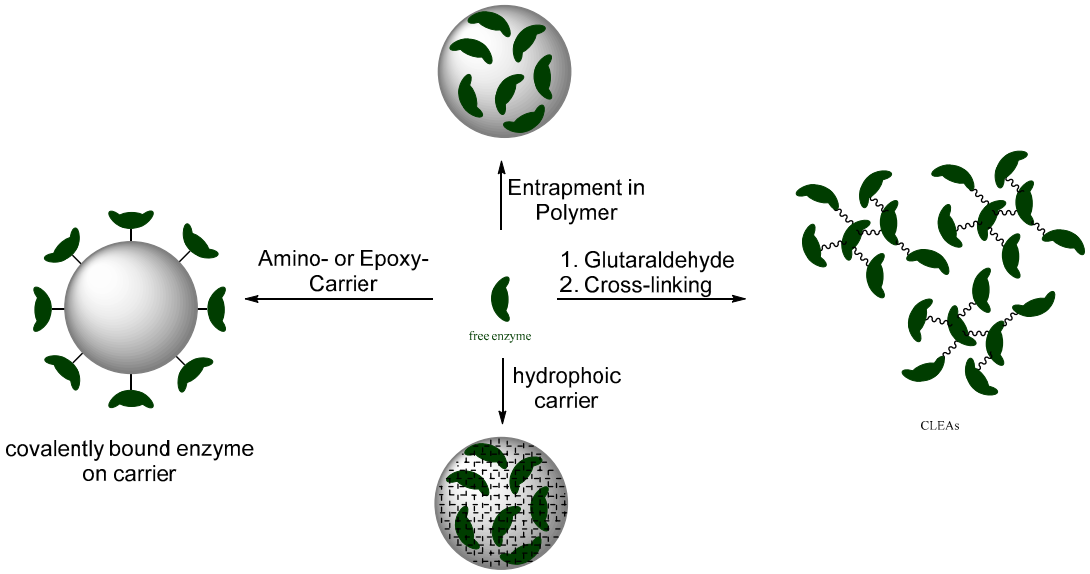
Abstract: Immobilization of biocatalysts is a current topic in research enabling the easy recovery of catalysts from the reaction medium after the reaction, and it is often accompanied by a stabilization of the catalysts, which enables recycling. Within our ongoing research on the utilization of aldoxime dehydratases in the cyanide-free synthesis of nitriles through dehydration of readily available aldoximes, a screening of different immobilization methods for free enzymes was performed. The applied immobilization methods are based on covalent binding and hydrophobic interactions of the enzyme with the carrier material and whole-cell immobilization in calcium alginate beads with and without subsequent coating. In our study, we found that the immobilization with purified free aldoxime dehydratases from OxdRE (*Rhodococcus erythropolis*) and OxdB (*Bacillus* sp. strain OxB-1) leads to high immobilization efficiencies, but also to a strong loss of activity with a residual activity of <20%, regardless of the carrier material used. However, when using whole cells for immobilization instead of purified enzymes, we could increase the residual activity significantly. *Escherichia coli* BL21(DE3)-CodonPlus-RIL OxdRE and OxdB whole cells were entrapped in calcium alginate beads and coated with silica using tetraethylorthosilicate (TEOS), leading to immobilized catalysts with up to 75% residual activity and a higher stability compared to the free whole cells. Even after three rounds of recycling, which corresponds to a 3 d reaction time, the immobilized OxdB whole cells showed a residual activity of 85%.

Keywords: aldoxime dehydratase; calcium alginate beads; dehydration; immobilization; nitrile

1. Introduction

Since biocatalysts have emerged as a valuable alternative to metal- and organo-catalysts [1–6], it is of major interest to develop highly efficient and stable biocatalysts, which in the best case can be reused. This can, for example, be realized by immobilization of the biocatalyst on a carrier or by entrapment in polymers or hydrogels. Such a heterogenized catalyst can be separated from the reaction medium more easily, usually by filtration. In comparison, whole-cell catalysts are often separated by centrifugation [7–9] or dissolved enzymes in an aqueous reaction medium which usually cannot be isolated. The free enzymes often lead to problems during the isolation of the product, especially if extraction is used. Extraction often leads to precipitation of the enzymes due to the denaturating effect of organic solvents, and the precipitate forms an interphase between the organic and aqueous phase [1–6,10–12]. This often makes downstream-processing tedious, thus causing lower isolated yields. In addition, in many cases, the biocatalyst cannot be reused after such an extractive work-up. Thus, for an improved process, a suitable immobilization method for the use of most biocatalysts is desirable. There are many different immobilization methods known, and different biocatalyst formulations can be used. On the one hand, free enzymes (used as crude extract or in the purified form) can be used, while

on the other hand, also whole-cell catalysts can be immobilized by various techniques. The application of whole-cell catalysts often has the positive effect that the cell membrane shields the enzymes from disturbing influences such as pH or solvents in the reaction medium. Immobilization of free enzymes can be performed using different strategies. An overview of different established immobilization techniques for enzymes is shown in Scheme 1.



Scheme 1. Immobilization strategies of enzymes.

The first strategy is heterogenization by crosslinking of the enzymes. The most prominent example are CLEAs (cross-linked enzyme aggregates) [13], which are formed by a preactivation of primary amino functionalities on the enzyme surface, such as the side chain of lysine, by glutaraldehyde and subsequent crosslinking of free primary amino groups with glutaraldehyde-bound amino groups to form heterogenized enzyme clusters. A second strategy of enzyme immobilization is a covalent binding of enzymes to carriers [14]. This can be performed, for example, by either usage of epoxy-carrier material, which reacts with side chains of amino acids of the enzyme surface, or by usage of amino carriers, which are preactivated by glutaraldehyde and react with amino side chains similar to the formation of CLEAs. A third technique is a strategy in which enzymes are bound non-covalently, for example, by hydrophobic interactions to carrier material with hydrophobic residues [14] or His-tagged enzymes on specific carriers [15], which often has the benefit of a weaker impact than covalent binding. Moreover, with free enzyme immobilization, whole-cell catalysts can also be immobilized. Many methods are known, for example immobilization of whole cells by entrapment in polymers, hydrogels, or other materials. To give an example for the entrapment of biocatalysts in polymers, von Langermann et al. developed a technique of whole-cell immobilization in polyurethane, which is a suitable heterogenized biocatalyst with a higher stability when compared to free whole cells [16–18]. Whole-cell catalysts (or free enzymes [19,20]) in an aqueous medium can also be immobilized by entrapment in superabsorber, yielding in a solid aqueous phase which can be used in an organic solvent as reaction medium and easily separated by filtration [21]. Although this technique allows biocatalytic reactions in organic solvents, it has the disadvantage that this immobilized biocatalyst cannot be used in aqueous medium, as it would lead to a leaching of the absorbed biocatalyst out of the superabsorber. Furthermore, the organic solvent usually has a deactivating effect on the enzymes; thus, the heterogenized catalyst cannot be reused [22]. Enzyme immobilization can also be performed by entrapment in calcium alginate beads. This immobilization technique was recently applied by the Patel group to combine a pig liver esterase with a Grubbs catalyst—both of which need different reaction mediums [23–26]. In this study, we focused on the immobilization of aldoxime dehydratase (Oxd), which has only been immobilized in

superabsorber to date. This limits the use of Oxd immobilizates in pure organic mediums. The purpose of this project was to broaden the usability of immobilized Oxds also to aqueous reaction systems by usage of other immobilization techniques.

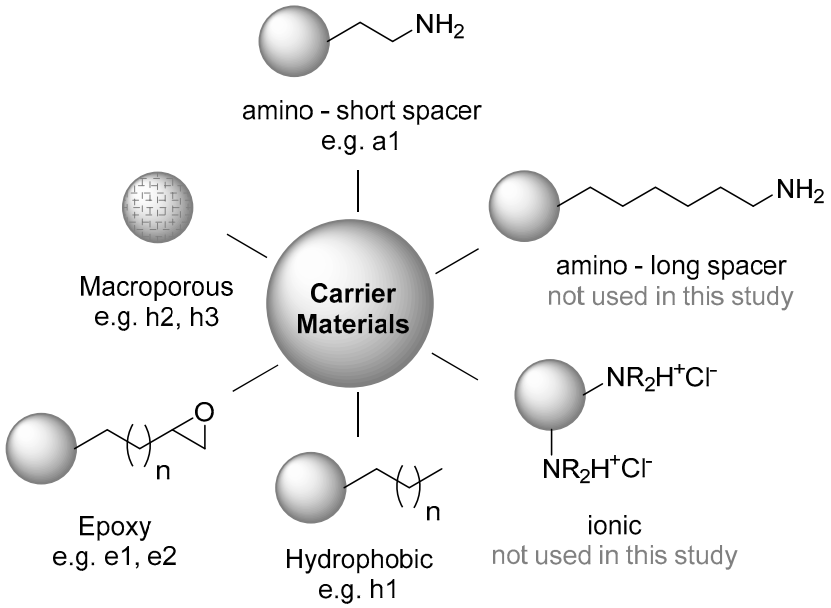
2. Results

The investigation of suitable immobilization strategies for Oxds was performed with two different enzymes, namely OxdB from *Bacillus* sp. OxB-1 and OxdRE from *Rhodococcus erythropolis*, which already emerged as an efficient catalyst for aldoxime dehydration, especially for aliphatic substrates [21,27,28]. We focused on the dehydration of *n*-octanaloxime to *n*-octanenitrile for our standard activity assays, since this substrates is accepted by both enzymes. In the first step, we investigated the immobilization of isolated enzymes (purified by Ni-NTA (Ni-nitrilotriacetic acid beads) affinity chromatography) on the different carriers listed in Table 1.

Table 1. Immobilization carrier used for the immobilization of OxdB (*Bacillus* sp. OxB-1) or OxdRE (*Rhodococcus erythropolis*) purified enzymes.

	Carrier Material	Abbreviation	Pore Size/Å	Purolite No.
1	Octadecyl methacrylate	h1	500–700	Lifetech ECR8806
2	Macroporous styrene	h2	900–1100	Lifetech ECR1090
3	Divinylbenzene/Methacrylate	h3	200–300	Lifetech ECR1030M
4	Amino C2 methacrylate	a1	600–1200	Lifetech ECR8309F
5	Epoxy methacrylate	e1	300–600	Lifetech ECR8204
6	Epoxy/butyl methacrylate	e2	400–600	Lifetech ECR8285

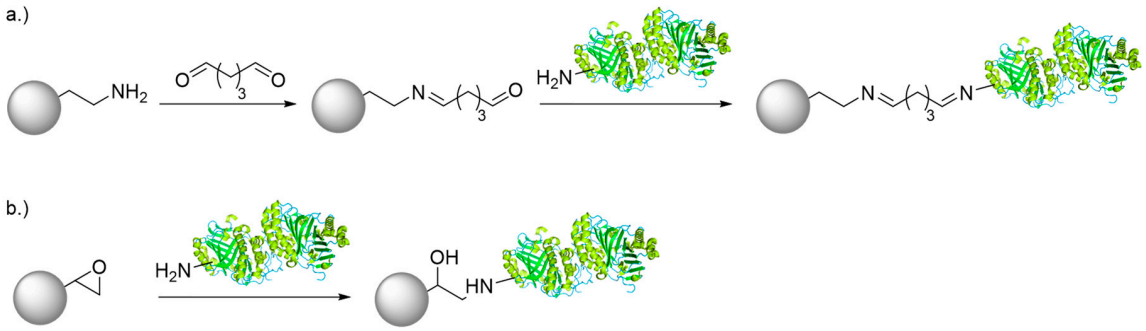
Depending on the carrier material, the immobilization is based on different interactions between the carrier and the enzyme. A schematic presentation of the interactions between immobilization carriers and enzymes is summarized in Scheme 2.



Scheme 2. Schematic overview of carrier materials used in this study.

Carriers h1–h3 are hydrophobic resins that non-covalently immobilize proteins via physical adsorption. We chose h1, h2 and h3 as hydrophobic carriers because they differ in surface area (h1: >80 m^2/g, h2: >750 m^2/g and h3: > 90m^2/g) and because of the pore sizes of the resins (see Table 1). Besides non-covalent immobilization, we also tested carriers which covalently bind proteins. We

chose one amino carrier (a1), which has a short ethylene spacer between resin and amino functionality. The amino group is preactivated by glutaraldehyde, which afterwards can covalently react with (for example) amino functions of the protein (Scheme 3a). Lastly, two epoxy carriers were tested. Here, we chose epoxy methacrylate (e1) and epoxy/butyl methacrylate (e2), which differ in the spacer length between resin and epoxy functionality and pore size. Epoxy resins react under formation of a covalent bond with (for example) amino functionalities of the protein (Scheme 3b).



Scheme 3. (**a**) Protein immobilization on amino carriers after preactivation of the resin with glutaraldehyde and (**b**) protein immobilization on epoxy carriers. X-ray structure shown is the crystal structure of OxdRE [29].

After immobilization of purified OxdB and OxdRE on the different carriers, the immobilization efficiency and residual activity of the protein in comparison with the purified Oxds were determined. Immobilization efficiencies were measured by determination of protein concentration of the purified enzyme solution in phosphate buffer (PPB) used for the immobilization and of the supernatant after immobilization (Figure 1).

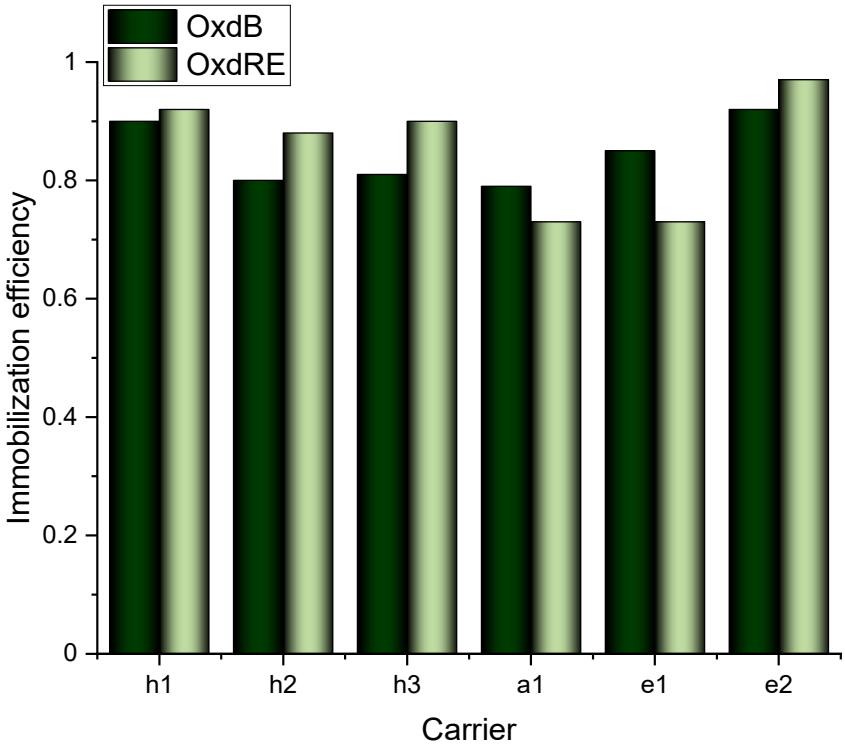
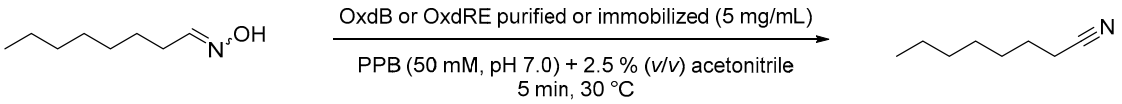


Figure 1. Immobilization efficiency of purified OxdB and OxdRE on different carriers.

Immobilization efficiencies were found to be very high in all cases and for both proteins. These immobilization efficiency values between ~80–90% indicate that nearly all the protein in the solution

was immobilized on the carriers. *n*-octanaloxime was used as a substrate to determine the activity of the immobilized enzymes in comparison with purified OxdB and OxdRE (Figure 2).



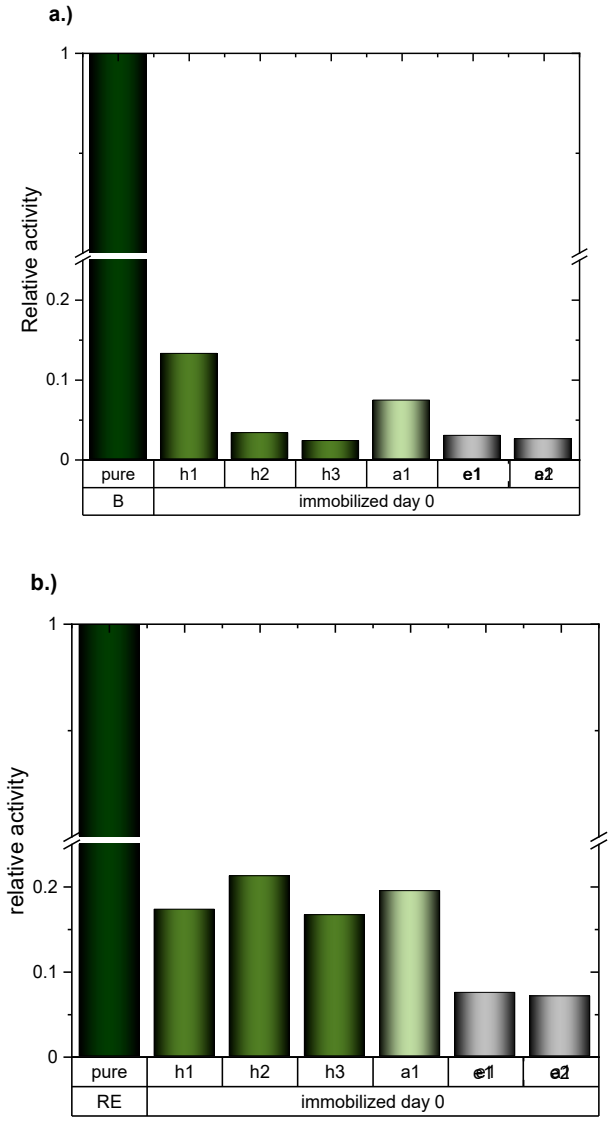


Figure 2. Residual activity of (**a**) OxdB and (**b**) OxdRE immobilizates in comparison with purified OxdB or OxdRE.

Residual activities after immobilization on different carrier materials were found to be in ranges of ~5–10% of the activity of purified OxdB or OxdRE. Especially in the case of OxdB, the carrier material showed a strong impact on the residual activity. In the cases of h1 and a1, the residual activity was still approximately 10% of the activity of purified enzyme, whereas the epoxy carriers e1 and e2 as well as h2 and h3 showed lower residual activities of approximately 5%. In the case of OxdRE, the impact of the carrier material was found to be weaker than OxdB. Only the epoxy resins e1 and e2 showed lower residual activities of ~7%. The amino carrier a1 and the hydrophobic and macroporous carriers h1–h3 showed residual activities of ~15–20% compared to the purified enzyme. Since h1

and a1 carriers showed acceptable results for OxdB and OxdRE, these two carriers were chosen for further experiments.

A storage stability assay was performed using purified OxdB and OxdRE enzymes and h1 and a1 immobilizates of both. Purified enzyme and immobilizates were stored at 4 °C for 6 and 32 d, and the activity was examined (Figure 3).

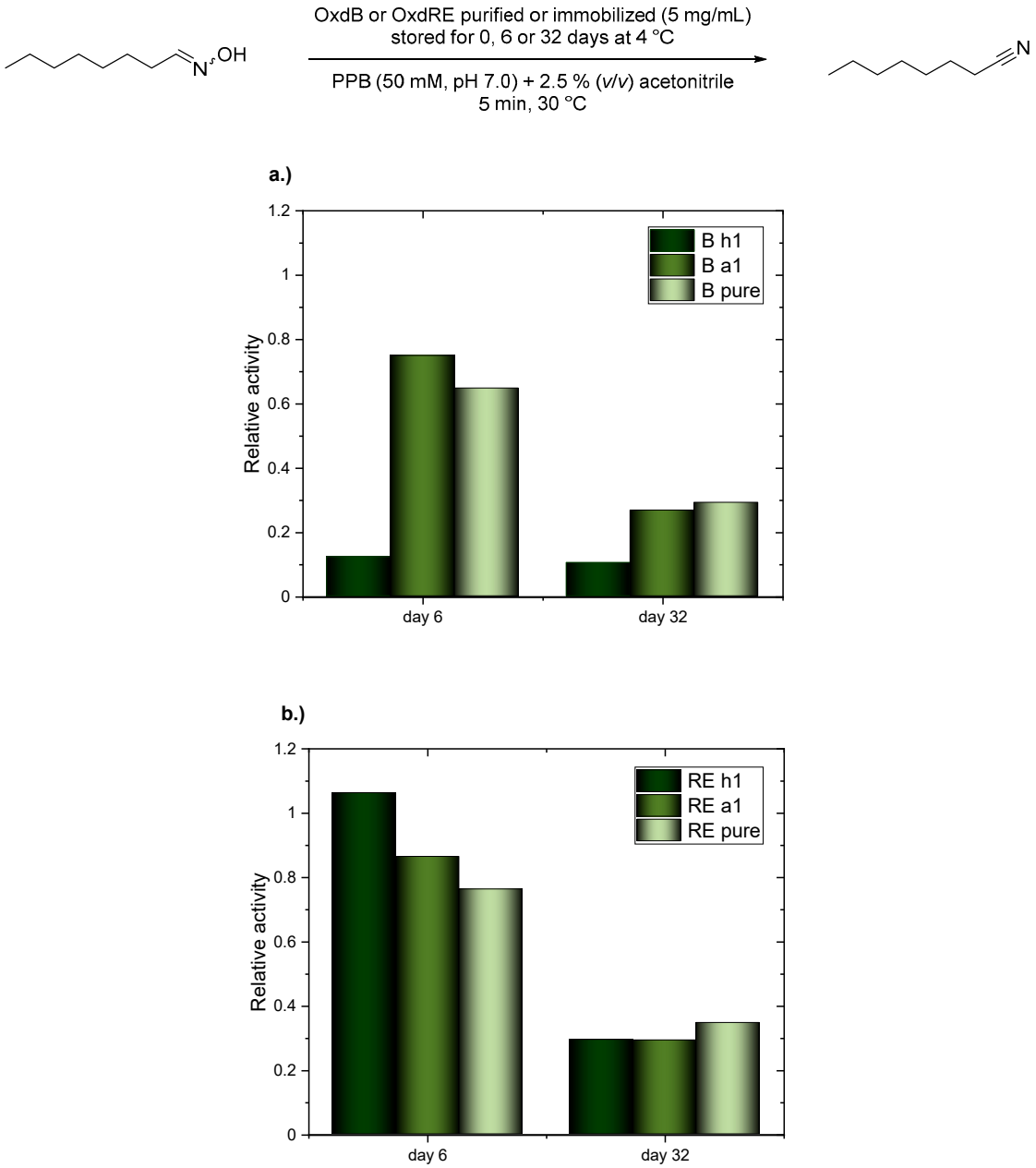
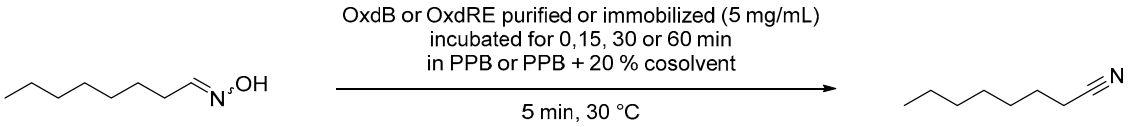
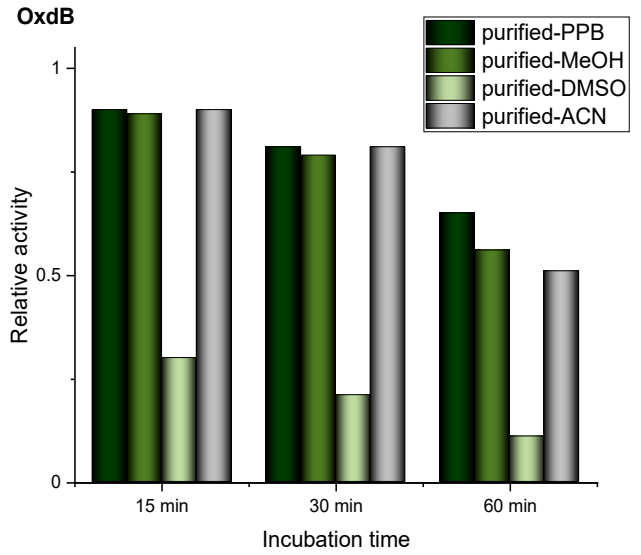


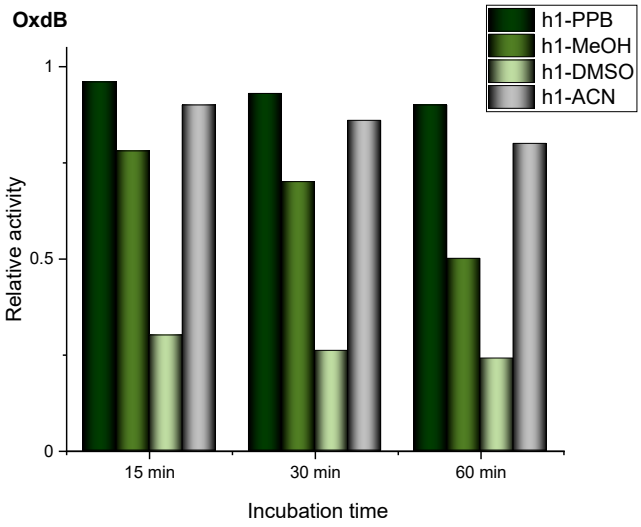
Figure 3. Storage stability assay of purified (**a**) OxdB and (**b**) OxdRE enzymes and h1 and a1immobilizates. The relative activity data refer to the activity measured on the first day (day 0).

The storage stability assay was performed in triplicates and the results show that the residual activity after different storage times at 4 °C for 6 d is often higher for the immobilized enzyme compared to that for the purified enzyme. In detail, the a1 immobilizates of OxdB are more stable, while for OxdRE, h1 immobilization seems to be more beneficial. After 32 d of storage, however, the activity dramatically decreases in all cases to ~20% or lower.

Following this, a cosolvent study was performed using purified OxdB and OxdRE enzymes and h1 and a1 immobilizates (Figure 4). The effects of immobilization on the stability of OxdRE and OxdB against organic solvents should be tested. Therefore, the purified enzymes or immobilizates were incubated for different times in different cosolvents (20% (*v*/*v*)) or PPB; afterwards, the activity of the conversion of *n*-octanaloxime to *n*-octanenitrile was determined.







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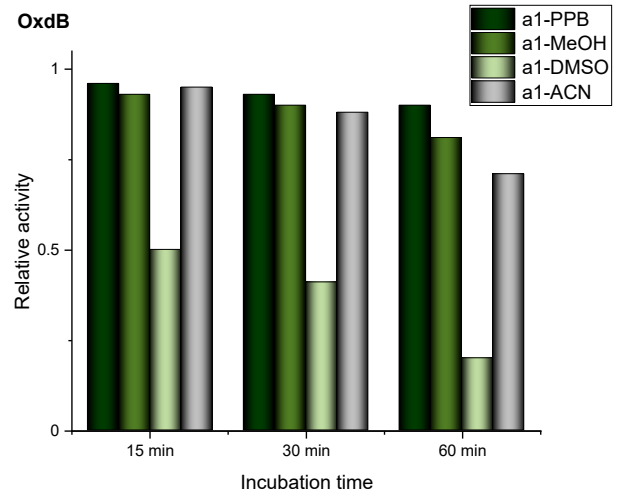

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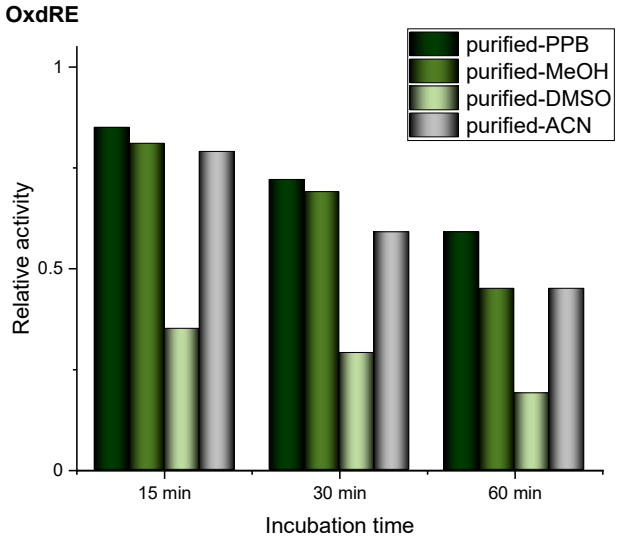


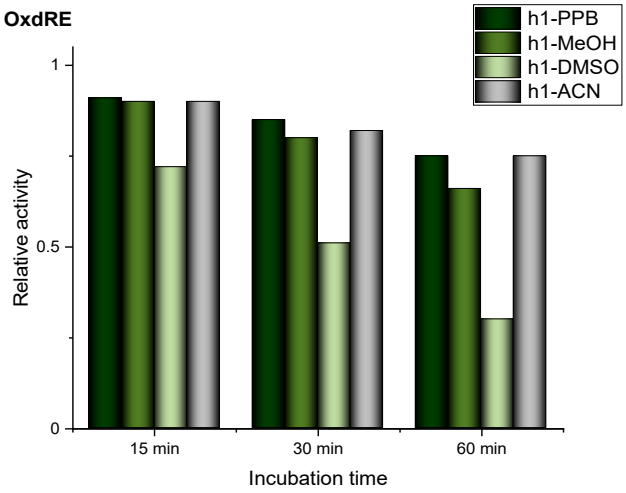


Figure 4. *Cont.*

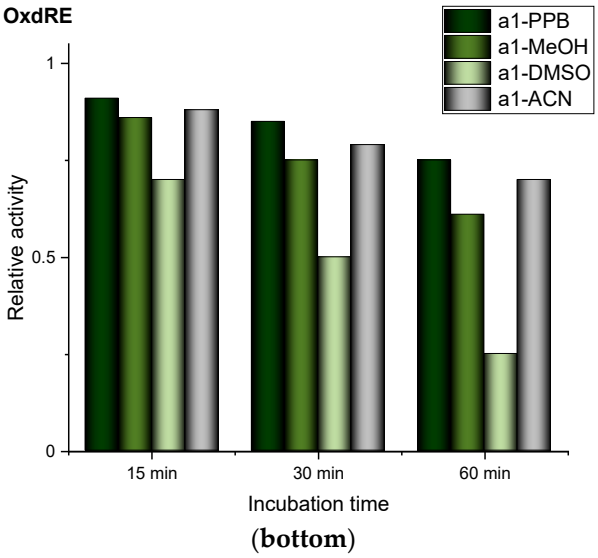


Figure 4. Cosolvent study of immobilized OxdB (**top**) and OxdRE (**bottom**).

In this cosolvent study, the activity of beads or purified enzymes without incubation was normalized to a relative activity of 1, and all measured activities after incubation were compared to this value. We found that OxdB and OxdRE immobilized enzymes were not much more stable after incubation in different cosolvents than purified enzymes. Dimethylsulfoxide (DMSO) seems to be the most unsuitable solvent for both enzymes and acetonitrile (ACN) the most favorable, while methanol deactivates the enzymes more. Unfortunately, even after a short incubation time of 1 h, a decrease in activity is found in all cases. Even without any co-solvent, a strong decrease in activity, with ~65% residual activity for OxdB and ~50% for OxdRE, of the purified enzymes was observed after just 1 h. The immobilizates show a stabilizing effect for all solvents. However, the deactivation of DMSO is strong even when using immobilizates. Although the immobilization seems to have a stabilizing effect on the enzymes, the activity of the immobilizates is very low in comparison with the free enzymes (~20% residual activity). These low activities are even decreased by the usage of organic solvents, which makes the use of purified enzyme or the tested immobilizates unsuitable for application in organic synthesis.

Therefore, we further investigated immobilization of OxdB and OxdRE whole-cell catalysts. Our group already found that Oxd whole cells can successfully be immobilized in superabsorber and used in organic media very efficiently [22]. A disadvantage of this system is that superabsorber-immobilized cells cannot be used in aqueous reaction medium due to the leaching of cells out of the superabsorber. Another immobilization technique that is also suitable for the immobilization of whole cells is based on sodium alginate, which can be premixed in an aqueous solution with the cells of interest and dropped into $CaCl_2$ solution which hardens alginate. When applying this immobilization technique to OxdB and OxdRE whole cells, first, we needed to perform a buffer screening because Oxds are normally used in PPB as buffer, which destabilize alginate beads. After we switched the standard PPB buffer to HEPES buffer, which showed similar activity to PPB, we immobilized OxdB and OxdRE whole cells in alginate beads. They were prepared by dropping a sodium alginate solution premixed with the whole-cell catalysts and buffer (HEPES, 50 mM, pH 7) into a $CaCl_2$ solution to harden the beads. The beads were filtered, washed with HEPES buffer and used afterwards for biotransformation of *n*-octanaloxime to *n*-octanenitrile (Figure 5).

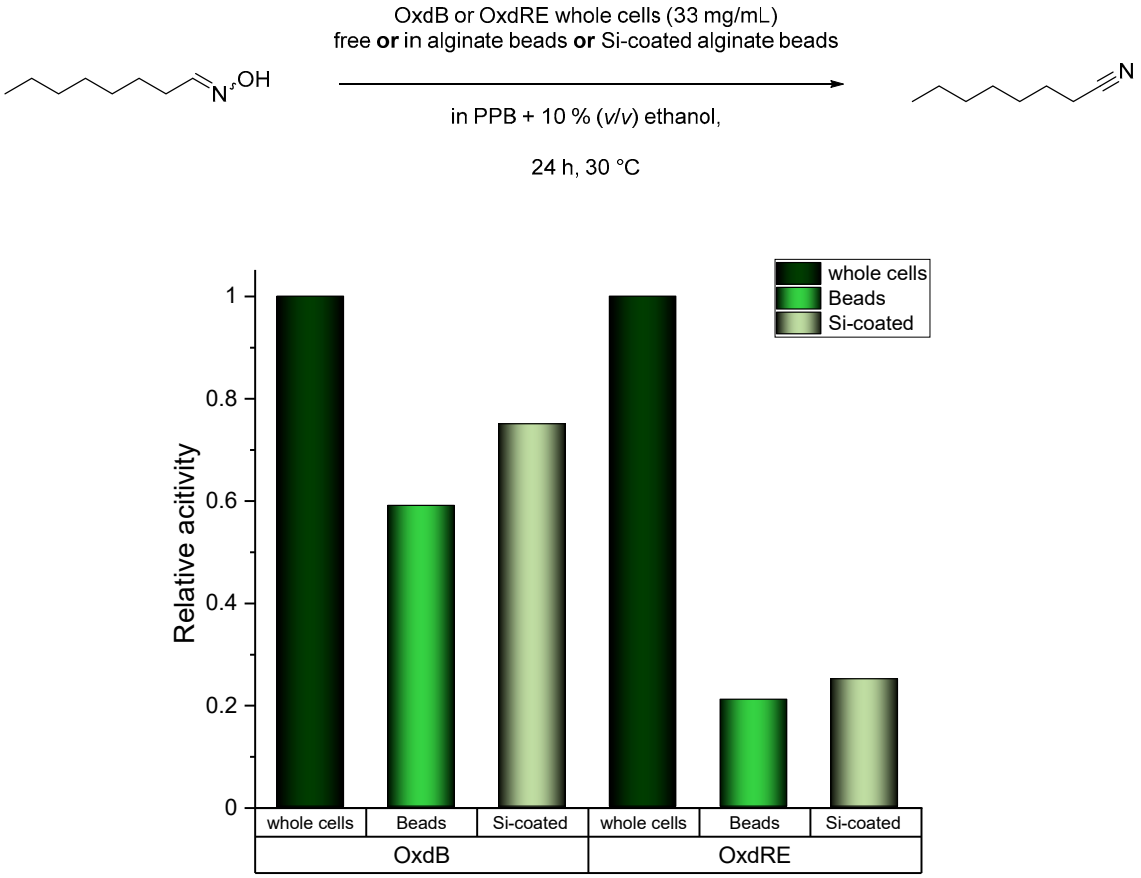
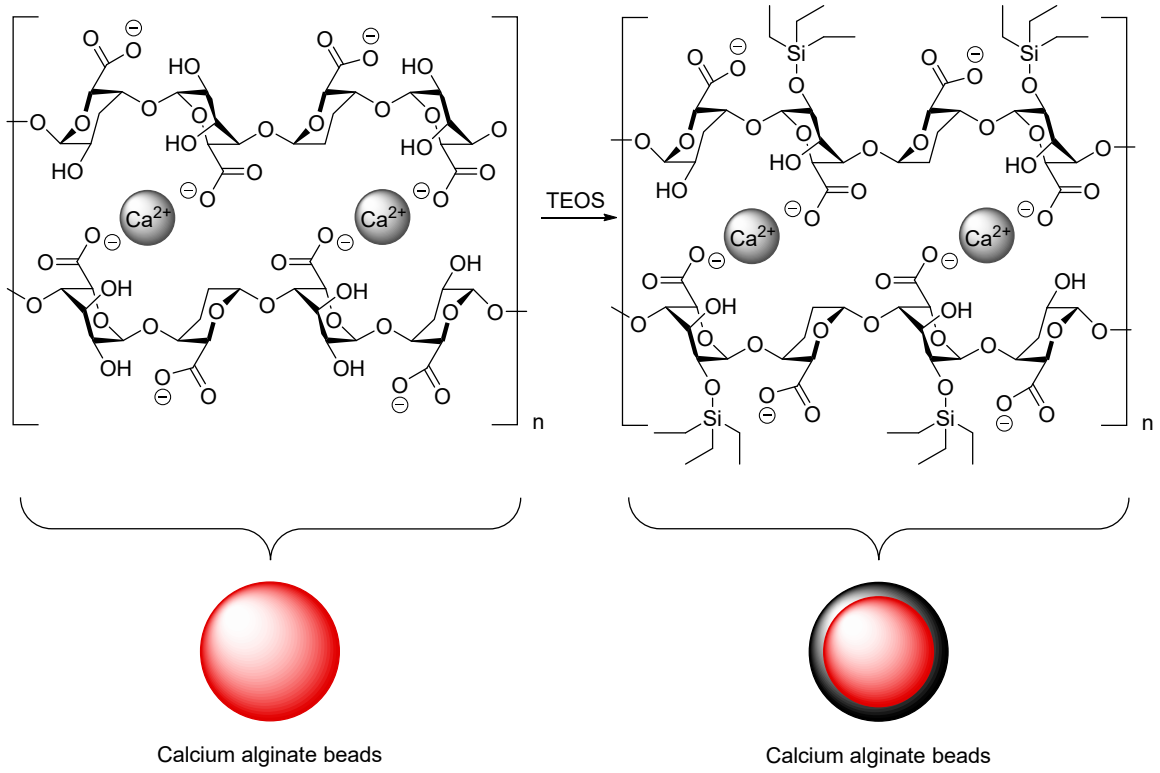


Figure 5. Comparison of OxdB and OxdRE whole cells and OxdB and OxdRE whole cells immobilized in alginate beads and immobilized in alginate beads coated with tetraethylorthosilicate (TEOS; Si-coated). Activities of OxdB and OxdRE free whole-cell catalyst were set as 100%.

The activity of OxdB whole cells and immobilized OxdB whole cells was determined. As shown in Figure 5, both whole-cell catalysts are still active after immobilization in alginate beads, however, the activity drops to approx. 60% in case of OxdB and approx. 20% in case of OxdRE in comparison with the free whole-cell catalysts.

Since the surface of calcium alginate is very hydrophilic and might negatively influence the diffusion of *n*-octanaloxime into the beads, we further investigated a coating of the beads with tetraethylorthosilicate (TEOS) (Scheme 4).



Scheme 4. Silica-coating of calcium alginate beads using TEOS.

This coating is performed after immobilization of OxdB or OxdRE in alginate beads as previously described by stirring the beads overnight in a solution of TEOS in *n*-hexane. After this "silica-coating", the surface of the beads is more hydrophobic and the substrate may diffuse into the beads more easily. The silica-coated (Si-coated) beads were also tested for the conversion of *n*-octanaloxime and compared to the results of free whole cells and uncoated alginate-immobilized whole cells (Figure 5).

Indeed, the silica-coating of the beads seems to have an impact on the activity of the immobilized whole cells. For both enzymes, the residual activity is higher after silica-coating compared to the uncoated beads; however, the residual activity of OxdRE is still much lower when compared to OxdB (~20% residual activity for OxdRE and ~75% residual activity for OxdB). This finding is very surprising because Oxds (also in whole cells) are normally found to be unstable in organic media, while in this case, stirring of the beads overnight in pure *n*-hexane with TEOS leads to immobilized cells that remain active. Thus, this result motivated us to test the activity of the beads also in a pure organic solvent (Figure 6).

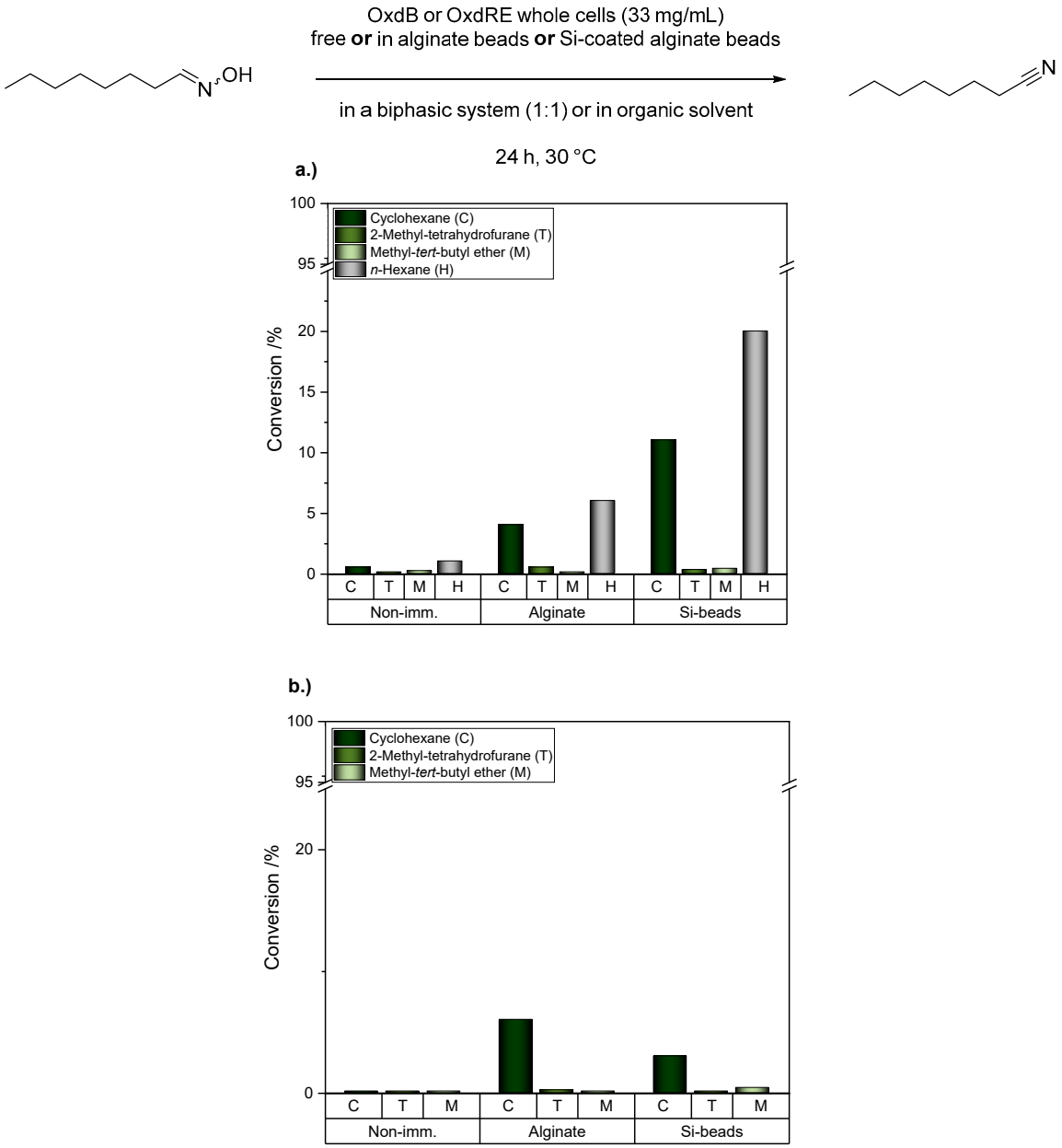


Figure 6. Dehydration of n-octanaloxime using immobilized whole cells in calcium alginate beads (alginate) or Si-coated calcium alginate beads (Si-beads) in pure organic medium compared to free whole cells in a biphasic reaction medium of OxdB (**a**) and OxdRE (**b**). In the y-axis an interruption from 25 to 95% is inserted.

It is already known that free Oxd whole cells lose their activity by usage in pure organic solvent or in a biphasic reaction medium [22]. For comparison reasons, we also applied free Oxd whole cells in a biphasic reaction medium compared to immobilized cells in alginate beads and Si-coated beads. As expected, we could not obtain any conversion in the biphasic approach in all tested solvents for free Oxd whole cells, namely cyclohexane (C), 2-methyl-tetrahydrofurane (T), methyl-*tert*-butyl ether (M) and *n*-hexane (H). In the case of the OxdRE immobilizates, we found conversion in cyclohexane as solvent; however, the conversions were very low and no difference between the coated and the uncoated beads were observed. In the case of OxdB immobilizates we could reach ~10% conversion by usage of cyclohexane as solvent for Si-coated beads. Even with the uncoated beads, a conversion of ~5% was observed. As we used *n*-hexane as solvent for TEOS coating, we further investigated *n*-hexane as solvent and found a conversion of 20% by using Si-coated OxdB immobilizate. This result

shows in principle that the beads are more suitable in organic solvents than free whole cells, especially in very unipolar solvents like cyclohexane or *n*-hexane. This might be caused by a shielding effect of the alginate beads. Generally, we were interested in utilizing the whole-cell immobilizates in an aqueous reaction medium, since we already established a method for the usage of Oxd whole cells in organic medium with a superabsorber. Therefore, we investigated the stability of the beads (calcium alginate beads and Si-coated beads) in buffer containing ethanol as cosolvent. Normally, aldoximes are not very soluble in an aqueous reaction medium, wherefore a cosolvent is needed. In previous studies, it was found that ethanol is a very suitable solvent for the use in combination with Oxd whole-cell catalysts [21]. The activity of free Oxd whole cells and those immobilized with alginate and Si-coated was tested in HEPES buffer with 10% or 50% ethanol as solvent. The free whole cells or the beads were incubated for 24 h in this mixture, and then biotransformations with *n*-octanaloxime were performed with these pretreated catalysts (Figure 7). We normalized the results on the activity of the free whole cells or immobilized whole cells without incubation.

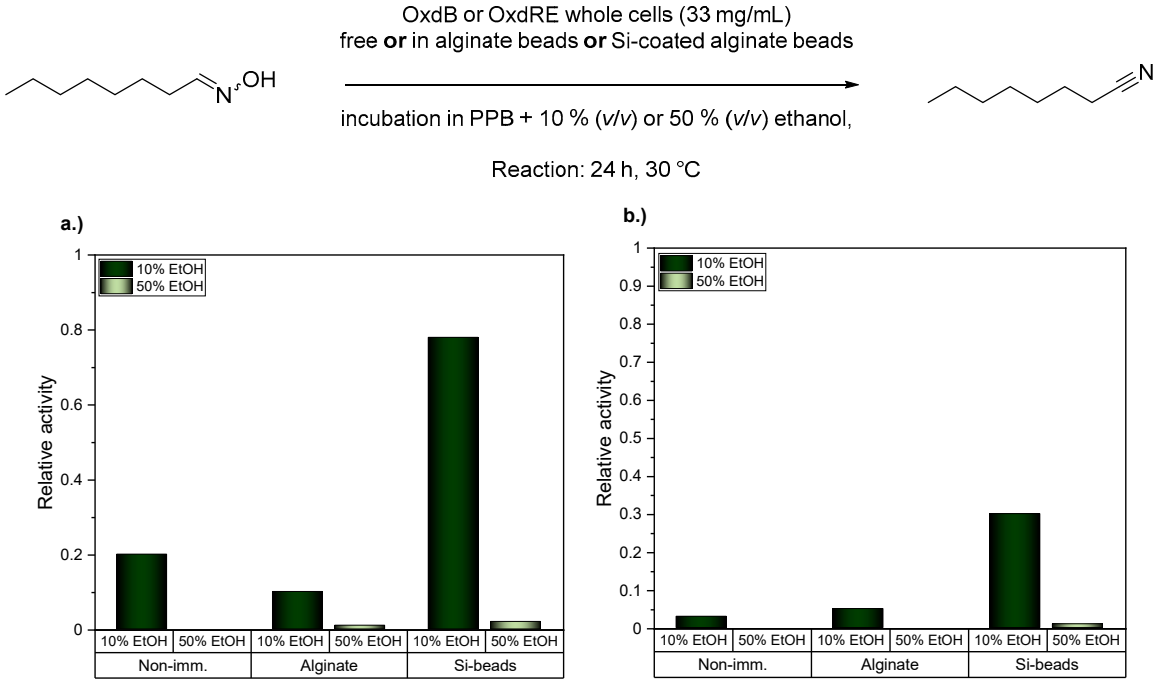


Figure 7. Incubation of (**a**) OxdB and (**b**) OxdRE whole cells (free, non-immobilized), immobilized in calcium alginate (alginate) or in Si-coated calcium alginate (Si-beads)) in 10% or 50% ethanol in HEPES. The relative activity in comparison with standard activity of dehydration is shown.

We found a very interesting stabilizing effect of the Si-beads in comparison with the free whole cells. While for the whole cells only 20% (OxdB) or ~5% (OxdRE) residual activity was observed after 24 h incubation time in buffer with 10% ethanol, ~80% (OxdB) or ~30% (OxdRE) residual activity was found for the Si-beads. These experiments show that a stabilizing effect of the beads, especially of the coated beads, is obtained. It is very surprising that after the TEOS coating overnight in *n*-hexane residual activity and an enhanced stability for both of the catalysts (OxdB and OxdRE) is observed, whereas usually only a few minutes of incubation of the whole cells in organic solvents leads to a dramatical loss of activity [22].

Based on these findings, we then performed a recycling study using OxdB whole cells immobilized in Si-coated alginate beads. We did not perform a recycling study for the OxdRE immobilizates because the previous experiments demonstrated higher stability and residual activity of OxdB after immobilization. In our recycling experiment, the conversion of *n*-octanaloxime to *n*-octanenitrile in the first round (88%) was normalized to 1, and all experiments with the recycled catalyst were compared to this value (Figure 8).

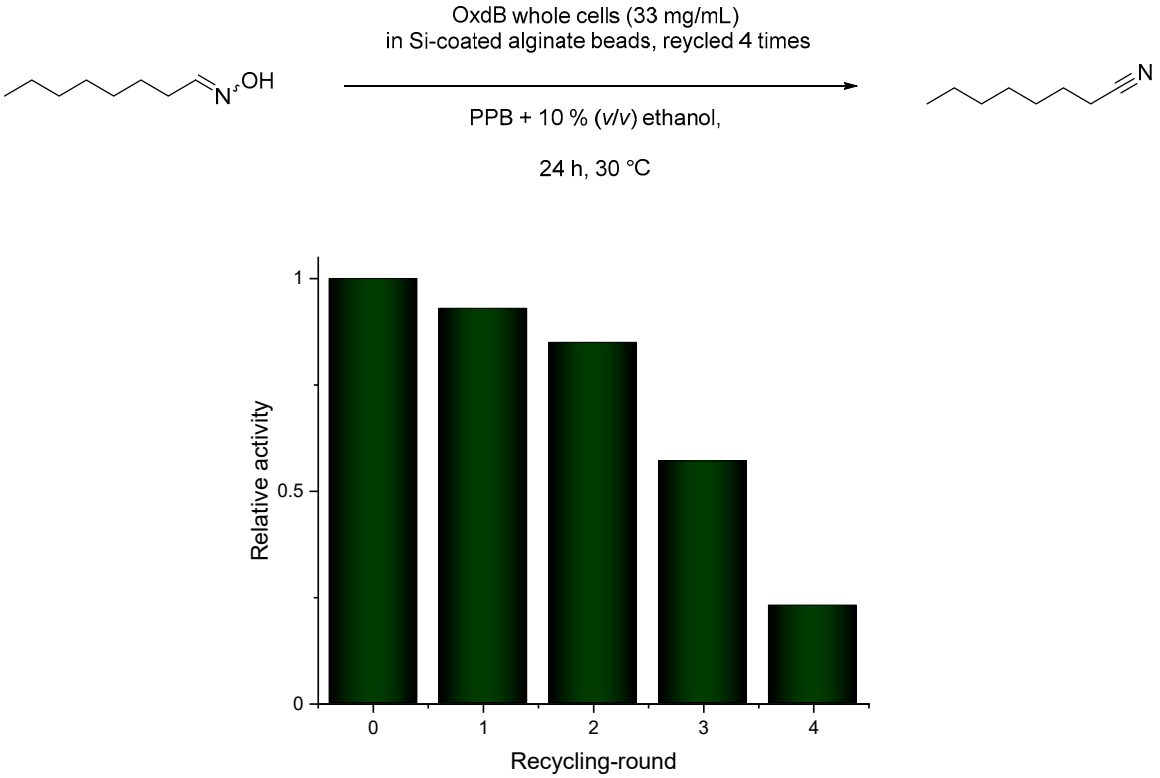


Figure 8. Recycling study of OxdB immobilized in calcium alginate beads coated with TEOS (Si-beads).

As shown in Figure 8, a loss of activity during the recycling study is observed; however, within the first three recycling steps, more than 85% residual activity is obtained. After five rounds of recycling, only ~20% residual activity of the catalyst is left.

3. Discussion

Different immobilization techniques of Oxds were tested using purified Oxds and Oxds in whole cells. We focused on two enzymes, namely OxdB from *Bacillus* sp. OxB-1 and OxdRE from *Rhodococcus erythropolis*. Both are active for the dehydration of *n*-octanaloxime to *n*-octanenitrile, which was used as a standard substrate in every activity assay. First, we investigated different immobilization carriers, which immobilize free enzymes, by hydrophobic interactions or covalently. We chose three different hydrophobic carriers (h1, h2 and h3), one amino carrier (a1) which is preactivated by glutaric aldehyde; and two epoxy carriers (e1 and e2). It was found that the residual activity after immobilization with high immobilization efficiencies (~80–90%) was <20% in all cases. The strong loss of activity for all carriers is probably due to the instability of the purified enzyme at elevated temperatures. We could show that even an incubation of the purified enzymes for 1 h at 30 °C in buffer leads to a loss of activity of 50%. During immobilization, the enzymes are shaken for 24 h hours at temperatures above room temperature (rt) (see Supporting Information), which partly explains the severe loss of activity. Although the remaining activities after immobilization of the free enzymes were very low, an additional cosolvent screening was performed to determine the stability of the immobilizates in comparison with free purified enzymes. The immobilization of the enzymes seems to have a small stabilizing effect with and without the co-solvent. These results led us to the conclusion that purified Oxds are not very stable and that immobilization of the enzymes has a positive effect on the stability, while the activity is drastically decreased. However, the strong decrease in activity during immobilization makes it difficult to use in organic chemistry, and we decided to switch our investigations to the immobilization of whole cells, since it was already found that Oxds are more stable in the whole cells compared to the purified enzymes [27]. Using an immobilization technique and comparing the use of calcium alginate

beads to calcium alginate beads coated with TEOS, we successfully immobilized OxdB and OxdRE in whole cells with residual activities of up to ~70%. Compared to the immobilization of purified enzymes, this residual activity is remarkable. For both enzymes, we could observe higher activities for the Si-coated beads in comparison with the uncoated beads, which is probably due to a better diffusion of the substrate in the beads. The alginate beads have a very hydrophilic surface, which makes diffusion of the hydrophobic substrate *n*-octanaloxime difficult. The Si-coating seems to mask the hydrophilic surface and make them more hydrophobic, which makes diffusion easier. Since TEOS coating to gain Si-coated alginate beads is performed in *n*-hexane as solvent, we performed reactions in pure organic solvents using immobilized cells compared to free cells. Conversion was found only in the case of OxdB in *n*-hexane or cyclohexane as solvent, leading us to the conclusion that the alginate beads are not suitable for use in organic solvents. Superabsorber immobilization of Oxd whole cells was already successfully established for use in organic solvents; thus, we did not further investigate the use of alginate beads in organic media [22]. It was found that the alginate beads, especially the Si-coated beads, are significantly more stable in ethanol-containing buffer with ~80% residual activity after 24 h incubation time in 10% ethanol of OxdB in Si-coated beads compared to ~20% for the free cells. Recycling of the beads led to decreased residual activity; however, the immobilizates can be used three times before a decrease in the activity of <85% is observed.

4. Material and Methods

Material for cell cultivation and aldoxime dehydratase (Oxd) expression was purchased by Carl Roth (Karlsruhe, Germany) (Antibiotics, LB- and TB-premixed medium, D-glucose, D-lactose). Buffer salts were obtained from VWR (Radnor, PA, USA) and Carl Roth. Ni-NTA beads were purchased from Fisher Scientific (Waltham, MA, USA). Further chemicals were purchased by Sigma Aldrich (St. Louis, MO, USA), Alfa Aesar (Ward Hill, MA, USA), VWR Chemicals or TCI Germany (Eschborn, Hesse, Germany).

Oxd expression and purification: *Escherichia coli* BL21-CodonPlus(DE3)-RIL (aldoxime dehydratase from *Rhodococcus erythropolis* (OxdRE)) or BL21(DE3) (aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB)) cells harboring the plasmids with the Oxd-genes (for sequences see Supporting Information) were stored as glycerol stocks at −80 °C. A sample from the glycerol stocks for each Oxd was plated on LB (Lysogeny broth Luria/Miller) agar containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol (OxdRE) or 100 µg/mL carbenicillin (OxdB), and was incubated for 12 to 18 h at 37 °C. Pre-cultures were prepared in 5 mL LB medium containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol (OxdRE) or 100 µg/mL carbenicillin (OxdB) using a single colony from the LB agar plate. The cultures were incubated for 12 to 18 h at 37 °C and 180 rpm. The main cultures for Oxd expression were performed using TB (Terrific broth) autoinduction medium (50 mL 20 g/L lactose solution, 5 mL 50 g/L d-glucose solution and 445 mL TB medium (purchased from Carl Roth) in a 500 mL Erlenmeyer flask). Then, 50 µg/mL kanamycin and 34 µg/mL chloramphenicol (OxdRE) or 100 µg/mL carbenicillin (OxdB) were added to the medium. The main cultures were inoculated with 1% (5 mL) of the relating pre-cultures and incubated for 1 h at 37 °C and 120 rpm. After 1 h incubation at 37 °C, the OxdB cultures were cultivated at 30 °C for 72 h and 120 rpm, and the OxdRE cultures were cultivated at 15 °C for 72 h and 120 rpm. Cell harvest was performed at 4000 xg for 15 min at 4 °C. Cells were washed three times with lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole) and afterwards resuspended in 2x mass of the bio wet weight (bww) of the cells in the lysis buffer. Cells were disrupted by sonication (5 times, 1 min, 15–20% output, Sonoplus Ultraschall-Homogenisator HD 2070 (Bandelin electronic GmbH & Co. KG, Berlin, Germany)), and crude extracts were obtained by centrifugation for 45 min at 25,000× *g* and 4 °C. Oxd purification was performed using Ni-NTA affinity chromatography. Oxds were eluted using elution buffer (20 mM Tris-HCl, 300 mM NaCl, 150 mM imidazole) and rebuffered to 50 mM potassium phosphate buffer (PPB) (pH 7.0). The protein concentrations of the purified enzyme and crude extracts were determined by Bradford assay in comparison with a bovine serum albumin (BSA) standard curve. Expression and purification success was analyzed by SDS-PAGE (Sodium

dodecyl sulfate polyacrylamide gel electrophoresis) using a 12% separation gel in comparison with a protein marker (PageRuler Prestained (Thermo Fisher)) (SDS-PAGE, see Supporting Information). Pure fractions of protein purification were combined and rebuffered to PPB (50 mM, pH 7.0) before usage for immobilization studies.

Immobilization of purified enzymes: Different carriers were chosen for the immobilization study of OxdB and OxdRE (see Supporting Information). Except for minor deviations in the incubation time, immobilization was conducted according to the instructions of Purolite. For immobilization, the respective resin (46 mg) was placed in an Eppendorf tube and washed with PPB (46 µL, 50 mM, pH 7.0). Then, in the case of the hydrophobic resins and the epoxy resins, the purified enzyme solution (184 µL) was added. The amino resin, however, had to be activated before the enzyme solution was added. For this purpose, glutaraldehyde solution (184 µL of a 25% aqueous solution) was added to the washed amino resin (46 mg), 2% in 50 mM PPB pH 7.0) and incubated at 600 rpm and 20 °C for 1 h. The activated amino resin was then diluted with PPB (pH 7.0), and the enzyme solution (184 µL) was added. The protein load was adjusted to 6%. The preparations were then incubated at approx. 300 rpm and 20 °C. After the immobilization, the supernatant was removed, and the immobilizates were washed with PPB (pH 7.0) and NaCl solution. For each carrier material, a different concentration of PPB for washing the carrier, for re-buffering the enzyme solution and for washing the immobilizate was used. The used buffers and the different incubation times are shown in the Supporting Information.

Determination of immobilization efficiencies: Immobilization efficiencies were determined by comparison of the protein concentration (determined by Bradford assay), the purified enzyme solution used for the immobilization and the supernatant after the immobilization.

Determination of water content in immobilizates: To determine the solvent content, approx. 5 mg of each immobilizate of OxdB and OxdRE were balanced and lyophilized after freezing for approx. 24 h. The immobilizates were then balanced again. Further details may be found in the Supporting Information.

Activity assay for purified or immobilized enzymes: To the enzyme (purified enzymes or immobilizates, 50 µL, 5 mg/mL final concentration) PPB (437.5 µL, 50 mM, pH = 7.0) was added. After an incubation time of 5 min at 30 °C and 1400 rpm, octanaloxime (12.5 µL, final concentration: 5 mM) in acetonitrile (final concentration: 2.5% (*v*/*v*)) was added. The conversion to octanenitrile was determined after 5 min at 30 °C and 1400 rpm by extraction with ethyl acetate (500 µL) and analyzation of the organic phase by gas chromatography. The data were compared to standard curves of octanaloxime and octanenitrile, giving conversions from which activities in $U/mg_{protein}$ were determined (see Supporting Information).

Cosolvent study: To the enzyme solution (50 µL, 5 mg/mL final concentration) or the equivalent amount of immobilized enzymes, PPB (350 µL, 50 mM, pH = 7.0) and cosolvent (87.6 µL and 98 µL) were added. After different incubation times of 0 min, 15 min, 30 min and 1 h at 30 °C and 1400 rpm, octanaloxime (12.5 µL, final concentration of 5 mM) in the respective cosolvent (2.5% (*v*/*v*) final concentration) was added. After 5 min of reaction, ethyl acetate (500 µL) was added and vortexed, and the organic phase was analyzed by gas chromatography (GC). As cosolvent, methanol, dimethylsulphoxide and acetonitrile were used

Immobilization in calcium alginate beads: The cell suspension of OxdB and OxdRE (333 g/L, 9 mL) in HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) buffer (50 mM, pH 7.0) was mixed with sodium alginate solution (9 mL; 4% (*w*/*w*) in HEPES). The solution was dropped into a calcium chloride solution (200 mM) in HEPES buffer using a flow rate of 2 mL/min. Following this, the beads were stirred for a further 30 min before they were washed with HEPES to remove residual $CaCl_2$. The sodium alginate beads were stored in HEPES buffer at 4 °C.

Coating of calcium alginate beads with TEOS: The calcium alginate beads were produced as previously described. Accordingly, the beads were covered with n-hexan before tetraethyl orthosilicate (TEOS) was added (7.5 mL). The suspension was stirred for 22 h at room temperature. The beads were filtered and washed with n-hexan and HEPES to remove residual TEOS.

Activity assay of free whole cells and immobilized whole cells: Octanaloxime was prepared as described in a previous publication [1]. The activity assay of whole cells was performed on a 10 mL scale in round-bottom flasks. HEPES buffer (9 mL; 50 mM; pH = 7) and octanaloxime in ethanol solution (1 M; 1 mL, 100 mM final concentration) were added. While stirring, the biocatalyst OxdB (final concentration of free whole cells: 16.5 mg/mL; final concentration of immobilized whole cells: 33 mg/mL) or OxdRE (final concentration of free and immobilized whole cells: 33 mg/mL) were added. The reaction was performed at 30 °C for 24 h. After the reaction, whole-cell biocatalysts were removed by centrifugation (5000× *g*, 2 min) and aqueous phases and cells were extracted with ethyl acetate (3x 15 mL). The combined organic phases were analyzed by GC. Immobilized whole cells were filtered and washed with HEPES buffer and ethanol. The filtrate was extracted with ethyl acetate (3x 15 mL), and the organic phase was analyzed by GC. In the case of the recycling study, the immobilized cells were isolated as described above and directly used for another biotransformation using the same conditions.

Biotransformation in pure organic medium: Free whole cells were used in a biphasic reaction medium consisting of HEPES buffer (7.5 mL) and organic solvent (7.5 mL). The final concentration of the free whole cells of 33 mg/mL was used. Immobilizates (alginate beads or Si-coated beads) were directly applied to the organic solvent (15 mL) with a whole cell concentration of 33 mg/mL. The reactions were performed for 24 h at 30 °C with an octanaloxime concentration of 100 mM before the organic phases were analyzed by GC.

Stability assay of free whole cells and immobilized whole cells in the presence of ethanol as cosolvent in HEPES buffer: Free whole cells (33 mg/mL final concentration) or immobilizates (33 mg/mL final concentration) were incubated in HEPES buffer (50 mM, pH 7.0) including 10% or 50% (*v*/*v*) ethanol (total volume 15 mL) for 24 h at 30 °C before addition of octanaloxime (final concentration of 100 mM). The reaction was performed for 24 h and 30 °C before the same work-up was performed as described above. The organic phases after extraction were analyzed by GC.

5. Conclusions

Within this study, we tested several immobilization techniques for enzymes such as epoxy- and amino-substituted resins and hydrophobic carriers, however, the residual activity for both tested enzymes (OxdB and OxdRE) was found to be very low. Therefore, we switched to the immobilization of OxdB and OxdRE in whole cells by entrapment in calcium alginate beads. After this, immobilization and coating with TEOS, high residual activities with up to ~75% were obtained. Using these immobilizates, a stability study in ethanol-containing buffer was performed, and a higher stability to free whole cells was found. In a recycling study of OxdB whole cells immobilized in calcium alginate beads covered with TEOS, over three cycles of the activity remained at >80% when compared with the starting activity. Thus, in conclusion, we found an applicable immobilization technique after which the immobilizates are usable in aqueous reaction medium, without dramatical loss of activity and higher stability compared to free whole cells, making it possible to reuse the biocatalyst and enable a simple work-up.

Supplementary Materials: The Supplementary Materials with the Oxd sequences as well as experimental protocols and analytical data are available online at http://www.mdpi.com/2073-4344/10/9/1073/s1.

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