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Novel immobilization routes for the covalent binding of an alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541

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ABSTRACT

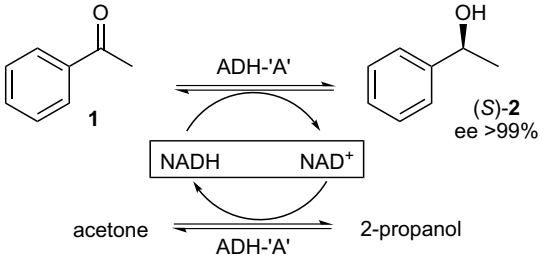
Immobilization of the alcohol dehydrogenase ADH-'A' from *Rhodococcus ruber* DSM 44541 has been performed with different amino-functionalized carrier materials. The procedure included the activation of the carrier with glutaraldehyde and subsequent covalent binding to the enzyme. The porous glass beads TRISOPERL® and TRISOPOR®, magnetic particles, and detonation nanodiamonds were used as carriers in these experiments. In all cases, the immobilization was successful with almost quantitative immobilization yields; subsequently the activity for the reduction of acetophenone was lower compared to the activity of the free biocatalyst. Activity yields of 40% and 60% were obtained. The immobilized biocatalysts showed high stabilities in repetitive batches.

1. Introduction

An increased demand for optically active compounds for the chemical, pharmaceutical, and agrochemical industries has recently developed. Besides established chemical methods involving transition metal catalysts and organic solvents, more environmentally friendly biocatalytic approaches using oxidoreductases (EC 1) are of increasing importance.[1] For example, alcohol dehydrogenases are able to catalyze the stereoselective reduction of aldehydes or prochiral ketones, with remarkable chemo-, regio-, and stereoselectivities.[2]

The NADH-dependent alcohol dehydrogenase ADH-'A' from *Rhodococcus ruber* DSM 44541 is overexpressed in *Escherichia coli* and can be used for the asymmetric reduction of various substrates, such as alkyl- and aryl-substituted ketones or *meso*-diketones (Scheme 1).[3–5] The product is usually formed with an enantiomeric excess (ee) of >99%. Partially purified ADH-'A' showed impressive operational stability in the presence of high concentrations of organic compounds. For instance, it is still active in 2-propanol in concentrations of up to 99% v/v, which is needed for the cofactor regeneration of NADH.[6] Further positive effects are the shift in the equilibrium to the desired product and the increased solubility of substrate and product in the aqueous phase[6] (Scheme 1).

On an industrial scale, continuously operated reactor setups are favored due to the high volumetric productivity and the prevention of setup times associated with batch reactions. However, less than a quarter of all biotechnological processes conducted on an industrial scale are operated in a continuous mode.[7] For the development of a continuous process, recycling of the valuable biocatalyst is necessary. This is possible by immobilizing the biocatalyst.



Scheme 1. Asymmetric reduction of acetophenone **1** to 1-(*S*)-phenyl-ethanol **2** in a substrate-coupled approach.

The chemical or physical fixation of enzymes on supporting carriers can be achieved by different methods: adsorption on suitable surfaces, covalent binding using linking reagents, encapsulation or entrapment in gel-like materials, and cross-linking of enzymes or combinations thereof.[8–13] Out of these variants, the covalent binding method features some distinct advantages compared to the other approaches. Firstly, the covalent bond between the enzyme and carrier prevents a loss of catalyst during the reaction. Furthermore, in comparison with the encapsulation and entrapment of enzymes, the particle size is defined prior to

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immobilization and in many cases the particles are more stable against shear forces.

In the past, most of the reported processes involving immobilized alcohol dehydrogenases were based on adsorption processes. Only a few examples using covalent binding are described.[13–15] Herein, we report the covalent immobilization of the ADH-'A' from *R. ruber* DSM 44541 on different carrier materials.

2. Results and discussion

The immobilization of the ADH-'A' was performed using three different types of carriers: porous glass beads, magnetic carriers and nanodiamonds. In all cases, the carrier material was already amino-functionalized. Therefore, an immobilization procedure was chosen by employing the linear dialdehyde glutaraldehyde (2.5% v/v) as a linker.[16] In the first step, the respective carrier was treated with the linker to yield active aldehyde groups on the carrier's surface. To remove an excess of the linker, the carriers were washed with buffer and dried at 65 °C. These activated particles were mixed with the enzyme solution at 4 °C. After the immobilization procedure, the remaining aldehyde functions were treated with glycine (100 mM, pH 7.5) and washed again with buffer.

In the two or three repetitive batches, the variance of the activity was in the range of measurement precision. For this reason, the estimation of deactivation constants or half-life times would not give confident results.

The immobilized biocatalyst was utilized in the model reaction, which is the stereoselective reduction of acetophenone **1** to 1-(*S*)-phenylethanol **2** that was performed in (repetitive) batches. The reaction medium was the potassium phosphate buffer (100 mM, pH 7). An ee of >99% was reached with all supports.

2.1. Porous glass beads

VitraBio TRISOPERL® and TRISOPOR® porous glass carriers were used for the investigations.[16–18] These particles with a size of 100–200 μm were amino-functionalized and had a pore size of about 100 nm or 50 nm, respectively. During the immobilization process, vacuum was applied to achieve a better degassing of the pores, and therefore an easier exchange between pore content and surrounding solution. The binding of the glutaraldehyde to the amino groups on the carrier surface resulted in a color change from white to red, due to the structure of the resulting imino moiety.

To compare the immobilized enzyme with the free biocatalyst, the reduction of acetophenone was performed in batch reactions with both systems (Fig. 1). Furthermore, the immobilized ADH-'A' was reused twice in additional batch reactions. Between these repetitive batches, the particles were washed five times with a phosphate buffer (100 mM, pH 7).

No conversion higher than 91% was reached due to the thermodynamic properties of the substrate-coupled system.[3]

The immobilization yield of the biocatalyst was almost quantitative in view of the residual activity in the supernatant (TRISOPERL®: 99%/TRISOPOR®: 100%). However, the activity of the immobilized enzyme was considerably lower, as it decreased from 26.3 U for the free enzyme to 11.8 U (45%; 0.059 U $mg^{-1}_{carrier}$) or 16.2 U (62%; 0.081 U $mg^{-1}_{carrier}$) for the TRISOPERL® or TRISOPOR® carriers, respectively. As expected, due to the resulting larger surface area in the case of particles with a smaller pore size (TRISOPOR®), a higher activity was observed when compared to the other particles.

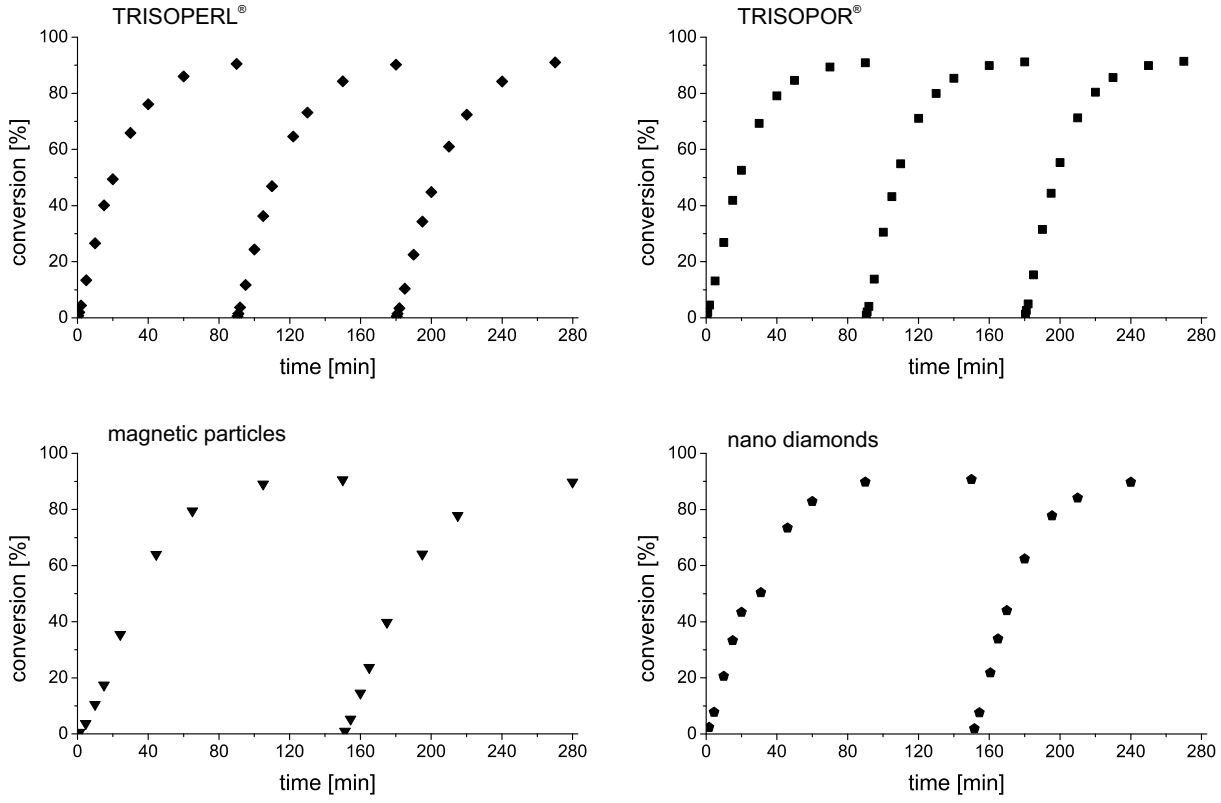

Figure 1. Conversion-time plots for the reduction of acetophenone catalyzed by ADH-'A' immobilized on TRISOPERL® (♦), TRISOPOR® (■), magnetic particles (▼) or nanodiamonds (⬟). Conditions: 80 mM acetophenone, 18% v/v 2-propanol, 1 mM NADH in 100 mM phosphate buffer, pH 7, 0.145 mg mL^{-1} ADH-'A', carriers and volume: TRISOPERL®, TRISOPOR®: 200 mg/10 mL, magnetic particles: 14 mg/1 mL, nanodiamonds: 20 mg/1 mL. Recycling of the immobilized carrier: separation by centrifugation, washing with 100 mM phosphate buffer (5×).

The repetitive batches of the ADH-'A' on TRISOPERL® or TRISOPOR® particles showed almost no loss in activity (Fig. 1).

2.2. Magnetic carriers

An alternative to porous glass particles is the application of magnetic carriers. In comparison to classical non-magnetic carriers, the magnetic properties enable simpler reaction control: the particles can be separated from a reaction mixture even in the presence of other suspended solids by applying a magnetic field. They can also be kept in suspension by magnetic stabilization and applied as a fluidized bed.[19–23]

Amino-functionalized magnetite (Fe_3O_4) containing magnetic carriers (1 μm) from *ATG:biosynthetics* was used for the immobilization of the ADH-'A'. The procedure was as outlined above. For the determination of the immobilization yield, the supernatant, washing solutions, and immobilized biocatalyst were subjected to activity tests. The calculated immobilization yield of 86% is lower when compared to the yield of the porous glass beads. The activity yield for the magnetic carriers was about 49%. A repetitive batch with the carrier-bounded enzyme showed almost no change in activity. The conversion-time plots were nearly identical.

2.3. Nanoparticles

In addition to the classic porous glass beads and the magnetic particles a novel carrier type, nano-size detonation diamonds,[24–27] was tested. The already amino-functionalized particles (agglomerate size 550 nm, prior to chemical functionalization: primary particles of 4–5 nm, agglomerates of ~300 nm)[25] enabled the application of the common immobilization route.

The use of nanodiamonds or carriers on a nano-scale in general offers some distinct advantages. Diffusion limitation, which is a known issue especially in the case of porous carriers or entrapped enzymes, is considerably low due to the small sizes of the particles. Furthermore, they offer a high surface to volume ratio and are easily suspendable in solutions.

Despite these advantages, there remains the challenge in the handling of these particles. Standard separation techniques such as centrifugation or filtration are more complicated or even impossible. This problem can be overcome by the use of membrane approaches.

The immobilization procedure was similar to the porous glass beads and the magnetic particles. The immobilization of the ADH-'A' on nanodiamonds yielded a biocatalyst with an activity of 0.071 U mg^{-1}. In a repetitive batch, the activity decreased to 0.063 U mg^{-1}, but as the shapes of the conversion-time plots are nearly identical, this decrease may result from measurement errors. The immobilization yield was >99.9% and the activity yield 49%.

3. Conclusion

The alcohol dehydrogenase ADH-'A' from *R. ruber* was successfully immobilized on different amino-functionalized supports: porous glass beads, magnetic particles, and nanodiamonds. A covalent binding was achieved by derivatizing the carrier and enzyme with glutaraldehyde.

The results of all the experiments performed are similar to each other and reproducible (Fig. 2). Very good immobilization yields were achieved with the porous glass beads TRISOPERL® and TRISOPOR® and with the nanodiamonds. The experiments with the magnetic carriers gave a slightly lower value. The activity yields are lower than the immobilization yields in all cases. It is unknown if the activity decrease was induced by deactivation during the immobilization process, thermal deactivation or by enzyme losses.

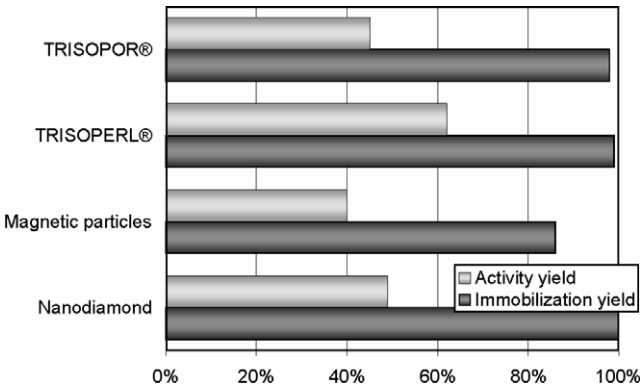

Figure 2. Activity and immobilization yields for different carrier materials.

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