Enzyme Immobilization on Synthesized Nanoporous Silica Particles and their Application in a Bi-enzymatic Reaction

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The application of enzymes presents a great advantage regarding highly selective reactions; however, it involves also challenges due to their sensitivity. Immobilization offers one strategy to overcome those challenges enabling enzyme stabilization, as well as retention. In the present study, covalent attachment on hydrophilic amino-functionalized carriers is found to be the most promising immobilization method for the investigated reaction system. To achieve this, a novel method for preparation of silica particles with subsequent amino-functionalization is developed to prepare spherical carriers for enzyme immobilization, whereby high porosities are obtained based on polymerization. With these particles, immobilization of an alcohol dehydrogenase and a formate dehydrogenase is realized with residual activities of 70 and 80% after 12 consecutive batches, respectively. The two immobilized enzymes are used in the reduction of cinnamyl aldehyde with in situ cofactor regeneration, obtaining a conversion of 100% and up to 10-fold higher turnover numbers compared to the free enzyme.

Introduction

Biocatalytic processes are promising alternatives to conventional chemical reactions, if they fulfill certain requirements like less byproduct formation, less intermediates, environmentally friendly and sustainable reactions. In addition, the application of enzymes offers different advantages like reactions at ambient temperature, pH and pressure, as well as high stereo and regioselectivities. Limitations using these catalysts like the need of cofactors or the recyclability can be overcome with an efficient cofactor regeneration and simplified downstream processing, which are key techniques to develop biocatalytic processes. Thereby, enzyme immobilization plays a major role, since it enables an easier recovery and recyclability of these often cost intensive catalysts, as well as possible stabilization in the reaction. Since there is not one immobilization method suitable for all biocatalysts, various factors and numerous procedures have to be considered establishing a suitable immobilization method for the applied enzyme and reaction system.[2]

The variety of different immobilization techniques can be divided into three main approaches: non-covalent binding, covalent binding and the entrapment of enzymes. Non-covalent binding via adsorption or ionic interaction is a simple attachment to a carrier; however, enzyme leaching is often an issue, due to weak interactions of the enzyme and the carrier. In contrast, covalent immobilization onto carriers is well-known for a tight fixation of the enzyme resulting in very good residual activities applying the immobilized enzyme in consecutive batches.[3] In addition, the multipoint-covalent attachment of the enzyme to the carrier enables prevention of denaturation through unfolding.[4] In comparison to covalent immobilization onto a carrier, cross-linking, as a very easy method, realizes strong binding of enzyme molecules by linking components. However, the distortion of the conformation and chemical modifications can influence the active center and are disadvantages of this method. A further approach is enzyme entrapment. This method generates stable enzyme preparations with high immobilization yields, nevertheless, leaching and diffusion limitation are reported as main problems using this technique.[5] Other interesting methods are the formation of enzyme-inorganic crystal hybrid catalysts including enzyme-inorganic crystal hybrid nanoflowers and enzyme–metal organic framework composites. These techniques provide self-assembly systems by one-step co-precipitation via complexation of the enzyme molecule and the metal component. Nevertheless, these metals can affect the enzyme activity as well.[6]
However, not only the method itself, also the choice of support is of great importance for the effectiveness of enzyme immobilization. The properties of the material like polarity, charge and dielectric constant have a strong influence affecting activity and stability. Based on this, increased stability by immobilization of enzymes allows inertness against reaction conditions and mechanical stability and therefore the application in different reactor types.[7] Furthermore, the used material should exhibit appropriate biocompatibility and biodegradability, as well as good regeneration feasibility for re-use of the carrier.[8] An increasing number of immobilized enzymes are applied in chemical industry, due to easier separation of the biocatalyst to simplify the downstream processing for economy improvement. For example, Merck developed a new adsorptively immobilized CalB preparation with a significantly increased stability in comparison to Novozym 435® for the production of odanacatib, a cathepsin K inhibitor.[9] In contrast, Tate and Lyle immobilized an epimerase on ion exchange resin realizing the production of the sweetener allulose in a continuous fixed bed reactor. Since March 2017 they are selling this calorie sweetener manufactured in their patented process.[10]

In the study presented, the immobilization of two commercially available enzymes, the alcohol dehydrogenase 97 L (ADH) expressed by C. boidinii (Sigma Aldrich), is under investigation for application in a model reaction system with in situ cofactor regeneration (Scheme 1). This system is of interest, because it can be applied for synthesis of flavors as well as fragrances and can be applied for synthesis of flavors as well as fragrances and derivates. The ADH is reducing cinnamaldehyde (1) to cinnamyl alcohol (2), while the FDH is regenerating the cofactor NADH. Different immobilization techniques were screened to find a suitable method with high immobilization and activity yield, for application in this bi-enzymatic reaction system. The ADH and FDH were immobilized using the method with the best results and characterized regarding surface area and recyclability. Both immobilized enzymes were applied in the bi-enzymatic reaction and compared with their free state.

Results and Discussion

Screening of different immobilization procedures

A screening of immobilization methods was carried out using the reduction of cinnamyl aldehyde to cinnamyl alcohol catalyzed by the ADH to find a suitable technique. Investigated methods were: covalent immobilization on hydrophobic and hydrophilic carriers, entrapment in alginate beads and formation of cross-linking enzyme aggregates (CLEAs). The screened immobilization techniques were investigated regarding activity and immobilization yield, results shown in Figure 1.

Entrapment was investigated using sodium alginate and chitosan for stabilization, based on the investigations of Zhou and co-workers.[1] The high immobilization yield reported in the mentioned study was reproduced in the present screening. Lower chitosan concentrations resulted in even higher immobilization and activity yields, 90 and 1 % respectively (Figure 1, entrap. A–C). In contrast, Zhou et al. reported a residual activity of 62 %. However, a disintegration of the matrix was observed, which resulted in an activity loss of 60 % after 6 batches.[11]

For CLEA preparation, different precipitation methods were tested to find a suitable one (data not shown). The best results were obtained using 70 % (w/v) ammonium sulfate and follow-up cross-linking with glutaraldehyde to achieve CLEAs. The CLEAs reached a high immobilization yield of 74 % and the highest activity yield of 7.3 % in comparison to all investigated techniques in this study. However, the particles showed poor stability, abrasion and re-dissolution occurred during activity measurements. In contrast, Hu et al. reported a residual activity of an ADH CLEA with a higher yield of 41 %.[12]

Commercially available hydrophobic and hydrophilic carriers from Purolite® were tested with different pre-functionalizations, according to the method of the supplier. As hydrophobic

![Figure 1. Influence of immobilization method on activity and immobilization yield. Highlighted in red: Synthesized silica particles. (MA – methacrylate, TP – Trisoperc®, entrap. A–C – 0.025, 0.25, 0.5 % chitosan, CLEA – cross-linked enzyme aggregates) Cond.: The immobilizations were carried out according to the described methods with Tris–HCl buffer pH 8, 0.1 M and phosphate buffer pH 8, 0.1 M, rt, cADH = 0.5 mg/mL. Activity: V = 100 mL, cNADH = 0.1 mM, cCinnamylaldehyde = 5 mM.](image)

Scheme 1. Reduction of cinnamaldehyde (1) to cinnamyl alcohol (2) with in situ cofactor regeneration. (ADH – alcohol dehydrogenase, FDH – formate dehydrogenase)
materials two carriers made of methacylate with epoxy-functionalization (ECR204F and ECR206F) were used and one with amino-functionalization (ECR309F). All three carriers showed a comparable low activity and a low immobilization yield; however, using the amino-functionalized carrier the highest immobilization yield of 22% was reached. Since the properties of the carrier can have a significant influence on the immobilization of the enzyme, a hydrophilic carrier was investigated as well. The application of this carrier showed a 3-fold higher activity yield and in addition, an immobilization yield as twice as high. A comparable immobilization behaviour for glass beads was already described by Goldberg et al. in our research group, whereby TRISOPERL® beads were used for ADH immobilization as well. Nevertheless, a higher residual activity of 45% was reported. The higher activity can be based on the enzyme and reaction system, but also on particles with already opened epoxy-groups and therefore less active functional groups for enzyme immobilization. For further investigation of the influence of buffer salts on immobilization, phosphate buffer was tested using hydrophilic carriers. This resulted in an increase in immobilization yield from 42 to up to 55% and a 3.4-fold higher activity yield was determined. Beside the conformational changes during covalent immobilization, the hydrophobicity of the surface area of the enzyme has a major influence on the activity, which became obvious during the screening in this study. Cao assumed that the immobilized enzyme is either active in aqueous or organic solvent, depending on the properties of the support, e.g. immobilization on hydrophilic carriers could show a higher activity in aqueous media. Supports with polyhydroxyl groups, e.g. glass and polysaccharides, have the advantage, that these groups are poor leaving groups and need a pre-activation in contrast to e.g. usage of epoxy-functionalized hydrophobic carriers, where the ring opening could occur over time. Therefore, several functionalization methods for amino-functionalized carriers, e.g. with 3-aminopropyltriethoxysilane (APTES), vinyltriethoxysilane (VTES) or glutaraldehyde, are known to result in different immobilization yields and retained activities.

Summarizing the immobilization screening, the reaction system as well as the nature of the applied enzyme plays an important role for activity retention. For subsequent investigations of the reduction of cinnamyl aldehyde in the bi-enzymatic reaction, CLEAs were not further evaluated, due to unstable aggregates. Entrapped enzyme in alginate showed a very low activity, possibly because of diffusion limitation, so that the method was also not taken into account. Using ADH covalently immobilized on hydrophobic carriers resulted in the lowest immobilization and activity yield of all procedures. A higher activity was yielded by covalent immobilization via Schiff base formation on hydrophobic covalent supports using amino-functionalized carriers. Covalent immobilization on hydrophilic carriers showed a comparable high immobilization yield with a significantly higher activity of the ADH in comparison to hydrophobic supports and entrapment, so that this method was highly promising for the application in the present process.

Preparation of silica carrier

The commercial hydrophilic silica particles showed the best results during the screening of immobilization, however, these carriers are not on the market anymore. Functionalized mesoporous silica is already established for enzyme immobilization offering pores with a suitable pore size for diffusion of the enzyme and the reaction compounds, as well as functional groups for immobilization. Due to the very promising results, hydrophilic spherical silica beads were synthesized based on the condensation of tetraethylorthosilicate (TEOS) and functionalized according to Wörmeyer et al., shown in Scheme 2.

For this, TEOS was hydrolyzed under acidic conditions and subsequently polycondensated with a base to obtain a highly porous material. The synthesis of spherical particles was realized
by dropping the solution into a column filled with a mixture of rapeseed oil/heptane/n-butylamine (0.81/0.16/0.03 (v/v)) and with a 1 cm heptane phase on top for reduction of the surface tension, shown in Scheme 3. The homogenous oil/heptane mixture was important for slowly sinking particles, to harden in the outer phase until reaching the bottom avoiding deformations. After complete condensation, the obtained spherical particles were washed and functionalized in two steps with APTES as linker and glutaraldehyde (GA) as spacer. Subsequently, the synthesized and functionalized particles were tested regarding enzyme immobilization. In respect to the ADH, an immobilization yield of 40% and activity yield of up to 4% were reached, highlighted in Figure 1 (red frame). In comparison to reported yields using hydrophilic carriers, the obtained results were rather low in this study. However, compared to immobilization on functionalized mesoporous silica described by Lei et al. with an immobilization yield of 3% and the immobilization methods described above, the immobilized enzyme on silica particles is in the mid-range of immobilization yield and show one of the highest activity yields. The results were comparable to commercial hydrophilic carrier preparations presenting a promising alternative for the applied enzyme, so that method is of great interest for the reduction of cinnamyl aldehydes (cf. Figure 1).

Due to the polycondensation, the carriers show a high porosity with a surface area of approximately 750 m² and a pore diameter of 5 nm. Those values were revealed by measurements of the adsorption isotherms with the BET (Brunauer-Emmet-Teller) method. In Figure 2, unfunctionalized particles were compared with functionalized ones (APTES and GA) and particles with immobilized ADH to see possible influences. A significant decrease in surface area was observed between unfunctionalized and functionalized particles, which is resulting from the binding of APTES and GA. A similar trend was reported by Gopinath and Sugunan, who investigated the adsorptive immobilization of an α-amylase on the grafted hydrophilic natural mineral montmorillonite. They reported also a slight decrease of surface area by functionalization with APTES and GA. With immobilization of the enzyme a further decrease was measured by Gopinath and Sugunan, whereby it does not change significantly in the present study. The determined pore diameter is increasing with functionalization and showed an additional rise with the linking of the enzyme. This might be due to a blockage of smaller pores by functionalization with APTES and GA leading to the decrease of the surface. However, this effect did not occur in the study of Gopinath and Sugunan. They compared also different enzyme concentrations for immobilization and observed a similar behaviour as for the functionalization with APTES and GA. With an increasing enzyme loading on the montmorillonite, they found a decrease in the surface area. However, with the prepared silica particles a 2 and 4 times higher surface area than reported for the particles of Gopinath and Sugunan was obtained and half of the pore diameter. In addition, BET measurements of self-assembled asparagine nanoflowers investigated by Wu et al. with 32 m²/g also in a much lower surface area and with 112.5 nm in an 8-fold higher pore size. The silica particles prepared during our investigations offer a high surface for multi-point attachment during covalent immobilization in comparison to other techniques.

Even if the retained activity is lower than for reported techniques, the enhanced stability and therefore the recycling as the most interesting property of immobilized enzymes, beside its activity, was investigated with the ADH as well as the FDH applied in their corresponding reactions (cf. Scheme 1). Twelve consecutive batches for the ADH and the FDH were carried out using the same immobilized enzyme preparation, which were stored at room temperature in buffer in between. The batches showed slight fluctuations, however, both enzymes were used with a retained activity of 70% and 83% for the ADH and FDH, respectively. The deviations of the 12 batches using the single enzymes could result from different handlings, as well as the deviations of the measurements itself, so that no
reliable activity calculations were possible regarding kinetics (cf. S1).

The comparable high activities after 12 consecutive batches were obtained over one month with no further loss in activity, showing the excellent suitability for the application in the target reactions (cf. Figure 3).

The main objective of this study was to use the immobilized enzyme for the reduction of cinnamyl aldehyde to cinnamyl alcohol with in situ cofactor regeneration. The application of immobilized versus free ADH/FDH applied in the bi-enzymatic reaction is shown in Figure 4. In both reactions 100% conversion of the substrate was reached, however, the reaction progress deviates. Here, it has to be considered that the amount of enzyme for the free reaction was calculated based on the theoretically immobilized amount onto the carriers. The immobilized enzyme can show a lower retained activity based on e.g. changes in enzyme conformation. Diffusion limitation plays an additional role for immobilized enzymes, differentiated between internal and external diffusion limitation and can cause the deviations seen in Figure 4. Studies of possible internal diffusion restrictions in the case of synthesized silica particles did reveal any significant limitation (cf. S1).

However, the reactions using free and immobilized enzymes were performed in a batch reactor with an overhead stirrer, whereby external diffusion limitation can occur using immobilized enzymes. For further investigations, different reactors with an increased flow through the particles should be used to decrease a possible external diffusion limitation and to increase the reaction rate for the immobilized enzymes.[22]

The direct comparison of the cinnamyl aldehyde reduction using free and immobilized enzyme can be made using the total turnover number (TTN). This value describes the ratio of converted substrate to applied catalyst and the calculated values for both enzymes of the reaction cascade are shown in Table 1. Based on the lower amount of applied FDH (m = 0.38 mg) the TTN is higher than for the ADH (m = 2.8 mg). For the ADH and the FDH a significant increase in the TTN value after immobilization was obtained due to the successful application in 12 consecutive reactions and despite the observed activity loss, caused by immobilization. The ADH showed an 8-fold increase to 34.7 gcat/gsubstrate and the immobilized FDH showed a 10-fold increase to 303.1 gcat/gsubstrate. Since for both enzymes high conversion after 12 batches were obtained, the TTN values could increase more if the immobilized enzymes are applied in more consecutive batch reactions.

Tufvesson and co-worker described the allowable cost contribution of the used catalyst and the corresponding range of required productivity for different applications, e.g. for bulk chemicals or pharmaceuticals.[23] The aromatic components investigated in this study are possible cosmetic ingredients and belong to the group of specialty chemicals. With the immobilization of the FDH with a TTN of over 300 gcat/gsubstrate values in the target region of specialty chemicals are enabled, if the price of the catalyst is within 100–500 €/kg. However, the price of the biocatalyst is always dependent on the market volume and same immobilized enzymes can be used in the production of pharmaceutical and bulk chemicals, so that the catalyst production is shared.

The synthesis of cinnamyl alcohol with in situ cofactor regeneration was subsequently performed in 3 batches using the same immobilized enzymes. In Figure 5 it can be seen, that the conversions are decreasing with the number of batches. This decreased recyclability of the enzymes in comparison to the recycling experiments of the individual immobilized enzyme (cf. Figure 3) can result from an influence of bi-enzymatic reaction on the single enzymes. For example, a rapid deactiva-

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**Table 1. Comparison of TN and TTN of free and immobilized enzyme.**

| Enzyme | TN free | TN immobilized | TTN immobilized |
|--------|---------|----------------|------------------|
| ADH    | 4.3     | 4.3            | 34.7             |
| FDH    | 30.4    | 30.4           | 303.1            |

TTN were calculated based on substrate conversion and applied enzyme amount. For the immobilized enzymes the theoretical amount of immobilized enzyme used and the residual activity during the 12. Batch in the recycling experiments.
A further reason can be the different diffusion behaviour of the single components. For example, cinnamyl alcohol provides a higher hydrophobicity (logP = 1.82) than the substrate cinnamyl aldehyde (logP = 2.00). Based on the different properties of the components, they can exhibit different interactions with the carrier resulting in different mass transports into, through and out of the particle. Nevertheless, the recyclability for the enzymes applied in the reduction of cinnamyl aldehyde with in situ cofactor regeneration needs still an optimization to obtain high conversion in consecutive batches applying the bi-enzymatic reaction.

**Conclusion**

In the present study, a screening of different immobilization techniques was performed and showed that hydrophilic carriers with amino-functionalization resulted in high immobilization and activity yields for the applied enzymes. A method to prepare hydrophilic spherical particles was developed based on a polymerization reaction. Preparation of the particles could be easily scaled up, showing a high potential for commercial processing.

The catalysts for the investigations, the alcohol dehydrogenase 97 L, was purchased from c-Lecta (Germany) and the formate dehydrogenase from *Candida boidinii* from Sigma-Aldrich (Germany).

Sodium formate, cinnamyl aldehyde, butylamine, tetraethyl orthosilicate were bought from Sigma-Aldrich (Germany), whereby NAD+, NADH, sodium alginate, chitosan, hydrochloric acid, glutaraldehyde, ethanol, heptane, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were purchased from Roth (Germany) and APTES from TCI (Germany).

**Immobilization procedures**

**Covalent immobilization**

For this immobilization, the commercial resin ECR309F from Purolite® was applied and a Tris–HCl buffer (0.1 M, 2 mM MgCl₂, pH 7) was used. The experiments were carried out at 25 °C. First, the resins were washed 3 times for equilibration with a resin/buffer ratio of 1:1 (w/v). Afterwards the carrier was pre-functionalized with 2% (v/v) glutaraldehyde with a resin/solution ratio of 1:1 (w/v) for 60 min at 25 °C. The resin was suspended in an ADH solution of 0.5 mg/mL with a resin/solution ratio of 2.5:1 and stirred for 18 h with an overhead stirrer. The solution was filtered (0.45 μm) and washed 3 times with 50 mL buffer and with 50 mL of 0.5 M NaCl.

**Epoxy-functionalized hydrophobic carriers**

For this immobilization, the resins ECR204F and ECR206F from Purolite® were applied and the immobilization performed with Tris–HCl buffer (0.1 M, 2 mM MgCl₂, pH 7) at 25 °C. The immobilization was carried out in the same way as the immobilization onto amino-functionalized carriers, but without glutaraldehyde. The resins were suspended directly into the enzyme solution and stirred for 18 h with an overhead stirrer and the solution was left in buffer at room temperature. This emphasizes the highly promising properties of these immobilized enzyme preparations, which were also investigated for the cofactor dependent reduction of cinnamyl aldehyde. With free and immobilized enzymes 100% conversion was reached, with different reaction rates. The TTN of the applied immobilized enzymes is up to 10-fold higher based on their recyclability, so that the process is more cost-efficient than using the free enzymes. However, applying the enzymes in consecutive batches of the bi-enzymatic reaction an inactivation is occurring.

Recyclization of biocatalysts is a key prerequisite for industrial application resulting in an enhanced economic efficiency of large-scale processes. The promising results for enzyme immobilization of the individual enzymes during this study are based on the efficient recycling resulting in a high amount of produced cinnamyl alcohol. With this, the investment for the used enzymes can be decreased significantly.

**Experimental Section**

**Materials**

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afterwards for immobilization without stirring for 22 h at room temperature. Afterwards the resins were filtered and washed buffer.

**Amino-functionalized hydrophilic carrier**

The immobilization on hydrophilic carrier was carried out with the glass beads TRISPERL® (particle size 100–200 μm, pore size 100.46 nm) using the method of Goldberg et al.[13] 200 mg beads were pre-activated in 10 mL 2.5 % glutaraldehyde solution prepared with phosphate buffer (0.1 M, pH 7) under vacuum (60 mbar) for 30 min and subsequently 50 min under ambient pressure. Thereby the color was changed from white to reddish. The beads were washed with 200 mL deionized water to remove the excess of the linker and dried at 65 °C for 4 h. The beads were suspended in ADH solution 0.5 mg/mL dissolved in phosphate buffer (100 mM, pH 7). The immobilization was carried out under vacuum (60 mbar) at 4 °C for 35 min and stirred further under ambient pressure at 4 °C. Subsequently the resins were filtered and washed 5 times with 100 mL phosphate buffer and suspended in 10 mL glycine buffer (0.1 M, pH 7.5). After 3 h, the filtered particles were washed 5 times with 10 mL phosphate buffer (0.1 M, pH 7.5). In addition, this immobilization method was also carried out with a Tris–HCl buffer (0.1 M, 2 mM MgCl₂, pH 7).

**Cross-linked enzyme crystals**

CLEAs were prepared according to the protocol described by Hu et al.[14] First, the ADH was precipitated adding 70 % (w/v) ammonium sulfate to the enzyme solution in 1 mL. The solution was incubated at 4 °C for 2 h. Afterwards, the mixture was centrifuged at 13,000 rpm for 15 min at 4 °C. The precipitated enzyme was cross-linked with 2.5 % (v/v) glutaraldehyde solution with a ratio of 0.5:1 (w/w) glutaraldehyde/protein. The mixture was shaken for 90 min at 25 °C and centrifuged afterwards at 5,000 rpm for 10 min. The solution above the obtained pellets was decanted and the pellets washed 3 times with 5 mL buffer.

**Entrapment**

The ADH was entrapped according to the method described by Zhou et al.[15] The different conditions used for the entrapment are shown in Table 2.

The sodium alginate was added to 50 mL deionized water and was shaken for 2 h at 30 °C to obtain a homogenous solution. A 0.5 mg/mL ADH solution was dissolved in Tris-buffer (0.1 M, 2 mM MgCl₂, pH 7) and mixed with an equal volume of the alginate solution at ambient temperature. As curing bath, a solution of 2 % (w/v) calcium chloride (CaCl₂) was prepared in the chitosan solution, which was dispersed in 5 % acetic acid and mixed at 7,000 rpm for 30 min. The ADH-alginate mixture was added dropwise (20 cm height) into the CaCl₂ bath using a 10 mL syringe and a 18G needle. The beads were cured for 6 h at 4 °C and washed afterwards 3 times with 10 mL of a 0.9 % (w/v) NaCl solution. The beads were treated for 2 h with a 0.5 % glutaraldehyde solution and finally washed with deionized water (3 × 10 mL).

**Preparation of silica particles**

For the preparation of the silica particles a mixture of 1 mol TEOS, 4 mol water, 2.4 mol ethanol and 10−5 mol HCl was prepared and stirred for 30 minutes, afterwards it was dropped into a mixture of rape seed oil, butyl amine and n-heptane (890 mL/178 mL/33 mL) via a syringe pump (60 mL syringe, 10 mL/min) and the prepared particles were cured overnight.

For functionalization, 100 g APTES (3-Aminopropyl)triethoxyxilane) were dissolved in 50 mL ethanol and 10 g particles were incubated at 50 °C for 48 h. The particles were washed with ethanol and stored in water. For cross-linking the particles were treated with a 2.5 % (v/v) glutaraldehyde solution for 1 h at 30 °C and washed afterwards with water for 30 min to remove residual glutaraldehyde. The enzyme immobilization onto the particles was carried out with a solution of 0.5 mg/mL ADH dissolved in phosphate buffer (0.1 M, pH 8), whereby the particles were incubated for 2 h at 25 °C. Subsequently, they were washed with water to remove unbound enzyme.

**Determination of immobilization and activity yield**

Immobilization yields were determined spectrophotometrically according to the Pierce Protein Assay. 10 μL of the sample was mixed with 150 μL of the Pierce solution and incubated for 5 minutes. Subsequently, the absorbance was measured at 562 nm and the protein concentration determined via a calibration of bovine serum albumin (BSA) as standard. The immobilization efficiency was determined by subtraction of the protein content of the solution after immobilization and all washing solutions from the starting ADH solution applied for immobilization.

The enzyme activity was determined based on NADH, which was quantified spectrophotometrically at 360 nm and 30 °C. The yield was calculated based on the measured activity of the immobilized protein and the theoretical activity, which was possible based on the immobilization yield.

**BET measurements**

The measurements of the specific surface area and the pore diameter were performed according to Raman et al.[23] The samples were degassed prior to the investigations for 24 h at 348 K. The data was determined using Brunauer-Emmet-Teller (BET) method and low-temperature nitrogen adsorption-desorption analysis (Quanatromer Nova 3000e) between p/p₀ range of 0.05–0.30.[24]

**Recycling of ADH and FDH**

For the measurement of recyclability of the immobilized enzymes, a 3-neck flask was used and the solution was stirred continuously with an overhead stirrer. The reactions were carried out in 25 mL phosphate buffer (0.1 M, pH 8) at 30 °C using 2 g of the immobilized enzyme. The measurement of the ADH was performed with 5 mM cinnamyl aldehyde and 0.1 mM NAD⁺, whereby the FDH was investigated with 200 mM sodium formate and 0.1 mM NAD⁺. The conversions were measured spectrophotometrically based on the NADH consumption or production at a wavelength of 360 nm.

**Cofactor regeneration**

For the investigation of the in situ cofactor regeneration both enzymes, ADH and FDH, were applied in their free and immobilized form. The reactions were carried out with the same set-up, which

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**Table 2. Immobilization conditions for entrapment.**

| Immobilization time [h] | Cₐlginate (w/v) [%] | Cₐlginate (w/v) [%] | Cₐd₀ (w/v) [%] |
|-------------------------|-------------------|-------------------|----------------|
| 6                       | 0.25              | 4                 | 2              |
| 6                       | 0.05              | 3                 | 2              |
| 6                       | 0.5               | 3                 | 2              |
was used for the recyclability measurements. Both reactions were carried out in 25 mL phosphate buffer (0.1 M, pH 8) at 30 °C and with 3.5 mM cinnamyl aldehyde, 100 mM sodium formate and 0.15 mM NADH. For the reactions with immobilized enzymes 5 g of FDH particles and 1 g of ADH particles were used. Based on the calculations for the immobilization of the enzymes onto the carriers 2.8 mg ADH and 0.38 mg FDH were used for the reaction of the free enzymes.

For the reaction monitoring 500 μL samples were taken and mixed gently with 500 μL p-xylene. After centrifugation 300 μL were measured via gas chromatography with an HP-5 column (Agilent Technologies, United States) and helium as carrier gas. The temperature was increased from 120 °C with 40 °C/min to 160 °C holding for 2 min, and was increased again with 40 °C/min 320 °C holding for 2 min.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Biocatalysis · Cofactor regeneration · Immobilization · Aromatic compounds · Alcohol dehydrogenase

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