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Covalent immobilization of benzoylformate decarboxylase from *Pseudomonas putida* on magnetic epoxy support and its carboligation reactivity

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ABSTRACT

Epoxy attached magnetic nanoparticles were prepared and used as solid support for covalent immobilization and stabilization of benzoylformate decarboxylase (BFD, E.C. 4.1.1.7) from *Pseudomonas putida*. A three-step immobilization/stabilization procedure is applied. The enzyme is firstly covalently immobilized under mild experimental conditions (e.g. pH 7.0, no added $MgSO_4$ and 20 °C). Secondly, the enzyme is immobilized under more drastic conditions (higher pH values, higher ionic strengths, etc.) to facilitate an increase in effective concentration of the enzyme on the support near the epoxide reactive sites. Thirdly, the remaining epoxy groups are blocked to stop any additional interaction between the enzyme and the support. With more drastic conditions, the loading of enzyme can be increased from 1.25 to 6.70 mg enzyme per gram of support. The covalently bounded enzyme was characterized in terms of its activity and stability for the formation of (*S*)-2-hydroxypropiophenone (2-HPP). The activity of the immobilized BFD was determined to be 53.0% related to the activity of the free enzyme. The immobilized biocatalyst retained 95% of its original activity after five reaction cycles.

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1. Introduction

Immobilized enzymes have been increasingly used in industrial bioprocesses due to the ease in separation of enzymes from the reaction solutions and their reuse, thereby increasing the turnover number of biocatalysts and reducing the operating costs [1]. The attachment of enzymes onto solid supports through covalent bonds is a commonly used method for immobilization of enzymes [2]. It provides distinct advantages, such as prevention of enzymes leakage from the support and improvement of enzyme stability through multipoint covalent attachment [3,4]. It was increasingly appreciated that the supports made a great difference to the immobilization efficiency in terms of the loading capacity of the support, activity recovery and operational stability of the immobilized enzymes [5,6].

Among a number of supports available for covalent binding of enzymes, those containing reactive epoxy groups on the surface have been paid great attention [7–9]. Epoxy supports present many advantages; for example, they are very stable, allowing for long-term storage, prolonged transport from manufacturer to consumer, and extended enzyme-support reaction periods.

Attachment of very diverse ligands, such as proteins [10,5], nucleic acids [11] and various small molecules [12,13] to the support via nucleophilic addition to the epoxide ring has been successfully performed. The reactive groups on the affinity ligand are any nucleophilic primary or secondary amine, sulfhydryl group or, less commonly, a hydroxyl group [12]. When both the support and the affinity ligand are stable toward alkaline conditions, the addition of the affinity ligands is conducted under basic conditions. When the nucleophilic group is an amino group, the pH of the reaction is usually above nine, where the amine is nonprotonated.

Because of the low reactivity of amino and hydroxyl groups at neutral pH, the epoxy-activated phases have been criticized as being unreactive compared to other activated phases [14]. A technique has been described to overcome this problem of poor reactivity at neutral pH [10,5,11,15]. In the presence of high concentrations of certain salts, efficient immobilization of proteins [5,15] and nucleic acids [11] at neutral pH has been reported. The increased coupling reactivity was explained as resulting from a salt-induced association between the macromolecule and the surface of the affinity support, thereby increasing the effective concentration of the nucleophilic groups on the macromolecules near the

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epoxide reactive sites. Other investigators [16,17] have used conditions at high salt concentrations for the efficient coupling of proteins to silica-based epoxide affinity phases with the retention of biological activity. Enzyme immobilization on epoxy-activated beads is believed to occur by the following two stepmechanism: (i) enzyme adsorption onto the bead surface, and (ii) formation of a covalent bond [18,19].

In recent years, functionalized magnetic nanoparticles (MNPs) have attracted much attention as supports for the immobilization of many bioactive substances, such as proteins, peptides, enzymes, and antibodies [20]. MNPs can be easily recovered from media by applying a magnetic field. When they are used for enzyme immobilization, particularly in batch reactors and continuous-flow stirred-tank reactors, this method facilitates the separation of enzymes from the product after the completion of the reaction in order to permit the reuse and recycling of the enzyme [21,22].

Benzoylformate decarboxylase (BFD, E.C. 4.1.1.7) from *Pseudomonas putida*, whose native function is to decarboxylase benzoylformate to benzaldehyde in the mandalate pathway, has been widely investigated concerning its application in enzymatic C—C-bond formation. Wilcocks and Ward reported the carboligase activity of BFD from *P. putida* for the first time, introducing the catalyst as an efficient alternative for the enantioselective synthesis of (*S*)-2-hydroxyketones [23]. Its stereoselectivity is highly dependent on the structure of the substrate aldehydes [24]. For synthetic purposes, the enzyme also accepts aldehydes as donors, instead of α-ketoacids, to give the corresponding 2-hydroxyketones with (*S*)-configuration [25]. BFD which has been characterized and cloned to an overexpressing *Escherichia coli* with a histidine tag is suitable for new immobilization applications [26]. Until now, only few investigations were published regarding the immobilization of BFD, whereas numerous results were documented in literature regarding the influence of immobilization on the stability and activity of enzymes, e.g. [27,28]. However, to the best of our knowledge, there were no reports of the magnetic epoxy supports for BFD immobilization.

In this work, the synthesis of 2-hydroxypropiophenone (2-HPP) starting from benzaldehyde and acetaldehyde was catalyzed by covalently immobilized BFD on magnetic epoxy support. The focus is set on the question how far the immobilization influences the performance of the enzyme. Therefore, the carrier-bound enzyme was characterized in terms of its activity and stability.

2. Materials and methods

2.1. Chemicals and materials

All chemicals used were of analytical grade, commercially available and used without any purification. Recombinant *E. coli* SG13009 strain carrying the histidine tagged BFD gene was a kind gift of Dr. Martina Pohl from CLIB-Graduate Cluster Industrial Biotechnology, Germany [29]. Chemicals used in production of BFD such as isopropyl-β-D-galactopyranoside (IPTG) and antibiotics were all of molecular biology grade and obtained from AppliChem.

2.2. Characterization

Silica coated magnetic nanoparticles were re-dispersed in pure water by sonication for 10 s. The particles were re-dispersed in pure water by sonication for 10 s and a drop of suspension was placed onto SPI Double Copper Grids 100/200. The particles were analyzed by transmission electron microscopy (TEM) (JEOL 2100F, Japan) for particle size and morphology.

Fourier transformed infrared (FT-IR) spectra were measured on a Thermo Scientific Nicolet IS10FT-IR spectrometer (USA). Sixteen scans were collected at a resolution of 4 cm^{-1}.

BFD-catalyzed reactions were monitored by thin layer chromatography (TLC) on silica gel (E. Merck, Darmstad). Spots were detected by both UV-absorption and phosphomolybdicacid (PMA) staining. The products were identified by ^{1}H NMR and ^{13}C NMR spectra (BRUKER DPX 400 MHz) using tetramethylsilane (TMS) as an internal standard and deutero-chloroform as the solvent. Enantiomeric excess values were determined via HPLC analysis (Agilent 1100 series, Chiralpak AD).

2.3. Synthesis of magnetite and silica-coated magnetite nanoparticles

Synthesis of magnetite nanoparticles and those functionalized with epoxy groups were previously reported by our group [30,31].

Briefly, magnetite (Fe_3O_4) nanoparticles were prepared by co-precipitating Fe^{2+} and Fe^{3+} ions under alkaline conditions by ammonia solution [32]. Fe_3O_4 particles were then coated with silica using the sol–gel method [32].

One gram of wet silica coated Fe_3O_4 particles were reacted with 10 mL of 5% 3-glycidoxypropyltrimethoxysilane (GPTMS) in toluene at room temperature overnight. After the coupling reaction, the modified magnetic nanoparticles were removed from the solution with the help of a permanent magnet and rinsed thoroughly with toluene and ethanol to remove physically adsorbed silane. Finally, they were freeze-dried. When prepared in this way, the surfaces of the magnetic nanoparticles had exposed active epoxy groups.

The schematic illustration for the preparation steps of epoxy-functionalized magnetic nanoparticles is shown in Scheme 1.

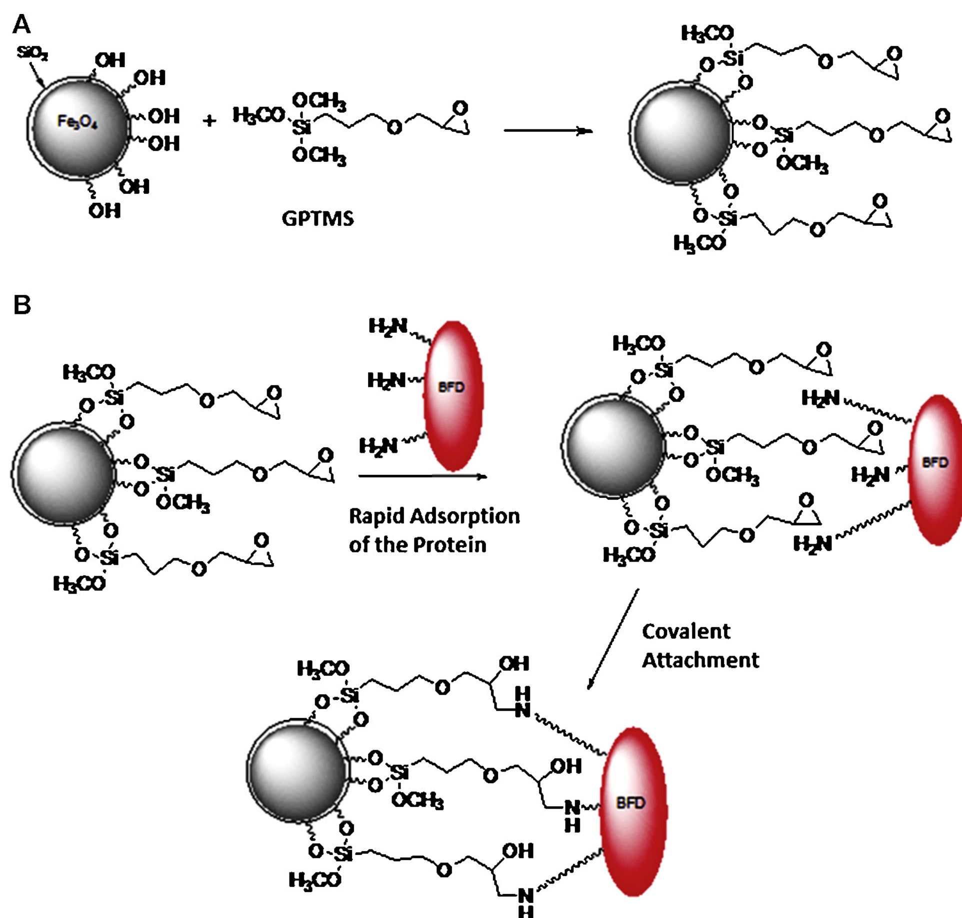
2.4. Immobilization of BFD

2.4.1. Preparation of recombinant BFD

Recombinant *E. coli* SG13009 strain carrying pKK233-2 plasmid with hexahistidine tagged BFD gene was used in this study. Cells were grown in 100 mL LB (luria broth) medium at 37 °C until the optical density reached 0.5 at 600 nm, and 1 mM isopropyl-β-D-thiogalactopyranosid (IPTG) was added for induction. The cells were grown for an additional 6 h at 25 °C prior to harvesting by centrifugation. Then, the cells were resuspended in 10 mL 50 mM potassium phosphate buffer (pH: 7.8, 10 mM NaCl and 20 mM imidazole), disrupted by sonication and the cell debris was removed by centrifugation. To purify the cell-free extract by Ni^{2+}-NTA affinity chromatography (Invitrogen®), the column was previously equilibrated in buffer B (50 mM potassium phosphate pH 7.8, 100 mM NaCl, 0.1 mM ThDP). After the column was washed with buffer B containing 10 mM imidazole, the enzyme was eluted with buffer B containing 200 mM imidazole. The fractions of highest purity were pooled and the buffer was exchanged for storage buffer (100 mM potassium phosphate buffer, pH 6.0, 1 mM $MgSO_4$, 0.5 mM ThDP) using a Hi-Trap™ Sephadex G-25 desalting columns (Amersham). The protein was concentrated using amicon 5K ultra centrifugal filters (Millipore) and lyophilized for long term preservation. 1 mg lyophilisate contains 0.89 ± 0.04 mg BFD.

2.4.2. Activity assays

BFD activity was determined by calculating the initial rate of 2-HPP formation. 10 mg lyophilized His-tagged BFD was dissolved in 5 mL reaction buffer (0.5 mM TPP, 2.0 mM $MgSO_4$, in 50 mM potassium phosphate buffer at pH: 7.5) to prepare enzyme solution. Reaction solution contained 4.5 mL buffer solution and 0.5 mL of enzyme solution rigorously mixed via magnetic stirrer at 200 rpm where the substrate concentrations were 40 mM benzaldehyde and



Scheme 1. Schematic illustration of the preparation steps for (A) magnetic epoxy support. (B) Adsorption and covalent attachment of His-tagged BFD to magnetic epoxy support.

400 mM acetaldehyde to initiate the 2-HPP synthesis for 30 min at 20 °C. At 1 min time intervals 100 μL sample was withdrawn from the reaction mixture and quenched by addition of a stop-buffer (90% acetonitrile, 5% H_2O, and 5% H_3PO_4) at the ratio of 2:1 (sample:stop-buffer, v/v) followed by intense mixing. The amount of product (2-HPP) formed was analyzed by HPLC analysis (Agilent 1100 series, Chiralpak AD column) [33].

2.5. Immobilization

To identify factors affecting immobilization efficiency, BFD was immobilized under different experimental conditions (initial enzyme concentration in the range of 1.0–4.0 mg BFD/mL, pH values of the immobilization medium varying from 7.0 to 9.0, ionic strength from no added $MgSO_4$ to 2.0 M, and the duration of immobilization up to 24 h). The reported results were the average values of three independent experiments.

BFD immobilization was carried out as follows. 8 mg lyophilized His-tagged BFD was dissolved in 4 mL immobilization buffer (0.05 M potassium phosphate buffer at different pH values). About 0.1 g magnetic epoxy support was mixed with 4 mL enzyme solution. The mixture was shaken at room temperature for 24 h at 160 rpm. At intervals of 1 or 2 h, 0.1 mL aliquots of the supernatant and 100 mg of suspended support were withdrawn by magnetic separation to determine the amount and the activity of the immobilized enzyme on the support. After enzyme immobilization, the immobilized BFD was washed with 1 mol/L $(NH_4)_2SO_4$ and 0.1 mol/L, pH 8.0 phosphate buffer, respectively, to remove non-covalently bound enzyme from the supports. The amount of immobilized enzyme bound to the support was determined spectrophotometrically by the difference between the amount of BFD added and the amount of protein in the supernatant and the washes. The enzyme concentration was determined by the Bradford method using bovine serum albumin as a standard protein [34]. After the immobilization of the enzyme, the residual epoxy groups on the support were quenched with 5 mL of a glycine buffer (3 M, pH 8.5) during 12 h at 20 °C to prevent further non-specific reaction, and the magnetic epoxy support attached with BFD was separated from the medium by a magnetic separation device. Finally, it was used for synthesis of (*S*)-2-hydroxy-1-phenylpropanone [(*S*)-2-HPP].

2.6. Synthesis of (S)-2-hydroxy-1-phenylpropanone [(S)-2-HPP] [35]

A substrate solution (pH 7.5; 50 mM potassium phosphate; 2 mM magnesium sulfate; 0.5 mM thiamine diphosphate; 40 mM benzaldehyde; 400 mM acetaldehyde) was prepared. The immobilized enzyme solution with a volume of 0.5 mL containing 100 mg of dry carrier was added to the 4.5 mL prepared substrate solution. Reaction was carried out in a total reaction volume of 5 mL for 30 min at 20 °C. Sampling was done after 30 min by stopping the stirring, allowing separation of the magnetic support attached with BFD from the medium by a magnetic separation device, and taking a volume of 300 μL and analyzed by HPLC. Work-up was performed as previously described [36]. Viscous oil, *ee* 92 ± 4%; $[\alpha]_D^{22} = -85.1$ (c 2.0, $CHCl_3$), HPLC (Chiralpak AD): 90:10 Hexane/2-propanol, 0.8 mL/min, UV detection at 250 nm, Rt (*S*) = 11.5, Rt (*R*) = 14.1; δ_H

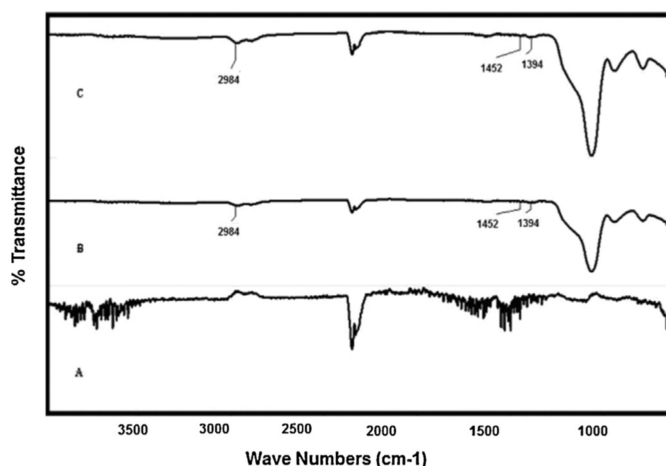


Fig. 1. (A) Fe_3O_4/SiO_2, (B) epoxy attached particles and (C) pure 3-glycidoxypropyltrimethoxysilane (GPTMS).

(400 MHz; $CDCl_3/CCl_4$; Me_4Si) 7.9 (2H, m, Ph), 7.40–7.60 (3H, m, Ph), 5.1 (1H, q, J = 6.0 Hz, CH), 3.8 (1H, br s, OH). δ_C (100 MHz; $CDCl_3/CCl_4$; Me_4Si) 201.7, 134.3, 134.0, 128.9, 128.7, 69.2, 22.0.

The reaction with free and immobilized enzyme was carried out according to the procedure described in the literature [33].

2.7. Stability and reusability of immobilized BFD

For the investigation of the stability of the immobilized BFD, repetitive batch experiments were performed. Therefore, the reaction solution consisted of 4.5 mL buffer solution and 0.5 mL immobilized enzyme solution (dry carrier 100 mg) containing 40 mM benzaldehyde and 400 mM acetaldehyde. Each batch was conducted for 60 min with four washing steps in between which again took 15 min. For each period, the immobilized enzyme was separated with the help of a magnet and washed two times with potassium phosphate buffer at pH 7.5. Each washing step of collected immobilized enzyme was done with 1 mL of fresh buffer and incubated with fresh substrate for the next batch reaction. In total, five repetitive batches were performed and the whole experiment was done in 6 h. The reaction temperature was maintained at 20 °C. Moreover, washed-out solutions were analyzed by Bradford assay. No leakage of enzyme was determined in any kind of reaction performed.

3. Results and discussion

3.1. Characterization

In order to illustrate the successful graft of epoxy group onto the magnetic nanoparticles, FT-IR spectrometry was performed. Fig. 1 shows the FTIR spectra of Fe_3O_4/SiO_2 nanoparticles and the nanoparticles reacted with glycidoxypropyltrimethoxysilane (GPTMS) together with the spectrum of pure GPTMS. Although the characteristic adsorption peak of the epoxy group at around 1150 cm^{-1} overlapped with the strong absorption of the silica, the alkyl C—H stretching vibration band at 2984 cm^{-1} and its bending vibration bands at 1394 cm^{-1} and 1452 cm^{-1} were clearly visible in the spectra of the modified nanoparticle (B) and pure GPTMS (C) [37–41]. All these informations referenced above confirm the chemical modification of the surface of Fe_3O_4/SiO_2 with epoxysilane.

The image in Fig. 2 shows the TEM bright field micrograph for the Fe_3O_4/SiO_2 magnetic nanoparticles. As can be seen from the TEM image, the average size of the nanoparticles is approximately 10 nm.

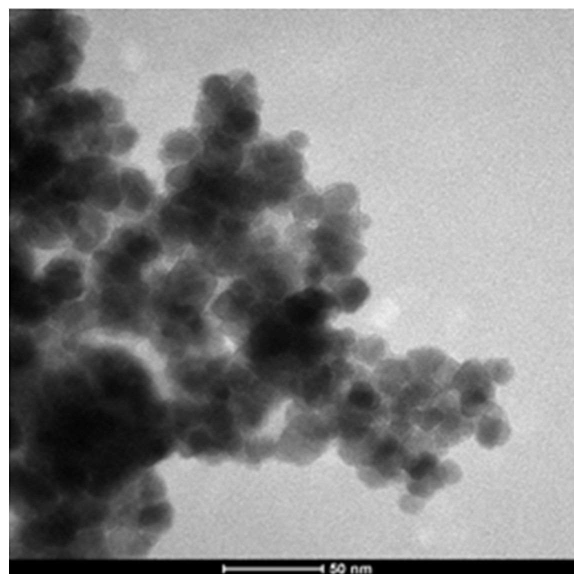


Fig. 2. TEM graphs of Fe_3O_4/SiO_2 magnetic nanoparticles.

3.2. Factors controlling binding of His-tagged BFD onto the magnetic epoxy support

In the present work, His-tagged BFD from *P. putida* was covalently attached onto the magnetic epoxy support. In order to achieve high enzyme loading and activity recovery, the immobilization conditions were optimized concerning enzyme concentration, pH, ionic strength and coupling time were investigated. The maximal enzyme loading and enzyme activity were investigated under the optimal immobilization parameters.

3.2.1. Effect of coupling time

The effect of coupling time on the immobilization efficiency at different concentrations onto the magnetic epoxy support under mild reaction conditions (0.05 M potassium phosphate buffer, pH 7) is presented in Fig. 3. The amount of immobilized enzyme increased during the first 5 h continuously with the incubation time. After 5 h binding of the enzyme to the surface of the carrier seemed to be complete. Enzyme loading increased with enzyme concentration and the maximum amount of immobilized enzyme was measured as 1.35 mg/g support in 4.0 mg/mL initial enzyme concentration.

3.2.2. Effect of ionic strength

In order to examine the effect of ionic strength on the immobilization efficiency, experiments were performed using different $MgSO_4$ concentrations. As demonstrated in Fig. 4, the enzyme loading onto the magnetic epoxy support increased as the ionic strength and enzyme concentration increased up to 1.25 M and 3.5 mg/mL, respectively. The maximum enzyme loading was found to be 5.2 mg/g support in 3.5 mg/mL initial enzyme concentration at ionic strength of 1.25 M $MgSO_4$.

This finding indicated that the amount of the BFD immobilized onto the magnetic epoxy support clearly depends on the ionic strength and initial enzyme concentration used for the immobilization procedure [17]. Covalent immobilization of enzymes onto the epoxy supports is believed to follow a two-step binding mechanism which involves first an adsorption of the enzyme onto the magnetic epoxy support surface and then the chemical reaction between the epoxy groups of support and nucleophilic groups of the enzyme forming covalent bonds [5]. The magnetic epoxy

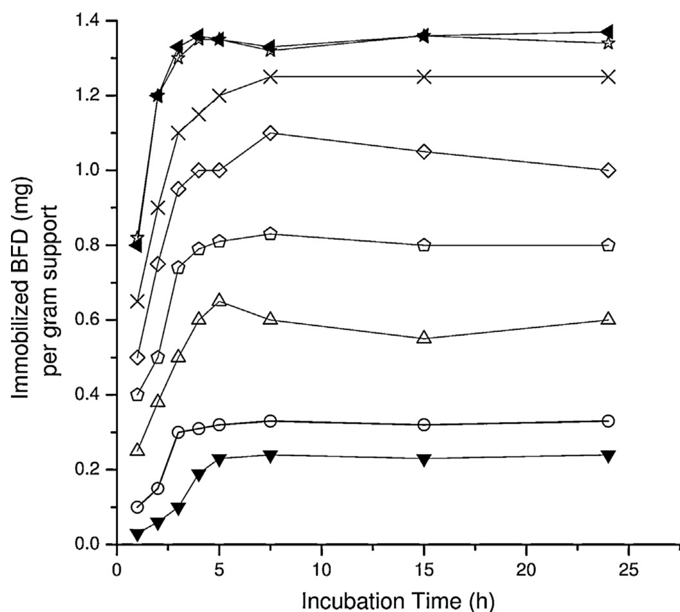


Fig. 3. Effect of the coupling reaction time on the amount of BFD immobilized onto the magnetic epoxy support under mild reaction conditions (0.1 g magnetic epoxy support; 4 mL enzyme solution; 0.05 M potassium phosphate buffer, pH 7) at a different benzoylformate decarboxylase concentrations ((▼): 1.0 mg/mL; (O): 1.5 mg/mL (△): 2.0 mg/mL; (⬠): 2.5 mg/mL; (◊): 3.0 mg/mL; (X): 3.5 mg/mL; (⋆): 4.0 mg/mL; (◄): 4.5 mg/mL).

support is hydrophobic, and the hydrophobic interaction was the main driving force for the adsorption of the His-tagged BFD from aqueous phase to the support. Therefore, high ionic strength should be favorable to the access of BFD to the reactive sites of magnetic epoxy support and, hence, benefits for the covalent binding of the enzyme onto the support [42].

3.2.3. *Effect of the environmental pH*

The pH of the immobilization medium was altered between 7.0 and 9.0, and the resulting effect on the BFD loading was

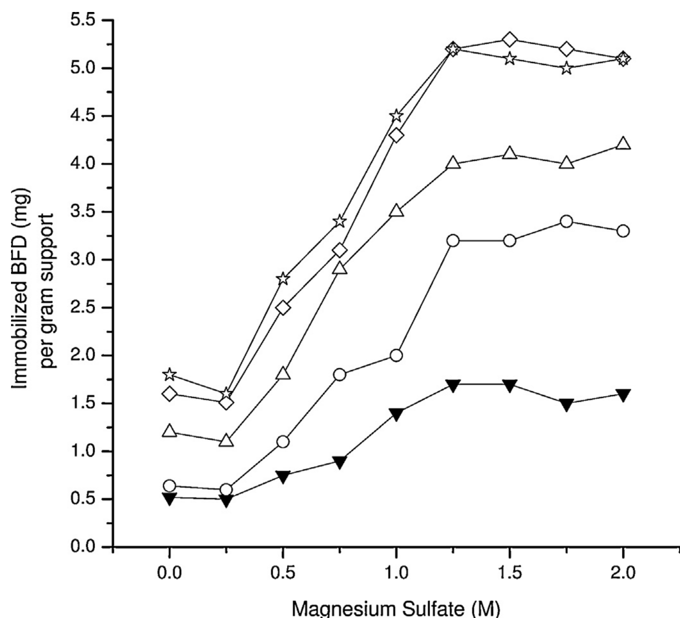


Fig. 4. Effect of ionic strength on the amount of immobilized BFD (0.1 g magnetic epoxy support; 0.05 M potassium phosphate buffer, pH 7) at a different benzoylformate decarboxylase concentrations ((▼): 2.0 mg/mL; (O): 2.5 mg/mL (△): 3.0 mg/mL; (◊): 3.5 mg/mL; (☆): 4.0 mg/mL).

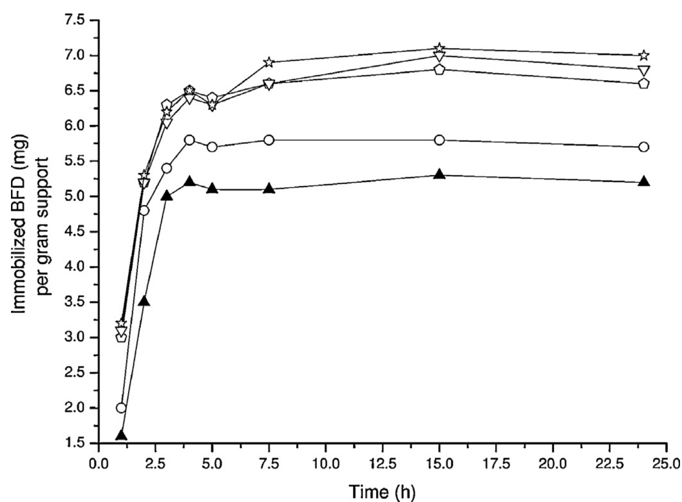


Fig. 5. Effect of pH on the amount of BFD attached to the magnetic epoxy support using an initial BFD concentration of 3.5 mg/mL in the presence of 1.25 M $MgSO_4$ (0.1 g magnetic epoxy support; 4 mL enzyme solution). (▲): pH 7; (O): pH 7.5; (⬠): pH 8.0; (△): pH 8.5; (☆): pH 9.0.

demonstrated in Fig. 5. The amount of the enzyme on magnetic epoxy support varied from 5.2 ± 0.2 to 6.7 ± 0.1 mg/g support in the pH range of 7.0–9.0, implying that the enzyme loading on the support was slightly influenced by environmental pH. Slight increasing of the enzyme loading was observed at pH 8.5 and 9.0 while we expected to see an increase of the binding capacity. The reason for this could probably be the decomposition of silica coating material. The best result was found at pH 8.0.

To determine the effect of the incubation time on the activity of the immobilized enzyme and in the supernatant, 40 mL 3.5 mg/mL His-tagged BFD solution in phosphate buffer, pH 7.5, was incubated with the magnetic epoxy support (1 g). At the indicated time points, 4 mL samples were withdrawn and supernatant and enzyme immobilized on the support were separated by a magnetic separation device. The activity in the supernatant was analyzed (see Fig. 6).

To determine the activity of immobilized enzyme, the magnetic support was washed three times with 5 mL portions of 50 mM phosphate buffer, pH 8.0, the remaining epoxide groups on the support were blocked with 5 mL of a glycine buffer (3 M, pH 8.5) during

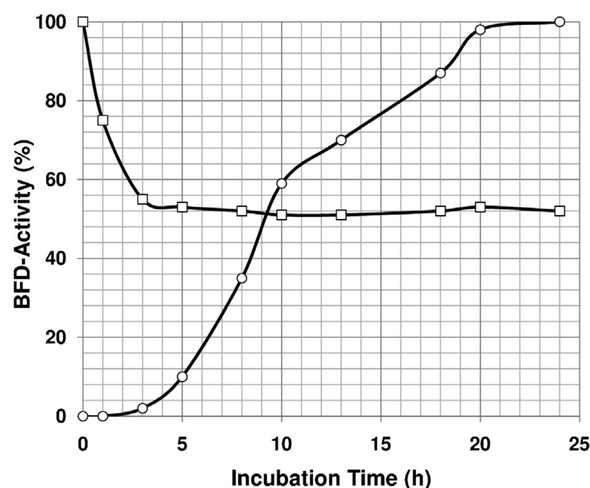
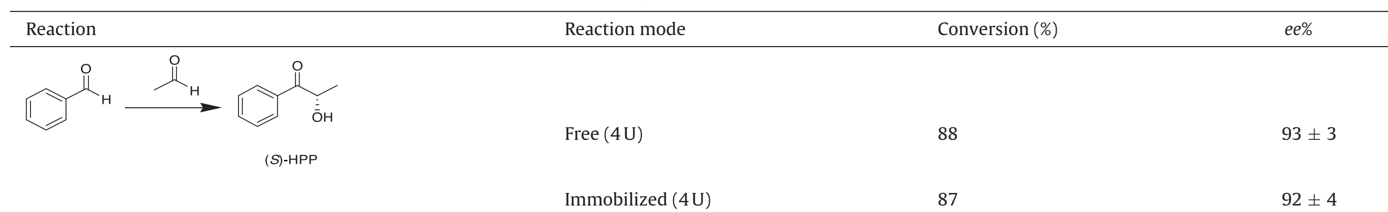


Fig. 6. Squares: the activity of the BFD that remains in the supernatant during incubation with the magnetic support at different incubation times. Circles: the activity of immobilized BFD on the magnetic epoxy support after different incubation times. The highest activity was given as 100%.

Table 1
Conversion and enantiomeric excess (*ee*) values for the immobilized BFD catalyzed reaction.

Reaction	Reaction mode	Conversion (%)	*ee*%
	Free (4 U)	88	93 ± 3
	Immobilized (4 U)	87	92 ± 4

12 h at 20 °C to prevent further non-specific reactions and then, the magnetic epoxy support attached to enzyme was separated from the medium by a magnetic separation device, and washed three times with 50 mM phosphate buffer, pH 7.5, and used for activity experiments.

Fig. 6 shows that we did not observe covalent immobilization of the enzyme up to 3 h on a magnetic-epoxy support. After 5 h of incubation time, the covalently immobilized enzyme activity increased with the incubation time. These results suggest that previous physical adsorption of the enzyme is necessary to obtain covalent immobilization of the enzyme molecules on the magnetic-epoxy support. This demonstrates that, in the first stages of the immobilization, a fraction of the enzyme was physically adsorbed on the support, but not covalently immobilized. Thus, after 20 h at pH 8.0, most of the enzyme was covalently attached to the support. The highest activity was given as 100% (in Fig. 6).

3.3. Synthesis of (S)-2-hydroxy-1-phenylpropanone [(S)-2-HPP]

BFD is effective in cross acyloin reaction of benzaldehyde with acetaldehyde to form (*S*)-2-HPP. As reported earlier in the literature, the benzaldehyde/acetaldehyde ratio was very important for the product distribution [36]. Equal amount of donor and acceptor aldehyde furnished mixture of (*R*)-benzoin and (*S*)-HPP but low concentration of benzaldehyde in the presence of excess acetaldehyde resulted in the formation of (*S*)-2-HPP with 96% *ee* and 84% yield [36].

Carboligation reactions were performed for the formation of the cross acyloin product HPP with free and immobilized BFD to identify potential effects caused by the immobilization. The enzyme immobilized magnetic epoxy support results in an activity decrease from 1.85 to 0.58 U/mg enzyme, which is 53% of the activity of free enzyme determined before immobilization. The result given for activity analysis is an average value based on values from independent duplicate assays. The *ee* of (*S*)-2-HPP synthesized by immobilized enzyme is determined to be 92 ± 4% (*S*)-HPP which is in good agreement with the *ee* yielded by free enzyme [93 ± 3% (*S*)-HPP]. In Table 1, the results of the immobilization are summarized.

The stability of BFD immobilized on the magnetic epoxy support during batch synthesis of (*S*)-2-HPP was determined by the repeated use of the same biocatalyst for five reaction cycles. Experimental conditions were given in Section 2.7. As shown in Fig. 7, the residual activity of the immobilized BFD slightly decreased gradually with the increasing number of reaction cycles, and it retained 95% of initial activity after five batches of repeated use. Conversion for five repeated reusability experiment has been found as 87%, 86%, 85%, 85% and 83%, respectively. This result indicated that the immobilized enzyme was very stable under operating conditions, which would be an advantage for industrial application.

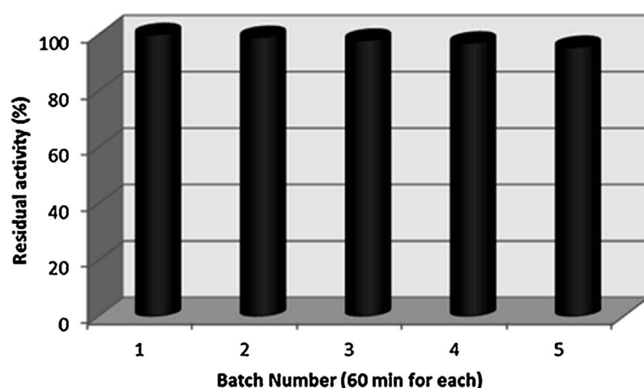


Fig. 7. Operational stability of BFD on the magnetic epoxy support during batch synthesis of (*S*)-2-HPP. The reaction solution consisted of 4.5 mL buffer solution (50 mM potassium phosphate, pH 7.5; 2 mM magnesium sulfate; 0.5 mM thiamine diphosphate) and 0.5 mL immobilized enzyme solution (dry carrier 100 mg) containing 40 mM benzaldehyde and 400 mM acetaldehyde. Each batch was conducted for 60 min with four washing steps in between which again took 15 min. In total, five repetitive batches were performed and the whole experiment was done in 6 h. The reaction temperature was maintained at 20 °C. Conversion for five repeated recycling experiments was 87%, 86%, 85%, 85%, and 83%, respectively.

4. Conclusion

To identify an adequate biocatalyst for a specific reaction, immobilization is one possibility to further improve its properties. The immobilization allows easy recycling, improves the enzyme performance, and it often enhances the stability of the enzyme. In this work, the immobilization of the benzoylformate decarboxylase (BFD) from *P. putida* was accomplished on magnetically responsive magnetic epoxy support following a two-step mechanism; that is, the protein is physically adsorbed and then the covalent reaction takes place. The total amount of enzyme loading and activity recovery was dependent on the immobilization conditions including initial enzyme concentration, pH, ionic strength and coupling time. Under optimum conditions, the amount of BFD immobilized on the magnetic epoxy support reached 6.7 mg/g, and the immobilized enzyme retained 53.0% of its initial activity. The immobilized BFD showed excellent performance in (*S*)-2-HPP production. The *ee* of (*S*)-HPP synthesized by immobilized enzyme is determined to be 92 ± 4% which is in good agreement with the *ee* yielded by free enzyme [93 ± 3% (*S*)-HPP]. The benefit of the immobilization is illustrated by the repetitive use of this biocatalyst (retained 95% of its original activity after five reaction repeats).

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