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Carboligation reactivity of benzaldehyde lyase (BAL, EC 4.1.2.38) covalently attached to magnetic nanoparticles

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ABSTRACT

Epoxy-functionalized Fe_3O_4–SiO_2 core–shell magnetic nanoparticles (epoxy-M-support) were prepared by modification with glycidyloxypropyltrimethoxysilane (GPTMS) and characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM), and fourier transform infrared spectroscopy (FTIR) methods. Pure histidine-tagged recombinant benzaldehydelyase (BAL, EC 4.1.2.38) was efficiently immobilized onto the epoxy-M-support with covalent binding. An immobilized BAL epoxy-M-support system was tested to catalyze the self and cross condensation reactions of aldehydes, and the kinetic resolution of racemic acyloins. The acyloin products were obtained in high yield and with high enantiomeric excesses (⩾98% ee). The carboligation reactivity of the immobilized enzyme was comparable to that of free enzyme-catalyzed reactions. The covalent immobilization offers high enzyme activity and stability (at least 5 repeats without losing its activity).

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1. Introduction

Benzaldehydelyase (BAL, EC 4.1.2.38), a thiamine pyrophosphate (TPP) dependent enzyme, isolated from *Pseudomonas fluorescens Biovar I*, was first reported by Gonzales and Vicuna.[1,2] The authors showed that this strain can grow on benzoin as the sole carbon and energy source. The structure of BAL at a 2.6 Å resolution has been reported by Schulz et al., who found that BAL is a homotetramer consisting of three domains, Dom-α, Dom-β, and Dom-γ for each monomer.[3] Residues from Dom-γ interact with the diphosphate moiety of thiamin diphosphate (ThDP) and magnesium, while those from Dom-α of a neighboring subunit bind to the pyrimidine ring of ThDP. Four ThDP molecules were located within the protein as well as four magnesium ions. The active center is defined by the thiazolium ring of ThDP, which sits in a deep pocket opening to the outer surface of the tetramer.[3,4]

Benzaldehydelyase is an important biocatalyst for enantioselective carboligation reactions.[5] The enzyme can produce chiral α-hydroxyketones, which are indispensable building blocks in the synthesis of several interesting compounds. Many novel reactions of the native and recombinant enzyme have been reported, in which the applicability of BAL has already been extended through a wide range of substrates.[6]

Immobilization is one of the most efficient methods for increasing the cost effectiveness of enzymatic reactions.[7,8] The stability and reusability of the immobilized enzyme bring several advantages. Functionalized magnetic nanoparticles (MNPs) have been extensively used in the immobilization of many bioactive substances, such as proteins, peptides, enzymes, and antibodies.[9] 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.[10]

Immobilized metal-chelate affinity chromatography is a well-developed tool for the industrial-scale purification of proteins fused to poly-His-tags.[11–17] In some cases, the adsorption of a poly-His-tagged protein on the chelate support is relatively strong and could be used for enzyme immobilization.[18] However, the reversibility of the binding process may be a drawback when used to develop an industrial-scale immobilization procedure. The undesired release of the metals to the reaction media may also become a problem in many cases.

One of the most suitable methods for the industrial-scale immobilization of proteins is based on epoxy supports.[19–24] Epoxy supports have many advantages; for example, they are very stable, thus allowing for long-term storage, prolonged transport from manufacturer to consumer, and extended enzyme-support reaction periods. Moreover, they can bind to different moieties of proteins (amine, thiol, and hydroxyl groups), yielding very stable protein-support bonds (secondary amine, thioether, and ether). Moreover, the remaining epoxy groups can be easily blocked after

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enzyme immobilization with different compounds, yielding an inert surface. More recently, it has been reported that epoxy supports may be used for enzyme stabilization via an intense enzyme-support multipoint covalent attachment by controlling incubation conditions.[25,26]

For biomolecule immobilization, MNPs need to be functionalized by surface modification to present reactive groups to the biomolecules. To date, the most commonly employed method for the surface modification of MNPs is silanization. Silica coating on MNPs (core–shell structure) allows these materials to retain the desired magnetic or optical properties. In the meantime, the silica shell makes them more biocompatible and easier to functionalize.[27–29]

Epoxysilane agents are classical compounds that are applied widely to a variety of polymer composite materials in order to enhance the stability and integrity of the polymer/inorganic interfaces.[28,30,31] Epoxy activated surfaces are also very attractive systems for the covalent immobilization of biomolecules.

The attachment of very diverse ligands, such as proteins,[32,33] nucleic acids,[34] and various small molecules,[35,36] to the support via nucleophilic addition to the epoxide ring has been carried out successfully. The reactive groups on the affinity ligand are any nucleophilic primary or secondary amine, sulfhydryl group or, less commonly, a hydroxyl group.[35] When both the support and the affinity ligand are stable under alkaline conditions, the addition of the affinity ligands can be carried out under basic conditions. When the nucleophilic group is an amino group, the pH of the reaction is usually above nine, therefore the amine is unprotonated.

Due to 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.[37] A technique has been described for overcoming this problem of poor reactivity at a neutral pH.[32–34,38] In the presence of high concentrations of certain salts, the efficient immobilization of proteins[38,33] and nucleic acids[34] 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, thus increasing the effective concentration of the nucleophilic groups on the macromolecules near the epoxide reactive sites. Other investigators[39,40] have used conditions at high salt concentrations for the efficient coupling of proteins to silica-based epoxide affinity phases with retention of biological activity, as evidenced by the affinities of the bound moieties for the mobile phase components. Enzyme immobilization on epoxy-activated beads is believed to occur by the following two step mechanism: (i) enzyme adsorption onto the bead surface, and (ii) a covalent bond being formed.[25,26]

In our previous work,[41] we have efficiently immobilized BAL on Co^{2+}-NTA functionalized γ-Fe_2O_3 magnetic particles. These magnetically responsive particles can be used for the direct immobilization and easy separation of enzymes from a crude extract in order to obtain a pure immobilized enzyme.[41–43]

Herein we aim to increase the enzyme stability and activity that gives high yields during acyloin condensation reactions through the covalent immobilization of BAL on a magnetically responsive epoxy support system. With this in mind, we herein exploit epoxysilane chemistry as a convenient method for the activation of MNPs and protein immobilization. Epoxysilane-modified MNPs were fully characterized. Experimental conditions for surface modification and protein immobilization were investigated and optimized. A BAL-epoxy-M-support system was applied for the ligase activity of the BAL during the self condensation of benzaldehyde and the cross condensation of benzaldehyde and acetaldehyde starting from racemic benzoin via C–C bond breaking and bond forming reactions. The cross condensation reactions led to the formation of (*R*)-2-hydroxy-1-phenyl-propanone and (*S*)-benzoin.

2. Results and discussion

2.1. Characterization

Epoxy-functionalized Fe_3O_4–SiO_2 core–shell magnetic nanoparticles (epoxy-M-support) were characterized by FT-IR. Figure 1 shows the FT-IR spectra of Fe_3O_4–SiO_2 nanoparticles, which reacted with glycidyloxypropyltrimethoxysilane (GPTMS) together with the spectrum of pure GPTMS molecules. Although the characteristic band of the epoxy group at approximately 1150 cm^{-1} overlapped with the strong absorption of the bare silica, the alkyl C–H stretching vibration band at 2984 cm^{-1} and its bending vibration bands at

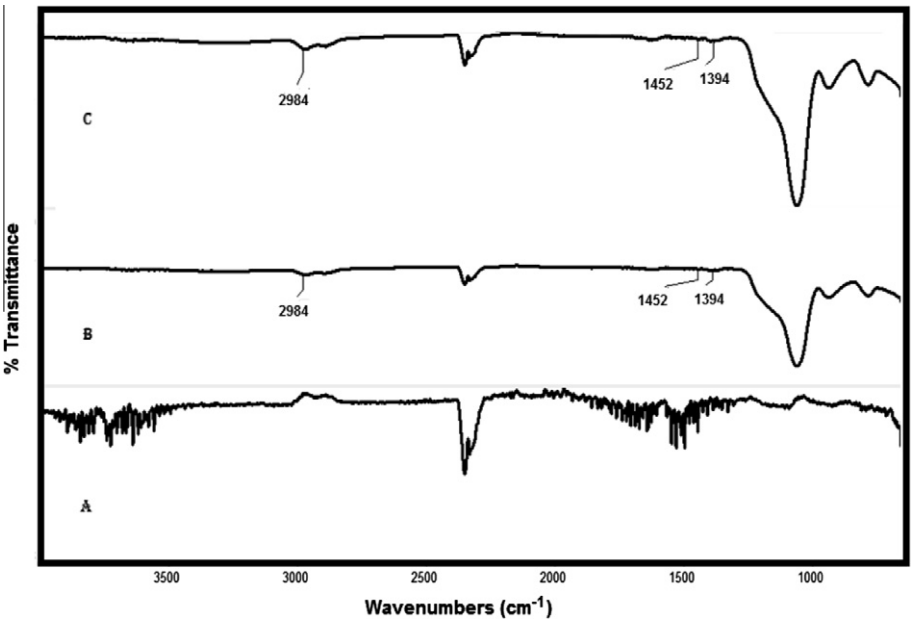


Figure 1. FT-IR spectra for: (A) Fe_3O_4–SiO_2 core–shell magnetic particles; (B) epoxy-M-support; (C) pure 3-glycidoxypropyltrimethoxysilane (GPTMS).

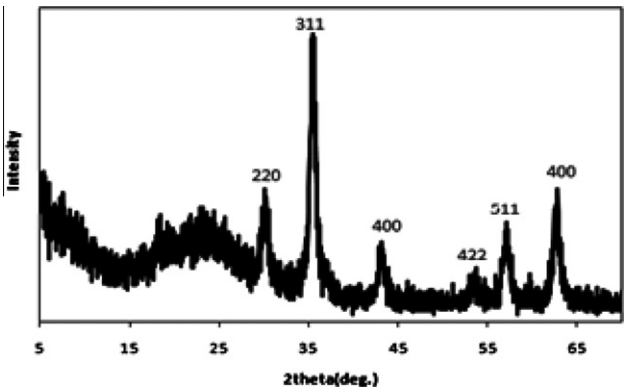


Figure 2. X-ray diffraction pattern for Fe_3O_4–SiO_2 core–shell magnetic nanoparticles.

1394 cm^{-1} and 1452 cm^{-1} were clearly visible in the spectra of the modified nanoparticle (B) and pure GPTMS (C).[44–48] The results indicate that epoxysilane was attached successfully to the surface of the Fe_3O_4–SiO_2 core–shell magnetic nanoparticles.

The crystal structure of core–shell magnetic nanoparticles was investigated by XRD analysis (Fig. 2). The main peaks occur at 2θ = 30.16°, 35.48°, 42.99°, 52.90°, 56.95°, and 62.70°, which correspond to (220), (311), (400), (422), (511), and (440), respectively. It is apparent that the diffraction pattern of our magnetic silica nanoparticles is close to the standard pattern for crystalline magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) with an inverse spinel structure.[49]

However, we cannot clearly distinguish between these two types of iron oxides. Since they have very similar magnetic properties, their identification is not important herein. It is reasonable to believe that our sample would be a mixture of Fe_3O_4 and γ-Fe_2O_3 because of the open-to-atmosphere preparation and handling procedures during which some Fe_3O_4 would change to γ-Fe_2O_3. The average size of the magnetite nanoparticles coated with silica was estimated as approximately 11.8 nm by using the Scherrer equation[50] ($d = 0.9\lambda/\Delta(2\theta)\cos\theta$, where d is the crystalline domain size, $\Delta(2\theta)$ is the width at half maximum of the strongest peak (311), and λ is the X-ray wavelength).

The image in Figure 3 shows the TEM bright field micrograph for the Fe_3O_4–SiO_2 core–shell magnetic nanoparticles. As it can be seen from the TEM image, the average size of the nanoparticles is approximately 11 nm. These results show that the size of the core–shell magnetic nanoparticles is in agreement with the size determined by XRD using the Scherrer equation.

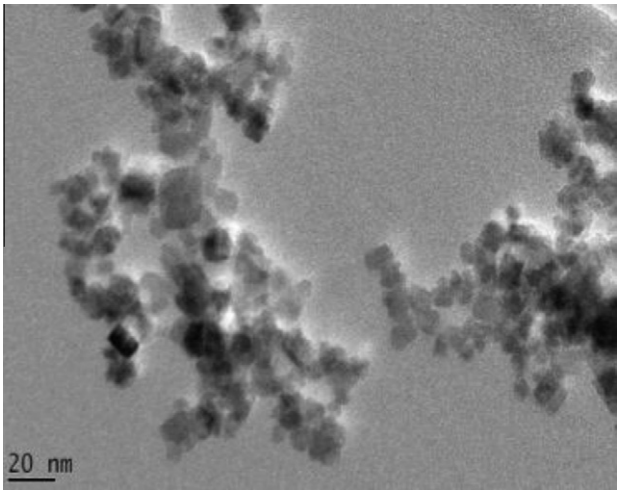


Figure 3. TEM graphs of Fe_3O_4–SiO_2 core–shell magnetic nanoparticles.

2.2. Immobilization

The nanoparticles consisting of an Fe_3O_4 core and epoxy-functionalized silica shell (epoxy-M-support) were prepared and used to immobilize benzaldehydelyase (BAL, EC 4.1.2.38) (see Section 4). In order to obtain an immobilized biocatalyst with a high amount of enzyme loading and activity, the effects of the immobilization efficiency under varying immobilization conditions such as enzyme concentration, pH, ionic strength, and coupling time were investigated.[33,38,40]

The determination of the BAL adsorption onto epoxy-M-support does not provide information on the BAL that is chemically linked versus physically adsorbed. In order to determine the physically adsorbed BAL on an epoxy-M-support, the BAL-epoxy-M-support was treated with a phosphate buffer (pH 7.5) at 37 °C, followed by Triton X-100 1% solution (see Section 4). This method allowed the quantitative extraction of physically adsorbed BAL from the epoxy-M-support. The enzyme concentration of the supernatants and the amount of enzyme in the washes were checked by the Bradford method.[51] Physically adsorbed BAL on the epoxy-M-support was found to be ~0.1 mg BAL/g epoxy-M-support.

2.2.1. Effect of initial enzyme concentration

After equilibration of the epoxy-M-support with a coupling solution (50 mM phosphate buffer pH 7.5 containing 1.5 M magnesium sulfate), which improves the ease of adsorption of the enzyme on the support,[13–20] the HIS-tagged BAL was incubated with the epoxy-M-support in order to achieve immobilization of the enzyme. Different amounts of pure BAL in the coupling solution were incubated with the resin under the specified conditions. The samples of the supernatant were taken at certain time intervals and the maximum enzyme amount immobilized onto the epoxy-M-support was determined from the amount of unbound enzyme using Bradford's method.[51] Since the enzyme-epoxy-M-support system was highly responsive to the magnetic field (Fig. 4), samples were withdrawn after the slurry of the reaction was clarified by the application of an external magnet.

Figure 4. Response of the BAL-epoxy-M-support biocatalyst to the magnetic field.

The immobilized enzyme was separated each time by the magnet; we used a desorption procedure for removing the physically adsorbed BAL. The effect of the initial BAL concentration on the amount of the enzyme attached onto the epoxy-M-support is shown in Figure 5A. No increase in the amount of immobilized enzyme was observed after 5 h. The amount of immobilized enzyme proportionally increased with an increase of the initial

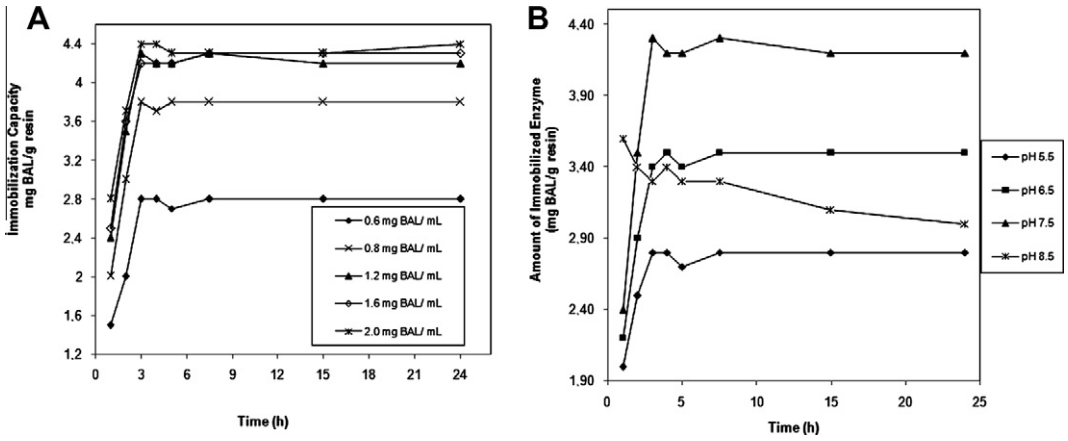


Figure 5. Effects in the immobilization media (50 mM of phosphate buffer pH 7.5 containing 1.5 M magnesium sulfate) on the amount of BAL attached to the epoxy-M-support (A) initial BAL concentration (B) environmental pH.

enzyme concentration in the bulk solution from 0.6 to 2.0 mg/mL. The maximum amount of immobilized enzyme per gram of support was found to be 4.3 mg.

2.2.2. Effect of the environmental pH

Since BAL can work within the pH range of 5–9, the pH values 5.5, 6.5, 7.5, and 8.5 were employed in order to determine the best pH value for the loading of epoxy-M-support and the stability of the BAL-epoxy-M-support system. The pH of the immobilization medium was altered between 5.5 and 8.5, while other conditions of the medium were fixed as 2.0 mg BAL/mL, 50 mM phosphate buffer, containing 1.5 M magnesium sulfate at 30 °C. The effect of the environmental pH on the covalently immobilized BAL is shown in Figure 5B. The results show that the enzyme loading on the support was influenced by the environmental pH.[26] A slight increase of the enzyme loading was observed from pH 5.5–7.5 and the best result was found at pH 7.5. A slight decrease of the binding capacity was observed at pH 8.5 while we expected to see an increase of the binding capacity. The reason for this could be decomposition of the silica coating material.

2.2.3. Effect of the ionic strength

In order to examine the effect of the ionic strength on the immobilization efficiency, experiments were performed using a variety of $MgSO_4$ concentrations. As observed in Figure 6, the enzyme loading onto the epoxy-M-support increased as the ionic strength increased, and the maximum amount of immobilized enzyme per gram of support was found to be 4.4 mg at an ionic strength of 1.75 M $MgSO_4$. This finding indicated that the amount of the BAL immobilized onto the epoxy-M-support clearly depends on the ionic strength of the buffer used for the immobilization procedure.[33]

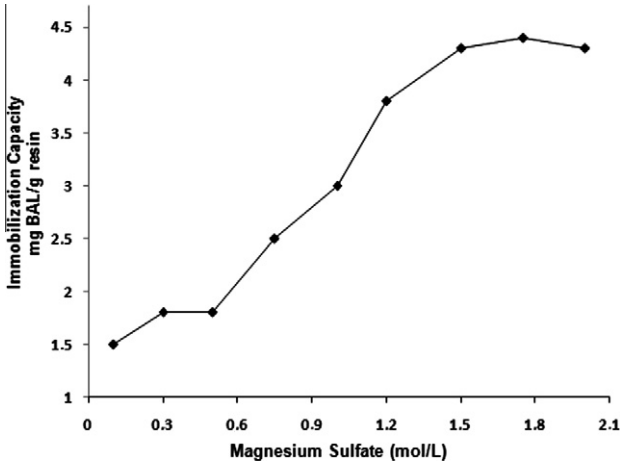


Figure 6. Effect of ionic strength on the amount of immobilized BAL.

Covalent immobilization of the enzymes onto the epoxy supports was believed to follow a two-step binding mechanism (Fig. 7A and B), which involved first an adsorption of the enzymes onto the support surface, and then a chemical reaction between the epoxy groups of the supports and the nucleophilic groups of the enzyme forming covalent bonds.[25,26,33] Hydrophobic interactions were the main driving force for the adsorption of the enzyme from the bulk aqueous phase to the support. Therefore, a high ionic strength should be favorable to the access of BAL to the reactive sites of the epoxy-M-support and, hence, benefits for the covalent binding of the enzyme onto the support.

The results (see Figs. 5 and 6) revealed that the immobilization conditions had a decisive influence on the immobilization efficiency of the BAL onto the epoxy-M-support and the maximum BAL amount covalently immobilized was determined as 4.4 mg per gram of epoxy-M-support. The optimal conditions for the enzyme immobilization were found to be 100 mg epoxy-M-support, 2.0 mg/mL initial BAL concentration, 1.75 mol/L $MgSO_4$ in 50 mM phosphate buffer, and 3.5 h incubation time at pH 7.5.

2.3. Benzoin condensation reaction

The benzoin condensation reaction of immobilized BAL was conducted in a batch synthesis (Fig. 8). A benzoin condensation reaction was performed with different benzaldehyde concentrations (Fig. 9) which were determined by measuring the benzoin concentration by HPLC.[41–43] Benzaldehyde amounts were chosen as 40 mM, 60 mM, and 80 mM, and the enzyme activities were determined as 3.44 U, 5.3 U, and 7.5 U for the BAL-epoxy-M-support system. Although the use of 100 mM of benzaldehyde was reported as being inhibitory,[43] the best enzyme activity was determined as 10 U when 100 mM of benzaldehyde was used in our support system. When 200 mM and 300 mM of benzaldehyde were applied, we observed that the enzyme activities decreased to 1 U and 0.7 U due to the enzyme inactivation and inhibition caused by the reaction product. A similar observation was also reported by Drager et al.[43] with benzaldehyde concentration during the BAL immobilized batch reaction; this phenomenon was also explained by product inhibition of the ligase reaction. The enzyme activities determined were higher, even when using 40 mM of benzaldehyde,

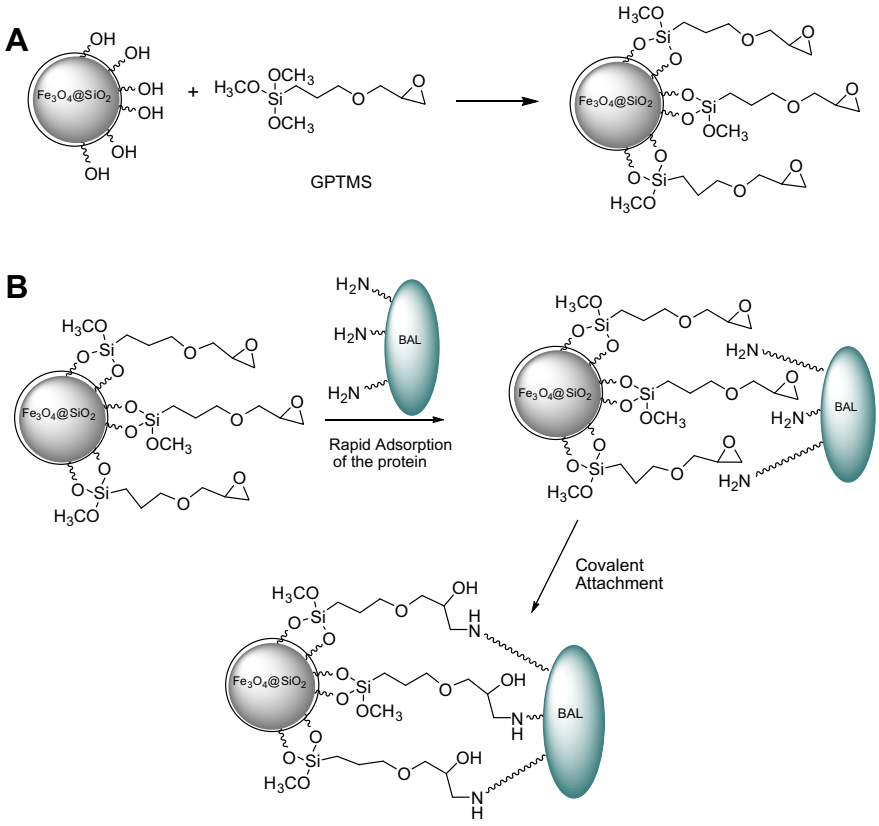


Figure 7. Schematic illustration of the preparation steps for (A) epoxy-M-support (B) adsorption and covalent attachment of BAL to epoxy-M-support.

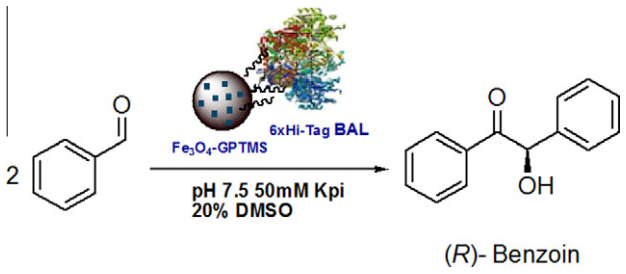


Figure 8. Covalently immobilized BAL catalyzed benzoin condensation reaction.

compared to the literature values due to the increased stability of the immobilized enzyme on the epoxy-M-support through covalent attachment and using DMSO.[42] The free enzyme specific activity was determined as 3.4 U according to the benzoin condensation reaction in 50 mM of potassium phosphate buffer at pH 7.5 (0.15 mM TPP, 2.5 mM $MgSO_4$, 20% DMSO, 80 mM benzaldehyde).

In addition to the improved enzyme activities, the immobilized enzyme systems also have the advantage of the reusability of the recovered enzyme (Fig. 10), which is the result of the improved stability of the enzyme on the surface. This stability can be achieved through covalent interactions between the enzyme and the support. Although the enzymes can be recovered when immobilization was affinity supported, an enzyme-support system gives limited reusability and, after the 4th trial, a decrease in the enzyme

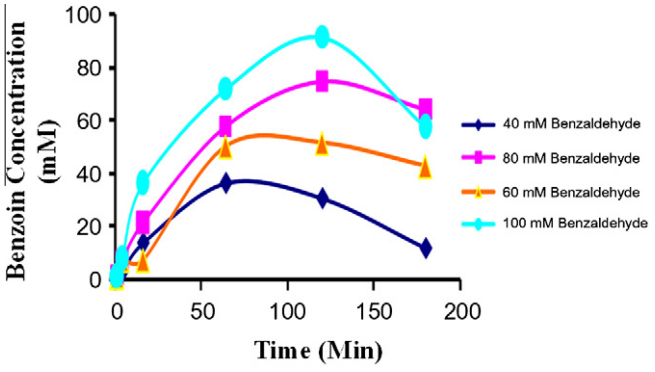


Figure 9. Time course of the change in the benzoin concentration during the reaction of immobilized BAL at different benzaldehyde concentrations (50 mM phosphate buffer; 2,5 mM Mg^{2+}, 0.1 mM ThDP, 30 °C, 20% DMSO).

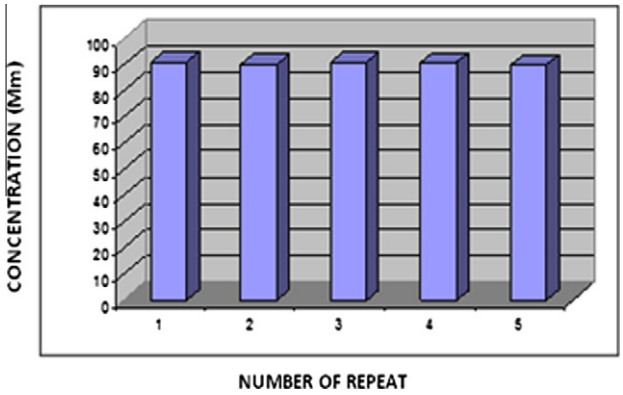


Figure 10. Concentration of benzoin after a repeated set of experiments with immobilized BAL.

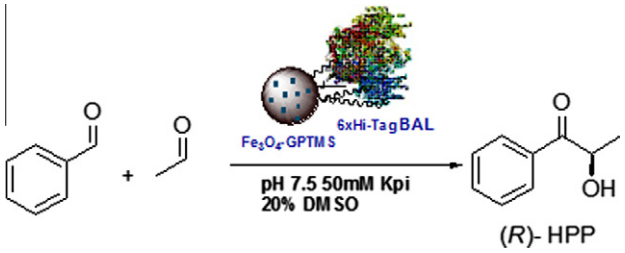


Figure 11. Synthesis of 2-hydroxypropiophenone (HPP).

activities was reported.[41–43] When the BAL enzyme was immobilized onto the epoxy-M-support through covalent interactions, a decrease in the activity was not determined even after the 5th trial, which brings about increased conversions and selectivities during carboligation reactions. As a result, the recovery of the enzyme was compensated with increased reusability through covalent interactions.

2.4. Synthesis of 2-hydroxypropiophenone (HPP)

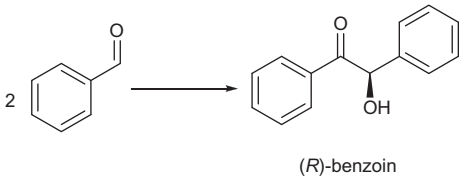
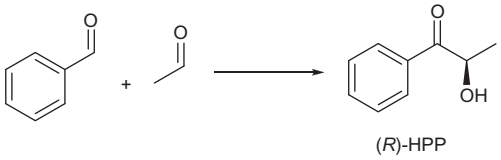
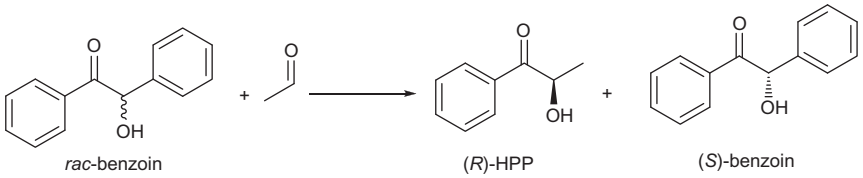
The lyase activity of the BAL enzyme was tested for the synthesis of HPP (Fig. 11), which is an important starting material for the synthesis of cytoxazone, the side chain of taxol and 5-methoxyhydrocarpin, which has multidrug pump inhibitor activity.[26,52] This cross condensation reaction between benzaldehyde and acetaldehyde was carried out with both BAL-epoxy-M-support and free enzyme systems (see Table 1). For the BAL-epoxy-M-support system, the same amount of enzyme was loaded onto the support and the maximum conversion was achieved. The progress of the reaction was monitored by TLC and HPLC analyses.

2.5. Kinetic resolution of *rac*-benzoin

In our previous publications,[5] we showed that BAL is also able to accept (*R*)-benzoin as a substrate to catalyze the C–C bond cleavage followed by carboligation in the presence of acetaldehyde. Accordingly, (*R*)-benzoin was reacted with BAL in the presence of acetaldehyde and the reaction was monitored by HPLC equipped with a chiral column. The addition of the corresponding acetaldehyde resulted in the formation of HPP in high ee and yields (see Table 1). Repeating this reaction with *rac*-benzoin afforded HPP and (*S*)-benzoin in an almost enantiomerically pure form (98% ee) after the separation of the products by column chromatography via C–C bond cleavage followed by C–C bond formation (Fig. 12 and Table 1).

Herein we have performed the carboligation reactions with free and immobilized enzymes. The reactions were carried out until the

Table 1
Yields and enantiomeric excess (ee) values for the immobilized BAL catalyzed reactions

Reaction	Reaction mode	Yield (%)	ee%	Recovery [(*S*)-benzoin]	
				Yield (%)	ee%
 (*R*)-benzoin	Free	89	>99		
	Immobilized	85	>96		
 (*R*)-HPP	Free	94	>98		
	Immobilized	90	>96		
 *rac*-benzoin (*R*)-HPP (*S*)-benzoin	Free	47	>99	46	>98
	Immobilized	44	>94	43	>98

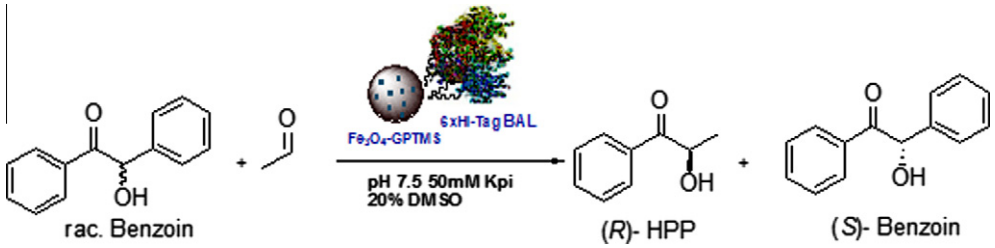


Figure 12. Kinetic resolution of *rac*-benzoin.

desired conversions were achieved. These representative reactions were screened with a different amount of immobilized enzyme for obtaining the maximum amount of product formation (the reaction was monitored by HPLC, see Table 1). We used 10 U of enzyme immobilized on 250 mg of epoxy-M-support. The reaction was repeated with free enzyme upon obtaining the same results. The reactions performed with free enzymes were started with 20 U of enzyme purified as described in previous work[41] and freshly applied for the reactions. In order to obtain the same yield with the immobilized system, the addition of fresh enzyme was necessary as reported earlier.[2,52]

3. Conclusion

Herein, BAL, a multimeric enzyme, was immobilized on the magnetically responsive epoxy-M-support through covalent interactions with the involvement of its subunits that increases the rigidity of the protein, thus promoting further enzyme stabilization and preventing dissociation.

A three-step immobilization/stabilization procedure was applied: (1) the BAL was hydrophobically adsorbed on the supports at high ionic strength (1.5 M magnesium sulfate). (2) There was an 'intermolecular' covalent reaction between the adsorbed BAL and the support. (3) The hydrophobic surface of the epoxy-M-support was hydrophylized by reaction of the remaining groups with 3 M glycine solution in order to reduce unfavorable enzyme-support hydrophobic interactions.

This strategy produced an enhanced enzyme stability and resulted in an increase in the enzyme activity to the highest value, which has not been previously reported for this class of enzymes. This system was used for the representative BAL catalyzed enantioselective homo and cross coupling of aldehydes via acyloin reactions via C–C-bond formation and bond cleavage in high chemical yield and enantiomeric excess ($\geqslant$96% ee, 90% conversion). This reaction can be used for the synthesis of key synthons for pharmaceutically important compounds. The covalent immobilization offers high enzyme activity and stability (at least 5 repeats without losing its activity). These results are a good demonstration of the fact that the covalent attachment of the enzyme has been achieved.

4. Experimental

4.1. Materials

All of the chemicals that were used in the immobilization studies were purchased from Sigma–Aldrich including 3-glycidoxypropyltrimethoxysilane (GPTMS), ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), ammonium hydroxide (25% [w/w]), tetraethyl *ortho* silicate (TEOS), and 2-propanol. The *Escherichia coli* BL21 (DE3) pLysS strain that was used to produce the recombinant BAL was purchased from Invitrogen®. For the carbo-ligation reactions, the substrates benzaldehyde, acetaldehyde, and racemic benzoin were obtained from Sigma. All of the other materials were of analytical grade and commercially available.

4.2. Characterization

Silica coated magnetic nanoparticles were redispersed in pure water by sonication for 10 s. The particles were redispersed 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 detected by transmission electron microscopy (TEM) (JEOL 2100 F, Japan) for particle size and morphology. Fourier transformed infrared (FT-IR) spectra were measured on a Thermo Scientific Nicolet IS10 FT-IR spectrometer (USA). Sixteen scans were collected at a resolution of 4 cm^{-1}. The crystal structure of the films was determined by X-ray diffraction using Hanawalt's method. A Rigaku Ultima X-ray diffractometer/PW 3710 equipped with Cu Kα radiation was used as a diffractometer.

The BAL-catalyzed reactions were monitored by thin layer chromatography (TLC) on silica gel (E. Merck, Darmstad). The detection of spots was performed by both UV-absorption and phosphomolybdic acid (PMA). The products synthesized were identified by ^{1}H NMR, and the ^{13}C NMR spectra were recorded by BRUKER DPX 400 MHz by using tetramethylsilane (TMS) as an internal standard and deutero-chloroform as a solvent. The reactions were followed by HPLC analysis (Agilent 1100 series).

4.3. Synthesis of epoxy-functionalized Fe_3O_4–SiO_2 core–shell magnetic nanoparticles

Magnetite nanoparticles were prepared by the chemical coprecipitation method.[28] A complete precipitation of Fe_3O_4 was achieved under alkaline conditions, while maintaining a molar ratio of Fe^{2+}:Fe^{3+} = 1:2 under a nitrogen gas environment to prevent critical oxidation. In order to obtain 1 g of Fe_3O_4 nanoparticles, 0.86 g of $FeCl_2{\cdot}4H_2O$ and 2.36 g of $FeCl_3{\cdot}6H_2O$ were dissolved under a N_2 atmosphere in 40 mL of deaerated deionized water with vigorous stirring (1000 rpm). While the solution was being heated to 80 °C, 5 mL of ammonium hydroxide was added. After 30 min, the resulting magnetite nanoparticles were obtained by putting the vessel on an Nd–Fe–B permanent magnet and the supernatant was decanted. The nanoparticles were washed with deionized water six times (50 mL each time) to remove the unreacted chemicals.

The Fe_3O_4 nanoparticles were coated with silica using the sol–gel method.[28] Typically, 30 mg of superparamagnetic Fe_3O_4 nanoparticles were dispersed in 80 mL of 2-propanol and 6 mL of deionized water by sonication for approximately 10 min. Then, under continuous mechanical stirring, 7 mL of ammonium hydroxide and 1 mL of TEOS were added consecutively to the reaction mixture. The reaction was allowed to proceed at room temperature for 12 h under continuous stirring. The resultant product was obtained by magnetic separation with the help of the permanent magnet and was thoroughly washed with deionized water six times (50 mL each time). The Fe_3O_4–SiO_2 nanoparticles were separated by magnetic separation.

One gram of wet Fe_3O_4–SiO_2 nanoparticles was reacted with 10 mL of 5% GPTMS in toluene at room temperature overnight. After the coupling reaction, the modified magnetic nanoparticles were removed from the solution with the help of the permanent magnet and rinsed thoroughly with toluene and ethanol to remove the physically adsorbed silane. Finally, they were freeze-dried. When prepared in this way, the surfaces of the magnetic nanoparticles had exposed active epoxy groups that could react readily with the amino groups of proteins.

4.4. Preparation of recombinant BAL

BAL was grown through culturing the *E. coli* BL21(DE3)pLyS strain containing pUC19-BAL by using 1.5 L of the Luria Broth (LB) medium that contained the necessary antibiotics (35 μL/mL Chloramphenicol and 100 μL/mL Amphicilin) in a 2 L fermentor (New Brunswick BioFlo110) at 37 °C. The BAL induction was initiated by the addition of isopropyl-β-D-thiogalactopyronoside (IPTG). Then, 6 h after the induction, the cells were harvested via centrifugation at 4 °C. The broken cells were lyophilized for 36 h.

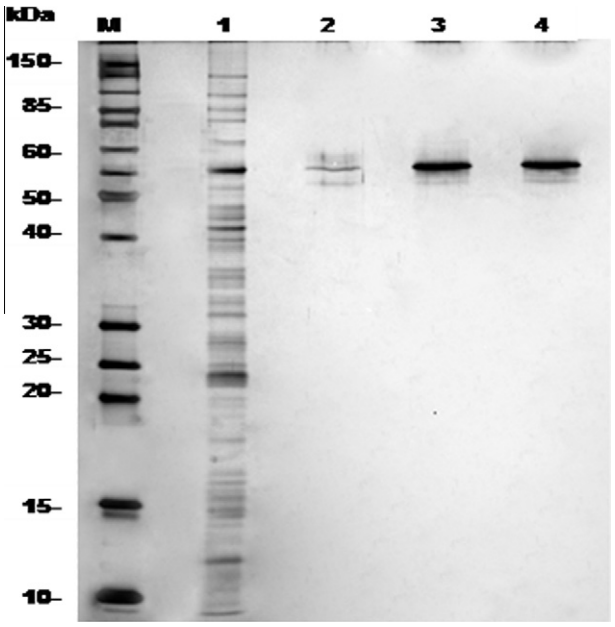


Figure 13. SDS–PAGE analysis of the purified 6Xhis tagged BAL. M, SDS–PAGE molecular weight; lane 1, crude extract of *E. coli* BL21(DE3)pLysS containing BAL_{HIS}; lanes 2–4, protein eluted from 100, 700, 1500 μL crude extract loaded resin (50 μL sample from eluents).

4.4.1. Activity assays

One unit activity of BAL is defined as the amount of enzyme necessary to catalyze the formation of 1 μmol of benzoin per minute under the standard conditions (30 °C, pH: 7.5). The BAL catalyzed benzoin condensation reactions were analyzed with HPLC equipped with the appropriate chiral column.

4.5. Purification of BAL

BAL was purified as described in previous work[41] and the purity was checked by SDS page as shown in Figure 13 and used for further covalent immobilization.

4.6. Immobilization

The epoxy-functionalized Fe_3O_4–SiO_2 core–shell magnetic nanoparticles (epoxy-M-support) were washed in order to activate with a 5 mL coupling solution (50 mM phosphate buffer pH 7.5 containing 1.5 M magnesium sulfate) at 30 °C for 1 h. The epoxy-M-support was settled with the help of a magnet and the supernatant was withdrawn.

Next, 100 mg of the epoxy-M-support was incubated with 10 mL of 2.0 mg/mL BAL into the coupling solution (50 mM phosphate buffer pH 7.5 containing 1.5 M magnesium sulfate) at 30 °C with gentle shaking (160 rpm) for 24 h. Periodically, 0.1 mL aliquots of supernatant were withdrawn for assay in order to determine the amount of immobilized enzyme. The amount of immobilized enzyme was calculated by the difference between the amount of enzyme in the supernatant before and after the immobilization procedure. The enzyme content was determined by the Bradford method using bovine serum albumin as a standard protein.[51]

In order to observe the factors affecting immobilization efficiency, BAL was immobilized under different experimental conditions (initial enzyme concentration in the range of 0.6–2.0 mg BAL/mL, pH values of the immobilization medium varying from 5.5 to 8.5, ionic strength from 0.1 to 2.0 M, and the duration of immobilization (from 30 min to 24 h). The results reported were the average values of three separate experiments.

In order to determine the physically adsorbed BAL, immobilized BAL on epoxy-M-support (50 mg) was incubated for 30 min in 3 mL of 50 mM phosphate buffer pH 7.5, at 37 °C. It was then separated by a magnetic separation device and washed three times with 5 mL portions of 50 mM phosphate buffer pH 7.5. Then recovered immobilized BAL was incubated for 30 min in 3 mL of 1% Triton X-100 solution at 37 °C and washed three times with 5 mL portions of 1% Triton X-100 solution. Enzyme desorbed from the resin with 50 mM phosphate buffer pH 7.5 and Triton X-100% solutions was measured by the Bradford method using bovine serum albumin as a standard protein.[51] The amount of protein bound to the support was determined spectrophotometrically by the difference between the amount of protein added and the amount of protein in the supernatant and the washes.

After the covalent enzyme immobilization, the remaining epoxide groups on the support were blocked with 5 mL of a glycine solution (3 M in 10 mM phosphate buffer pH 7.5) for 12 h at 20 °C to prevent further non-specific reactions and then, the epoxy-M-support attached to BAL was separated from the medium by a magnetic separation device, and washed three times with 50 mM of phosphate buffer pH 7.5 stored at 4 °C.

4.7. Representative reactions with covalently immobilized BAL

The reactions with the free enzyme were carried out according to the procedure described in the literature.[6]

4.7.1. Synthesis of (*R*)-2-hydroxy-1,2-diphenylethan-1-one[5]

BAL was immobilized to epoxy-M-support by following the procedure described above. Then, the 250 mg BAL-epoxy-M-support system was equilibrated with 5 mL of reaction buffer [0.15 mM thiamine pyrophosphate (TPP), 2.5 mM $MgSO_4$, 50 mM pH 7.5 phosphate buffer, 20% DMSO] twice and the supernatant was removed. The reaction was initiated with the addition of 100 mM of benzaldehyde and incubated under the appropriate conditions (120 rpm, 37 °C) overnight. The reaction was monitored with thin layer chromatography (TLC). After 40 min (checked by TLC), the reaction mixture was extracted with ethyl acetate (3 × 30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to give the desired compound (90% conversion, >96% ee). $[\alpha]_D^{22} = -112$ (*c* 1.5, CH_3COCH_3). HPLC (Chiralpak AD) R_t (*R*): 27.1 min, R_t (*S*): 34.5 min. δ_H (400 MHz; $CDCl_3/CCl_4$; Me_4Si) 7.8 (2H, d, *J* = 7.8 Hz, Ph), 7.4 (1H, t, *J* = 7.5 Hz, Ph), 7.3 (2H, t, *J* = 7.6 Hz, Ph), 7.1–7.2 (5H, m, Ph), 5.7 (1H, d, *J* = 5.9 Hz, CH), 4.42 (1H, *J* = 5.9 Hz, OH). δ_C (100 MHz; $CDCl_3/CCl_4$; Me_4Si) 198.7, 139.1, 133.8, 133.6, 129.1, 129.0, 128.6, 128.5, 127.7, 76.2.

4.7.2. Synthesis of (*R*)-2-hydroxy-1-phenyl propanone[5]

At first, 250 mg of BAL-epoxy-M-support was equilibrated with 5 mL of reaction buffer (0.15 mM TPP, 2.5 mM of $MgSO_4$, and 50 mM of pH 7.5 phosphate buffer, 20% DMSO) twice. The reaction was initiated by the addition of 100 mM of benzaldehyde and 500 mM of acetaldehyde. The reaction was monitored by TLC (26 h) and the mixture was extracted with chloroform (3 × 30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to give the desired compound (90% conversion, 96% ee). $[\alpha]_D^{22} = +83.5$ (*c* 2.0, $CHCl_3$). The enantiomeric excess of the product was determined with a chiral AD column (90:10/hexane:isopropanol, 1 mL/min, 254 nm, retention time 12.54 for (*R*)-2-hydroxy-1-phenyl propanone. δ_H (400 MHz; $CDCl_3/CCl_4$; Me_4Si) 7.9 (2H, dd, *J* = 1.4, 8.2 Hz, 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) 202.7, 134.4, 134.0, 128.9, 128.7, 69.2, 22.0.

4.7.3. Kinetic resolution of racemic benzoin: synthesis of (*R*)-2-hydroxy-1-phenylpropanone [(*R*)-2 HPP] and (*S*)-benzoin from racemic benzoin and acetaldehyde[5]

At first, 0.3 mmol *rac*-benzoin and 20 mmol of acetaldehyde were added to 5 mL of the reaction medium, which was prepared for the 250 mg BAL-epoxy-M-support. The reaction was performed at 30 °C and 4 mM of acetaldehyde was added at the 30 and 120 min marks. The reaction was monitored by TLC (32 h). The mixture was extracted three times with chloroform and separated after which the products were analyzed by NMR. The determination of the enantiomeric excess of benzoin was performed by HPLC analysis [Chiralpak AD, 90:10 hexane: isopropanol, 1 mL min^{-1}, 254 nm, retention time for (*R*)-2-hydroxy-1-phenylpropanone: 12.45 min, 38% conversion, 94% ee; for (*S*)-benzoin: 33.35 min, 45% conversion, 98% ee].

4.8. Stability and reusability of the immobilized BAL

In order to assess the stability of the covalently immobilized BAL and their reusability for carboligation processes, the benzoin condensation reaction was carried out for 5 consecutive batches with the same immobilized enzyme. Briefly, 100 mg of immobilized BAL was incubated with 5 mL of 60 mM of benzaldehyde solution at 30 °C and pH 7.5, (0.15 mM TPP, 2.5 mM $MgSO_4$, 50 mM phosphate buffer, 20% DMSO). For each period, the immobilized enzyme was separated with the help of a magnet, washed twice with potassium phosphate buffer at pH 7.5, and incubated with fresh substrate for the next batch reaction. The residual activity of the immobilized biocatalyst was determined via determination of the benzoin concentration by HPLC after each batch of the reaction.

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