Functionalized Poplar Powder as a Support Material for Immobilization of Enoate Reductase and a Cofactor Regeneration System^S

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In this study, functionalized poplar powder (FPP) was used as a support material for the immobilization of enoate reductase (ER) and glucose-6-phosphate dehydrogenase (GDH) by covalent binding. Under optimal conditions, the immobilization efficiency of ER-FPP and GDH-FPP was 95.1% and 84.7%, and the activity recovery of ER and GDH was 47.5% and 37.8%, respectively. Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) analysis indicated that FPP was a suitable carrier for enzyme immobilization. ER-FPP and GDH-FPP exhibit excellent thermal stabilities and superior reusability. Especially, ER-FPP and GDH-FPP enable the continuous conversion of 4-(4-Methoxyphenyl)-3-buten-2-one with NAD⁺ recycling. While the immobilization strategies established here were simple and inexpensive, they exploited a new method for the immobilization and application of ER and its cofactor recycling system.

Keywords: Enoate reductase, functionalized poplar powder, covalent immobilization, cofactor regeneration system

Introduction

Enoate reductases (ERs), from the 'Old Yellow Enzyme' family of flavor proteins, are widely distributed in microorganisms—particularly in bacteria and lower fungi—and in plants [1, 2]. They are able to reduce a wide range of activated alkenes, such as α , β -unsaturated aldehydes, ketones, carboxylic acids and derivatives (esters, lactones, cyclic imides, and cyclic acid anhydrides), and α , β -unsaturated nitroalkene, employing nicotinamide [NAD(P)H] as a cofactor, and providing products with a variety of biotechnological and pharmaceutical applications [3–6]. Despite the remarkable synthetic potential of ERs, the wide spread use of purified ERs in bio-catalysis is limited due to the high cost of the cofactors [7]. So, it is necessary to establish cofactor regeneration system for preparative applications of ER.

Natural or engineered whole-cell systems can allow in situ cofactor recycling, and provide a natural environment for enzyme [8]. However, bioconversions using whole cells have some drawbacks such as substrate or product toxicity

to the cells, poor permeability of substrate or product, formation of by-product, and difficult isolation of products following catalysis [9]. The immobilization of a catalytic enzyme and a cofactor-regenerating enzyme can provide an enhanced stability of the enzymes, simplify product isolation, and reduce the protein contamination from substrate or product [10, 11]. The improved enzyme performance can also result in lower enzyme costs and increase their potential for use as industrial catalysts [12]. Herein, covalent binding is employed for the immobilization of ER and its cofactor-recycling partner enzyme GDH, where the enzymes can remain stable while bound on carriers due to a strong binding force between them [13].

Nowadays, an array of available materials such as agarose [14, 15], chitosan [16, 17], copolymer carriers [18, 19], glass beads [20], magnetic poly (ionic liquids) [21], and acrylic and styrenic resins [22, 23] are being used as insoluble carriers to provide the most straightforward route for covalent immobilization of enzymes. Nevertheless, from an industrial perspective, the cost of preparing immobilized enzymes needs to be considered even if we can establish an

efficient immobilization strategy to obtain immobilized enzymes with excellent stabilities and reusability [10, 12]. Commodity chemical materials generally have very low added value, leaving very little space for the costs of immobilized enzymes [13]. Therefore, cheap immobilization carriers and protocols together with high reusability are the key factors that will determine whether the preparation of immobilized enzyme is successful, or whether it can be effectively used in industrial fields. On a global scale, lignocellulose is one of the most abundant substances in nature [24]. Agricultural residues such as cornstalk, pasture, wheat straw, rice husk, and poplar powder (PP) are common renewable lignocellulose resources and their low cost [25] makes them highly suitable as enzyme immobilization materials when compared with the commodity carriers.

In the present study, we explore the potential of PP as a carrier for the covalent immobilization of ER and GDH. We pretreated PP to expose the cellulose, and then the cellulose components were functionalized for covalent anchoring of the enzymes. Characterization of untreated PP, functionalized PP (FPP), and immobilized ER-FPP were done by SEM and EDS. Furthermore, the thermal stabilities of ER-FPP and GDH-FPP, the continuous catalytic reduction of 4-(4-Methoxyphenyl)-3-buten-2-one, and the reusability of ER-FPP and GDH-FPP were investigated. The aim of this study is to construct simple and cost-effective immobilization systems for ER and GDH.

Materials and Methods

Materials

Bovine serum albumin (BSA), NADH, and NAD⁺ were purchased from Roche (China). Glucose, ammonium sulfate, lactic acid, formic acid, acetic acid, choline chloride, sodium hydroxide, dimethyl sulfoxide, epichlorohydrin, sodium thiosulfate, ethanediamine, glutaraldehyde, and sodium metaperiodate were purchased from Sinopharm Chemical Reagent Co., Ltd (China). Dextran (MW 70,000) was purchased from Energy Chemical (Shanghai, China). Polyethyleneimine solution (PEI, MW~1,300) was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd (China). One hundred meshes of cornstalk, pasture, wheat straw, rice husk, and poplar powder were purchased from XingYi Deep Processing Factory (China). 2-Cyclohexen-1-one was purchased from Chengdu Best Chemical Reagent Co., Ltd (China). The substrate 4-(4-Methoxyphenyl)-3-buten-2-one and the product 4-(4-Methoxyphenyl)-butan-2-one were synthesized and purified in our laboratory [26]. ER and GDH were constructed and expressed in our laboratory [26]. The deep eutectic solvents (DESs) were prepared in our laboratory [27]. The other chemicals used in this study were of analytical grade.

Pretreatment of Lignocellulosic Biomass

In this study, lignocellulosic biomass consisting of cornstalk, pasture, wheat straw, rice husk, and poplar powder (PP) was selected for use as support materials for enzyme immobilization. The biomass (1.0 g) was added in a vial with DES (10.0 g). The DESs were utilized in 1:1 mole ratios of choline chloride: lactic acid (ChCl: LA), choline chloride: formic acid (ChCl: FA), and choline chloride: acetic acid (ChCl: AA), respectively. The vials were placed into a water bath set at 80°C for 2 h. After that, the samples were filtered to separate the pre-treated biomass from the DES. The isolated biomass was then put into another vial with 15 ml of deionized water, and shaken at 50°C for 20 min, to remove the residual DES. The water-washing process was repeated 3 times. The pretreated biomass was next placed in a drying oven set at 85°C for 24 h prior to weighing, and was later evaluated by measuring the activation degree.

Activation Degree Assay for Pretreated Biomass

The pretreated biomass (100 mg) was mixed with sodium hydroxide solution (0.4 M, 2.0 ml), dimethyl sulfoxide (2.0 ml) and epoxy chloropropane (2.0 ml). After the mixture was stirred for 6 h at room temperature, the biomass was washed with acetone and dried. Next, the biomass (50 mg of dry weight) was mixed in sodium thiosulfate solution (1.4 M, 6.0 ml) with phenolphthalein indicator (5.0 g/l, 30 µl). After 2 h, dilute hydrochloric acid was used to titrate the reaction solution. The activation degree (A) of the pretreated biomass was calculated with the following equations:

$$A = \frac{cv}{m}$$

m: pre-treated biomass amount (mg); *c*: molar concentration of hydrochloric acid (µM); *v*: volume of hydrochloric acid consumed in titration (µl).

Functionalization of Pretreated PP

The pretreated PP (1.0 g of dry weight) and sodium periodate (NaIO₄, 2.0 g) were mixed in deionized water (50 ml), and the oxidation was carried out in darkness at 25°C for 22 h. The oxidized PP (OPP) was washed 3 times with alcohol to remove the unreacted NaIO₄. Then OPP was added in aqueous solution of PEI (8 mM, 50 ml) or ethanediamine (150 mM, 50 ml) to perform the amination reaction at 25°C for 3 h. After the aminated OPP was washed 2 times with 50 ml of deionized water, it was further added in deionized water containing glutaraldehyde (50 mM, 50 ml) or oxidized dextran (40 µM, 50 ml), and the reactions took place at 25°C for 1 h. The functionalized PP (FPP) was washed 2 times with 50 mM of Tris-HCl (pH 7.5, 50 ml), and used in the subsequent experiments.

Immobilization of ER and GDH with FPP as a Support Material

ER (10 U) or GDH (20 U) was mixed with FPP (1.0 g) in Tris-HCl buffer (50 mM, pH 7.5, 6.0 ml). The mixtures were kept at 4°C for 3 h with shaking. Then the immobilized ER and GDH were collected by centrifugation (10,614 ×g, 5 min, 4°C), and washed 3

times with Tris-HCl buffer (50 mM, pH 7.5). Parameters such as enzyme additive amount (10–60 mg), pH of Tris-HCl buffer (5.5–9.5), and immobilization time (1–4 h), were optimized during the immobilization process. The assay and calculation of protein amounts and enzymatic activities of ER and GDH immobilized on the functionalized biomass, and the immobilization efficiency (%) and activity recovery (%) of ER-FPP and GDH-FPP, were based on our previous paper [26].

Morphological Characterization of Untreated PP, FPP and ER-FPP

The morphology of untreated PP, FPP and ER-FPP was characterized with a scanning electron microscope (JEOL JSM6700F, Japan) in a field-emission FEI Inspect F operated at 3 kV. Samples were suspended in ethanol for ultrasonic dispersion, dried, and fixed onto silicon wafers. The samples were then immediately sputter-coated with platinum before observation.

The changes of elemental components on the surface of FPP and FPP-ER were analyzed by peripheral energy dispersive spectroscopy (EDS, JEOL JSM-7800F/ Oxford X-Max 80 SDD Detector).

Thermal Stabilities of ER-FPP and GDH-FPP

To study the thermal stabilities of free and immobilized ER and GDH, their residual enzymatic activities were measured after incubation in a Tris-HCl buffer (50 mM, pH 7.5) at 30°C and 50°C for a certain period of time. The maximum specific activity values of free and immobilized enzyme under initial conditions were regarded as 100%, and the ratio of the specific activity obtained under other conditions to the maximum specific activity is referred to as the relative activity.

Continuous Reduction of 4-(4-Methoxyphenyl)-3-Buten-2-One by ER-FPP and GDH-FPP

The substrate 4-(4-Methoxyphenyl)-3-buten-2-one (50 μ M), glucose (2.0 mM) and NAD⁺ (100 μ M) were added in Tris-HCl buffer (50 mM, pH 7.5, 2.0 ml) with ER-FPP (3.5 U) and GDH-FPP (7.0 U), and the resulting mixture was stirred at 30°C. After every 8 h, new substrate (50 μ M) and glucose (2.0 mM) were added in the reaction system, and this step was repeated six times. The reaction samples of each round were removed and injected into the high-performance liquid chromatography (HPLC) system for analysis. The reduction of 4-(4-Methoxyphenyl)-3-buten-2-one was monitored through HPLC (P230p, Dalian Elite Analytical Instruments Co., Ltd) using an Elite C18 column. The mobile phase was a mixture of methanol/deionized water (60/40, v/v) at a flow rate of 0.8 ml/min. UV detection at 215 nm was employed for quantification. The retention time of the product 4-(4-Methoxyphenyl)-butan-2-one was 10.2 min, which was confirmed based on its standard (purity >99.5%), and the conversions were confirmed by external standard method.

Reusability of the Mixture of ER-FPP and GDH-FPP

The reaction system (2.0 ml) consisted of ER-FPP (3.5 U), GDH-FPP (7.0 U), 4-(4-Methoxyphenyl)-3-buten-2-one (0.5 mM), glucose

(50 mM), and NAD⁺ (1.0 mM) in Tris-HCl buffer (50 mM, pH 7.5). Each cycle lasted for 3 h at 30°C, followed by separation of ER-FPP and GDH-FPP from the reaction system by centrifugation. After thorough washing with Tris-HCl buffer (50 mM, pH 7.5), the mixture of ER-FPP and GDH-FPP was re-suspended in a fresh reaction system, and repeatedly used for the next reaction cycle under the same reaction conditions. The relative ER activities of the ER-FPP and GDH-FPP mixture were calculated by regarding the enzyme activity of ER in the first cycle as 100%.

Results and Discussion

Pretreatment of Lignocellulosic Biomass

Herein, five lignocellulosic biomasses including cornstalk, pasture, wheat straw, rice husk and poplar powder, were selected as support materials for the immobilization of ER and GDH by covalent binding. The immobilization is mainly based on the exposed hydroxyl groups of cellulose on biomass. However, lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin, and cellulose is wrapped tightly by lignin, which makes it hard to use directly [28]. So, it is necessary to pretreat the biomass to separate lignin from cellulose sufficiently. DESs have been recently reported as having novel abilities to dissolve the lignin packaged in cellulose, thereby exposing the cellulose [29, 30].

DESs consist of an ammonium or phosphonium salt, such as choline chloride, and a hydrogen-bond donor (HBD). The properties of DESs can be easily changed by adjusting the type and molar ratios of the salt and HBD [30]. Here, choline chloride (ChCl), and a HBD including formic acid (FA), lactic acid (LA), acetic acid (AA) were used to prepare DESs, and the molar ratio of ChCl and HBD was firstly set at 1:1. The selected biomass could be pretreated by DESs to different extents. DESs could show high solubility for lignin, while cellulose is found to be immiscible, and the lignin solubility could show a clear trend with the increase of the molar ratio of ChCl: HBD. The observed phenomena were similar to those reported by other groups [30]. The contents of cellulose hydroxyl groups exposed on biomass could be evaluated by measuring the activation degree. As shown in Fig. 1A, in the presence of ChCl:LA (1:1), the activation degree for all of the biomasses was higher than that in ChCl:FA (1:1) and ChCl:AA (1:1). This finding was attributed to the possible variations in the network formed by hydrogen bonds, leading to different dissolved behaviors of the DESs [30]. The different activation degree was also observed in the same DES, and the activation degree of cornstalk, pasture, wheat straw, rice husk and PP pretreated by ChCl:LA (1:1) was 0.26, 0.32, 0.41, 0.45, and 0.51,

Fig. 1. Pretreatment of lignocellulosic biomass by DESs (A) and effects of ChCl: LA with different molar ratios on pretreatment of PP (B).

respectively. Because the activation degree of the biomasses was proportional to their respective content of cellulose hydroxyl groups, PP with high activation degree (0.51) indicated that its available hydroxyl group content was also high. So, PP was selected as a carrier for the subsequent immobilization of ER and GDH.

The effects of different molar ratios of ChCl: LA on PP pretreatment were then investigated. Fig. 1B shows that the activation degree of PP was improved with the increase of the molar ratios of ChCl: LA from 1:1 to 1:9, and a maximum activation degree of 0.77 was achieved at a molar ratio of 1:9 of ChCl: LA. It might be ascribed to the decreased viscosity of the DES with the increase of the amount of LA, which was beneficial to the mass transfer between the DES and PP, and it might be effective in disrupting inter- and intra-molecular hydrogen bonding in biomass [29]. A further increase in molar ratio to 1:11

slightly decreased the activation degree of PP. One possible explanation is that a small amount of cellulose might be dissolved simultaneously in the presence of a large amount of LA [30].

Functionalization of Pretreated PP and Covalent Immobilization of ER and GDH

After PP was pretreated, a large number of cellulose hydroxyl groups were exposed, however, the hydroxyl groups could not directly react with the amine groups on the enzymes to form covalent bonding. But cellulose hydroxyl groups can be used as an anchor point for functionalization. After such modification, the covalent binding between functionalized PP and enzyme can be significantly enhanced. Considered from the view of simple operation and effective cost, NaIO_4 was firstly used to oxidize the hydroxyl groups on cellulose, introducing

Table 1. Functionalization of pre-treated PP and covalent immobilization of ER and GDH.

Functional conditions of pre-treated PP	Enzymes (20 mg)	Immobilization efficiency (%)	Activity recovery (%)	
NaIO_4 -oxidized PP	ER	18.0	N.D.	
PEI (8 mM) + glutaraldehyde (50 mM)	ER	26.1	5.1	
PEI (8 mM) + oxidized dextran (40 μM)	ER	28.5	14.8	
Ethanediamine (150 mM) + oxidized dextran (40 μM)	ER	20.2	6.4	
Ethanediamine (150 mM) + glutaraldehyde (50 mM)	ER	92.5	31.3	
Ethanediamine (150 mM) + glutaraldehyde (50 mM)	GDH	74.5	26.3	
			ER	GDH
Ethanediamine (150 mM) + glutaraldehyde (50 mM)	ER+GDH	85.7	10.2	9.6

N.D.: not detectable.

Scheme 1. Scheme of functionalization of pretreated PP and covalent immobilization of ER and GDH.

aldehyde groups on PP, and then NaIO_4 -oxidized PP (OPP) was directly used to immobilize ER. The results in Table 1 show that OPP was weak in binding to ER, and the immobilization efficiency of ER-OPP was only 18.0%.

With the above results, we considered further introducing an amine spacer arm on OPP, which might be beneficial to the immobilization of enzymes. Ethylenediamine or PEI were firstly selected to react with the aldehyde groups on OPP, and then glutaraldehyde or oxidized dextran were used as a modifier to react with the amine groups, to finally obtain the functionalized PP (FPP) with an elongated spacer arm with the exposed aldehyde groups. Subsequently, the obtained FPP was used as a support material for the immobilization of ER and GDH. The functionalization of pretreated PP and the covalent immobilization of enzymes was listed in Scheme 1.

Table 1 shows that OPP was functionalized by a different combination of amine and aldehyde modifier, which can lead to different immobilization results. When PEI was selected as an amine modifier, whether glutaraldehyde or oxidized dextran was used, both the immobilization efficiency and activity recovery of ER-FPP were poor. For the combination of ethylenediamine and oxidized dextran, the immobilization results of ER-FPP were also not ideal. But it was found that ethylenediamine and glutaraldehyde were the excellent combination, and the immobilization efficiency and the activity recovery of ER-FPP were improved to 92.5% and 31.3%, respectively. Under the same immobilization conditions, an immobilization efficiency of

74.5% and activity recovery of 26.3% for GDH-FPP can also be obtained.

Because ER is a reductase, it is necessary to prepare co-immobilized ER and GDH, forming an in situ cofactor recycling system. In this case, the cofactor would be effectively recycled between ER and GDH. The available cofactor concentration on the carrier would be high, which could be easily used by ER in catalyzed reactions [31]. Based on this consideration, the co-immobilization of ER and GDH was carried out using FPP as a supporting material by covalent bonding. Unfortunately, both immobilization efficiency and activity recovery were much lower than that in single-immobilization of ER and GDH. So, ER-FPP and GDH-FPP were selected to be further investigated.

The concentration of modifiers had a significant effect on immobilization efficiency and activity recovery of ER-FPP and GDH-FPP. As shown in Fig. 2, the activity recovery of ER and GDH in ER-FPP or GDH-FPP was increased with an increase in the concentration of ethylenediamine (from 100 mM to 300 mM) and glutaraldehyde (from 25 mM to 100 mM). At ethylenediamine concentration at 300 mM and glutaraldehyde concentration at 100 mM, the excellent immobilization efficiency and activity recovery of ER-FPP were 95.5% and 44.1%, and that of GDH-FPP were 83.6% and 36.8%, respectively. With further increase in the concentration of ethylenediamine and glutaraldehyde to 500 mM and 200 mM, respectively, the activity of ER and GDH in ER-FPP and GDH-FPP was decreased, which might be due to the high level of functional groups of

Fig. 2. Effects of the concentration of ethylenediamine (A) and glutaraldehyde (B) on immobilization of ER and GDH: activity recovery of ER-FPP (■) and GDH-FPP (▒); immobilization efficiency of ER-FPP (△) and GDH-FPP (▽).

ethylenediamine and glutaraldehyde, which led to excessive crosslinking of PP themselves and could not effectively provide binding sites for enzymes [32–34]. But all in all, FPP with simple pretreatment and functionalization, can be used as an excellent carrier for the immobilization of ER and GDH.

Further Immobilization Optimization of ER-FPP and GDH-FPP

Given that the immobilization efficiency and activity recovery of the immobilized ER and GDH can be easily affected by the environment, the major variable parameters, such as pH of Tris-HCl buffer, enzyme amount, and immobilization time were carefully optimized, and the data were listed in supplementary material. In all the optimized parameters, the pH of Tris-HCl buffer was an especially important parameter, which is because the established immobilization strategies were the formation of covalent bonds between aldehyde groups on FPP and lysine residues on enzyme. So, the immobilization process is sensitive to the environmental pH, and the pH of Tris-HCl buffer had a certain influence on the formation of imine [35]. When the buffer pH was over 7.5, the immobilization efficiency and activity recovery of ER-FPP and GDH-FPP were all decreased. Especially at high pH of 9.5, the immobilization efficiency and activity recovery of ER-FPP were only 41.6% and 7.2% respectively, while that of GDH-FPP were only 41.9% and 2.6%, respectively (Fig. S1b). It might be because high alkaline pH could weakened the electrophilicity of the carbonyl groups in the aldehyde groups on FPP, which was unfavorable in reacting with the amine groups on protein surfaces,

resulting in the decrease of the reaction activity. Also, the decreased activities of enzyme itself at high pH of Tris-HCl buffer might in turn also have a close relationship with the poor immobilization results of ER-FPP and GDH-FPP.

The activity recovery of ER-FPP and GDH-FPP was also significantly influenced by enzyme amount and immobilization time (Figs. S1a and S1c, respectively). The influence trends of various parameters were similar to each other and the activity recovery of ER-FPP and GDH-FPP increased with ER and GDH amount from 10 mg to 30 mg, as did the immobilization time from 1 h to 3 h. Further increasing the values of all corresponding parameters decreased the activity of ER-FPP and GDH-FPP, which might be due to the aggregation of the immobilized ER and GDH, reducing the accessibility of enzyme particles to substrates. The maximum immobilization efficiency of 95.1% and 84.7%, and activity recovery of 47.5% and 37.8% for ER and GDH, respectively, was achieved at Tris-HCl buffer (50 mM, pH 7.5), ER amount (30 mg), GDH amount (30 mg), and immobilization time (3 h).

Morphological Characterization of Untreated PP, FPP and ER-FPP

SEM reveals that the morphologies of PP were varied with the changing processes of pretreatment, functionalization and immobilization. The untreated PP had a smooth surface, and the lignocellulosic structure was complete (Fig. 3A). For PP pretreated by DESs, the surface became loose and coarse, and displayed a ‘peeling’ appearance (Fig. 3B). Combined with experimental data, we think the lignin of PP can be effectively removed by DES. After the pretreated PP was functionalized by ethylenediamine and

Fig. 3. SEM images of untreated PP (A); pretreated PP (B); FPP (C); ER-FPP (D). EDS images of the full extent of FPP (E) and ER-FPP (F). Distribution of S element on the surface of FPP (G) and ER-FPP (H).

glutaraldehyde, a porous, crosslinked blocky structure with on the FPP surface was observed (Fig. 3C), which has a close relationship with the functionalized modification. When ER was covalently bound to FPP, the surface pore size of FPP became smaller (Fig. 3D).

To further confirm the successful binding of ER onto FPP, EDS was used to analyze and compare the sulfur (S) element distribution on the surface of FPP and ER-FPP. On the basis of the full extent of FPP (Fig. 3E) and ER-FPP (Fig. 3F), it was observed that large amounts of S elements were distributed on ER-FPP (Fig. 3H), and not on FPP (Fig. 3G). Because S elements are characteristic elements of protein, the results indicated that ER had been successfully immobilized on FPP.

Thermal Stabilities of ER-FPP and GDH-FPP

The thermal stabilities of free and immobilized ER and GDH were examined because of their importance in industrial applications. Free and immobilized ER and GDH were firstly incubated at a temperature of 30°C for 10 h, where 30°C was selected because the subsequently continuous conversions of 4-(4-Methoxyphenyl)-3-buten-2-one were carried out at 30°C. Figs. 4A and 4B shows that free ER and GDH had lost 30% and 50% of their initial activity after 10 h of heat treatment, respectively, whereas ER-FPP and GDH-FPP still maintained their nearly entire initial activity. In order to further confirm the immobilized enzymes could exhibit excellent thermal stabilities, a much high tempera-

ture of 50°C was subsequently used. After 8 h of heat treatment, ER-FPP and GDH-FPP kept 82.3% and 55.7% of their initial activity, respectively, whereas free ER had approximately only 9.2% of its initial activity, and free GDH had already lost all its activity after one hour (Figs. 4C and 4D). The enhanced thermal stabilities of immobilized ER and GDH have a close relationship with the immobilization, which can create a much more suitable microenvironment for enzyme, thus making the enzyme denaturation difficult.

Continuous Conversions of 4-(4-Methoxyphenyl)-3-Buten-2-One

To certify whether ER-FPP and GDH-FPP can effectively recycle the cofactor, the optimized immobilized enzymes were used to test the recycling system in continuous conversions of 4-(4-Methoxyphenyl)-3-buten-2-one to prepare 4-(4-Methoxyphenyl)-butan-2-one, which is an important fine chemical and is used extensively in several consumer products such as raspberry scent, food additives and insect attractants as well as in the preparation of melanin formation inhibitors [36]. 4-(4-Methoxyphenyl)-3-buten-2-one was supplied to the system in 50 μ M increments to a total of 350 μ M. NAD⁺ (100 μ M) was supplied to initiate the reaction. The coupling of ER and GDH activities was evidenced by the continuous formation of 4-(4-Methoxyphenyl)-butan-2-one for 56 h without further cofactor addition. The results in Fig. 5 show that the reaction conversion increased

Fig. 4. Thermal stabilities of free and immobilized ER and GDH: incubation at 30°C (A) and (B); incubation at 50°C (C) and (D).

steadily in the first five additions of substrate, and after the sixth addition of substrate, the conversion rate increased slowly. Despite this, the total reaction conversion could

still reach to 30.6%, and 107 μM of 4-(4-Methoxyphenyl)-butan-2-one could be finally obtained, which indicated that the immobilization strategy established here can effectively achieve a cofactor regeneration system.

Fig. 5. Continuous conversions of 4-(4-Methoxyphenyl)-3-buten-2-one by ER-FPP and GDH-FPP.

Recovery and Reusability of ER-FPP and GDH-FPP

Generally, free enzymes are difficult to recover and reuse. Recovery and reusability of ER-FPP and GDH-FPP are therefore important aspects to be tested in this study. We studied the durability of immobilized ER and GDH through a repeated batch reduction of 4-(4-Methoxyphenyl)-3-buten-2-one at 30°C. After 3 h for each run, the immobilized enzyme mixture was filtered off, washed with Tris-HCl buffer, and reused with fresh substrates. The relative ER activities of the mixture of ER-FPP and GDH-FPP after several times of recycling were illustrated in Fig. 6. The mixture of ER-FPP and GDH-FPP could retain over 80% of its initial ER activity in the first eight cycles, and 61.4% of its initial ER activity could still be observed after fourteen cycles. These obtained results indicate that covalent

Fig. 6. Recovery and reusability of ER-FPP and GDH-FPP.

immobilization ensures the reusability of biocatalyst, and allows the biocatalyst to be continuous recycled.

In summary, covalent immobilized ER-FPP and GDH-FPP were successfully constructed with FPP as a support material. The thermal stability and reusability of ER-FPP and GDH-FPP were superior to that from free ER and GDH. The immobilized ER and GDH can also be applied in the continuous conversions of 4-(4-Methoxyphenyl)-3-buten-2-one, and the cofactor regeneration system can be effectively recycled. This study exploited a simple and cost-effective method of immobilization and application for ER and its cofactor recycling system.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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