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Simultaneous Purification and Immobilization of Histidine-tagged *Bacillus licheniformis* Aldehyde Dehydrogenase on Metal Affinity Magnetic Beads

Huei-Fen Lo[1]*, Yan-Zhi Chen[1], Meng-Chun Chi[2] and Long-Liu Lin[2]*

[1]Department of Food Science and Technology, Hungkuang University,
34 Chungchie Road, Taichung City 433, Taiwan.
[2]Department of Applied Chemistry, National Chiayi University,
300 Syuefu Road, Chiayi City 60004, Taiwan.

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In this research, His Mag Sepharose Ni magnetic beads were employed to simultaneously purify and immobilize histidine-tagged *Bacillus licheniformis* aldehyde dehydrogenase (His-*Bl*ALDH). The optimal conditions for the adsorption of His-*Bl*ALDH on the matrix were 24.6 U/mg adsorbent, incubation at 4 °C for 1 h and the addition of 500 mM NaCl into the crude cell-free extract, and the bound enzyme could be efficiently eluted by 20 mM phosphate buffer (pH 7.4) containing 500 mM NaCl and 500 mM imidazole. Free His-*Bl*ALDH was active in temperature range of 30 - 40 °C and had an optimum of 37 °C, while its thermal stability was improved as a result of immobilization. The immobilized enzyme was recycled six times without an obvious loss of the dehydrogenase activity. Up to 20 days of storage, the preserved activity of free and immobilized enzyme was found to be 2.1% and 78.2%, respectively. Collectively, this magnetic adsorbent may be useful for a novel purification-immobilization of His-*Bl*ALDH.

Key words Aldehyde dehydrogenase, *Bacillus licheniformis*· His tag, Mag Sepharose Ni, Enzyme immobilization.

Immobilized enzymes are currently the object of considerable interest because of the expected benefits over soluble enzymes or alternative technologies. The ability to retain or recover enzymes allows biocatalyst separation from product, thereby permitting continuous processes, and prevents carry-through of protein or activity to subsequent process steps[1]. In spite of the obvious advantages of enzyme immobilization[2,3], it is estimated that only 20% of biocatalytic processes involve the use of immobilized enzymes[4]. However, a number of interesting new developments indicate that enzyme immobilization has entered an exciting new phase[5].

The selected approach of immobilized enzymes should be able to stabilize the target macromolecules and allow easier diffusion of substrates and products. Stabilization of enzymes against various forms of inactivation has been accomplished by a multitude of immobilization strategies, including covalent coupling, adsorption, microencapsulation, polymer entrapment, chemical aggregation, and bioaffinity[2,3,6]. Among the immobilization procedures, adsorption of enzymes onto insert supports is particularly useful since it can facilitate product separation, and sometimes

* To whom all correspondence should be addressed.
E-mail: hflo@sunrise.hk.edu.tw;
llin@mail.ncyu.edu.tw

help to improve the stability of biocatalysts[7,8]. Owing to the conventional procedures for enzyme immobilization are usually problematic and laborious, the interested proteins are virtually fused to affinity tags to endow them with specific binding ability to their unnatural ligands[9]. This approach is of great interest for simultaneous purification and immobilization of industrially important enzymes and offers several advantages of bioprocess, such as reversible adsorption of biocatalysts to the insert support, proper exposure of the enzyme active site, and the lack of substrate diffusion barriers[10].

The immobilized metal ion affinity chromatography (IMAC) technique is commonly used to separate and purify proteins[9, 11]. IMAC is based on the affinity of the surface functional groups of the target protein to the chelated metal ions, such as Zn^{2+}, Co^{2+} and Ni^{2+}, of the insert matrix. Protein-matrix interactions are assumed to arise mainly through the imidazole group of histidine group and, to a lesser extent, the indoyl group of tryptophan[12]. In this regard, a variety of IMAC adsorbents have developed as new selective materials for enzyme immobilization[7, 8, 13, 14]. Even though the target protein may not have any surface exposed metal chelating amino acids, a His tag can be attached to either N- or C-terminus of the biomolecule through genetic engineering methods[9]. With this construction, the simultaneous purification and immobilization of His-tagged enzyme on IMAC adsorbent become a pervasive approach for commercial applications.

Aldehyde dehydrogenases (ALDHs; aldehyde: $NAD(P)^+$ oxidoreductase, EC 1.2.1.5) are a group of enzymes that catalyze the conversion of aldehydes to the corresponding acids by means of an $NAD(P)^+$-dependent virtually irreversible reaction. Aldehydes are cytotoxic and mutagenic compounds that are present in our environment as a result of industrial activities[15], although they could also be formed endogenously during the metabolism of amino acids, vitamins, lipids and carbohydrates[16]. Since the dehydrogenase activity plays a chief role in detoxification of these compounds, a variety of ALDHs have been studied extensively[17-20]. In the genome of *Bacillus licheniformis*, more than 10 *ALDH* genes have been identified[20], and one of these genes, *ybcD*, encodes an ALDH of 488 amino acid residues has been cloned and over-expressed in recombinant *Escherichia coli* [21]. Biophysical studies of histidine-tagged *B. licheniformis* ALDH (His-*Bl*ALDH) revealed that it is a tetrameric enzyme with the resistant characteristics of water-miscible organic solvents[22]. Mutagenesis and kinetic analyses further verified that the conserved Glu255 and Cys289 residues are essential for the catalytic reaction of His-*Bl*ALDH[23]. Beyond these observations, it is worth noting that the recombinant enzyme prefers NAD^+ as a cofactor in the oxidation reaction of aliphatic aldehydes[21]. Although role of ALDHs in metabolic pathways have been extensively studied, there are still only very few research papers dealing with the immobilization of ALDH on a certain type of matrix, including controlled-pore glass[24], sol-gel layer[25], nylon-tubing matrix[26], and glass beads[27]. In this study, the commercial available metal affinity magnetic beads were employed for the single-step purification and immobilization of His-*Bl*ALDH. The immobilized enzyme could be recovered magnetically from the reaction mixture and used repeatedly. Also, thermal and storage stabilities of the recombinant enzyme were greatly improved by the immobilization process.

MATERIAL AND METHODS

Materials

Luria-Bertani (LB) medium was brought from Difco Laboratories (Detroit, MI, USA). The enzyme assay reagents, propionaldehyde, DTT, NAD^+ and NADH, were acquired from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). His Mag Sepharose™ excel was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Protein assay reagents, acrylamide, bis-acrylamide, TEMED, and ammonium persulfate were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Unless indicated otherwise, all other chemicals were commercial products of analytical or molecular biological grade.

Preparation of the crude enzyme extract

To overexpress His-*Bl*ALDH, *E. coli* M15 cells carrying pQE-*ybcD*[21] were grown aerobically in 100-ml LB medium supplemented with antibiotics ampicillin (100 μg/ml) and kanamycin (25 μg/ml) until OD_{600} reached 1.0. Isopropyl-β-D-thiogalactopyranoside was added to a final

concentration of 0.1 mM and the culture was cultivated at 37 °C in a shaking incubator for another 12 h. Bacterial cells were then harvested by centrifugation, resuspended in 3-ml of binding buffer (20 mM sodium phosphate, 500 mM NaCl, and 5 mM imidazole; pH 7.4), and sonicated on ice for 5 min (30-s bursts and pulses). After removal of cell debris by centrifugation, 600 µl (~25 mg) His Mag Sepharose™ excel magnetic beads were added to the supernatant for the binding of His-*Bl*ALDH and the mixture was stirred with an end-over-end shaker for 30 min. Thereafter, the conjugated matrix was recovered magnetically and washed three times with 3 ml of the binding buffer. The bound His-*Bl*ALDH was either eluted from His Mag Sepharose™ excel magnetic beads with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 500 mM imidazole or stored at 4 °C until further use.

Transmission electron microscopy (TEM)

The sample for TEM analysis was prepared by placing a drop of the solution of magnetic beads dispersed in ethanol on a Formvar covered copper grid and evaporated in air at ambient temperature. The morphology and average size of the magnetic beads were examined by TEM with a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan). For each sample, over 100 beads from different parts of the grid were used to estimate the mean diameter of the magnetic matrix.

Gel electrophoresis and determination of protein concentration

Sodium dodecyl sulfate – polyarylamide gel electrophoresis (SDS – PAGE) was carried out with 4% polyacrylamide stacking and 12% polyacrylamide separating gels. The protein samples in dissociating buffer (2% SDS and 5% 2-mercaptoethanol) were heated at 100 °C for 5 min before electrophoresis. The electrophoresed gels were then stained with 0.25% Coomassie brilliant blue R-250 dissolved in 50% methanol – 10% acetic acid and destained in a solution of 30% methanol – 10% acetic acid.

Protein concentration was estimated by the Bio-Rad protein assay reagent based on the Bradford dye-binding procedure [28] using bovine serum albumin as a standard protein.

Measurement of dehydrogenase activity

Dehydrogenase activity was assayed by the procedure described previously[21]. The reaction mixture consisting of 20 mM sodium phosphate buffer (pH 7.4), 1 mM DTT, 2 mM propionaldehyde and 4 mM NAD^+ was firstly equilibrated at 37 °C for 5 min. The reaction was then initiated by adding a suitable dilution of enzyme solution. Following incubation at the same temperature for 20 min, enzyme activity was measured by monitoring the reduction of NAD^+ to NADH at 340 nm, and the formation of NADH was determined with $\Delta\varepsilon_{340}$ = 6.22 mM^{-1} cm^{-1}. One unit of dehydrogenase activity is defined as the amount of enzyme required to reduce 1 µmol of NAD^+ to NADH in 1 min. All enzyme assays were performed in triplicate and the data were expressed as mean values.

Effect of temperature on free and immobilized enzymes

Effect of temperature on free and immobilized enzymes was examined. The enzyme samples were subjected to different temperatures ranging from 20 - 80 °C at pH 7.4 for activity assays. The experiments were carried out in triplicate and the data were expressed as mean values.

Thermal stability of free and immobilized enzymes was performed in 1 ml of 20 mM sodium phosphate buffer (pH 7.4) at different temperatures (20 – 80 °C). After 30 min incubation, the residual activity was determined under the standard assay conditions. The experiments were carried out in triplicate and the data were expressed as mean values.

Effect of pH on free and immobilized enzymes

Effect of pH on the dehydrogenase activity was determined by incubating free and immobilized enzymes separately at different pHs from 4.0 up to 12.0 at 37 °C for 15 min. At the end of incubation, the residual activity of each sample was measured under the standard assay conditions.

Free and immobilized enzymes in 20 mM NaOAc-HOAc buffer (pH 4.0-5.0), 20 mM sodium phosphate buffer (6.0-8.0), or 20 mM glycine-NaOH buffer (pH 9.0 -12.0) were incubated at ambient temperature for 30 min, and the residual activity was determined according to the assay conditions.

Determination of kinetic constants

The K_M and k_{cat} values of free and immobilized enzymes were determined by measuring the dehydrogenase activity at various concentrations of NAD^+ and a fixed concentration

of 10 mM propionaldehyde. To estimate the kinetic constants, a Lineweaver – Burk plot was fitted using GRAF4WN softwares. All the reactions were performed at least three times.

Reusability of the immobilized enzyme

The immobilized His-*Bl*ALDH was repeatedly used for the oxidation of propionaldehyde in batch reaction. Enzyme-conjugated magnetic beads (~20 mg) in 1 ml of 20 mM sodium phosphate buffer (pH 7.4) containing 1 mM DTT, 2 mM propionaldehyde and 4 mM NAD^+ were shaken (100 rpm) at 37 °C for 15 min each time. The residual activity was subsequently determined under the standard assay conditions. Before the next cycle of reaction, the enzyme-matrix complex was washed twice with 1 ml of 20 mM sodium phosphate buffer (pH 7.4) and reused for another run. The experiments were performed in triplicate and the data were expressed as mean values.

Storage stability of free and immobilized enzymes

The storage stability of free and immobilized enzymes was assessed at 4 °C for a period of time. At a specific time interval, aliquots were withdrawn to measure the dehydrogenase activity under the standard assay conditions. The experiments were performed in triplicate and the data were expressed as mean values.

RESULTS AND DISCUSSION

Immobilization and elution of His-*Bl*ALDH on the magnetic beads

Earlier, we have constructed an expression plasmid containing a truncated *B. licheniformis ALDH* gene for the functional expression of His-*Bl*ALDH[21]. With this construction, the expressed enzyme contains 10 additional amino acid residues at its N-terminus, which facilitates the binding of the recombinant protein by the metal-affinity magnetic beads (Fig. 1a). A preliminary study was cautiously performed to evaluate the binding of His-*Bl*ALDH on His Mag Sepharose Ni magnetic beads. Time course of His-*Bl*ALDH adsorption on the matrix was recorded at 4 °C and it was found that the adsorbed dehydrogenase activity reached a saturation level beyond 1 h of incubation (Fig. 2). In this regard, the enzyme adsorption was conducted for 1 h in the further experiments.

Incorporation of salt into the immobilization process was necessary due to the consideration of ions strength[29]. It was reported that ions strength would bring about the shelter effect and reduce the interactive relationship between the amino acid residue of proteins and the solid supports[30, 31]. The hydrophobic

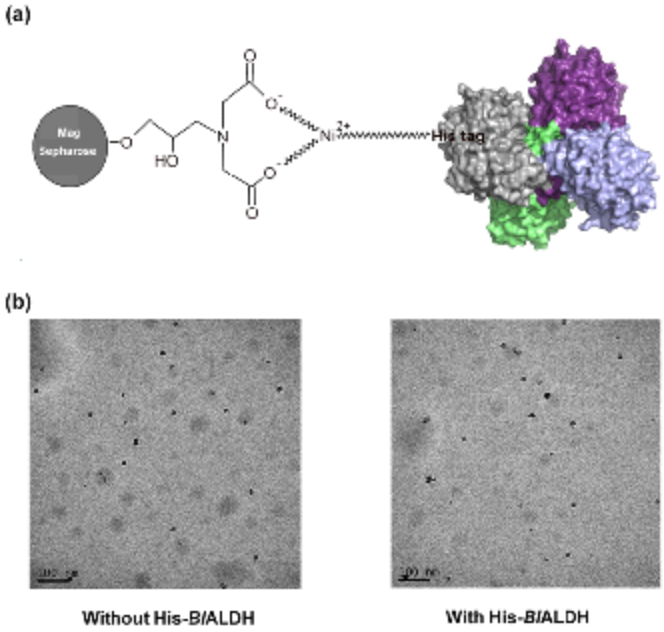

Fig. 1. Schematic illustration of His-*Bl*ALDH immobilization (a) and TEM micrograms of the magnetic beads (b)

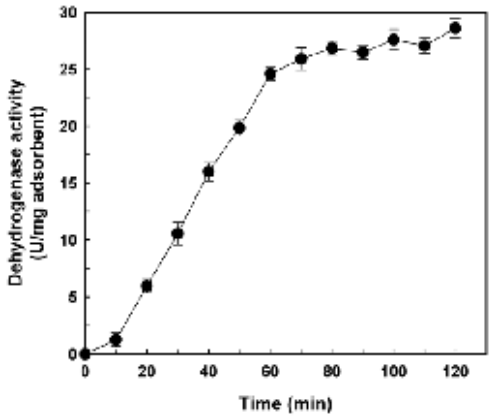

Fig. 2. Time course of crude His-*Bl*ALDH directly immobilized on the magnetic beads

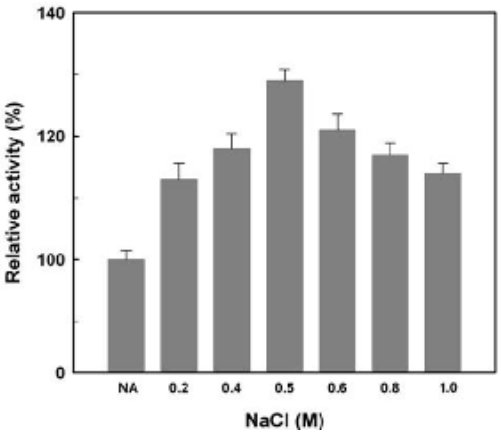

Fig. 3. Effect of NaCl concentration on the immobilization of His-*Bl*ALDH

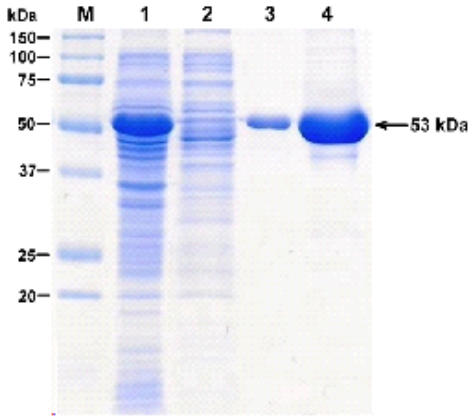

Fig. 4. SDS – PAGE analysis of the cell-free enzyme extract and the magnetic beads bound with His-*Bl*ALDH. M, protein size marker; 1, the cell-free extract; 2, the filtrate; the eluted His-*Bl*ALDH; 4, the bound His-*Bl*ALDH

interaction might also contribute to the binding of His-*Bl*ALDH on the magnetic beads. Higher ion strength is likely to make more proteins randomly adsorbed on the matrix through the hydrophobic binding and leads to low selectivity[32]. In contrast, the lower electrostatic force would decrease the adsorption of impure proteins and therefore increase the purification fold. Accordingly, effect of NaCl concentrations on His-*Bl*ALDH adsorption was investigated. As shown in Fig. 3, the matrix adsorbed a highest amount of dehydrogenase activity in the presence of 500 mM NaCl. SDS – PAGE analysis further displayed that His-*Bl*ALDH in the crude extract was mostly adsorbed by the magnetic beads (Fig. 4, lane 2). Higher or lower salt concentrations caused a reduced adsorption of His-*Bl*ALDH. The possible explanation for these findings may be due to the addition of NaCl at a suitable concentration provides shelter effect to suppress ion – ion interaction and leave more opportunity for the binding of His-*Bl*ALDH on the magnetic beads[30,33].

The typical TEM micrograms for the magnetic beads without and with bound His-*Bl*ALDH are shown in Fig. 1b. The TEM microgram showed that His Mag Sepharose Ni magnetic beads were essentially monodisperse and had a mean diameter of 15.8 ± 1.6 nm. After the binding of His-*Bl*ALDH, the beads remained discrete and had a diameter similar to that of the unbound ones. These observations indicate that the adsorption process do not significantly change the molecular size of the magnetic beads.

The reaction rates at several concentrations of NAD^+ and a fixed concentration of propionaldehyde were determined for free and immobilized enzymes. The calculated K_M and k_{cat} values for His-*Bl*ALDH were 2.01 mM and 8.34 s^{-1}, respectively, while the immobilized enzyme exhibited 52.6% increase in K_M and 2.7-fold decrease in k_{cat}. Moderate increases in K_M value upon enzyme immobilization have frequently been reported, especially for the insoluble/solid matrix[34-36]. Only marginal increase in the K_M value after immobilization of His-*Bl*ALDH confirms that enzyme-substrate binding continues to be efficient in spite of the molecular nature of the substrates.

The specific dehydrogenase activity for the enzyme-matrix conjugate was 24.6 ± 5.6 U/mg adsorbent. SDS – PAGE of the enzyme-matrix

conjugate confirmed the binding of His-*Bl*ALDH on the magnetic beads (Fig. 4, lane 4). It is worth noting that the bound His-*Bl*ALDH could be easily eluted from the magnetic beads by 20 mM sodium phosphate (pH 7.4) containing 500 mM NaCl and 500 mM imidazole (Fig. 4, lane 3). The recovery yield for the adsorption – elution process was approximately 84%. Taken these observations together, it can be concluded that adsorption – elution of His-*Bl*ALDH on His Mag Sepharose Ni magnetic beads is an efficient process for the simultaneous purification and immobilization of this enzyme.

Effects of temperature on free and immobilized enzymes

Fig. 5(a) shows the temperature dependence of the dehydrogenase activity of free and immobilized enzymes. The optimal temperature for free His-*Bl*ALDH was found at 37 °C. The immobilization process did not significantly change the optimal reaction temperature. This means that the binding of His-*Bl*ALDH would essentially retain the native enzyme's conformation.

Thermal stability of free and immobilized His-*Bl*ALDHs was also determined by incubating the tested samples in a temperature range of 20 - 80 °C for 30 min and subsequently assaying their residual activity. Fig. 5(b) shows that both free and immobilized enzymes had a different profile of thermal stability. The residual activity of immobilized enzyme was consistently higher that that of free His-*Bl*ALDH when the incubation temperatures were set below 45 °C. It is believed that the surface immobilization of enzyme on magnetic supports can somehow increase the thermal stability, probably by increasing its molecular rigidity[37], thus preventing any undesirable change in the molecular structure caused by heating. Stability improvement of enzymes as function of temperature has also been observed for other biocatalysts immobilized on different types of magnetic supports[7, 36, 38].

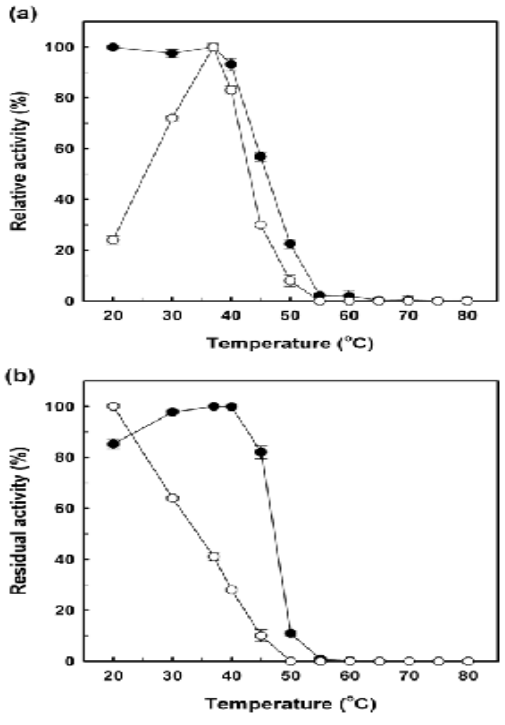

Fig. 5. Effect of temperature on activity and stability of free (a) and immobilized
(b) enzymes. Closed circles, temperature dependence; Open circles, thermal stability

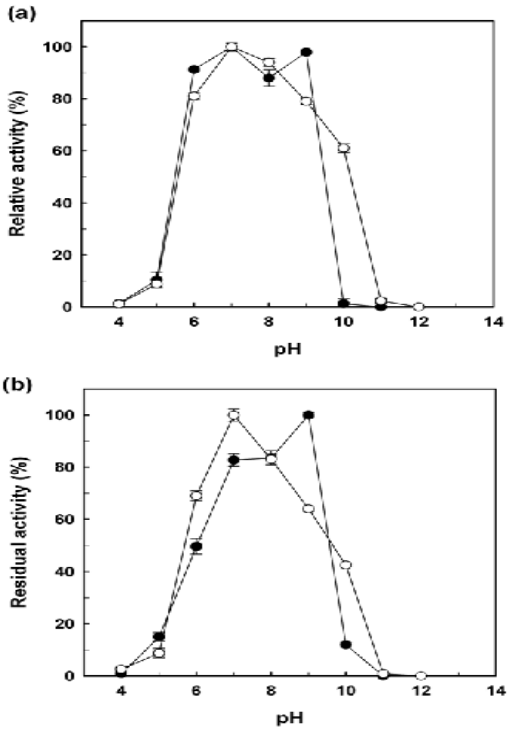

Fig. 6. Effect of pH on activity and stability of free (a) and immobilized
(b) enzymes. Closed circles, pH dependence; Open circles, pH stability

Effects of pH on free and immobilized enzymes

Fig. 6(a) shows the pH dependence of the dehydrogenase activity of free and immobilized enzymes. The performance of enzyme activity was highly affected by pH values. Free His-*Bl*ALDH exhibited an optimal pH range of 6.0-9.0. Its activity decreased rapidly when the pH value approached 5.0 or 10.0. The pH optimum for the immobilized His-*Bl*ALDH exhibited a little shift with respect to that of free enzyme. Additionally, the immobilized enzyme displayed a higher relative activity under the alkaline pH value as compared with that of free His-*Bl*ALDH. Possibly, the configuration of His-*Bl*ALDH is fixed on the surface of magnetic beads and leads to an increase in the enzymes' tolerability to pH variance in its surroundings. It would be ideal if the immobilized enzyme could be active over a wide range of pH values.

Recyclability of the immobilized His-*Bl*ALDH

The repeated usability of the immobilized enzyme was examined in batch operation mode. Although the dehydrogenase activity of the immobilized His-*Bl*ALDH decayed with the increase of recycle numbers, the enzyme-matrix conjugate retained more than 92.3% of its initial activity until 10 cycles (Fig. 7). There was still approximately 36.7% activity remaining even after 25 consecutive repeated uses. The loss of dehydrogenase activity during the operational steps may be related to the enzyme inactivation caused by protein denaturation. This indicates that immobilization of His-*Bl*ALDH on the magnetic beads has good durability and magnetic recovery. Except that, the high operational stability can significantly reduce the operation cost in practical applications of this enzyme.

Storage stability of free and immobilized enzymes

The economics of industrial enzyme bioprocesses depend mostly on the biocatalyst production cost[39]. Long-term storage of industrial value-added enzymes without a significant loss of biological activity has attracted considerable attention in recent years[40]. In this regard, free and immobilized enzymes were stored in a sodium phosphate buffer solution (20 mM, pH 7.4) at 4 °C for 30 days. The storage stability was monitored by determining the dehydrogenase activity profiles of free and immobilized enzymes every day up to 30 days. As shown in Fig. 8, the dehydrogenase activity of immobilized His-*Bl*ALDH exhibited a slower decrease with respect to free enzyme. The immobilized His-*Bl*ALDH retained approximately 57.3% of the initial activity during the 30-day period of storage, while the free enzyme lost completely its initial activity beyond 22 days. This result indicates that the stability of His-*Bl*ALDH is improved considerably as a consequence of enzyme immobilization.

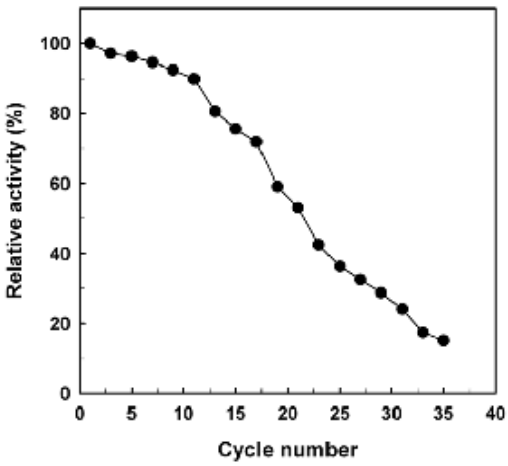

Fig. 7. Reuseability of the immobilized enzyme

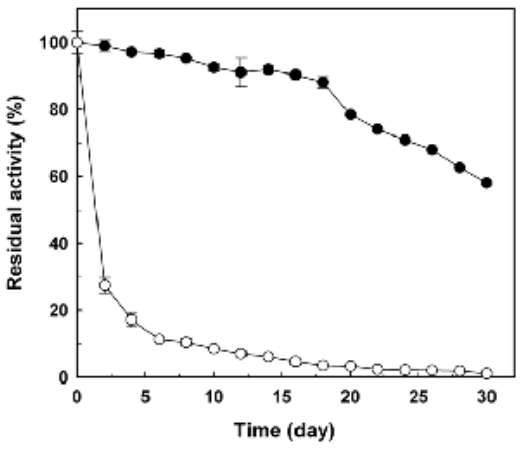

Fig. 8. Storage stability of free and immobilized enzymes. Closed circles, immobilized His-*Bl*ALDH; Open circles, free His-*Bl*ALDH

CONCLUSIONS

In this study, the commercially available magnetic beads were employed for the simultaneous purification and immobilization of His-*Bl*ALDH. The enzyme-matrix conjugate exhibited a very high stability in both the repeated use and long-term storage tests. All these results suggest that this is a promising technique for His-*Bl*ALDH immobilization. Due to its easy processing and highly specific selectivity, this approach is expected to be a pervasive alternative for the industrial applications of His-*Bl*ALDH.

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