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His-tagged Horse Liver Alcohol Dehydrogenase: Immobilization and application in the bio-based enantioselective synthesis of (*S*)-arylpropanols

Daniela Quaglia [a], Matteo Pori [b], Paola Galletti [b], Enrico Emer [b], Francesca Paradisi [a,**], Daria Giacomini [b,*]

[a] *Centre for Synthesis and Chemical Biology, UCD School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland*
[b] *Dipartimento di Chimica "G. Ciamician", Via Selmi 2, University of Bologna, 40126 Bologna, Italy*

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ABSTRACT

The novel histidine-tagged Horse Liver Alcohol Dehydrogenase (His-HLADH-EE) was successfully purified and covalently immobilized onto a solid support in a one-step procedure through a metal-directed technique. A full characterization of the immobilized enzyme was carried out. Effects of pH, temperature and organic co-solvents were deeply investigated and they showed a shift in the optimum pH with respect to the free form as well as increased stability to temperature and solvents. The immobilized His-HLADH-EE proved to be effective as catalyst in the reduction of aliphatic and aromatic aldehydes. Application of the free and immobilized His-HLADH-EE to the chemo-enzymatic synthesis of (*S*)-Profenols demonstrated enhanced enantioselectivity and high reusability of the immobilized form. The achievement of a robust and effective immobilization of an alcohol dehydrogenase substantiated the use of biocatalytic reduction in the synthesis of primary alcohols and valuable chiral intermediates especially for pharmaceutical industries.

1. Introduction

Dehydrogenases represent an important class of co-factor dependent redox enzymes that have been successfully employed for the synthesis of chiral alcohols, hydroxy-acids, or aminoacids [1,2]. Alcohol dehydrogenases (ADH) belong to that class and reversibly catalyze the reduction of aldehydes or ketones to the corresponding primary or secondary alcohols, respectively. Despite the dramatic increase in production of optically active compounds integrating biocatalysis, redox enzymes [3–5] are less used for industrial processes than hydrolytic enzymes, such as Lipases for instance [6].

Hurdles in industrial applications of dehydrogenases concern the need for expensive cofactors, low stability and susceptibility to organic solvents, thus limiting their effectiveness on lipophilic substrates poorly soluble in aqueous media. With the introduction of isolated and purified enzymes, recombinant DNA technology and the development of efficient cofactor regeneration techniques [7], some of those hurdles can be overcome. Protein immobilization has been shown as a further step in addressing other important issues that enzymes must face with in order to be used on an industrial scale, namely re-using of the biocatalyst and stability [8,9]. Interestingly, immobilization has also been found to concurrently enhance enzyme properties [10]. Where the enzyme is immobilized within a porous solid (such as a protein aggregate or crystal, an inert porous support) some factors contributing to enzyme inactivation, such as aggregation, adsorption onto hydrophobic surfaces and auto-proteolysis are minimized [11].

While immobilization is widely reported for enzymes such as hydrolases, significantly fewer cases are documented for redox enzymes [12,13]. However, a general strategy applicable to the immobilization of every enzyme is not yet available and the selection of a method strongly depends on the nature of the enzyme and of the carrier of choice [14]. A very reliable procedure has been reported by the group of Guisan which exploits both ionic interaction and covalent immobilization [15–17].

Among the variety of immobilization strategies, methods based on affinity ligands or bio-specific recognitions are quite attractive because the activity of the immobilized biomolecule is generally preserved, the efficiency of the specific immobilization can be high,

* Corresponding at. Dipartimento di Chimica "G.Ciamician" Via Selmi, 2, University of Bologna, 40126 Bologna, Italy. Tel.: +39 051 209 9528; fax: +39 051 209 9456.
** Corresponding at. Centre for Synthesis and Chemical Biology, UCD School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland. Tel.: +353 1 7162967; fax: +353 1 716 2501.
E-mail addresses: francesca.paradisi@ucd.ie (F. Paradisi), daria.giacomini@unibo.it (D. Giacomini).

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making it possible to work with low biomolecule concentrations, the nonspecific adsorptions are limited, and the stability of the biomolecule is usually improved. Recent examples, specifically on alcohol dehydrogenases, are those reported by Bolivar et al. [13,18] and Rocha-Martin et al. [19].

Horse Liver Alcohol Dehydrogenase (HLADH) is a zinc-containing enzyme, successfully used in biocatalysis [20–22]. Native HLADH is found in two isoforms, E and S, which leads *in vivo* to the formation of a dimeric enzyme of mixed composition (EE, ES and SS) [23]. While the E subunit is specific for ethanol, the S subunit recognizes steroidal substrates. Following our interest in the use of enzymes for the synthesis of bioactive molecules [24–28] we applied HLADH to the enantioselective synthesis of (2*S*)-2-arylpropanols starting from the parent racemic aldehydes *via* the activation of an efficient dynamic kinetic resolution (DKR) process [29–31]. A DKR process combines a selective kinetic resolution and *in situ* fast racemization of the unreacted enantiomer. This approach allows for the conversion of both enantiomers of the racemic substrate into a single enantiomer of the product and overcomes the 50% yield limitation in classical kinetic resolution whenever the racemization rate successfully competes with that of the resolution reaction [32]. In this chemo-enzymatic reaction the commercial HLADH preparation and a recombinant EE-enzyme were used with similar results in enantioselectivities and yields. The products (2*S*)-arylpropanols are useful intermediates in flavour manufacture and were oxidized to (2*S*)-2-arylpropionic acids [33], active ingredients of the Profen class of non-steroidal anti-inflammatory agents (NSAIDs).

Native HLADH has been immobilized in the past onto different supports with various degree of success by co-polymerization [34] adsorption [35] and more recently by covalent linkage [36,37].

Recently, we reported on the production and characterization of His(6)-tagged Horse Liver Alcohol Dehydrogenase (His-HLADH-EE) [38].

Here we describe a method for its immobilization, which took advantage of the increased metal affinity due to the poly-histidine tag. The immobilized His-HLADH-EE (imHis-HLADH-EE) thus obtained showed enhanced properties in terms of stability, pH and temperature profiles, presence of organic co-solvents, and reusability. Finally we report its application in the bio-reduction of aliphatic and aromatic aldehydes; in particular it showed excellent results in the enantioselective reduction of some racemic Profenals yielding, with a complete unprecedented enantioselectivity, enantiopure (*S*)-Profenols such as (*S*)-ibuprofenol, and (*S*)-naproxenol, valuable intermediates in the synthesis of (*S*)-Ibuprofen and (*S*)-Naproxen, benchmark drugs in the class of NSAIDs.

2. Materials and methods

2.1. Materials, strains, vectors and culture conditions

All chemical reagents, unless otherwise stated, were purchased as analytical grade from Sigma–Aldrich or TCI and used without further purification. Aldehydes were commercial or prepared as reported in ref. [31]. Alcohols were commercial or prepared from the corresponding aldehydes by reduction with $NaBH_4$ in methanol. All spectra were consistent with reported data. NAD^+ was purchased from Apollo Scientific Ltd, Stockport, U.K. Staining and de-staining was performed using the Stain/DeStain-Xpress protein detection kit (Enzolve Technologies, Ltd., Ireland). *Escherichia coli* BL21 competent cells were purchased from Novagen (Germany). Transformed *E. coli* strains were generally cultured in Luria–Bertani (LB) agar and in LB broth, both containing ampicillin (100 μg/mL) at 37 °C, shaking at 250 rpm. *E. coli* strains harbouring a pRSETb-EqADH-E vector were cultured in PG (minimal media) agar and in Auto Induction broth, both containing ampicillin (100 μg/mL) at 37 °C, shaking at 250 rpm. Sepabeads EC-EP/S were kindly donated by Resindion SRL (Binasco, Milan, Italy). Morphological investigation was carried out with a Philips XL-20 Scanning Electron Microscope operating at 15 kV coupled with Energy-Dispersive X-ray Spectrometer. Samples were air-dried, then sputter-coated with carbon 60 s prior to examination.

2.2. Expression and purification of His-HLADH-EE

His-HLADH-EE gene inserted in vector pRSETb was expressed and the protein purified as previously reported [38]. Crude protein concentration was determined by Bradford protein assay dye reagent (Bio-Rad Laboratories GmbH, Germany) with bovine serum albumin as a standard. Pure protein concentrations were determined by UV absorption at 280 nm using the absorbitivity reported in the literature (0.441 mL/(mg cm) for His-HLADH-EE) [39].

2.3. Immobilization of His-HLADH-EE

Sepabeads EC-EP/S (a commercially available rigid methacrylic polymer matrix with diameter ranging between 100 to 300 μm, activated with epoxy groups in a ratio of 100 μmol/g of wet resin) were derivatized with iminodiacetic acid (IDA) and $CoCl_2$ following the procedure reported in literature by Guisan, allowing a modification of around 5% of the epoxy-groups in the support [17], 1 g of beads was shaken at room temperature for 2 h in 2 mL of support modification buffer (0.1 M sodium borate, 2 M iminodiacetic acid, pH 8.5). The derivatized resin, rinsed with double distilled water, was then re-suspended in 5 mL of metal containing solution (0.05 M sodium phosphate buffer pH 6.0, 1.0 M NaCl and 5 mg/mL of $CoCl_2$) and shaken at room temperature for 2 h. The resin, rinsed again with double distilled water, was then put in contact with His-HLADH-EE (1 mg of enzyme per 1 g of resin, in storing buffer pH 8.5, room temperature if not otherwise stated) and the mixture was gently shaken at room temperature over 24 h. The resin was then thoroughly washed using a desorption buffer (20 mM Na_2HPO_4-NaH_2PO_4, 50 mM EDTA, 0.5 M NaCl, pH 7.4, as per IMAC purification procedure) [38] and water to achieve complete removal of the cobalt first and of the residual EDTA after. The un-reacted epoxides were neutralized using a blocking buffer (3 M glycine, pH 8.5, 4 mL per g of beads) over 20 hrs at room temperature, with gentle shaking. The immobilized enzyme was thoroughly washed and routinely stored in buffer (Tris–HCl, pH 8.5). To achieve immobilization using different metals the following salts were used: $NiCl_2 \times 6H_2O$, $CuSO_4 \times 5H_2O$ (5 mg/mL, 5 mL for 1 g of beads, allowing a 2 hrs contact with the derivatized beads). The metal solutions were prepared using buffer 50 mM Na_2HPO_4-NaH_2PO_4, pH 6.0. Immobilization has been performed on purified enzyme, if not otherwise stated.

2.4. Activity assays

Spectrophotometric activity measurements were based on the substrate-dependent absorbance change of NADH at 340 nm and routinely done in reaction mixtures (1 mL for the soluble enzyme and 5 mL for the immobilized) at 25 °C, using a Varian Cary 50 Scan UV-visible spectrophotometer equipped with a Cary single cell Peltier temperature controller. For the immobilized enzyme the reaction mixture was shaken at 25 °C, 250 rpm and the absorbance at 340 nm was recorder every minute as single readings. To test the recyclability of the enzyme after each cycle (9 min in total duration) the resin was washed thoroughly with buffer and a new reaction was set up. Unless otherwise stated, the reaction mixture for the oxidative step contained NAD^+ (1 mM), ethanol (4 mM), enzyme sample (appropriate amount) and up to 1 mL of 0.1 M sodium pyrophosphate buffer, pH 8.8. The buffer was equilibrated at 25 °C prior to the assay. One unit of HLADH corresponded to the amount of enzyme required to reduce 1 μmol of NAD^+ per min at 25 °C. For the pH stability test the following buffers were used: 50 mM Na_2HPO_4–NaH_2PO_4 buffer pH 6.5, 50 mM Tris–HCl pH 8.5. To investigate the optimum pH of reaction the following buffers were used: 50 mM Na_2HPO_4–NaH_2PO_4 buffer pH 6.5, 7.5, 50 mM Tris–HCl buffer pH 8.5, 0.1 M pyrophosphate buffer or 50 mM Glycine–NaOH buffer pH 8.8, 50 mM Glycine–NaOH buffer pH 9.5, 10.5, 50 mM Na_2HPO_4–NaOH buffer pH 11.0, 11.5, 50 mM Glycine–NaOH buffer, 50 mM KCl–NaOH buffer pH 12.5.

A pH 9.5 was used to investigate the optimum reaction temperature for the oxidation of EtOH (4 mM) to acetaldehyde and temperatures between 25 °C and 55 °C were tested. The pH values of the buffers were always adjusted at the temperature at which the experiment was carried out. To test the stability at different solvents the enzymes were incubated in 10 (free enzyme) and 20% (free and immobilized enzymes) of solvent (CH_3CN, THF, DMSO and methanol were used) in 50 mM Tris–HCl, pH 8.5 buffer with a total volume of 0.2 mL in all cases. For the immobilized enzyme, 50 mg of resins were used in each sample, while the free enzyme was always tested in a concentration of 0.7 mg/mL. The activity was recorded at time zero and after 24 h in the usual conditions. To test the inactivation upon contact with metals, the free enzyme was incubated at room temperature for 24 h in a 3.5 mM metal solution which is the equivalent ratio of metal to enzyme in the immobilization step. Samples with different metals ($CoCl_2$, $NiCl_2$ and $CuSO_4$) were checked for activity over 24 h and compared to a control sample.

2.5. General procedure for enzymatic reduction of aldehydes

2.5.1. Synthesis

Method A: into a vial stirred on an orbital shaker (140 rpm) at room temperature, all reagents were added in the following order: 0.5 mL of a 5 mM solution of the starting aldehyde in CH_3CN, 0.146 mL of EtOH (0.5 M), 0.5 mL of a 0.1 mM solution of NADH freshly prepared in the appropriate 0.1 M buffer, then 0.1 M buffer to reach a total final volume of 5 mL and the chosen amount of enzyme.

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Method B: biphasic system, into a vial stirred on an orbital shaker (140 rpm) all reagents were added in the following order: 2.5 mL of a 5 mM solution of the starting aldehyde in the organic solvent, 0.146 mL of EtOH (0.5 M), 0.5 mL of a 0.1 mM solution of NADH freshly prepared in the appropriate 0.1 M buffer, 0.1 M buffer to reach a total final volume of 5 mL and the chosen amount of enzyme.

Immobilized enzyme recycling. Into a vial stirred on an orbital shaker (140 rpm) all reagents were added in the following order: 0.5 mL of a 5 mM solution of the starting aldehyde in CH_3CN, 0.146 mL of EtOH, 0.5 mL of a 0.1 mM solution of NADH freshly prepared in the appropriate 0.1 M buffer, 0.1 M buffer to reach a total final volume of 5 mL and the chosen amount of enzyme. After the conversion of the starting aldehyde the solution was removed and the enzyme was gently washed with the same buffer (3 mL × 5 mL). The enzyme was reused for the reaction with the same starting conditions.

2.5.2. Analysis of products

Formation of arylpropanols and alcohols was monitored by reverse phase HPLC or GC-FID analysis: at different reaction times, aliquot samples were filtered, diluted and directly injected.

Method 1, for alcohols **2a**, **2c**–**2g**: reverse phase HPLC on Agilent Technology HP1100, column ZORBAX-Eclipse XDB-C8 5 μm, 4.6 mm × 150 mm Agilent Technologies. The compounds were eluted with CH_3CN–H_2O, flux 0.5 mL min^{-1}. Method 1: 50% of CH_3CN and 50% of H_2O in 25 min.

Method 2, for alcohol **2b**: reverse phase HPLC on Agilent Technology HP1100, column ZORBAX-Eclipse XDB-C8 5 μm, 4.6 mm × 150 mm Agilent Technologies. Elution program from 70% to 100% of CH_3CN in 15 min, then 100% of CH_3CN for 10 min T = 30 °C.

Method 3, for alcohols **2i** and **2h**, GC-FID Agilent Technologies 6890 using gradient from 50 °C to 280 °C over 30 min, column HP5 5%Ph-Me silicon.

Calibration curves (five dilutions, each in triplicate) were used for quantitative analysis of arylpropanols, while for the other alcohols the reaction was evaluated by the disappearing of the starting aldehyde.

Enantiomeric ratios of 2-arylpropanols were determined by HPLC analysis on chiral columns as described in the reference [31].

3. Results and discussion

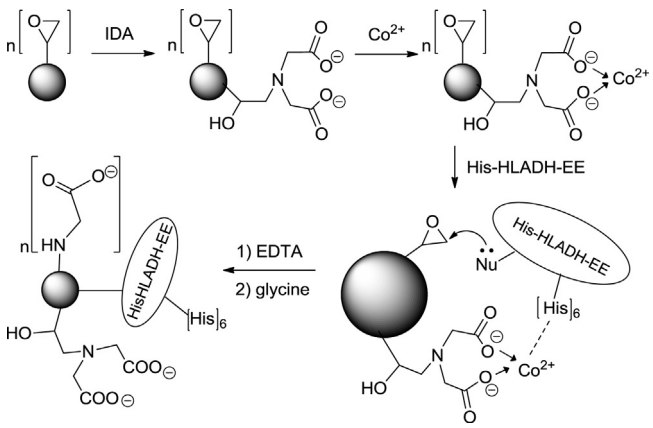
3.1. His-HLADH-EE immobilization

A series of immobilization methods for HLADH have been screened in our lab with the intention of maximizing enzyme stability and reusability. Attempts to cross-link HLADH-EE to derivatized glass beads, ferromagnetic particles and polystyrenes "scavenger" resins did not lead to successful results with a significant loss of activity upon immobilization. Bolivar et al. also reported on the significant difficulties faced when multimeric enzymes are immobilized; loss of activity and low operational stability are among the main issues [13].

A metal-directed strategy for the immobilization of His-tagged ADHs had never been attempted, being the production of His-ADHs still rare. We then decided to attempt such immobilization on our newly developed histidine tagged HLADH-EE with the aim of broadening the strategies available for the stabilization of multimeric dehydrogenases.

The choice of an epoxy resin as starting support appeared to be the most appropriate as previous literature reported on the broad application of these types of derivatized beads for enzyme immobilization [15–17]. The epoxy resin (Sepabeads EC-EP/S) was first reacted with iminodiacetic acid to convert about 5% of the epoxides on the surface of the beads and then treated with a suitable metal ion solution for the complexation on the resin. His-HLADH-EE obtained with the procedure recently reported [38], was then added and a selective interaction between the poly-histidine tag and the metal allowed for a quick complexation, followed by a reaction between nucleophilic residues on the protein surface (Lys, Cys, or Ser) and the unreacted epoxy residues on the beads to give a successful covalent immobilization. The metal ion was then removed by washing with an EDTA solution. To ensure that no reactive epoxide remained, the beads were finally treated with glycine as capping agent (Scheme 1).

The optimization of the loading conditions and characterization of the immobilized biocatalyst were performed with purified



Scheme 1. Reaction sequence for the immobilization of His-HLADH-EE.

His-HLADH-EE samples. However, the method was valuably applied with the same efficiency, to the crude *E. coli* cell extract obtained from the expression of His-HLADH-EE, allowing to bypass the purification step at the same time [17]. In all cases, activity was tested on the standard reaction of ethanol oxidation monitoring the formation of NADH by UV spectrophotometry at 340 nm.

3.1.1. Studies on the conditions of loading

The efficiency of immobilization, measured as the activity retained by the enzyme on the resin, is dependent on whether the beads are metal-derivatized or not, and on the metal ion used in the process. In the absence of a metal-derivatization of the resin, upon incubating the enzyme for 24 h, only partial immobilization was achieved (Fig. 1a). Upon resin derivatization using $CoCl_2$, the immobilized His-HLADH-EE retained up to 60% of its original activity (free pure enzyme before immobilization: 1.3 U/mg, immobilized enzyme: 0.8 U/mg). When other metals were used the activity of the immobilized enzyme decreased in the order: $NiCl_2$ > $CuSO_4$ and the best conditions were obtained with $CoCl_2$ (Fig. 1a). To evaluate enzymatic inactivation as a consequence of potential metal leaching, the effect of the metal ions on the free His-HLADH-EE was also assessed. Straight after the addition of the metal to the enzyme solution, the following was observed: with cobalt ion the enzyme retained 96% of activity, with nickel ion 83% and with copper ion an immediate complete loss of activity was observed (Fig. 1b). The values for cobalt and nickel ions decreased only slightly over the next 24 h of incubation following the same trend. While total leaching of the metal is unlikely, it is evident that copper ions have a lethal effect on the biocatalyst. These results are in accordance with the retention of activity observed upon derivatization of the resin with the different metals. Cobalt ions allow for the best retention of activity after immobilization as it is also the least detrimental of the metals for the enzyme.

Furthermore, when the immobilization procedure was directly performed on the cell lysate, the use of a metal ion allowed for the selective immobilization of His-HLADH-EE from the crude, as previously reported [15]. In this case, comparing the initial specific activity of the cell lysate (0.09 U/mg) with the activity of the immobilized enzyme (0.84 U/mg of enzyme, or U/g of resin) and considering a retention of activity of about 60% after immobilization, a purification fold of 15 was estimated. This matches the results previously reported for the standard purification method of His-HLADH-EE [38].

Loading studies using $CoCl_2$ as immobilization metal with increasing amounts of His-HLADH-EE were also performed. During this experiment 1–5 mg of enzyme per g of resin were loaded, but the increment in activity was not proportional to the amount of enzyme, which eventually led to inactivation for ratios higher

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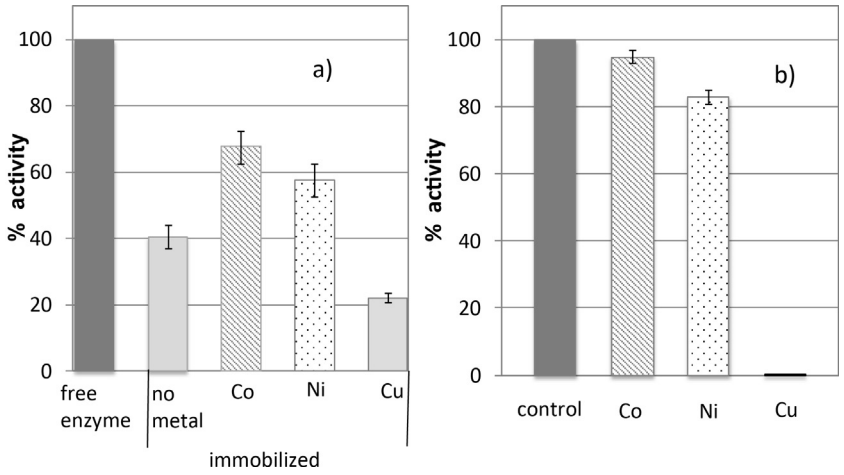

Fig. 1. (a) Activity of the immobilized His-HLADH-EE when different metal ions (or no metal) were used for the immobilization process. 100% of activity was that observed for the free enzyme prior to immobilization. (b) Study on the inactivation of the free His-HLADH-EE by the same metals (3.5 mM) used for immobilization compared to a control where no metal was added. Activity was recorded after 24 h under standard methodology.

than 2 mg of enzyme per gram of resin. It is possible to hypothesize that with increased enzymatic concentration in the sample, aggregation on the porous surface of the beads effectively diminishes the maximum loading capacity leading to apparent inactivation of the biocatalyst as observed by Pessela et al. [17]. The loss in activity could also be partially attributed to diffusional limitations. This concept is known as mass transfer resistance and it is common when porous supports are used for immobilization. An indication of the presence of this effect is the non-proportional dependence of the activity of the immobilized derivative to the enzyme load [40]. Furthermore it has been observed that the immobilized enzyme is much less temperature dependent if compared to its free counterpart. The specific activity of both enzymes increases with increasing temperature, but to a lesser extent in the case of the immobilized biocatalyst.

3.2. Characterization of immobilized His-HLADH-EE

3.2.1. SEM analysis

Resin beads before and after immobilization of His-HLADH-EE were investigated by scanning electron microscopy (SEM-EDS) to detect changes of the surface during the coupling procedure (Fig. 2).

Beads supported with His-HLADH-EE have dimension from 100 to 300 μm and maintained a spherical regular shape (Fig. 2a). They are individually separated and interestingly did not present any aggregation phenomena. The absence of aggregates in resin-enzyme beads is quite important as they exhibit the maximum contact area for biocatalysis. In Fig. 2a inset of a single bead with the enzyme bound was presented as an example. Microscopic observations carried out at a higher magnification (20,000×) of three resin samples are reported in Fig. 2b) final stage with the

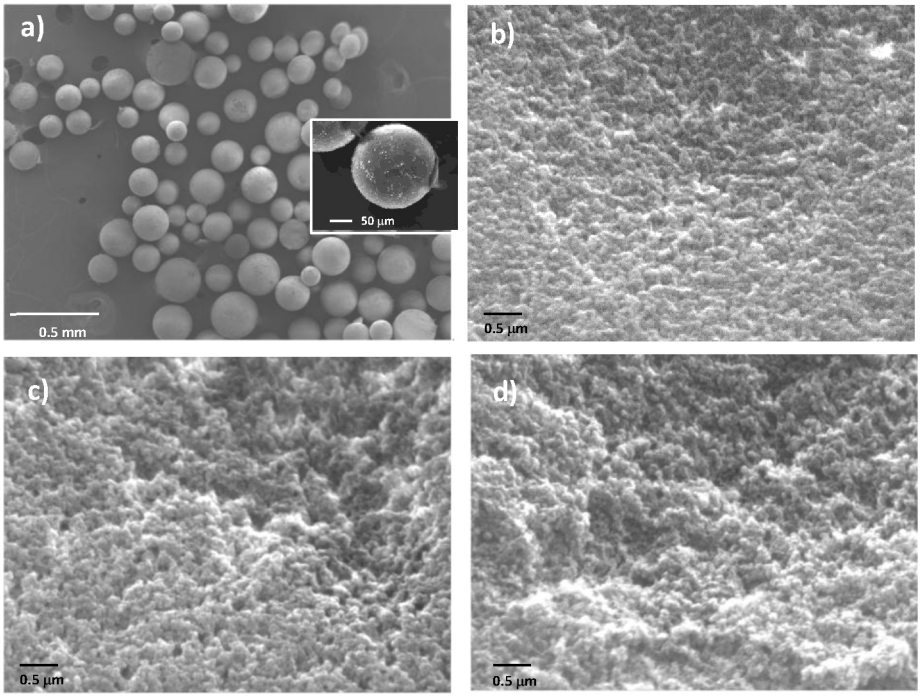

Fig. 2. SEM micrographs of resin beads (a) with His-HLADH-EE, (b) with His-HLADH-EE at higher magnification, (c) early treatment with iminodiacetic acid, (d) after treatment with Co^{2+}.

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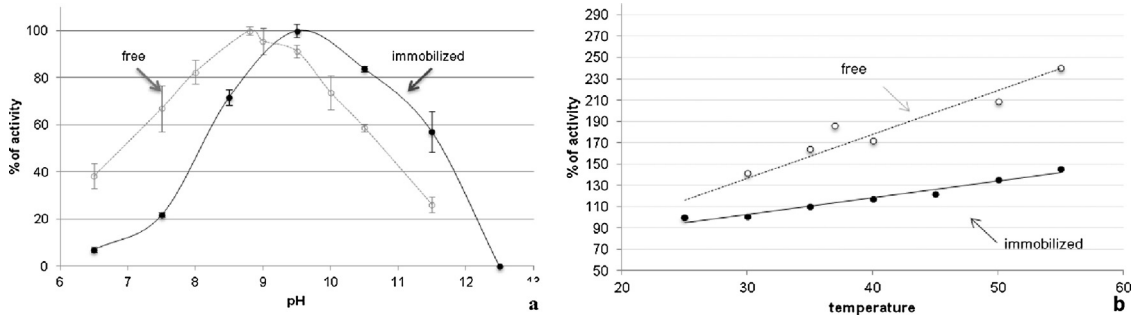

Fig. 3. (a) Optimal pH screening for both free His-HLADH-EE and immobilized His-HLADH-EE between 6.5 and 12.5 for ethanol oxidation. (b) Activity screening at different temperatures (25–55 °C). Maximum activity (100%) is equivalent to 0.6 U/mg for the free enzyme and 1.43 U/mg for the immobilized form.

enzyme bounded and after EDTA washing; (c) resin treated with diaminoacetic acid; (d) resin after complexation with Co^{2+}. Images (c) and (d) showed an increased surface roughness with respect to (b). In the resin sample with micrograph (d) the presence of cobalt was confirmed by the presence of its lines k_α 6.92 keV and k_β 7.65 keV by EDS analysis. In the sample image b), resin-enzyme final stage, no lines of cobalt were detected thus confirming the absence of Co^{++} and excluding metal-contamination of the immobilized enzyme.

3.2.2. pH, temperature, and reusability

Temperature and pH were investigated both for the purpose of determining whether the immobilized His-HLADH-EE had a higher tolerance than the free enzyme to broader temperature and pH ranges, and to determine the optimal pH and temperature for the catalysis (Fig. 3).

The pH optima were determined for the immobilized enzyme by performing the ethanol oxidation reaction between pH 6.5 and 12.5 (Fig. 3a). The immobilized His-HLADH-EE showed the usual bell-shaped curve but interestingly, with respect to the free His-HLADH-EE, it showed a shift in the optimal pH towards alkaline values. The best pH appears to be 9.5 but 60% of activity is still observed at pH 11.5.

The immobilized His-HLADH-EE and the free enzyme showed a linear increase in activity with increasing temperature between 25 °C and 55 °C (Fig. 3b), though the free enzyme presented a more dramatic increment than the immobilized counterpart [38]. Above 55 °C the UV traces were no longer reliable, probably due to ethanol evaporation and enzyme instability.

The free and immobilized enzymes were then also incubated at 37 °C at two different pHs. At pH 8.5 both the immobilized and the free His-HLADH-EE were stable at 37 °C over 160 h, at pH 6.5 the immobilized enzyme was by far more stable overtime than the free counterpart which lost 50% of activity after only 15 h. The immobilized enzyme is routinely stored at 4 °C and we observed that 60% of activity was still retained after 2 months, comparable with the free enzyme [38].

A sample of imHis-HLADH-EE was also assessed for activity over several oxidative cycles. Even after 10 cycles using the same immobilized sample, the specific activity (S.A.) remains virtually unchanged going from 0.45 U/mg to 0.35 U/mg. The slight drop in S.A. can be attributed to the loss of immobilized sample during the analysis (adherence to different vessels) and the rinsing procedures.

3.2.3. Stability in the presence of organic co-solvents

It is well established that enzymes can express catalytic activity in organic media [41,42]. The use of an organic co-solvent in biocatalysis could be necessary whenever the substrate of the bioreaction is quite lipophilic and its solubility in the aqueous buffer solution is poor. It is thus important to test the tolerance of our free and immobilized His-HLADH-EE for little amounts of organic co-solvents such as CH_3CN, THF, DMSO, and MeOH. It is important to underline that methanol is not a substrate for HLADH, for

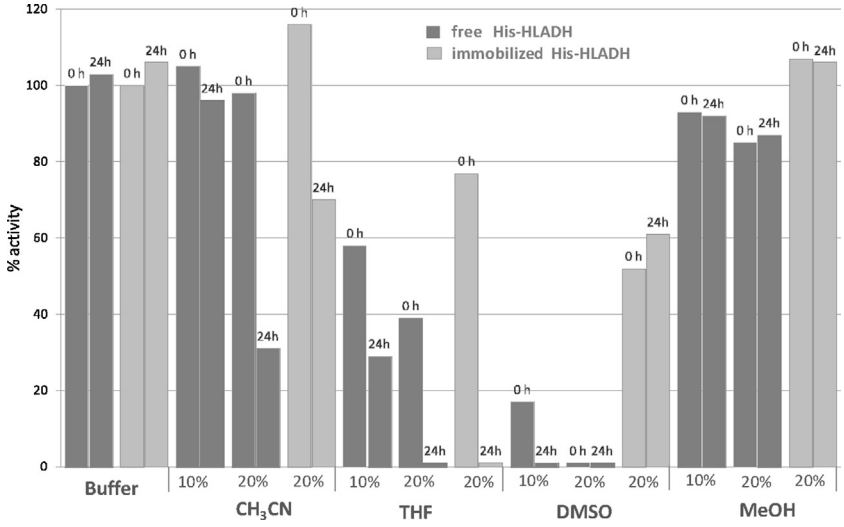

Fig. 4. Stability of the free and immobilized enzyme in different solvents at 10 and 20% concentration in 50 mM Tris–HCl, pH 8.5 buffer. The experiment is performed at 25 C, over 24 h and activity determined per standard procedure. The volume of each sample is 0.2 mL containing either 50 mg of imHis-HLADH-EE or 0.7 mg/mL of His-HLADH-EE.

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this reason it could be considered purely as a solvent [43–46]. The behaviour of the free and immobilized His-HLADH-EE in the presence of a 10 or 20% of co-solvents was compared to that in 50 mM Tris buffer solution, pH 8.5 at time zero and after 24 h (Fig. 4). In all cases the activity was normalized to time 0 in buffer alone. The free His-HLADH-EE well tolerated a 10% of CH_3CN but it was significantly affected when the co-solvent was increased to 20% with a loss of almost 70% of activity within 24 h. Interestingly, the immobilized His-HLADH-EE tested with 20% of CH_3CN showed a substantial increment in activity with respect to the free enzyme, and it was significantly more stable retaining over 70% activity after 24 h. The immobilization had an important stabilizing effect when DMSO was used. The free His-HLADH-EE did not tolerate even 10% of DMSO, while the immobilized form retains 60% of activity after 24 h in 20% of solvent. THF was still poorly tolerated, while MeOH minimally affected the stability of free His-HLADH-EE and a slightly increase of activity of the immobilized form was observed.

3.3. His-HLADH-EES catalyzed reduction of aldehydes

Alcohol dehydrogenases are valuable catalysts used in bioreduction of aldehydes to get alcohols [47]. Feasibility of the new His-HLADH-EE to efficiently work in the synthesis of aromatic and aliphatic primary alcohols and, in particular, in the enantioselective synthesis of 2-arylpropanols was tested [48].

3.3.1. Synthesis of 2-arylpropanols (Profenols)

Immobilized His-HLADH-EE was screened in the reduction of arylpropanals **1a–c.** All the reactions were performed utilizing a coupled-substrate approach employing ethanol as co-substrate to regenerate the co-factor NADH.

Using racemic 2-phenypropanal (**1a**) as model substrate we analyzed the influence of enzyme amount and pH on reaction yields, data are reported in Fig. 5. Almost a quantitative yield was obtained in phosphate buffer at pH 7.5 with 10% CH_3CN as co-solvent to favor aldehyde solubilization, whilst increasing the pH the yields diminished. The pH optima in this case is lower than that above

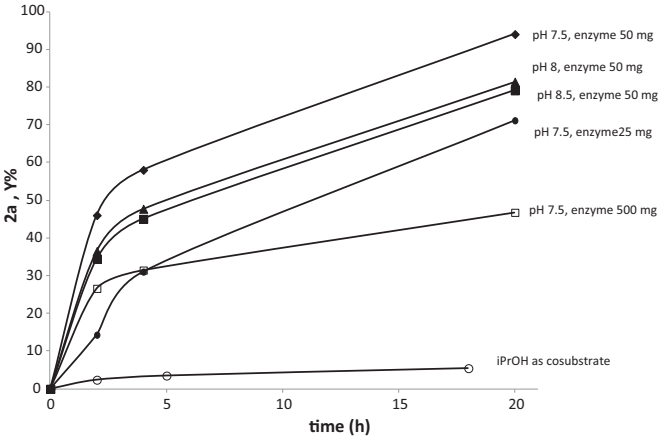
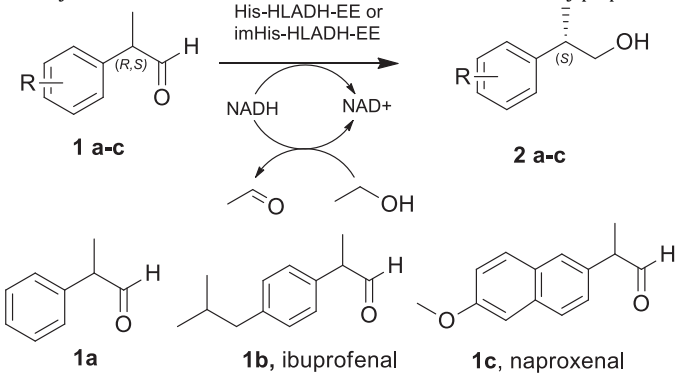


Fig. 5. Influence of pH and enzyme amount on enzymatic reduction of **1a** (0.5 mM) to give **2a** using immobilized His- HLADH-EE in 5 mL solution of phosphate buffer and CH_3CN 10% with catalytic NADH. ♦,▲, ■, ●, □ ethanol as co-substrate; ○: pH 7.5, 50 mg of enzyme and isopropanol as co-substrate.

reported for the oxidation of ethanol (Fig. 3). It must be considered that in those conditions a DKR process is in action so yields over 50% are indicative of an efficient interplay of the enzymatic reduction with the racemization of the starting aldehyde [31]. With regards to the enzyme amount, we tested the reaction on **1a** (0.5 mM) with 500, 50 and 25 mg of immobilized His-HLADH-EE (0.4, 0.04 and 0.02 units according to the results reported above) finding the best results with 50 mg amount. Surprisingly, higher amounts of enzyme acted unfavorably on reaction course. The use of isopropanol as co-substrate for NADH recycling through oxidation to acetone has also been tested but it resulted ineffective (Fig. 5 empty circle data).

Activity data for both free and immobilized His-HLADH-EE on selected racemic 2-arylpropanals are reported in Table 1. The reaction conditions (pH, temperature, substrate concentration,

Table 1
Activity data of free and immobilized His-HLADH-EE on selected 2-arylpropanals **1a–c**.



Ent.	Ald.	Enzyme	Solvent	Alcohol Y%				(S/R)
				2 h	4 h	18 h	Final yield (time, h)	
1	**1a**	His-HLADH-EE	CH_3CN 10%	56	74	90	100 (42)	>99/1
2	**1b**	His-HLADH-EE	CH_3CN 10%	33	39	64	61 (42)	>99/1
3	**1c**	His-HLADH-EE	CH_3CN 10%	56	65	92	100 (66)	>99/1
4	**1a**	imHis-HLADH-EE	CH_3CN 10%	46	53	77	88 (42)	>99/1
5	**1b**	imHis-HLADH-EE	CH_3CN 10%	18	21	37	34 (92)	>99/1
6	**1c**	imHis-HLADH-EE	CH_3CN 10%	16	20	63	79 (42)	>99/1
7	**1a** (5 mM)	imHis-HLADH-EE	MTBE 50%	3	6	54	82 (42)	>99/1
8	**1a** (5 mM)	imHis-HLADH-EE	Hexane 50%	10	10	39	84 (72)	>99/1

Experimental conditions: aldehyde 0.5 mM (entries 1 to 6 method A), 0.01 mM NADH, 0.5 M EtOH, 0.1 M phosphate buffers pH 7.5, 27 °C, V = 5 mL, enzyme amount 50 mg. Entries 7 and 8 method B.

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co-factor and co-substrate amounts) have been chosen on the basis of the results above reported.

Using the free His-HLADH-EE in a 10% mixture of CH_3CN in phosphate buffer as solvent, excellent reaction yields were obtained in the case of 2-phenylpropanal and naproxenal while ibuprofenal gave poorer yields. An analogous trend has been observed with immobilized His-HLADH-EE even if the reaction rate decreased and the alcohol was not obtained quantitatively due to acetophenone-like by-products that 2-arylpropanals are known to form with prolonged reaction time [31]. Notably, with both free and immobilized enzyme, the enantioselectivity was always total for the *S* configuration and no traces of *R* enantiomers were detected. This is peculiar of His-HLADH-EE, because, as we already reported, the commercial HLADH gave lower enantiomeric ratios of arylpropanols depending on co-solvents. It is not generally observed that the creation of a fusion His-tag protein alters the substrate stereospecificity, though the enzyme may become less effective and, at times, less stable. One study performed on two lipases, reported on the influence of the *N*-terminal poly-histidine-tag on the regio- and stereoselectivity of the recombinant enzymes, showing that while the efficiency of the catalysts was substantially unaffected, both the regio- and enantio- preference were in fact significantly altered [49]. This is the first time such a result is seen in oxidoreductases. A possible explanation in this case is that the poly-histidine tag could somewhat affect the enzyme flexibility and therefore the conversion rate of the least favorable enantiomer.

We exploited the advantages of enzyme immobilization by performing the enzymatic reduction of 2-phenylpropanal in a biphasic system with immiscible organic solvents, in particular we used *tert*-butylmethylether (MTBE, entry 7) and hexane (entry 8). In these cases we could successfully use higher concentration of aldehyde with yields and enantioselectivity comparable to those obtained with homogeneous buffer/co-solvent mixtures.

The recyclability of the immobilized His-HLADH-EE was tested in the reduction of a solution of **1a** (5 mM) in acetonitrile 10% and data are reported in Fig. 6.

Before re-use, the enzyme was accurately washed with phosphate buffer and re-charged with 0.5 mL of a 5 mM solution of **1a** in CH_3CN. Yields and rate stay constant until the third cycle, during the fourth cycle we recorded quantitative yields but a decrease in reaction rate whereas from the fifth cycle the reaction remained incomplete.

To shed light on the main factors that lead to enzyme inactivation after the fourth cycle, we analyzed the effect of increasing the concentration of the starting aldehyde on reaction yields (Fig. 7).

Other conditions being equal, we recorded lower yields when the initial aldehyde concentration was increased from 0.5 mM to 5 mM till 25 mM thus suggesting that the starting material inhibits the enzyme activity at millimolar concentration. Unfortunately the inhibition effect appears irreversible, after washing the imHis-HLADH-EE just used in a first run with 5 mM aldehyde concentration and running it in a second cycle with a lower

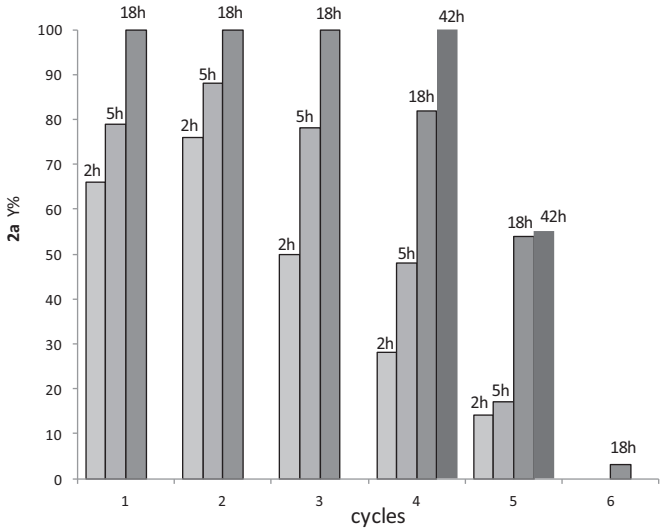


Fig. 6. Recyclability of one sample of imHis-HLADH-EE (50 mg) on the enzymatic reduction of 2-phenylpropanal **1a** to give 2-phenylpropanol (**2a**) at different reaction times (method A).

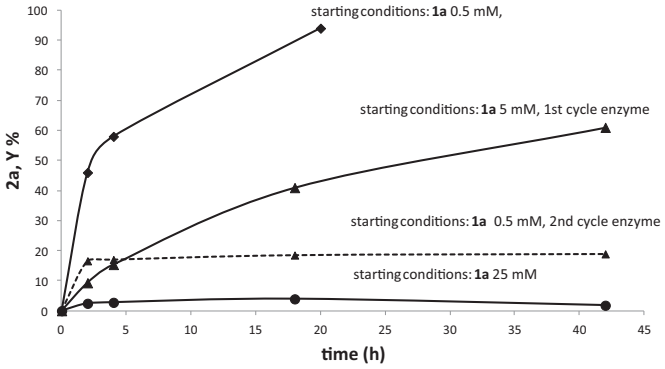
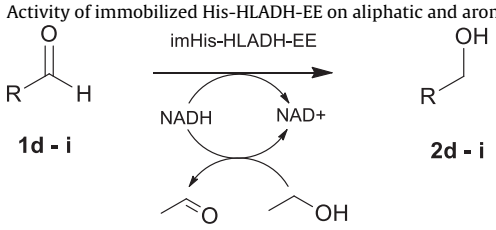


Fig. 7. Influence of starting aldehyde concentration on enzymatic reduction of **1a** to **2a** using imHis-HLADH-EE (50 mg) in phosphate buffer and CH_3CN 10% with catalytic NADH, ethanol as co-substrate (method A).

Table 2
Activity of immobilized His-HLADH-EE on aliphatic and aromatic aldehydes.



Ent.	Aldehyde	Aldehyde name	Alcohol	Yield % (time, h)
1	**1d**	Benzaldehyde	**2d**	100 (5)
2	**1e**	p-Methoxybenzaldehyde	**2e**	95 (20)
3	**1f**	p-Nitrobenzaldehyde	**2f**	100 (5)
4	**1g**	Cinnamaldehyde	**2g**	100 (2)
5	**1h**	Cyclohexanecarboxyaldehyde	**2h**	100 (20)
6	**1i**	Octanal	**2i**	100 (5)

Experimental conditions: aldehyde 0.5 mM, 0.01 mM NADH, 0.5 M EtOH, 0.1 M phosphate buffers pH 7.5, 27 °C, *V* = 5 mL, enzyme amount 50 mg, CH_3CN 10%, method A.

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aldehyde concentration, a satisfying enzymatic activity was not restored and yields were very low (dotted curve in Fig. 7). Combining together low reaction yield data obtained with a higher concentration of aldehyde and enzyme we can hypothesize that some amine residues on the enzyme reacted with the substrate carbonyl function.

3.3.2. Enzymatic reduction of aliphatic and aromatic aldehydes

Investigating the scope of the bio-reduction, the activity of immobilized His-HLADH-EE on aliphatic and aromatic aldehydes to give primary alcohols is reported in Table 2. Aromatic aldehydes (entries 1–3) were quantitatively transformed into the corresponding benzylalcohols with *p*-methoxybenzaldehyde reacting slower than benzaldehyde and *p*-nitrobenzaldehyde. Aliphatic aldehydes (saturated, entry 5 and 6, and unsaturated, entry 4) yielded the corresponding alcohols quantitatively.

For what concerns the activity on other carbonyl substrates, we tested the activity of both free and immobilized His-HLADH-EE on several ketones and keto-esters such as acetophenone, 4-nitroacetophenone, 2-phenylcyclohexanone, 2-chlorocyclo hexanone, 4-*t*-butylcyclohexanone, β-ionone, 4-*N*-benzyl piperidone, benzyl acetoacetate and 2-phenylpyruvic acid without detecting the alcohol formation. The chemo-selectivity of His-HLADH-EE towards aldehydes is also confirmed by the inefficiency of isopropanol in NADH recycling, as above described (Fig. 5, empty circle data set). On the other hand, in an early report Klibanov observed reduction of ketones by the commercial HLADH [20]. This is likely to be due to the presence of multiple HLADH isoforms in the commercial enzyme at the time and strengthens the result of a higher chemoselectivity of the new His-HLADH-EE.

4. Conclusions

The histidine-tagged Horse Liver Alcohol Dehydrogenase (His-HLADH-EE) was successfully purified and immobilized onto a solid support by means of a metal-directed technique. Effects of pH, temperature and organic co-solvents on the imHis-HLADH-EE were deeply investigated and they showed a shift in the optimum pH with respect to the free form as well as an increased stability to temperature and organic co-solvents. Application of the free and immobilized His-HLADH-EE to the chemo-enzymatic synthesis of (*S*)-Profenols demonstrated enhanced enantioselectivity in the dynamic kinetic resolution process and high reusability of the immobilized form. The imHis-HLADH-EE proved also to be effective in the chemo-selective reduction of aliphatic and aromatic aldehydes. The achievement of a robust and effective immobilization of an alcohol dehydrogenase realized the scope to get a sustainable bio-reduction of aldehydes to valuable industrial intermediates.

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