

SUPPORTING INFORMATION

Continuous flow biocatalysis: production and in-line purification of amines by immobilised transaminase from *Halomonas elongata*

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1 - Materials and methods

1.1. Expression and purification of the HEWT in *E. coli*

Protein expression and purification was performed following previously reported protocols.¹

1.2. Protein immobilisation

1 g of Sepabeads® EC-RP/S (Resindion S.r.l.) immobilisation support were treated with 2 mL of modification buffer (0.1 M of sodium borate and 2 M of iminodiacetic acid in Tris-HCl buffer 50 mM pH 8.5) under gentle shaking for two hours at room temperature. The sample was then filtered and washed with distilled water and metal containing solution (1 M of sodium chloride and 5 mg/mL of metal cation in phosphate buffer 50 mM pH 6) applied for two hours. 2 mL of enzyme solution, prepared assuming a ratio of 1 mg of enzyme per 1 g of beads, were added and kept under agitation for 24 hours. For the flow experiment, the immobilisation was developed using up to 5 mg per g. The beads were filtered and washed thoroughly with desorption buffer (0.05 M of EDTA and 0.5 M of sodium chloride in phosphate buffer pH 7.4 20 mM) and distilled water, 4 mL of blocking buffer (3 M of glycine in phosphate buffer pH 8.5 50 mM) were added and the suspension left under agitation for 20 hours. Finally, the beads were washed, collected and conserved in 2 mL of Storage Buffer (pH 8, 50 mM phosphate, 0.1 mM PLP). The imm-HEWT was routinely stored at 4 °C.

1.3. Spectrophotometric enzymatic assays

A kinetic assay derived from Schatzle, *et al.*² was used as standard enzymatic assay following the procedure applied in Cerioli, *et al.*¹

The activity of immobilised HEWT was determined by weighing an appropriate amount of imm-HEWT (25-50 mg) into a 25 mL reaction tube with cap, followed by the addition of 10 mL reaction mixture (50 mM phosphate pH 8.0, containing 2.5 mM pyruvate, 2.5 mM (S)-(-)-1-phenylethylamine, 0.1 mM PLP and 0.25 % DMSO). The immobilised enzyme reaction mixture was shaken at 25 °C, 250 rpm and the absorbance at 245 nm was recorded every minute as single readings using Brand UV-cuvettes. The imm-HEWT specific activity (U/g) is defined as μmol of acetophenone formed for minute for grams of immobilised enzyme. For the initial imm-HEWT characterisation the 1 mg per g of resin ratio was used.

1.4. Imm-HEWT catalysed batch reactions

The batch reactions using the imm-HEWT were performed in 1.5 mL micro centrifuge tubes; 500 μ L reaction mixture in 50 mM phosphate buffer pH 8.0, containing 10 mM amino acceptor substrate, amino donor substrate (concentration reported in Tab 1), 0.1 mM PLP, and 50 mg of imm-HEWT (5 mg/g) was left under gentle shaking at 37 °C. 10 μ L aliquots were quenched with trifluoroacetic acid (TFA) 0.2% every hour and then analysed by HPLC equipped with a Supelcosil LC-18-T column (250 mm x 4.6 mm, 5 μ m particle size; Supelco, Sigma-Aldrich, Germany). The compounds were detected using an UV detector at 210 nm, or 250 nm after an isocratic run with 25% acetonitrile/75% water with TFA 0.1% v/v at 25 °C with a flow rate of 1 mL/min. The retention times in minutes are: (S)-(-)-1-phenylethylamine (5.3 min), acetophenone (15.2 min), *p*-nitrobenzaldehyde (12.2 min), (*p*-nitrophenyl)methanamine (4.4 min), vanillin (5.7 min), vanillylamine (3.7 min), cinnamaldehyde (16.9 min), cinnamylamine (6.6 min).

1.5. Imm-HEWT catalysed flow reactions

The continuous flow biotransformations were performed using a R2+/R4 flow reactor commercially available from Vapourtec[®] equipped with an Omnifit[®] glass column (6.6 mm i.d x 100 mm length) filled with 1.0 g of imm-HEWT (5 mg/g). A 20 mM amino acceptor substrate solution (solution A, for *p*-NO₂-benzaldehyde 10 mM solution, Scheme S.1) in phosphate buffer (50 mM, pH 8.0) containing 0.1 mM PLP and a 1 M amino donor solution (solution B, Scheme S.1) were prepared. The percentage of cosolvent (*i.e.*, DMSO) is reported in the text (Table 1). The two solutions were mixed in a T-piece and the resulting flow stream was directed into the column packed with the biocatalyst (packed bed reactor volume: 0.78 mL). The flow rate was varied and optimised. The exiting flow stream was analysed by HPLC using the conditions reported in paragraph 1.4.

1.6. In-line purification procedure and product isolation

Under optimised conditions (Table 1, entry 2, total flow rate: 0.39 mL/min), an in-line basification to pH 11 was performed by using an inlet of 1N NaOH aqueous solution (flow rate: 0.20 mL/min) that was mixed to the exiting reaction flow stream using a tee junction. The resulting aqueous phase was extracted in-line using a stream of EtOAc (flow rate: 0.59 mL/min) and a Zaiput liquid/liquid separator. The organic phase, containing the amine and possible traces of unreacted aldehyde, was passed through a column packed with a polymer supported benzylamine (PS-BZA) (Scheme S.1). The organic solvent was evaporated to yield the desired amine. The purity of the amine was assessed by HPLC and ¹H NMR. ¹H NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (*J*) are expressed in Hz.

(*p*-nitrophenyl)methanamine.⁴ Yellow oil; ¹H NMR (300 MHz, CDCl₃): 4.06 (2H, s), 7.55 (2H, d, *J* = 8.4), 8.20 (2H, d, *J* = 8.4).

(*E*)-3-phenylprop-2-en-1-amine (cinnamylamine).⁵ Slightly yellow oil; ¹H NMR (300 MHz, CDCl₃): 3.48 (2H, d, *J* = 4.8), 6.25 (1H, dt, *J* = 4.8, 15.6), 6.52 (1H, d, *J* = 15.6), 7.20–7.40 (5H, m).

1.7. Flow synthesis of vanillylamine

An Omnifit® glass column (6.6 mm i.d × 100 mm length) was packed with 1.0 g of imm-HEWT (5 mg/g). A 20 mM vanillin solution (solution A, Scheme S.2) in phosphate buffer (50 mM, pH 8.0) and 10% DMSO containing 0.1 mM PLP and a 1 M alanine solution (solution B, Scheme S.2) were prepared. The two solutions were mixed in a T-piece and the resulting flow stream was directed into the column packed with the biocatalyst (packed bed reactor volume: 0.78 mL, residence time: 2 min, T = 37 °C). The exiting flow stream was directed into a column (10 mm i.d × 100 mm length) packed with A-15 resin (packed bed reactor volume: 3.8 mL) and then collected into a separated flask from which was newly pumped through the imm-HEWT packed column (Scheme S.2) after mixing with the amine donor solution. After 5 cycles a 50% conversion was achieved. The vanillylamine was then recovered as the salt from the A15 resin by flowing through the A-15 column 1N HCl.

(4-hydroxy-3-methoxyphenyl)methanaminium chloride (vanillylamine hydrochloride).⁶ ¹H NMR (300 MHz, DMSO-*d*₆): 3.75 (s, 3H), 3.85 (m, 2H), 6.75 (1H, d, *J* = 8.1), 6.82 (1H, dd, *J* = 1.9, 8.1), 7.10 (1H, d, *J* = 1.9), 8.20 (3H, bs), 9.18 (1H, s).

2 - Metals screening to optimise HEWT immobilisation

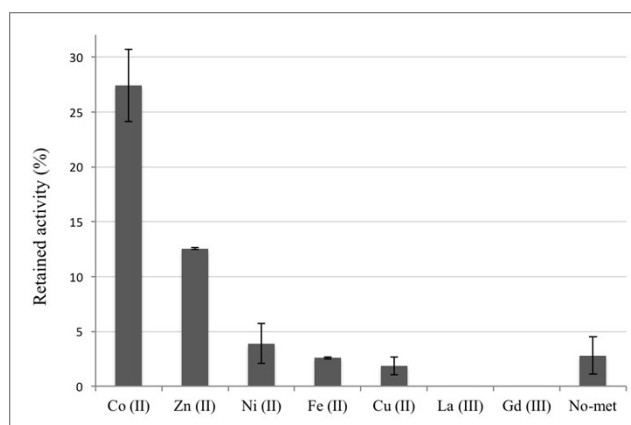


Fig. S.1: Activity of imm-HEWT when different metal ions (or no metal) were used for the immobilisation process; 100% corresponds to 6.6 U/mg of the free enzyme. The best result was obtained with cobalt (II), which yielded a complete immobilisation of the protein with no detectable activity (or even inactive, denatured protein) in the supernatant. Nickel (II), iron (II), and copper (II) gave unsatisfactory results, possibly due to toxicity or excessive affinity. Based on the patent WO2013179072A1 from Poppe et al. lanthanides have been applied in IMAC applications³, however in our hands neither lanthanum (La³⁺) or gadolinium (Gd³⁺) showed any affinity despite longer incubation times. Interestingly, the supernatant after addition of HEWT to both La³⁺ and Gd³⁺ beads had virtually unchanged activity (no toxicity detected).

3 - imm-HEWT enhanced activity and stability

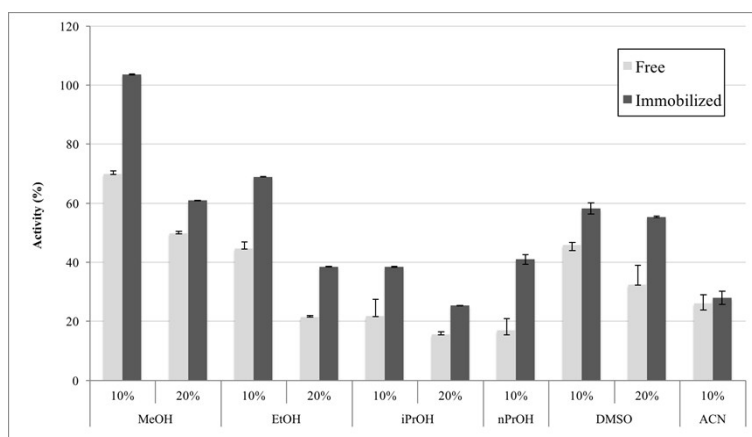


Fig. S.2: Stability of the free (light columns) and imm-HEWT (dark columns) in different co-solvents at 10 and 20% concentration in 50 mM Phosphate, pH 8.0 buffer. With 10% methanol, the activity was comparable with the control (103.5%), however, with 20% the activity dropped by a third. With ethanol and propanol, increased stability was observed for the immobilised preparation. Results for the imm-HEWT with 20% shows almost twice the stability of the free form.

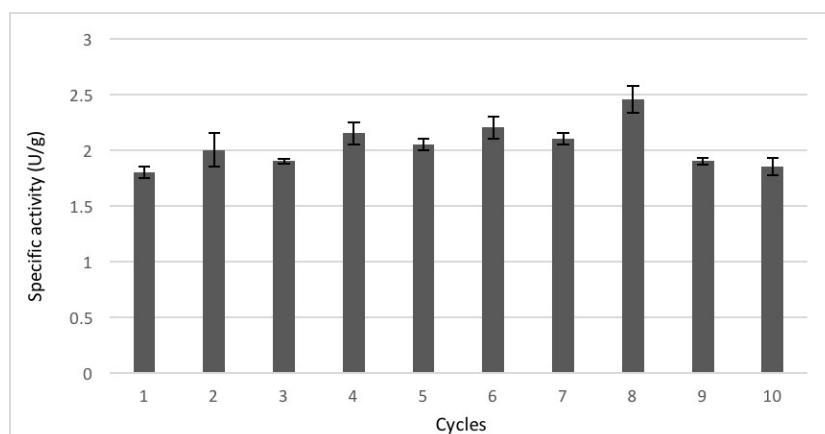


Fig. S.3: Reusability profile of the immobilized HEWT after ten reaction cycles. The experiment was conducted repeating the activity assay ten times; every time the imm-HEWT was isolated from the exhausted mixture and used in the following run. After 10 cycles using the same immobilised sample, the specific activity remained stable between 1.8 and 2.0 U/g.

4 - One-step purification and immobilisation

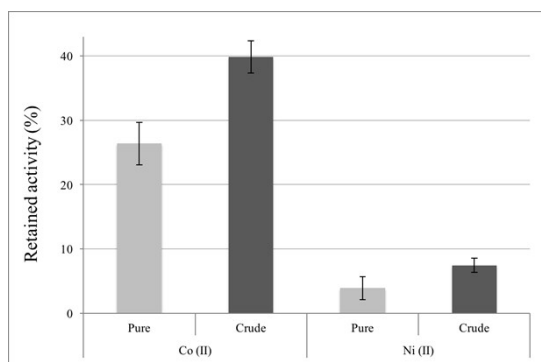
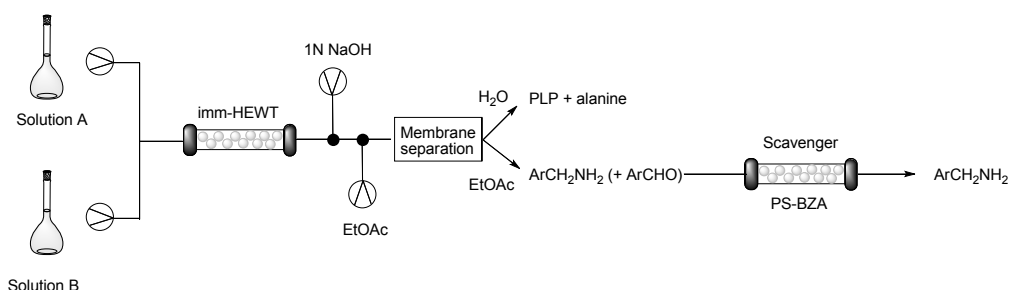
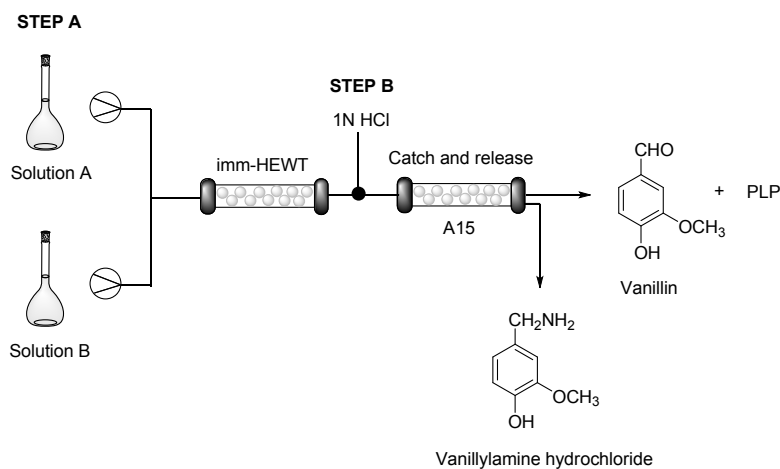


Fig. S.4: Retained activity for immobilized pure HEWT and crude lysate, performed both with cobalt (II) and nickel (II).

5 – Schematic representation of flow reaction set ups exploiting the imm-HEWT



Scheme S.1: Solution A: 20 mM amino acceptor substrate solution (10 mM solution in the case of *p*-NO₂-benzaldehyde) in phosphate buffer (50 mM, pH 8.0) containing 0.1 mM PLP. The percentages of cosolvent (i.e., DMSO) are reported in the text (Table 1). Solution B: 1 M amino donor (alanine) solution in phosphate buffer (50 mM, pH 8.0). *T* = 37 °C, *P* = atm.



Scheme S.2: Solution A: 20 mM vanillin solution in phosphate buffer (50 mM, pH 8.0) and 10% DMSO containing 0.1 mM PLP. Solution B: 1 M alanine solution in phosphate buffer (50 mM, pH 8.0). *T* = 37 °C, *P* = atm.

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