# Electronic supplementary information (ESI)

## Self-sufficient asymmetric reduction of β-ketoesters catalysed by a novel and robust thermophilic alcohol dehydrogenase co-immobilized with NADH

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# Content

# Supplementary Figures

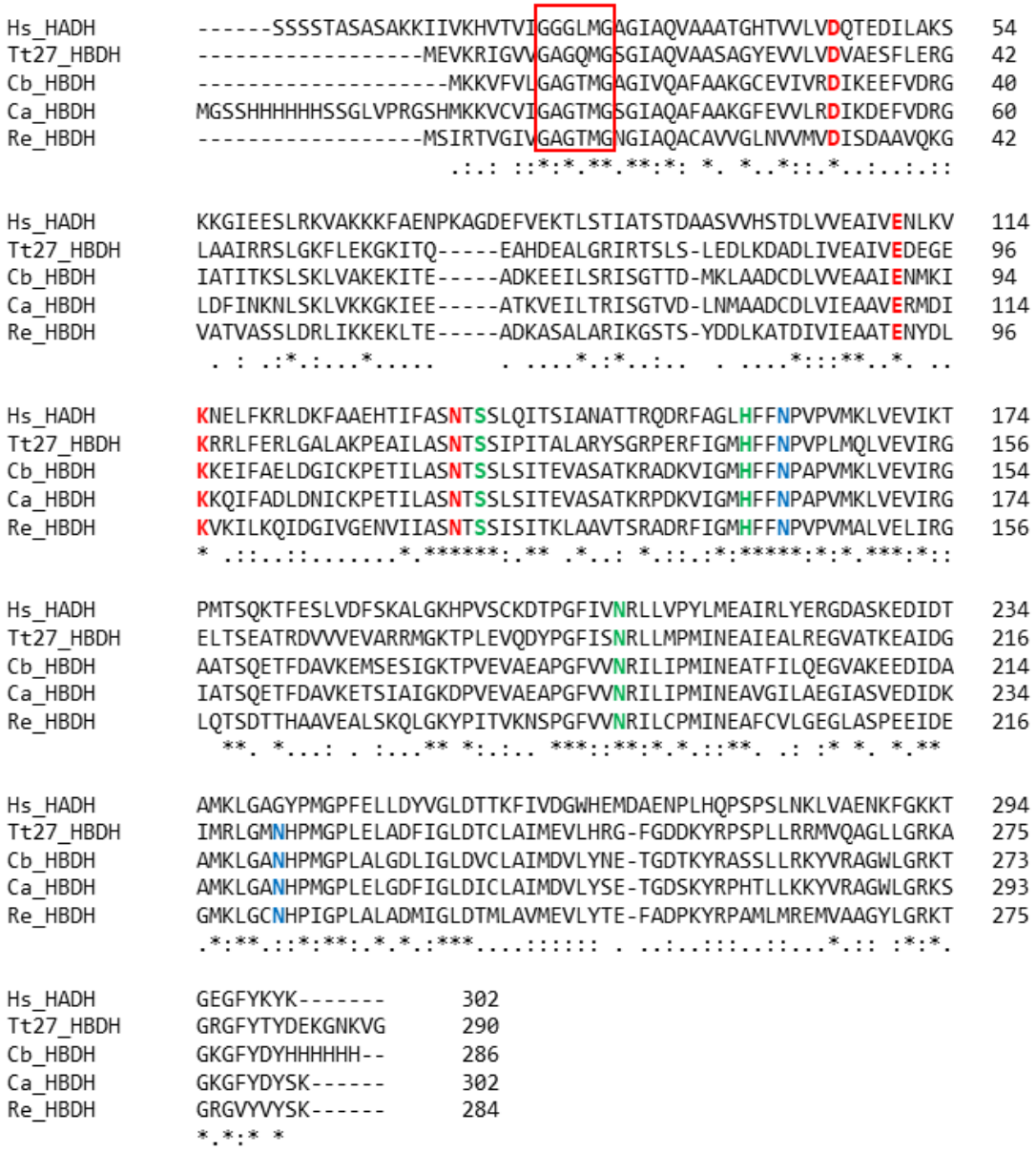


**Figure S1. Multiple protein alignment of different 3-hydroxyacyl-CoA dehydrogenases.** The sequences were aligned using Clustal Omega algorithm. The green bolded residues correspond to the catalytic triad. The red bolded residues correspond to those involved in cofactor binding. The blue bolded residues correspond to those involved in acetoacetyl-CoA binding. The nucleotide binding-motif (G-x-G-x-x-G) is highlighted in the red box.

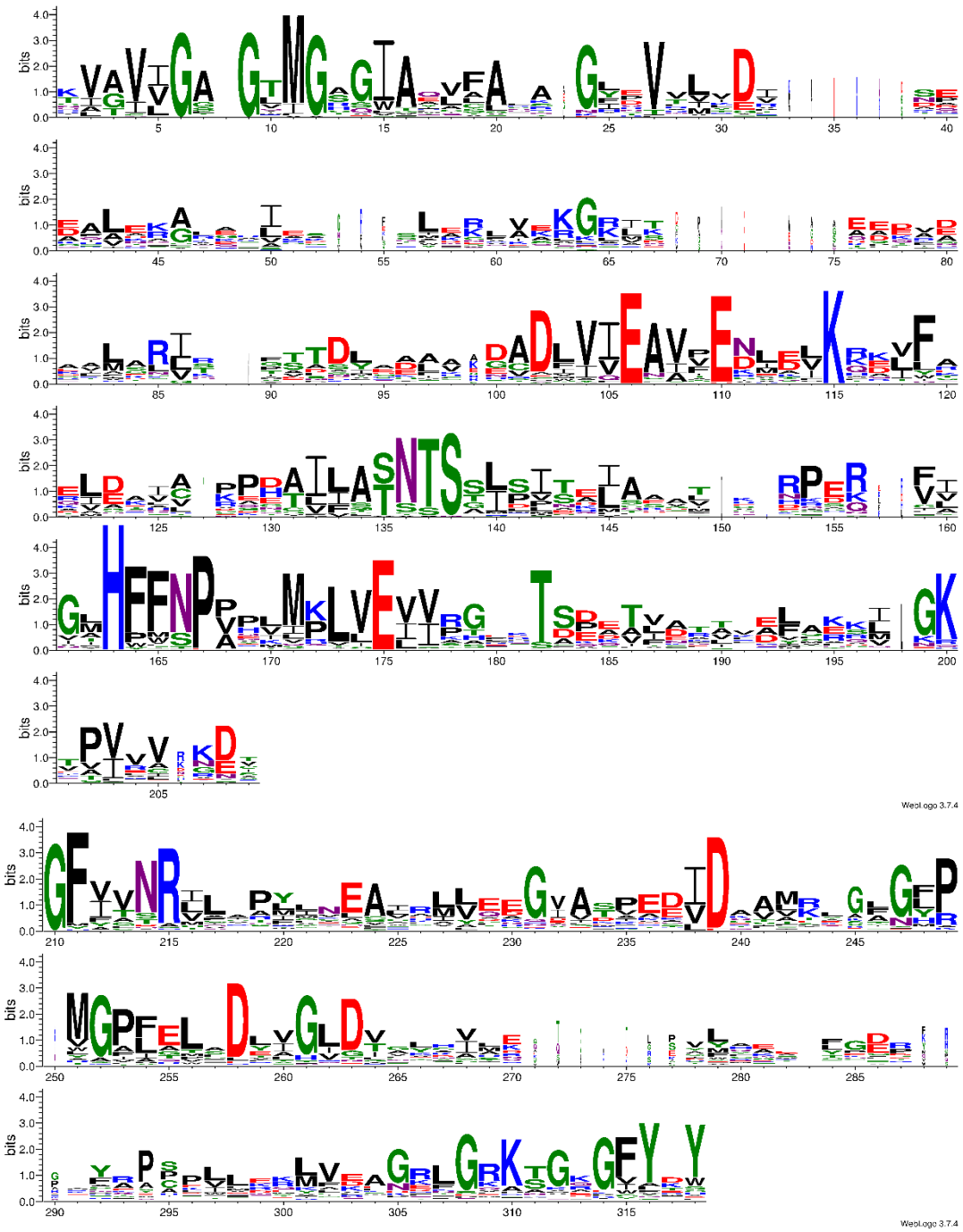


**Figure S2. Sequence logo of 3HCDH_N and 3HCDH Pfam families.**

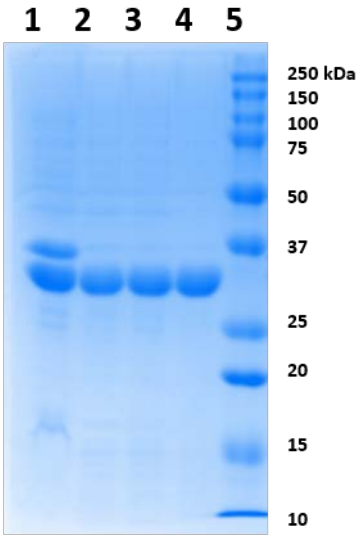


**Figure S3. SDS-PAGE of the expression and purification of Tt27-HBDH.** Lane 1: Crude extract. Lane 2: Soluble fraction after cell disruption by sonication. Lane 3: Total fraction after thermal shock: 30 min at 70 ºC. Lane 4: Pure Tt-ADH: soluble fraction after thermal shock treatment (molecular weight 31.78 kDa). Lane 5: Molecular weight marker (BioRad Precision Plus Protein Dual Color Standard).

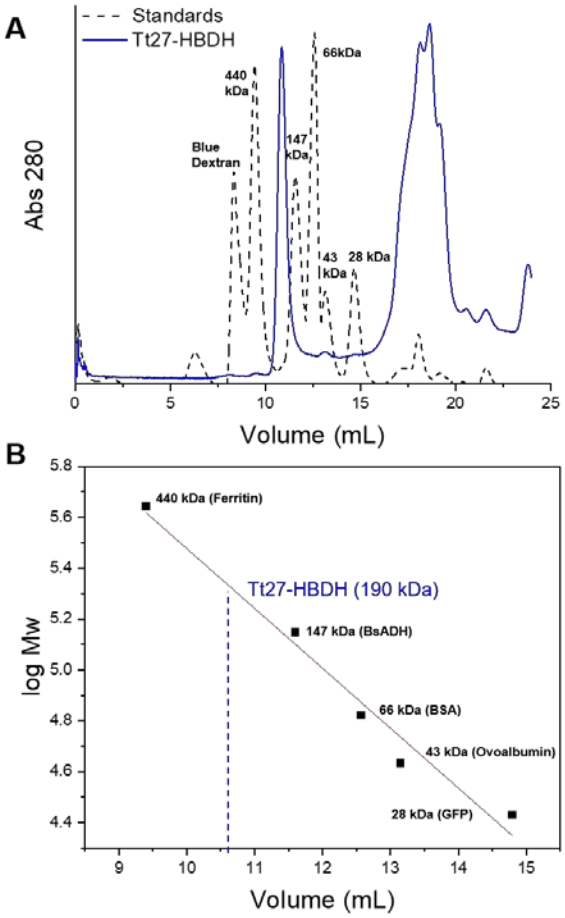


**Figure S4. Molecular weight determination of Tt27-HBDH by Size Exclusion Chromatography.** (A) SEC was performed to determine molecular weight of soluble and pure Tt27-HBDH. A sample of 1 mg/ml of Tt27-HBDH purified by thermal shock in Tris-HCl buffer 25 mM at pH 7 was analysed by SEC. The molecular weight was calculated using a protein/polymer standard calibration curve: blue dextran (2000 kDa), ferritin (440 kDa), alcohol dehydrogenase from *Bacillus stearothermophilus* (147 kDa), bovine serum albumin (63 kDa), Ovoalbumine (43 kDa) and GFP (28 kDa). (B) A logarithmic calibration curve was obtained from elution volume data of the different proteins. With the equation: Mw = $\mathbf{10}^{(-0.2353*\boldsymbol{Elution\ Volume})+7.830}$ an apparent Mw of 190 kDa was predicted for the Tt27-HBDH.

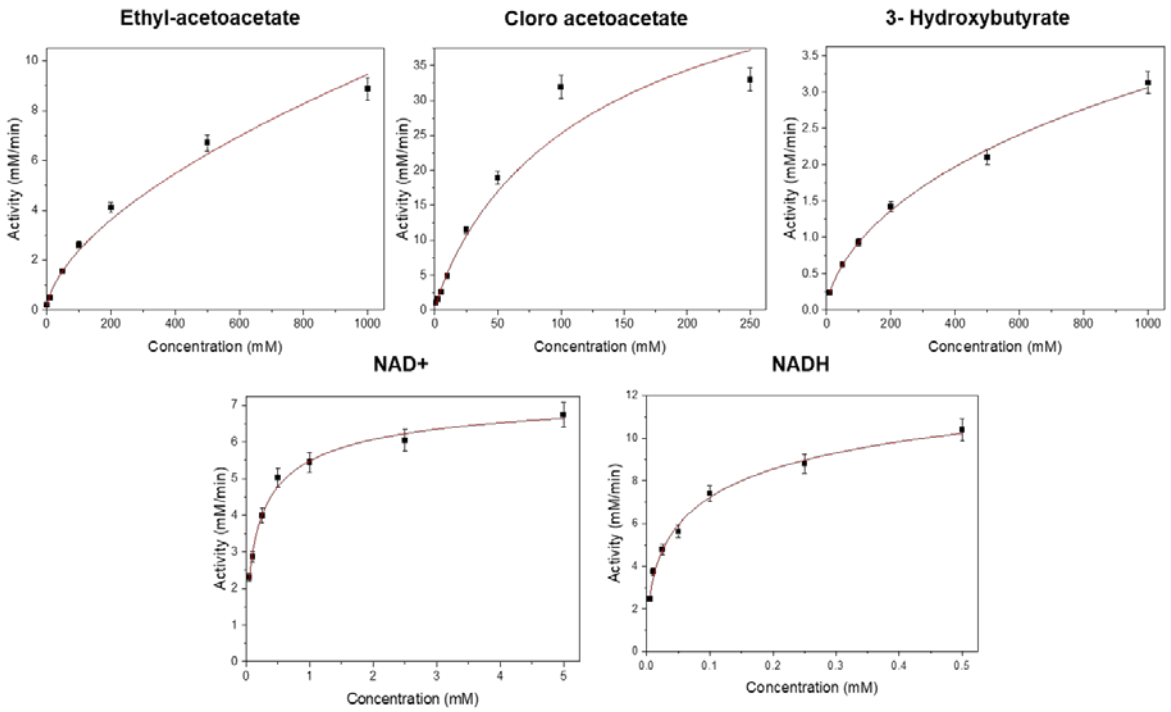


**Figure S5. Michaelis-Menten curves of Tt27-HBDH for different substrates or cofactors.** Oxidative steady-state kinetics were calculated towards different concentrations of ethyl (*S*)-3-hydroxibutyrate (0.01-1 M) and $NAD^+$ (0.01-5 mM). Reductive steady-state kinetics were calculated towards different concentrations of ethyl acetoacetate (0.001-1 M), ethyl 2-chloroacetoacetate (1-250 mM) and NADH (0.005-0.5 mM). The activities for each substrate concentration were done by triplicate, resulting in a mean value for each substrate concentration. All mean activities were plotted and adjusted to a Michaelis-Menten model using Origin Pro8 software.

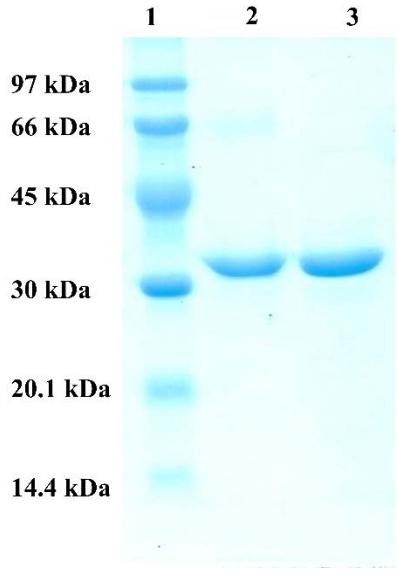


**Figure S6. SDS-PAGE analysis of the Ag-G (3h) biocatalyst.** Lane 1 – Molecular weight markers, Lane 2 Ag-G (3h) biocatalyst, Lane 3 – Soluble Tt27-HBDH.

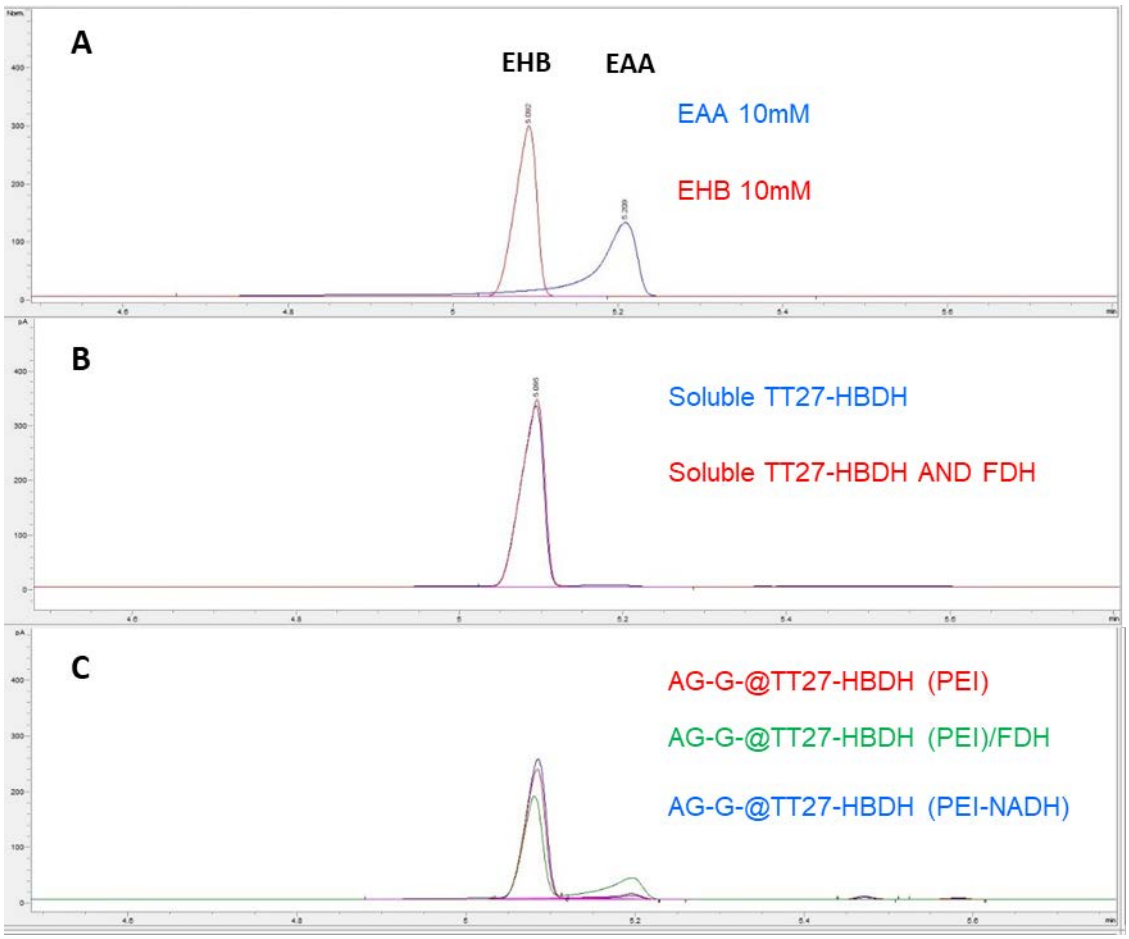


**Figure S7. GC-FID Chromatograms of:** (A) Ethyl acetoacetate (EAA) and ethyl-hydroxybutyrate (EHB) 10 mM standards, retention times: 5.20 and 5.07 min, respectively. (B) Products from a 24 h batch reaction of soluble monoenzymatic (TT27-HBDH) and bi-enzymatic (TT27-HBDH and FDH. (C) Products from batch reactions at 24 h with the monoenzymatic biocatalysts: Ag-G@-TT27-HBDH(PEI), Ag-G@TT27-HBDH(PEI-NADH) and the bi-enzymatic Ag-G@TT27-HBDH(PEI)/FDH. The reaction mixes were: for the monoenzymatic system: 10 mM ethyl-acetoacetate, 5% isopropyl alcohol, 1 mM NADH in 10 mM Tris-HCl pH 8.0. For the bi-enzymatic systems: 10 mM ethyl-acetoacetate, 5% acetonitrile, 1 mM NADH in 10 mM Tris-HCl pH 8.0 and 20 mM ammonium-formate.

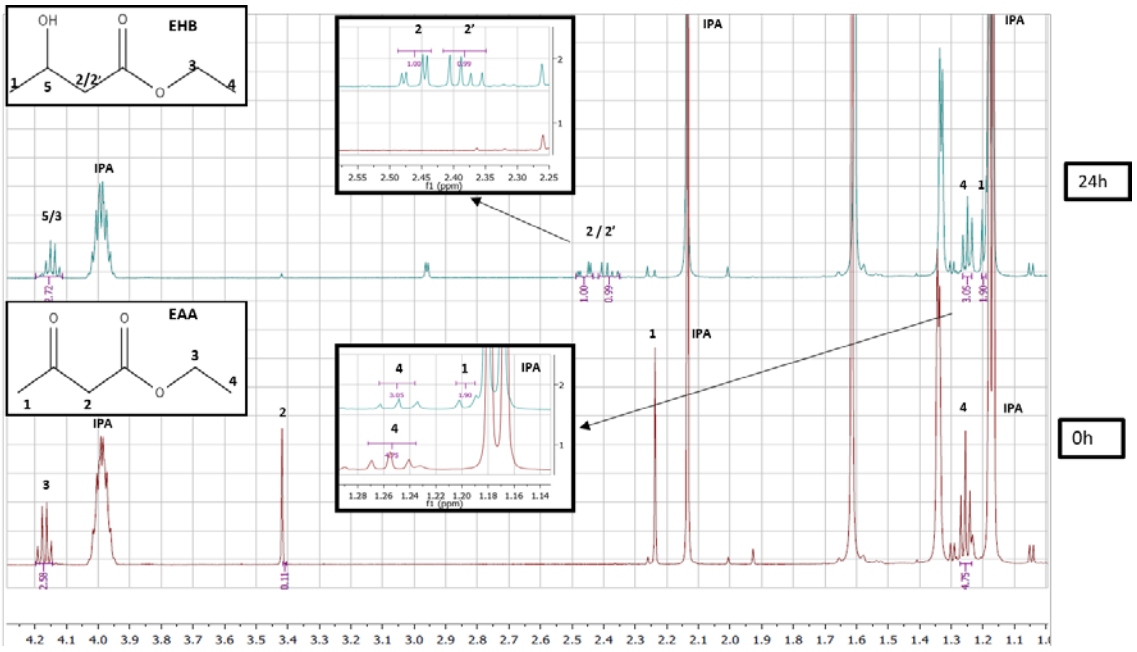


**Figure S8. $^{1}$H NMR (500 MHz, Chloroform-*d*) spectrum of initial reaction mix and final product of a 24 h batch reaction with Ag-G@TT27-HBDH(PEI-NADH)**

**Reaction at t=0h:** $^{1}$H NMR (500 MHz, Chloroform-*d*) δ 4.17 (q, *J* = 7.2 Hz, 3H), 1.26 (t, *J* = 7.2 Hz, 5H). **Final product at t=24h :** $^{1}$H NMR (500 MHz, Chloroform-*d*) δ 4.14 (q, *J* = 7.1 Hz, 3H), 2.46 (dd, *J* = 16.5, 3.3 Hz, 1H), 2.38 (dd, *J* = 16.4, 8.9 Hz, 1H), 1.26 – 1.24 (m, 3H), 1.20 (s, 3H).

We also performed the $^{1}$H NMR spectrum of the substrate and product standards: **Ethyl-acetoacetate (EAA) 10 mM:** $^{1}$H NMR (500 MHz, Chloroform-*d*) δ 4.17 (q, *J* = 7.2 Hz, 2H), 2.24 (s, 2H), 1.26 (t, *J* = 7.1 Hz, 3H). **Ethyl-3-hydroxy-butyrate (EHB) 10 mM**: $^{1}$H NMR (500 MHz, Chloroform-*d*) δ 4.21 – 4.12 (m, 3H), 2.47 (dd, *J* = 16.4, 3.4 Hz, 1H), 2.39 (dd, *J* = 16.5, 8.8 Hz, 1H), 1.25 (t, *J* = 7.2 Hz, 3H), 1.20 (d, *J* = 6.3 Hz, 3H).

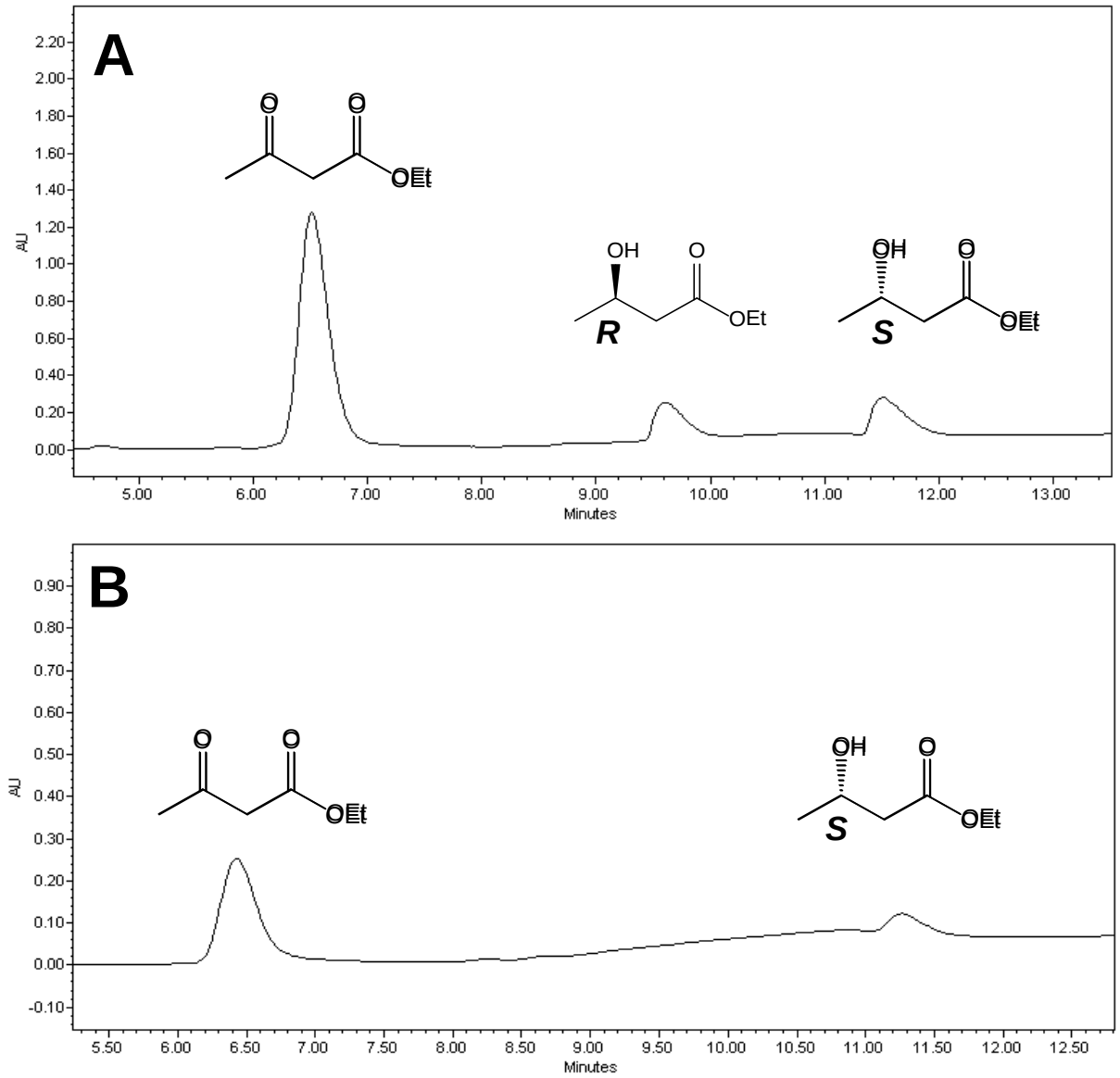


**Figure S9. Chiral HPLC chromatogram.** (A) Standards 1 (RT = 6.24 min), *S*-2 (RT = 9.75 min) and *R*-2 (RT = 11.56 min). (B) Chiral HPLC chromatograms of enzymatic reaction sample. 1 (RT = 6.24 min), *S*-2 (RT = 9.75 min) and *R*-2 (RT = 11.56 min).

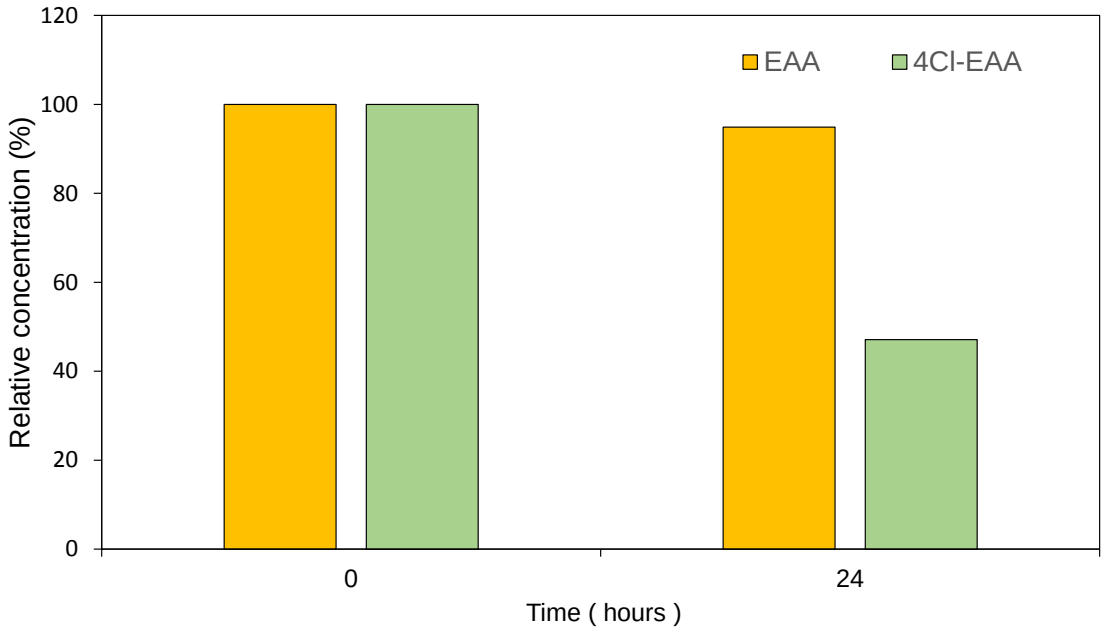


**Figure S10. Spontaneous decarboxylation of EAA and 4-Chloro-EAA under reaction conditions.** A solution of 10 mM of each β-ketoester, 10 mM Tris HCl, 5% 2-propanol at pH 8 was incubated for 24 hours at 25 ºC. Samples were withdrawn at 0 and 24 hours and analyzed by GC.

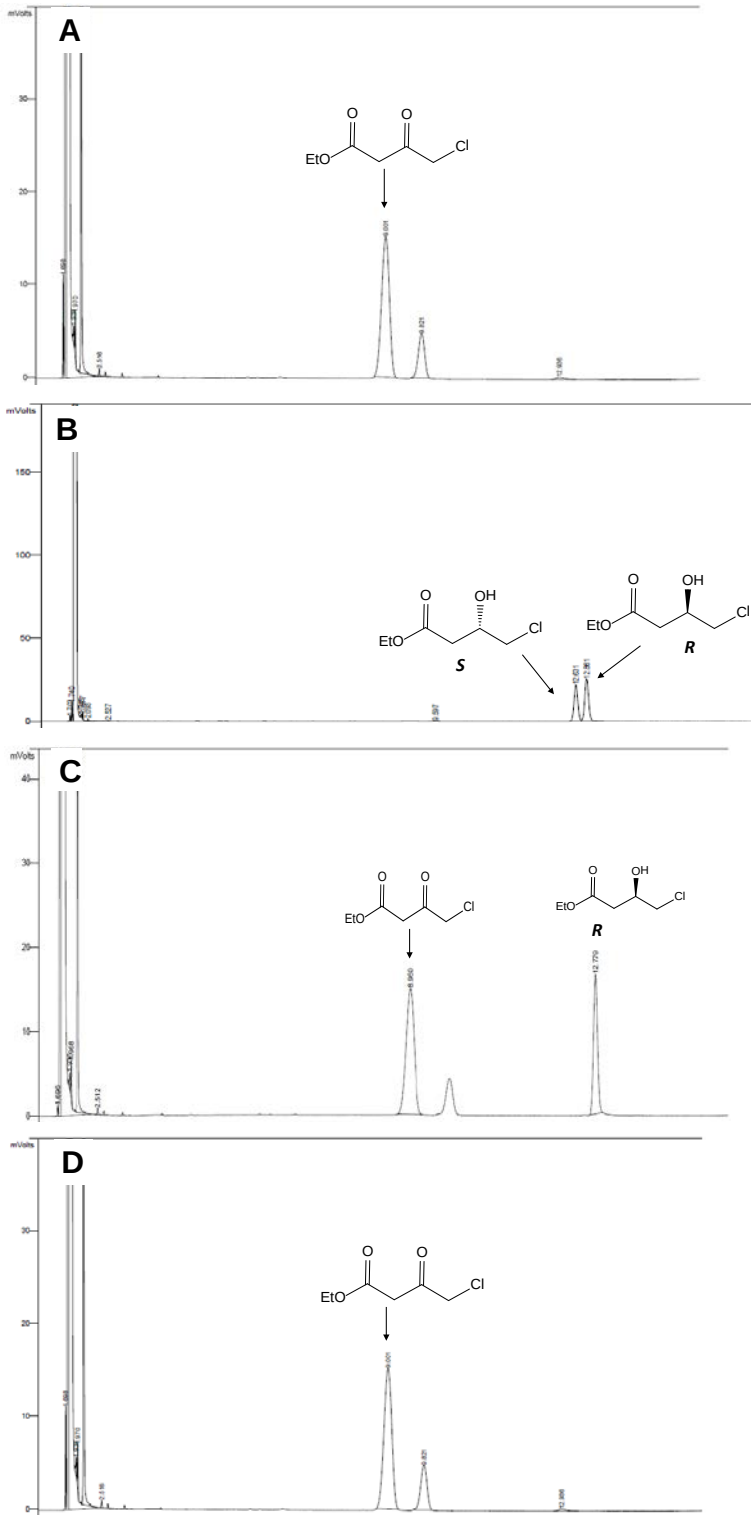


**Figure S11. Chiral GC chromatograms of chlorinated derivatives.** (A) Standard of ethyl-4-chloroacetoacetate (RT = 8.98 min), (B) Standards of ethyl-(*R*)-4-chloro-3-hydroxybutyrate (RT = 12.86 min) and ethyl-(*S*)-4-chloro-3-hydroxybutyrate (RT = 12.63 min). (C) Chiral GC chromatograms of an enzymatic reaction sample and (D) a control reaction sample without enzyme.

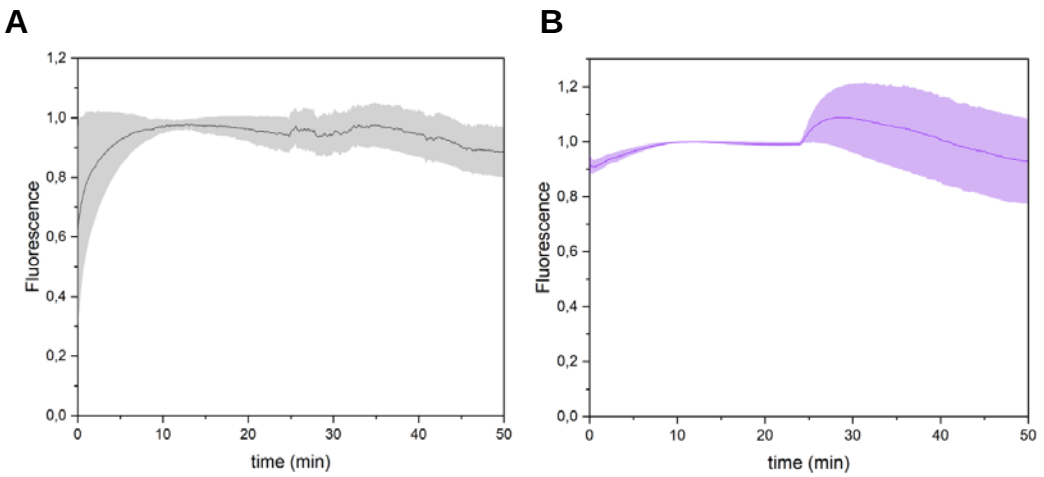


**Figure S12. Time courses of NADH immobilization on different beads.** The minimum number of beads analysed was n=5 and its incubation under reaction conditions in absence of EAA (A) and after the addition of 2-propanol at 25 min (B)

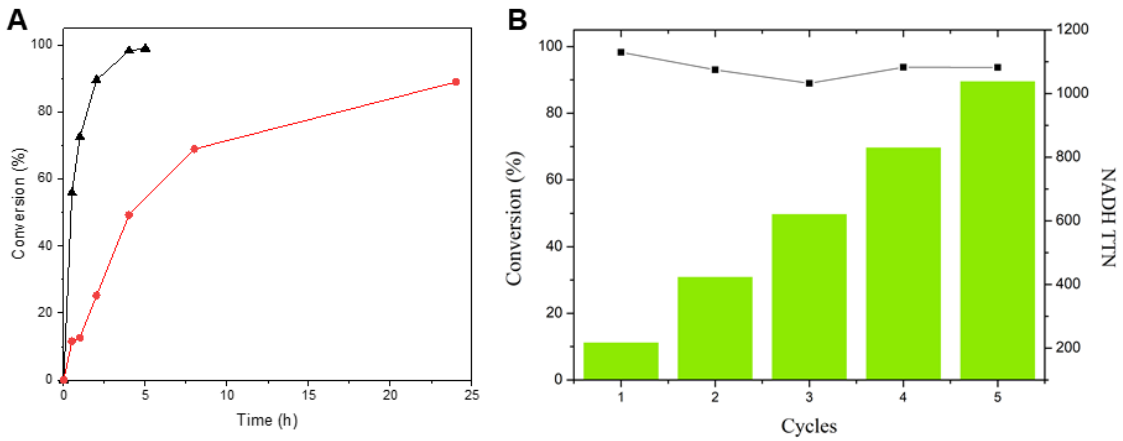


**Figure S13. Reaction course and TTN of 200 mM ethyl acetoacetate asymmetric reduction of by high-loaded AG-G@-TT27-HBDH(PEI-NADH).** (A) The incubation temperatures were 25 ºC (red circles) or 60 ºC (black triangles). A 1:5 (w/v) biocatalyst suspension was incubated with a reaction mix of 200 mM of ethyl-acetoacetate, 2-propanol 5% (0.66 M) in 10 mM Tris-HCl at pH 8.0, for (5-24) h at (60-25) ºC with orbital agitation and a total reaction volume of 1 mL. (B) Conversion and cofactor accumulated turnover number during 5 cycles of 4 h batch reactions. In black squares is represented the percentage of conversion of the asymmetric reduction of EAA catalyzed by AG-G@-TT27-HBDH (PEI) (32.3 mg enzyme per gram of support). Reactions were carried as in panel A. Green bars represent the cofactor accumulated TTN after each consecutive batch reaction cycle.

# Supplementary Tables

**Table S1. Co-immobilization parameters of TT27-HBDH and FDH**

| Ag-G@ TT27-HBDH(PEI)/FDH | Ψ% (P) | Load (U/g) / (mg/g) | iSA (U/mg) | Recovered activity (U/g) / (%) |
|---|---|---|---|---|
| **Tt27-HBDH** | 91.3 | 0.91 / 11.04 | 0.035 | 0.39 / 43.0 |
| **FDH** | 15.8 | 1.58 / 0.89 | 0.25 | 0.22 / 14.3 |

**Ψ, Immobilization Yield (%):** 100*(Protein concentration in crude fraction – Protein concentration in flow through)/ Protein concentration in crude fraction. **Load (U/g)/(mg/g):** Enzymatic activity (U/g of biocatalyst) / (mg of immobilized enzyme / g of biocatalyst). **iSA, Immobilized Specific Activity (U/mg):** Recovered activity of immobilized enzyme / mg immobilized enzyme. **Recovered activity: (U/g)** Enzyme activity / g of biocatalyst, and relative recovered activity (%) means the quotient between the specific activities of immobilized enzyme and the soluble one.

**Table S2. Immobilization of NADH on Ag-G@TT27-HBDH(PEI).**

| | **µmol NADH /g biocatalyst** |
|---|---|
| Offered | 10 |
| Flow through | 2.3 |
| Eluted in washes | 2.1 |
| Adsorbed in biocatalyst | 5.6 |

Ag-G@-TT27-HBDH(PEI) was incubated with 10 µmol of NADH per g of biocatalyst. NADH presence was analyzed after incubation and 2 washes with Tris-HCl buffer 10mM.