Supporting information

Enantiodivergent Biosynthesis of β-Hydroxy esters by Self-Sufficient Heterogeneous Biocatalysts in Continuous flow.

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Materials and Methods

Materials.

Polyethyleneimine branched (PEI) (Mw ~25 kDa), polyallylamine (PAH) solution in H_2O (Mw ~65 kDa, 10 wt.%), isopropanol, ethyl acetoacetate (99%) (1a), ethyl 2-methylacetoacetate (90%) (1b); ethyl 2-ethylacetoacetate (90%) (1c); ethyl 2-chloroacetoacetate (95%) (1d); ethyl 4-chloroacetoacetate (95%) (1e); iminodiacetic acid (IDA), and other reagents and solvents of analytical grade were purchased from Sigma-Aldrich (St. Louis, IL, USA).). Methyl 3-oxopentanoate (99%) (1g); ethyl 3-oxopentanoate (98%) (1h) were purchased from Thermo-Fischer (MA, USA). Agarose microbeads 6BCL (50-150 μm diameter) were purchased from Agarose Bead Technologies (Madrid, Spain). Reduced Nicotinamide-adenine-dinucleotides sodium salts (NAD(P)H) were purchased from GERBU Biotechnik GmbH (Heidelberg, Germany). Bradford protein assay dye reagent was purchased from BIORAD (Bio-Rad. Hercules, CA, USA) μ-Slides 8 well glass bottom was purchased from Ibidi (Planegg, Germany).

Expression and purification of TtHBDH and LkKRED

The (*S*)-3-hydroxybutyryl-CoA dehydrogenase from *Thermus thermophilus* HB27 (TtHBDH) and the Ketoreductase from *Lactobacillus kefir* were expressed and purified, as described elsewhere.[1, 2] Briefly, a pET28b plasmid encoding the corresponding enzymes (Table S1) were transformed in BL21 (DE3) E. coli cells. Colonies were picked and grown in LB medium containing kanamycin (30 μg mL^{-1}). When an optical density of 0.6 was reached, 1 mM of Isopropyl-β-thiogalactopyranoside (IPTG) was added to induce the protein expression. The induced cultures continued for 3 h at 37 °C for TtHBDH or 12 h at 21°C for LkKRED, then the bacteria were harvested by centrifugation. Next, We resuspended the cells in 25 mM phosphate buffer at pH 7 for TtHBDH and a complex buffer (100 mM Tris buffer, 200 mM NaCl, 1 mM $MgCl_2$) at pH 7 for LkKRED. We lysed the bacteria through sonication and the cell debris was discarded by centrifugation (10000 g for 15 min). For TtHBDH, the soluble crude protein extract

was incubated at 70 °C for 45 min to purify the thermophilic enzyme through thermal shock, as previously described.[1] The pellets of denatured mesophilic proteins were discarded after centrifugation (10000 g for 30 min). For LkKRED the soluble crude protein extract was purified through immobilisation with metal affinity chromatography using agarose microbeads. (ABT, Madrid, Spain) functionalised with Co^{2+} chelates. The protein was eluted with a solution of 100 mM Tris-HCl, 200 mM NaCl, 1 mM $MgCl_2$ and 500 mM imidazole at pH 7. The imidazole was removed from purified enzymes by gel filtration using a PD-10 column (GE Healthcare, Chicago, IL).

Determination of enzyme concentration.

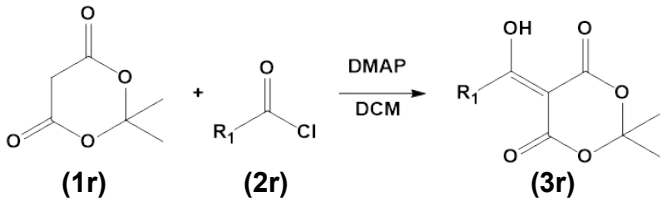
The concentration of soluble enzymes (TtHBDH and LkKRED) was determined during the processes of purification and immobilisation using the Bradford protein assay (Bio-Rad, CA). A standard curve was established using a commercial solution of bovine serum albumin (Sigma-Aldrich, St. Louis, IL). To measure the concentration, 5 µL of the soluble enzyme solution was combined with 200 µL of diluted Bradford reagent, and the mixture was incubated for 5 minutes at room temperature. The absorbance was then measured at 595 nm.

Enzyme activity Spectrophotometric assay

The enzymatic activities of soluble enzymes were determined in 96-well plates by monitoring the NAD(P)H absorbance at 340 nm. Five microliters of enzyme solution were incubated with 200 µL of a solution of 10 mM of 1a and 0.2 mM NAD(P)H in 25 mM phosphate buffer pH 7 at 30 °C. One enzyme unit (U) was defined as the amount of enzyme required to reduce 1 µmol of NAD(P)H per minute under given conditions and considering an $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$ for NADH at 340 nm

Synthesis of β-keto esters derivates

Concerning the synthesis of the non-commercially available substrates, meldrum acid (1r) and acyl chlorides (2r) were used as starting materials. Depending on the product needed, two

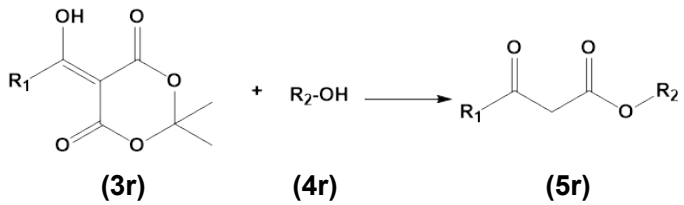


different acyl chlorides were used, acetyl chloride or propionyl chloride (Reaction 1)

Reaction 1: Reaction between meldrum acid and acyl chloride.

In a 250 mL round-bottom flask equipped with a dropping funnel and under a nitrogen atmosphere, meldrum acid (1 eq., 41.4 mmol) and 4-dimethylaminopyridine (DMAP) (2 eq., 82.2 mmol) were dissolved in 60 mL of anhydrous dichloromethane (DCM). The mixture was cooled down to 0 °C, and then the acyl chloride dissolved in 20 mL of anhydrous dichloromethane was added dropwise. The mixture was stirred for 24 hours at 25°C.

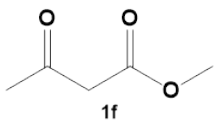
The reaction was quenched with 80 mL of 1M hydrochloric acid solution, observing that two phases were formed. The aqueous phase was extracted 2 times with dichloromethane. The organic phases were gathered, washed with 10 mL of NaCl saturated solution and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo to yield the product (3r) as an orange solid, which was used for the next step without further purification (Reaction 2).



Reaction 2: Esterification of 3r.

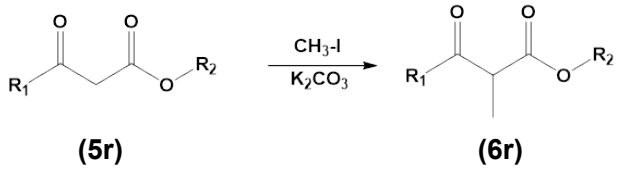
In the second reaction, by adding the corresponding alcohol (4r) at the appropriate temperature, an alcoholysis took place: under reflux the acyl meldrum (3r) was broken, affording the corresponding β-keto ester (5r), as well as carbon dioxide and acetone as by-products. This reaction was done in a round-bottom flask equipped with a condenser. The compound 3r was dissolved in 100 mL of MeOH or EtOH and the reaction was constantly stirred for 16 hours and heated to reflux (for EtOH to 90 °C, for MeOH to 70 °C). Then the mixture was cooled down and the solvent was removed under vacuum.

The crude was purified by column chromatography (Hexane/Ethyl acetate: from 80:20 to 70:30) and the product was obtained as a pale yellow oil. Overall, the products showed in the Scheme 1S were obtained.[3, 4]



Scheme 1S: Obtained product from the synthesis.

The last synthesis was the addition of a methyl group on the α-carbon of the ethyl-3-oxopentanoate. The reaction showed in Reaction 3, was the used procedure for the synthesis of both.

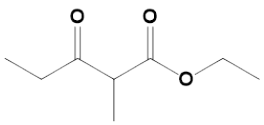


Reaction 3: Methylation in the α carbon.

For this reaction, 5 g of the β-keto ester (5r) (1 eq., 38.4 mmol) was dissolved in 50 mL of anhydrous acetone under a nitrogen atmosphere. Then, one equivalent of potassium carbonate (1

eq., 38.4 mmol), previously dried in the vacuum oven for 48 hours at 80 °C was added. The mixture was stirred for 15 minutes at room temperature. Afterwards, methyl iodide (1 eq., 38.4 mmol) was added. The reaction was stirred under reflux (75 °C) for 24 hours and checked by TLC.

For the extraction 60 mL of diethyl ether was added; the mixture was filtered off with filter paper, and then the product was concentrated under a vacuum to obtain a pale yellow liquid (6r). The product was used without further purification. The obtained products are shown in Scheme S2.



1i

Scheme S2: Obtained products from methylation synthesis.

Finally, the synthesised compounds 1f and 1i were analysed by NMR (Figures S15-S16).

Determination of kinetic parameters

To determine the kinetic parameters of the enzymes, the redox activity was measured by monitoring the absorbance at 340 nm of NADH in the case of the TtHBDH and NADPH for LkKRED. The general enzymatic assay involved the reduction of a solution containing different concentrations (0.1-500 mM) of the tested substrates, 0.2 mM of either NADH or NADPH and 25 mM of sodium phosphate buffer at pH 7. Reactions were monitored by measuring absorbance at 340 nm, after adding 5 µL of soluble enzyme to 200 µL of the reaction solution in a 96-well plate. Each substrate concentration was assayed in triplicate, and the mean value for each substrate concentration was calculated. All mean activities were plotted and adjusted to a Michaelis-Menten or Michaelis-Menten with inhibition models using Origin Pro software.

Functionalisation of agarose beads

Agarose macroporous microbeads (AG) 6BCL (50-150 µm diameter, 200 nm pore sise) were functionalised following different strategies to immobilise both enzymes:

Glyoxyl-activated supports.

The activation was prepared as previously described using agarose 6BCL. Briefly, agarose was first activated with glyceryl groups by incubation at alkaline pH with glycidol. Then the obtained glyceryl groups were oxidised with 20 mM $NaIO_4$ (1 hour of incubation). An approximate group density of 100 μmol of glyoxyl groups per gram of agarose was obtained. [5]

Epoxy - Iminodiacetic acid – Cobalt chelates activated supports (AG-Co^{2+}/E).

6 BCL AG microbeads were initially activated with epoxy groups by incubation with an aqueous solution of 2 M Epichlorohydrin, 1 M NaOH, 0.088 M $NaBH_4$ and 3.6 M Acetone for 16h. The obtained agarose epoxy (AG-E) was then incubated with 0.5M iminiodiacetic acid (IDA) at pH 11 for 30 min at room temperature and orbital shaking to produce agarose activated with epoxy and IDA groups (AG-E/IDA). Finally, the beads were incubated with an aqueous solution of $CoCl_2$ (30 mg mL^{-1}) in a 1:10 (*w:v*) ratio with orbital shaking, to produce AG-Co^{2+}/E.

Construction of ssHB: Enzyme and cofactor immobilisation protocols

The process of constructing a ssHB involves multiple stages, including enzyme immobilisation, post-immobilisation polymer coating, and cofactor immobilisation.

ssHB of TtHBDH:

Immobilisation on glyoxyl agarose (AG-G)

The immobilisation was carried out by mixing a solution of 100 mM sodium bicarbonate pH 10 containing around 25 mg mL^{-1} of purified soluble TtHBDH with AG-G beads in a 1.10 (w*/v) ratio.* The suspension was gently stirred at 25 °C for 3 h and subsequently filtered. The concentration of immobilised enzyme was determined by subtracting the concentration of the supernatant from the offered enzyme concentration (measured through Bradford protein assay).

Post-immobilisation coating

After the immobilisation, TtHBDH@AG-G was incubated with a solution of 10 mg mL^{-1} of branched polyethyleneimine (PEI) (25 kDa) in 100 mM sodium bicarbonate pH 10 and incubated at 25 °C for 1 h under gentle stirring. Then, 1 mg mL^{-1} of solid $NaBH_4$ was added and

incubated for 30 min at 4 ºC to covalently immobilise the PEI and the enzyme on the support to yield (PEI)TtHBDH@AG-G. the completely reduced biocatalyst was vacuum-filtered and washed with an excess of 10 mM Tris-HCl buffer at pH 7.

Cofactor immobilisation

The previously prepared (PEI)TtHBDH@AG-G was incubated with a freshly prepared solution of 1 mM NADH in 10 mM Tris-HCl pH 7 for 1 h at 4 °C. Finally, the heterogeneous biocatalyst with the immobilised cofactor (TtHBDH@ssHB) was washed with 2 volumes of 10 mM Tris-HCl pH 7 buffer. The supernatants of the cofactor immobilisation and the washing steps were analysed by UV-vis to quantify the amount of cofactor that was immobilised and then released during the washing steps.

ssHB of LkKRED:

Immobilisation on AG-$Co^{2+/}$E:

LkKRED was immobilised directly from the soluble crude protein extract in complex buffer (100 mM Tris-HCl buffer, 200 mM NaCl, 1 mM $MgCl_2$) at pH 7. The protein solution was incubated with AG-$Co^{2+/}$E in a ratio of 1:10 (*w/v*) for 1 h at 4°C, then the suspension was filtered and the supernatant was collected for further analysis. Then the heterogeneous biocatalyst was washed with the same buffer. The concentration of immobilised enzyme was determined by subtracting the concentration of the supernatant from the offered enzyme concentration (measured through Bradford protein assay).

Post-immobilisation coating:

The immobilised enzyme (LkKRED@AG-$Co^{2+/}$E) was incubated with a solution of polyallylamine 65 kDa (PAH) of 10 mg mL^{-1} at pH 8 in a 1:10 (*w/v*) ratio for 1 h at room temperature as previously described. [6]

Cofactor immobilisation

The previously prepared (PAH)LkKRED@AG-$Co^{2+/}$E was incubated with a freshly prepared solution of 1 mM NADH in 10 mM Tris-HCl pH 7 for 1 h at 4 °C. Finally, the heterogeneous

biocatalyst with the immobilised cofactor (LkKRED@ssHB) was washed with 2 volumes of 10 mM Tris-HCl pH 7 buffer. The supernatants of the immobilisation and the washing step were analysed by UV-Vis to quantify the amount of cofactor that was immobilised and released during the washing steps.

Calculation of immobilisation parameters

The immobilisation parameters characterised in this study (Table 2) were calculated as follows:[7]

- **Load** is the mass (mg) of enzyme immobilised per gram of support.

$$Load\ (mg\ g^{-1}) = (offered\ enzyme\ (mg\ mL^{-1}) -\ enzyme\ in\ supernatant\ (mg\ mL^{-1}))\ x\ \frac{immobilization\ volume\ (mL)}{mass\ of\ support\ (g^{-1})} \quad [1]$$

- **Immobilisation yield (Ψ)** is the percentage of the offered enzyme that is immobilised on the support. The concentration of enzyme offered or remaining in the supernatant after immobilisation was calculated by the Bradford protein assay.

$$\Psi\ = 100\ x \frac{offered\ enzyme\ (mg\ mL^{-1}) - enzyme\ in\ supernatant\ (mg\ mL^{-1})}{offered\ enzyme\ (mg\ mL^{-1})} \quad [2]$$

- **Recovered activity (Ar)** is defined as the measured enzyme activity per gram of carrier and is expressed in U g^{-1}. The determination of the immobilised enzyme activity was done as described in the enzyme activity assay section but with minor modifications: 10µL of a 1.10 (*w/v*) suspension of the heterogeneous biocatalyst were placed in the well instead of the 5 µL of the soluble enzyme.
- **Immobilised specific activity (iSA)** is defined as the activity per mg of immobilised enzyme.

$$iSA\ (U\ mg^{-1}) =\ A_r(U\ g^{-1})\ x\ Load\ (mg\ g^{-1}) \quad [3]$$

- **Relative specific activity (%)** is defined as the ratio between iSA and the free enzyme specific activity

$$rSA\ \% =\ \frac{iSA}{soluble\ enzyme\ SA}\ x\ 100 \quad [4]$$

- **Immobilised cofactor:** Amount of cofactor (µmol) immobilised per gram of support.

$$A_{340} = c_{NAD(P)H} \cdot l \cdot \varepsilon_{NADH} \rightarrow \mu mol_{\ NAD(P)H} = \frac{A \cdot Vol}{l \cdot \varepsilon} \quad [5]$$

$$Immobilized\ cofactor \left(\frac{\mu mol\ NAD(P)H}{g\ Het.\ Biocatalyst}\right) = \frac{\mu mol\ NADH_{\ offered} - \mu mol\ NADH_{\ flowtrought} - \mu mol\ NADH_{\ washed}}{g\ Heterogeneous\ Biocatalyst} \quad [6]$$

LkKRED@ssHB Batch Reaction course

To obtain the reaction course of the asymmetric reduction of 1a by LkKRED@ssHB, 100 mg of solid LkKRED self-sufficient heterogeneous biocatalyst were placed in a micro chromatographic column (Biospin TM, BIO-RAD). The reaction was triggered by adding 1 mL of reaction mixture composed of 200 mM of ethyl acetoacetate (1a), and 5% (v/v) of isopropyl alcohol in a 10 mM Tris-HCl buffer at pH 7.0. Several samples were withdrawn at different times from the reaction bulk by vacuum filtration but never removing more than 5% of the total reaction volume. The samples were analysed by GC-FID as described in the following section.

GC-FID analysis

We analysed the reaction samples using a gas chromatograph with a flame ionisation detector (GC-FID). Each sample was extracted (liquid-liquid) with dichloromethane in a 1:1 (*v:v*) ratio, the aqueous phase was discarded. 50 mg of anhydrous $MgSO_4$ were added to the organic phase to dry the samples. Analyses were carried out in an Agilent 8890 gas chromatography system as described previously.[1] Briefly, we used a column of (5%-phenyl)-methylpolysiloxane (Agilent, J&W HP-5 30 m × 0.32 mm × 25 μm), helium as the carrier gas (1.5 mL min^{-1}) and equipped with an FID detector. The temperatures of the injector and FID detector were 280 °C and 300 °C, respectively. The separation of compounds was carried out by two sequential temperature ramps: the initial temperature (60 °C) was maintained for 2 min and progressively increased up to 160

°C at a rate of 10 °C min^{-1}. Then, the column temperature was increased to 20 °C min^{-1} for 4 min until 250 °C and maintained for 4 min. Retention times were 3.4 min for ethyl acetoacetate (1a) and 3.29 min for ethyl 3-hydroxybutyrate (2a).

Chromatographic Yield and Space-Time Yield calculation

Some parameters such as Chromatographic Yield (CY) (%), space-time yield (STY), were calculated according to the following equations:

$$CY\ (\%) = \frac{\mu mol\ product}{\mu mol\ product + \mu mol\ substrate}\ x\ 100 \quad [7]$$

$$STY\ (g\ L\ h^{-1}) = \frac{Product\ concentration\ (g/L)}{\tau\ (h)} \quad [8]$$

Chiral GC-analysis

The enantioselectivity of LkKRED@ssHB in the reduction of 1a was determined by GC-FID. 500 µL of the reaction sample or a solution of enantiopure commercial standards (100mM (*R*) or (*S*) ethyl 3-hydroxybutyrate) were incubated with 500 µL of ethyl acetate for liquid-liquid extraction. 200µL of the extracted sample were analysed by GC-FID as previously described{Orrego, 2023 #100} in an Agilent 8890 System using a Beta DEXTM 120 Capillary Column (30 m x 0.25 mm x 0.25 µm) and helium as carrier gas. The temperature of the injector and FID detector was 280°C and 300°C respectively. the initial temperature (80 °C) was progressively increased up to 150 °C at a rate of 2 °C min^{-1}, then it was maintained for 5 minutes before increasing the temperature up to 200 ºC at a 20 °C min^{-1} rate, the oven temperature was maintained at 200 ºC for 2 minutes. The retention times were 10.85 (ethyl (*S*)-3-hydroxy butyrate) and 10.95 for (ethyl (*R*)-3-hydroxy butyrate).

Kinetic parameters of ssHB

To determine the Michaelis-Menten parameters in the reduction of 1a, 50 mg of solid either TtHBDH or LkKRED self-sufficient heterogeneous biocatalysts (~ 25 $mg_{protein} \times g_{carrier}^{-1}$) were placed in a micro chromatographic column (Biospin TM, BIO-RAD). The reaction was triggered by adding 500 µL of reaction mixture composed of different concentrations (1-1000 mM) of 1a, 5% (*v/v*) of isopropyl alcohol in a 10 mM Tris-HCl buffer at pH 7. For TtHBDH@ssHB reactions were incubated for 0.5 h at 50 °C under gentle orbital agitation. To obtain always less than 30 % conversion, in the case of LkKRED@ssHB, reaction times were modified depending on substrate concentration: 2 min for reactions with low substrate concentrations (1, 5 mM); 5 min for medium substrate concentrations (10, 20, 50 mM) or 10 min for reactions with high substrate concentration (100, 250, 500, 1000mM). The reactions were then stopped by vacuum filtration and analysed by GC-FID as described above.

Set-up of continuous flow reactions

For analyzing the performance of ssHBs in a continuous flow process, we built two packed bed reactors (PBR) by packing 1 g of solid TtHBDH ssHB or 0.5g of LkKRED ssHB in 9 mm diameter columns with a final reactor volume (Vr) of 1.4 mL or 0.7 mL respectively. In the case of TtHBDH PBR, three reaction mixtures containing different amounts of 1a (50, 200 or 1000 mM); Isopropanol (5, 5 or 10 % (*v/v)* respectively*)* but always in 10 mM Tris-HCl pH 7 buffer were pumped through the PBR with a syringe pump 11-PLUS, Harvard apparatus (Massachusetts) at different flow rates (0.01, 0.05, 0.1, 0.2 mL min^{-1}). Each column was flushed with 2 column volumes for each flow rate to ensure the perfect equilibration of the PBR. The eluted volume was passed through an online spectrophotometer (360 nm) (Essi tech, Slovenia), collected in different aliquots, and analysed by GC-FID as previously described. The same procedure was applied to the LkKRED@ssHB PBR reactor at a fixed substrate (1a) concentration of 200 mM (5% (*v/v*) isopropanol in 10mM Tris-HCl pH 7), maintaining the flow rate variations.

The residence times (τ) of the different conditions were analysed with the following equation:

$$\tau\ (min) = \frac{Reactor\ volume\ (mL)}{Flow\ rate\ (mL\ min^{-1})} \quad [9]$$

Simulation of reaction kinetics of SSHBs.

The conversion of substrate 1a (A) catalyzed by TtHBDH@ssHB and LkKRED@ssHB was simulated by using a kinetic model coupled to reactor mass balance. The reaction kinetic equations and kinetic models used are shown in the following table:

Model	Reactor kinetic equation	Kinetic model
S + P inhibition (Batch reactor and ideal plug flow reactor)	$\tau = C_{A0}\int_0^{x_A}\frac{dX_A}{r}$ [10]	$r = \frac{V_{max}}{C_A(1+\frac{C_A}{K_s}) + K_m(1+\frac{C_{A0}-C_A}{K_p}}$ [11]
S + P inhibition + full axial dispersion Flow reactor with full axial dispersion)	$\tau = \frac{C_{A0}}{r}X_A$ [12]	$r = \frac{V_{max}}{C_A(1+\frac{C_A}{K_s}) + K_m(1+\frac{C_{A0}-C_A}{K_p}}$ [13]

The results were expressed in terms of conversion versus dimensionless reaction time, Da_I, which is the first Damköhler number given by the ratio between the reaction residence time (τ) and the characteristic reactor time ($\tau_{characteristic}$).

The characteristic reaction time ($\tau_{characteristic}$) is given by the ratio of substrate concentration to v_{max} and indicates the minimum time to reach 100% conversion if the enzyme kinetics would respond to a zero reaction order.

$$Da_I = \frac{\tau}{\tau_{characteristic}} = \frac{\tau\, V_{max}}{C_{A0}} \quad [14]$$

The kinetic constant values are described in Table S4.

The numerical integration of the reactor kinetic equation was carried out by using the software Berkely Madonna (Version 10.4.2) by using a 4th-order Runge-Kutta algorithm. A single

response fitting was performed by using the tool "curve fit" for the calculation of the product inhibition kinetic constant.

Product inhibition determination

50 mg of solid either TtHBDH or LkKRED self-sufficient heterogeneous biocatalysts (~25 $mg_{protein} \times g_{carrier}^{-1}$) were placed in a micro chromatographic column (Biospin TM, BIO-RAD). The reaction was triggered by adding 500 µL of reaction mixture composed of different concentrations (1-500mM) of ethyl 3-hydroxybutyrate (2a), 200 mM of ethyl acetoacetate (1a), 5% (*v/v*) of isopropanol in a 10 mM Tris-HCl buffer at pH 7.0. To always obtain conversions below 30 %, TtHBDH@ssHB reactions were incubated for 0.5 h at 50 °C under gentle orbital agitation and LkKRED@ssHB reactions were incubated for 10 min at 25 ºC. The reactions were then stopped by vacuum filtration and analysed by GC-FID.

Operational stability of Continuous flow reactions set-up

For analyzing the performance of ssHBs in long operations, we prepared packed bed reactors as described above with 1g of either TtHBDH@ssHB or LkKRED@ssHB (Vr = 1.4 mL). The reaction mixture (200 mM 1a, 5% (*v/v*) Isopropanol; 10 mM Tris-HCl buffer pH 7) was pumped through the reactor with a peristaltic pump, previously calibrated, at a maintained flow rate of 0.05 mL min^{-1} with an average retention time (τ) of 28 minutes. The collected aliquots were analysed by GC-FID as described above and the following parameters were calculated:

$$Specific\ Productivity\ (SP) = \frac{STY}{[enzyme]} \quad [15]$$

$$TTN_{Enzyme} = \frac{mol\ Product}{mol\ Enzyme} \quad [16]$$

$$TTN_{NAD(P)H} = \frac{mol\ Product}{mol\ NAD(P)H} \quad [17]$$

In the operation of TtHBDH@ssHB, the reaction crude obtained was purified with three extractions with diethyl ether 1:1 (*v/v*). The organic phases were gathered and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo, and the crude was purified by column chromatography (Hexane/Ethyl acetate: from 90:10 to 80:20).

Post-operation ssHB analysis

After the long operation, the used TtHBDH@ssHB was carefully extracted from the reactor to enable segregation into the inlet, intermediate, and outlet sections. These samples were subjected to two procedures: firstly, the evaluation of the remaining enzyme activity as previously outlined, and secondly, the lixiviation of the remaining cofactor by incubating 1.10 (*w/v*) with 1M NaCl. Following the lixiviation of the cofactor, all the samples of the heterogeneous biocatalyst were incubated with 1 mM NADH as previously described, to re-immobilise fresh NADH and test the activity in continuous flow operation under identical conditions as described earlier.

UPLC-Ms analysis of eluted NADH

The solution with the lixiviated cofactor was analyzed by UPLC-MS. Ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS). using an Acquity UPLC equipped with a photodiode array detector (PDA) and a time-of-flight mass spectrometer (ESI-TOF) LCT Premier XE from Waters. The gradient elution buffers were (A) 100 mM ammonium formate and (B) acetonitrile. The following gradient program was used at a 0.3 mL min^{-1}: from 0 to 1 min, isocratic at 95 % A; from 1 to 14 min, gradient to 80 % A; from 14 to 15 min, gradient to 10 % A; from 15 to 17 min, isocratic at 10 % A; from 17 to 17.5 min, gradient back to 95% A; from 17.5 to 20 min, stabilisation at 95% A. 5 µL of samples were injected and NADH and its degradation fragments were detected at 340 nm and its molecular mass were confirmed by ESI-TOF MS. Mass spectrometry detection was performed using a time-of-flight mass spectrometer (ESI-TOF) LCT Premier XE with an electrospray ionisation source, working in positive mode. The MS range was between m/z 100 and 1000 Da. The capillary and cone voltages were set at

3000 and 100 V, respectively. The desolvation gas temperature was 220 °C, and the source temperature was 120 °C. The desolvation gas flow was set at 600 L h^{-1}, and the cone gas flow was set at 50 L h^{-1}. Quantification and data analysis were done in Masslynx version 4.1.

In-operando under microscope setup

We build a reactor by modifying one of the ends of a channel μ-slides Luer $I^{0.4}$ (Ibidi, Gräfelfing, Germany) with dimensions: length 50 mm; width 5 mm. height: 0.4 mm with a 0.45 μm filter to prevent beads lixiviation. Then, we pumped a 1.20 (w/v) suspension of TtHBDH@ssHB in 10 mM Tris-HCl buffer pH 7 through the channel until we filled it with biocatalyst, obtaining a packed bed reactor (Vr = 0.1 mL). The reaction mixture (200mM 1a, 5% (*v/v*) Isopropanol; 10 mM Tris-HCl buffer pH 7) was pumped through the reactor with a syringe pump 11-PLUS, Harvard apparatus (Massachusetts), previously calibrated, at a maintained flow rate of 0.003 mL min^{-1} with an average retention time (τ) of 30 minutes. The reactors were operated at 30 ºC or 50 ºC for 24 h and then analysed with epifluorescence microscopy. The autofluorescence of NADH was followed using epifluorescence microscopy with a ZEISS Axio Observer microscope(Carl Zeiss, Germany) and an excitation Colibri 5 led system of λ_{ex} = 385 nm.

Green Metrics calculation and formula

The sustainability of the synthesis of ethyl 3-hydroxybutyrate in the long-term continuous flow operations with TtHBDH@ssHB and LkKRED@ssHB was determined by calculating the E factor with the following parameters and equations:

- Product mass: Amount of product obtained after the whole continuous process operation.

- Waste mass: the total sum of the mass of reagents, water and catalyst involved in the process minus the product mass.

$$E\ factor = \frac{Waste\ mass}{Product\ mass} \quad [18]$$

The dissected E factor was obtained by division of the mass of each contributor by the mass of the products according to the following equations:

$$E\ factor\ reagents = \frac{Reagents\ mass}{Product\ mass} \quad [19]$$

$$E\ factor\ water = \frac{Water\ mass}{Product\ mass} \quad [20]$$

$$E\ factor\ catalyst = \frac{Catalyst\ \ mass}{Product\ mass} \quad [21]$$

Supporting Tables

Table S1: Enzyme sequences

Enzyme	Sequence
(S)-3-hydroxybutyryl-CoA dehydrogenase from *Thermus thermophilus* HB27 **(TtHBDH)**	MGEVKRIGVVGAGQMGSGIAQVAASAGYEVVLVDVAESFLERGLAAIRRSLGKFLEKGKITQEAHDEALGRIR TSLSLEDLKDADLIVEAIVEDEGEKRRLFERLGALAKPEAILASNTSSIPITALARYSGRPERFIGMHFFNPVPLM QLVEVIRGELTSEATRDVVVEVARRMGKTPLEVQDYPGFISNRLLMPMINEAIEALREGVATKEAIDGIMRLGM NHPMGPLELADFIGLDTCLAIMEVLHRGFGDDKYRPSPLLRRMVQAGLLGRKAGRGFYTYDEKGNKVGL

Ketoreductase from *Lactobacillus kefir* **(LkKRED)**	MGSSHHHHHHSSGLVPRGSHMTGFTAANTTYTLNNGVRIPAVGFGTFANEGAKGETYAAVKKALEVGYRHL DCAWFYQNEDEVGQALAEFLENHKDVKREDIFICTKVWNHLHEPEDVKWSLQNSLDKLKVDYVDLFLIHWPI AAEKDEATNMPKIGPDGKYIIKKELTENPEPTWRAMEDLVDAGKTRSIGVSNWTIPGLQKLLKFARIKPTVNQI EIHPFLPNTELVEFCFKNQIIPTAYSPLGSQNQVPSTGERVRDDPTLKAVAERSGHNLAQVLLAWGLRRGYVV LPKSSTPSRIESNFQIPVLRDEDFKAIQEVAKGRHCRFVNMKDTFGYDVWPEESDGQLKQE

Table S2: Kinetic parameters of simulations

		TtHBDH	LkKRED
K_M	mM	65,6	7,03
CAo	mM	200	200
K_S	mM	584	418,6
V_{max} reactor batch	mM h^{-1}	117,9	118
V_{max} reactor Flow dispersion	mM h^{-1}	926,4	927
K_P	mM	117	30

Table S3: Comparative metrics of different ssHBs in asymmetric reductions in continuous flow found in the literature.

		COFACTOR	Media	STY ($g\ L^{-1}\ h^{-1}$)	TTN_{enzyme} ($mol\ mol^{-1}$)	$TTN_{cofactor}$ ($mol\ mol^{-1}$)
a	https://doi.org/10.1002/ange.202211912	NADP+	Buffer / Org. Solv.	5,54*	Nd	59204
b	https://doi.org/10.1002/cssc.202201654	NADP+	Buffer	46,3*	Nd	4800
c	https://doi.org/10.1002/cctc.201500766	NADH	Buffer	1*	90000	655
d	https://doi.org/10.1002/anie.201810331	NAD	Buffer	5*	nd	14000
e	https://doi.org/10.1021/acsnano.5b01278	NADH	Buffer	nd	Nd	9,4
f	https://doi.org/10.1002/anie.201609758	NAD+	Buffer	1,8	30000*	85
g	https://doi.org/10.1002/adsc.202000058	NADPH	Buffer/ Org. Solv.	121	nd	12855
h	https://doi.org/10.1038/s41929-019-0353-0	NAD+	Buffer	10,75*	1700*	10839
TtHBDH	this work	NADH	Buffer	37,5**	146000	2984
LkKRED	this work	NADP+	Buffer	37.4**	937354	27240

* Calculated from available data in publication. ** Average during operation time

Table S4: Metrics of the continuous synthesis of (*R*) and (*S*) enantiomers of Ethyl 3-hydroxybutyrate.

[a]This range represents the lower limits reported by Messier and Woodley in their mass metric analysis for high- and medium-priced chemicals.

	TtHBDH	LkKRED	Reference[a]
Titre* (mass product/reaction volume)	19,5 g L^{-1}	24,7 g L^{-1}	10–50 g L^{-1}

(Average)			
Rate** (mass product/reaction volume/reaction time)	49 - 27 g L^{-1} h^{-1}	49.5 – 25.3 g L^{-1} h^{-1}	1–10 g L^{-1} h^{-1}
Yield (mass product/mass substrate) (Average)	95 - 49 %	90.2 - 46 %	> 90%
Specific yield*** (mass product/mass enzyme)	>101,5 g $g_{protein}^{-1}$	1169 g $g_{protein}^{-1}$	50 – 500 g $g_{protein}^{-1}$
TTN enzyme (mol mol^{-1})	146532	937354	10000
TTN NADH (mol mol^{-1})	2984	27240	1000
E factor	52,5	57.8	30

Supporting Figures

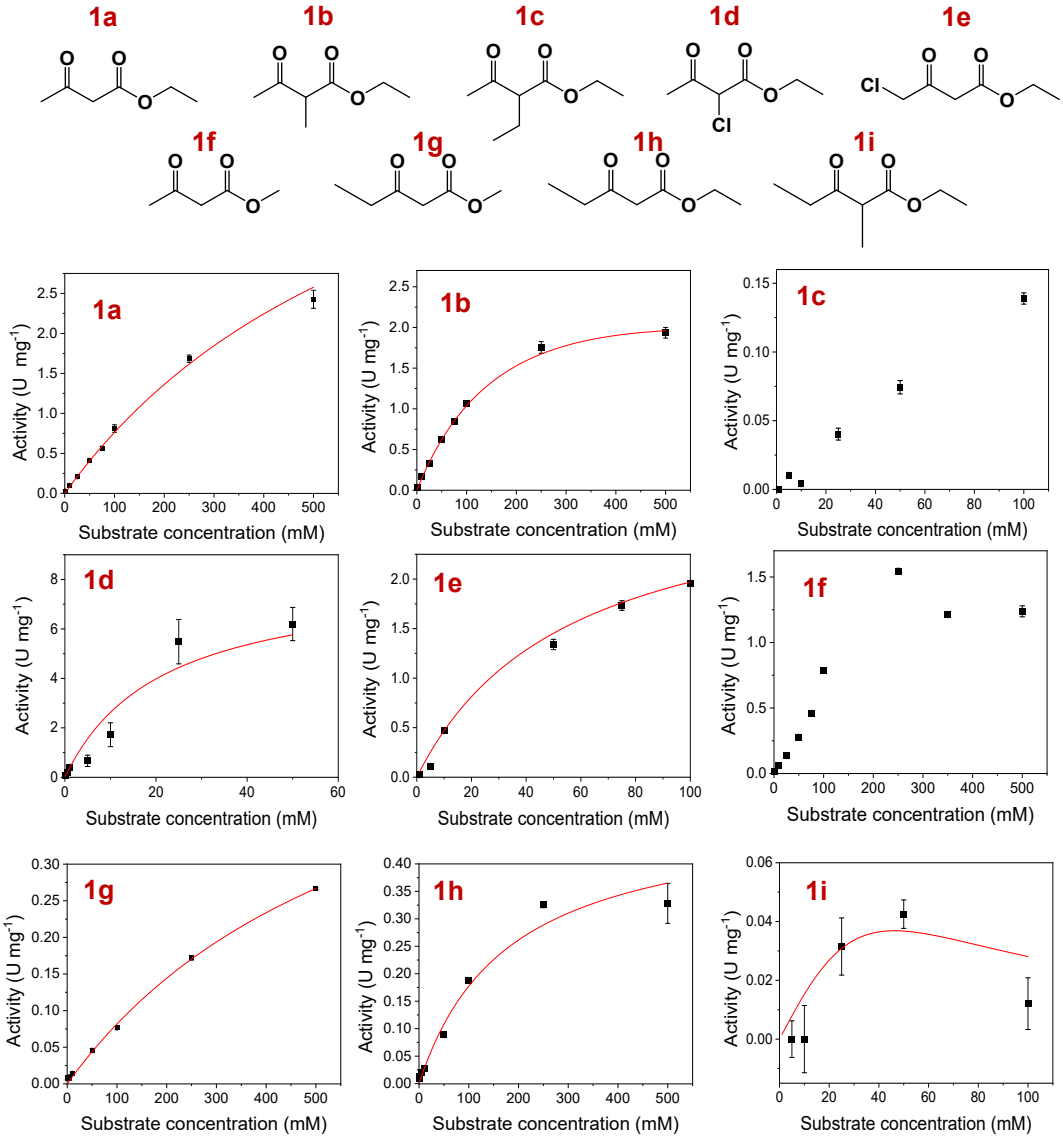


Figure S1. Michaelis-Menten curves of TtHBDH for different β-keto esters.

Reductive steady-state kinetics were calculated towards different concentrations of substrates (0.1-500 mM), with 0.2 mM of NADH in 25 mM sodium phosphate buffer at pH 7. The activities for each substrate concentration were done by triplicate at 30 ºC, resulting in a mean value for each substrate concentration. All mean activities were plotted and adjusted to a Michaelis-Menten or Michaelis-Menten with inhibition models using Origin Pro software.

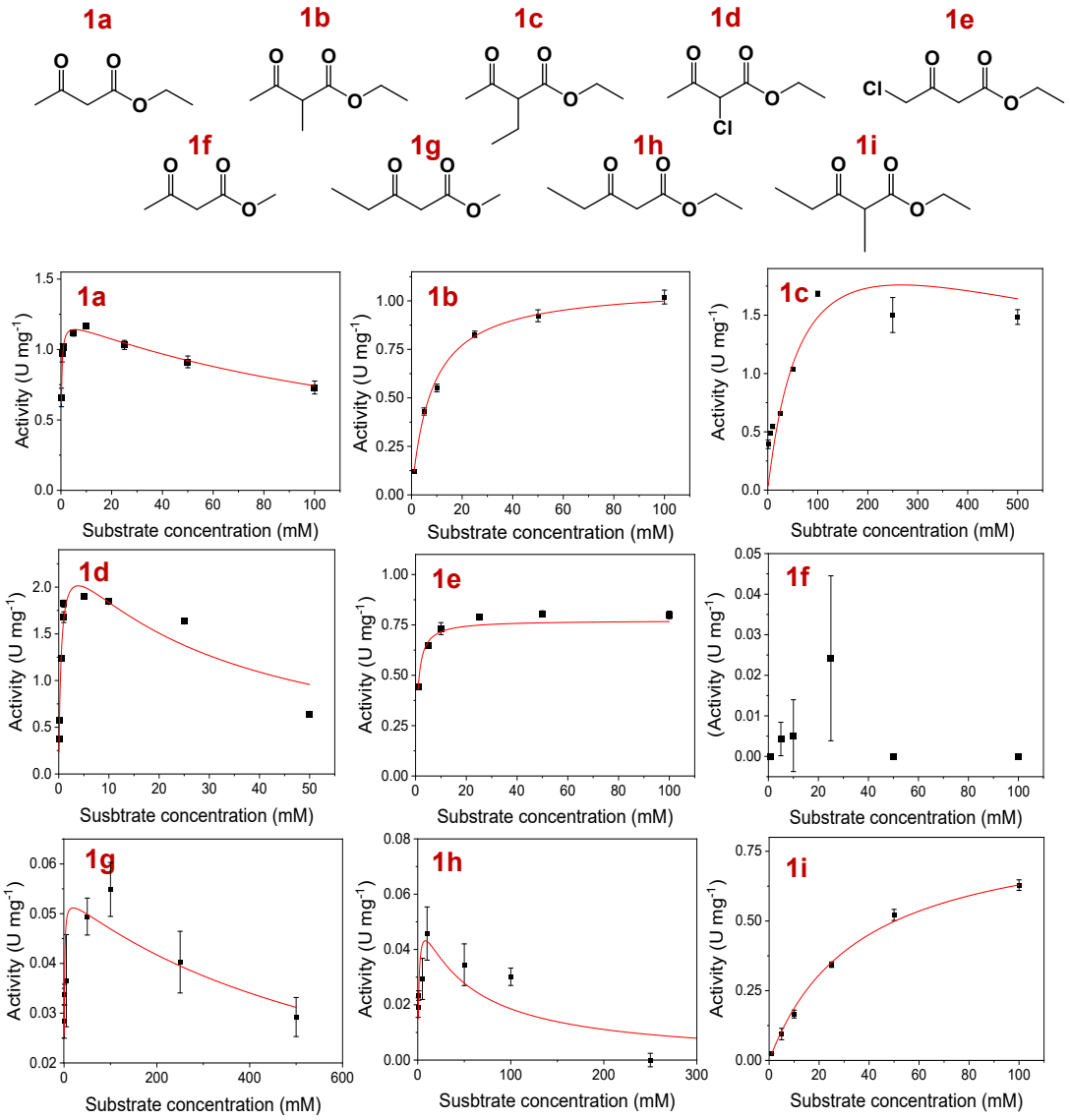


Figure S2. Michaelis-Menten curves of LkKRED for different β-keto esters.

Reductive steady-state kinetics were calculated towards different concentrations of substrates (0.1-500 mM), with 0,2 mM of NADPH in 25 mM sodium phosphate buffer at pH 7. The activities for each substrate concentration were done by triplicate at 30 ºC, resulting in a mean value for each substrate concentration. All mean activities were plotted and adjusted to a Michaelis-Menten or Michaelis-Menten with inhibition models using Origin Pro software.

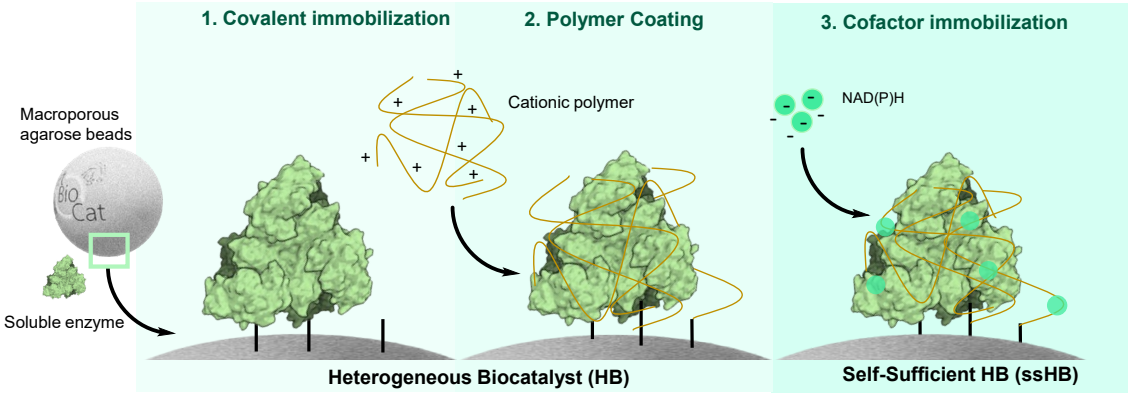


Figure S3. Construction steps of a Self-Sufficient Heterogeneous Biocatalyst.

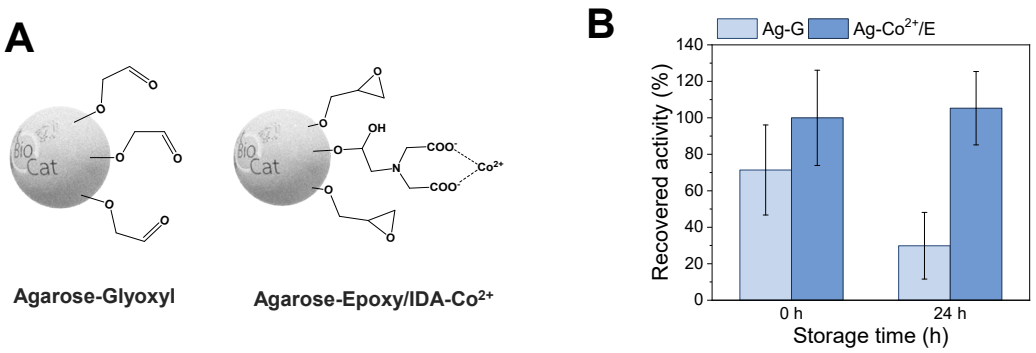


Figure S4. Agarose functionalisation and stability of immobilised LkKRED.

(A) Functionalisation of the agarose-porous beads employed in the construction of the ssHB with glyoxyl groups or with epoxy groups and cobalt chelates. (B) Recovered activity of immobilised LkKRED after 24 h storage at 4 ºC. The activity was measured with spectrophotometric assay as described in the methods.

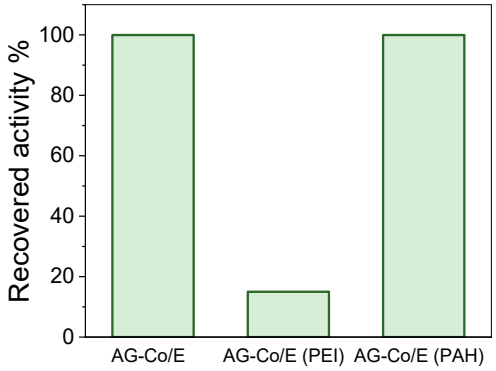


Figure S5. Recovered activity of immobilised LkKRED after post-immobilisation coating.

The enzymatic activity of LkKRED immobilised and coated with different cationic polymers was analysed in the reduction of 1a. The activity was measured with spectrophotometric assays as described in Materials and Methods.

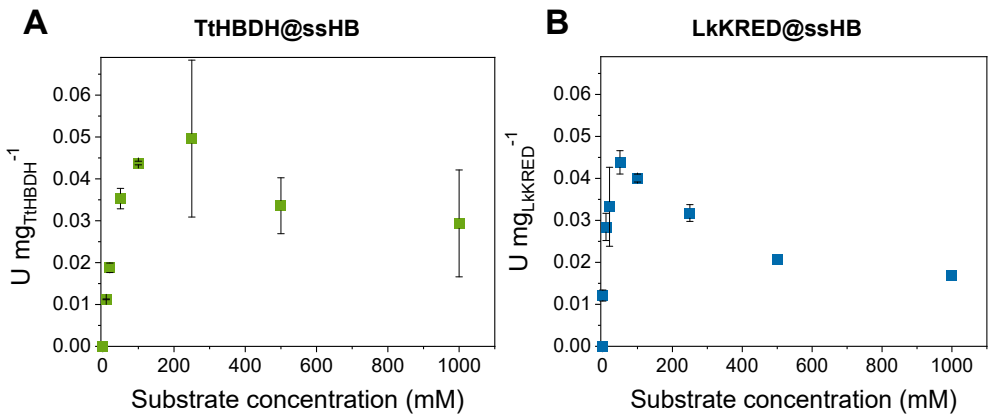


Figure S6. Michaelis-Menten curves of TtHBDH (A) and LkKRED (B) ssHBs.

Reductive steady-state kinetics were calculated by incubating 100 mg of ssHBs with solutions with different concentrations of ethyl acetoacetate (1a) (1-1000 mM) in 10 mM Tris-HCl buffer at pH 7. The cofactors (NADH for TtHBDH and NADPH for LkRED) were immobilised at 6.5 and 8.1 μmol_{NADH} per gram of ssHB. The reactions were incubated for 30 min (TtHBDH) or 10 min (LkKRED) and made by duplicate. All mean activities were plotted and adjusted to a Michaelis-Menten or Michaelis-Menten with inhibition models using Origin Pro software (fitting not shown).

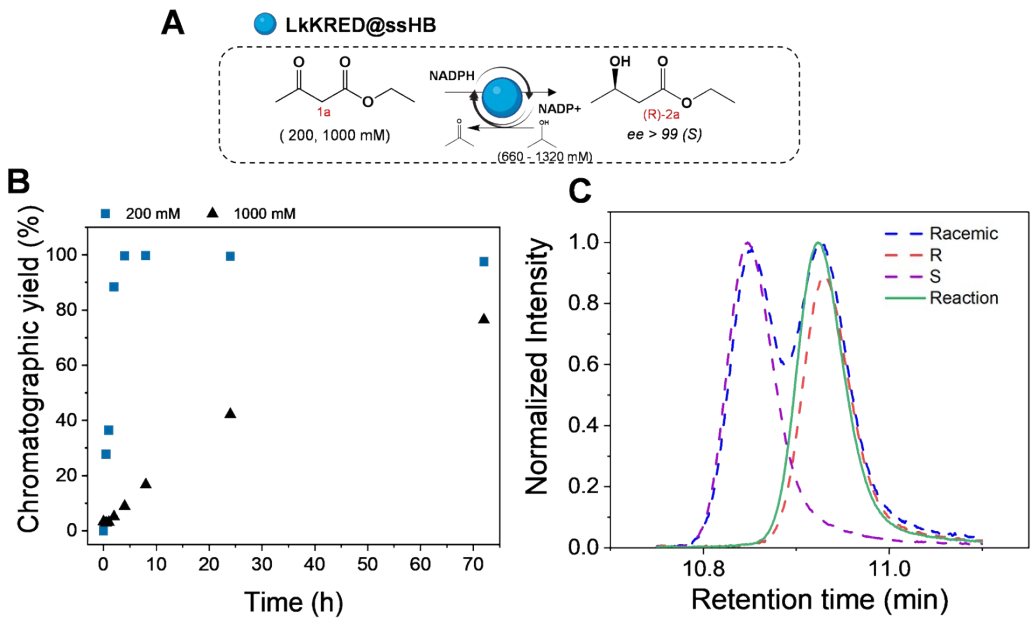


Figure S7. Reaction scheme, Reaction course and Chirality analysis of LkKRED@ssHB reduction of 1a.

(A) Reaction scheme of asymmetric reduction of 1a by LkKRED self-sufficient heterogeneous biocatalyst with cofactor recycling based on Isopropanol oxidation. (B) 100 mg of the ssHB described in Table 2 were incubated for 72 h with 1 mL of a solution of (200-1000 mM) ethyl acetoacetate (1a) in 10 mM Tris-HCl buffer at pH 7. Several aliquotes of less than 5% of the total reaction volume were withdrawn and analysed by GC-FID as described in materials and methods. (C) The final product of the previous reactions in addition to racemic, (*S*)- and (*R*) ethyl 3-hydroxybutyrate (2a) standards were derivatised as described in materials and methods and analyzed by chiral GC-FID.

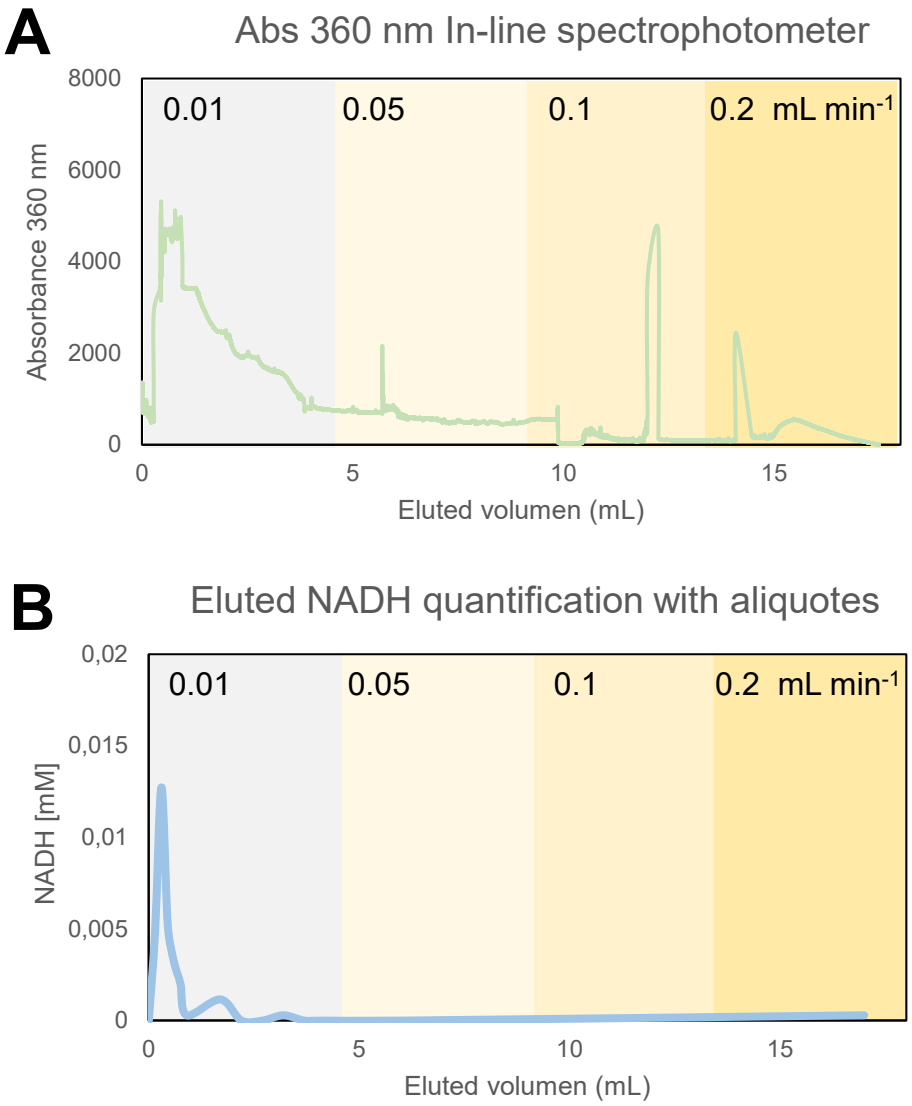


Figure S8. Eluted NADH during continuous flow optimisation 200 mM 1a reduction at different flow rates with TtHBDH ssHB

A) Absorbance (360 nm) detected by In-line spectrophotometer during continuous flow experiments. B) Concentration of NADH eluted vs eluted volume measured by determination of Abs 340 nm in the collected aliquots.

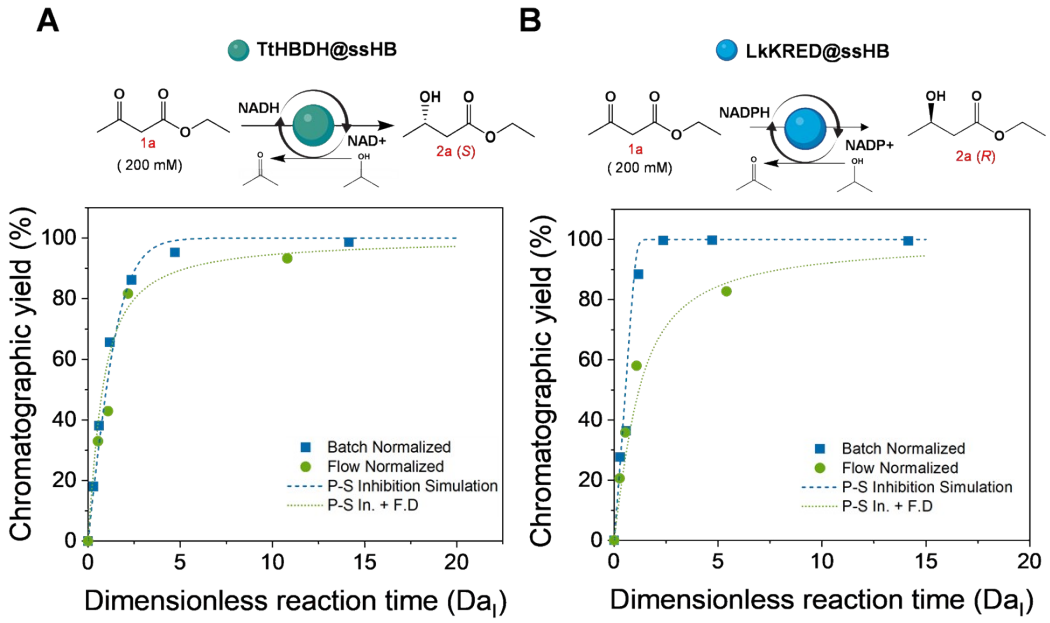


Figure S9. Comparison of simulated (dashed line) and real (square/circle) data of 1a conversion

To enable a proper comparison between batch and continuous flow reactions we plotted the conversion vs. dimensionless reaction time (first Damköhler number) calculated dependent on reactor type and residence time. We compared the real data of the TtHBDH (A) or LkKRED (B) ssHBs in batch reactions (circles) or packed bed reactor (squares) with the first-order reaction simulated employing a kinetic model of Michaelis-Menten with substrate and product inhibition in the absence (blue dashed line) or presence (green dots line) of flow dispersion. These results were simulated using the kinetics constants obtained for the immobilised enzymes.

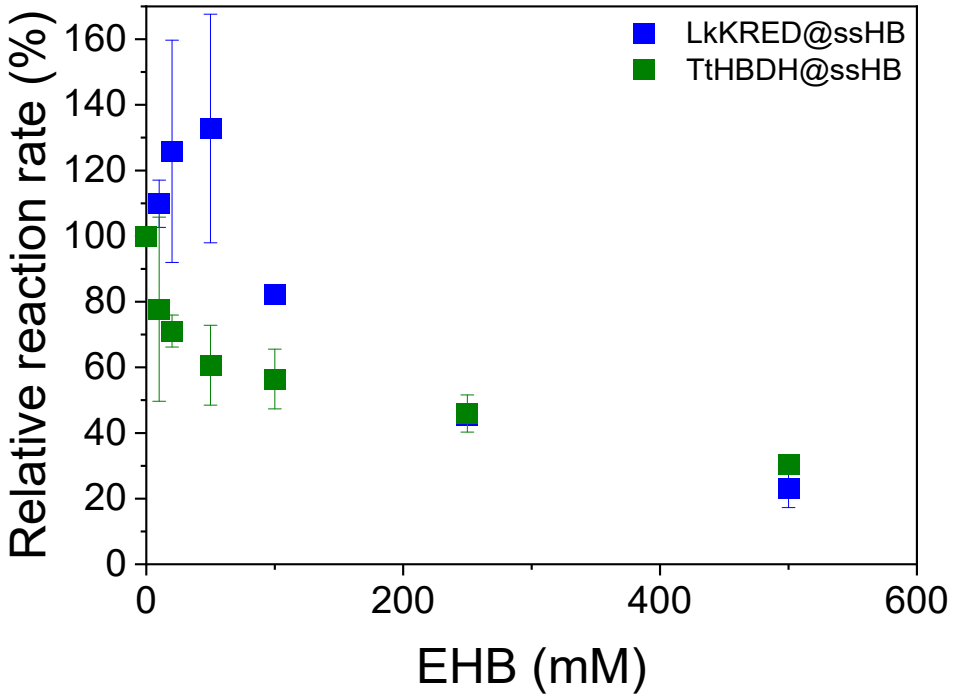


Figure S10. Relative reaction rate of TtHBDH (Green) and LkKRED (Blue) ssHB in the presence of different concentrations of 2a.

100 mg of the ssHB were incubated for 30 min with 1 mL of a solution of ethyl acetoacetate (1a) (200 mM) in 10 mM Tris-HCl buffer at pH 7, in the presence of different concentrations of 2a (10-500 mM). The reaction was stopped by filtration and analysed by GC-FID.

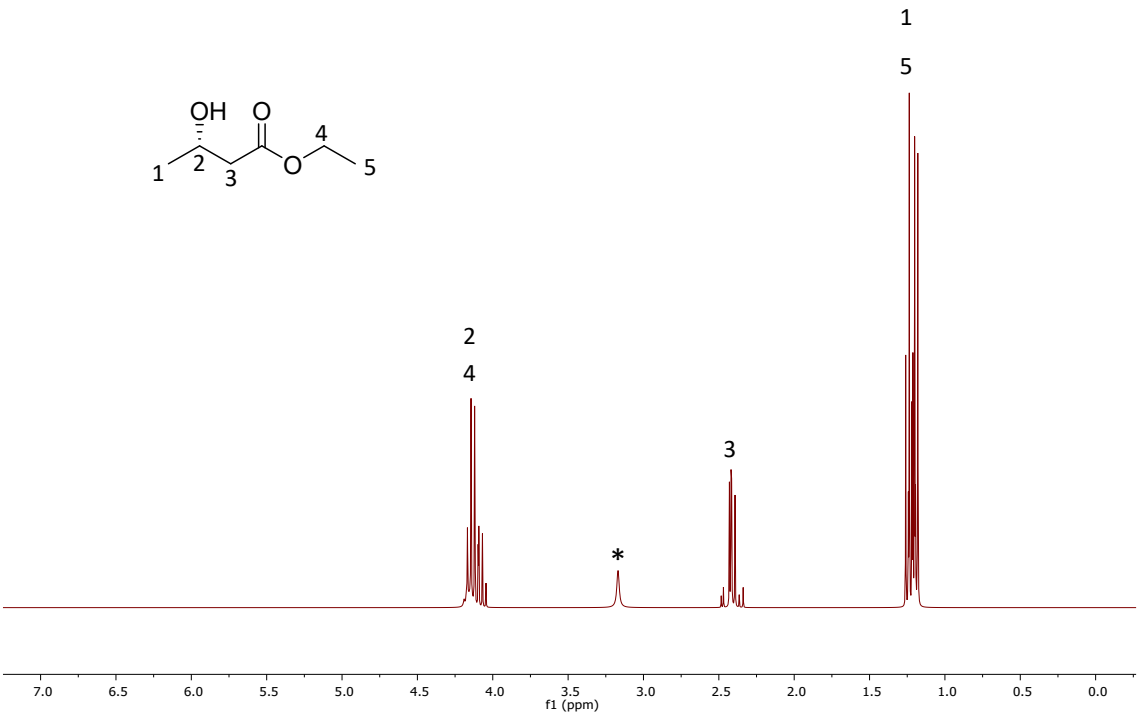


Figure S11. ^{1}H NMR of purified ethyl (*S*)-3-hydroxybutyrate (2a) (300 MHz, Chloroform, 298 K)

^{1}H NMR (300 MHz, Chloroform, 298 K) δ 4.22 – 4.03 (m, 3H), 2.49 – 2.32 (m, 2H), 1.28 – 1.14 (m, 6H). * Correspond to the C3 of the starting material (**1a**)

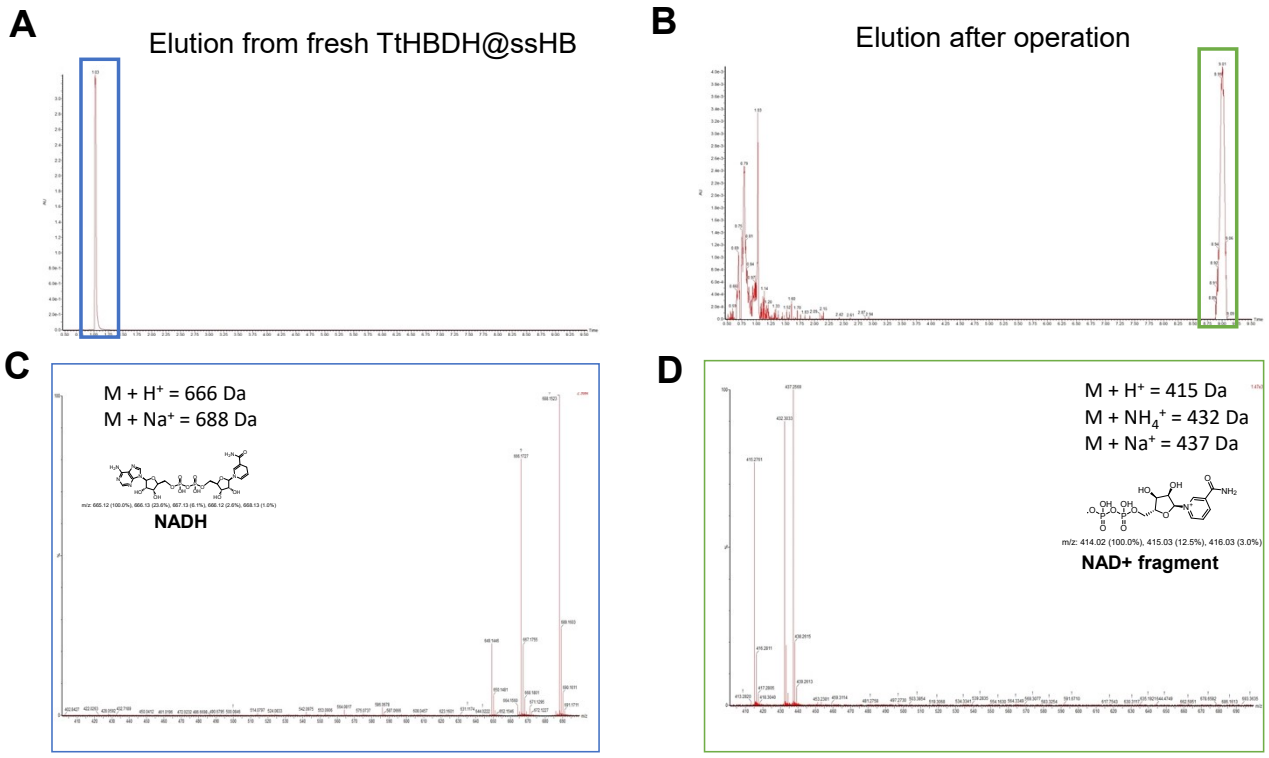


Figure S12. UPLC-Ms analysis of eluted NADH

UPLC-MS chromatograms at 340 nm of the supernatants recovered after incubation with 1M NaCl of fresh (A) and used (B) TtHBDH@ssHB. Positive ion TOF-MS spectra of peaks squared in blue from A (C) or in green from B (D).

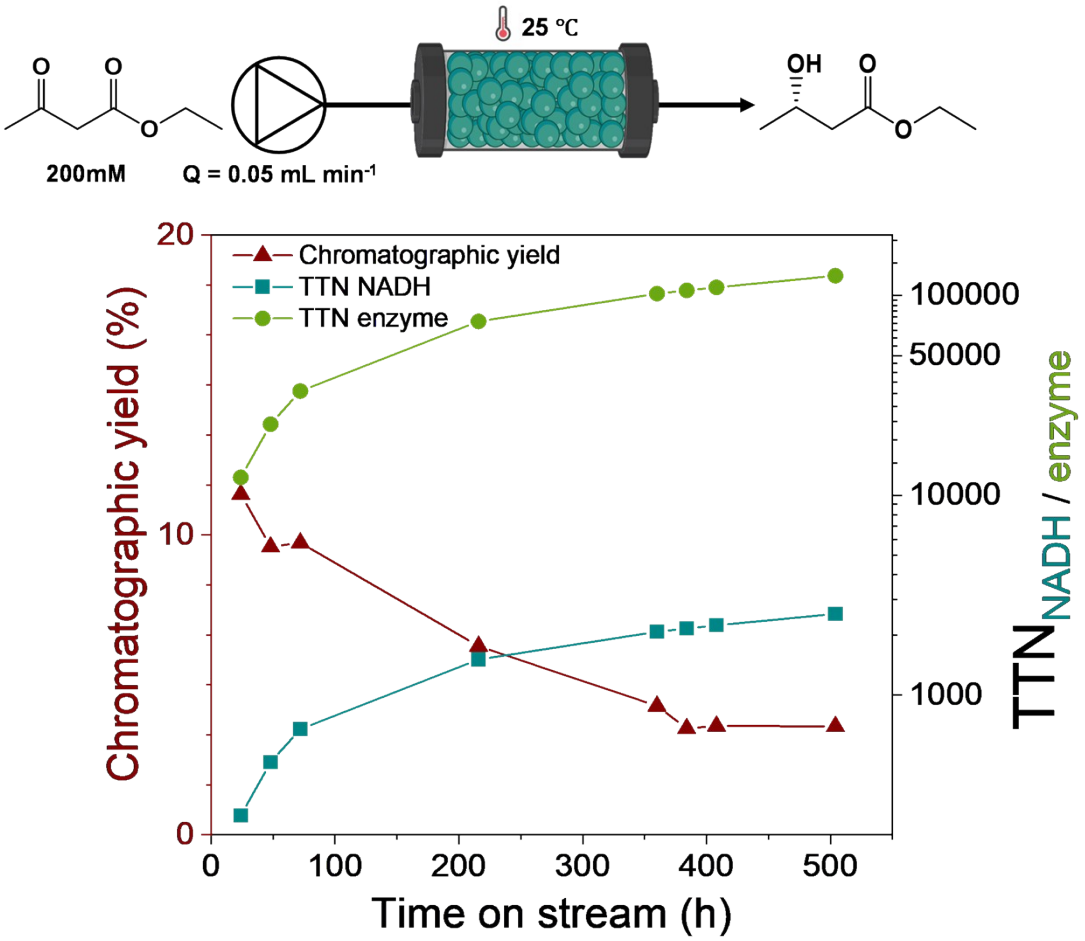


Figure S13. Continuous operation of TtHBDH@ssHB at 25ºC

Chromatographic Yield vs TTN of enzyme and cofactor of TtHBDH@ssHB in the reduction of 200mM of **1a**.

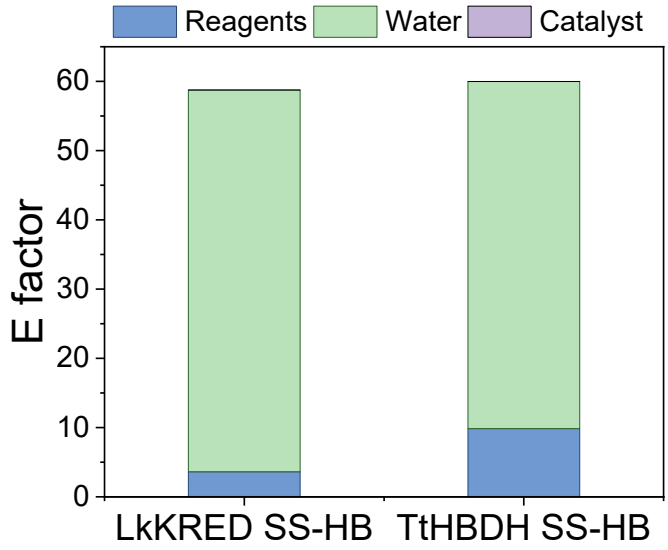


Figure S14. Dissected E factor

Dissected E factor of the continuous synthesis of *R*- and *S*- enantiomers of ethyl 3-hydroxybutyrate (2a) by TtHBDH and LkKRED ssHBs in continuous flow operation.

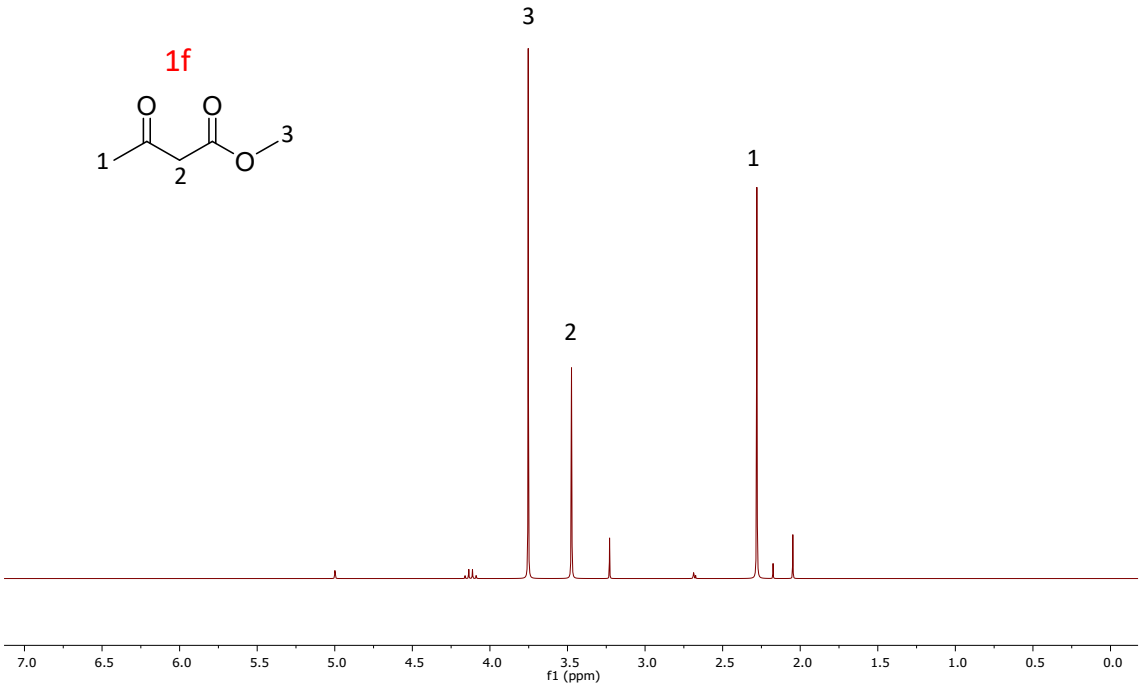


Figure S15. ^{1}H NMR of 1f (300 MHz, Chloroform, 298 K)

^{1}H NMR (300 MHz, Chloroform, 298 K) δ 3.75 (s, 3H), 3.47 (s, 3H), 2.28 (s, 3H).

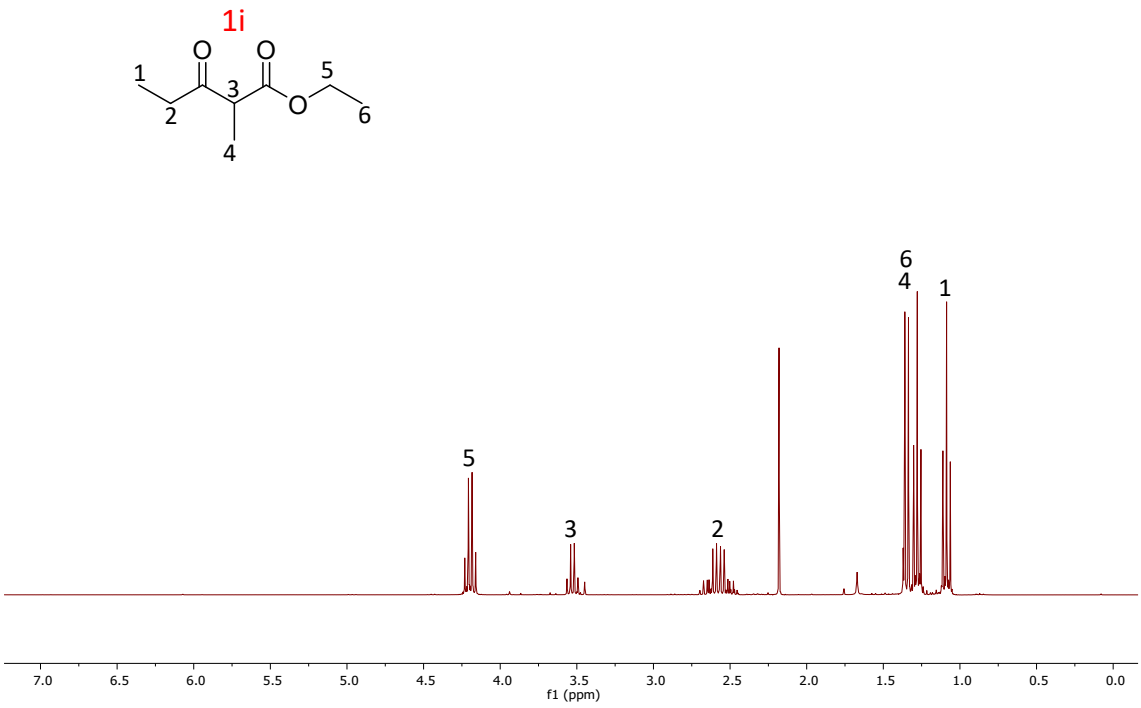


Figure S16. ^{1}H NMR of 1i (300 MHz, Chloroform, 298 K)

δ 4.20 (q, *J* = 7.1 Hz, 2H), 3.59 – 3.42 (m, 1H), 2.71 – 2.43 (m, 2H), 1.38 – 1.20 (m, 6H), 1.07 (d, *J* = 7.2 Hz, 3H).

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