E. coli Nickel-Iron Hydrogenase 1 Catalyses Non-native Reduction of Flavins: Demonstration for Alkene Hydrogenation by Old Yellow Enzyme

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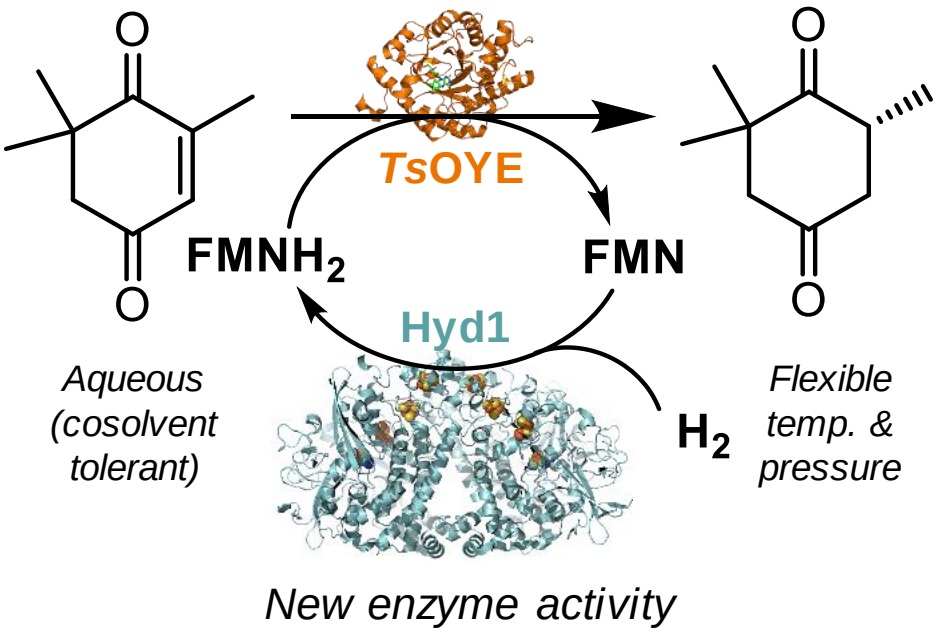
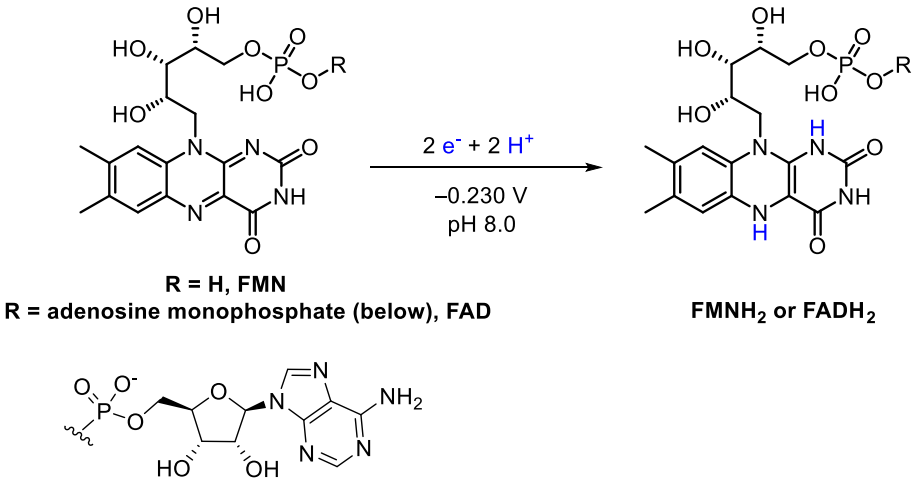


Abstract: A new activity for the [NiFe] uptake hydrogenase 1 of Escherichia coli (Hyd1) is presented. Direct reduction of biological flavin cofactors FMN and FAD is achieved using H_2 as a simple, completely atom-economical reductant. The robust nature of Hyd1 is exploited for flavin reduction across a broad range of temperatures (25–70 °C) and extended reaction time. The utility of this system as a simple, easy to implement $FMNH_2$ regenerating system is then demonstrated by supplying reduced flavin to an Old Yellow Enzyme to support asymmetric alkene reductions with up to 100% conversion. High Hyd1 turnover frequencies (up to 340 s^{-1}) and total turnover numbers (>10,000,000) during flavin recycling show the promise of this biocatalytic system.

As the need to make chemical manufacturing more sustainable becomes urgent, academic and industrial fields increasingly turn to biotechnology.[1] Enzymes provide many advantages over other catalysts: they are renewable, biodegradable, nonhazardous, and provide high selectivity. The once-limited scope of known enzyme reactions has rapidly expanded, aided by enzyme engineering and ongoing discovery and characterisation of new enzymatic functions.[2,3]

Many useful enzymes for biotechnology employ flavin moieties (e.g. FMN, FAD; Scheme 1) as prosthetic groups or cofactors. These include halogenases (chlorination, bromination, iodination),[4] ene-reductases (activated alkene reduction),[5] and flavoprotein monooxygenases (epoxidations, hydroxylations, Baeyer-Villiger oxidation).[6] Potential applications of these enzymes are natural product and pharmaceutical synthesis,[7] biodegradation of environmental pollutants,[8] and non-native light-driven reactions (Figure S2).[9] These reactions require one equivalent of the reduced cofactors $FMNH_2$ or $FADH_2$. To lower cost and waste, a catalytic quantity of more stable oxidised FMN/FAD is supplied, and is reduced (Scheme 1) *in situ* by means of photochemistry, electrochemistry, metal-catalysis or biocatalysis (Figure S2).[4,5,10]

In general, biocatalysed cofactor recycling is the most straightforward option for coupling with flavoenzyme reactions because the alternative catalysts can face biocompatibility challenges (e.g. mutual inactivation, mismatched ideal solvent, pH or temperature).[4,11] A common, yet cumbersome, strategy is to regenerate the flavin using an NAD(P)H-dependent reductase which produces $FMNH_2$ or $FADH_2$ at the expense of NAD(P)H[12] or its analogues.[13] A catalytic quantity of the reduced nicotinamide cofactors must in turn be regenerated due to their high cost. This is typically achieved *via* glucose dehydrogenase-catalysed oxidation of glucose, which elevates cost, waste and downstream processing.[14] The complexity of currently-available recycling systems for reduced flavins may explain the under-utilisation of flavoenzymes in biotechnology, despite the important reactions they catalyse.[15]



Scheme 1. Oxidised (left) and reduced (right) FMN or FAD cofactors

Alternatively, H_2 has previously been demonstrated for cleaner enzymatic NADH cofactor recycling.[16,17] The soluble hydrogenase from *Cupriavidus necator* (formerly *Ralstonia eutropha*) natively uses H_2 to provide electrons for NAD^+ reduction at a prosthetic flavin cofactor.[17] Reduction of flavin substrates by this enzyme under H_2 has also been reported,[18] presumably at the NAD^+ binding site. However, this enzyme is complex to express and lacks stability at elevated temperatures.[19,20] This inspired us to test whether a simple hydrogenase (Figure 1) could be suitable for H_2-driven flavin reduction. The thermodynamic potential for the H^+/H_2 couple (−0.472 V, pH 8) relative to the flavin potential (−0.230 V, pH 8, Scheme 1),[21] makes reduction of flavin by H_2 thermodynamically

favourable. We selected *E. coli* [NiFe]-hydrogenase (Hyd1), which is a good H_2 oxidiser[22,23] and well-characterised in terms of X-ray crystal structures[24,25] and spectroscopy.[23,26] Hyd1 is natively expressed in *E. coli* and, unlike many hydrogenases,[27] it is O_2-tolerant[23] and active over a wide pH range.[28] Like other uptake hydrogenases, the basic unit of Hyd1 is a heterodimer of the large subunit (HyaB) housing the [NiFe] active site, and the small subunit (HyaA) housing the iron-sulfur cluster electron transfer relay. Natively, Hyd1 is coupled to a cytochrome electron acceptor, and exists as a homodimer of HyaAB units. The isolated enzyme comprises predominantly dimeric HyaAB[29] and our preparation lacks the cytochrome (Figure S1).

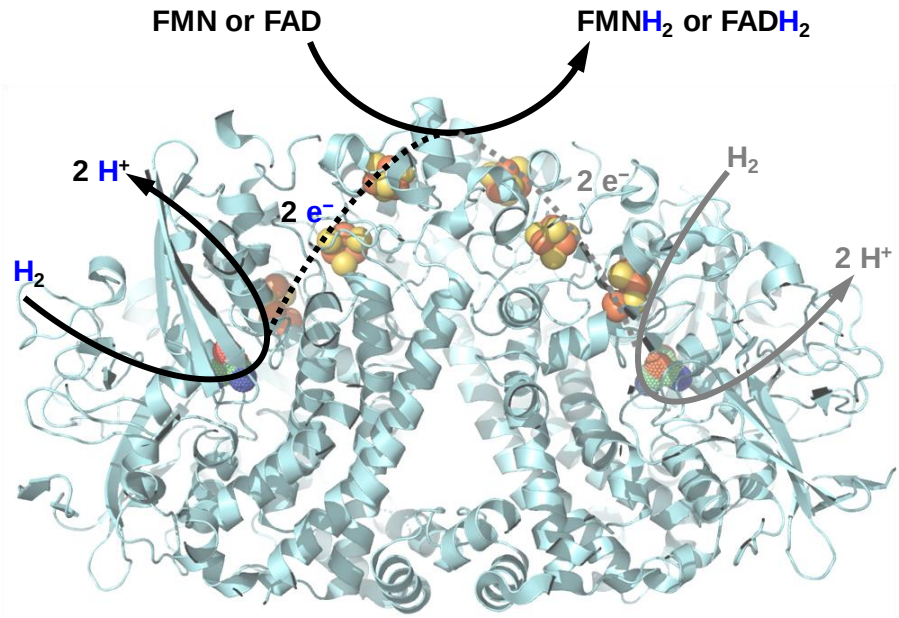


Figure 1. Two-electron flavin reduction by Hyd1. H_2 oxidation at the [NiFe] active site (green, red, blue) provides 2 electrons that are transferred to the surface of the protein *via* FeS clusters (yellow, orange). The figure, showing the homodimer of HyaAB units, was prepared using PyMOL™ 2.3.4 (PDB: 6FPW).

The H_2 oxidation activity of Hyd1 is typically measured using the artificial electron acceptor benzyl viologen in colourimetric assays.[28] Electrons from H_2 oxidation at the [NiFe] active site (Figure 1) are relayed through FeS clusters where, evidence suggests, benzyl viologen reduction occurs rather than directly at the [NiFe] active site.[30] Herein, we demonstrate that both FMN and FAD can accept electrons from H_2 oxidation by Hyd1 to generate $FMNH_2$ and $FADH_2$, and show that Hyd1 can be used as an effective $FMNH_2$ regeneration system to support asymmetric alkene reduction by an Old Yellow Enzyme (OYE)-type ene-reductase.

Figure 2 shows the results of *in situ* UV-visible spectrophotometric assays to explore FMN and FAD reduction by Hyd1 (38 µg, produced and isolated in accord with S1.2, Supporting Information) under H_2 (General Procedure A, Supporting Information). The flavin moiety of FMN gives λ_{max} at 445 nm and FAD at 450 nm, both of which bleach upon two-electron reduction (Figure 2A–B; see Figure S8 for spectra of fully reduced FMN).[31,32] The decrease in [oxidised flavin] over time was used to calculate initial enzyme activity (Figure 2C–D). Control experiments indicated that omission of Hyd1 or H_2 led to negligible flavin reduction (Figures S3–S4).

Upon addition of Hyd1, a lag phase was observed during FMN and FAD reduction, which is attributed to the well-characterised H_2-dependent activation phase for aerobically purified Hyd1.[23] Later experiments (when indicated) used Hyd1 that was first activated under a H_2 atmosphere.[33] The lag phase was followed by a decrease in absorbance consistent with

$FMNH_2$/$FADH_2$ formation, and clear isosbestic points at 330 nm corroborate a lack of side products. Specific initial activities for FMN and FAD reduction (76 and 32 nmol min^{-1} mg^{-1} Hyd1, respectively) were determined during the linear reaction phase. The higher activity for reduction of FMN compared with FAD cannot be attributed to thermodynamic driving force since both cofactors have similar reduction potentials,[21] but could relate to the cofactors' ability to interact at the protein surface.

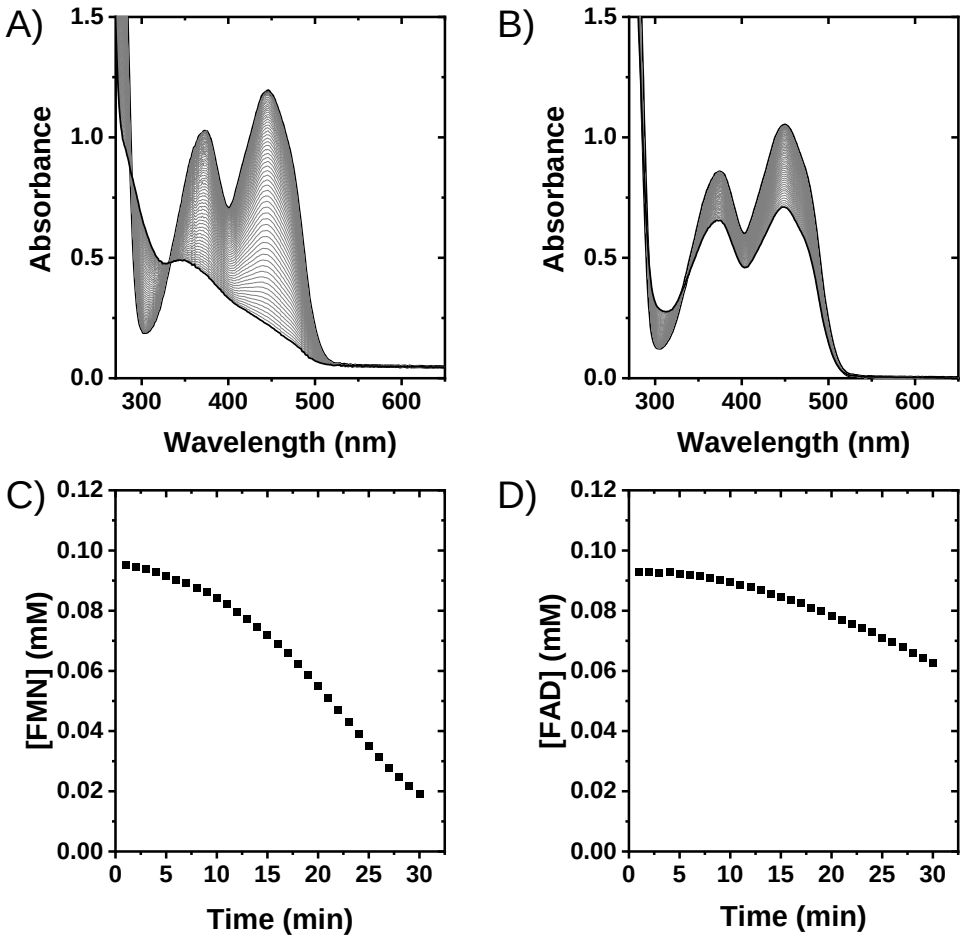


Figure 2. Activity assay for H_2-driven Hyd1 reduction of flavin measured by *in situ* UV-visible spectroscopy. A) Hyd1 reducing FMN. B) Hyd1 reducing FAD. C) Calculated [FMN] based on λ_{max} = 445 nm (ε = 12.50 mM^{-1} cm^{-1}). D) Calculated [FAD] based on λ_{max} = 450 nm (ε = 11.30 mM^{-1} cm^{-1}). Reaction conditions: General Procedure A in Tris-HCl buffer (50 mM, pH 8.0, 25 °C).

Hyd1 is known to be robust which inspired us to test H_2-driven flavin reduction activity at different temperatures (25–70 °C, General Procedure A). Percentage conversion of FMN and FAD to the reduced forms after 30 min reaction time increased with temperature (Figure 3), though FMN reduction was not enhanced past 60 °C. This suggests that Hyd1 is likely to open new doors to cofactor recycling for flavoenzymes with optimal activity at higher temperatures.

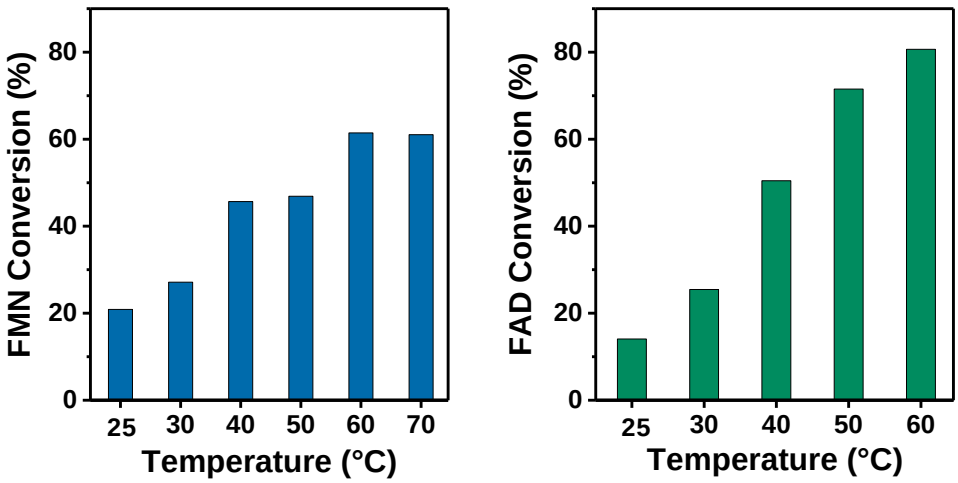
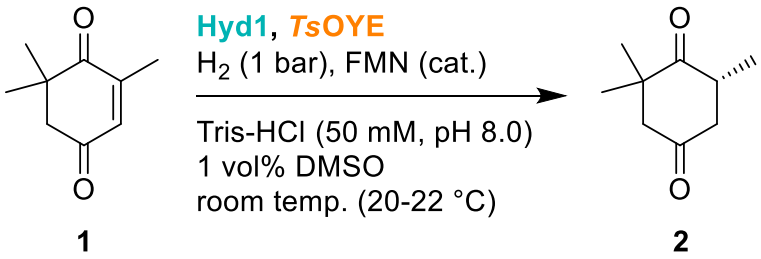


Figure 3. Hyd1-catalysed flavin reduction at different temperatures. Reaction conditions: General Procedure A (Supporting Information) in phosphate buffer (50 mM, pH 8.0). Conversion was calculated after 30 min using UV-visible spectroscopy.

In order to demonstrate the utility of Hyd1 in biotechnologically-relevant flavin recycling, we coupled Hyd1-catalysed flavin reduction with the OYE-type ene-reductase from *Thermus scotoductus, Ts*OYE,[34,35] to catalyse enantioselective reduction of ketoisophorone (**1**) to (*R*)-levodione (**2**, Scheme 2). Reactions were conducted according to General Procedure B (Supporting Information) and monitored using chiral-phase GC-FID after extraction of the mixture into ethyl acetate (Figure S9). Enantiomeric excess (*ee*) was always >99% at the first time point but decreased to 86–92% from slow racemisation under alkaline conditions. Control experiments confirmed that each component is required for conversion (Table S1).



Scheme 2. Two-enzyme asymmetric alkene hydrogenation.

Quantitative conversion and the highest Hyd1 turnover frequency (TOF, 344 s^{-1}) were achieved with 0.5 mM FMN and 2 mM **1** (entry 1, Table 1). This TOF compares with or improves upon two-component NAD(P)H:flavin reductases.[15,36]

When 0.1 mM FMN was used with varying [**1**] (entries 2–5), up to 97 FMN turnovers (TN) were achieved. This is comparable to the FMN TN reported for a formate-driven homogeneous Rh-catalysed method for $FMNH_2$ recycling coupled to *Ts*OYE.[34] That system required careful balance between enzyme and Rh-catalyst loading to prevent non-enantioselective alkene reduction by $FMNH_2$ or $[Cp^*Rh(bpy)H]^+$, which was not an appreciable issue with our biocatalytic system (Table S1).

The highest Hyd1 total turnover number (TTN, 10,246,000) was achieved using 10 mM **1** (entry 4). This TTN is of an appropriate order of magnitude for industrial catalysis,[37] but there remains room for further optimisation to that end. At 20 mM **1**, Hyd1 TTN and conversion were boosted using 4 bar H_2, which also improved Hyd1 TOF from 88 s^{-1} to 108 s^{-1} (compare entries 5–6).

Table 1. H_2-driven enzymatic alkene reduction under various conditions

Entry	[**1**] (mM)	[FMN] (mM)	Conv. to **2** (%)[a]	Hyd1 TTN[b]	FMN TN[b]
1	2	0.5	100	2,142,000	4
2	2	0.1	100	2,105,000	20
3	5	0.1	95 {100}	5,263,000	50
4	10	0.1	62 {97}	10,246,000	97
5	20	0.1	24 {37}	7,822,000	74
6[c]	20	0.1	{44}	9,332,000	88

Reaction conditions: General procedure B using consistent catalyst loadings. [a]Chiral-phase GC conversion to **2** at 15 h {and 24 h}. [b]Hyd1 total turnover number (mol **2** per mol Hyd1) and FMN turnover number (mol **2** per mol flavin) were determined at the end of the reaction. [c]4 bar H_2.

Like Hyd1, *Ts*OYE has enhanced activity at elevated temperatures,[34] therefore entry 4 was replicated at 35 °C (data not tabulated): Hyd1 TOF nearly doubled to 160 s^{-1} and full conversion was achieved after 24 h, however GC-FID showed that some of **1** and **2** likely evaporated.

To test stability over time, Hyd1 (57 µg) was activated under H_2 at 22 °C for 58 h, then incubated in 0.08 mM FMN under H_2 (1 bar) in a sealed vessel for 62 h. Upon release of H_2, $FMNH_2$ partially oxidised under the N_2 atmosphere to 0.05 mM FMN (determined using UV-visible spectroscopy). The Hyd1 and FMN/$FMNH_2$ solution was placed back under H_2, and full reduction to $FMNH_2$ was noticed after 3.5 h (see Figure S8), which demonstrates appreciable Hyd1 stability over 125 h (>5 days).

The simplified, H_2-driven biocatalysed flavin recycling method coupled with *Ts*OYE led to high Hyd1 TOF and TTN (2–10 million) that correspond with commercial grade enzymes.[38] Further modifications to Hyd1, which is tolerant of mutagenesis,[25,33] might enhance the non-native activity. Additionally, process development is underway to improve industrially-relevant metrics such as cofactor TN. This proof of concept work shows that the robust Hyd1, tolerant to a range of conditions, is a promising catalyst to bring clean flavin recycling into biotechnology.

Acknowledgements

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Keywords: Cofactor recycling • biocatalysis • flavoenzyme • hydrogenation • asymmetric catalysis

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Supplementary Information for

E. coli Nickel-Iron Hydrogenase 1 Catalyses Non-native Reduction of Flavins: Demonstration for Alkene Hydrogenation by Old Yellow Enzyme.

Contents

S1. Reagents

S1.1. General reagents

Buffer salts (Sigma-Aldrich), FAD (disodium salt, ≥98%, Cayman Chemical Company), and FMN (monosodium salt dihydrate, Applichem Panreac) were all used as received. Ketoisophorone (**1**, 2,6,6-trimethyl-2-cyclohexene-1,4-dione) was purchased from Sigma-Aldrich with ≥98% purity. (6*R*)-Levodione ((*R*)-**2**), was obtained by Baker's yeast fermentation and was a gift from Dr. Adrie Straathof from the Delft University of Technology. GC standard *rac*-**2** was prepared following literature procedure.[1] All aqueous solutions were prepared with deoxygenated MilliQ water (Millipore, 18 MΩcm).

S1.2. Enzymes

The hydrogenase (*E. coli* hydrogenase 1, Hyd1) was produced by homologous over-expression of the genes encoding the structural subunits of the enzyme and key maturases. After Hyd1 overexpression under anaerobic bacterial growth, the enzyme was isolated following published protocols (described in the caption to Figure S1).[2] The *Thermus scotoductus* ene-reductase of the Old Yellow Enzyme family (*Ts*OYE)[3,4] was produced and purified following published protocols, and stored at −20 °C as a 200 µM solution in MOPS-NaOH buffer (20 mM, pH 7.0).

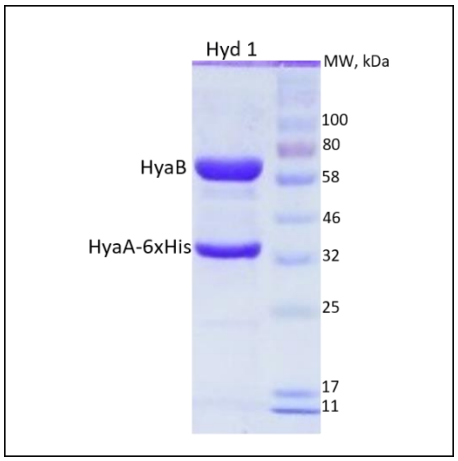

Figure S1. SDS-PAGE analysis of purified Hyd1.

Protein composition and purity in the Hyd1 samples used during all experiments, except those in Section S4.2. After overexpression, the Hyd1 enzyme was purified by Immobilised Metal Affinity Chromatography in a Nickel-NTA prepacked column. After elution with imidazole buffer, the protein was further purified by Size Exclusion Chromatography using a HiLoad 16/600 Superdex 200 pg gel-filtration column (GE Healthcare, UK). Subsequently, the enzyme was buffer-exchanged into Tris HCl buffer (20 mM Tris-HCl pH 7.2, 350 mM NaCl, 0.02% Triton X, 1 mM DTT), concentrated and stored at −80°C.

S2. Analytical tools

S2.1. UV-visible spectroscopy to monitor flavin reduction

UV-visible spectra were recorded by a Cary 60 spectrophotometer with a cell holder (Agilent) and a Peltier accessory for temperature control using a quartz cuvette (path length 1 cm, cell volume 1 mL, Hellma). The indicated buffer was used to take a baseline scan. In some of the experiments, there was a uniform shift of the baseline across the entire spectral region (200–800 nm), which was corrected for during data processing. The concentration of FMN was directly calculated based on the absorbance at λ = 445 nm (ε = 12.50 mM^{-1} cm^{-1}) and FAD based on the absorbance at λ = 450 nm (ε = 11.30 mM^{-1} cm^{-1}). The decrease in [oxidised flavin] over time was determined in order to calculate specific initial enzyme activity (discounting any lag phase).[5]

S2.2. Chiral phase GC-FID to monitor alkene reductions

Column: CP-Chirasil-Dex CB (Agilent), 25 m length, 0.25 mm diameter, 0.25 μm (film thickness), fitted with a guard of 10 m undeactivated fused silica of the same diameter

Carrier: He (CP grade), 170 kPa (constant pressure)

Inlet temperature: 200 °C

Injection conditions: Splitless with split flow 60 mL/min, splitless time 0.8 mins, purge 5 mL/min. Injection volume = 0.5 μL.

Detection: FID (H_2 = 35 mL/min, air = 350 mL/min, makeup N_2 = 40 mL/min, temp = 200 ˚C)

Oven heating profile:

Time (minutes)	Temperature
0 → 5	Hold at 70˚C
5 → 30	Ramp to 120 ˚C at 2 ˚C/min
30 → 36	Ramp to 180 ˚C at 10 ˚C/min
36 → 45	Hold at 180 ˚C for 5 minutes

Compound retention times (reduction of **1***)*:

Time (minutes)	Compound
12.27	Ketoisophorone (**1**)
12.68	(*R*)-Levodione (**2**)
12.80	(*S*)-Levodione (**2**)

S3. Experimental procedures

All experiments were carried out in a glovebox (Glove Box Technology Ltd) under a protective N_2 atmosphere (O_2 < 0.1 ppm). Stock solutions of FAD and FMN were prepared using deoxygenated buffer. Different concentrations of stock solutions of **1** were prepared in DMSO such that DMSO was 1 vol% in the final reaction mixture.

S3.1. General Procedure A (Flavin reduction)

The indicated volume of Tris-HCl buffer (50 mM, pH 8.0) or phosphate buffer (50 mM, pH 8.0) was added to a UV-visible quartz cuvette, which was placed in the cell holder and allowed to warm to the indicated temperature (pre-set on the Peltier accessory) for 5 min. A baseline was recorded using the UV-visible spectrophotometer (see S2.1). A solution of 0.1 mM flavin (unless otherwise noted) in the designated buffer was next prepared in the cuvette, which was then capped with a rubber septum that was pierced with two needles to provide a gas inlet and outlet. An H_2-line was then connected and bubbled through the flavin solution *via* the inlet needle for 10 minutes. The needle was then moved up to the headspace through which a continuous H_2 flow was supplied. About 0.4 mL of the flavin solution was then used to transfer the designated quantity of Hyd1 into the cuvette using a syringe and needle, and the needle and syringe rinsed by drawing solution in and out of the cuvette. The assay was carried out by taking one scan (200–800 nm) every 30 seconds over 30 minutes.

S3.2. General Procedure B (Alkene reduction)

Using a syringe and needle, 600 µL of H_2-saturated Tris-HCl buffer (50 mM, pH 8.0, 25 °C) was transferred to a centrifuge tube (Eppendorf, 1.5 mL) that contained the required quantities of FMN and **1** in DMSO (1 vol% DMSO in total reaction mixture). A portion of this solution (approx. 0.2 mL) was used to transfer Hyd1 (57 µg, activated under H_2 for 3–15 h) and *Ts*OYE (145 µg) into the reaction tube in sequence *via* a needle and syringe. The lid of the centrifuge tube was pierced once with a needle, capped, and placed in a Büchi Tinyclave pressure vessel which was then charged to the designated pressure of H_2. The pressure vessel was then removed from the glovebox and wrapped in aluminum foil to exclude light in order to prevent photodecomposition of the FMN, flavoenzyme, or both.[6] The vessel was placed on a Stuart® mini see-saw rocker set to 30 oscillations/min. The extent of conversion and enantiomeric excess (%*ee*) of (*R*)**-2** was determined by chiral GC-FID (General Procedure C).

S3.3. General Procedure C (Preparing samples for chiral GC analysis)

Aliquots (25 µL) of reaction mixture were taken for analysis at 1 h and 15 h (and 24 h when indicated) then extracted into 200 µL EtOAc with 2 mM undecane as an internal standard. The biphasic solution was centrifuged to separate out any solids (12,000 × *g*, 2 min), then 150 µL of the EtOAc layer was removed, dried over Na_2SO_4 and 75 µL of the solution was taken for GC analysis (see S2.2).

S4. Supplementary data and results

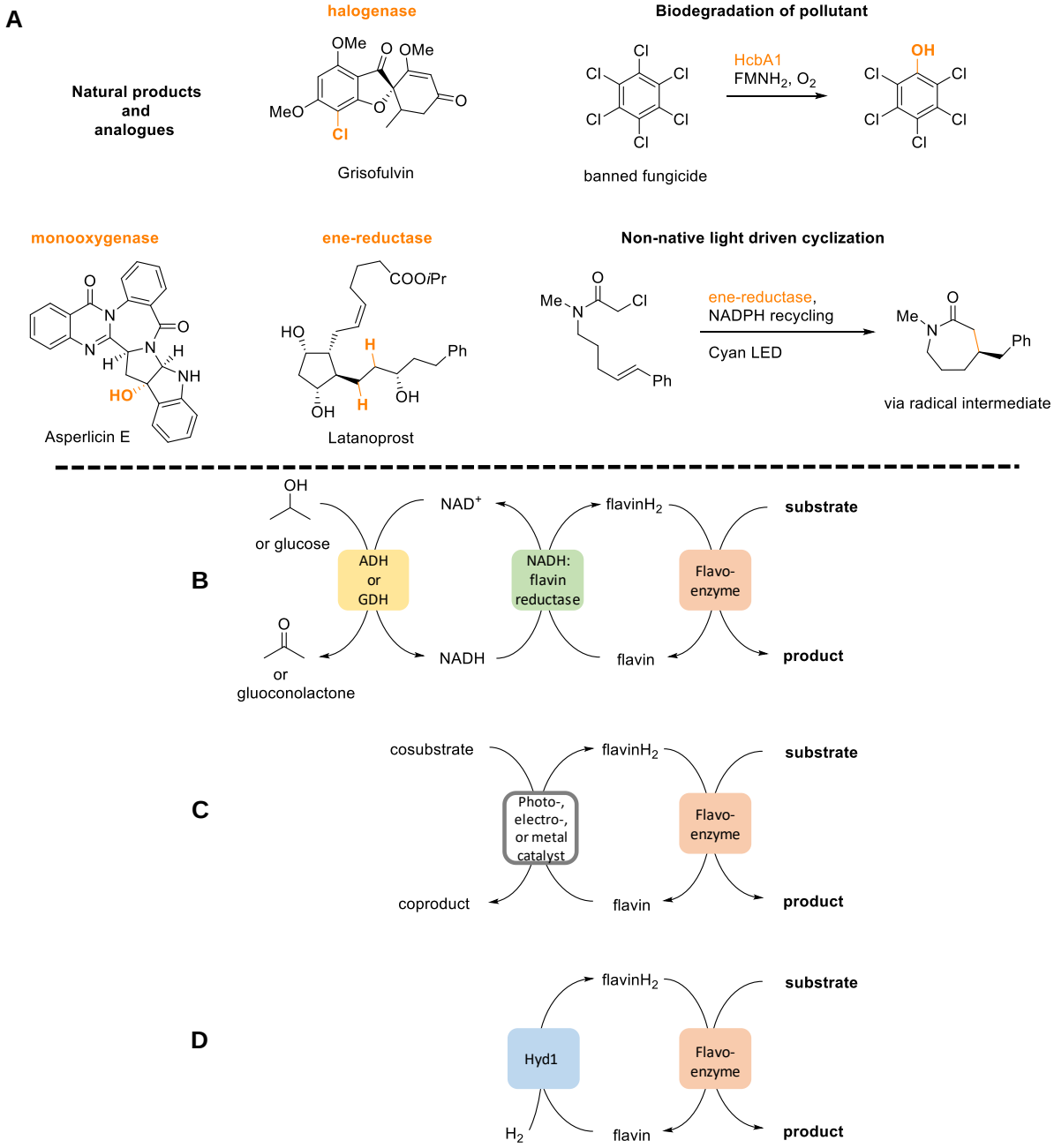

Figure S2. Current applications and methods of flavin recycling

A. Examples of flavoenzymes applied toward natural products and analogues,[7–9] degradation of an environmental pollutant,[10] and a non-native light-driven cyclisation.[11] **B.** Current enzymatic flavin regeneration methods rely on NAD(P)H, which itself is continually regenerated using expensive, carbon-based sacrificial reductants. **C.** Other catalytic methods for flavin recycling tend to rely on cosubstrate additives. **D.** (This work) A simplified H_2-driven direct flavin reduction method using Hyd1 enzyme.

S4.1. Control experiments to confirm role of Hyd1 and H_2 in flavin reduction

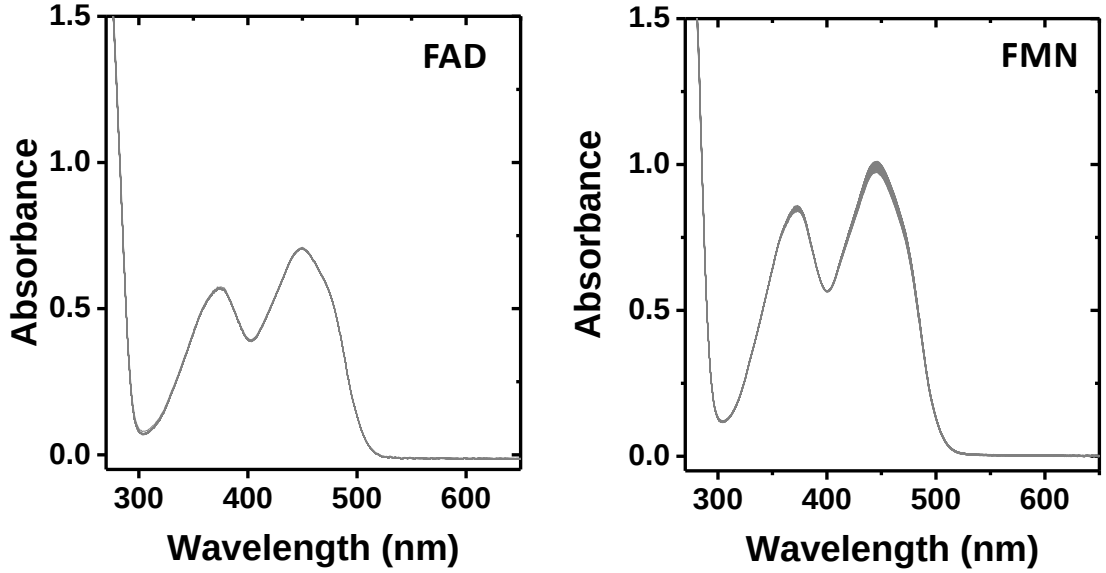

Figure S3. Background flavin reduction in absence of H_2
Reaction conditions: 800 µL scale, 0.1 mM flavin in Tris-HCl buffer (50 mM, pH 8, 25 °C), 40 µg Hyd1, 25 °C controlled by Peltier accessory. The Hyd1 specific activity for FAD and FMN reduction during this control reaction was 0.06 nmol min^{-1} mg^{-1} and 2.08 nmol min^{-1} mg^{-1} respectively.

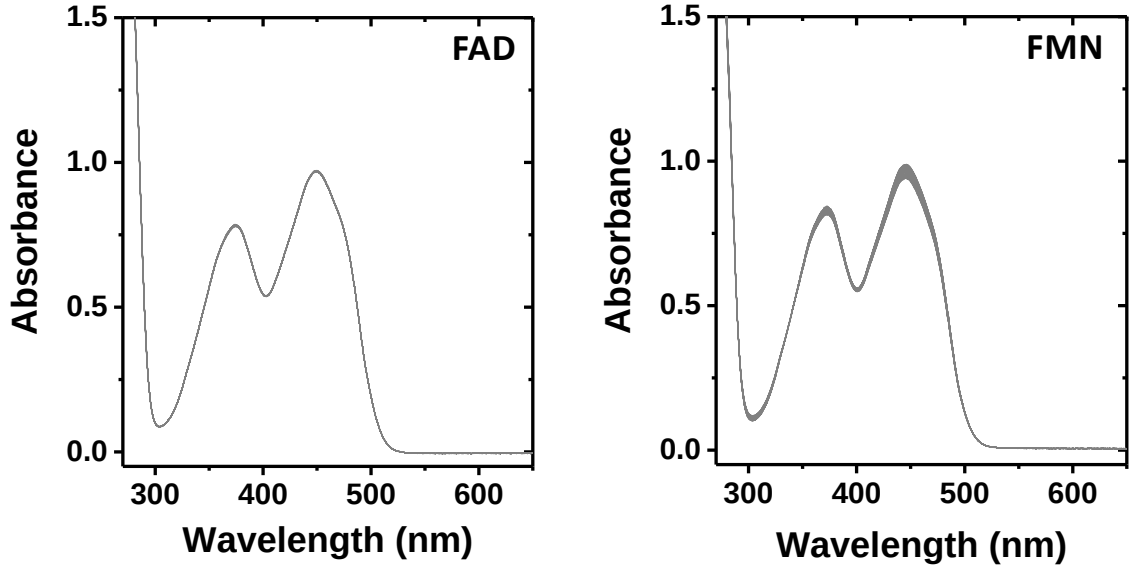

Figure S4. Background flavin reduction in absence of Hyd1
Reaction conditions: 800 µL scale, 0.1 mM flavin in Tris-HCl buffer (50 mM, pH 8, 25 °C), H_2 flow (cuvette head space), 25 °C controlled by Peltier accessory. The overall decrease in [FAD] and [FMN] amounts to 0.000 mM and 0.005 mM after 30 minutes respectively.

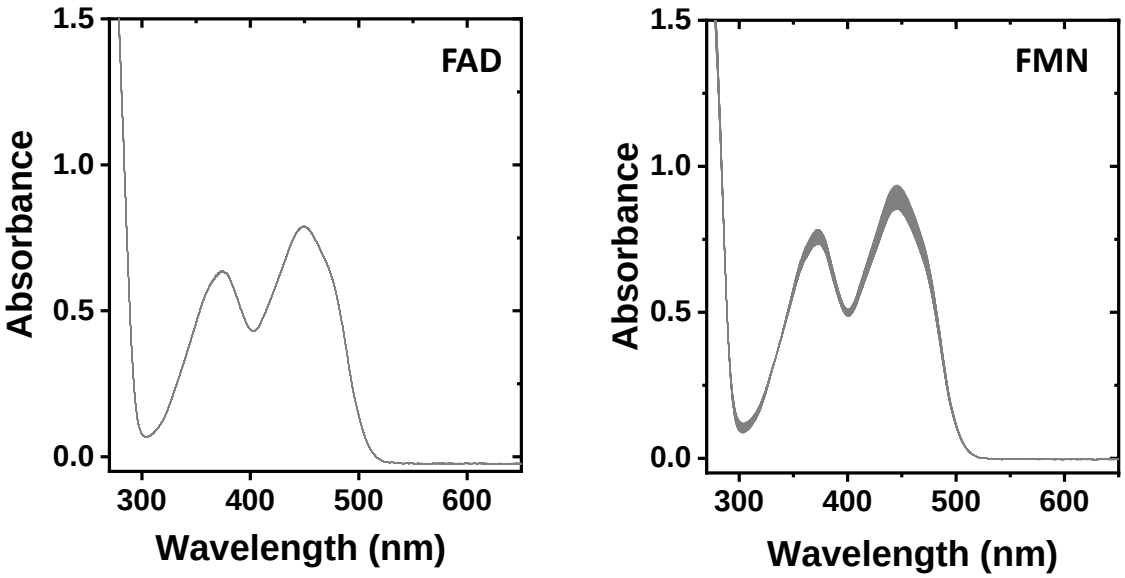

Figure S5. Background flavin reduction in the absence of H_2 and Hyd1
Reaction conditions: 800 µL scale, 0.1 mM flavin in Tris-HCl buffer (50 mM, pH 8, 25 °C), 25 °C controlled by Peltier accessory. The overall decrease in [FAD] and [FMN] amounts to 0.000 mM and 0.080 mM after 30 minutes respectively.

S4.2. Reduction of FMN under H_2 using Hyd1 enzyme fractions that contain or are lacking the cytochrome

Experiments were conducted to determine whether the presence of the partner cytochrome, HyaC, impacts the flavin reduction activity. As shown in Figures S6 and S7, the rate of FMN reduction was not affected significantly by the presence or absence of cytochrome. Hyd1 for these experiments was provided by Wangzhe Li and Sophie Kendall-Price (University of Oxford), with advice from Dr Rhiannon Evans (University of Oxford). Hyd1 with cytochrome was separated by gel filtration. Hyd1 without cytochrome was prepared from a strain lacking the hyaC gene.

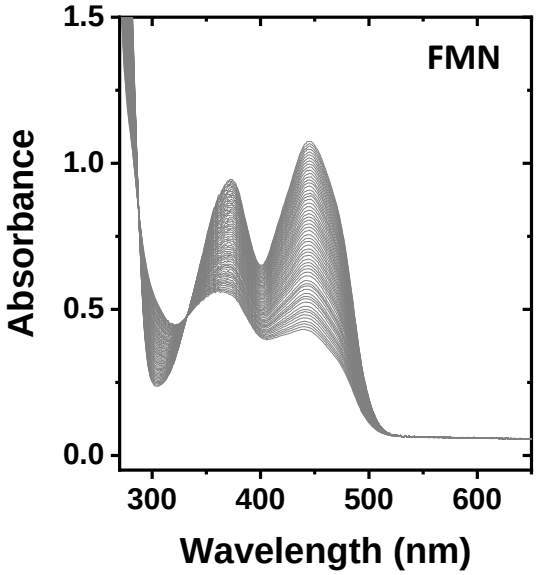

Figure S6. Flavin reduction in the presence of H_2 using a Hyd1 sample that does not contain the cytochrome

Reaction conditions: 800 µL scale, 0.1 mM FMN in Tris-HCl buffer (50 mM, pH 8, 25 °C), 40 µg Hyd1 expressed without the cytochrome gene (activated for 16 h under H_2), 25 °C controlled by Peltier accessory. The Hyd1 specific activity observed for FMN for this reaction was 47.5 nmol min^{-1} mg^{-1}.

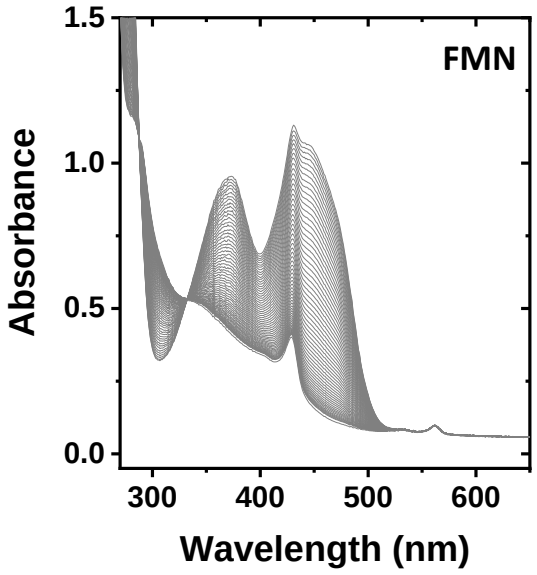

Figure S7. Flavin reduction under the presence of H_2 using Hyd1 containing the cytochrome
Reaction conditions: 800 µL scale, 0.1 mM FMN in Tris-HCl buffer (50 mM, pH 8, 25 °C), 60 µg Hyd1 containing the cytochrome subunit (activated for 17 h under H_2), 25 °C controlled by Peltier accessory. In this case, an absorbance change for oxidised vs reduced cytochrome is overlaid on the spectral changes from the FMN, giving rise to a sharp peak at around 425 nm.[12] The Hyd1 specific activity observed for FMN reduction in this reaction was 51.6 nmol min^{-1} mg^{-1}.

S4.3. Complete reduction of FMN to $FMNH_2$

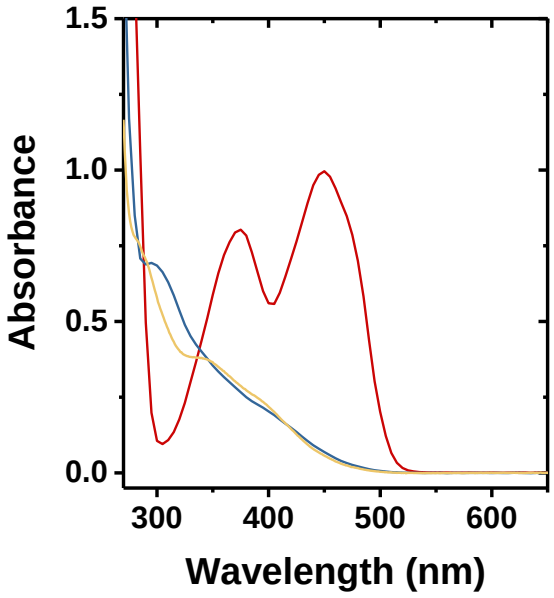

Figure S8. UV-visible spectra of FMN (red) and $FMNH_2$ produced by Hyd1 under H_2 (yellow) or sodium dithionite (blue).
Reaction conditions for FMN reduction by Hyd1 (yellow): 800 µL scale, 0.1 mM FMN in Tris-HCl buffer (50 mM, pH 8, 25 °C), H_2 flow (cuvette head space), 57 µg Hyd1, 25 °C controlled by Peltier accessory. The full reduction of FMN by Hyd1 was completed during the experiment designed to test the stability of Hyd1 over time (>5 days).
Reaction conditions for FMN reduction by sodium dithionite (blue): 800 µL scale, 0.1 mM FMN in Tris-HCl buffer (50 mM, pH 8, 25 °C), 0.15 mM sodium dithionite, 25 °C controlled by Peltier accessory.

S4.4. Control experiments for H_2-driven ketoisophorone reduction

Control experiments were performed to see if Hyd1 (entry 1) or *Ts*OYE (entry 2), alone, could lead to **2**. In addition, similar experiments were done in the absence of FMN (entry 3) or no enzyme (entry 4). The control experiments demonstrated the need for each reaction component for the reaction to be successful. The results of the experiment are shown in Table S2.

Table S2. Control experiments for H_2-driven ketoisophorone reduction

Entry	FMN	*Ts*OYE	Hyd1	Conversion to 2 (%)
1	✓	-	✓	0
2	✓	✓	-	0
3	-	✓	✓	0
4	✓	-	-	0

Reaction conditions: 600 µL scale, 0.1 mM FMN, 57 µg Hyd1, *Ts*OYE (145 µg), 10 mM **1**, Tris-HCl buffer (50 mM, pH 8.0, 25 °C), 1 vol% DMSO at ambient temperature in pressure vessel (1 bar H_2), 24 h.

S4.5. Exemplary chiral-phase GC-FID spectra of enzymatic H_2-driven reduction of ketoisophorone to (*R*)-levodione

Reduction of **1** (entry 4, Table 1) was carried out and the reaction mixture was analysed by chiral GC-FID according to General Procedure C (see S3.3). Conversion to **1** and the enantiomeric excess (%*ee*) were calculated based on the peak area of their respective peaks as shown in Figure S9.

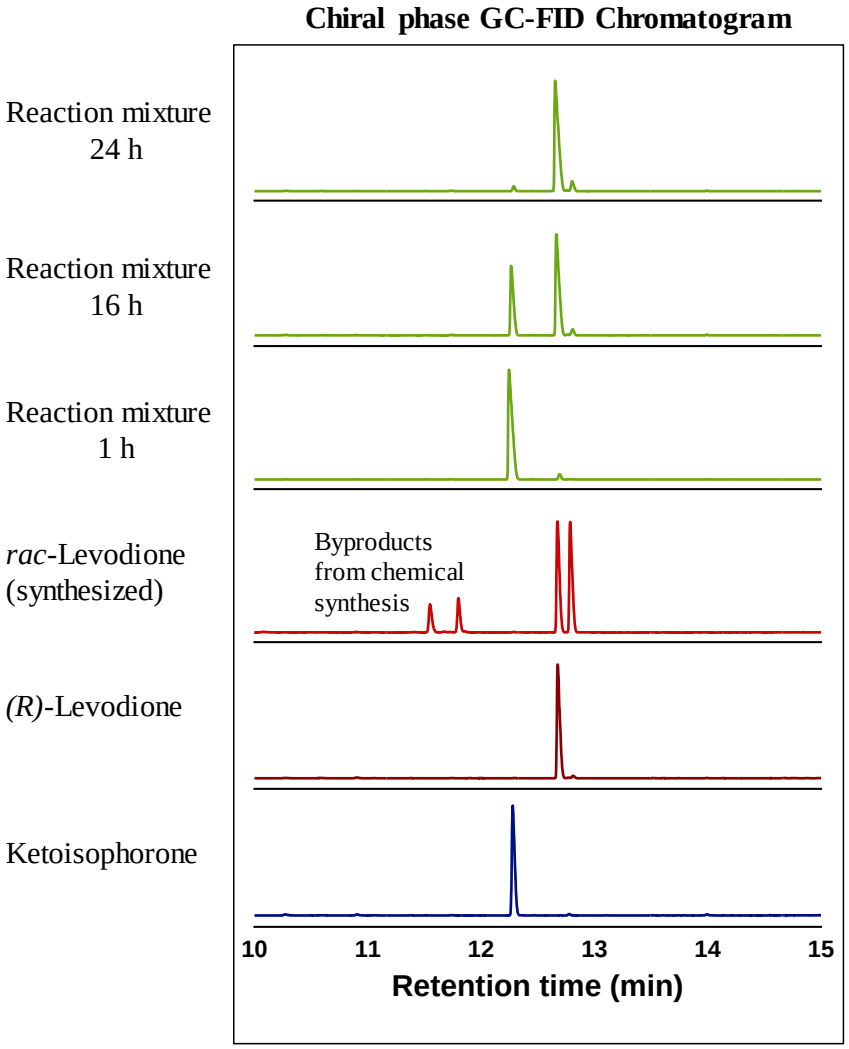

Figure S9. GC-FID results of ketoisophorone reductions (green). Ketoisophorone (purple, commercially available in ≥98% purity), (rac)-levodione (red, synthesised following literature procedure)[1] and (R)-levodione (burgundy, obtained from Baker's yeast fermentation) standards were diluted using EtOAc with 2 mM undecane as internal standard.

S5. Supporting References

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