Research Article
doi.org/10.1002/cctc.202501548

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H_2-Driven Flavin Recycling Supports Biocatalytic Halogenation by Flavin-Dependent Halogenase

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We report a simplified, H_2-driven method for operating biocatalytic halogenation by a flavin-dependent halogenase, PyrH, which enables intensification of biocatalytic conversion of L-tryptophan to its 5-halo product. Flavin-dependent halogenases are gaining traction in biotechnology as their substrate scope is expanded by enzyme discovery and engineering, but their application remains impeded by a particularly complex electron transfer chain and the fact that full conversion is generally only achieved at sub-millimolar substrate concentrations. Here, we apply nickel-iron hydrogenase and ambient pressure H_2 in place of the $NAD(P)^+$, glucose, glucose dehydrogenase and reductase which are normally used to supply reduced FAD to halogenases. Together with controlled delivery of O_2, which is needed for generating a hypohalous acid intermediate, we achieve full conversion of 5.5 mM tryptophan, with a PyrH total turnover number of 275, and PyrH turnover frequency of 0.76 min^{-1} over a 6 h reaction, comparable with rates sustained only for short reaction times using the conventional glucose-driven system. This should help to facilitate application of flavin-dependent halogenases in fine chemical synthesis.

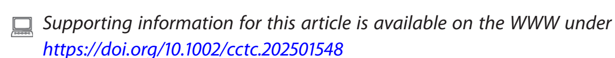
1. Introduction

Biocatalysis is becoming established in fine chemical manufacturing because of its ability to achieve functional-group selective transformations and enantiopure products under mild reaction conditions. Unfortunately, biocatalytic oxidations or reductions are typically dependent upon the expensive nicotinamide cofactors, NADH or NADPH. In order to utilise these cofactors in a cost-effective way, robust glucose dehydrogenases (GDHs) have been developed to recycle the reduced cofactors at the expense of glucose oxidation. However, using a C_6 sugar as stoichiometric, sacrificial reductant for biocatalytic transformations gives poor atom economy. H_2 or electrons (electrosynthesis) are preferable reductants as the chemical industry seeks more sustainable and economic processes.[1,2,3] This has propelled development of a range of H_2-driven biocatalytic strategies which exploit hydrogenases for NAD(P)H recycling.[4] Hydrogenases also show non-native activity for reduction of flavin cofactors, including flavin adenine dinucleotide (FAD) to the hydroquinone form, $FADH_2$.[5,6] This has been used for reductant supply to ene reductases and nitro-reductases.[5,6,7]

Flavin-dependent (FD)-halogenases catalyse chlorination, bromination and iodination of a range of organic substrates. Halogen substituents are prevalent in pharmaceuticals and agrochemicals because of their ability to tune biophysical properties of molecules, and their role in molecular assembly, as handles for metal-catalyzed cross coupling.[8,9] Halogenation is typically reliant on inorganic oxidants or halogen gases, but control over the site or extent of halogenation is limited; byproducts contribute to environmentally-harmful halogenated waste. These concerns have brought attention to enzymatic halogenation, and in particular the FD-halogenases, which offer good site-selectivity, relatively adaptable substrate scope, and halide transfer from benign sodium halide salts.[10–12] Their substrate scope is being further expanded through protein engineering of new reactivities and discovery of new enzymes.[13–16]

The mechanism of FD-halogenases involves reaction of $FADH_2$ with O_2 to form a flavin-bound peroxo intermediate within the enzyme. This reacts with halide to generate free hypohalous acid which travels through a tunnel from the flavin binding site to the organic substrate binding site where the halogenated product is formed.[17,18] During typical operation, a reductase provides a continuous supply of $FADH_2$ by coupling NAD(P)H oxidation to flavin reduction. This is supported by the well-established GDH/glucose NAD(P)H recycling system to complete a three-enzyme cascade, Scheme 1A. The complexity of this system has held back practical application of FD-halogenases, and several alternatives have been explored for supply of $FADH_2$, including transfer hydrogenation from formate by an organometalic catalyst, photocatalytic regeneration or use of artificial cofactors.[19–21] Even with effective supply of reduced flavin, FD-halogenases are generally limited to low substrate concentrations,[22–24] and they are prone to inactivation over several hours of operation, although the reasons remain uncertain.[25] Catalase is often added to FD halogenase reactions to mitigate damage by H_2O_2 leaked from the enzyme,[26,27] but it remains uncertain whether this is necessary.[28]

We hypothesized that it should be possibl to exploit hydrogenase/H_2-driven FAD reduction to simplify operation of



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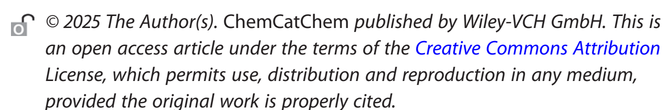
Supporting information for this article is available on the WWW under https://doi.org/10.1002/cctc.202501548



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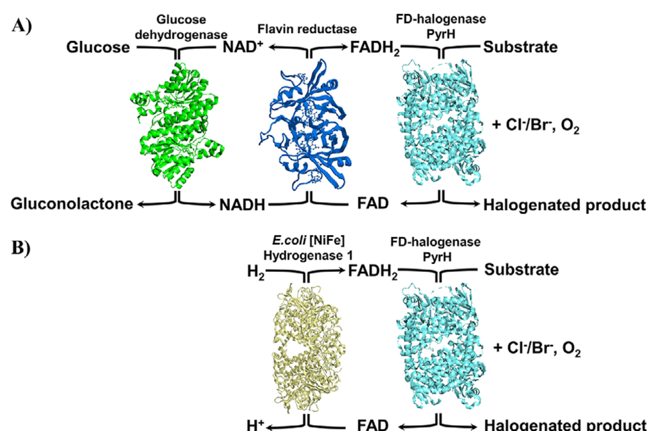

Scheme 1. FD-halogenase operation. A) Conventional operation in which NADH and $FADH_2$ are recycled via two enzymes, GDH and reductase (Fre), to support catalysis by the FD-halogenase PyrH which requires $FADH_2$, O_2 and halide source. B) This work: Hyd-1 uses H_2 for recycling $FADH_2$ under safe H_2 S25/ O_2 mixtures to supply to PyrH.

FD-halogenases avoiding the need for a carbon-based reductant, Scheme 1B. Although many hydrogenases are sensitive to O_2,[29] and hence would be unsuitable for this role, O_2-tolerant nickel-iron (NiFe) hydrogenases are known, and here we select the robust *E. coli* hydrogenase 1 (Hyd-1).[5,30] This hydrogenase sustains activity in the presence of low O_2, albeit at a slightly diminished rate since some electrons from H_2 oxidation are diverted to reduction of O_2 at the active site.[30] Activity remains good at $< 3\%$ O_2 in H_2.[31] H_2 / O_2 mixtures are noncombustible in regimes far from stoichiometry,[32] and we chose H_2-rich / O_2-lean operating conditions to maximise electron delivery to FAD by hydrogenase and minimise non-productive, reoxidation of reduced flavin by O_2.

2. Results and Discussion

To test the feasibility of combining biocatalytic halogenation with Hyd-1/H_2 flavin recycling, we chose the FD-halogenase from *Streptomyces rugosporus*, PyrH, which natively catalyzes site-selective chlorination of L-tryptophan, **1**, to give 5-chloro-tryptophan, **2** (Figure 1A) as part of the synthesis of the antibiotic, pyrroindomycin B.[33] PyrH also catalyzes bromination of **1** to 5-bromo-tryptophan (**3**), at slower rate.[28,33] Isolated, recombinant PyrH was first tested using *E. coli* flavin reductase (Fre), GDH, glucose, FAD and NAD^+ (see Section S3.1) and shown to have activity of 2.4×10^{-4} $\mu mol.s^{-1}.mg^{-1}$ ($0.83\ min^{-1}$), comparable to previous reports.[30]

Reactions were then tested as shown in Figure 1A in a septum-capped vial under H_2 with Hyd-1 in solution. Precision redox balancing is needed in any cascade involving FD-halogenases because of the competing direct oxidation of $FADH_2$ by O_2 which leads to waste of reducing equivalents – the so called "oxygen dilemma".[34] During initial screening we employed pulsed delivery of O_2, whereby an aliquot of gaseous O_2 was injected into the reaction vessel headspace approximately every 5 minutes via a syringe to give 0.5% O_2 (Figure 1B).

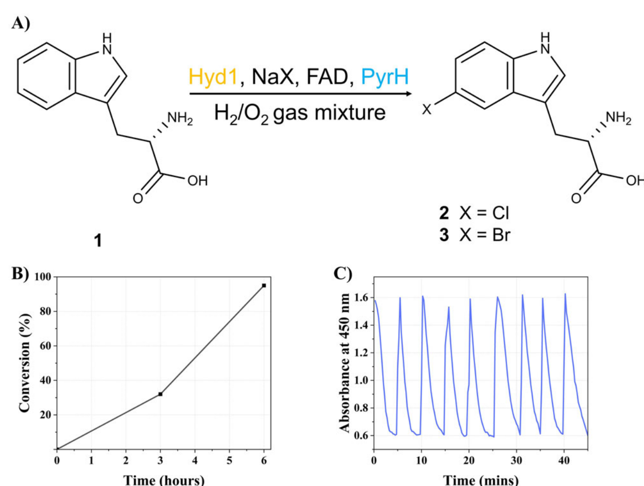

Figure 1. A) Reaction scheme for chlorination or bromination of L-tryptophan (Trp) by PyrH, with reduced FAD produced by Hyd-1 under H_2 injected with dilute O_2. B) Timecourse for Trp conversion as in (A), under H_2 with pulsed delivery of 0.5% O_2 every 5 min. C) Oxidation state of FAD monitored via absorbance at 450 nm during a reaction analogous to (B), run in a cuvette. Reaction conditions: potassium phosphate buffer (50 mM, pH 7.0), 30 mM NaCl, 0.5 mM L-tryptophan, 5.8 µM Hyd-1 and 20 µM PyrH (See Section S3.2).

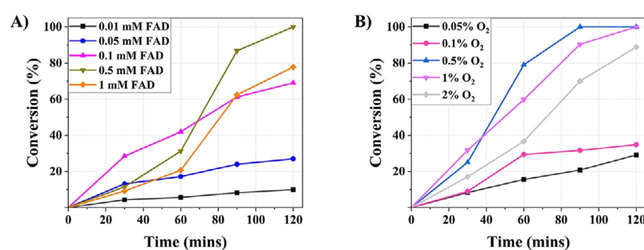

Figure 2. A, B) Timecourse for chlorination by PyrH with Hyd-1/H_2, used to determine: A) optimal FAD concentration with 2% O_2 pulses each 5 min; and B) optimal %O_2 pulse level, at 0.5 mM FAD. Two of these series were conducted in triplicate (Figures S9 and S15). Reaction conditions: potassium phosphate buffer (50 mM, pH 7.0), 30 mM NaCl, 0.5 mM L-tryptophan, 5.8 µM Hyd-1 and 20 µM PyrH, see Section S3.2. Conversion was calculated using HPLC data by comparison to analytical standards (see Section S4.3).

The reaction solution was allowed to completely decolorise between injections of O_2 (indicative of complete reduction of the flavin) before the next pulse was delivered to restore the O_2 level in the headspace. This caused a yellow color (indicative of oxidized flavin) to return to the solution. This was confirmed by running an analogous reaction in a cuvette with in situ monitoring at 450 nm, a wavelength at which oxidized FAD absorbs strongly, Figure 1C. Decreases in absorbance can be attributed to Hyd-1 mediated FAD reduction, while rapid increase in absorbance is associated with re-oxidation of reduced FAD by O_2-dependent PyrH activity in conjunction with unproductive oxidation of $FADH_2$ by O_2, which together lead to full re-oxidation of FAD during each pulse cycle.

We next explored the optimal level of FAD and O_2 for halogenation of **1** with Hyd-1/H_2 flavin recycling, Figure 2. Samples of the reaction mixture were taken at 30 min intervals for analysis, allowing calculation of % conversion of **1** to **2** (see Section S4.3). Figure 2A shows the timecourses for halogenation by PyrH,

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Table 1. Enzymatic chlorination at different concentrations of **1**, with either GDH/glucose or Hyd-1/H_2 for $FADH_2$ recycling.[a]

Entry	Flavin recycling system	Conc. 1 [mM]	Time [mins]	Conv.[%][b]	PyrH TTN[c]	PyrH TOF[d] [min^{-1}]
1	GDH/glucose	0.5	30	100	25	0.83
2	Hyd-1/H_2	0.5	30 (90)	25 (100)	6.25 (25)	0.21 (0.28)
3	GDH/glucose	5.5	360	23.6	65	0.18
4[e]	GDH/glucose	5.5	360	26.3	65	0.20
5[f]	Hyd-1/H_2	5.5	360	100	275	0.76
6[g]	Hyd-1/H_2	2.5	180	100	125	0.69

[a] For reaction conditions see Section S3.
[b] Conversion to **2** monitored by HPLC.
[c] PyrH total turnover number (mol **2** per mol PyrH).
[d] PyrH Turnover frequency (mol **2** per mol PyrH per min).
[e] Conducted under N_2 with pulses of 0.5% O_2.
[f] Entry 5 was conducted in triplicate (Figure S23).
[g] Entry 6 was conducted under 0.5% O_2 in H_2 using a balloon above the reaction mixture.

operated with 2% O_2 injections into a H_2 headspace, over a range of FAD concentrations. The lowest of these concentrations is below the level needed to fully reconstitute PyrH with flavin, and hence low activity is to be expected in this case. The best conversion (100% after 2 h) was achieved at 0.5 mM FAD, with no improvement offered by stepping up to 1 mM FAD. An effective Michaelis-Menten constant (K_M) of Hyd-1 for FAD was previously measured as 0.86 ± 0.54 mM.[5] The fact that 0.5 mM FAD proves optimal for the PyrH reaction probably reflects a compromise between the level required for its effective reduction by Hyd-1 and the extent to which reducing equivalents may be wasted on direct reoxidation of $FADH_2$ by O_2 at high flavin concentrations. A lag phase was observed in the reactions run at higher FAD, possibly due to a delay in activating Hyd-1 after exposure to a pool of oxidised flavin, since the hydrogenase can undergo a reversible oxidative inactivation. Another series of experiments was then conducted to establish an optimal O_2 level. The 0.5% O_2 reaction gave the fastest rate, with full conversion of 0.5 mM **1** into **2** within 90 min (Figure 2B). Again, this likely achieves a compromise: here, between the O_2 level needed for efficient catalysis by PyrH and the competing rate of unproductive flavin reoxidation.[34] Higher O_2 concentrations will likely also slow FAD reduction by Hyd-1 due to partial inactivation of the hydrogenase by O_2.

We then confirmed that the range of activity reported for PyrH is maintained when it is used with Hyd-1/H_2. Bromination of **1** (Figure S21), gave full conversion to **3** within 140 min, representing approximately 75% of the turnover frequency of chlorination by PyrH under analogous conditions, consistent with a previous report.[33] PyrH has also been shown to halogenate the non-indolic substrate, anthranilamide, **4** to give exclusively 5-chloro-anthranilamide, **5,** reaching 46% conversion after a 30 min reaction using the GDH/glucose system, compared to 100% **2** formed from **1** in the same time.[35] Consistent with this, reactions run in triplicate with **4** and the Hyd-1/H_2 system (Figure S25) reached full conversion to **5** by 180 min – double the time taken to reach full conversion of **1** under similar conditions (Figure 2B).

To test whether the reactions using Hyd-1/H_2/0.5% O_2 could be intensified, we compared to GDH/glucose operation of PyrH at different concentrations of **1** (Table 1). Full conversion of 0.5 mM **1** was achieved with the GDH/glucose system within just 30 min (Entry 1), with the Hyd-1/H_2 reaction taking longer (Entry 2). However, when PyrH with the GDH/glucose system was pushed harder, at 5.5 mM **1**, only 1.3 mM **2** was formed after 6 h (Entry 3). With the Hyd-1/H_2 system at 5.5 mM **1**, performance was significantly better: full conversion in 6 h (Entry 5).

The timecourses associated with Entries 3 and 5 are shown in Figure 3 and confirm the sustained performance with Hyd-1/H_2. Although PyrH still appears active after this reaction, we did not extend to higher substrate concentrations due to poor water solubility of product **2** which complicated reaction analysis beyond 5.5 mM. To check whether low O_2 explained the improved performance of PyrH with Hyd-1/H_2 compared to GDH/glucose, we tried operating PyrH with GDH/glucose under N_2 with pulses of 0.5% O_2 each 5 min, however this gave only slightly improved conversion (compare Entries 4 and 3 in Table 1; see Figure S8). For scaled reactions, pulsed delivery of O_2 may be inconvenient, so we tested a PyrH reaction using Hyd-1/H_2 under a constant atmosphere of 0.5% O_2 in H_2, and encouragingly, this also gave full conversion of **1** (Table 1, Entry 6).

Catalase is sometimes added to FD-halogenase reactions to remove H_2O_2 byproduct by disproportionation,[26] but no difference was seen when catalase was used in reactions with either GDH/glucose or Hyd-1/H_2 (see Figure S6 and S7). GDH/glucose is known to cause pH change due to gluconic acid formation, which could account for the poor activity of PyrH with GDH/glucose at higher substrate loadings, and would require continual pH adjustment in scaled reactions. A reaction using GDH/glucose in air at 1 mM **1** showed pH change from 7.4 to 5.2 over 3 h (see Section S4.7), whereas a similar reaction using Hyd-1/H_2 was at pH 7.2 after 3 h, with full conversion to **2**. This shows an important advantage of the Hyd/H_2 flavin recycling system in allowing pH-stable operation over a number of hours.

The Total Turnover Number (TTN) of 275 for PyrH calculated from Table 1, Entry 5 compares favorably to literature TTNs for

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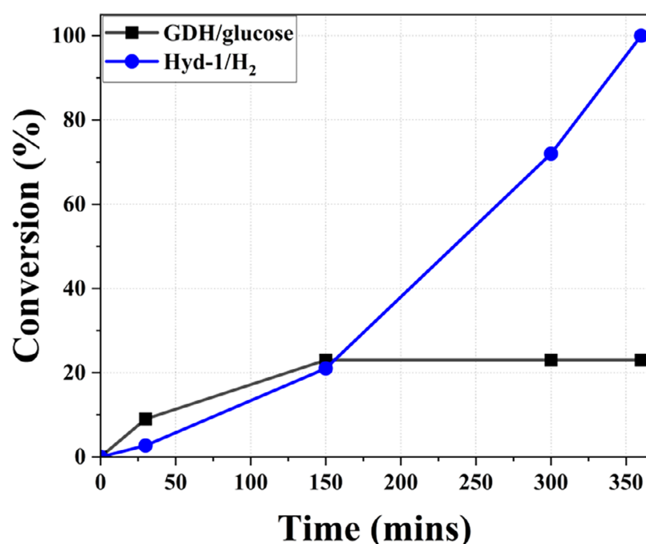

Figure 3. Timecourses for PyrH chlorination at 5.5 mM Trp using Hyd-1/H_2 versus GDH/glucose. Other conditions: see Section S3.1 or S3.2.

FD-halogenases which are typically below 200, as noted by Frese and Seewald.[36] The PyrH turnover frequency (TOF) for operation with Hyd-1/H_2 of 0.76 min^{-1} over the 6 h reaction (Table 1 Entry 5) is close to TOFs measured over short reactions with the glucose/GDH system in this study (Entry 1) and in literature reports on PyrH[35] and for other FD-halogenases with cofactor recycling,[36] but the GDH/glucose system was unable to sustain this rate over more than 2.5 h (Figure 3). The PyrH TOF using the Hyd-1/H_2 system surpasses alternative chemoenzymatic or photocatalytic systems including transfer hydrogenation from formate to FAD by a (penta-methylcyclopentadienyl)rhodium-bipyridine complex (0.0023 min^{-1} using *Pseudomonas fluorescens* Trp 7-halogenase),[19] photochemical regeneration of $FADH_2$ with ethylenediaminetetraacetic acid (EDTA) as sacrificial reductant (0.012 min^{-1} with PyrH),[20] or with BNAH (1-benzyl-1,4-dihydronicotinamide) as sacrificial reductant for FAD (0.17 min^{-1} with PyrH, or 0.32 min^{-1} with another FD-halogenase, Thal).[21]

3. Conclusion

In conclusion, running biocatalytic halogenations with $FADH_2$ recycling by hydrogenase/H_2 offers an atom-efficient alternative to GDH/glucose operation of FD-halogenase, avoiding the costly cofactor NAD^+ and the waste generated from glucose. Our system cuts out one enzyme from the cascade, by replacing GDH and Fre with Hyd-1. Beneficially, PyrH also shows better performance over several hours when run with Hyd-1/H_2, achieving substrate conversions that exceed published values, with no need for pH adjustment. Delivering O_2 and reducing equivalents in a mixed-gas feed opens up this biocatalytic process to a range of reactors developed for hydrogenation or oxygenation reactions, including the bubble column reactor for batch operation,[37] or the tube-in-tube reactor for continuous flow.[38] Therefore this simplified mode of operation for FD-halogenase should propel the uptake of these important enzymes in industrial biotechnology.

Acknowledgments

We are grateful to Prof Jason Micklefield for providing a plasmid for expression of *E. coli* reductase, Fre; to Dr Tim Sudmeier for helping to establish HPLC protocols; to Dr Stephen Carr and Plastida Synteng Umwi for helping with production of Hyd-1; and to Jiachuan Peng for developing the control code used for mass flow controller pulse sequences. This research was supported financially by Engineering and Physical Sciences Research Council (EPSRC) IB Catalyst award EP/N013514/1, European Research Council CoG-819580 (BiocatSusChem) and awards from the Biotechnology and Biological Sciences Research Council (BBSRC) BB/X002624/1 and E3B NIBB BB/S009787/1 to Kylie A. Vincent. Kylie A. Vincent is also grateful for support from EPSRC-SCHEMA hub, EP/Z532782/1.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Supporting Information

The Supporting Information contains further details of experimental methods, additional experimental results, and additional reference citations.

Keywords: Biocatalysis · Flavin recycling · Flavin-dependent halogenase · Halogenation · Hydrogenase

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Manuscript received: October 26, 2025
Accepted manuscript online: November 21, 2025
Version of record online: ■■, ■■

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