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Scalable Bioreactor Production of an O_2-Protected [FeFe]-Hydrogenase Enables Simple Aerobic Handling for Clean Chemical Synthesis

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The enzyme CbA5H, a [FeFe]-hydrogenase from *Clostridium beijerinckii*, has previously been shown to survive exposure to oxygen, making it a promising candidate for biotechnological applications. Thus far [NiFe]-hydrogenases are typically considered for such applications, due to the superior O_2-tolerance and therefore simplified enzyme handling. However, methods for production of [FeFe]-hydrogenases are generally more successful than for other classes of hydrogenases, therefore in this work we focus on demonstrating scalable CbA5H production, and report results with active enzyme prepared in bioreactors (up to 10 L) with >20-fold improvement in purified enzyme yield. We then go on to confirm excellent H_2/H^+-cycling activity of the air-purified protein, highlighting that CbA5H can be prepared and isolated without the need for complex and expensive infrastructure. Next, we demonstrate good stability of the air-purified CbA5H both in solution assays, and as a heterogenous catalyst system when immobilized on a carbon support. Finally, we successfully implement this enzyme within previously demonstrated biotechnologies for flavin and NADH recycling, highlighting its relevance in chemical synthesis, and we demonstrate production of an important API precursor, 3-quinuclidinol at >0.4 g scale in standard benchtop hydrogenation infrastructure, with >100,000 CbA5H turnovers over 18 operational hours.

Introduction

The use of H_2 gas as a clean source of energy is of increasing interest across a number of disciplines, and H_2 is already well established as a 100% atom efficient reductant in chemistry, for example in catalytic hydrogenation reactions which account for *ca* 14% of all chemical steps.[1] Hydrogenase enzymes are responsible for biological H_2 oxidation to form two protons (H^+) and two electrons (e^-) and are of interest as alternative catalysts to typical metals for H_2/H^+ cycling.[2a–c]

Two of the most widely studied classes of these H_2 cycling enzymes are [NiFe]- and [FeFe]-hydrogenases, named for the metals in their active site. There are a number of excellent review articles describing the origins, production, characterization and application of these enzymes.[2d–g]

We have previously explored application of hydrogenase enzymes in driving highly selective biocatalytic hydrogenation reactions. In each case the hydrogenase catalyzes H_2 oxidation, with electrons then transferred directly to flavin recycling,[3] via a carbon support to an NAD^+ reductase for NADH[4] (or NAD^2H)[5] recycling using our "H_2BioCat" system (Figure 1), or via a carbon support for nitro group reduction.[6] To date, all

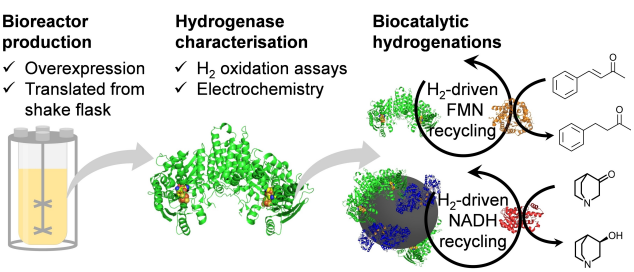

Figure 1. Production, characterization, and exploitation of CbA5H for biocatalytic chemical synthesis. The green ribbon shows the partial homodimer crystal structure of CbA5H (PDB: 6TTL).

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examples have been carried out using [NiFe]-hydrogenases (for H_2 oxidation activity) coupled to further redox partner(s). This is most significantly due to the superior O_2-stability (structural integrity of the enzyme in the presence of O_2) or O_2-tolerance (ability to operate in the presence of low or moderate O_2) of many [NiFe]-hydrogenases. This aids aerobic isolation, handling and semi-anaerobic processes, allowing operation of these biocatalyst systems without the need for specialist infrastructure such as anaerobic glove boxes. The most significant bottleneck to more widespread application of such enzymes are current limitations in scalable production strategies.

In contrast, there are more advanced methods for production of [FeFe]-hydrogenases. Many of these enzymes not only lose catalytic activity in the presence of O_2, but can be irreversibly inactivated on very short exposure to O_2 from the air. Therefore the requirement for rigorously anaerobic isolation, purification and application almost entirely precludes their use outside of fundamental academic research to-date.

The enzyme CbA5H from *Clostridium beijerinckii* has been recently identified as the only known [FeFe]-hydrogenase able to spontaneously survive exposure to oxygen, by forming a protected overoxidized species called H_{inact}. CbA5H can be isolated fully aerobically and it can be reactivated to full activity after exposure to oxygen.[7] Spectroscopic and structural studies have demonstrated that this ability is intrinsic to the enzyme,[8] and this unique feature is linked to a conformational change that allows a conserved cysteine residue to reversibly bind the catalytic center (H-cluster) protecting it from O_2 attack. This is in stark contrast to the irreversible damage suffered by most other known [FeFe]-hydrogenases, with the only exception of DdH from *Desulfovibrio desulfuricans*, where supplementation of exogenous sulfide is required according to a recently characterized protection mechanism.[9]

This unusual stability towards O_2, combined with more advanced enzyme production capability, makes CbA5H a compelling hydrogenase to take forwards in applications such as biocatalytic hydrogenation for chemical synthesis. In this work, we demonstrate overexpression of the [FeFe]-hydrogenase, translation from shake flask to bioreactors, and biochemical and electrochemical characterization (Figure 1). The knowledge acquired then directs exploitation of CbA5H in two biocatalytic applications: direct enzyme-mediated flavin reduction coupled to selective alkene reduction, and incorporation into the "H_2BioCat" technology for H_2-driven NADH reduction (Figure S1) coupled to ketone reduction.

Results and Discussion

CbA5H Overexpression in Different Cell Hosts: *Escherichia coli* versus *Clostridium beijerinckii*

Simple and effective overproduction of enzymes is essential for uptake in industrial applications. For this reason, we have designed and assessed two novel expression plasmids for CbA5H (Figure S2). The first plasmid uses *E. coli* as the expression host and it is based on well-established methods[10] that require the co-expression of CbA5H with HydE, HydF and HydG maturases from *C. acetobutylicum* that have been previously demonstrated to be very effective in inserting the H-cluster in [FeFe]-hydrogenases from other organisms.[7] All genes were cloned in IPTG-inducible cassettes, as follows: the *CbA5H* gene in a pET21 vector (pET-CbA5H); the *CaHydE, CaHydF and CaHydG* genes in a modified pCDFDuet-1 vector (pEFG). Despite CbA5H being O_2-stable, HydEFG maturases are not, so anaerobic conditions are still needed during overexpression.

The second plasmid uses *C. beijerinckii* as the expression host and it is based on a modular vector that has been recently proven successful in the closely related solvent producer *C. acetobutylicum* ATCC 824.[11] The CbA5H gene was cloned under the control of a strong constitutive thiolase promoter (pClos-CbA5H) in a replicative vector backbone suitable for *C. beijerinckii* NCIMB 8052.[12]

Following small-scale growth (0.5 liter), cells can be harvested aerobically, and affinity purification can also be performed aerobically without any particular precaution. Purified CbA5H displayed high specific activity from both new plasmids (Table 1). The purification yields are significantly higher than previous results at similar scale. In particular, the new *E. coli* plasmid resulted in a 4-fold increase in yield, and the new *C. beijerinckii* plasmid resulted in an 8-fold increase. The specific activity was not significantly different. This suggests that the H-cluster supply in *E. coli* by *C. acetobutylicum* maturases HydEFG is not limiting CbA5H assembly. Similarly, this observation suggests that the native expression levels of HydEFG maturases in *C. beijerinckii* are sufficient to supply H-cluster to the recombinantly overexpressed CbA5H, and additional genetic modifications are not required in this strain.

This is the first report of CbA5H overexpression in *C. beijerinckii*, thus direct comparison with previous research is not possible. However, CaHydA1 has been overexpressed in *C. acetobutylicum* before, resulting in much lower yield both when the gene was expressed from a plasmid (0.4 mg/L)[13] and genome-integrated (1 mg/L).[14]

Overproduction Scale-up

In order to maximize the overproduction of CbA5H for future industrial applications, the performances of the new *E. coli* plasmid have been assessed in a bioreactor. Bioreactors offer numerous advantages over flasks, as all growth parameters can be conveniently controlled. Furthermore, bioreactors are scalable, and offer the possibility to demonstrate that large scale production of a given enzyme is possible. Assessing the performance of CbA5H production in a bioreactor is a credible predictor of performance at larger scales, enabling real world industrial applications.[15]

CbA5H overproduction in a bioreactor was optimized at 1-liter scale (Table 1). The possibility to precisely control the fermentation process over time has led to longer post-induction times that are not feasible at smaller scale in our flask + bottle protocol. As such, we have extended the post-induction time to

Table 1. Summary of CbA5H overproduction scale-up.

Plasmids	Host	Process conditions	OD_{600} at harvest	Purification yield (mg/L)	Specific activity H_2 evolution (U/mg)[a]	Reference
See ref [7]	*E. coli* Rosetta2(DE3)	0.5-L Flask + bottle (TB media)	n.d.	1.5 ± n.d.	751 ± 91	[7] [b]
pET-CbA5H + pEFG	*E. coli* BL21(DE3) ΔIscR	0.5-L Flask + bottle (TB media)	1.4	6.3 ± 0.2	737 ± 69	This work
pClos-CbA5H	*C. beijerinckii* NCIMB 8052	0.5-L Bottle (TYA media)	4.1	12.7 ± 0.9	784 ± 83	This work
pET-CbA5H + pEFG	*E. coli* BL21(DE3) ΔIscR	1-L bioreactor (TB media)	1.8	24.0 ± 0.4	665 ± 73	This work
pET-CbA5H + pEFG	*E. coli* BL21(DE3) ΔIscR	1-L bioreactor (M9 media)	1.9	5.3 ± 1.0	432 ± 10	This work
pET-CbA5H + pEFG	*E. coli* BL21(DE3) ΔIscR	10-L bioreactor (TB media)	3.9	29.5 ± 0.3	332 ± 15	This work

Specific details on the process conditions and methods are available in the experimental section. All experiments were carried out at least in biological duplicates, except the 10-L experiment which was a single run. All values are mean values ± standard deviations from three independent measurements. [a] 1 unit corresponds to 1 μmol H_2 released per minute at 37 °C in 100 mM Tris·HCl pH 8 supplemented with 150 mM NaCl. [b] Two alternative heterologous expression methods have been recently reported for CbA5H, but purification yield and specific activity are not disclosed.[8a,b]

48 hours (Figure S3). When working in rich TB media, this has led to CbA5H preparations that have high specific activity and a purification yield that is unprecedented (24 mg/L), 3.8 times higher than at small scale. We have also explored the possibility of performing the process in a minimal media (M9), which would be more desirable at larger scale due to lower cost and standardized composition. While M9 media also resulted in a significant amount of active CbA5H, its performances were lower than in TB media, suggesting that further optimization of the minimal media composition or fermentation operation may be required in future work. Finally, to test the potential for further scaled up production, we performed one test run at to 10-liter scale (Table 1), confirming that high yield of enzyme can be obtained (20-fold improvement over previous reports) but at lower specific activity. We hypothesize that this may be due to operational conditions that are not optimal at this scale (e.g. mixing speed causing cellular stress during post-induction anaerobic phase, suboptimal heat transfer, or incompatibility with the redox DO probe in this specific bioreactor).

With respect to improved purification yields, we note that these do not correlate directly with cell density at harvest (measured as OD_{600}, Table 1), suggesting that the specific operational conditions play a crucial role in determining higher or lower protein accumulation.

Overall, our results demonstrate for the first time that CbA5H production is potentially scalable and that yields can be significantly improved by optimizing the conditions in a bioreactor at 1-L scale. We are planning to explore and optimize further the scale up of the process.

Enzyme Characterization

As expected, the FTIR spectrum of aerobically purified CbA5H displayed the previously characterized H-cluster species, H_{inact} (Figure S4), which is inactive but O_2 stable. We characterized CbA5H for its H_2 uptake (oxidation) activity, based on methyl viologen reduction, in the perspective of its exploitation in biocatalysis. CbA5H reactivation occurs *in situ*, during the reaction due to the presence of the natural substrate H_2, however this results in a significant lag phase of up to 13 minutes at pH 6, while higher pH values resulted in a faster reactivation (Figure 2a). As previously demonstrated,[7] faster and more consistent reactivation can be achieved by pre-treatment with reducing agents, such as H_2 or sodium dithionite (NaDT) (Figure 2b). Following reactivation, the H_2 oxidation turnover frequencies (TOF) are not significantly different between the pre-activated ($TOF_{H2} = 57.5 \pm 13.8\ s^{-1}$ $TOF_{NaDT} = 69.3 \pm 19.3\ s^{-1}$) or non-reactivated samples ($TOF = 54.7 \pm 10.5\ s^{-1}$). These results demonstrate that CbA5H reactivation readily occurs when oxygen is removed and can be accelerated by reductive treatment, however this is not essential for catalytic function to be re-instated.

Further characterization experiments have all been carried out after pre-activation with H_2. CbA5H was active for H_2 oxidation in a broad pH range (Figure 2c), with an optimum at pH 8.5. The enzyme was also active across a broad temperature range (Figure 2d), with an optimum at 37 °C. An activation energy of 24.3 kJ/mol was calculated (Figure S5), which is similar to CpI and CpII.[16]

Recently, CbA5H stability towards oxygen has been questioned when the enzyme has been repeatedly exposed to air.[17] For this reason, we further investigated the long-term stability of CbA5H (Figure 3). When stored at room temperature under air, CbA5H loses activity on the time scale

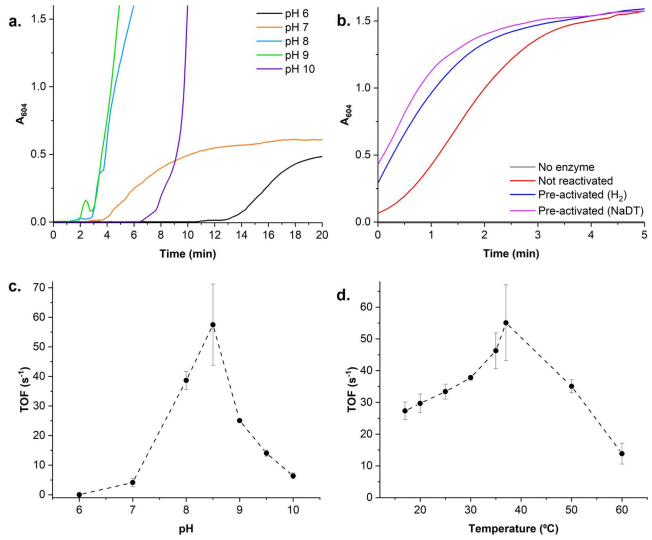


Figure 2. Characterization of H_2 uptake (oxidation) activity by CbA5H, based on the reduction of methyl viologen (1 mM). Reduction of methyl viologen was monitored over time in a mixed buffer set at the pH indicated in the legend, saturated with H_2. **a**. *In situ* enzyme reactivation after aerobic purification, assayed at 37 °C. **b**. Enzyme reactivation following reductive pretreatment with either H_2 or sodium dithionite (NaDT) at pH 8.5, 37 °C. **c**. pH activity profile of CbA5H-catalysed H_2 oxidation at 37 °C, following pre-activation with H_2. **d**. Temperature activity profile of CbA5H-catalysed H_2 oxidation at pH 8.5, following pre-activation with H_2. TOF = turnover frequency (µmol_H_2 per µmol_CbA5H per second). All values are mean values ± standard deviations from three independent measurements.

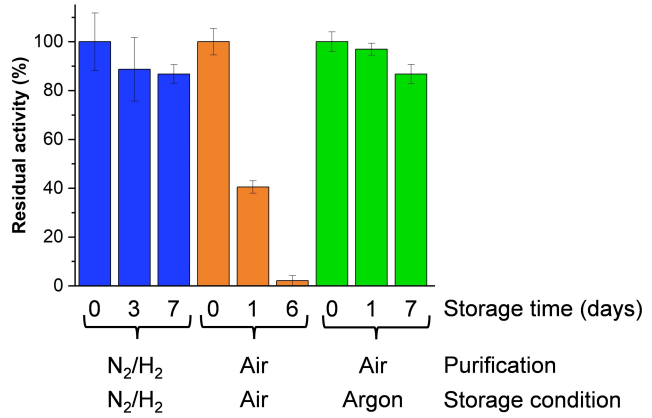


Figure 3. Characterization of retained activity of CbA5H over 7 days at room temperature, comparing purification and storage atmospheres. All values are mean values ± standard deviations from three independent measurements of H_2 evolution activity.

of a day, confirming that the enzyme is able to withstand O_2 exposure but not indefinitely, consistent with previously reported spectroscopic data.[8a] Activity loss is directly related to exposure to oxygen, because when samples are stored at room temperature in an anaerobic glove box (N_2/H_2 atmosphere) or in sealed vials sparged with an inert gas (Ar), the activity is preserved (> 80 % residual activity). Overall, these results demonstrate that short term handling of CbA5H under air is possible, for up to 8 hours, *i.e.* for harvesting of cells, enzyme purification, immobilization and setting up of biocatalytic reactions (see below), while long term storage can be achieved by sparging the samples with inert gas. The ability to purify and handle this hydrogenase under aerobic conditions has advantageous implications for designing and using the biocatalyst in industrial applications.

Application in Hydrogenase-Driven Flavin Recycling

We have previously reported that under an H_2 atmosphere, *E. coli* [NiFe]-Hyd1 reduces FMN and FAD cofactors to $FMNH_2$ and $FADH_2$, respectively.[3a] This likely occurs at the surface of the protein with electron transfer via a series of FeS clusters from H_2 oxidation at the [NiFe] active site.

To test if CbA5H was also a suitable biocatalyst for H_2-driven flavin reduction, we mixed CbA5H with H_2-saturated flavin solutions and monitored reduction spectrophotometrically (Figure 4). CbA5H was able to reduce both FAD (TOF = 1.48 ± 0.24 s^{-1}) and FMN (TOF = 3.03 ± 0.31 s^{-1}).

The CbA5H-catalysed H_2-driven FMN reduction was next implemented as a cofactor recycling system to continually supply a commercial ene-reductase (ENE-108, Johnson Matthey) with $FMNH_2$, which in turn reduces 4-phenyl-3-buten-2-one (**1**) to 4-phenyl-2-butanone (**2**), with chemoselectivity toward the alkene (Scheme 1). Ene-reductases are typically supplied with reduced NAD(P)H cofactor, recycled at the expense of glucose via glucose dehydrogenase (or other

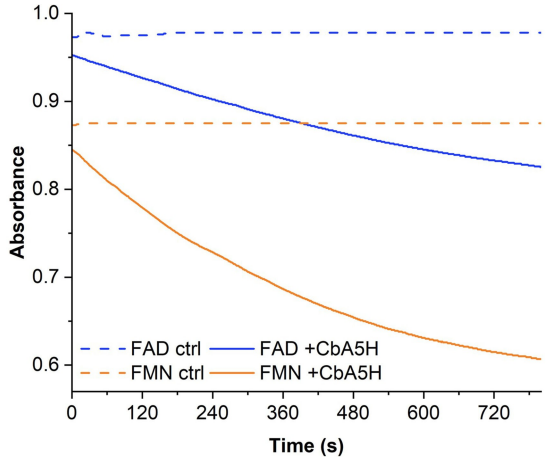
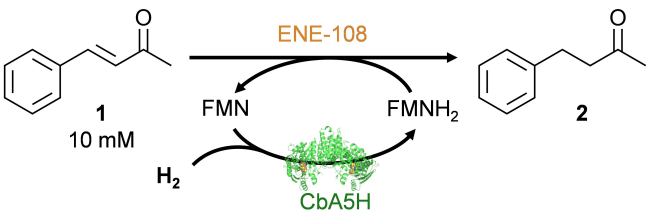


Figure 4. H_2-driven flavin reduction by CbA5H. Reduction of flavins (0.1 mM aH2 at 37 °C. FMN reduction was monitored at 445 nm. FAD reduction was monitored at 450 nm. The dashed lines show controls "ctrl" which were run in the absence of CbA5H.



Scheme 1. Reduction of **1** to **2** using CbA5H (0.17 mg/mL), ENE-108 (0.5 mg/mL) and FMN (0.5 mM) in 10 % v/v DMSO in Tris-HCl (50 mM, pH 8.0) at 35 °C under H_2 (1 bar). See S2.4 for more details.

carbon-intensive recycling systems) when implemented in vitro. We have previously coupled the Hyd1-catalyzed H_2-driven flavin recycling to commercial ene-reductases, which provided a highly atom and cofactor efficient alternative. Here we demonstrate this system using CbA5H as a more scalable hydrogenase alternative. This cofactor recycling system was tested in a round bottom flask on a 4 mL scale on the benchtop by stirring CbA5H (0.69 mg) and ENE-108 (2 mg) in a degassed solution of **1** (10 mM) and FMN (0.5 mM) in 10% v/v DMSO in Tris-HCl (50 mM, pH 8.0) under a steady flow of H_2 at 35 °C. Timepoints taken during the reaction were analyzed for conversion using HPLC (see details in S2.4, Supporting Information). After 1 hour and 4 hours, the conversion to **2** was 19% and 43%, respectively (Table S1). The calculated TOF = 0.20 s^{-1} based on the concentration of **2** in the reaction solution at 1 hour was an order of magnitude lower than the TOF of 3.03 s^{-1} seen during H_2-driven FMN reduction by CbA5H (Figure 4), which suggests that the ENE-reductase alkene reduction step was rate-limiting (*e.g.* due to replacing the more-typical NADH cofactor with $FMNH_2$).

The concentrations of both **1** and **2** in the reaction solution dropped after stirring overnight (see Table S1), therefore overnight conversion was not applicable. This loss of organic compounds could be indicative that compound evaporation occurred. In the future, evaporation might be prevented through optimization of the reaction set up (*e.g.* lowered temperature, sealed pressure vessel).

This is a promising proof-of concept demonstration of a "clean" biocatalytic system that removes the need for glucose-driven NAD(P)H recycling in order to drive the alkene reduction, which in principle generates less waste and simplifies downstream processing.

Enzyme Immobilization and Stability

To determine if CbA5H is a suitable hydrogenase in our previously developed H_2-driven NADH recycling biocatalyst system, "H_2BioCat",[4,18] we first tested for effective adsorption of CbA5H on BP2000 carbon support, which led to ≥99% immobilization efficiency (free protein was undetectable with the Bradford assay in the supernatant after immobilization, see Figure S6). Catalytic activity of CbA5H immobilized on BP2000 was retained, with a TOF of 1.64 ± 0.50 s^{-1} for H_2 oxidation (Figure S7). This is lower activity than the enzyme in solution, likely due to mass transfer limitations, and with a longer lag phase. Due to the additional complexity of analyzing immobilized CbA5H on carbon via mediated assays (*i.e.* the mediator sticking to carbon, carbon sedimentation), we next investigated CbA5H immobilized on a carbon electrode via protein film electrochemistry for its "shelf-life" under anaerobic and aerobic atmospheres.

Aerobically purified CbA5H was immobilized on to two separate pyrolytic graphite edge electrodes following previously published protocols,[19] and each enzyme film was characterized using cyclic voltammetry (see S2.5.1 for details). The electrochemical experiments show reversible H_2/H^+ cycling by CbA5H (Figure S8). The electrodes were then stored in either anaerobic or aerobic conditions, and tested each day for five days. While the enzyme film displayed significant levels of film loss, Figures S8–S9 show that CbA5H did retain activity after being stored anaerobically in a glovebox (< 3 ppm O_2) over the course of five days, giving us confidence that the immobilized air-purified enzyme would behave similarly to the solution enzyme on anaerobic storage.

Application in Biocatalytic Hydrogenation of 3-Quinuclidinone to (3R)-Quinuclidinol

With the promising activity and stability results from both solution assays and CbA5H immobilized on carbon, this hydrogenase was incorporated into the "H_2BioCat" system. This relies on co-immobilizing a hydrogenase and NAD^+ reductase on a conductive carbon support (*e.g.* BP2000) which together catalyze H_2-driven NADH generation (shown in detail in Figure S1). Our previous work on "H_2BioCat" has predominantly used *E. coli* [NiFe]-hydrogenases Hyd1[18,20] and Hyd2.[4,21] Here, CbA5H was co-immobilized with the NAD^+ reductase from *R. eutropha* "I64A" on BP2000, which led to >99% protein immobilization. This H_2-driven NADH recycling was coupled with the *R*-selective quinuclidinone reductase from *Agrobacterium tumefaciens* "*At*QR" for biocatalytic hydrogenation of 3-quinuclidinone (**3**) to 3-quinuclidinol (**4**, Figure 5), an important chemical building block that is present in Solifinacin and other commercial chemicals.[5,18]

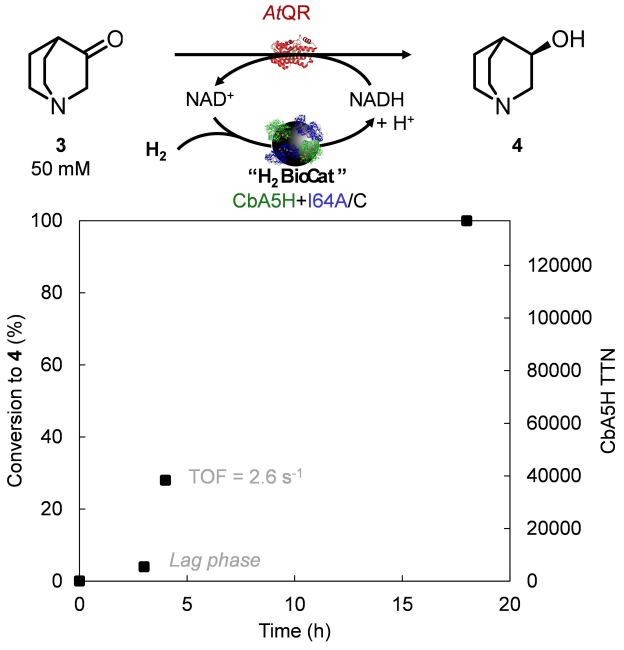


Figure 5. Biocatalytic hydrogenation of 3-quinuclidinone (**3**, 50 mM) to 3-quinuclidinol (**4**) using NAD^+ (1 mM), "H_2BioCat" (CbA5H + I64A/C, 0.6 mg/mL) and *At*QR (1.0 mg/mL) in Tris-HCl (50 mM, pH 8.0) under H_2 (1 bar) at 30 °C. 100% conversion was determined after 18 hours, which corresponds with CbA5H total turnover number (TTN) of 135,300. Turnover frequency ("TOF" = mmol_**4** per mmol_CbA5H per second) was calculated at 4 hours.

The biocatalytic hydrogenation system was tested for feasibility on the benchtop to prepare **4** on a 3.8 mL scale, and timepoints were taken throughout the course of the reaction and analyzed using ^{1}H NMR spectroscopy (details in S2.6 in the Supporting Information).

Figure 5 shows different conversion rates to **4** at three hours (4%) and four hours (28%), which suggests that the "H_2BioCat" system faced a lag phase, likely due to the aerobic isolation of CbA5H. In the future, this might be overcome by pre-activating the CbA5H, as shown in Figure 2b. In spite of this, it was gratifying that the TOF reached 2.6 s^{-1} at the four hour time point. The I64A TOF was also determined (2.2 s^{-1}), which is on the same order of magnitude to our previously report (TOF = 1.4 s^{-1}) when I64A was co-immobilized with Hyd2 to supply NADH to a different alcohol dehydrogenase for acetophenone reduction.[4] While the two systems cannot be directly compared, this does show that CbA5H is a suitable alternative hydrogenase to our previous "H_2BioCat" system.

After stirring for 18 hours, the reaction went to >99% conversion, which corresponds with the generation of >49.5 mM **4** and CbA5H total turnover number (TTN) of 135,300. It has been suggested that this TTN typically makes an enzyme cost-effective for pharmaceutical chemical production.[22]

To take advantage of the scaled-up CbA5H production, the biocatalytic hydrogenation of **3** was next implemented at a preparative scale in a round bottom flask on the benchtop (66 mL, 538 mg of 3-quinuclidinone-HCl; see details in S2.6.2 in the Supporting Information). When only 12% conversion was achieved after 19.5 hours, we increased the temperature from 30 to 35 °C, and stir rate from 250 to 680 r.p.m. (Figure S11). Following this, the conversion steadily increased, giving 80% conversion and a theoretical yield of 432 mg **4** after a further 24 hours. We attribute the low conversions in the first 19.5 hours to the initially lower stir rate, which likely led to insufficient gas-liquid-solid mixing. This is a common challenge encountered when scaling up hydrogenation reactions in round bottom flasks, in which the interfacial area decreases as vessel size increases.[23] In spite of the slow start, this reaction demonstrates that the CbA5H was active during two days of stirring (in line with retained activity after one day under anaerobic storage conditions, Figure 3 and Figure S9). Overall, benchtop catalyst handling was straightforward, with similar considerations to that of typical heterogeneous hydrogenation catalysts (*e.g.* Pd/C) in which N_2-purged solutions provide sufficient catalyst protection. Future work with this NADH recycling system would aim to take advantage of the stable CbA5H activity over time, for example implementation in continuous flow hydrogenation.

Conclusions

In this work we demonstrate production of the [FeFe]-hydrogenase CbA5H in scalable bioreactors and study the air-purified enzyme for activity and stability in standard solution assays. The results show that this enzyme is able to tolerate significant exposure to O_2, making it an important candidate for further research, for example to bring together the advantages of simplified [FeFe]-hydrogenase production and straightforward aerobic handling of O_2-tolerant [NiFe]-hydrogenases (*e.g. E. coli* Hyd1). The CbA5H enzyme was then evaluated for H_2 oxidation activity and stability when immobilized on either a carbon support or electrode, further confirming its suitability for application within HydRegen's heterogeneous "H_2BioCat" technology for NADH recycling. When the CbA5H was incorporated into the "H_2BioCat" system for 3-quinuclidinol production, more than 100,000 turnovers were achieved in 18 hours, and the system was also used to generate >0.4 g of chemical product. Overall, the results presented here highlight scalable methods for production and application of CbA5H, giving confidence for further research into this enzyme for clean and sustainable industrial chemical synthesis.

Experimental Section

Carbon black particles (Black Pearls 2000, "BP2000", Cabot Corporation) and NAD^+ (Prozomix) were purchased and used as received. All other chemicals were obtained from Sigma-Aldrich (now Merck), unless otherwise stated and used without further purification. Details about ENE-108, I64A and *At*QR enzymes are in the S1.2 of the Supporting Information.

Cloning. DNA oligos were sourced from Integrated DNA Technology (Belgium). All PCR amplifications were performed with NEB Q5 DNA polymerase, and cloning was performed with the NEB HiFi DNA Assembly kit.

The *CbA5H* gene was amplified by PCR from *C. beijerinckii* NCIMB 8052 genomic DNA and modified with a C-terminal Twin-Strep-Tag, preceded by a TEV cleavage site. The modified gene was assembled into a pET21 vector (between NdeI/XhoI), yielding pET-CbA5H for the heterologous expression in *E. coli*. An alternative construct with a C-terminal 6HisTag was also generated.

The pPM12 vector[11] was modified to replace the chloramphenicol resistance cassette with a spectinomycin resistance cassette (aad9).[12] Subsequently, the modified *CbA5H* gene was assembled (between NdeI/NheI) under the control of a thiolase promoter (yielding pClos-CbA5H for overexpression in *C. beijerinckii*).

The *CaHydE* gene cassette (including its independent T7 promoter) was amplified by PCR from the pCaE2 vector, and subsequently assembled into the pCaFG vector,[10a] upstream of the *CaHydF* gene, yielding pEFG.

The final vectors were verified by DNA sequencing. pET-CbA5H was co-transformed with pEFG into *E. coli* BL21(DE3) ΔIscR.[24] pClos-CbA5H was transformed into *E. coli* C600RK2, this was then used to conjugate pClos-CbA5H into *C. beijerinckii* NCIMB 8052 as previously described. During conjugation, *C. beijerinckii* was grown on RCM (13 g/L yeast extract, 10 g/L Peptone, 5 g/L glucose, 1 g/L soluble starch, 5 g/L sodium chloride, 3 g/L sodium acetate, 0.5 g/L Cysteine hydrochloride, 15 g/L Agar), supplemented, when appropriate, with 250 μg/mL spectinomycin and 10 μg /ml trimethoprim (to counter select against *E. coli*).[25]

Media, cell growth and small-scale overexpression. Heterologous overexpression at small-scale in *E. coli* was performed as previously described (flask + bottle method).[7,26] Briefly, *E. coli* was grown in 500 mL TB media (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L

glycerol, 2.2 g/L KH_2PO_4, 9.4 g/L K_2HPO_4) supplemented with 0.5 mM ammonium ferric citrate, 100 μg/mL carbenicillin, 50 μg/mL streptomycin and 30 μg/mL kanamycin. After initial aerobic growth to OD_{600} ~0.8, the culture temperature was lowered to 20 °C, argon was sparged, and overexpression was induced by adding 0.5 mM IPTG, 0.5 % w/v D-glucose, 2 mM L-cysteine and 25 mM sodium fumarate. Overexpression at small-scale in *C. beijerinckii* was achieved by growing the cells anaerobically (bottle method) in 500 mL TYA media (50 g/L glucose, 2 g/L yeast extract, 6 g/L tryptone, 0.5 g/L KH_2PO_4, 3 g/L ammonium acetate, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$) supplemented with 250 μg/mL spectinomycin until late exponential phase. Small-scale experiments were performed as previously described in conventional Erlenmeyer flasks (*E. coli* aerobic growth) and Schott bottles equipped with appropriate tubing (*E. coli* anaerobic post-induction, or *C. beijerinckii* anaerobic growth). All experiments were performed at least in biological duplicates.

Overexpression in a bioreactor. Scale up experiments were performed at 1-liter scale in batch fermentations. Inocula for batch fermentations were developed by inoculating glycerol stocks (20 μL) of *E. coli* strain into either TB medium (100 mL) (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 2.2 g/L KH_2PO_4, 9.4 g/L K_2HPO_4) supplemented with 100 μg/mL carbenicillin, 50 μg/mL streptomycin and 30 μg/mL kanamycin, or M9 medium (100 mL) (15 g/L KH_2PO_4, 33.9 g/L Na_2HPO_4, 5 g/L NH_4Cl, 2.5 g/L NaCl, 2.5 g/L ammonium citrate) supplemented with 100 μg/mL carbenicillin, 50 μg/mL streptomycin and 30 μg/mL kanamycin and grown overnight at 37 °C.

The cultures were used to inoculate either TB fermentation medium (as above but also supplemented with 0.5 mM ammonium ferric citrate) or M9 medium (as above but also supplemented with 0.5 mM ammonium ferric citrate and 3.6 mL of fermentation trace elements containing 22.3 g/L $Na_2EDTA \cdot 2H_2O$, 10.03 g/L $FeCl_3$, 0.5 g/L $CaCl_2 \cdot 2H_2O$, 0.18 g/L $ZnSO_4 \cdot 7H_2O$, 0.18 g/L $CoCl_2 \cdot 6H_2O$, 0.16 g/L $CuSO_4 \cdot 5H_2O$ and 0.15 g/L $MnSO_4 \cdot H_2O$) at 1 L scale in a BioFlo 115 Fermenter vessel (2 L) (Eppendorf UK). Cultures were grown at 37 °C, and the pH was maintained at pH 7.2 by automated addition of acid and base. The air flow rate was 1 L/min and the dO_2 was maintained at 30 % of saturation by automatic control of the stirrer speed between 300–1000 r.p.m. Once an OD_{600} of 0.6–0.8 was reached the air supply was replaced with nitrogen supplied at 1 L/min to obtain anaerobic conditions and the temperature reduced to 20 °C. Expression of CbA5H was induced by the addition of IPTG (0.5 mM). Sodium fumarate (25 mM), glucose solution (0.5 %) and L-cysteine (2 mM) were also added at induction. The fermentation proceeded for 48 hours after which biomass was harvested for enzyme purification. All experiments were performed at least in biological duplicates. A larger scale experiment was also performed in a Fermac 360 fermenter vessel (10 L) utilizing TB as medium (Electrolab Biotech UK Ltd).

Enzyme purification. CbA5H was purified aerobically by affinity chromatography using Cytiva StrepTrap HP 5 mL prepacked columns. *E. coli* cells were lysed on ice for 1.5 hours by chemical treatment with 1 mg/mL lysozyme and 0.5 % v/v Triton X-100 in 100 mM Tris-HCl, 150 mM NaCl, pH 8, supplemented with cOmplete protease inhibitor (Roche) and Benzonase nuclease. *C. beijerinckii* cells were lysed at 37 °C for 1 hour with 1 mg/mL lysozyme in 100 mM Tris-HCl, 150 mM NaCl, pH 8, supplemented with cOmplete protease inhibitor (Roche) and Benzonase nuclease and additionally sonicated for 5 minutes. After binding on the affinity column and extensive washing, CbA5H was eluted with 5 mM desthiobiotin in 100 mM Tris-HCl, 150 mM NaCl, pH 8. Desalting was performed with Cytiva PD-10 columns and sample concentration was performed by ultrafiltration on Vivaspin 20 concentrators (30 kDa MWCO). Protein concentration was determined by the Bradford assay using BSA as a standard.

Enzyme characterization. H_2 evolution activity was assayed by gas chromatography, as previously reported,[26] utilizing an Agilent 7820 A gas chromatographer equipped with a purged packed (PP) inlet, Carboxen-1010 PLOT column (30 m×0.53 mm I.D.) and thermal conductivity detector (TCD). H_2 uptake (oxidation) activity was assayed spectrophotometrically on a Shimadzu UV2600 spectrometer, as previously reported.[10a] Reactions were set up at 37 °C in H_2-saturated buffer containing an appropriate electron acceptor. A mixed buffer (35 mM each MES, Tris, CHES) adjusted to pH 8.5 was used for all assays, except for the pH dependance experiments where the pH was adjusted within the range 6–10. 1 mM Methyl viologen (ε_{604} = 13,600 M^{-1} cm^{-1}) was used for standard H_2 uptake assays; 0.1 mM FAD (ε_{450} = 11,300 M^{-1} cm^{-1}) or 0.1 mM FMN (ε_{445} = 12,500 M^{-1} cm^{-1}) were used for flavin reduction assays. Prior to assays, the aerobically purified CbA5H enzyme was reactivated by incubating under a H_2 atmosphere (≥ 10 minutes), or by adding 10 mM sodium dithionite under an Ar atmosphere.

H_2-driven $FMNH_2$ recycling with CbA5H and ENE-108. A round bottom flask equipped with a stir bar was sealed with a rubber septum with two needles inserted. The flask was charged with a 3.5 mL solution of **1** (10 mM) and FMN (0.5 mM) in 10 % v/v DMSO in Tris-HCl (50 mM, pH 8.0). This was sparged with N_2 for 1 hour via one of the needles, then the gas line was switched to H_2 and sparged for 1 hour while stirring at 35 °C. The needle used for sparging was then moved out of the reaction solution and into the flask headspace. A solution of CbA5H (0.69 mg, 0.5 mL) was thawed under a balloon of N_2, placed under a steady flow of H_2 for 5 minutes in order to reactivate the enzyme, and added to the reaction flask via a degassed needle and syringe. A 0.5 mL portion of the reaction solution was used to dissolve and transfer ENE-108 powder (2 mg) into the reaction solution, via degassed needle and syringe. The reaction was stirred under a steady flow of H_2 at 35 °C. Reaction progress was monitored using HPLC (detailed in S2.4 in the Supporting Information).

Immobilization of CbA5H. Carbon black (BP2000) was suspended in Tris-HCl buffer (50 mM, pH 8.0, 20 °C) to make a 20 mg/mL slurry in a 1.5 mL centrifuge tube. Larger carbon agglomerates were dispersed using a pipette, then sonicated 4×15 min which gave an ink-like slurry. A 250 μL portion of BP2000 slurry (5 mg) was mixed with 100 μL of CbA5H solution (0.71 mg) and incubated for 1 hour. The percent of protein immobilization onto carbon black was determined by centrifuging twice (12,000× *g*) and the supernatant was analyzed in a Bradford assay. The immobilized CbA5H was then characterized using the methyl viologen-mediated H_2 uptake assays at pH 8 as described above. CbA5H was also immobilized on pyrolytic graphite edge and evaluated using protein film electrochemistry for activity and stability (see full method in S2.5.1).

Biocatalytic hydrogenation of 3-quinuclidinone to 3-quinuclidinol. "H_2BioCat" catalyst slurry was prepared on the benchtop under an atmosphere of N_2 by co-immobilizing CbA5H with an NAD^+ reductase "I64A" (a construct of the NAD^+-reducing soluble hydrogenase from *R. eutropha* with inactive hydrogenase following a single amino acid substitution, I64A, in the hydrogenase large subunit;[27] purified similarly to methods described previously) on BP2000, and analyzed for extent (%) of enzyme immobilization. The isolation and purification of I64A and *At*QR followed previously published protocols (further details in S1.2).

The "H_2BioCat" was then used for biocatalytic hydrogenation of 3-quinuclidinone (**3**): A round bottom flask equipped with a stir bar

was sealed with a rubber septum, and two needles were inserted. The flask was charged with 2.8 mL of a solution containing 3-quinuclidinone-HCl (50 mM) and NAD^+ (1 mM) in Tris-HCl (50 mM, pH 8.0). This was sparged with N_2 for 30 minutes via one of the needles while stirring at 30 °C. The freshly prepared "H_2BioCat" slurry was transferred to the stirred solution via a needle and syringe, giving a loading of 0.6 mg/mL. *At*QR was added to the reaction mixture, to give an enzyme loading of 1.4 mg/mL. The gas through the inlet needle was then switched from N_2 to H_2, and the flask was stirred at 850 r.p.m. under a steady flow of H_2 at 30 °C for 18 hours. Reaction progress was monitored using ^{1}H NMR spectroscopy (detailed in S2.6.1).

A preparative-scale (66 mL) biocatalytic hydrogenation of 3-quinuclidinone-HCl (538 mg) was also implemented using the above conditions and loadings (substrate, cofactor, and catalysts). The only difference was volume and stir rate (250–680 r.p.m.). See the S2.6.2 for more experimental details about this reaction.

Supporting Information

The authors have cited additional references within the Supporting Information.[28]

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Conflict of Interests

HydRegen is a limited commercialising biotechnologies for sustainable chemical manufacturing. In this work, HydRegen collaborate with Dr Morra under Innovate UK funding (project number 10065700), to consider novel hydrogenase enzymes. This research was not part of a commercial relationship and was carried out for R&D purposes.

Data Availability Statement

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

Keywords: hydrogenase · cofactor recycling · industrial biotechnology · biocatalysis · sustainable chemistry

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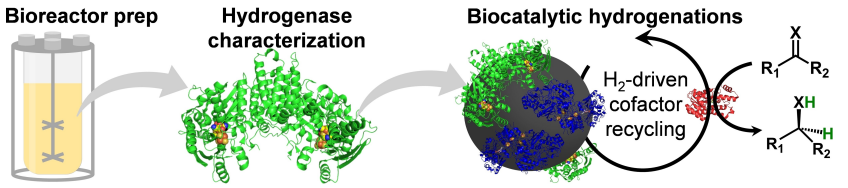
RESEARCH ARTICLE



The enzyme CbA5H from *Clostridium beijerinckii*, the only known [FeFe]-hydrogenase able to survive exposure to oxygen, was evaluated here in biotechnologies for sustainable chemical synthesis. Enzyme production was optimized in a scalable bioreactor.

CbA5H was purified aerobically, characterized using benchtop activity assays, and used to drive biocatalytic hydrogenations for example in the preparation of pharmaceutical fragment (3)-quinuclidinol at 0.4 g scale.

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Scalable Bioreactor Production of an O_2-Protected [FeFe]-Hydrogenase Enables Simple Aerobic Handling for Clean Chemical Synthesis