Biological hydrogen storage and release through multiple cycles of bi-directional hydrogenation of CO_2 to formic acid in a single process unit

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Summary

Hydrogen is a promising fuel in a carbon-neutral economy and many efforts are currently undertaken to produce hydrogen. One of the challenges is a safe and easy way to store and transport the highly explosive gas. One option that is intensively analyzed by chemists and biologists is the conversion of hydrogen and CO_2 to the liquid organic hydrogen carrier formic acid. Here, we demonstrate for the first time that a bio-based system, using the bacterium *Acetobacterium woodii* as biocatalyst, allows multiple cycles of bi-directional hydrogenation of CO_2 to formic acid in a single bioreactor. The process was kept running over two weeks producing and oxidizing 330 mM formic acid in sum. Unwanted side product formation in form of acetic acid was prevented through metabolic engineering of the organism. The demonstrated process design can be considered as a future "bio-battery" for the reversible storage of electrons in form of H_2 in the versatile compound formic acid.

Keywords: carbon capture, hydrogen storage, whole-cell catalysis, acetogenic bacteria, fermentation, bioreactor, hydrogenation of CO_2, formate oxidation, hydrogen-dependent CO_2 reductase

Introduction

Concerns about climate change and global warming have prompted research to replace fossil-fuel based energy carriers with the coincident need to maintain the world's energy demand.[1-3] Molecular hydrogen has been considered as an attractive, alternative energy carrier which can be produced environmentally friendly and renewable. If the applied hydrogen is produced from renewable energy sources (i.e., solar-, wind-, hydro-, geothermal power) by water splitting and not *via* traditional routes such as steam reforming and partial oxidation of coal, oil and natural gas, no net CO_2 is generated in the production process.[4-6] Furthermore, hydrogen is a strong candidate for energy/electricity storage in an environment with excess renewable energy supply. However, due to handling and storage concerns of the highly volatile H_2 gas, the catalytic process of direct hydrogenation of CO_2 has attracted more and more attention in recent years.[7-9] The process does not only allow hydrogen energy conversion and storage on a large scale, it further provides a feasible avenue for carbon dioxide capture and storage. The necessary CO_2 molecule can be derived from sources such as industrial flue gas but direct carbon capture and storage (CCS) technologies also enable CO_2 capture from air; a technology that is intensively studied but still requires optimization.[10-12] The process of hydrogen-dependent CO_2 reduction allows the conversion and storage of H_2 and CO_2 in the versatile compound formic acid/formate that is one likely product of the mentioned process.[9,13,14] Formic acid does not only belong to the group of liquid organic hydrogen carriers (LOHCs), moreover, the compound fulfills the necessary requirements for a putative sustainable formate bioeconomy in future.[15] The field of applications for renewably produced formic acid is diverse since formic acid can be used as bulk chemical, microbial feedstock or can even be applied directly in formic acid fuel cells (DFAFC).[15-17]

Chemical catalysts can also facilitate the interconversion of H_2 and CO_2 to formic acid, however, mostly requiring noble metals and/or extreme conditions in the reaction process which

make the conversion economically unattractive.[13,18,19] But aside from chemical catalysts, also biological solutions arise. Recently, a soluble, biotechnological interesting enzyme complex was identified in the obligate anaerobic group of acetogenic bacteria, named as hydrogen-dependent CO_2 reductase (HDCR).[20,21] The enzyme complex consists of four subunits, harboring a [FeFe]-hydrogenase, a molybdenum/tungsten-*bis* pyranopterin guanosine dinucleotide (Mo/W-*bis* PGD) cofactor containing formate dehydrogenase as well as two electron transferring subunits (Figure 1). So far, the HDCRs were purified from the mesophilic and thermophilic acetogenic bacteria *Acetobacterium woodii* and *Thermoanaerobacter kivui*, respectively, but bioinformatic analysis of available genome data indicates the presence of HDCR-like enzymes also outside of this bacterial group.[20,21] The purified and characterized HDCR enzymes catalyze the direct hydrogenation of CO_2 to formic acid with remarkable catalytic rates, outcompeting chemical catalysts under comparable moderate reaction conditions.[22] Noteworthy, the activity is fully reversible and can be affected by the substrate concentrations and prevailing reaction conditions, thus, facilitating the release of stored hydrogen in another catalytic dehydrogenation reaction. The two challenging reactions of hydrogen-dependent CO_2 reduction to formate as well as the "reverse reaction" of formate-driven H_2 production are catalyzed with almost identical catalytic rates.[20,21] Since the purified enzyme is sensitive to O_2, a HDCR-based whole-cell system was established in serum bottle and bioreactor scale, again, demonstrating the remarkable bi-directionality of the HDCR-based system.[20,23-25] In the applied system, the HDCR reaction was decoupled from the Wood-Ljundahl pathway (WLP) of CO_2 fixation by lowering the intracellular amount of ATP using specific inhibitors or ionophores. Here, the antibiotic monensin was identified to be a cheap and efficient uncoupler of the *A. woodii* bioenergetics. The substance works as a carboxylic ion-selective ionophore (especially Na^+/H^+ antiporter) which facilitates electroneutral, monovalent ion transport across the cell membrane. Therefore, the substance destroys the Na^+ gradient across the cytoplasmic membrane that drives ATP synthesis in *A. woodii*. Without

84 cellular ATP, the ATP-dependent further conversion of formic acid to acetic acid in the WLP
85 of acetogenic bacteria is blocked (Figure 1). As a result, the typical intermediate formic acid
86 which is produced by many acetogens transiently during acetogenesis from H_2 and CO_2
87 becomes the predominant product.

88 So far, hydrogen-dependent CO_2 reduction to formate as well as formate/formic acid driven H_2
89 and CO_2 production were only studied separately from each other. In this study, we describe
90 and implement a new process design for biological hydrogen storage and release through
91 multiple cycles of bi-directional hydrogenation of CO_2 to formic acid in one single bioreactor
92 by using the same biocatalyst.

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Results

Multiple cycles of bi-directional hydrogenation of CO_2 to formic acid in a single bioreactor

Since the upscaling feasibility of the HDCR-based whole-cell system was recently proven in stirred-tank bioreactors (STR),[25] the same bioreactor type was chosen in this study to investigate the bi-directional hydrogenation of CO_2 to formic acid in a single bioreactor by using our established whole-cell system from *A. woodii*. A H_2 storage period (day period) of 8 h and a H_2 production period (night period) of 16 h were assumed in the experiment to simulate potential H_2 production times by solar power *via* solar panels. This ratio was based on the fact that in southern Germany the average hours of sunshine during summer time are about 7-8 h, thereby, resulting in a time period of 8 h to force hydrogen-dependent CO_2 reduction to formic acid. In the remaining time period (16 h), the produced formic acid was aimed to be re-oxidized to release the stored H_2. To test whether H_2 storage is bi-directional in one bioreactor, non-growing, resting cells suspended in buffer were used. These cells convert substrates to products with high rates but are not able to grow since essential nutrients are missing. *A. woodii* cells were grown in 20 L complex medium with fructose to the end of the exponential growth phase and resting cells were prepared. The bioreactor contained 50 mM K-phosphate buffer at pH 7.0 with additional 15 µM of the metabolic uncoupler monensin that blocks further reduction of formic acid to acetic acid by the cell (Figure 1).[25] The pH was kept in the range of 5.9 to 8.1 by titration with NH_4OH or H_3PO_4. The experiment was started by adding resting cells to a final cell protein concentration of 1 mg/mL to the bioreactor which was flushed with H_2, CO_2 and N_2. As expected, resting cells immediately started to produce formic acid from H_2 and CO_2 with a production rate of 4.3 (± 1.6) mmol g^{-1} h^{-1} (Figure 2A).

During the "day-period" of 8 h, 28 mM formic acid were produced *via* direct hydrogenation of CO_2. Then, the gas composition was switched to 100% N_2 in the "night period"; by sparging with 100% N_2 the chemical equilibrium was pushed towards hydrogen formation from formic

120 acid (Figure 2B). Formic acid oxidation proceeded with a rate of 2.3 (± 0.8) mmol g^{-1} h^{-1}.
121 During the 16 h "night period", the formic acid concentration decreased from initial 28 mM to
122 4.9 mM. The formic acid-driven gas production in the "night period" as well as the H_2-
123 dependent CO_2 reduction in the "day-period" could also be observed in the off-gas flow rate.
124 Here, only a qualitative but not quantitative plot can be shown for the off-gas (Figure 2C). This
125 is owed to the used bioreactor set-up since a 1.5 L headspace was applied in the bioreactor to
126 allow potential foaming. Additionally, a low gas-flow rate of 10 mL/min was applied to reduce
127 gas wasting and gas dilution during H_2 production. The applied set-up allowed a diffusive back
128 mixing of the head space additional to a prolonged mean retention time of the gases which
129 distorted the data for quantitative analysis. Nevertheless, the qualitative plot clearly showed
130 that the off-gas flow was reduced during the day period due to H_2 and CO_2 consumption. In
131 contrast, more gas was released from the bioreactor in the night period due to formic acid-
132 driven H_2 and CO_2 production. The effect of alternating gas mixtures, mainly based on varying
133 CO_2 concentrations, could also be seen in the pH profile (Figure 2D). Prior to cell
134 supplementation, CO_2 saturation was achieved in the liquid phase in the bioreactor. Therefore,
135 the initial pH drop was mainly based on the production of formic acid from H_2 and CO_2. After
136 the gas composition was switched to 100% N_2, formic acid was re-oxidized and dissolved H_2
137 as well as CO_2 were flushed out of the reactor. The pH value shifted to more alkaline conditions.
138 In a new cycle of H_2 storage, dissolving CO_2 concentrations as well as formic acid production
139 relocates the pH value to a more neutral state.

140 Since the process enabled the bi-directional storage and release of H_2, the bioreactor application
141 was continued. Over the first 96 h (4 day/night cycles) the reaction kinetics of formic acid
142 formation and formic acid oxidation were monitored (Figure 3A). As can be seen in Figure 3B,
143 the specific activities for both reactions remained almost constant over the time. After 4 cycles,
144 83 and 75% of the initial formic acid formation and formic acid oxidation rate, respectively,
145 were present. Therefore, the system was kept running over 2 weeks which corresponds to 15

146 day/night-cycles or 360 h process time. The amount of produced formic acid per day-period
147 decreased with increasing process time (Figure 4A). At the same time, acetic acid formation
148 increased (Figure 4B). The unwanted side product acetic acid was only produced in traces
149 (around 2 mM) during the first 96 h of fermentation but increased up to 23 mM at the end
150 (t_{360} h). In the last day/night cycle 20% of the initial formic acid formation and formic acid
151 oxidation rate was still present. In sum 330 (± 85) mM formic acid was produced and oxidized
152 by direct hydrogenation of CO_2 within 15 day/night cycles in a single bioreactor. The
153 corresponding pH profile, optical density and total cell protein concentration were also
154 monitored (Figure 4C, D). Here, the optical density decreased slightly at the beginning of the
155 experiments and stayed constant for the rest of the application. As expected, the total cell
156 protein concentration behaved similar to the optical density. Noteworthy, 87% of the initial cell
157 concentration remained after 2 weeks of application, indicating a solid robustness of the
158 *A. woodii* cell system under the given conditions. Moreover, the chosen bioreactor set-up
159 required only 25 mM of phosphoric acid and even no addition of base was needed in the entire
160 process.

161 **Repetitive monensin addition to ensure product specificity**

162 The observed acetic acid formation is not wanted in the process of H_2 storage and H_2 release in
163 and from formic acid. Since acetic acid is only produced from H_2 and CO_2 if the energy state
164 of resting cells is not fully diminished (Figure 1), a putative loss of efficacy over time of the
165 used ionophor monensin was assumed. Therefore, monensin was repetitively added every 72 h
166 to the bioreactor broth. In this approach, the entire bioreactor set-up and the process parameters
167 (gas flow, stirrer speed, gas composition, duration of day/night cycling etc.) were identical to
168 the previous experiment with the exception of the repetitive addition of monensin. The behavior
169 of the bioreactor process as well as product formation were comparable to the previously shown
170 process (Figure S1). Again, formic acid formation was constant for the first 4 day/night cycles,

but decreased afterwards whereas acetic acid formation increased. To check whether the bioreactor buffer has still an uncoupling ability due to the presence of sufficient amounts of active monensin, some bioreactor broth was taken at the end of the process to check for its uncoupling ability. Therefore, *A. woodii* cells were removed from the bioreactor broth and the recovered cell-free bioreactor buffer was used for another cell suspension experiment with freshly prepared *A. woodii* cells. No additional monensin was added to the cell-free bioreactor buffer to check its ability to uncouple fresh cells and to force formic acid production. Indeed, the cell-free bioreactor buffer was able to uncouple *A. woodii* cells which showed a specific formic acid production rate of 29 mmol g^{-1} h^{-1} (Figure S2). The control experiment with new buffer and added monensin (15 µM) showed a specific activity of 19 mmol g^{-1} h^{-1}. This clearly shows that the ionophore monensin is still active in the bioreactor broth after 360 h of process time and that other reasons seem to be responsible for the reactivation of the *A. woodii* cell metabolism and the formation of acetic acid.

Genetic modification of *A. woodii* for bi-directional H_2 storage

A gene knock-out of an enzyme in the Wood-Ljungdahl pathway (WLP) for CO_2 reduction to acetic acid was one likely way to prevent the conversion of the formic acid produced to acetic acid and to force formic acid accumulation. Therefore, the genes encoding a central enzyme, the methylene-tetrahydrofolate (THF) reductase, were deleted (Δ*metVF*). This strain lacks two (*metV*, allocated locus tag Awo_c09300; *metF*, Awo_c09310) of three (*metV*; *metF*; *rnfC2*, Awo_c09290) genes coding for the methylene-THF reductase enzyme complex which catalyzes the formation of a THF-bound methyl group of N^5-methyl-THF from N^5,N^{10}-methylene-THF in the methyl-branch of the WLP (Figure 1).[26] The generation, genotype and phenotypic characterization of this mutant will be described elsewhere. If the methylene-THF reductase enzyme is lacking, the further conversion of H_2 and CO_2 to acetate should be blocked. To check for product formation, resting cells of *A. woodii* Δ*metVF* were prepared and the

196 conversion of H_2 and CO_2 was investigated in serum bottle experiments in the absence of
197 monensin. Indeed, resting cells of *A. woodii* Δ*metVF* converted H_2 and CO_2 exclusively to the
198 end product formic acid (Figure S3A). After 120 min, 12.7 mM formic acid were produced
199 with a specific formic acid formation rate of 18 mmol g^{-1} h^{-1} and no acetate could be detected.
200 Also, the "reverse reaction", formate-driven H_2 production, was studied in resting cells of the
201 Δ*metVF* mutant (Figure S3B). When 300 mM of Na-formate were added to the cells, no acetic
202 acid was produced and the oxidation of 167 mM formic acid resulted in the release of 164 mM
203 H_2 after 22 h. The specific H_2 production rate (qH_2) was around 36 mmol g^{-1} h^{-1}. Next, the bi-
204 directional hydrogenation of CO_2 to formic acid was investigated in a single bioreactor by using
205 resting cells of the *A. woodii* Δ*metVF* strain as biocatalysts. The entire process parameters were
206 as before. Resting cells of the Δ*metVF* mutant showed a comparable catalytic performance as
207 the *A. woodii* wild type strain (Figure 5). The Δ*metVF* mutant was able to produce formic acid
208 from H_2 and CO_2 with a production rate of 3.0 (± 0.4) mmol g^{-1} h^{-1} during the "day-period".
209 After 8 h 23 mM formic acid was produced. In the "night period", the cells oxidized the
210 produced formic acid from the reactor broth with a specific rate of 1.7 (± 0.2) mmol g^{-1} h^{-1}. In
211 16 h, the formic acid concentration decreased to 5.4 mM. As a result of bi-directional
212 hydrogenation of CO_2, 220 (± 5) mM of formic acid was produced and oxidized in total in the
213 entire bioreactor process. After 2 weeks of process time, 30% of the initial formic acid
214 formation rate as well as 98% of the initial total cell protein concentration were still present
215 (Figure S4). The entire process consumed 17 mM of phosphoric acid and no base was needed.
216 Noteworthy is that in contrast to wild type cells only traces of acetic acid (0.88 ± 0.22 mM)
217 were produced after two weeks.

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Discussion

In this study, we investigated biological hydrogen storage and release in a single bioreactor using *A. woodii* cells as biocatalysts. For this approach, *A. woodii* is superior over, for example, bacteria that use the formate hydrogen lyase (FHL) for formate oxidation. FHL has a strong bias towards formate oxidation, the physiological reaction, whereas the reverse reaction is only possible with appreciable rates under harsh conditions.[27,28] In contrast, HDCR containing acetogenic bacteria grow on formate[29] as well as on H_2 + CO_2 and, therefore, the enzyme must catalyze both reactions with high (identical) rates. Since the equilibrium constant for H_2-dependent CO_2 reduction to formic acid is close to one, minor changes in substrate/product concentrations, pH and temperature can influence the chemical equilibrium. For example, varying gas compositions affected either the production or the degradation of formic acid in our single bioreactor. The observed rates for both reactions were multiple times lower than previously reported rates,[25] mainly due to reaction limitations (i.e., substrate limitations) in the bioreactor process. We decided to use a "realistic" and reduced gas flow rate to reduce the gas waste stream and to avoid an excessive dilution of the produced H_2. Additionally, it has to be kept in mind that a change in gas flow rates has an effect on the change in pH values due to the contribution of CO_2. Of course, in a more applied process, the non-captured H_2 and CO_2 of the day-period can be recycled and can be fed back into the bioreactor.

Alternatively, air-captured CO_2 or captured and stored CO_2 in form of bicarbonate can be used as CO_2 source in our process in the future. Previously, it was shown that resting cells of *A. woodii* convert $KHCO_3$ and H_2 into formic acid.[20] This conversion is possibly due to the presence of a fast and soluble carbonic anhydrase enzyme which catalyzes the interconversion of bicarbonate in CO_2 and H_2O.[30] *A. woodii* cells even showed the highest carbonic anhydrase activity of different investigated acetogens.[31] In such an approach, the necessary bicarbonate can be obtained from a process called carbon capture and storage by mineral carbonation

(CCSM). In this route of CO_2 sequestration, CO_2 is stored in the form of bicarbonate by carbonation of carbonate minerals.[32,33]

The demonstrated bioreactor set-up in this study allowed multiple cycles of bi-directional interconversion of formic acid and H_2/CO_2. Interestingly, the uncoupling effect of monensin on *A. woodii* cells could not be maintained over the whole process time. Since *A. woodii* cells started to produce acetic acid from H_2 and CO_2 in the late phase of the process, Na^+-dependent energy conservation seemed not to be impaired anymore by addition of monensin. But the loss of function of monensin could be experimentally excluded. The activity of freshly prepared *A. woodii* cells uncoupled by the bioreactor buffer had even more than 100% of the control activity, mainly due to the fact that the re-used bioreactor buffer had a more alkaline pH (pH 7.94) which favors formic acid production. However, acetic acid formation leads to the assumption that *A. woodii* cells may adapt to the antibiotic substance. In general, Gram-positive bacteria are considered to be more sensitive to monensin than Gram-negative bacteria, but monensin adaptation is not a new phenomenon. Pure culture studies of three isolated cattle rumen bacteria have been demonstrated to exhibit a long lag phase prior to growth in the presence of monensin.[34] The three investigated Gram-positive bacteria *Enterococcus faecium*, *Enterococcus faecalis* and *Clostridium perfringens* developed monensin resistance through altered cell wall characteristics, showing a thickening of the cell wall or the extracellular polysaccharide (glycocalyx) layer. Similarly, the Gram-positive, amino acid fermenting bacterium *Clostridium aminophilum* could be adapted to monensin.[35] It was even shown that adapted cultures can subsequently grow in even higher concentrations of monensin.[36,37]

Another approach to redirect product formation in an organism is by metabolic engineering. In this study, genetic modification of *A. woodii* cells were applied to prevent unwanted side-product formation such as acetic acid in the bioreactor process. The addition of ionophores or antibiotic substances would not be desirable in future demo/pilot scale approaches, therefore,

autotrophic acetate formation was blocked by the deletion of the methylene-THF reductase encoding genes. Of course, the knock-out of formyl-THF synthetase genes would be the most obvious way to prevent the further conversion of formic acid in the WLP (Figure 1). Unfortunately, *A. woodii* has two isogenes (*fhs1*, Awo_c09260; *fhs2*, Awo_c08040) encoding formyl-THF synthetases and a double knock-out could, so far, not be obtained.

A potential application concept for future bi-directional H_2 storage by using the described bioreactor approach is shown in Figure 6. Excess energy can be used to produce "green" H_2 by water splitting. The necessary renewable energy can be obtained, for example, from sunlight during daytime but other renewable energy sources are also conceivable. H_2 as well as air-captured CO_2 (*via* CCS technologies) can then be converted to the LOHC formic acid in a single bioreactor under ambient pressure and temperature. Here, *A. woodii* cells are used as efficient whole-cell biocatalysts to drive this reaction. The accumulated formic acid can later be re-oxidized in the same bioreactor with the help of the same biocatalyst to release the stored H_2 in times of power deficiencies. The produced H_2 can be separated from the hydrogen-lean CO_2 molecule by existing methods for gas separation.[38,39] CO_2 can be recycled and can afterwards be used in another H_2 storage cycle. Thus, the designed process unit can be considered as a future "bio-battery" for the reversible storage of electrons in form of H_2 in the versatile compound formic acid.

The reversible electrocatalysis of a formate dehydrogenase and a hydrogenase from *D. vulgaris* was previously demonstrated using a semiartificial FHL concept.[40] These systems were immobilized on an electrochemical device to achieve reversible formate/H_2 interconversion. However, the novelty in this study is the shown bi-directionality of the entire process to allow H_2 storage and release over several cycles in a bio-based system. That renewably produced hydrogen *via* water splitting can allow for CO_2 fixation/reduction was previously demonstrated

in an inorganic–biological hybrid system using *Xanthobacter autotrophicus* or *Ralstonia eutropha*.[41,42]

In summary, we could prove the feasibility of bi-directional hydrogenation of CO_2 to formic acid in a single bioreactor by using our established whole-cell system based on the acetogenic bacterium *A. woodii*. Here, the H_2 and CO_2 stored and captured during the day period could be released in the night period by the same biocatalyst in the same process unit. As far as we know, it is the first time that a bio-based system allows multiple cycles of bi-directional interconversion of formic acid and H_2/CO_2 in a single bioreactor. This could allow storage of locally produced energy/H_2 in form of the non-toxic, non-environmental harmful LOHC formic acid and release of H_2 in the same environment. The safety concerns as well as the need for valuable raw materials and metals could be less critical compared to already existing energy storage technologies. In future, a switch to less energy intense bioreactor types such as bubble column bioreactors would be the preferred way to improve the total energy balance of the system. Many influencing factors of the investigated process itself but also the dependence from global/local political regulations effects the viability of a process. However, the unique process design described here combines two chemically challenging reactions which could be an integral part of a future hydrogen economy to combat global warming and to solve the renewable energy demand of the growing world population.

Experimental procedures

Resource availability

Lead contact

Further information and requests for resources and materials should be directed to and will be fulfilled by the lead contact, Volker Müller (vmueller@bio.uni-frankfurt.de).

Materials availability

The materials in this study will be made available upon reasonable request.

Data and code availability

The datasets generated in this study are available from the lead contact on reasonable request.

Organism and cultivation

Acetobacterium woodii (DSM 1030) wild type was cultivated at 30 °C under anoxic conditions in carbonate-buffered medium[43] using 1 L flasks (Müller-Krempel, Bülach, Switzerland) with 500 mL media or in 22 L flasks (Glasgerätebau Ochs; Bovenden-Lenglern, Germany) with 20 L media. The medium was prepared under anoxic conditions as described before.[44,45] To grow the *A. woodii metVF* deletion strain, 50 mg/l uracil and 50 mM glycine-betaine was added to the medium. Fructose (20 mM) was used as growth substrate for all cultivations and cell growth was followed by measuring the optical density at 600 nm with an UV/Vis spectrophotometer.

Preparation of resting cells and cell suspension experiments

Resting cells of *A. woodii* wilde type and Δ*metVF* were prepared as described before.[20] In resting cells, biomass formation/cell division does not occur but cells are still fully metabolic active. The cells were washed and resuspended in K-phosphate buffer (50 mM K-phosphate,

20 mM KCl, 4 µM resazurin, 2 mM DTE, pH 7.0). The total cell protein concentration of the cell suspension was determined according to Schmidt et al.[46] and the cells were directly used for the subsequent experiments. To determine the conversion of H_2 + CO_2 into formate by resting cells of *A. woodii*, 120 mL serum bottles (Glasgerätebau Ochs GmbH, Bovenden-Lenglern, Germany) containing pre-warmed buffer (50 mM K-phosphate, 20 mM KCl, 4 µM resazurin, 2 mM DTE, pH 7.0) under a N_2 atmosphere were incubated with cell suspensions at the protein concentration stated. The final liquid volume in the serum flasks was 10 mL and the resting cells were incubated for at least 10 min at 30 °C prior to the start of the experiment. The reaction was started by changing the head space to a H_2 + CO_2 (80:20%, [v/v]) atmosphere with 1 bar overpressure. If necessary, the ionophore monensin (as sodium salt; dissolved in EtOH) was added prior to the reaction start and liquid samples were taken over the time to analyze the formation of acetic acid and formic acid.

Multiple cycles of bi-directional hydrogenation of CO_2 to formic acid in a single bioreactor

The bioreactor experiments were carried out in Biostat Aplus bench-top reactors from Sartorius (Melsungen, Germany) with a working volume of 1.5 L as described before.[25] Each bioreactor was equipped with micro sparger, baffles, two Rushton-impeller, pH-probe (Hamilton, Bonaduz, Switzerland), temperature probe and a redox potential probe (Hamilton, Bonaduz, Switzerland) (Figure 7). The temperature of the buffer (50 mM K-phosphate, 20 mM KCl, 2 mM DTE, pH 7.0) was maintained at 30 °C, using a cooling finger and heating sleeve. The permitted pH-range of the bioreactor experiments was from pH 5.9 to 8.1 and was achieved by titration with H_3PO_4 (4 M) and NH_4OH (4 M). The gas flow rate was maintained during the "day/night cycles" at a constant value of 10 mL/min using a digital mass-flow controller (Bronkhorst High-Tech, Ruurlo, Netherlands). The supplied gas composition varied in the stoichiometry of H_2, CO_2 and N_2 in dependence of the catalytic cycle. During the "day period" (duration: 8 h; H_2 storage process) a gas composition of 45% H_2, 45% CO_2 and 10% N_2 [v/v]

was used (Nippon Gases, Germany). During the "night period" (duration: 16 h; H_2 production process) a gas composition of 100% N_2 [v/v] was used. The gas switch was made manually without an interruption of the existing gas flow. The installed micro sparger ensured the formation of microbubbles to enhance mass transfer between gaseous and aqueous phase.[47] The headspace of the bioreactor was at atmospheric pressure and the gas-liquid mixing was achieved by using a stirrer set-up with two Rushton-impeller at 400 rpm. The bioreactor buffer was prepared under aerobic, non-sterile conditions and oxygen was removed by subsequent sparging with 45% H_2, 45% CO_2 and 10% N_2 [v/v] for about 16 h. After the achievement of anoxic conditions and CO_2 saturation in the liquid phase, 2 mM DTE and 15 µM monensin were added as indicated. The reaction was started by adding *A. woodii* cell suspension to a final cell protein concentration of 1 mg/mL to the bioreactor. Samples (2 mL) were taken at defined time points for product analysis as well as OD and total cell protein determination. A single liquid sample of 3 mL reactor broth was taken and discarded prior to the bioreactor sampling to account for the dead volume of the sampling line. The samples were centrifuged (18,000 × g, 8 min, room temperature) to remove cells and the supernatant was frozen at −20 °C until further off-line analysis.

Inhibitory effect of reactor buffer on fresh cell suspensions

Fresh cells suspensions of *A. woodii* were prepared as described in the section above. The reaction buffer for cell suspension experiments was obtained from the bioreactor approach at time point 360 h. Therefore, 60 mL cell suspension of each bioreactor was transferred to a 120 mL anoxic serum bottle (Glasgerätebau Ochs GmbH, Bovenden-Lenglern, Germany) at the end of the fermentation process. Afterwards, buffer and cells were separated *via* centrifugation (11,500 × g, 15 min, 4 °C) under anoxic conditions. The supernatant was taken as reaction buffer in the subsequent serum bottle experiment with a total cell protein

concentration of 1 mg/mL of the freshly prepared *A. woodii* cells. The serum bottle experiments were performed as described above.

Analytical methods

The concentrations of formic acid and acetic acid were measured by high-performance liquid chromatography using a 1260 Infinity II LC System (Agilent Technologies, Santa Clara, CA, USA) as described before[48] or by using a commercially available formic acid and acetic acid determination kit (Boehringer Mannheim/R-Biopharm AG, Mannheim/Darmstadt, Germany) following the instructions of the manufacturer. Bioreactor off-gas analysis was conducted *via* a Micro-GC (Inficon, Bad Ragaz, Switzerland) which was equipped with two measurement modules containing different analytical columns. The analytical conditions and columns were used as described before.[49] The total cell protein concentration of the prepared cell suspensions was determined according to Schmidt et al.[46]

Chemicals

All chemicals were supplied by Sigma-Aldrich (St. Louis, USA) and Carl Roth GmbH & Co KG (Karlsruhe, Germany). All premixed gases for cell suspension experiments were purchased from Nippon Gases Europe (Düsseldorf, Germany). Pure gases such as N_2 (purity of 5.0) were purchased from Air Liquide (Paris, France).

Supplemental information

Supplemental information can be found online.

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Author contributions

V.M designed and supervised the research, analyzed the data and wrote the manuscript. F.M.S designed the research, performed the experiments, analyzed the data and wrote the manuscript. J.M. generated the mutant strain. F.O. supervised the fermentation.

Declaration of interests

Goethe-University Frankfurt and V.M possess a patent on the HDCR based whole-cell system for storing gaseous hydrogen through producing methanoate (patent number: EP2816119).

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Figure legends

Figure 1. HDCR-based whole-cell catalysis and the bioenergetics and biochemistry of the metabolism in *A. woodii*. The gaseous substrates H_2 and CO_2 can diffuse across the cell membrane where the HDCR enzyme hydrogenates the CO_2 molecule to formic acid. Formic acid is then bound to the cofactor tetrahydrofolic acid and reduced to a methyl group and then condensed with carbon monoxide, derived by reduction of a second molecule of CO_2, and coenzyme A to acetyl-CoA which is further metabolized to acetic acid. If the energy state of the cell is reduced by the ionophore monensin, which destroys the sodium ion potential across the cytoplasmic membrane required for ATP synthesis, the ATP-dependent further conversion of formic acid in the Wood-Ljungdahl pathway (WLP) is blocked. Another way to prevent formic acid conversion to acetic acid is by metabolic engineering. The knock-out of the methylene-THF reductase coding genes prevents further formic acid conversion in the methyl branch of the WLP. The exact mechanism of formic acid uptake and excretion is so far not known. The HDCR enzyme is the key enzyme for HDCR-based whole-cell catalysis to convert H_2 and CO_2 into formate and *vice versa*. HDCR, hydrogen-dependent CO_2 reductase; HydA2, [FeFe]-hydrogenase; FdhF2, molybdenum containing formate dehydrogenase; HycB2/3, electron-transferring subunits; HydABC, electron-bifurcating hydrogenase; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; THF, tetrahydrofolate; HCO-THF, formyl-THF; HC-THF, methenyl-THF; H_2C-THF, methylene-THF; H_3C-THF, methyl-THF; Rnf, ferredoxin:NAD^+ oxidoreductase; CoFeSP, corrinoid-iron-sulfur-protein; Fd^{2-}, reduced ferredoxin. Transparent pink box indicates the methyl and carbonyl-branch of the WLP.

Figure 2. Process development during day/night cycles for biological hydrogen storage and release in a single bioreactor. Resting cells were transferred into the bioreactor at a final concentration of 1 mg/ml in K-phosphate buffer (50 mM K-phosphate, 20 mM KCl, 2 mM

DTE, pH 7.0). A continuous gas flow rate of 10 mL min^{-1} with 45% H_2, 45% CO_2 and 10% N_2 (day period) or 100% N_2 (night period) was applied. The reaction temperature was kept at 30 °C in the liquid phase and the stirrer speed was set at 400 rpm. Monensin (15 µM) was added as uncoupling agent. A) Formate production from H_2 and CO_2 during day period, B) formate oxidation during night period, C) qualitative off-gas graph over the first 48 h and D) corresponding pH course over two day/night cycles. The shown data from the off-gas graph is from one representative experiment out of three independent replicates. All other data points are mean ± SD, N = 3.

Figure 3. Kinetics of multiple cycles of bi-directional hydrogenation of CO_2 to formic acid in a single process unit. A) Formic acid formation and formic acid oxidation over 4 day/night cycles in the first 96 h of process time and B) the corresponding specific activity of formic acid production (black bars) and formic acid oxidation (grey bars). 100% of the activity corresponds to a formic acid production rate of 4.3 mmol g^{-1} h^{-1}. The formic acid oxidation rate of 100% is similar to 2.3 mmol g^{-1} h^{-1}. All data points are mean ± SD, N = 3.

Figure 4. Long-term application of bi-directional interconversion of formic acid and H_2/CO_2 in a single process unit. Shown are A) 15 formic acid formation/oxidation cycles of the entire process (360 h) and B) corresponding side-product formation profile of acetic acid. Additionally, C) optical density at 600 nm, total cell protein concentration and D) pH were monitored over the entire process. Empty triangles up, optical density at 600 nm; empty triangles down, total cell protein concentration. All data points are mean ± SD, N = 3.

Figure 5. Multiple cycles of bi-directional hydrogenation of CO_2 to formic acid in a single bioreactor using *A. woodii* Δ*metVF*. Formic acid formation and formic acid oxidation as well as acetic acid production is shown for two weeks of fermentation with multiple day/night cycles. In total 220 mM formic acid was formed and oxidized. A more detailed process kinetic is shown for the initial day- (blue box) and night-period (green box). The specific formic acid formation and oxidation rate were 3.0 mmol g^{-1} h^{-1} and 1.7 mmol g^{-1} h^{-1}, respectively. Triangels, formic acid; squares, acetic acid. All data points are mean ± SD, N = 3.

Figure 6. Schematic cycle of future bi-directional H_2 storage *via* direct hydrogenation of CO_2 to formate using a single bioreactor. Excess energy generated from renewable sources can be used to produce H_2 by water splitting. Captured CO_2 and H_2 are then converted into the LOHC formate by whole-cell catalysis in a single process unit. The same bioreactor system and biocatalyst are used for overnight hydrogen release from the LOHC system. The produced H_2 can ensure off-grid power supply in energy-lean times and CO_2 can be recycled and re-used in another H_2 storage cycle. The use of sunlight is just exemplary and different sources can be used to produce renewable electricity in dependence of the local conditions, therefore, making different scenarios more likely and more beneficial. All icons were taken from freepik from flaticon.com.

Figure 7. Scheme of a batch operated stirred-tank bioreactor with continuous gas supply. Whole- cells of *A. woodii* were used as biocatalysts in a batch operated stirred-tank bioreactor to reversibly convert H_2 and CO_2 into formic acid. During the day period (8 h) a gas composition of 45% H_2 + 45% CO_2 + 10% N_2 was fed into the bioreactor. During the night period (16 h) the gas composition was switched to 100% N_2. pHIRC, pH indicator recording

and control; pO_2IRC, pO_2 indicator recording and control; TIRC, temperature indicator recording and control; ORPIR, oxidation reduction potential indicator and recording; GC, gas chromatography.

Figures

Figure 1

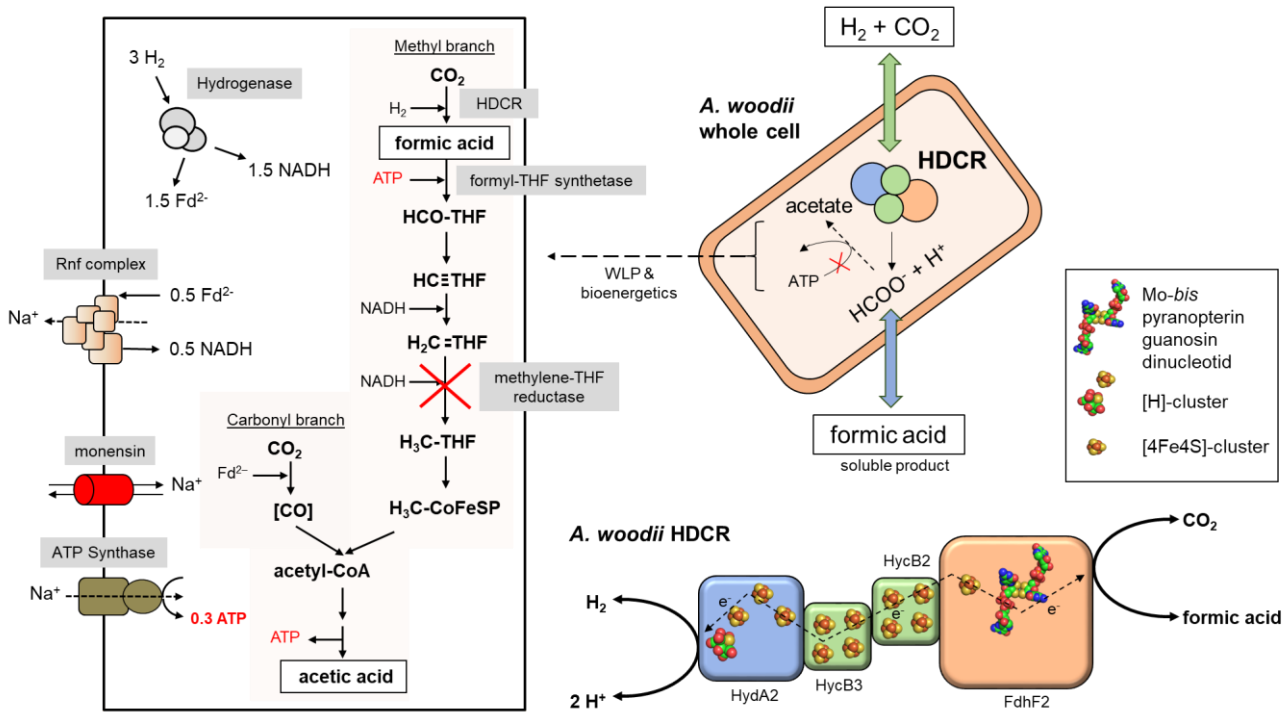


Figure 2

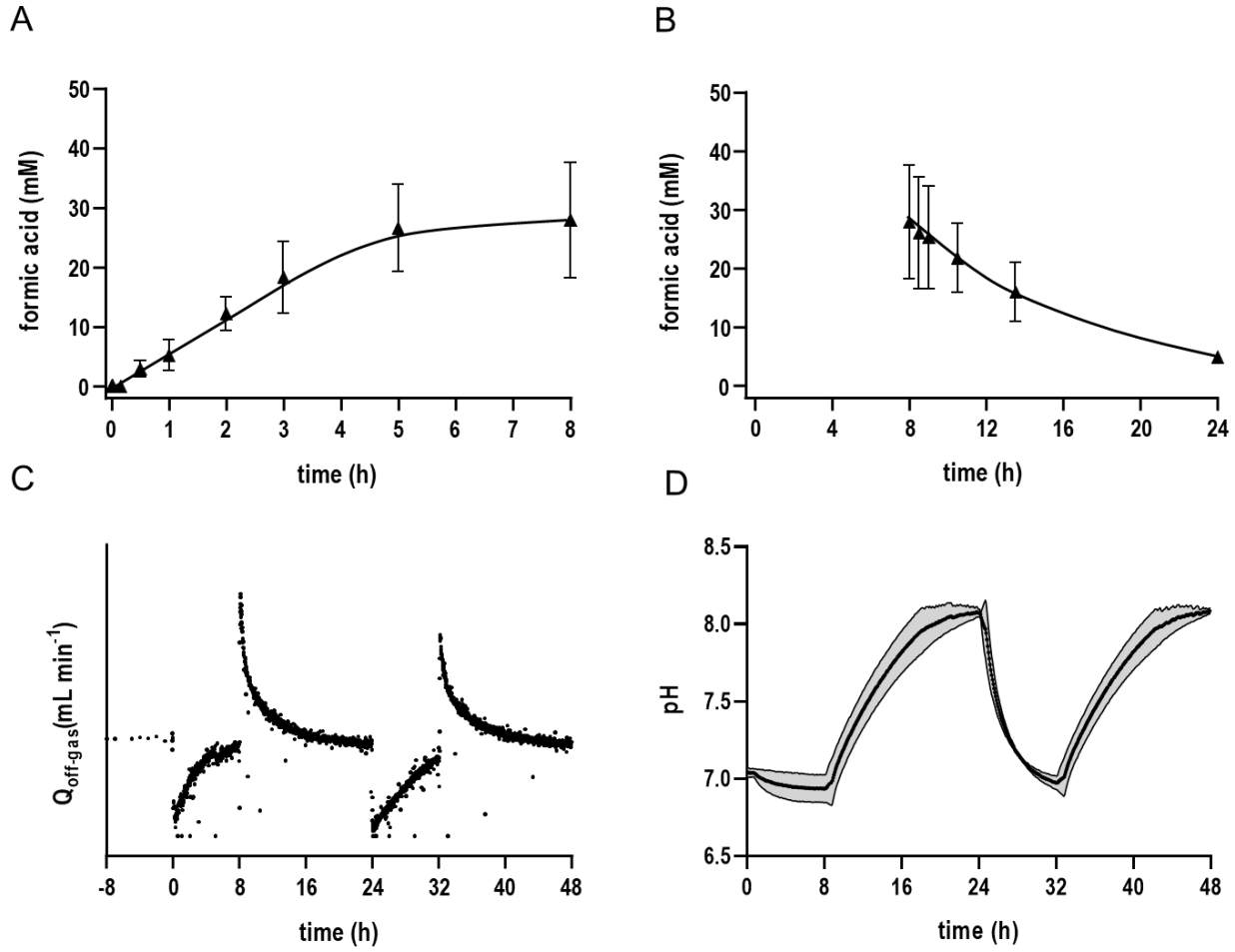

A
formic acid (mM)
time (h)
B
formic acid (mM)
time (h)
C
$Q_{off-gas}$(mL min^{-1})
time (h)
D
pH
time (h)

Figure 3

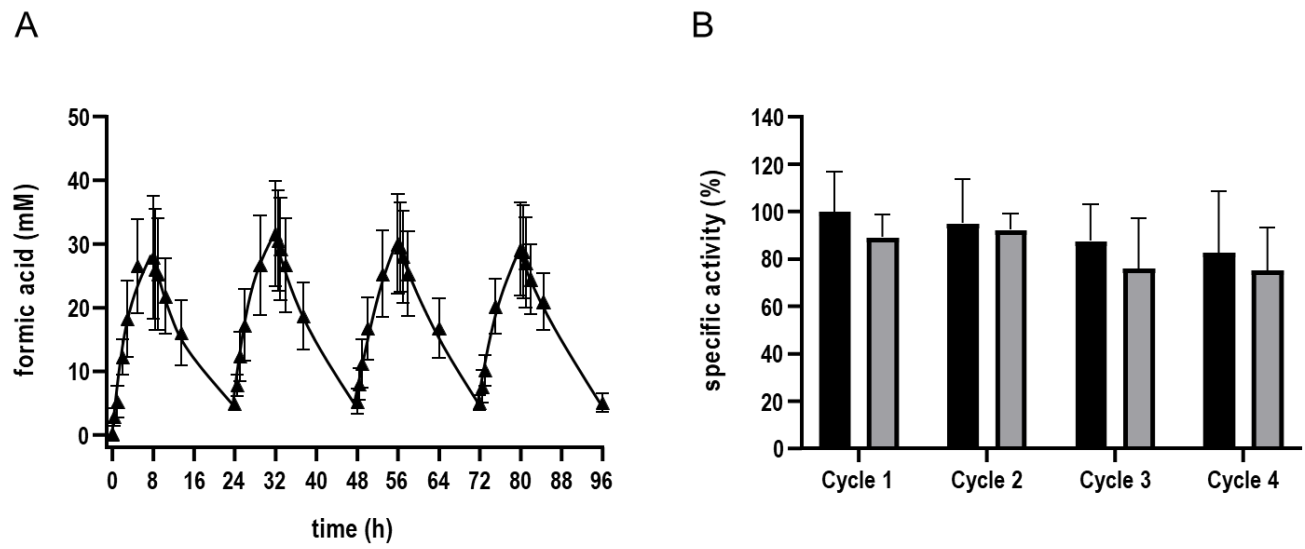

A
formic acid (mM)
50
40
30
20
10
0
0 8 16 24 32 40 48 56 64 72 80 88 96
time (h)
B
specific activity (%)
140
120
100
80
60
40
20
0
Cycle 1
Cycle 2
Cycle 3
Cycle 4

Figure 4

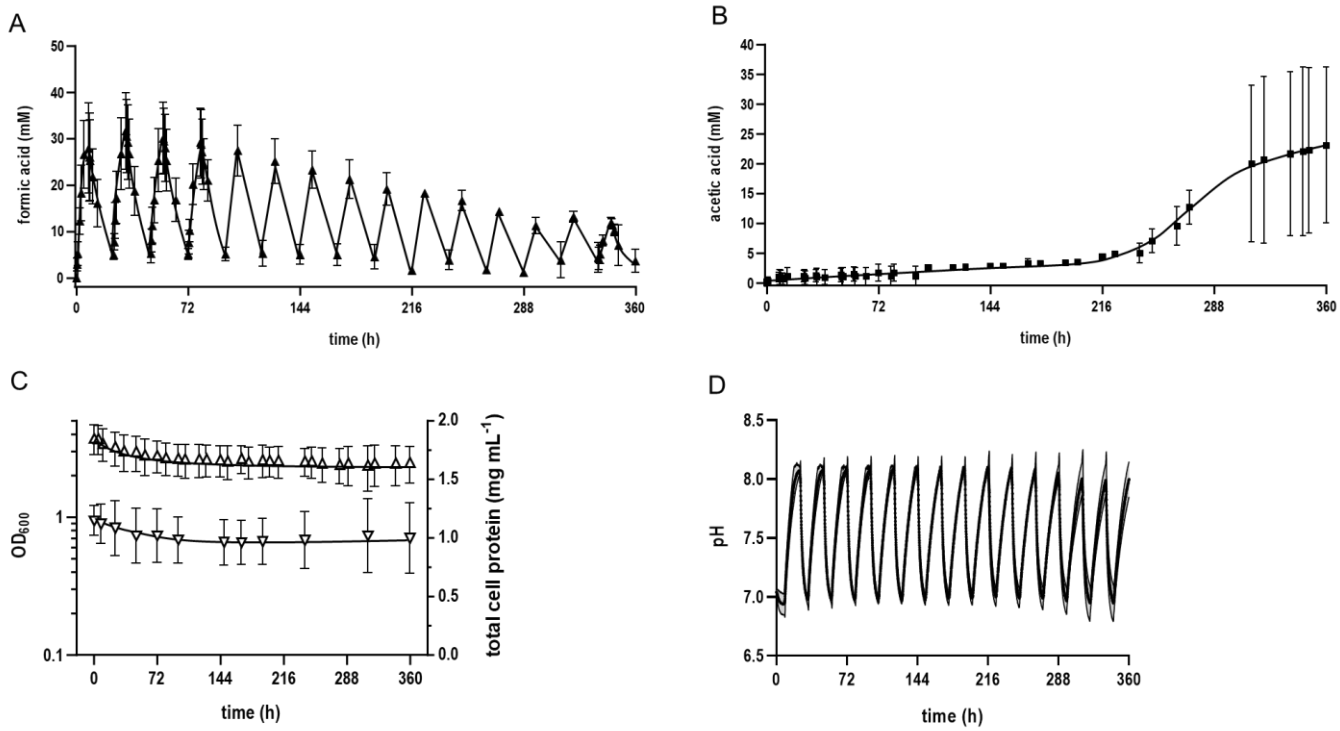

A
formic acid (mM)
time (h)
B
acetic acid (mM)
time (h)
C
OD600
total cell protein (mg mL-1)
time (h)
D
pH
time (h)

Figure 5

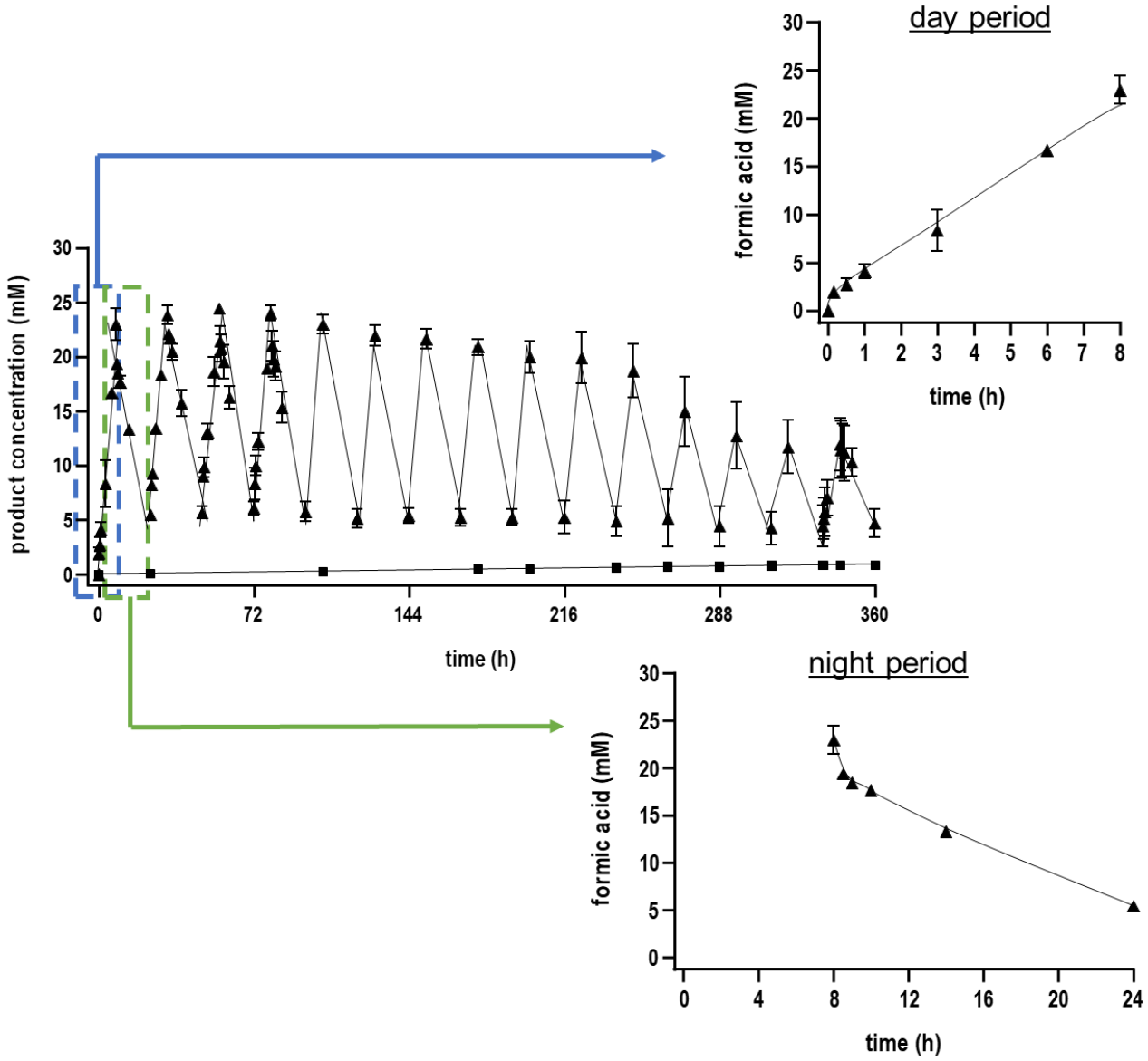

day period
formic acid (mM)
time (h)
product concentration (mM)
time (h)
night period
formic acid (mM)
time (h)

Figure 6

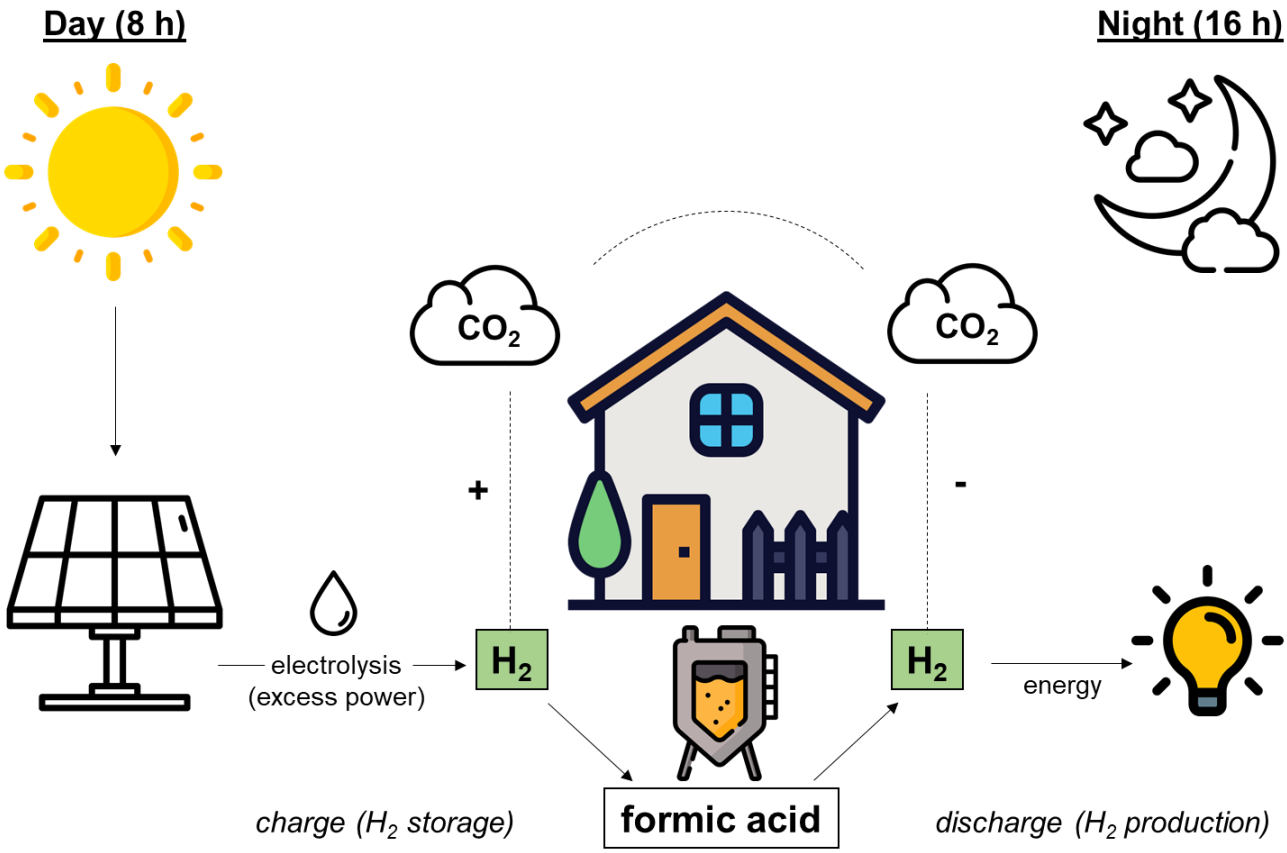
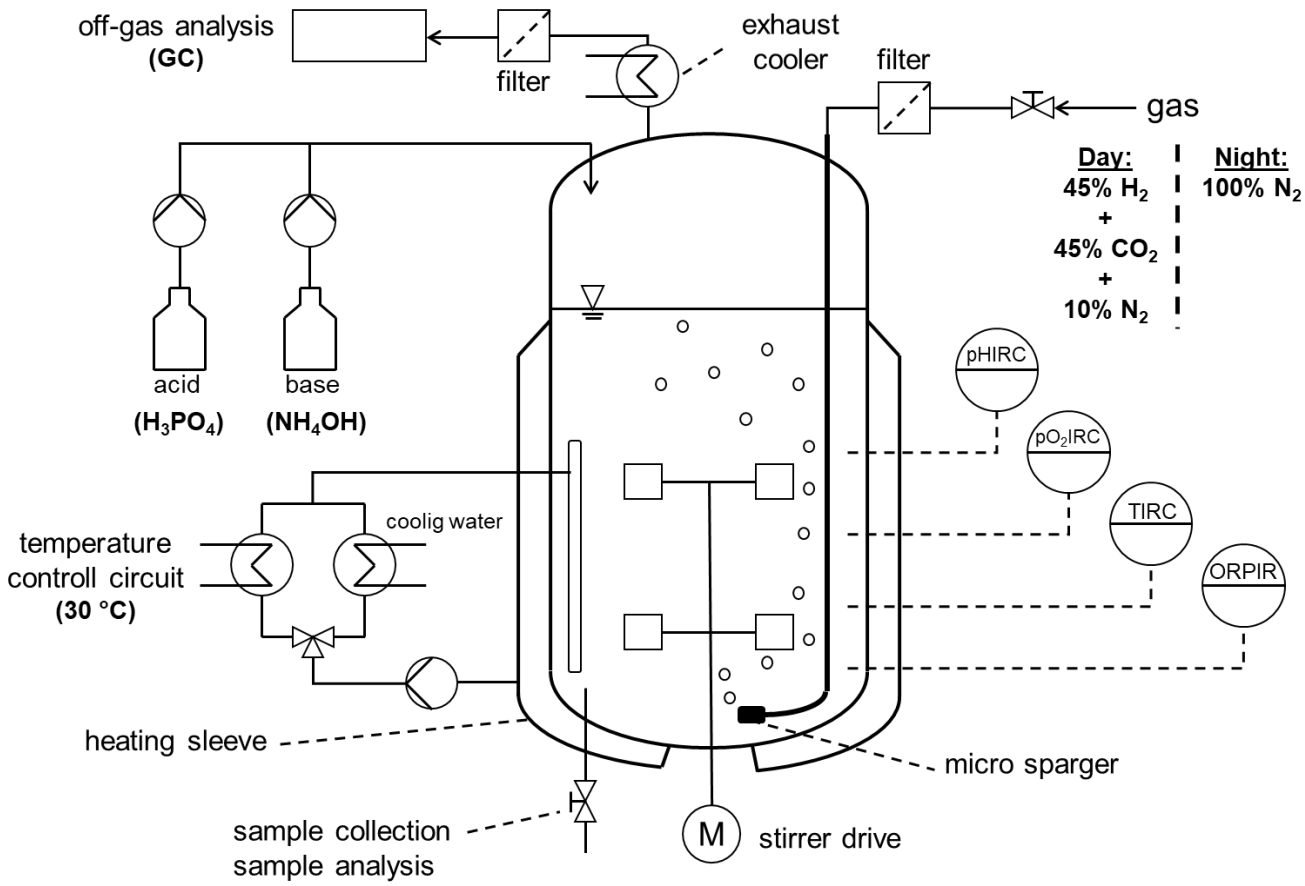

Day (8 h)
Night (16 h)
CO2
CO2
+
-
electrolysis
(excess power)
H2
H2
energy
formic acid
charge (H2 storage)
discharge (H2 production)

Figure 7


off-gas analysis
(GC)
filter
exhaust
cooler
filter
gas
Day:
45% H2
+
45% CO2
+
10% N2
Night:
100% N2
acid
(H3PO4)
base
(NH4OH)
pHIRC
pO2IRC
TIRC
ORPIR
coolig water
temperature
controll circuit
(30 °C)
heating sleeve
micro sparger
sample collection
sample analysis
M
stirrer drive

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767 **Supplementary data**

768 **Biological hydrogen storage and release through multiple cycles of bi-**

769 **directional hydrogenation of CO_2 to formic acid in a single process unit**

770

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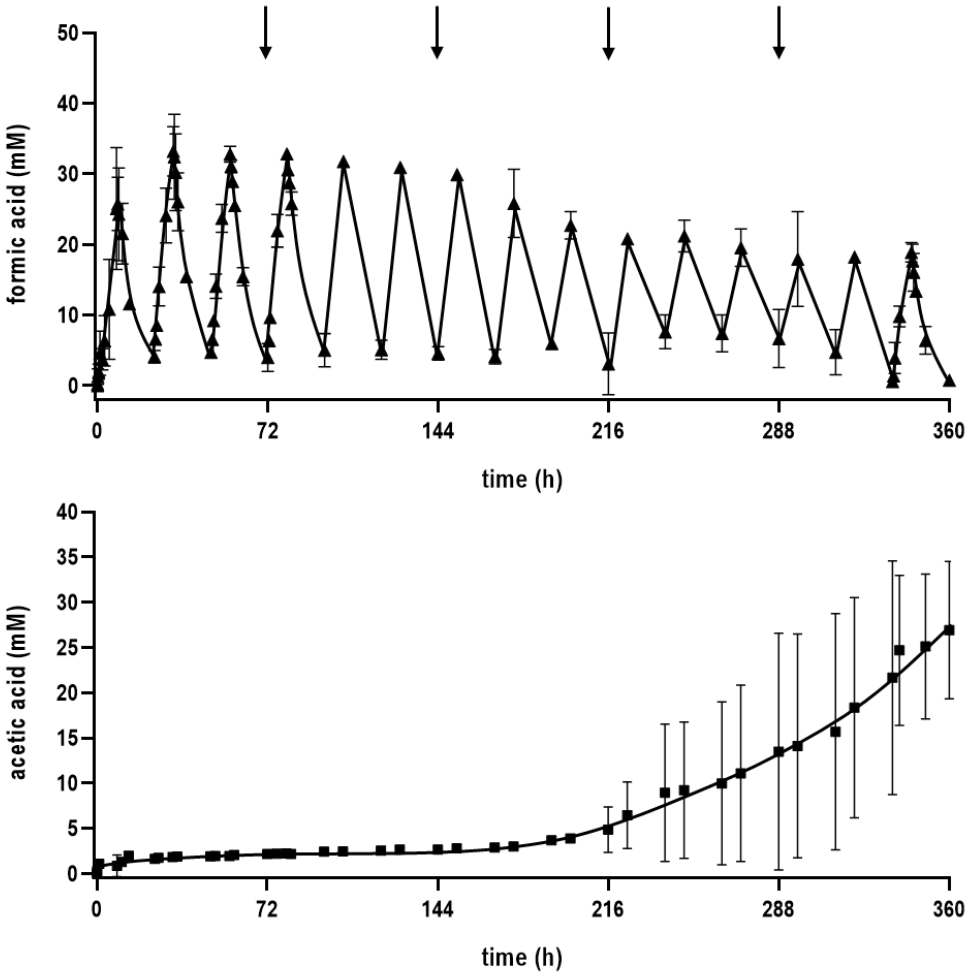

Figure S1. Long-term application of bi-directional hydrogenation of CO_2 to formic acid in a bioreactor with repetitive addition of monensin. Shown are A) 15 formic acid formation/oxidation cycles of the entire process (360 h) and B) corresponding side-product formation profile of acetic acid. Every 72 h, 15 µM of monensin was added to the bioreactor broth indicated by black arrows. All data points are mean ± SD, N = 3.

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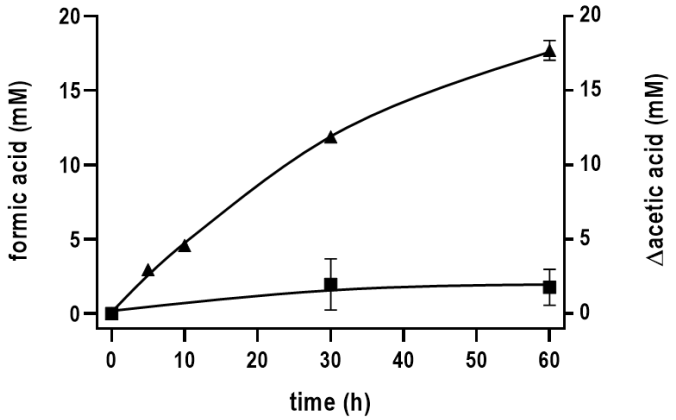

	serum bottle experiment (fresh buffer)	serum bottle experiment (re-used bioreactor buffer)
Specific formic acid production rate [mmol g^{-1} h^{-1}]	19	29

805
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807 **Figure S2. Uncoupling effect of used bioreactor buffer on fresh cell suspensions of *A. woodii*.**
808 Serum bottle experiments were performed using the bioreactor buffer (at t_{360}h) relieved from cells by
809 centrifugation without the addition of new monensin. Freshly prepared *A. woodii* cells were transferred
810 into the spent buffer to determine their ability to convert H_2 and CO_2 (80:20%, 1×10^5 Pa overpressure)
811 to formic acid. Due to residual acetic acid in the spent buffer, the difference of formed acetic acid (Δ
812 acetic acid) to the initial acetic acid is shown. Prior to the start of the experiment 27 mM of acetic acid
813 was present. Triangles, formic acid; squares, acetic acid. All data points are mean ± SD, N = 2.

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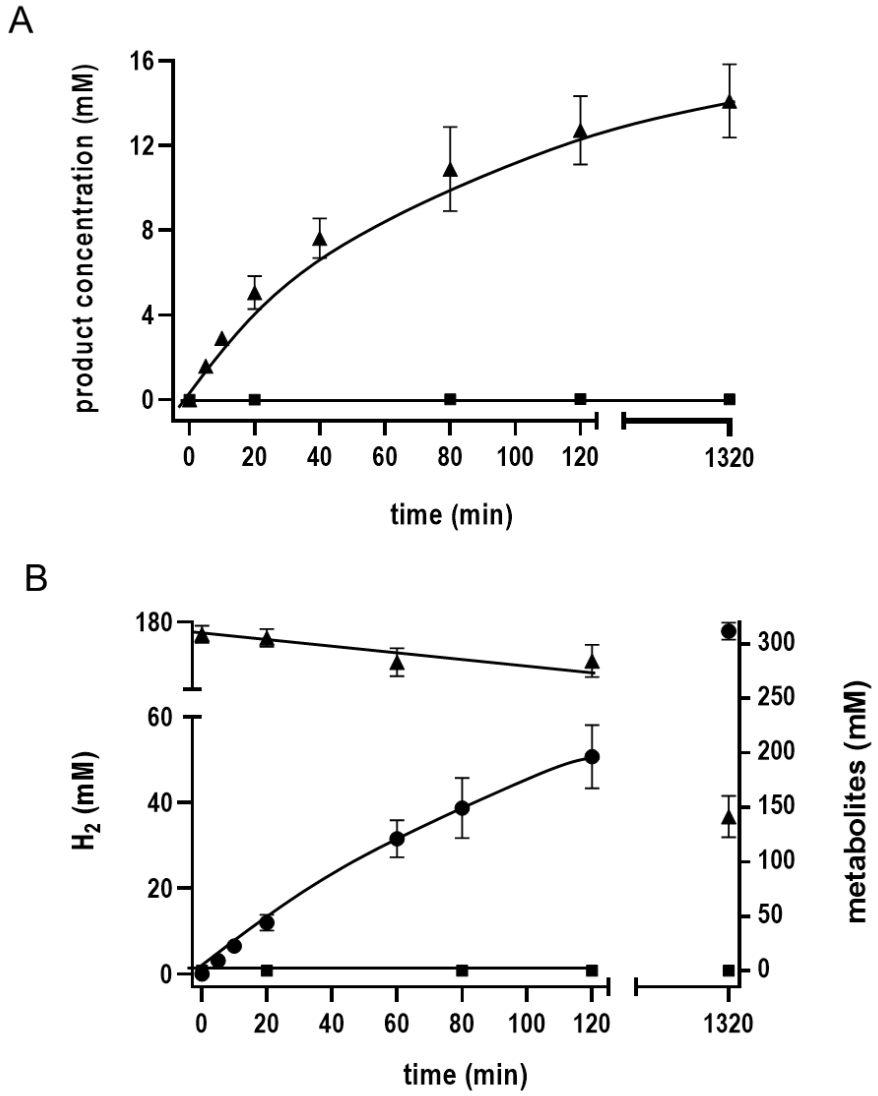

Figure S3. H_2-dependent CO_2 reduction and formate-driven H_2 production in resting cells of *A. woodii* Δ*metVF*. A) Resting cells (1 mg/mL) of *A. woodii* Δ*metVF* were resuspended in K-phosphate buffer (50 mM K-phosphate, 20 mM KCl, 2 mM DTE, pH 7.0) buffer with a H_2 + CO_2 (80:20%, 1 × 10^5 Pa overpressure) atmosphere or B) in the same K-phosphate buffer containing 300 mM sodium formate. The product and metabolite concentrations were determined. Triangles, formic acid; squares, acetic acid; circles, H_2. All data points are mean ± SD, N = 3.

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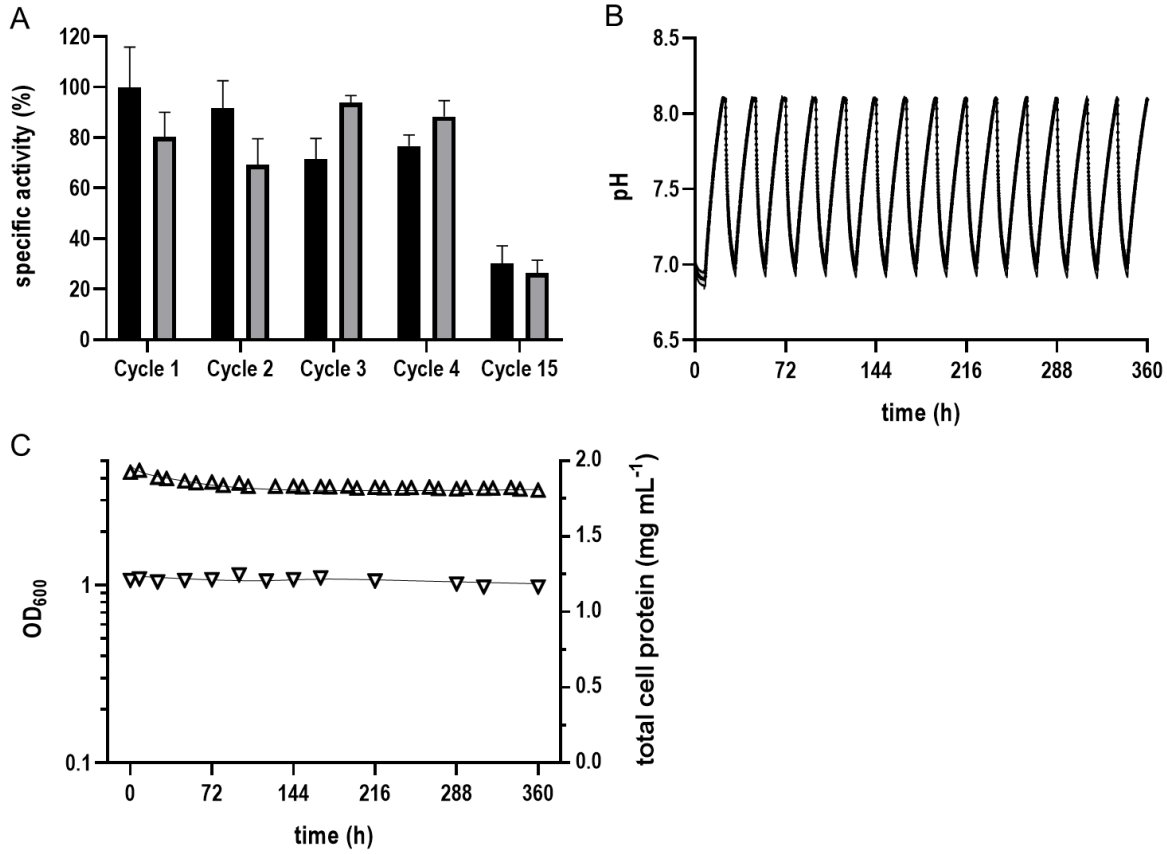

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831 **Figure S4. Overview over the catalytic activity, optical density and pH in a bioreactor with**
832 ***A. woodii* Δ*metVF* cells performing multiple cycles of bi-directional hydrogenation of CO_2 to**
833 **formic acid.** A) Specific activity of formic acid production (black bars) and formic acid oxidation (grey
834 bars). 100% of the activity corresponds to a formic acid production rate of 3.0 mmol g^{-1} h^{-1} and a formic
835 acid oxidation rate of 1.7 mmol g^{-1} h^{-1}. B) pH profile of the entire fermentation. 17 mM of phosphoric acid
836 and no base was needed as pH correcting agent in the entire process. C) Optical density at 600 nm and
837 total cell protein concentration. Empty triangles up, optical density at 600 nm; empty triangles down,
838 total cell protein concentration. All data points are mean ± SD, N = 3.

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