Efficient whole cell biocatalyst for formate-based hydrogen production

Patrick Kottenhahn, Kai Schuchmann and Volker Müller*

Abstract

Background: Molecular hydrogen (H₂) is an attractive future energy carrier to replace fossil fuels. Biologically and sustainably produced H₂ could contribute significantly to the future energy mix. However, biological H₂ production methods are faced with multiple barriers including substrate cost, low production rates, and low yields. The C1 compound formate is a promising substrate for biological H₂ production, as it can be produced itself from various sources including electrochemical reduction of CO₂ or from synthesis gas. Many microbes that can produce H₂ from formate have been isolated; however, in most cases H₂ production rates cannot compete with other H₂ production methods.

Results: We established a formate-based H₂ production method utilizing the acetogenic bacterium Acetobacterium woodii. This organism can use formate as sole energy and carbon source and possesses a novel enzyme complex, the hydrogen-dependent CO₂ reductase that catalyzes oxidation of formate to H₂ and CO₂. Cell suspensions reached specific formate-dependent H₂ production rates of 71 mmol g⁻¹ protein h⁻¹ (30.5 mmol g⁻¹ CDW h⁻¹) and maximum volumetric H₂ evolution rates of 79 mmol L⁻¹ h⁻¹. Using growing cells in a two-step closed batch fermentation, specific H₂ production rates reached 66 mmol g⁻¹ CDW h⁻¹ with a volumetric H₂ evolution rate of 7.9 mmol L⁻¹ h⁻¹. Acetate was the major side product that decreased the H₂ yield. We demonstrate that inhibition of the energy metabolism by addition of a sodium ionophore is suitable to completely abolish acetate formation. Under these conditions, yields up to 1 mol H₂ per mol formate were achieved. The same ionophore can be used in cultures utilizing formate as specific switch from a growing phase to a H₂ production phase.

Conclusions: Acetobacterium woodii reached one of the highest formate-dependent specific H₂ productivity rates at ambient temperatures reported so far for an organism without genetic modification and converted the substrate exclusively to H₂. This makes this organism a very promising candidate for sustainable H₂ production and, because of the reversibility of the A. woodii enzyme, also a candidate for reversible H₂ storage.

Keywords: Hydrogen production, Biohydrogen, Acetobacterium woodii, Formate dehydrogenase, Hydrogenase

Background

Fossil fuel limitation and increasing atmospheric CO₂ concentrations necessitate alternative energy carriers. Molecular hydrogen (H₂) is an attractive carbon-free alternative that can be converted to energy without CO₂ emission. It can be used as energy carrier for mobile applications (i.e., fuel cell powered vehicles) or as an intermediate energy storage system to store excess electrical energy that is produced in peak times from renewable sources [1]. Currently, H₂ is produced mainly from fossil fuels by steam reforming and thus unsustainable and environmentally harmful [2]. Hence, new H₂ production methods are required.

Biologically produced H₂ provides a promising alternative for a sustainable H₂-based energy economy. H₂ production by biological systems can generally be classified into four different mechanisms: direct and indirect biophotolysis, photofermentation, and dark fermentation [3]. From these processes, the latter mechanism has so far the highest H₂ evolution rates (HER). However, the major drawback of dark fermentations, e.g., from glucose, is the low H₂ yield per substrate consumed and the limitations of agricultural production of the substrate [4].
recently considered alternative substrate is formic acid/formate that could be produced from electrochemical reduction of CO₂ or from synthesis gas, a very flexible substrate that can derive as by-product from steel mills or from waste gasification [5–7]. Conversion of formate to H₂ proceeds according to the reaction:

\[ \text{HCOO}^- + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}_2 \quad \Delta G^\circ = +1.3 \text{kJ mol}^{-1}. \]

Microbial formate oxidation is catalyzed by multiple enzyme systems. Organisms such as some enterobacteria use a membrane-bound formate-hydrogen lyase system composed of membrane-associated hydrogenase and formate dehydrogenase subunits [8, 9]. Clostridiaceae or archaia such as Methanococcus can produce H₂ from formate by the action of separate cytoplasmic formate dehydrogenases and hydrogenases [10]. The observed HERs for these organisms are typically very low and do not reach the levels for H₂ production from other feedstocks [4]. One exception is the recently characterized organism Thermococcus onnurineus. This organism requires 80 °C for growth and formate-dependent H₂ formation reached HERs that outcompete other dark fermentations for the first time [11, 12]. H₂ production in this organism depends on a membrane-bound enzyme complex of formate dehydrogenase, hydrogenase, and Na^{+}/H^{+} antiporter subunits that couples H₂ formation to formate oxidation as well as energy conservation [13, 14].

A new enzyme of the bacterial formate metabolism has been discovered recently in the strictly anaerobic bacterium Acetobacterium woodii [15]. The enzyme named hydrogen-dependent CO₂ reductase (HDCR) was the first described soluble enzyme complex that reversibly catalyzes the reduction of CO₂ to formate with H₂ as electron donor. CO₂ reduction is catalyzed at ambient conditions with rates far superior to chemical catalysis [15–17]. Therefore, it could not only be used for H₂ production but, depending on the application, for H₂ storage as well. In the form of formate, the explosive gas could be stored and handled much easier and with an increased volumetric energy density [18]. H₂-dependent CO₂ reduction to formate by the HDCR has also been shown to be very efficient in whole cell catalysis with A. woodii [15]. However, the reverse reaction has not been addressed in detail so far.

In the present report, we describe the first characterization of formate-based H₂ production with an organism harboring an HDCR complex. The results show that A. woodii has H₂ production rates from formate of 66 mmol H₂ gₐCDW h⁻¹ at ambient temperatures that are among the highest reported so far for an organism without genetic modification. Therefore, A. woodii is an efficient catalyst for H₂ production and, considering the reversibility of the whole cell system, a potent catalyst for reversible H₂ storage. In addition, A. woodii can grow with formate as sole carbon and energy source making it possible to produce cell mass and H₂ with the same substrate.

**Results**

**H₂ production with resting cells**

The acetogenic bacterium A. woodii can utilize, among others, H₂ + CO₂, formate, or monosaccharides such as fructose as substrates for growth. In all three cases, acetate (or acetate + CO₂ in the case of formate) is the major end product [19, 20]. Recently, we could show that the addition of the sodium ionophore ETH2120 (sodium ionophore III) led to a complete inhibition of acetate formation from H₂ + CO₂ and the two gases were completely converted to formate [15]. This opened the possibility to utilize A. woodii as catalyst for H₂ storage. The hydrogen-dependent CO₂ reduction activity could be addressed to a novel enzyme complex of a formate dehydrogenase and hydrogenase, named HDCR. Experiments with the purified enzyme showed that the catalyzed reaction proceeds with almost the same rate in the reverse reaction as well, making A. woodii a potential candidate for formate-based H₂ production [15]. In this study, we analyzed this potential using whole cells of A. woodii. First, we grew the organism with fructose, a substrate to reach high cell densities relatively quickly (doubling time tₜ = 4.7 h compared to 11 h with formate as substrate), harvested the cells, and incubated them in reaction buffer at a protein concentration of 1 mg mL⁻¹ (corresponding to 2.3 mₐCDW mL⁻¹). After addition of sodium formate to a final concentration of 300 mM, the cells produced H₂ with an initial specific H₂ productivity (qH₂) of 52.2 ± 3 mmol g⁻¹protein h⁻¹ (22.5 mmol g⁻¹CDW h⁻¹) (Fig. 1). 0.6 mmol H₂ was produced from 2.14 mmol formate consumed leading to a yield of H₂ consumed per substrate consumed (Y(H₂/formate)) of 0.28 mol mol⁻¹. It was surprising to observe these high H₂ production rates since H₂ is typically no major product from cells growing on formate; however, Y(H₂/formate) was significantly decreased by the high amount of 0.45 mmol acetate produced alongside H₂. The produced acetate results from the assimilation of CO₂ or formate via the Wood–Ljungdahl pathway for autotrophic CO₂ fixation of A. woodii [20, 21]. As shown recently for the reverse reaction of formate formation from H₂ + CO₂, we tried to decrease acetate formation by addition of the sodium ionophore ETH2120. Acetate formation in A. woodii is coupled to a sodium ion gradient for energy conservation across the cytoplasmic membrane that can be specifically diminished by the sodium ionophore. In the presence of 30 μM ETH2120, the final amount of H₂ produced increased to 1.15 mmol from 1.68 mmol
formate consumed. At the same time, acetate formation decreased to a final amount of 0.17 mmol acetate. In summary, addition of ETH2120 increased \( Y_{(\text{H}_2/\text{formate})} \) to 0.68 mol mol\(^{-1}\). An alternative approach to the inhibition of acetate formation by ETH2120 is the depletion of the cells for sodium ions. In the CO\(_2\) reduction direction, sodium ion depletion showed the same effect on formate formation as ETH2120 but comes with much less cost for the fermentation. To test this for \( \text{H}_2 \) production, we added potassium formate instead of sodium formate. Initial \( \text{qH}_2 \) was identical to ETH2120 inhibited cells and the amount of \( \text{H}_2 \) produced was more than double compared to the control (Fig. 1). However, after 100 min we observed reassimilation of \( \text{H}_2 \) which decreased the product significantly. We interpret this result as an incomplete inhibition of sodium-dependent acetate formation due to sodium ion contamination in the potassium formate, which is 0.5% in \( \geq 99.0\% \) potassium formate used.

In the initial experiments, we used fructose-grown cells as catalysts. An advantage of \textit{A. woodii} is the wide range of possible growth substrates. Depending on the process and available substrate, cultivation of the cells on \( \text{H}_2 + \text{CO}_2 \) or directly on formate might be advantageous. \( \text{qH}_2 \) in cells grown on \( \text{H}_2 + \text{CO}_2 \) was almost identical to formate-grown cells; however, with 21 mmol g\(^{-1}\)protein h\(^{-1}\) only 67% of the \( \text{qH}_2 \) of fructose-grown cells was reached (Fig. 2a). pH dependency showed a decrease in \( \text{qH}_2 \) with increasing pH within the tested pH range of 6–9 (Fig. 2b). Highest \( \text{qH}_2 \) was observed at a pH of 6 with 37 mmol g\(^{-1}\)protein h\(^{-1}\) but a decrease in \( \text{qH}_2 \) (Fig. 3). Maximum specific \( \text{H}_2 \) production of 71 mmol g\(^{-1}\)protein h\(^{-1}\) (30.5 mmol g\(^{-1}\)CDW h\(^{-1}\)) was observed at a protein concentration of 0.5 mg mL\(^{-1}\). At the same time, increasing cell densities led to higher accumulation of acetate and less production of \( \text{H}_2 \), meaning that ETH2120 inhibition decreases at higher cell densities. In the next experiment, we tested inhibition of \( \text{H}_2 \) production by increased formate concentrations. We tested formate concentrations from 25 to 600 mM. Within this range, initial \( \text{H}_2 \) production rates did not change, with similar HERs up to 600 mM.

![Fig. 1](image1.png) **Fig. 1** \( \text{H}_2 \) production from formate by resting cells of \textit{A. woodii}. Cells were grown with 20 mM fructose, harvested in the exponential growth phase, and suspended in buffer (50 mM imidazole, 20 mM KCl, 20 mM MgSO\(_4\), 4 mM DTE, pH 7) to a final protein concentration of 1 mg mL\(^{-1}\) (corresponding to a CDW of 2.3 g L\(^{-1}\)) in anoxic serum bottles (gas phase 100% N\(_2\)). The bottles were incubated in a shaking water bath at 30 °C. At the beginning of the experiment, sodium formate, potassium formate, ETH2120, NaCl, and ethanol (solvent of ETH2120 as negative control) were added as indicated. Triangles down, 300 mM sodium formate, 20 mM NaCl, diamonds, 300 mM sodium formate, 20.5 mM ethanol, 20 mM NaCl, circles, 100 mM K-formate; triangles up, 100 mM sodium formate, 20.5 mM ethanol, 20 mM NaCl.

![Fig. 2](image2.png) **Fig. 2** Influence of the growth substrate (a) and pH (b) on \( \text{H}_2 \) production. a Cells were grown with 20 mM fructose (squares), 2 atm. \( \text{H}_2 + \text{CO}_2 \) (80:20 v:v), triangles, or 100 mM sodium formate (circles). The experiment was performed as described for Fig. 1 using 300 mM sodium formate, 30 µM ETH2120, and 20 mM NaCl. b Fructose-grown cells were suspended in buffer (25 mM MES, 25 mM Tris, 25 mM MOPS, 25 mM CHES, 20 mM KCl, 20 mM MgSO\(_4\), 4 mM DTE, 20 mM NaCl) at pH 6 (circles), pH 7 (squares), pH 8 (triangles), pH 9 (diamonds). The experiment was started by the addition of sodium formate to a final concentration of 300 mM.
sodium formate tested, demonstrating that formate is not inhibiting the catalyst even at high concentrations. Final H₂ concentrations increased with increasing initial formate concentrations (Fig. 4).

**H₂ production in batch fermentation**

The experiments described with resting cells showed that *A. woodii* is a promising catalyst for formate-dependent H₂ production at ambient temperatures. For these experiments, cells were grown, harvested under anoxic conditions, and incubated in anoxic reaction buffer. This procedure is labor-intensive and requires sophisticated techniques to maintain anoxic conditions. To optimize this procedure, we wanted to abolish the medium exchange and establish H₂ production directly in closed batch fermentation. Therefore, cells were grown with 20 mM fructose as substrate to mid-exponential growth phase (*t₀* = 4.7 h). At this point, formate was added with or without 30 µM ETH2120. Addition of the sodium ionophore led to an immediate growth arrest, whereas addition of formate alone had no effect on the growth rate (data not shown). After addition of formate, H₂ was produced with a HER of 7.9 mmol L⁻¹ h⁻¹ and a qH₂ of 65.9 mmol g⁻¹ CDW h⁻¹ (Fig. 5a). Without addition of ETH2120, the H₂ evolution rate was 4.5 mmol L⁻¹ h⁻¹ initially, but decreased significantly after 1 h. After addition of formate, acetate was still produced alongside H₂ when no ETH2120 was added (78.4 mmol L⁻¹ after 23 h) (Fig. 5b). In contrast, cells in the presence of ETH2120 did produce acetate only in marginal amounts as side product (0.3 mmol L⁻¹).

Next, we wanted to further optimize the system by generating cell mass directly from formate as substrate, therefore testing a system independent on carbohydrates and using formate for growth and H₂ production. Therefore, *A. woodii* was grown with 100 mM sodium formate (*t₀* = 11 h). These cultures already produced small amounts of H₂ during growth (around 2 mmol L⁻¹ before the switch to the production phase). To switch from growth to production phase, 15 mmol additional sodium formate (corresponding to 300 mM) was added. As in the case for fructose-grown cells, H₂ was produced immediately after addition of ETH2120 with a HER of 1.2 mmol L⁻¹ h⁻¹ and a specific production rate of 19 mmol g⁻¹ CDW h⁻¹. At the end of the fermentation, 25.1 mmol L⁻¹ H₂ was produced from 36.2 mmol L⁻¹ formate consumed when ETH2120 was added (*Y(H₂/formate) = 0.69 mol H₂ mol formate⁻¹*) (Fig. 6). Additional acetate was not produced after the addition of...
the ionophore. Without ETH2120, 18.6 mmol L\(^{-1}\) \(\text{H}_2\) and 17.0 mmol L\(^{-1}\) acetate were produced from 80.5 mmol L\(^{-1}\) formate. This results in a lower \(Y_{(\text{H}_2/\text{formate})}\) of 0.23 mol \(\text{H}_2\) mol\(^{-1}\) formate. In comparison to fructose-grown cells, the final amount of \(\text{H}_2\) produced was much lower, even though the same amounts of formate were supplied. This could be an effect of the conditions established by the cells during the growth phase, e.g., growth on fructose leads to an acidification of the medium, whereas growth on formate increases the pH. Further studies need to address the optimal media composition depending on the substrate used for the growth phase. Nevertheless, the experiments with growing cells demonstrate in each case that the metabolism of \(A.\ woodii\) can be specifically switched from growth and acetate formation to \(\text{H}_2\) production by interfering with the sodium ion gradient across the membrane and thus dramatically increasing the yield coefficient \(Y_{(\text{H}_2/\text{formate})}\).

**Discussion**

In this study, we examined the \(\text{H}_2\) production capacity of the anaerobic bacterium \(A.\ woodii\). This organism is a promising candidate for formate-based \(\text{H}_2\) production due to the recently identified reversible hydrogen-dependent \(\text{CO}_2\) reductase complex (HDCR), an enzyme able to reversibly reduce \(\text{CO}_2\) to formate with \(\text{H}_2\) as electron donor with so far exceptional catalysis rates. This enzyme catalyzes the first step in the Wood–Ljungdahl pathway, the pathway for \(\text{CO}_2\) fixation and energy conservation in this organism that has a wide substrate spectrum for growth ranging from monosaccharides, mono- and diols, \(\text{H}_2+\text{CO}_2\), and, especially important in this context, formate [20, 22]. However, without modification this organism produces mainly acetate as end product from most substrates [19]. As shown in this study, cells growing on formate produce
only very little H2. Addition of high concentrations of formate to cells growing on formate or fructose led to immediate H2 production; however, H2 production rapidly slowed down and acetate was still produced. A. woodii can use H2 + CO2 for growth and acetate formation, and therefore this result is not unexpected since H2 + CO2 is the product of formate oxidation by the HDCR complex [15] (Fig. 7). The HDCR is not connected to the metabolism by electron carriers such as NAD+/NADH and it seems, from the results here, that it catalyzes formate oxidation unregulated if the formate concentration increases suddenly even if this provides no advantage for the cell. The independence of the HDCR from other metabolic processes makes it feasible to inhibit the major pathways for substrate conversion and growth by still retaining HDCR activity. As shown before, a very specific target for inhibiting the metabolism is the sodium ion gradient across the membrane that is built up during acetate formation and is necessary for energy conservation and growth. We assume that formate is imported by the putative formate transporter FdhC2 (Awo_c08050) whose gene is in close proximity to the HDCR gene cluster. FdhC2 could couple formate import to the proton gradient.

![Fig. 7 Model of formate-dependent H2 production with A. woodii. Formate can be used by A. woodii as carbon and energy source. Formate could be taken up by the putative formate transporter FdhC2. It is then bound to the cofactor tetrahydrofolate (THF) and reduced to a cofactor-bound methyl group. To generate the required reducing equivalents, part of the formate is oxidized to H2 + CO2 catalyzed by the HDCR. H2 is further oxidized by an electron bifurcating hydrogenase and CO2 is reduced to carbon monoxide (CO) which is fused to the methyl group resulting in the formation of acetyl-CoA and subsequently acetate. The Rnf complex generates a sodium ion gradient driven by the electron transfer from reduced ferredoxin to NAD+ that is then used by a sodium ion-dependent ATP synthase to generate ATP. The sodium ionophore ETH2120 collapses the membrane potential which inhibits ATP formation and could lead to ATP hydrolysis by the now uncoupled ATP synthase. This in turn inhibits conversion of formate to acetate because the first reaction is ATP dependent, resulting in sole conversion into H2 + CO2. CHO-THF, formyl-THF; CH-THF, methenyl-THF; CH2-THF, methylene-THF; CH3-THF, methyl-THF; CoFeSP, Corrinoid iron-sulfur protein; Fd, ferredoxin]
due to the similarity of the primary structure to the formate transporter FocA of Escherichia coli or Salmonella typhimurium [23, 24] (Fig. 7). In the next step, formate is reduced via the Wood–Ljungdahl pathway and the necessary reducing equivalents for this process are generated by oxidizing part of the formate via the HDCR. Addition of the ionophore should inhibit the reductive formate pathway without influencing the HDCR activity. This should stop acetate formation and result in the accumulation of hydrogen. At the same time, collapsing the membrane potential should be advantageous for uptake of the negatively charged formate molecule. As demonstrated in this study, neutralizing this gradient by adding a sodium ionophore (we used ETH2120) proved to be an effective switch from acetate to H₂ production if formate is provided as substrate. It was possible to completely turn off acetate and biomass formation and reach yields (YH₂/formate) of 100%. Comparing the total amount of formate consumed with and without ETH2120 showed that formate utilization stopped earlier when cells were inhibited by the ionophore. However, in this case formate was completely converted to H₂ and this reaction is slightly endergonic (ΔG° = +1.3 kJ mol⁻¹). The equilibrium constant of this reaction is therefore only 0.6. In the absence of the sodium ionophore, formate is mainly converted to acetate. This reaction is highly exergonic (ΔG° = −110 kJ mol⁻¹ [25]) explaining the increased formate consumption. The thermodynamics of formate-based H₂ production might seem as a disadvantage; however, the reaction close to the thermodynamic equilibrium allows simple adjustment of the direction of the reaction without additional energy supply. H₂ can be produced from formate or stored in the form of formate without the input of much energy, a prerequisite for a reversible H₂ storage material. Another very attractive property of formate-based H₂ production is the complete conversion of the substrate to gaseous products. The substrate could be continuously supplied to the fermentation in the form of formic acid (at the same time providing a constant pH) resulting in the formation of H₂ + CO₂ only, circumventing any inhibition by dissolved products. Future studies need to address the long-term stability of the ionophore inhibited A. woodii system in such a continuous and pH-controlled fermentation. The price of the ionophore ETH2120 is a disadvantage considering the economic feasibility of the process. We used this compound to specifically study the effect of collapsing the membrane potential. However, with the gained knowledge that it is only necessary to inhibit the metabolism at any point it should be possible to identify other more inexpensive inhibitors. Alternatively, with the advent of genetic tools in acetogenic bacteria, mutations could be introduced to block key steps of the metabolism that stops acetate production and keeps the HDCR functional.

In summary, A. woodii and the corresponding enzyme HDCR turned out to be a very promising catalyst for formate-based H₂ production and storage, as it operates at ambient temperatures with very similar reaction rates in the forward and reverse reaction. The specific H₂ productivity (qH₂) from formate observed with whole cells of A. woodii (66 mmol g⁻¹ CDW h⁻¹) is among the highest reported at ambient temperatures for an organism without genetic modification, highlighting the H₂ production potential of this organism [4, 5]. Much higher qH₂ are reported at 80 °C utilizing the thermophile T. onnurineus [12]. This organism uses a different enzyme system for formate-based H₂ production, namely a membrane-bound enzyme complex consisting of a hydrogenase, formate dehydrogenase, and Na⁺/H⁺ antiporter subunits [13]. If T. onnurineus can also catalyze, the reverse reaction has not been shown so far. At ambient temperatures, the best results have been achieved using E. coli or other Enterobacteria such as Citrobacter in non-growing conditions [26]. Without genetic modification, E. coli has typically a low formate-dependent H₂ productivity. However, by metabolic engineering including overexpression of the formate-hydrogen lyase enzyme, deletion of inhibitory pathways such as uptake hydrogenases and process optimization, the H₂ productivity could be increased dramatically (144.2 mmol g⁻¹ h⁻¹ when products was removed continuously from the medium) [27, 28]. On the other hand, E. coli is inhibited by low concentrations of approximately 50 mM formate. This was addressed by using agar-embedded immobilized cells that were able to tolerate higher concentrations [29].

Conclusions
This study demonstrated that A. woodii is an efficient H₂ producer from the very flexible and inexpensive substrate formate. Together with our recent study on the reverse reaction, the results show that A. woodii can also be used as whole cell biocatalyst for the reversible storage of H₂, by binding it to CO₂ to produce formate and vice versa. Future studies need to address the process in a larger scale and in a continuous fermentation to analyze the stability and investigate alternatives to the expensive inhibitor ETH2120. Since any inhibition of the metabolism that does not affect the HDCR should be sufficient, other inhibitors or a genetic modification of the organism should be easy to find to improve the cost of the process.
Methods

Growth of *A. woodii*

*Acetobacterium woodii* (DSM 1030) was cultivated at 30 °C under anaerobic conditions. The defined carbonate buffered medium was prepared as described [30]. For closed batch fermentation, defined phosphate buffered medium was used and prepared as described [31]. Fructose (20 mM), formate (100 mM), or H₂ + CO₂ (80:20 [v/v]) was used as substrates. Growth was followed by measuring the optical density at 600 nm (OD₆₀₀).

Preparation of cell suspensions

The medium and all buffers were prepared using the anaerobic techniques described [32, 33]. All preparation steps were performed under strictly anaerobic conditions at room temperature in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) filled with 95–98% N₂ and 2–5% H₂ as described [30]. *A. woodii* (DSM 1030) was grown in carbonate buffered medium till late exponential phase, harvested by centrifugation, and washed two times with imidazole buffer (50 mM imidazole–HCl, 20 mM MgSO₄, 20 mM KCl, 4 mM DTE, 1 mg L⁻¹ resazurin, pH 7.0). Cells were resuspended in imidazole buffer and transferred to Hungate tubes. The protein concentration of the cell suspension was determined as described previously [34]. To remove remaining H₂ from the Hungate tube, the gas phase of the cell suspension was changed to N₂ and the cells were stored on ice until use. For the experiments, the cells were suspended in the same buffer to a concentration of 1 mg mL⁻¹ in 115-mL glass bottles. The bottles contained a final volume of 10 mL buffer under an N₂ atmosphere and were incubated at 30 °C in a shaking water bath. Samples for substrate/product determination were taken with a syringe, cells were removed by centrifugation (15,000 g, 2 min), and the supernatant was stored at −20 °C until further analysis. For determination of H₂, gas samples were taken with a gas tight syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) and analyzed by gas chromatography.

Closed batch fermentations

*Acetobacterium woodii* (DSM 1030) was grown at 30 °C in 50 mL phosphate buffered medium in 115-mL glass bottles containing an initial gas phase of 100% N₂. Samples for substrate/product determination were taken with a syringe and handled as described for the cell suspension experiments.

Determination of hydrogen, formate, and acetate

For determination of H₂, the gas samples were analyzed by gas chromatography on a Clarus 580 GC (PerkinElmer, Waltham, MA, USA). The samples were injected at 100 °C with nitrogen as carrier gas with a head pressure of 400 kPa and a split flow of 30 mL min⁻¹. The oven was kept at 40 °C and H₂ was determined with a thermal conductivity detector at 100 °C. The peak areas were proportional to the concentration of H₂ and calibrated with standard curves.

The concentration of formate was determined with an enzymatic assay using the formate dehydrogenase from *Candida boidinii* (Sigma-Aldrich, Munich, Germany). The assay contained in addition to the sample 1 U of enzyme in 50 mM potassium phosphate buffer (pH 7.5) and 2 mM NAD⁺. Formation of NADH was measured photometrically at 340 nm. Sodium formate was used for preparation of standard curves.

Acetate was measured using a commercially available enzymatic assay kit from R-Biopharm (Darmstadt, Germany).

Chemicals

All chemicals were supplied by Sigma-Aldrich Chemie GmbH (Munich, Germany) and Carl Roth GmbH & Co KG (Karlsruhe, Germany). All gases were supplied by Praxair (Düsseldorf, Germany).

Authors’ contributions

VM and KS designed and supervised the research, analyzed the data, and wrote the manuscript. PK performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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