Formate-driven H₂ production by whole cells of *Thermoanaerobacter kivui*

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**Abstract**

**Background:** In times of global warming there is an urgent need to replace fossil fuel-based energy vectors by less carbon dioxide (CO₂)-emitting alternatives. One attractive option is the use of molecular hydrogen (H₂) since its combustion emits water (H₂O) and not CO₂. Therefore, H₂ is regarded as a non-polluting fuel. The ways to produce H₂ can be diverse, but steam reformation of conventional fossil fuel sources is still the main producer of H₂ gas up to date. Biohydrogen production via microbes could be an alternative, environmentally friendly and renewable way of future H₂ production, especially when the flexible and inexpensive C₁ compound formate is used as substrate.

**Results:** In this study, the versatile compound formate was used as substrate to drive H₂ production by whole cells of the thermophilic acetogenic bacterium *Thermoanaerobacter kivui* which harbors a highly active hydrogen-dependent CO₂ reductase (HDCR) to oxidize formate to H₂ and CO₂ and vice versa. Under optimized reaction conditions, *T. kivui* cells demonstrated the highest H₂ production rates (qH₂ = 685 mmol g⁻¹ h⁻¹) which were so far reported in the literature for wild-type organisms. Additionally, high yields (Y(H₂/formate)) of 0.86 mol mol⁻¹ and a hydrogen evolution rate (HER) of 999 mmol L⁻¹ h⁻¹ were observed. Finally, stirred-tank bioreactor experiments demonstrated the upscaling feasibility of the applied whole cell system and indicated the importance of pH control for the reaction of formate-driven H₂ production.

**Conclusions:** The thermophilic acetogenic bacterium *T. kivui* is an efficient biocatalyst for the oxidation of formate to H₂ (and CO₂). The existing genetic tool box of acetogenic bacteria bears further potential to optimize biohydrogen production in future and to contribute to a future sustainable formate/H₂ bio-economy.

**Keywords:** Biohydrogen, Dark fermentation, Acetogenic bacteria, Bioreactor, qH₂, HER, Optimization, Scale-up, Hydrogen-dependent CO₂ reductase (HDCR)

**Background**

Molecular hydrogen is considered as an attractive, alternative energy carrier which can be produced environmentally friendly and renewable. If hydrogen is produced from renewable energy sources by water splitting, no net CO₂ is generated in the production process and H₂ can be regarded as a promising environmentally friendly fuel [1, 2]. However, traditional routes such as steam reforming and partial oxidation of coal, oil and natural gas are still the main producers of H₂ gas to date [3, 4]. Therefore, the production of H₂ from renewable, sustainable and environmentally friendly sources become more and more important. Biohydrogen production via microbial organisms has already been studied extensively over several decades, but is still an active field of research which comprises three major categories: (1) biophotolysis (direct and indirect), (2) photofermentation, and (3) dark fermentation [5–7]. In the latter one, the organisms do not rely on the availability of light and can utilize a vast variety of carbon sources to break them down into H₂ and most likely organic acids and alcohols. Since
dark fermentative H₂ production has high production rates and does not need direct solar input, the process possesses a greater potential for practical applications [8, 9]. Here, formic acid/formate is a very flexible and inexpensive substrate for H₂ production by fermentative microbes [7, 10]. The hydrogen content in formic acid is 4.4 wt% and the compound is regarded as liquid organic hydrogen carrier (LOHC) as well as feedstock chemical and microbial carbon source [11, 12]. Natural formate-trophic microorganisms are able to assimilate formate in their metabolism using either the Calvin cycle, the serine pathway or the Wood–Ljungdahl pathway (WLP) [12, 13]. Several acetogenic bacteria (i.e., Acetobacterium woodii, Clostridium ljungdahlii and Moorella thermoacetica) and methanogens can utilize formate via the WLP as sole energy and carbon source for growth [14, 15]. However, different microorganisms, especially of the Thermoanaerobacterales, Clostridiaceae and Enterobacteriaceae families are known to oxidize formate with the concomitant evolution of H₂ and CO₂ [8] and diverse enzyme systems are involved to catalyze the reaction. The enterobacterium Escherichia coli has become a workhorse for enhanced biohydrogen production due to a formate hydrogen lyase (FHL) system which catalyzes the disproportionation of formate to H₂ and CO₂ (Eq. 1) [16–18]:

\[
\text{HCOO}^- + H_2O \leftrightarrow \text{HCO}_2^- + H_2
\]

\[
\Delta G^0 = +1.3 kJ \text{ mol}^{-1}
\]  

The purified FHL consists of a membrane-associated [NiFe]-hydrogenase, a molybdenum-containing formate dehydrogenase as well as several electron-transferring iron–sulfur proteins [19]. The good genetic accessibility of the organism as well as different strategies such as heterologous gene expression, metabolic engineering, adaptive evolution and protein engineering were already applied to enhance H₂ production by E. coli [20–24]. Another example for hydrogen production from formate is the hyperthermophilic archaeon Thermococcus onnurineus. This archaeon has a similar membrane-bound enzyme complex as E. coli to mediate formate-driven H₂ production which is called Fdh-Mrp-Mbh enzyme complex. The enzyme consists of a formate dehydrogenase (Fdh) module, a membrane-bound hydrogenase (Mbh) module and a multisubunit Na+/H⁺ antiporter (Mrp) module. So far, the entire enzyme complex could not be purified from T. onnurineus, but the enzyme is involved in chemiosmotic energy conservation [25–27].

Recently, a soluble, biotechnological interesting enzyme complex that is involved in formate metabolism of acetogenic bacteria was purified from the thermophilic acetogenic bacterium Thermoanaerobacter kivui, named as hydrogen-dependent CO₂ reductase [28]. The enzyme complex consists of a formate dehydrogenase and a [FeFe]-hydrogenase subunit which are, most likely, connected by two electron-transferring, iron–sulfur subunits. So far, the HDCRs were only purified from the thermophilic T. kivui and the mesophilic acetogenic bacterium A. woodii, but bioinformatic analysis of available genome data indicates the presence of HDCR-like enzymes also outside of this bacterial group [28, 29]. The purified and characterized HDCR enzymes catalyze the direct hydrogenation of CO₂ to formic acid with remarkable catalytic rates, outcompeting chemical catalysts under comparable moderate reaction conditions [30]. But the enzymes do not only catalyze CO₂ reduction (H₂:CO₂ oxidoreductase activity), moreover, they catalyze the reverse reaction of formate oxidation (formate: H⁺ oxidoreductase activity) with almost identical rates. The thermophilic enzyme from T. kivui showed a H₂:CO₂ oxidoreductase activity and a formate: H⁺ oxidoreductase activity of 900 and 930 U/mg [28], respectively, demonstrating the reversible nature of the enzyme and the high potential of the organism in formate-based H₂ production. It is worth mentioning that the catalytic rates of the thermophilic HDCR are almost two orders of magnitude higher than the mesophilic enzyme from A. woodii [28].

For biotechnological applications, the HDCR-based conversion efficiency of H₂ and CO₂ to formate was already proven by resting cells of T. kivui and A. woodii in serum bottle and bioreactor scale [29, 31, 32]. But formate-driven H₂ production was so far only investigated for A. woodii cells [32, 33]. To take advantage of the multiple times higher reaction speed of the thermophilic enzyme, whole-cell catalysis for biohydrogen production from formate using the thermophilic acetogen T. kivui was addressed in this study.

**Results**

**H₂ production with resting cells**

To investigate the formate-driven H₂ production potential of resting cells from T. kivui, the organism was grown in complex medium with glucose as substrate, cells were harvested and washed, and were subsequently incubated in reaction buffer at a protein concentration of 0.6 mg mL⁻¹ (corresponding to 1.54 mgCDW mL⁻¹). After the addition of 150 mM sodium formate, H₂ production as well as substrate and metabolite concentrations were monitored over time (Fig. 1a). The cells started to produce H₂ with an initial specific H₂ productivity (qH₂) of 249 ± 51 mmol g⁻¹ h⁻¹ (162 mmol gCDW⁻¹ h⁻¹). 1.2 mmol H₂ was produced from 1.4 mmol formate consumed leading to a yield of H₂ produced per substrate consumed (Y_{(H2/formate)}) of 0.86 mol mol⁻¹. Interestingly, only traces
of acetate (2.4 ± 2.3 mM) were finally produced. Unlike in *A. woodii* uncoupling agents did not stimulate \( \text{H}_2 \) production (Fig. 1b) and did not affect the produced amount of acetate. These results were surprising since growing cultures of *T. kivui* are expected to disproportionate formate to acetate and \( \text{CO}_2 \) according to Eq. 2:

\[
4 \text{HCOOH} \leftrightarrow \text{CH}_3\text{COOH} + 2\text{CO}_2 + 2\text{H}_2\text{O} \tag{2}
\]

But in contrast to the expectations, formate was nearly completely oxidized to \( \text{H}_2 \) and \( \text{CO}_2 \) by resting cells. Therefore, it was of interest to study formate metabolism in growing cells. As shown before [15], *T. kivui* disproportionated formate according to Eq. 2 under different conditions (Additional file 1: Fig. S1). The utilization of 200 mM of formate resulted in the formation of 40 ± 5 mM acetate in average, yielding a stoichiometry of 5:1. This clearly shows, that *T. kivui* cells grew on formate according to Eq. 2. Cell suspensions of formate-grown cells behaved as cell suspensions from glucose-grown cells: from 300 mM of formate only traces of acetate (0.15 ± 0.02 mM) were formed and 160 ± 17 mM of hydrogen were produced (Additional file 1: Fig. S2).

Formate-driven \( \text{H}_2 \) production was characterized in detail using glucose-grown *T. kivui* cells. The \( \text{qH}_2 \) decreased with increasing pH (Additional file 1: Fig. S3). At a pH range of 5.5–7 the highest \( \text{qH}_2 \) of 245 mmol g\(^{-1}\) h\(^{-1}\) was observed, whereas under alkaline conditions (pH 9) only 29% of the activity was present. Temperature dependence of whole cell catalysis was tested from 30 to 80 °C (Additional file 1: Fig. S4). The temperature profile showed the highest \( \text{qH}_2 \) at 70 °C (\( \text{qH}_2 = 370 ± 84 \text{ mmol g}^{-1} \text{ h}^{-1} \)) which is close to the physiological growth temperature optimum of 66 °C of *T. kivui*. But at ambient temperatures of 30 °C, still 11% of the maximum \( \text{qH}_2 \) was achieved. In the next experiment, the effect of increasing formate concentrations (25 mM to 8 M) on \( \text{H}_2 \) productivity was investigated. Within this range, initial \( \text{H}_2 \) production rates were optimal at 600 mM sodium formate and decreased up to 8 M substrate (Additional file 1: Fig. S5). An exponential decrease of the maximum \( \text{qH}_2 \) of 394 ± 17 mmol g\(^{-1}\) h\(^{-1}\) (256 mmol g\(_{\text{CDW}}\)\(^{-1}\) h\(^{-1}\)) at 0.3 mg mL\(^{-1}\) was observed with increasing cell densities. On the other hand, the hydrogen evolution rate (HER) increased up to 521 ± 40 mmol L\(^{-1}\) h\(^{-1}\) at a cell concentration of 4 mg mL\(^{-1}\) (Fig. 2).

![Figure 1](image-url)  
**Fig. 1** \( \text{H}_2 \) production from formate by resting *T. kivui* cells in presence or absence of metabolic inhibitors. Resting cells (0.6 mg mL\(^{-1}\)) were added to preheated (60 °C) imidazole buffer (50 mM imidazole, 20 mM MgSO\(_4\), 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) under a \( \text{N}_2 \) atmosphere. a The reaction was started by addition of 150 mM of sodium formate. \( \text{H}_2 \) (black squares) and formate concentrations (black triangles up) are plotted against time. b 30 µM DCCD, TCS or ETH2120 or 20 µL ethanol was added to the serum bottles 10 min before the reaction was started by adding 150 mM of sodium, potassium or ammonium formate as indicated. The specific hydrogen production rate was calculated based on the first 15 min after start of the reaction. All data points are mean ± SD, \( N = 2 \).
Higher dissolved concentrations of O₂ led to a dramatic decrease in qH₂ (Additional file 1: Fig. S7).

Next, we used the optimal pH of 7 and an optimal temperature of 70 °C, but raised the formate and the protein concentration (Fig. 3). At 4 mg mL⁻¹ total cell protein and 600 mM formate, H₂ production was fast but came to completion after 25 min. The plateau reached was only 175 mM H₂, which is only 58% of the theoretical value. At lower protein and formate concentrations (0.3 mg mL⁻¹ total cell protein and 300 mM formate), hydrogen production was slower and came to an end at 100 mM H₂, again only 67% of the theoretical value. Formate oxidation leads to an alkalinization of the buffer and indeed, at the end of the experiment the pH was around 8.4–8.6. The results of qH₂ and HER are summarized in Table 1.

**H₂ production in batch-operated stirred-tank bioreactors**

To avoid pH effects on productivity, formate oxidation was investigated under controlled reaction conditions using batch-operated stirred-tank bioreactors with continuous gas sparging of 100% N₂. *T. kivui* cells were grown in 20 L of complex medium with glucose as growth substrate to the end of the exponential growth phase, and resting cells were prepared. The bioreactor contained 50 mM imidazole buffer and sodium formate was added to a final concentration of 600 mM. The reaction was started by adding resting cells to a final cell protein concentration of 0.6 mg mL⁻¹. Two different approaches were tested in the bioreactor experiments, either keeping the pH value constant at 7.2 by titrating with H₃PO₄ (Batch [1]) or by keeping the pH value unaffected during the whole experiment (Batch [2]).

As seen in Batch [1], formate oxidation started immediately after the addition of the cell suspension to the bioreactor (Fig. 4a) and H₂ and CO₂ evolution were also directly observed (Fig. 4b). At the beginning of the experiment, the amount of detected CO₂ was slightly lower than the amount of H₂ observed, but similar amounts were observed during the course of the experiment. This observation is caused due to the higher solubility of CO₂ in the liquid phase compared to H₂, since both gases are produced in stoichiometric amounts in the catalyzed reaction. Based on the first 2 h and a total cell protein concentration of 0.54 mg mL⁻¹, the specific H₂ productivity and formate oxidation rate were determined to be 147 and 192 mmol g⁻¹ h⁻¹, respectively (Fig. 4c).

**Table 1** qH₂ and HER of formate-based H₂ production by whole *T. kivui* cells under optimized reaction conditions

| Condition | H₂ production | HER |
|-----------|---------------|-----|
| 0.3 mg mL⁻¹ cells | 685 ± 87 | 205 ± 26 |
| 300 mM sodium formate | 999 ± 6 |
| 4 mg mL⁻¹ cells | 250 ± 2 |
| 600 mM sodium formate | 999 ± 6 |

*Cell suspension experiment in imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) at 70 °C and the final cell protein concentration and sodium formate concentration as indicated*
24 h of process time, 600 mM formate was consumed and 514 mM of H₂ was produced. Acetate was only formed in small amounts (8.6 ± 1.4 mM), resulting in a high \( Y_{\text{H₂/formate}} \) of 0.86 mol mol⁻¹ as also observed in serum bottle experiments. The pH value was kept constant at 7.2 during the whole process (Fig. 4a) and 316 mM of H₃PO₄ was fed into the bioreactor in total. This is in accordance with the expectations since H₃PO₄ is able to release two H⁺ at pH 7.2. Unfortunately, optical density as well as total cell protein concentration showed an exponential decay, indicating the lysis of cells over the time (Fig. 4d).

Different results were obtained in the control experiment without pH control (Batch [2]). Formate oxidation and hydrogen production started immediately after cell addition, however, the reaction sharply stopped after 6 h (Fig. 5a, b). At that time point no hydrogen was produced anymore and the formate concentration stayed constant in the bioreactor broth. Looking at the pH curve, the loss in activity seemed to be directly connected to the pH value (Fig. 5a). Due to formate oxidation, the pH value strongly increased from 7.2 to 8.4 in the first 6 h. After the catalytic activity of the cells was diminished, the change in pH slowed down, reaching a pH value of 8.9 after 34 h. This clearly indicates the dependence of the whole cell-based formate-driven hydrogen production on the pH. In contrast to Batch [1], similar amounts of CO₂ and H₂ were not observed in the off-gas analysis during the course of the experiment (Fig. 5b). This was based on the fact, that CO₂ is stored in the liquid phase in form of bicarbonate (HCO₃⁻) under more alkaline conditions. At pH 8–9 the bicarbonate anion is the predominant form. Optical density as well as total cell protein concentration showed a similar exponential decay as observed in Batch [1] (Fig. 5c).
Discussion

In this study, we investigated the biological hydrogen production from formate using the thermophilic acetogenic bacterium *T. kivui* as biocatalyst. Formate-based H₂ production was catalyzed by the HDCR enzyme. Since acetogenic bacteria can grow under lithoautotrophic conditions, a sufficient CO₂ reduction system is necessary to fix one CO₂ molecule in the methyl-branch of the WLP via formate dehydrogenases (CO₂ reductases). Here, CO₂ is reduced with two electrons to the intermediate formate which is further converted to acetate (Fig. 6). Different types of purified and characterized formate dehydrogenases of acetogenic bacteria clearly demonstrate the diversity in subunit composition and the diversity of reductants used. Reductants can be reduced ferredoxin as in case of *Clostridium pasteurianum* [34], NADPH as in case of *M. thermoacetica*, [35], a combination of reductants as in case of *Clostridium autoethanogenum* and *Eubacterium callanderi* KIST612 [36, 37], or even molecular H₂ as shown for the HDCR enzyme complex of *A. woodii* and *T. kivui* [28, 29]. The enzyme of the latter two organisms is not connected to the metabolism by an external cofactor as electron carrier. Additionally, a good reversibility of the HDCR enzyme complex is also required since the HDCR-containing bacteria *A. woodii* and *T. kivui* can also grow with methanol or formate, respectively, as sole carbon and energy source [15, 38, 39]. During organoheterotrophic conditions, the physiological function of the HDCR enzyme is formate oxidation to provide the necessary reducing equivalents for acetogenesis (Fig. 6). However, in both life-styles the main end product is acetate. For this reason, specific ionophores or uncoupling agents were used in previous studies of *A. woodii* and *T. kivui* to decouple the HDCR reaction of CO₂ reduction from the WLP. By lowering the intracellular amount of ATP, the ATP-dependent further conversion
of formic acid to acetic acid in the WLP of acetogenic bacteria is blocked and formate (in case of CO₂ reduction) or H₂ and CO₂ (in case of formate oxidation by A. woodii) became the predominant product(s). Interestingly, the situation is different in resting cells of T. kivui. Here, no specific ionophore nor metabolic uncoupler were needed to efficiently catalyze the oxidation of formate to H₂ and CO₂ by whole cells. Moreover, only small amounts of acetate were produced and a high Y(H₂/formate) could be reached. This behavior is highly unusual for formate utilizing acetogens since growth studies demonstrated the formation of acetate as the dominant product (Fig. 6). Putative adaptation and CO₂ concentration effects could be experimentally excluded. Since the elucidation of the formate metabolism in A. woodii and T. kivui is still in its infancy and little is known about formate import/export and the corresponding transporters, further metabolic, bioinformatic and genetic studies are necessary to reveal this enigma. However, future metabolic engineering of T. kivui by knocking-out responsible genes coding for WLP enzymes downstream of the HDCR reaction could further help to diminish unwanted side product formation in form of acetate. Here, the knock-out of the formyl-THF synthetase genes would be the most obvious way to prevent the further conversion of formic acid towards acetate in the methyl-branch of the WLP.

Nevertheless, the highest volumetric hydrogen production rate measured during the experiments described in this study was 999 mmol L⁻¹ h⁻¹ (using 4 mg/mL cell protein). This HER is higher than the highest HER described for other wild-type organisms like A. woodii

![Fig. 6 Model of the bioenergetics and biochemistry of acetogenesis from formate in T. kivui. HDCR, hydrogen-dependent CO₂ reductase; HydABC, electron-bifurcating hydrogenase; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; THF, tetrahydrofolate; HCO-THF, formyl-THF, HC-THF, methenyl-THF, H₂C-THF, methylene-THF, H₂C-THF, methyl-THF; Ech, energy-converting hydrogenase; Ech-MetFV, energy-converting hydrogenase complex coupled to methylene-THF reductase; CoFeSP, corrinoid-iron-sulfur-protein; Fd²⁻, reduced ferredoxin. The Ech-MetFV complex is hypothetical. The ion stoichiometries for the membrane proteins have not been determined experimentally. Adapted from Katsyv et al. [55]](image-url)
cells of the hyperthermophilic archaeon T. kivui showed more or less comparable specific H2 production potential of T. kivui cells in formate-driven H2 production. As far as we know, the demonstrated qH2 of T. kivui cells (685 mmol g\(^{-1}\) h\(^{-1}\)) are also among the highest rates which were so far reported in the literature for wild-type organisms. Looking into the thermophilic world of microbial H2-producers, only wild-type cells of the hyperthermophilic archaeon T. onnurineus showed more or less comparable specific H2 production rates 404 mmol g\(^{-1}\) h\(^{-1}\) as T. kivui cells [40]. Of course, biohydrogen production can even be further stimulated in future by homologous overexpression of the HDCR genes or by the identification and optimization of putative formate transporters to increase formate uptake rates into the cell. Enhanced hydrogen production from formate was already shown in high cell density cultures of T. onnurineus and in a T. onnurineus strain with a mutated formate transporter caused by adaptive evolution of the organism [41, 42].

Moreover, the HDCR complex can be regarded as a functional production unit to achieve H2 formation from formate in non-HDCR-containing organism as it was recently shown for E. coli [43]. That formate-driven H2 production can be achieved with non-formate-dependent H2 producer was also demonstrated for genetically engineered Pyrococcus furiosus cells, containing the Fdh-Mrp-Mbh enzyme complex of T. onnurineus NA1 [44]. Looking at ambient temperatures, E. coli is one of the most studied examples for microbial hydrogen production. Resting cells of E. coli K-12 (WT) showed a typically low formate-driven H2 productivity of 200 mmol g\(^{-1}\) h\(^{-1}\), but 2.8 times higher production rates could be achieved by overexpressing the FHL genes [10, 45]. Due to this overexpression and the use of high cell concentrations of 93 g/L the HER measured (23.6 g L\(^{-1}\) h\(^{-1}\), which equals 11,707 mmol L\(^{-1}\) h\(^{-1}\)) was significantly higher than in the wild type [10]. This shows the high potential of genetic engineering for the increase of hydrogen production in T. kivui.

Another advantage of using T. kivui is that it grows in mineral salt solutions, without addition of yeast extract or other additions such as vitamins. Here, we have used complex medium to grow the cells for the preparation of resting cells, but in further upscaling experiments, mineral medium can be used as well. In addition, cells can be grown on the inexpensive carbon source formate, rather than on glucose, and T. kivui even grows on syngas in mineral media [46], which would add, in addition, to a zero-carbon balance.

In this study, stirred-tank bioreactor experiments did not only demonstrate the upscaling feasibility of our applied system, moreover, limitations in closed-batch serum bottle experiments became apparent. Only a fraction of formate could be oxidized in bioreactor experiments without pH control compared to experiments with controlled pH due to the alkalinization of the buffer. Since sodium formate (and not the pure acid) was oxidized, the missing protons (H\(^{+}\)) to form H2 were taken up from the liquid phase. Thereby, the proton concentration in the liquid phase decreased and the pH value of the buffer became alkalinic. Since the investigated reaction of formate-based H2 production is close to the thermodynamic equilibrium, minor adjustments of the reaction conditions can affect the direction of the reaction. For example, if the pH is drifting to a more alkaline value (i.e., pH 8), the Gibbs free energy value gets much more positive (\(G > 0\); endergonic) and the equilibrium is strongly shifted to the side of the educts (\(K < 1\)). Because of that, the pH value is an important and critical parameter in the investigated reaction which has to be kept in mind to achieve a complete conversion of the substrate formate to the gaseous products H2 and CO2. Also high growth temperatures and prevailing environmental conditions (i.e., low H2 concentrations) change the thermodynamics in favor of formate oxidation and enables for example the hyperthermophilic archaeon T. onnurineus to grow at high temperatures by the oxidation of formate to H2 + CO2 [25]. The thermodynamics of the formate-oxidizing metabolism and the implications for H2 production were already discussed in greater detail [40]. In another example, growth of the sulfate-reducing bacterium Desulfovibrio vulgaris was coupled to formate-driven H2 production using a bioreactor with continuous gas sparging and without sulfate availability or syntrophic partners [47]. Enough energy can also be released in the formate oxidation reaction if the H2 concentration is kept at a low level. In nature, this can be achieved by the coupling to H2-consuming partner organisms which maintain a low-enough H2 partial pressure and which was demonstrated in a coculture of Moorella sp. strain AMP or Desulfovibrio sp. strain with a hydrogen-consuming methanogen [48].

**Conclusion**

This study demonstrated that whole cells of T. kivui can be used as biocatalysts to catalyze formate oxidation with concomitant evolution of H2 and CO2. The H2 productivity indicates the great potential of T. kivui cells for practical application in comparison to other H2-producing microbes. Because of the good reversibility of the HDCR reaction, the organism could be a central part in H2 storage/CO2 capture into formate as well as in hydrogen production from formate. Even if the demonstrated rates are among the highest rates reported, the existing genetic
tool box of acetogenic bacteria bears further potential to optimize biohydrogen production by *T. kivui* cells, thus, contributing to a future sustainable formate/H₂ bio-economy.

**Methods**

**Organism and cultivation**

*Thermoanaerobacter kivui* (DSM 2030) wild type was cultivated at 66 °C under anoxic conditions in 50 mL, 500 mL or 20 L media using 120-mL serum bottles or 1-L/22-L flasks (Glasgerätebau Ochs GmbH, Bovenden-Lenglern, Germany). The medium was prepared under anoxic conditions as described before [49, 50]. Media were either carbonate- and phosphate-buffered complex medium according to Leigh et al. [51] or complex medium without additional NaHCO₃ which was named as phosphate-buffered complex medium. As growth substrate 28 mM glucose or 200 mM sodium formate was used. Optical densities of growing cultures were determined at 600 nm.

**Growth experiments**

Growth experiments were performed in 120-mL serum bottles with 50 mL of the corresponding complex medium at 66 °C under anoxic conditions. Medium used was either carbonate- and phosphate-buffered complex medium with a gas phase of N₂ + CO₂ (80:20% [v:v]) or phosphate-buffered complex medium with a N₂ (100% [v:v]) or N₂ + CO₂ (80:20% [v:v]) gas phase. As growth substrate 200 mM of sodium formate was used. These media were either inoculated with a glucose-grown culture of *T. kivui* to an optical density of 0.05. Samples were taken every 2 h to determine the optical densities and pH values (pH-meter CG 825, SCHOTT Instruments GmbH, Mainz, Germany). Cells were pelleted by centrifugation (18,000g, 5 min, 4 °C) and the supernatant was used to determine acetate and formate concentrations.

**Preparation of resting cells**

Resting cells of *T. kivui* were prepared as described before [31]. Glucose-grown cells were harvested at the end of the exponential growth phase at OD 1.7–2.2 and formate-grown cells were harvested at OD 0.4–0.5 by centrifugation. Then, cells were washed two times and resuspended in imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7). All preparation steps were done in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). For storage at 4 °C a gas-tight Hungate tube with a N₂ atmosphere (100%) was used. The total cell protein concentration of the cell suspension was determined according to Schmidt et al. [52].

**Cell suspension experiments**

To characterize hydrogen production from formate by resting cells of *T. kivui*, 120-mL serum bottles (Glasgerätebau Ochs GmbH, Bovenden-Lenglern, Germany) were used with a total liquid reaction volume of 10 mL under a N₂ atmosphere (100%). Serum bottles were incubated in a shaking water bath to preheat the corresponding buffer to the temperature as indicated. Prior to the start of the reaction, resting cells were added to the buffer at the concentration as indicated and were incubated for 10 min. When metabolic inhibitors DCCD (N,N′,N′,N′-dicyclohexylcarbodiimide), TCS (3,3′,4,5-tetrachlorosalicylanilide), ETH2120 (N,N,N′,N′-tetracyclohexyl-1,2-phenylenedioxycarbamide; all dissolved in EtOH) were used, they were additionally added to the buffer. In case of O₂ sensitivity studies, corresponding amounts of atmospheric air (assuming an O₂ content of 21%) were added to the serum bottle head space and dissolved O₂ concentrations were calculated according to the law of Henry. Atmospheric pressure was ensured in the serum bottles prior to the start of the experiments. The experiments were started by adding the substrate (sodium, potassium or ammonium formate). If not otherwise stated, generally a temperature of 60 °C, a sodium formate concentration of 150 mM, a protein concentration of 0.6 mg mL⁻¹ and imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) was used. A different buffer composition (25 mM MES, 25 mM MOPS, 25 mM HEPES, 25 mM EPPS, 25 mM CHES, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin) was used to determine the pH optimum. The pH value of this buffer was set to 5–10 at room temperature. Gas samples were taken with a gas-tight syringe during the experiments to analyze hydrogen concentrations via gas chromatography as described before [28]. Liquid samples were taken over time and were subsequently centrifuged (18,000g, 5 min, 4 °C) to remove the cells. The supernatant was frozen till the formate and acetate concentrations were determined.

**Batch experiments in stirred-tank bioreactors**

The batch-operated stirred-tank bioreactor experiments were carried out in Biostat Aplus bench-top reactors from Sartorius (Melsungen, Germany) as described before [32]. The working volume was 2 L containing modified imidazole buffer (50 mM imidazole, 20 mM KCl, 2 mM DTE, pH 7). Each bioreactor was equipped with micro-sparger, baffles, two Rushton-impellers, pH-probe (Hamilton, Bonaduz, Switzerland), temperature probe and a redox potential probe (Hamilton, Bonaduz, Switzerland). The temperature of the buffer was maintained at 60 °C, using a cooling finger and heating sleeve. The stirring speed was...
400 rpm and a gas flow rate of 50 mL/min with 100% N₂ was applied using a digital mass-flow controller (Bronkhorst High-Tech, Ruurlo, Netherlands). The bioreactor buffer was prepared under aerobic, non-sterile conditions and oxygen was removed by subsequent sparging with 100% N₂ for about 16 h. The headspace of the bioreactor was at atmospheric pressure. 600 mM of anoxic sodium formate was added befor the reaction was started by the addition of resting cells to a final cell protein concentration of 0.6 mg mL⁻¹. The pH was either controlled at pH 7.2 via titration with 4 M H₃PO₄ during the experiment (Batch [1]) or was not controlled (Batch [2]). Liquid samples (2 mL) were taken at defined time points for HPLC measurement as well as OD and protein determination. The samples were centrifuged (18,000g, 8 min, room temperature) to remove cells and the supernatant was frozen at −20 °C until further off-line analysis.

**Analytical methods**

The concentrations of formate and acetate were measured 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 or by high-performance liquid chromatography (HPLC) as described before [53]. Bioreactor off-gas analysis was conducted via a Micro-GC (Inficon, Bad Ragaz, Switzerland) using the analytical conditions and columns as described before [54]. Gas samples from closed-batch serum bottle cell suspension experiments were analyzed via GC as described in [28]. The total cell protein concentration of the prepared cell suspensions was determined according to Schmidt et al. [52].

**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 and bioreactor applications were purchased from many (Air Liquide (Paris, France)). Such as N₂ (purity of 5.0) were purchased from Air Liquide (Paris, France). High-performance liquid chromatography, rpm: Rounds per minute; DCCD: N,N'-Dicyclohexylcarbodiimide, TCS: 3,3',5,5'-Tetraclorosalicylanilide;

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**Supplementary Information**

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**Additional file 1:**

Figure S1. Growth of *T. kivui* on formate in the presence of different concentrations of CO₂. Complex medium with 200 mM sodium formate was inoculated to an OD of 0.05 with glucose-grown culture of *T. kivui* and cultivated at 66 °C. a, b: carbonate/phosphate-buffered medium with N₂ + CO₂ (80:20% [v:v]) as gas phase, c, d: phosphate-buffered medium with N₂ (100% [v:v]) as gas phase, e, f: phosphate-buffered medium with N₂ + CO₂ (80:20% [v:v]) as gasphase. CDOM (empty circles), pH (black diamonds), formate (black triangles up), acetate (black circles).

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**Additional file 2:**

Figure S2. Effect of different formate concentrations on H₂ production by formate-grown resting cells of *T. kivui*. Resting cells (0.6 mg mL⁻¹) were added to preheated (60 °C) imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) under a N₂ atmosphere. The reaction was started by addition of 150 mM (black squares), 300 mM (black triangles up) or 2 M (black triangles down) sodium formate. The hydrogen concentration is plotted against the time. Acetate was only produced in traces (0.15 ± 0.02 mM). All data points are mean ± SD, N = 2.

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**Additional file 3:**

Figure S3. pH dependence of H₂ production. Resting cells (0.6 mg mL⁻¹) were added to preheated (60 °C) buffer (25 mM MES, 25 mM MOPS, 25 mM HEPES, 25 mM EPPS, 25 mM CHES, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin) under a N₂ atmosphere. The pH of the buffer was adjusted to the values of 5 to 10 at room temperature. The reaction was started by addition of 150 mM of sodium formate. Specific H₂ production rates were calculated based on the first 15 minutes after start of the reaction. All data points are mean ± SD, N = 2. Figure S4. Effect of varying temperatures on the H₂ productivity. Resting cells (0.6 mg mL⁻¹) were added to preheated imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) under a N₂ atmosphere. The reaction was started by adding 150 mM sodium formate. Specific H₂ production rates were calculated based on the first 15 minutes after start of the reaction. All data points are mean ± SD, N = 2.

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**Additional file 4:**

Figure S5. Dependence of the hydrogen production rate on the formate concentration. Resting cells (0.6 mg mL⁻¹) were added to preheated (60 °C) imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) under a N₂ atmosphere. The reaction was started by addition of 25 mM to 8 M of sodium formate. Specific H₂ production rates were calculated based on the first 10 to 15 minutes after start of the reaction. All data points are mean ± SD, N = 2.

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**Additional file 5:**

Figure S6. Influence of the storage time of resting *T. kivui* cells on the H₂ productivity. Resting cells (0.6 mg mL⁻¹) were added to preheated (60 °C) imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) under a N₂ atmosphere. The reaction was started by addition of 150 mM of potassium formate. Specific H₂ production rates were calculated based on the first 15 minutes after start of the reaction. All data points are mean ± SD, N = 2.

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**Additional file 6:**

Figure S7. Effect of different O₂ concentrations on the H₂ productivity. Resting cells (0.6 mg mL⁻¹) were added to preheated (60 °C) imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) under a N₂ atmosphere. The reaction was started by addition of 150 mM sodium formate. Specific H₂ production rates were calculated based on the first 15 minutes after start of the reaction. All data points are mean ± SD, N = 2.

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**Additional file 7:**

Figure S8. Effect of different O₂ concentrations on the H₂ productivity. Resting cells (0.6 mg mL⁻¹) were added to preheated (60 °C) imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 µM resazurin, pH 7) under a N₂ atmosphere. The reaction was started by addition of 150 mM sodium formate. Specific H₂ production rates were calculated based on the first 15 minutes after start of the reaction. All data points are mean ± SD, N = 2.

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**Abbreviations**

H₂: Hydrogen; CO₂: Carbon dioxide; O₂: Oxygen; LOHC: Liquid organic hydrogen carrier; WLP: Wood–Ljungdahl pathway; HDCR: Hydrogen-dependent CO₂ reductase; TFH: Tetrahydrofolate reductase; FHL: Formate hydrogen lyase; ATP: Adenosine triphosphate; Fdh: Formate dehydrogenase; Mbp: Multisubunit Na⁺/H⁺ antipporter; OD: Optical density at 600 nm; UV/vis: Ultraviolet/visible; X: Equilibrium constant; qH₂: Specific hydrogen production rate; HER: Volumetric hydrogen production rate; Y[H₂,formate]: Yield of H₂ produced per formate consumed; CDW: Cell dry weight; WT: Wild type; td: Doubling time; GC: Gas chromatography; HPLC: High-performance liquid chromatography; rpm: Rounds per minute; DCCD: N,N’-Dicyclohexylcarbodiimide; TCS: 3,3’,5,5’-Tetraclorosalicylanilide;
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Author contributions
VM and FMS designed and supervised the research, analyzed the data and wrote the manuscript. FMS also performed the fermentation experiments. YB performed the experiments, analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files. If additional information is needed, please contact the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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