



# Blending industrial blast furnace gas with H<sub>2</sub> enables *Acetobacterium woodii* to efficiently co-utilize CO, CO<sub>2</sub> and H<sub>2</sub>

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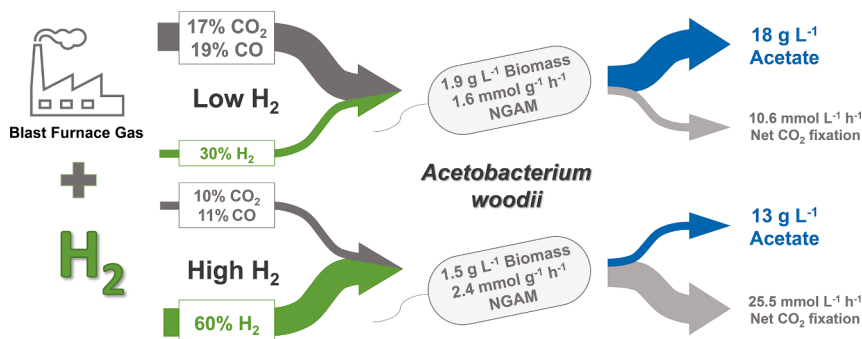
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## HIGHLIGHTS

- *A. woodii* can utilize industrial blast furnace gas for growth and acetate production.
- CO, CO<sub>2</sub> and H<sub>2</sub> can be co-utilized in batch and continuous cultivation.
- Maximum specific H<sub>2</sub> uptake rate of *A. woodii* is between 116 and 167 mmol g<sup>-1</sup>h<sup>-1</sup>.
- Blending blast furnace gas with H<sub>2</sub> enables acetate production at 14 mmol l<sup>-1</sup>h<sup>-1</sup>.
- Blending industrial gas with H<sub>2</sub> allows control of gas uptake and carbon distribution.

## GRAPHICAL ABSTRACT



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## ABSTRACT

In this study, the impact of gas composition (i.e. CO, CO<sub>2</sub> and H<sub>2</sub> partial pressures) on CO<sub>2</sub> utilization, growth, and acetate production was investigated in batch and continuous cultures of *A. woodii*. Based on an industrial blast furnace gas, H<sub>2</sub> blending was used to study the impact of H<sub>2</sub> availability on CO<sub>2</sub> fixation alone and together with CO using idealized gas streams. With H<sub>2</sub> available as an additional energy source, net CO<sub>2</sub> fixation and CO, CO<sub>2</sub> and H<sub>2</sub> co-utilization was achieved in gas-limited fermentations. Using industrial blast furnace gas, up to 15.1 g l<sup>-1</sup> acetate were produced in continuous cultures. Flux balance analysis showed that intracellular fluxes and total ATP production were dependent on the availability of H<sub>2</sub> and CO. Overall, H<sub>2</sub> blending was shown to be a suitable control strategy for gas fermentations and demonstrated that *A. woodii* is an interesting host for CO<sub>2</sub> fixation from industrial gas streams.

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## 1. Introduction

The use of CO<sub>2</sub> as a substrate is one possibility to reduce atmospheric CO<sub>2</sub> emissions, close global carbon cycles and to establish a circular economy (Bengelsdorf and Dürre, 2017; Liew et al., 2016b; Vees et al., 2020). One of the biggest industrial CO<sub>2</sub> emitters is the iron and steel industry, which accounts for 5% of anthropogenic CO<sub>2</sub> emission in the European Union ("Eurostat", 2018; Pardo and Moya, 2013). A variety of process gases are produced during the production process and blast furnace gas (BFG), formed during the reduction of iron ore to iron is one example for a by-product gas from the steel milling process (Hou et al., 2011; Molitor et al., 2016). Hydrogen is an important energy source for acetogenic gas fermentations and a promising future electron carrier that can be obtained from renewable resources (Das and Veziroglu, 2008; Hallenbeck and Ghosh, 2009; Yukesh Kannah et al., 2021).

Acetogenic bacteria are promising microbial hosts for industrial CO<sub>2</sub> fixation since they have the ability to fix carbon in the form of CO and CO<sub>2</sub> via the Wood-Ljungdahl pathway (WLP), where two molecules of CO<sub>2</sub> are reduced to acetyl-CoA using H<sub>2</sub> as an energy source (Bertsch and Müller, 2015a; Köpke and Simpson, 2020; Schoelmerich and Müller, 2020; Schuchmann and Müller, 2014). Acetate is the main product of acetogens, but the product spectrum of different acetogenic strains is broad and includes ethanol, 2,3-butanediol, butanol, butyrate and others (Liew et al., 2016b). Chemical production using acetogens therefore is a promising alternative to energy intensive chemical processes such as the Fisher-Tropsch synthesis (Munasinghe and Khanal, 2010; Takors et al., 2018). An industrial process for ethanol production from CO using *Clostridium autoethanogenum* has already been commercialized by the leading company LanzaTech Inc (Skokie, IL, USA).

*Acetobacterium woodii* is a homoacetogenic bacterium known to efficiently utilize CO<sub>2</sub> and H<sub>2</sub> (Kantzow et al., 2015; Schuchmann and Müller, 2014). In addition to autotrophic growth, *A. woodii* can utilize a broad variety of substrates including C1 components such as formate and methanol (Drake et al., 1997; Schuchmann and Müller, 2016; Wiechmann et al., 2020). During growth on H<sub>2</sub> and CO<sub>2</sub>, 0.3 mol ATP are formed per mol of acetate produced (Schuchmann and Müller, 2014). This low ATP gain allows *A. woodii* to grow at very low H<sub>2</sub> partial pressures and explains why cultivation on H<sub>2</sub> and CO<sub>2</sub> is much easier compared to *C. autoethanogenum*, which has a higher H<sub>2</sub> threshold (Mock et al., 2015). *A. woodii* was the first organism where energy conservation was fully elucidated (Schuchmann and Müller, 2014) and additional metabolic properties continue to be described (Westphal et al., 2018; Wiechmann et al., 2020). Acetate produced by *A. woodii* can either be used directly or upgraded into other valuable products (Novak et al., 2020; Novak and Pflügl, 2018). The production of acetate is advantageous over a mixed product spectrum due to controllability of the process (Schwarz et al., 2020). Growth and acetate production on CO<sub>2</sub> and H<sub>2</sub> have been characterized in detail (Demler and Weuster-Botz, 2011; Kantzow et al., 2015) and the mechanism of CO utilization has been described (Arantes et al., 2020; Bertsch and Müller, 2015b; Schwarz et al., 2020), but data on utilization of gas containing both CO and CO<sub>2</sub> is scarce. The presence of CO inhibits bacterial hydrogenases, in particular the hydrogen-dependent CO<sub>2</sub> reductase (HDCR) (Bertsch and Müller, 2015b; Ragsdale and Ljungdahl, 1984), which leads to delayed growth in the presence of CO. However, growth can be restored by the addition of formate, the product of the HDCR (Bertsch and Müller, 2015b). Although initially reported to grow on CO as the sole carbon and energy source, it has recently been shown that *A. woodii* does not grow on CO alone (Bertsch and Müller, 2015b; Genthner and Bryant, 1987).

In contrast to *A. woodii*, CO utilization of several clostridial species has been broadly studied, owing to their enhanced CO utilization properties (Cotter et al., 2009; Mahamkali et al., 2020; Mohammadi et al., 2012; Valgepea et al., 2018, 2017). Special attention has been given to the utilization of synthesis gas, which contains large amounts of CO and H<sub>2</sub> as well as CO<sub>2</sub> in varying minor quantities (Takors et al., 2018). Compared to CO<sub>2</sub> and H<sub>2</sub>, growth of *Clostridium ljungdahli* and

*C. autoethanogenum* on CO shows higher growth rates, increased ATP generation and the production of reduced compounds such as ethanol (Hermann et al., 2020; Mahamkali et al., 2020). However, CO utilization is usually accompanied by CO<sub>2</sub> production, which has been reduced by the additional supply of H<sub>2</sub> to CO-grown *C. autoethanogenum* (Valgepea et al., 2018). The addition of H<sub>2</sub> also shifted the product spectrum towards more reduced end products, highlighting that the feed gas composition can be considered a critical factor for enhancing the economics of gas fermentation processes. A recent study has shown that the addition of 2% CO enhanced fermentation of CO<sub>2</sub> and H<sub>2</sub> in *C. autoethanogenum* (Heffernan et al., 2020), while utilization of syngas containing high amounts of CO resulted in the formation of CO<sub>2</sub> (Valgepea et al., 2018, 2017).

Due to the toxicity mechanisms of CO, a process focussing on CO<sub>2</sub> fixation in CO-containing gas (e.g. BFG) needs to aim for limitation of CO in the liquid medium to achieve co-utilization of CO, CO<sub>2</sub> and H<sub>2</sub>. This CO limitation can be achieved due to low solubility of CO in combination with high biomass and thereby high CO uptake rates (Bertsch and Müller, 2015a).

This study aimed to determine the effect of the gas composition (i.e. CO, CO<sub>2</sub> and H<sub>2</sub> partial pressures) on CO<sub>2</sub> utilization, growth, and acetate production in *A. woodii*. Considering the gas composition of industrial BFG as a starting point, idealized gases with and without CO blended with different concentrations of H<sub>2</sub> were used. This approach allowed to draw conclusions about effects on growth and acetate formation mediated by both H<sub>2</sub> and CO availability. Moreover, it was hypothesized that H<sub>2</sub> blending could also be a useful tool to enable co-utilization of CO, CO<sub>2</sub> and H<sub>2</sub> and net CO<sub>2</sub> fixation. Indeed, H<sub>2</sub> blending and limitation of the inhibitory gas CO in continuous cultures enabled *A. woodii* to efficiently produce acetate from industrial BFG. Using a metabolic model, the effect of gas composition on intracellular carbon, redox and energy metabolism was studied. The gas fermentation strategy used here provides valuable information on acetogenic metabolism and shows potential in upgrading industrial gas pollutants into value-added products.

## 2. Materials and methods

### 2.1. Organism and media

*Acetobacterium woodii* DSM 1030 was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and used for all cultivations.

The medium for all cultivations is based on media 135 suggested by DSMZ. Changes in the medium included omitting Na<sub>2</sub>S \* 9 H<sub>2</sub>O and an increase in Ca-pantothenate and FeSO<sub>4</sub>\*7 H<sub>2</sub>O. Bicarbonate-buffered medium was used for the initial experiments in serum bottles investigating growth on BFG. This medium was later replaced by phosphate-buffered medium which was used in serum bottle experiments investigating the effect of formate and for all bioreactor cultivations. Bicarbonate-buffered medium contained per liter: 1 g NH<sub>4</sub>Cl, 0.1 g MgSO<sub>4</sub>\*7H<sub>2</sub>O, 2 g yeast extract, 0.33 g KH<sub>2</sub>PO<sub>4</sub>, 0.45 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g cysteine-HCl-H<sub>2</sub>O, 10 g NaHCO<sub>3</sub>, 0.25 ml sodium resazurin (0.2% w/v), 20 ml trace element solution DSMZ 141, and 10 ml vitamin solution DSMZ 141. In phosphate-buffered serum bottle medium NaHCO<sub>3</sub> was replaced by 1.76 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 8.44 g l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub> and 3.47 g l<sup>-1</sup> NaCl. For mixotrophic precultures, 5 g l<sup>-1</sup> fructose or formate were added from anoxic sterile stocks of 250 g l<sup>-1</sup>. The trace element solution was adapted from medium DSMZ 141 and contained per liter: 1.5 g nitrilotriacetic acid, 3 g MgSO<sub>4</sub> \* 7 H<sub>2</sub>O, 0.5 g MnSO<sub>4</sub>\*H<sub>2</sub>O, 1 g NaCl, 0.1 g FeSO<sub>4</sub> \* 7 H<sub>2</sub>O, 0.152 g Co(II)Cl<sub>2</sub> \* 6 H<sub>2</sub>O, 0.1 g CaCl<sub>2</sub> \* 2 H<sub>2</sub>O, 0.18 g ZnSO<sub>4</sub> \* 7 H<sub>2</sub>O, 0.01 g CuSO<sub>4</sub> \* 5 H<sub>2</sub>O, 0.02 g KAl(SO<sub>4</sub>)<sub>2</sub> \* 12 H<sub>2</sub>O, 0.01 g boric acid, 0.01 g Na<sub>2</sub>MoO<sub>4</sub> \* 2 H<sub>2</sub>O, 0.033 g Ni(II)SO<sub>4</sub> \* 6 H<sub>2</sub>O, 0.3 mg Na<sub>2</sub>SeO<sub>3</sub> \* 5 H<sub>2</sub>O and 0.4 mg Na<sub>2</sub>WO<sub>4</sub> \* 2 H<sub>2</sub>O. The vitamin solution was prepared according to DSMZ medium 141 and contained per liter: 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg thiamine-HCl, 5 mg riboflavin,

5 mg nicotinic acid, 5 mg D-Ca-pantothenate, 0.1 mg vitamin B12, 5 mg *para*-aminobenzoic acid and 5 mg lipoic acid. The pH of all media was adjusted to 7.0 using 5 M KOH. For bioreactor cultivations, the media composition was adapted: concentration of the phosphate buffer was reduced to 0.33 g KH<sub>2</sub>PO<sub>4</sub> and 0.45 g K<sub>2</sub>HPO<sub>4</sub>, trace element and vitamin concentrations (except for Ca-pantothenate which was added to a concentration of 1 mg l<sup>-1</sup> in the final medium according to (Godley et al., 1990)) were doubled and the iron concentration was increased to 26.9 mg l<sup>-1</sup> FeSO<sub>4</sub>\*7 H<sub>2</sub>O in the final medium (Demler and Weuster-Botz, 2011).

Media was prepared using anaerobic techniques as described previously (Hungate, 1969). All chemicals were purchased from Roth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) or Merck (Merck KGaA, Darmstadt, Germany).

## 2.2. Gases and compositions

Premixed gas containing 80% H<sub>2</sub> and 20% CO<sub>2</sub> was obtained from Air Liquide Austria GmbH (Schwechat, Austria) and used for routine cultivations such as precultures. Industrial blast furnace gas (BFG) was filled into gas cylinders directly on site at the blast furnace of voestalpine Stahl GmbH (Linz, Austria). The gas composition represents the annual average of the online quantification by the multi-analysis-system Advance Optima AO2000 (ABB Asea Brown Boveri Ltd, Zürich, Switzerland).

BFG was blended with H<sub>2</sub> (Messer Austria GmbH, Gumpoldskirchen, Austria) to obtain H<sub>2</sub> concentrations of 30% (low H<sub>2</sub> blend) and 60% (high H<sub>2</sub> blend) using two independent mass flow controllers (Eppendorf AG, Hamburg, Germany and CRANE Instrumentation & Sampling, Spartanburg, SC, USA). Idealized gases with and without CO containing the same amount of CO<sub>2</sub>, CO and H<sub>2</sub> as the corresponding BFG-H<sub>2</sub> blend were purchased from Messer Austria GmbH (Gumpoldskirchen, Austria).

## 2.3. Preparation of precultures

*A. woodii* was stored as anoxic stocks at -80 °C in 125 g l<sup>-1</sup> saccharose. For cultivations, these stocks were transferred into 125 ml serum bottles containing 50 ml medium with 5 g l<sup>-1</sup> fructose. For mixotrophic growth, the atmosphere was exchanged to 80% H<sub>2</sub> and 20% CO<sub>2</sub> and a total pressure of 2.5 bar was applied. *A. woodii* was cultivated in a rotary shaker at 30 °C and 200 rpm (Infors AG, Bottmingen, Switzerland). For further cultivation in serum bottles, 2 ml of the mixotrophic culture was transferred anaerobically to fresh medium. Batch experiments in bioreactors were inoculated with an exponentially growing culture obtaining an optical density at 600 nm (OD<sub>600</sub>) of 0.1. For cultivations on gas without CO mixotrophic precultures were used, whereas cultivations on gas containing CO were inoculated with a preculture previously grown on 5 g l<sup>-1</sup> formate and the high H<sub>2</sub> blend of the BFG.

## 2.4. Experiments in serum bottles

Growth experiments in 125 ml serum bottles were carried out using 50 ml medium. During the initial experiments for growth on the industrial flue gas, bicarbonate-buffered medium was used and later replaced by phosphate-buffered medium for experiments with formate. The atmosphere in the head space was exchanged by the desired gas composition and pressurized with a total pressure of 2.5 bar. The industrial flue gas was diluted with H<sub>2</sub> using two mass flow controllers (Brooks Instrument, Matfield, USA). Upon gas consumption, the headspace was refilled with the corresponding gas at 1.5 bar. Samples of 2 ml

were regularly taken and used for OD<sub>600</sub> and high-performance liquid chromatography (HPLC) measurements.

## 2.5. Cultivations in bioreactors

Bioreactor cultivations were carried out in duplicates in a DASbox® Mini Bioreactor system (Eppendorf AG, Hamburg, Germany) with a working volume of 200 ml at a temperature of 30 °C. The pH was initially set to 7.0, monitored by a pH electrode EasyFerm Plus K8 120 (Hamilton, Reno, NV, USA) and controlled by the addition of 2 M phosphoric acid and 5 M KOH with a MP8 multi pump module (Eppendorf AG, Hamburg, Germany). The agitator speed was routinely set to 600 rpm and adjusted to 300 and 1200 rpm to investigate gas liquid mass transfer. In both batch and continuous cultures, the medium was continuously sparged with 0.25 vvm (3 sl h<sup>-1</sup>) of the indicated gas. Microspargers made of sintered metal with a pore size of 10 µm (Sartorius Stedim Biotech GmbH, Göttingen, Germany) were used for improved gas transfer into the liquid phase. Prior to inoculation, the reactor medium was flushed with the appropriate gas for at least 3 h. During continuous cultivations, feed medium and a 1% (w/v) solution of antifoam (Struktol SB2020, Schill und Seilacher, Hamburg, Germany) were continuously added with the MP8 pump module at a rate of 10 ml h<sup>-1</sup> and 0.2 ml h<sup>-1</sup>, respectively. To keep the reaction volume constant, culture broth was continuously removed by peristaltic pumps (Ismatec SA, Glattburg, Germany) via a dip tube and the cultivation volume was maintained at 197 ± 11 ml. The feed bottles were continuously flushed with 6 sl h<sup>-1</sup> N<sub>2</sub> to maintain anaerobic conditions. Steady state conditions were examined after a minimum of three volume changes when biomass and acetate concentrations as well as gas uptake was constant. Samples were taken in regular intervals to measure OD<sub>600</sub> and estimate biomass growth. The culture broth was centrifuged at 14,000 rpm (21,913 g) for 5 min and the supernatant was used for HPLC analysis of acetate and formate.

## 2.6. Biomass determination

Biomass concentrations were determined gravimetrically by transferring 5 ml of cultivation broth into pre-weighed glass tubes. The tubes were centrifuged at 4,800 rpm (2,396 g) for 10 min, washed with 5 ml of distilled water and centrifuged again. The biomass was dried for at least 72 h at 105 °C. Gravimetric biomass determination was carried out at the end of the batch phase and at the cultivation end. The optical density at 600 nm (OD<sub>600</sub>) was measured in a spectrophotometer (Genesys™ 20, Thermo Fisher Scientific, Waltham, MA, USA) against a water blank. Gravimetrically determined biomass concentrations were correlated with OD<sub>600</sub> values (biomass = 0.43 \* OD<sub>600</sub>) and this correlation was used to estimate the biomass concentration for all other time points.

## 2.7. HPLC analysis

Substrates and products in the liquid medium were determined with an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules/CA, USA) using an Ultimate 3000 system (Thermo Scientific, Waltham/MA, USA). 4 mM H<sub>2</sub>SO<sub>4</sub> was used as a mobile phase and the column was operated at 60 °C at a flow rate of 0.6 ml min<sup>-1</sup> for 30 min. 10 µl sample were injected onto the column (Erian et al., 2018). Detection was performed using a refractive index (Refractomax 520, Thermo Fisher Scientific, Waltham/MA, USA) and a diode array detector (Ultimate 3000, Thermo Fisher Scientific, Waltham/MA, USA). Chromeleon 7.2.6 Chromatography Data System (Thermo Fisher Scientific, Waltham/MA, USA) was used for control, monitoring and evaluation of the analysis. 450 µl of culture supernatant were mixed with 50 µl of 40 mM H<sub>2</sub>SO<sub>4</sub>

and centrifuged for 5 min at 14,000 rpm (21,913 g) at 4 °C. The remaining supernatant was used for further analysis. Standards at defined concentrations of formate, acetate, ethanol and fructose were treated the same way. A 5-point-calibration was used for quantification.

## 2.8. GC analysis

The concentrations of H<sub>2</sub>, CO<sub>2</sub> and CO were determined using a gas chromatograph (Trace Ultra GC, Thermo Fisher Scientific, Waltham/MA, USA). 100 µl of sample were injected at 100 °C with a split ratio of 20 on a ShinCarbon ST 100/120 packed column (Restek Corporation, Bellefonte/PA, USA). The oven was kept at 30 °C for 6.5 min, temperature was increased by 20 °C min<sup>-1</sup> to 250 °C and kept constant for 1.5 min. Argon 5.0 (Messer Austria GmbH, Gumpoldskirchen, Austria) was used as a carrier gas at a flow rate of 2 ml min<sup>-1</sup>. Samples were analyzed with a thermal conductivity detector. The filament temperature, cell block temperature and transfer temperature were set to 370 °C, 240 °C and 200 °C, respectively. The combination of two electrical valves enabled offgas measurement of each of the four reactors in 2-hour intervals. Chromeleon 7.2.10 Chromatography Data System (Thermo Scientific, Waltham/MA, USA) was used for control, monitoring and evaluation of the analysis. The gas composition of each gas was determined before and after its use in the fermentation process and gas uptake rates were determined by calculating the difference to these reference gas concentrations.

## 2.9. Rate calculations and elemental balancing

In batch experiments, volumetric acetate production rates  $r_{Ace}$  [mmol l<sup>-1</sup>h<sup>-1</sup>] were calculated for every sampling point according to the equation:

$$r_{Ace} = \frac{c_{Ace,t} - c_{Ace,(t-1)}}{\Delta t} \quad [1]$$

where  $c_{Ace}$  represents the acetate concentration [mmol l<sup>-1</sup>] at each time point and  $\Delta t$  the time difference [h] between measurements. Specific production rates  $q_{Ace}$  [mmol g<sup>-1</sup>h<sup>-1</sup>] were calculated according to:

$$q_{Ace} = \mu \frac{c_{Ace,t} - c_{Ace,(t-1)}}{c_{BM,t} - c_{BM,(t-1)}} \quad [2]$$

where  $c_{BM}$  represents the biomass concentration [g l<sup>-1</sup>] at each time point and  $\mu$  the specific growth rate [h<sup>-1</sup>]. The highest value of these uptake rates calculated between individual sampling points is described as the maximum production rate. Total volumetric acetate production rates were calculated by considering the final acetate concentration after the fermentation and the total process time.

The volumetric gas uptake rates HUR, COUR, and CO<sub>2</sub>UR [mmol l<sup>-1</sup>h<sup>-1</sup>] were calculated after normalization of the gas composition using N<sub>2</sub> as inert gas. For the calculation of CO<sub>2</sub>UR during continuous cultivations, the amounts of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> accounting for saturation of fresh medium were considered using the solubility of CO<sub>2</sub>, the Henderson-Hasselbalch equation assuming a pK<sub>s</sub> of 6.2 and the feeding rate. The specific gas uptake rates  $q_{H_2}$ ,  $q_{CO}$  and  $q_{CO_2}$  were obtained by dividing HUR, COUR and CO<sub>2</sub>UR [mmol g<sup>-1</sup>h<sup>-1</sup>] by the biomass concentration.

In chemostats,  $r_{Ace}$  was calculated by multiplying the average acetate concentration from at least 3 data points from steady state conditions by the liquid dilution rate D. Specific rates for gas uptake and acetate production were obtained by dividing the volumetric rate by the average biomass concentration of steady state data points.

For elemental balancing, biomass was assumed to contain 50% (w/w) carbon and 0.6% (w/w) hydrogen. The degree of reduction (DoR) of biomass was assumed to be 4.15 mol electrons per mol of carbon (Rittmann et al., 2012). For hydrogen balancing, water production was assumed to correlate to substrate uptake with 0.5 mol water produced per mol hydrogen taken up, 0.5 mol water per mol formate and 1 mol

water per mol CO (Bertsch and Müller, 2015b; Schuchmann and Müller, 2014). The influence of water formation can be neglected for the calculation of DoR balances. All balances closed well without consideration of yeast extract.

## 2.10. Metabolic modeling and flux balance analysis

To model intracellular fluxes, we adapted a previously published *A. woodii* core model (Koch et al., 2019) consisting of 118 reactions. The model considers energy conservation and redox balancing as previously described for *A. woodii* (Schuchmann and Müller, 2014). The biomass composition was assumed to be similar to *C. autoethanogenum* (Valgepea et al., 2017).

Flux balance analysis (FBA) was done using the *CellNetAnalyzer* toolbox (Klamt et al., 2007; von Kamp et al., 2017). For all simulations, the measured specific rates for biomass formation, substrate uptake rates (CO, CO<sub>2</sub>, H<sub>2</sub>) and acetate were used to constrain the model. These measurements bear redundancies with respect to carbon and redox balances in the metabolic model and, therefore, fluxes had to be corrected prior to the actual FBA calculations to obtain a consistent system. This was done by minimizing the relative changes in the measured rates necessary to obtain a consistent flux scenario ("Check feasibility" function in *CellNetAnalyzer*). FBA requires the formulation of an objective function and most FBA studies use maximization of biomass synthesis. Since the growth rate was fixed to the experimentally observed value, the pseudo reaction that quantifies the non-growth associated ATP maintenance (NGAM) demand was maximized instead. In this way, an upper bound of ATP available for NGAM processes was obtained.

## 3. Results and discussion

### 3.1. Gas composition

Industrial BFG typically contains high amounts of N<sub>2</sub>, ~ 20% CO and CO<sub>2</sub>, and ~ 3% H<sub>2</sub> (Hou et al., 2011). To determine the exact composition, the gas was filled into gas cylinders at the stack of the steel production site and analyzed for its main components (Table 1). The gas composition of the gas did not differ greatly from literature data (Hou et al., 2011; Molitor et al., 2016).

Some components of the BFG, especially high concentrations of CO, might inhibit growth of *A. woodii*. Growth was not possible on BFG alone (Fig. 2b), which confirmed that in contrast to other organisms, utilization of CO as the sole carbon and energy source is not possible in *A. woodii* (Bertsch and Müller, 2015b). The hypothesis was that blending BFG with H<sub>2</sub> would enable growth, acetate formation and CO<sub>2</sub> fixation in the CO-containing gas. To this end, two H<sub>2</sub> blending ratios were chosen: low (30%) and high (60%) H<sub>2</sub>. In accordance, CO and CO<sub>2</sub>

**Table 1**

Composition of gases used in this study. Low and high H<sub>2</sub> gases were blended with 30% H<sub>2</sub> and 60% H<sub>2</sub>, respectively. n.d. = not determined; errors for CO<sub>2</sub>, H<sub>2</sub> and CO determination in gas mixtures are 2% relative.

Blast furnace gas from industrial steel production site						
Gas composition [%]	N <sub>2</sub>	CO <sub>2</sub>	CO	H <sub>2</sub>		
	48.5	22.7	25.2	3.6		
Gas blends used in this study						
Gas composition [%]	Calculated value			Actual value		
	CO <sub>2</sub>	H <sub>2</sub>	CO	CO <sub>2</sub>	H <sub>2</sub>	CO
Idealized, low H <sub>2</sub>	16.5	30.0	–	16.6	30.3	–
Idealized, high H <sub>2</sub>	9.4	60.0	–	9.6	60.0	–
Idealized with CO, low H <sub>2</sub>	16.5	30.0	18.3	16.7	29.7	18.6
Idealized with CO, high H <sub>2</sub>	9.4	60.0	10.4	9.5	60.1	10.6
BFG, low H <sub>2</sub> *	16.5	30.0	18.3	n.d.	n.d.	n.d.
BFG, high H <sub>2</sub> *	9.4	60.0	10.4	8.8	62.2	9.8
Standard	20.0	80.0	–	20.0	80.0	–



partial pressures were higher in the low  $H_2$  blend compared to the high  $H_2$  blend. To investigate the effect of the presence of CO as well as other potential inhibitors in the industrial flue gas, performance of *A. woodii* was additionally investigated using idealized gases. Concentrations of CO,  $CO_2$  and  $H_2$  in idealized gases were identical to those in BFG blended with the same amount of  $H_2$ . In one approach, the gas only consisted of  $CO_2$ ,  $N_2$  and  $H_2$ , which allowed to determine effects mediated by the presence of CO. In another approach, CO,  $CO_2$ ,  $N_2$  and  $H_2$  were present and the effect of other potential inhibitors could be studied. Table 1 gives an overview of all gas compositions used in this study.

### 3.2. Utilization of blast furnace gas requires adaptation phase

In a first step, the utilization of different gases by *A. woodii* was tested in serum bottle experiments. Growth and acetate production were compared using idealized gas and BFG at the low and high  $H_2$  blend. Performance was also monitored for a standard gas composition of 20%  $CO_2$  and 80%  $H_2$ .

While growth and acetate production started immediately on idealized and standard gas blends, the culture containing CO displayed a lag phase of  $\sim 100$  h (Fig. 1a and 1c). This adaptation phase could be reduced by transferring cells from a culture growing on CO-containing BFG (stage 1) to fresh medium (stage 2, Fig. 1b and 1d). This observation implies that *A. woodii* can be adapted to growth on BFG by sequential transfers. Maximum acetate production rates were similar in both cultivations, but the total volumetric acetate productivity was increased in stage 2 cultivations due to the reduction of the adaptation phase (Table 2). An adaptation phase of *A. woodii* grown on CO has previously been described and the delay was hypothesized to be a result of the time required for expression of enzymes involved in CO metabolism (Bertsch and Müller, 2015b). Conclusively, the efficient utilization of BFG is possible in *A. woodii* when cells have previously been adapted to growth on CO-containing gas.

### 3.3. Formate addition reduces adaptation phase during growth on CO

CO toxicity in acetogens is caused by an inhibitory effect on hydrogenases, i.e. the hydrogen dependent  $CO_2$  reductase (HDCR). The methyl branch of the WLP can alternatively be fed with formate instead

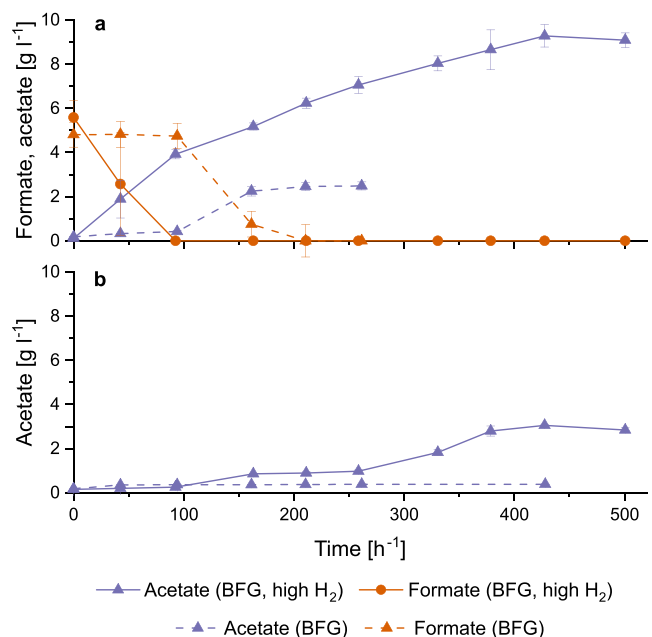


Fig. 2. Addition of formate circumvents adaptation phase of *A. woodii* during growth on CO-containing gas. Growth on industrial furnace gas with 60%  $H_2$  in phosphate-buffered medium is compared with (a) or without (b) the addition of 5 g  $l^{-1}$  formate. Results represent means and standard deviations from biological duplicates in serum bottle experiments.

of  $CO_2$  and  $H_2$ , which finally allows for growth and CO utilization (Bertsch and Müller, 2015b). To investigate whether the addition of formate can improve growth and production characteristics on BFG, further experiments were carried out in serum bottles. BFG alone and the high  $H_2$  blend BFG were used as carbon and energy source and cultures with and without formate were compared (Fig. 2).

While cultivations without formate displayed a growth delay of  $\sim 150$  h, no lag phase was observed when cultures were provided with formate. Growth and acetate production even continued in BFG with  $H_2$  after formate was depleted, indicating co-consumption of CO,  $CO_2$  and

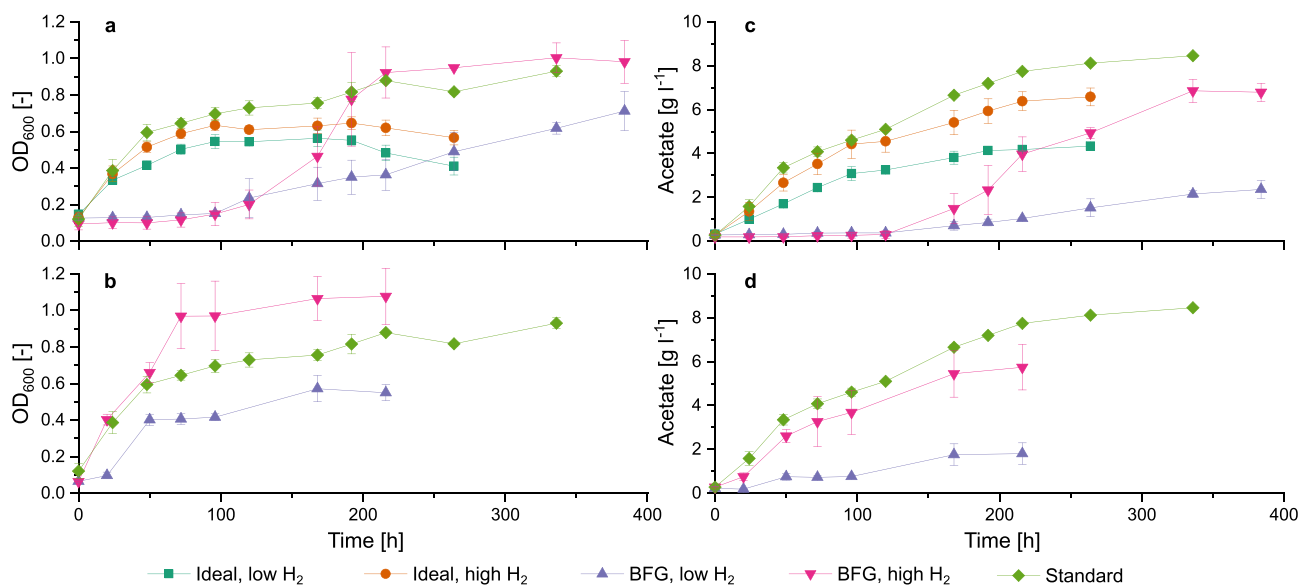


Fig. 1. Adaptation prevents growth delay in *A. woodii* during growth on CO containing gas. Industrial furnace gas (real) diluted with  $H_2$  was compared to idealized gas without CO and a standard mixture (80%  $H_2$ , 20%  $CO_2$ ). a and b inoculation from mixotrophically growing cells (Stage 1), c and d inoculation with adapted cells from the first stage (Stage 2). Bicarbonate-buffered medium was used. Results represent means and standard deviations from biological triplicates in serum bottle experiments.

**Table 2**

Maximum and total acetate productivity of *A. woodii* on gases of different compositions. Means and standard deviations of biological triplicates are shown in bicarbonate-buffered medium. After growth on CO-containing gas (stage 1), adapted cells were transferred to fresh medium (stage 2).

Gas blend	CO <sub>2</sub> [%]	H <sub>2</sub> [%]	CO [%]	$r_{\text{Ace, max}}$ [mmol l <sup>-1</sup> h <sup>-1</sup> ]	$r_{\text{Ace, total}}$ [mmol l <sup>-1</sup> h <sup>-1</sup> ]
Idealized, low H <sub>2</sub>	16.7	30.0	–	12.2 ± 0.1	7.9 ± 0.1
Idealized, high H <sub>2</sub>	9.5	60.0	–	21.8 ± 0.9	11.3 ± 0.9
BFG, low H <sub>2</sub> , stage 1	16.7	30.0	18.5	4.1 ± 0.5	2.2 ± 0.5
BFG, high H <sub>2</sub> , stage 1	9.5	60.0	10.6	27.2 ± 0.6	7.9 ± 0.6
BFG, low H <sub>2</sub> , stage 2	16.7	30.0	18.5	7.6 ± 0.5	3.7 ± 1.0
BFG, high H <sub>2</sub> , stage 2	9.5	60.0	10.6	24.7 ± 5.7	12.3 ± 2.5
Standard	20.0	80.0	–	29.3 ± 0.2	13.8 ± 0.2

H<sub>2</sub>. When CO was present as the sole carbon and energy source, acetate production ceased after formate depletion (Fig. 2a). More acetate was produced in experiments where formate was added, potentially due to a pH stabilizing effect of formate. Acetate titers in the experiment without formate are not comparable to initial experiments since phosphate-buffered instead of bicarbonate-buffered medium was used. Apart from differences in buffer capacities, the presence of bicarbonate potentially also influenced enzymes of the WLP or enzymes involved in energy conservation, as shown for *Thermoanaerobacter kivui* (Schwarz et al., 2020; Schwarz and Müller, 2020). Conclusively, the addition of formate to cultures of *A. woodii* containing CO in the gas phase reduced the lag-phase and enabled growth on BFG blended with H<sub>2</sub> after formate depletion.

### 3.4. Batch cultivations show feasibility of CO, CO<sub>2</sub> and H<sub>2</sub> co-utilization

In contrast to experiments in serum bottles, continuously supplied gas in bioreactor cultivations enhances substrate availability and improves productivity. To study whether growth of *A. woodii* on CO-containing BFG is also feasible in bioreactor cultivations and to

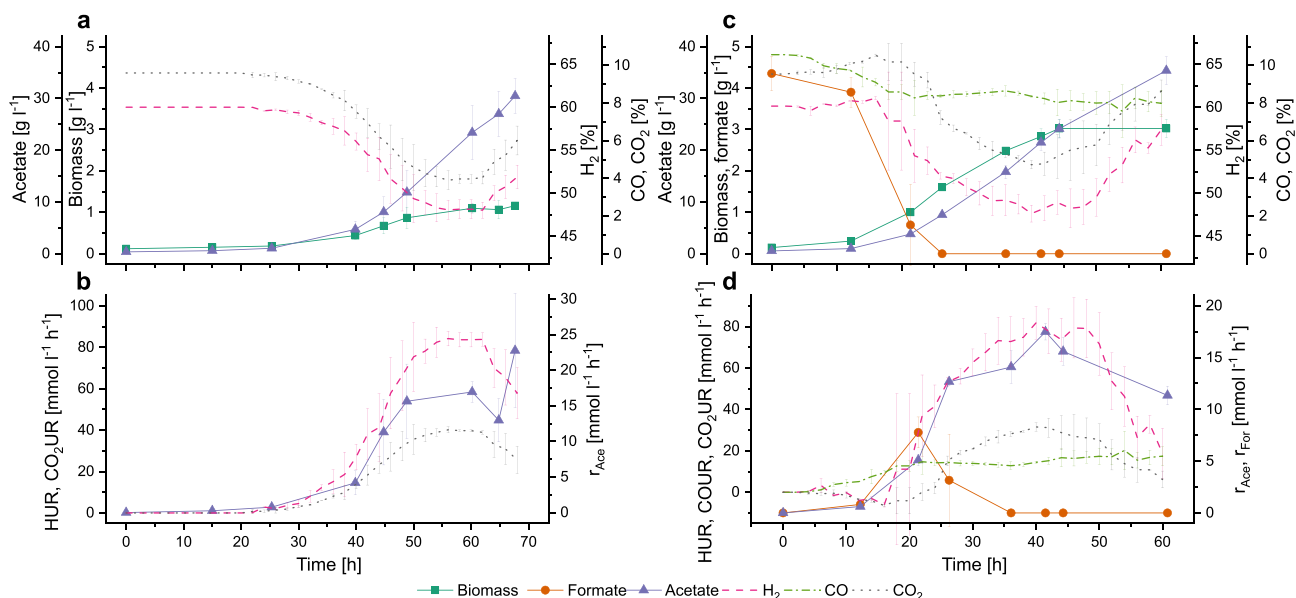
investigate acetate production and uptake characteristics of the individual gases, batch experiments were carried out. Since high H<sub>2</sub> blends were shown to result in superior production characteristics, this blend was chosen for cultivations with idealized gas with and without CO. Formate was added in cultivations using CO-containing gas to facilitate efficient growth.

During growth on gas with only CO<sub>2</sub> and H<sub>2</sub>, uptake rates of these two gases approximately represented a stoichiometric ratio of 2:1 (Fig. 3a and 3b). Gas uptake rates increased steadily until reaching their maximum after 50–60 h. High and inhibitory acetate concentrations in the culture broth might have been a reason for the subsequent decrease of gas uptake. Although biomass formation ceased in the late batch phase, acetate was still produced.

When CO was present in the gas, it was first co-utilized with formate, resulting in the production of CO<sub>2</sub> and H<sub>2</sub> (Fig. 3c and 3d). As soon as CO in the offgas dropped below 8.5% CO (from the initial 10.6%), CO<sub>2</sub> and H<sub>2</sub> uptake started. Subsequently, CO, CO<sub>2</sub> and H<sub>2</sub> co-utilization progressed and continued even after the depletion of formate. In accordance with cultivations on idealized gas without CO, the uptake rates of CO<sub>2</sub> and H<sub>2</sub> decreased after ~ 50 h of cultivation. Product inhibition by high acetate concentrations could have been responsible for these decreasing gas uptake rates. While inhibition by acetate has been described to occur between 8 and 12 g l<sup>-1</sup> acetate (Kantzow et al., 2015), in this study impaired gas uptake rates were only observed when acetate titers exceeded 20 g l<sup>-1</sup>.

The final biomass concentration was 3-fold higher in cultivations on gas containing CO, whereas the final acetate titer was increased by 16% (Table 3). Data from bioreactor cultivations therefore confirmed the previous observation that the utilization of CO is beneficial for biomass formation in *A. woodii* due to a 5-fold higher ATP yield (Bertsch and Müller, 2015a,b). Volumetric H<sub>2</sub> uptake rates were comparable in both cultivations, which suggests a limitation during the exponential growth phase. Increased biomass concentrations during cultivation on CO, CO<sub>2</sub> and H<sub>2</sub> in combination with comparable H<sub>2</sub> uptake rates generally resulted in decreased specific uptake rates on CO-containing gas.

The maximum specific growth rate ( $\mu_{\text{max}}$ ) was ~ 0.1 h<sup>-1</sup> on both gases (Table 3) and is comparable to values previously reported (0.112 h<sup>-1</sup> and 0.125 h<sup>-1</sup> on H<sub>2</sub>/CO<sub>2</sub> and formate-CO, respectively) (Bertsch and Müller, 2015b).



**Fig. 3.** Growth and acetate production in batch cultivations of *A. woodii* on idealized gas blends. Idealized gas without CO (a, b) and with CO (c, d) with 60% H<sub>2</sub> were compared. The gas composition (in %) in a and c is based on the gas flow rate of the ingas. Results represent mean values and standard deviations from biological duplicates of bioreactor cultivations.

**Table 3**

Fermentation characteristics of *A. woodii* cultivations in batches on idealized gas mixtures containing 60% H<sub>2</sub> with and without CO. Maximum volumetric and specific production (acetate) and uptake (H<sub>2</sub>, CO<sub>2</sub> and CO) rates are shown. Means and standard deviations were calculated from biological duplicates.

Gas blend	Titer [g l <sup>-1</sup> ]		Max. spec. growth rate [h <sup>-1</sup> ] $\mu_{\max}$	Maximum volumetric rates [mmol l <sup>-1</sup> h <sup>-1</sup> ]				Maximum specific rates [mmol g <sup>-1</sup> h <sup>-1</sup> ]				Balance [%]		
	Acetate	Biomass		r <sub>Ac</sub>	HUR	CO <sub>2</sub> UR	COUR	q <sub>Ac</sub>	q <sub>H2</sub>	q <sub>CO2</sub>	q <sub>CO</sub>	Carbon	Hydrogen	DoR
Idealized without CO, high H <sub>2</sub>	30.5 ± 3.3	1.16 ± 0.08	0.10 ± 0.03	22.8 ± 8.0	84.1 ± 4.2	40.3 ± 0.4	–	21.1 ± 2.0	82.9 ± 4.8	39.6 ± 0.3	–	112 ± 16	108 ± 3	108 ± 6
Idealized with CO, high H <sub>2</sub>	35.4 ± 2.7	3.02 ± 0.22	0.13 ± 0.01	17.5 ± 0.8	79.8 ± 13.8	31.2 ± 4.6	19.8 ± 1.3	9.9 ± 0.4	41.0 ± 13.5	17.5 ± 1.9	11.8 ± 1.3	87 ± 1	101 ± 7	82 ± 6

### 3.5. Continuous cultivations are limited in gas liquid mass transfer of H<sub>2</sub>

After demonstrating simultaneous CO, CO<sub>2</sub> and H<sub>2</sub> uptake in batch cultivations, this study aimed to characterize gas uptake, growth, and acetate formation under steady state conditions in continuous cultivations of *A. woodii*. Therefore, gas-limited cultures were established as a basis for solid characterization of the process performance. To study H<sub>2</sub> limitation, the influence of different H<sub>2</sub> partial pressures and gas-liquid mass transfer rates were first investigated independently from the presence of CO. Chemostats were performed on low (30%) and high (60%) H<sub>2</sub> blend idealized gas (without CO) at a dilution rate of 0.05 h<sup>-1</sup> (~50% of  $\mu_{\max}$ ).

Acetate was produced at a rate of 15.6 mmol l<sup>-1</sup>h<sup>-1</sup> at 600 rpm in the high H<sub>2</sub> blend, which is ~20% higher compared to the low H<sub>2</sub> blend and 60% of the rates observed in batch experiments. H<sub>2</sub> and CO<sub>2</sub> uptake rates were enhanced by 20% due to the improved availability of H<sub>2</sub> in the high H<sub>2</sub> blend. Gas transfer is typically proportional to partial pressures and several factors can influence hydrogen solubility (Demler and Weuster-Botz, 2011). Apart from media additives like e.g. antifoam, increased biomass concentrations were reported to decrease H<sub>2</sub> solubility (Cotter et al., 2009). Subsequently, it was examined whether cultivations at 600 rpm were limited in H<sub>2</sub> gas-liquid mass transfer by increasing and decreasing the stirrer speed to 1200 and 300 rpm, respectively.

Increasing the stirrer speed from 600 rpm to 1200 rpm improved gas-liquid mass transfer as indicated by enhanced gas uptake rates (Fig. 4). In both approaches at the high and low H<sub>2</sub> blend, the enhanced volumetric power input increased volumetric H<sub>2</sub> uptake and acetate production rates to 150%, while specific uptake rates stagnated or increased by 30% in the high and low H<sub>2</sub> blend, respectively (Table 4). A decrease of the stirrer speed from 600 rpm to 300 rpm drastically reduced H<sub>2</sub> uptake and acetate production to ~20%. Therefore, cultures were indeed H<sub>2</sub>-limited at a stirrer speed of 600 rpm.

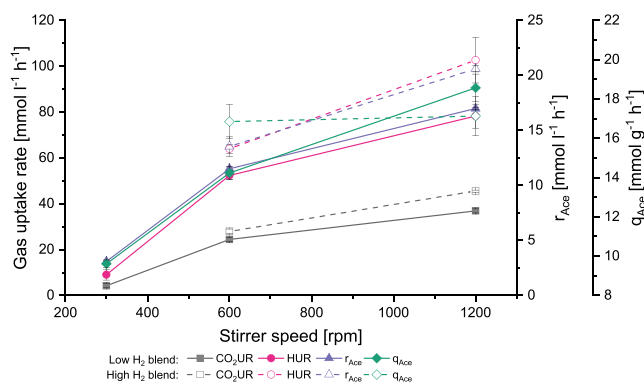
In addition to a limitation of the H<sub>2</sub> gas-liquid mass transfer,

continuous cultivations on H<sub>2</sub> and CO<sub>2</sub>, could be limited by the following mechanisms: (i) limitation in the liquid medium, e.g. nitrogen source or a trace element, (ii) CO<sub>2</sub> limitation, (iii) a physiological limitation, i.e. by reaching maximum uptake or production per cell or (iv) product inhibition. To exclude any other limitation the other four limitation possibilities were systematically examined. The cultivation performance was not restrained by neither yeast extract nor trace elements, since individual pulses of these components at steady state conditions showed no effect on gas uptake rates, biomass, or acetate production (data not shown). A limitation in gas-liquid transfer of CO<sub>2</sub> is not probable, because the CO<sub>2</sub> transfer rate is still 4.5-fold higher than the H<sub>2</sub> transfer rate under the conditions tested (pH<sub>2</sub> = 60%, pCO<sub>2</sub> ~ 10%). Since the acetate concentration as well as the specific H<sub>2</sub> uptake and acetate production rates were higher at 1200 compared to 600 rpm, a physiological limitation or product inhibition is also unlikely.

Production rates obtained in chemostat experiments (13.5 mmol l<sup>-1</sup>h<sup>-1</sup>) are within 7% to previous reports, where a slightly lower acetate productivity of 12.6 mmol l<sup>-1</sup>h<sup>-1</sup> (=302.4 mmol l<sup>-1</sup> d<sup>-1</sup> = 18.2 g l<sup>-1</sup> d<sup>-1</sup>) was observed during continuous cultivations of *A. woodii* (Kantow et al., 2015). There, acetate productivity but not acetate concentration was increased in a cultivation system with cell retention. In contrast to this study, H<sub>2</sub> uptake rates were not comparable in batch and chemostat experiments. Concomitantly, acetate concentrations reached ~22 g l<sup>-1</sup> in continuous cultivations (Kantow et al., 2015). Since product inhibition was observed at acetate concentrations above 20 g l<sup>-1</sup> in batch experiments of this study, it is possible that continuous cultivations described by Kantow et al. (2015) were limited by product inhibition rather than by H<sub>2</sub> transfer. When aiming for gas-limited processes, the focus should therefore be laid on achieving acetate concentrations below 20 g l<sup>-1</sup>. Conclusively, it was shown that autotrophic cultivations with *A. woodii* on H<sub>2</sub> and CO<sub>2</sub> are very flexible regarding the gas composition and gas-liquid mass transfer. Cultivations at 600 rpm at low and high H<sub>2</sub> blend were limited in H<sub>2</sub> availability, which makes these conditions suitable for further process characterization.

### 3.6. Dynamic gas-liquid mass transfer shifts allow to approximate maximum q<sub>H2</sub>

To determine the physiological limit of the gas fermentation system, this study aimed to approximate the maximum possible specific H<sub>2</sub> uptake rate. This important characteristic of the host strain can contribute to a better understanding of the physiology of *A. woodii* and facilitate further process development. To this end, a dynamic experiment with a shift from lower to higher volumetric power input and thus from lower to higher hydrogen transfer rate was performed. At low biomass concentrations, *A. woodii* cannot utilize all available H<sub>2</sub> and will grow at maximum q<sub>H2</sub> until cells are no longer physiologically limited. Since uptake rates of H<sub>2</sub> and CO<sub>2</sub> as well as acetate are stoichiometrically dependent (H<sub>2</sub> : CO<sub>2</sub> ~ 2:1, H<sub>2</sub> : acetate ~ 4:1), different approaches can be used to approximate the maximum q<sub>H2</sub>: directly via online measurement of HUR or indirectly via online measurement of CO<sub>2</sub> and off-line determination of acetate.



**Fig. 4.** Increased gas liquid mass transfer improves growth of *A. woodii* on CO<sub>2</sub> and H<sub>2</sub>. Mass transfer was improved by increasing stirrer speed. Results represent means and standard deviations of biological duplicates in steady state during continuous cultivation.

**Table 4**

Steady state data of *A. woodii* chemostat cultivations on idealized gas mixtures without CO under varying mass transfer. Means and standard deviations were calculated from biological duplicates. DoR = degree of reduction.

Idealized without CO	Stirrer speed [rpm]	Titer [g l <sup>-1</sup> ]		Volumetric rates [mmol l <sup>-1</sup> h <sup>-1</sup> ]			Specific rates [mmol g <sup>-1</sup> h <sup>-1</sup> ]			Yields [mol mol <sup>-1</sup> ]		Balance [%]		
		Biomass	Acetate	r <sub>Ace</sub>	HUR	CO <sub>2</sub> UR	q <sub>Ace</sub>	q <sub>H<sub>2</sub></sub>	q <sub>CO<sub>2</sub></sub>	Y <sub>Ace/CO<sub>2</sub></sub>	Y <sub>Ace/H<sub>2</sub></sub>	Carbon	Hydro-gen	DoR
Low H <sub>2</sub> blend	300	0.32 ± 0.02	3.7 ± 0.3	3.1 ± 0.1	9.0 ± 2.4	4.2 ± 1.6	9.6 ± 0.2	27.9 ± 9.6	12.3 ± 4.8	0.67 ± 0.29	0.36 ± 0.10	123 ± 35	104 ± 17	114 ± 36
	600	0.82 ± 0.02	13.3 ± 0.91	11.5 ± 0.2	52.3 ± 1.7	24.3 ± 0.9	14.2 ± 0.3	64.6 ± 1.3	30.1 ± 0.8	0.47 ± 0.02	0.22 ± 0.01	103 ± 4	99 ± 3	95 ± 4
	1200	0.92 ± 0.03	17.2 ± 0.1	17.0 ± 0.1	78.2 ± 5.2	36.3 ± 0.8	18.6 ± 0.7	85.6 ± 8.5	39.7 ± 3.1	0.47 ± 0.02	0.22 ± 0.01	100 ± 5	94 ± 3	93 ± 7
High H <sub>2</sub> blend	600	0.80 ± 0.01	15.6 ± 0.2	13.5 ± 0.9	64.0 ± 2.0	28.6 ± 1.0	16.9 ± 0.9	79.7 ± 1.2	35.6 ± 1.8	0.48 ± 0.05	0.21 ± 0.01	102 ± 10	93 ± 1	90 ± 3
	1200	1.20 ± 0.05	19.5 ± 0.2	20.6 ± 0.2	102 ± 10	46.2 ± 0.5	17.1 ± 1	85.2 ± 4.5	38.4 ± 2.1	0.45 ± 0.01	0.20 ± 0.02	96 ± 1	91 ± 5	87 ± 10

After an increase of the stirrer speed at 280.2 h, specific H<sub>2</sub> and CO<sub>2</sub> uptake instantly increased, remained constant for ~ 20 h and subsequently decreased (Fig. 5a). To correct for errors and variations in quantification of specific uptake rates, the volumetric rates were plotted over the biomass concentration during growth at maximum q<sub>H<sub>2</sub></sub> (Fig. 5b). Data was fitted by linear regression, where the slope represents the corresponding maximum specific rate. These fitted specific rates indicate that the maximum specific H<sub>2</sub> uptake rate q<sub>H<sub>2</sub>,max</sub> is between 116 and 167 mmol g<sup>-1</sup> h<sup>-1</sup> (Table 5). By setting the gas uptake rate to 0

mmol l<sup>-1</sup> h<sup>-1</sup> and solving the linear regression for the biomass concentration (X), the result represents the amount of biomass produced at no gas uptake. This biomass production can be attributed to the presence of yeast extract in the medium and was used to correct carbon balances of chemostat cultivations at low gas transfer rates (300 rpm). An approximation of the acetate production from yeast extract was performed accordingly.

Comparing the approximated q<sub>H<sub>2</sub>,max</sub> to steady state data on idealized gas revealed that all specific rates obtained at steady state conditions were lower than the possible maximum. Therefore, a physiological limitation of *A. woodii* in any chemostat cultivation in this study is unlikely.

### 3.7. *A. woodii* can efficiently utilize CO<sub>2</sub> in gases containing CO

After characterizing growth of *A. woodii* on CO<sub>2</sub> and H<sub>2</sub> containing gases, CO, CO<sub>2</sub> and H<sub>2</sub> utilization in BFG was studied and the effects of CO on *A. woodii* metabolism was determined. To this end, continuous cultivations were compared using idealized gas with CO at low and high H<sub>2</sub> blends and BFG at high H<sub>2</sub> blend.

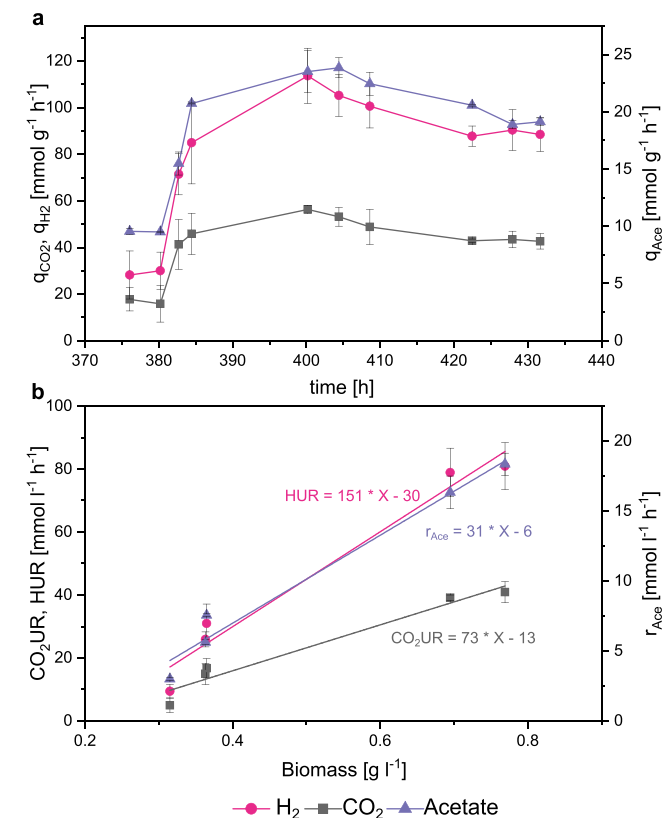
The transition from batch to continuous cultures was found to be crucial for successful growth on CO-containing gas. The optimal starting point for the continuous cultivation was after formate had been depleted, but when CO, CO<sub>2</sub> and H<sub>2</sub> were still co-utilized and before CO<sub>2</sub> and H<sub>2</sub> uptake decreased at the end of the batch. Since formate was not added to the feed, all cultivations on CO-containing gas were carried out autotrophically similar to chemostats without CO.

*A. woodii* was able to co-utilize CO, CO<sub>2</sub> and H<sub>2</sub> in all gas compositions and even at higher CO partial pressures (low H<sub>2</sub> blend, Table 6). A comparison of steady state data confirmed observations from batch experiments, where biomass increased with increasing CO partial pressure (decreasing pH<sub>2</sub>). Higher CO partial pressures decreased acetate titers, which was also reflected by the higher specific acetate productivity in

**Table 5**

Process characteristics of *A. woodii* obtained from mass transfer shift experiment. To increase gas transfer into the liquid phase, stirrer speed was increased from 300 to 1200 rpm. Data was obtained from linear regression of volumetric rates with biomass concentrations. Means and standard error of regression are shown.

Calculation for	Slope = specific gas uptake rate [mmol g <sup>-1</sup> h <sup>-1</sup> ]	R <sup>2</sup>	q <sub>H<sub>2</sub>, max</sub> [mmol g <sup>-1</sup> h <sup>-1</sup> ]	Intercept [mmol l <sup>-1</sup> h <sup>-1</sup> ]	Biomass at no gas uptake [g l <sup>-1</sup> ]
H <sub>2</sub>	151 ± 16	0.966	151 ± 16	-30.4 ± 8.7	0.20 ± 0.08
CO <sub>2</sub>	73 ± 9	0.957	146 ± 18	-13.3 ± 4.8	0.18 ± 0.09
Acetate	32 ± 3	0.974	128 ± 12	-5.6 ± 1.6	–



**Fig. 5.** Approximation of maximum q<sub>H<sub>2</sub></sub> using dynamic shift experiments of *A. woodii* on CO<sub>2</sub> and H<sub>2</sub>. a, specific uptake and production rates. Cultivation was shifted from low mass transfer (300 rpm) to high mass transfer (1200 rpm) at 380.2 h. b, volumetric CO<sub>2</sub> and H<sub>2</sub> uptake and acetate production from 380.2 h to 440.4 h (linear range). The data was fitted by linear regression (X = biomass). The slope represents the maximum specific rate and when X is set to zero, the biomass concentration at no gas uptake (and thus only from yeast extract in the medium) can be obtained. Results represent means and standard deviations of biological duplicates during continuous cultivation.



**Table 6**

Steady state data of *A. woodii* chemostat cultivations on gas mixtures containing CO. Stirrer speed was set to 600 rpm. Means and standard deviations were calculated from biological duplicates DoR = degree of reduction.

Gas blend	Titer [g l <sup>-1</sup> ]		Volumetric rates [mmol l h <sup>-1</sup> ]				Specific rates [mmol g <sup>-1</sup> h <sup>-1</sup> ]				Yields [mol mol <sup>-1</sup> ]		Balance [%]		
	Biomass	Acetate	r <sub>Acc</sub>	HUR	COUR	CO <sub>2</sub> UR	q <sub>Acc</sub>	q <sub>H2</sub>	q <sub>CO</sub>	q <sub>CO2</sub>	Y <sub>Acc/CO2</sub>	Y <sub>Acc/H2</sub>	Carbon	Hydrogen	DoR
Idealized with CO, low H <sub>2</sub> blend	1.86 ± 0.07	13.4 ± 0.7	12.6 ± 0.7	40.8 ± 2.5	22.1 ± 0.9	10.6 ± 0.2	6.8 ± 0.6	21.9 ± 0.6	11.9 ± 0.4	5.5 ± 0.1	0.37 ± 0.01	0.29 ± 0.01	89 ± 3	86 ± 6	95 ± 8
Idealized with CO, high H <sub>2</sub> blend	1.54 ± 0.12	17.8 ± 1.5	16.6 ± 0.7	71.6 ± 2.3	13.4 ± 1.4	26.2 ± 0.2	10.8 ± 0.4	46.6 ± 2.2	8.7 ± 0.2	17.1 ± 1.2	0.43 ± 0.01	0.23 ± 0.01	93 ± 1	87 ± 1	87 ± 1
BFG, high H <sub>2</sub> blend	1.27 ± 0.06	15.1 ± 0.7	14.3 ± 0.4	72.2 ± 8.0	12.5 ± 8.1	20.8 ± 5.7	11.1 ± 0.8	51.2 ± 13.0	9.5 ± 6.3	16.1 ± 5.5	0.44 ± 0.04	0.23 ± 0.05	97 ± 8	86 ± 1	81 ± 3

the high H<sub>2</sub> blend compared to the low H<sub>2</sub> blend. Uptake rates of CO, CO<sub>2</sub> and H<sub>2</sub> differed for the low and high H<sub>2</sub> blend. While HUR was 1.8-fold higher in the high H<sub>2</sub> blend of idealized gas with CO, COUR was 1.7-fold lower. These ratios correspond to the differences in partial pressures of the two gases, suggesting that cultures were limited in CO and H<sub>2</sub>. CO and H<sub>2</sub> limitation were also experimentally demonstrated as decreasing the stirrer speed from 600 rpm to 300 rpm resulted in decreased gas uptake rates (data not shown). Surprisingly, H<sub>2</sub> uptake rates were lower in high H<sub>2</sub> blends when gases did not contain CO (Table 4 and 6). Apart from the influence of biomass concentration on H<sub>2</sub> and CO solubility (Cotter et al., 2009; Rittmann et al., 2012), the presence of other gases could potentially influence gas uptake or solubility. Since H<sub>2</sub> was the limiting factor in all chemostats, the uptake of CO<sub>2</sub> was stoichiometrically coupled to H<sub>2</sub> utilization. Because CO was utilized as an additional carbon and energy source, total carbon turnover (i.e. CO and CO<sub>2</sub>) was higher compared to gases without CO. Utilization of CO reduced CO<sub>2</sub> uptake, but still enabled net CO<sub>2</sub> fixation even in the low H<sub>2</sub> blend with a high partial pressure of CO.

Cultivations of *A. woodii* on industrial BFG resulted in similar gas uptake rates compared to idealized gas with CO and the same H<sub>2</sub> blending ratio. Biomass and acetate production were slightly decreased, and acetate productivity reached 86% of the rate on idealized gas of the same composition. The specific acetate productivity remained constant due to identical H<sub>2</sub> and CO partial pressures. The slight difference in production rates on real and idealized gas with CO indicates that other inhibitors in the BFG could have had a minor influence on the performance of *A. woodii*. Gas uptake rates during the cultivation on BFG varied to a greater extent than on idealized gas with CO. These variations can probably be attributed to the *in situ* blending of two independent gas streams, whereas premixed gases were used for all other bioreactor experiments. The fact that *A. woodii* was not negatively influenced by these fluctuations renders the organism suitable for industrial applications, since similar deviations of the gas composition might also occur in large-scale fermentations.

During growth on CO<sub>2</sub> and H<sub>2</sub> alone, specific H<sub>2</sub> uptake rates of *C. autoethanogenum* were ~ 60% of those in *A. woodii* (Heffernan et al., 2020). Based on the comparison of specific CO and H<sub>2</sub> uptake rates, the high efficiency of H<sub>2</sub> and CO<sub>2</sub> utilization by *A. woodii* compared to *C. autoethanogenum* and *C. ljungdahlii* was therefore confirmed in this study (Hermann et al., 2020; Schuchmann and Müller, 2014; Takors et al., 2018). On the other hand, for gases with comparable CO and H<sub>2</sub> partial pressures, higher specific CO uptake rates were observed in *C. autoethanogenum*, while q<sub>H2</sub> was only 60% of the maximum reached using BFG in this study (Valgepea et al., 2018).

The fact that CO utilization in *A. woodii* is less efficient compared to other acetogens has long been described. Potential reasons include that *A. woodii* is more affected by the inhibition of hydrogenases by CO compared to other acetogens because the organism reduces CO<sub>2</sub> directly with H<sub>2</sub> instead of redox equivalents (NAD(P)H and/or reduced

ferredoxin) used by *C. autoethanogenum* and *C. ljungdahlii* (Bertsch and Müller, 2015a,b; Mock et al., 2015; Ragsdale and Ljungdahl, 1984; Schuchmann and Müller, 2014; Schwarz et al., 2020). In addition to hydrogenase inhibition, the efficiency of the CO dehydrogenase reaction has also been speculated to be responsible for efficient CO utilization (Liew et al., 2016a; Ragsdale et al., 1983). *T. kivui* could be adapted to growth on high concentrations of CO even though the organism also uses H<sub>2</sub> for CO<sub>2</sub> reduction to formate (Weghoff and Müller, 2016). Therefore, it was suggested that monofunctional CO dehydrogenases might play an important role in CO metabolism to quickly scavenge CO and prevent hydrogenase inhibition.

Fermentation of CO<sub>2</sub> and H<sub>2</sub> using *C. autoethanogenum* was found to be improved by adding small amounts of CO (Heffernan et al., 2020). While CO<sub>2</sub> and H<sub>2</sub> partial pressures were comparable to this study, CO concentrations in the gas were only 2%. In this study, net CO<sub>2</sub> fixation was achieved by simultaneous uptake of CO<sub>2</sub> and H<sub>2</sub> with CO at higher CO and lower H<sub>2</sub> concentrations of 18 and 30%, respectively. CO and H<sub>2</sub> partial pressures of 15 and 45%, respectively, comparable to this study promoted efficient CO utilization and a shift towards reduced metabolites in *C. autoethanogenum* but resulted in the production of small amounts of CO<sub>2</sub> (Valgepea et al., 2018).

Prior to this study, *A. woodii* had not been considered for fermentation of CO-rich industrial gases. Moreover, CO utilization at comparable gas compositions usually results in net CO<sub>2</sub> production (Hermann et al., 2020; Valgepea et al., 2018, 2017). Here, it was shown that *A. woodii* is highly flexible regarding co-utilization of CO, CO<sub>2</sub> and H<sub>2</sub> at different gas compositions allowing net CO<sub>2</sub> fixation. H<sub>2</sub> blending in combination with an efficient H<sub>2</sub> metabolism renders *A. woodii* a suitable host for CO<sub>2</sub> fixation from industrial gases even in the presence of CO.

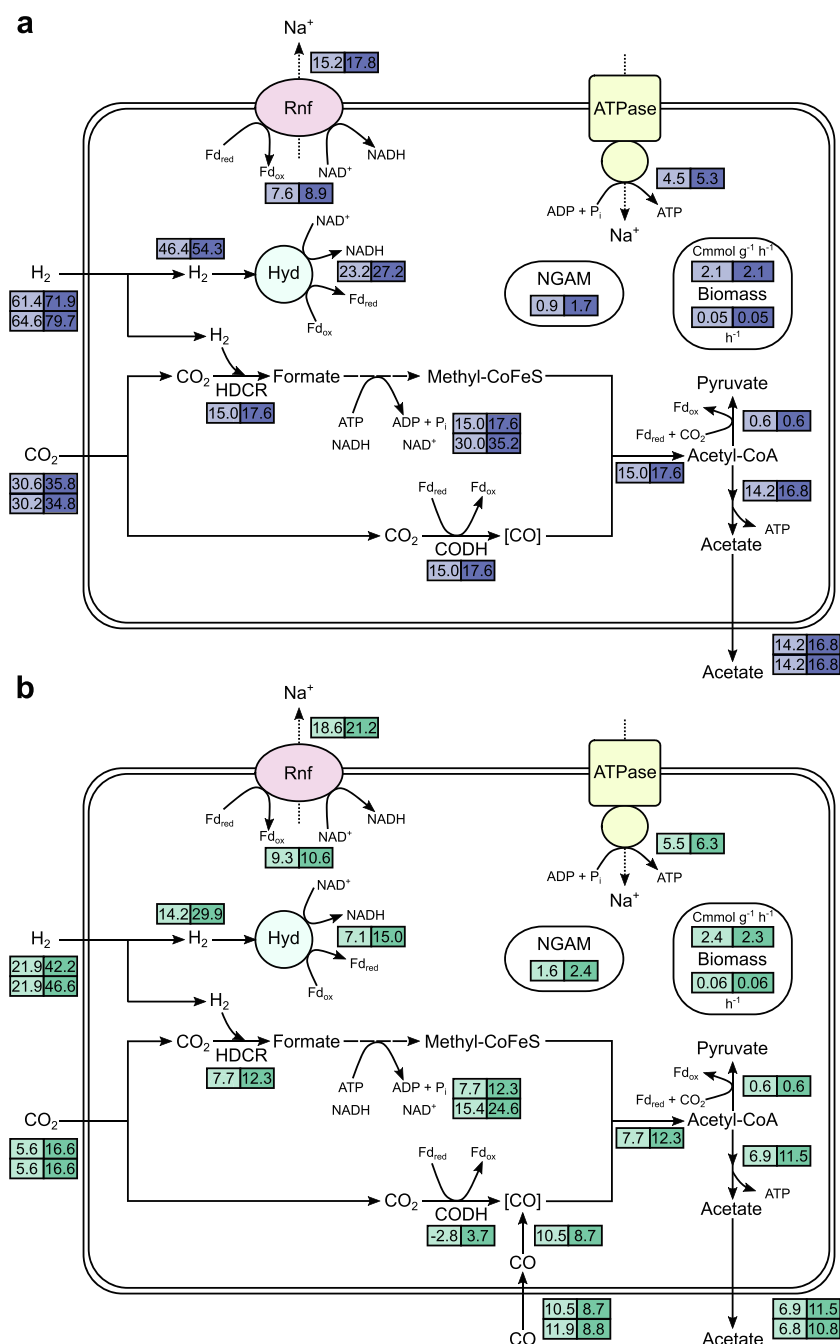
### 3.8. Intracellular flux distributions depend on H<sub>2</sub> and CO availability

To model intracellular flux distributions, specific uptake and production rates from chemostat cultivations on idealized gas with and without CO were. An *A. woodii* core model was used for FBA simulations. In typical FBA studies, maximization of biomass synthesis is used as objective function. Since the growth rate was fixed to the experimentally observed value, the pseudo reaction that quantifies the non-growth associated ATP maintenance (NGAM) demand was maximized instead. In this way, an upper bound of ATP available for NGAM processes was obtained. Fig. 6 summarizes the simulation results for intracellular fluxes of the carbon, redox and energy metabolism. Experimental data of biomass formation as well as specific uptake (CO, CO<sub>2</sub> and H<sub>2</sub>) and acetate excretion rates proved to be very consistent, since in all scenarios considered only small corrections for measured fluxes were necessary (Fig. 6). In terms of ATP production, the specific flux through the ATPase increased by ~ 20% for high H<sub>2</sub> compared to low H<sub>2</sub> for H<sub>2</sub>/CO<sub>2</sub> fermentations. ATP generation was further increased in the presence of CO regardless of the H<sub>2</sub> partial pressure. In detail, low and high CO partial

pressures increased flux through the ATPase by an additional 20% compared to the corresponding  $H_2/CO_2$  without CO. Likewise, the NGAM was lowest for the condition using low  $H_2$  without CO ( $0.9 \text{ mmol g}^{-1} \text{ h}^{-1}$ ) and highest for the high  $H_2$  in combination with CO ( $2.4 \text{ mmol g}^{-1} \text{ h}^{-1}$ ). Generally, higher maintenance ATP demands in the presence of high  $H_2$  or CO could be a result of additional protein synthesis required. Furthermore, higher acetate concentrations were observed when high  $H_2$  was used, which could also increase the ATP demand to maintain the proton gradient across the membrane.

Model simulations showed that at lower CO and high  $H_2$  partial pressures (i.e. high  $H_2$  blend), CO was only assimilated in the carbonyl branch, thus only serving as a carbon, but not as an electron source (Fig. 6b). Usually, CO serves as the main electron source during acetogenic growth on CO or syngas and  $CO_2$  produced is only partially utilized in the methyl branch (Hermann et al., 2020; Valgepea et al., 2018,

2017), which in this study was the case at higher CO and lower  $H_2$  partial pressures (i.e. low  $H_2$  blend). CO was therefore used both as a carbon and electron source, which resulted in increased availability of reducing equivalents, i.e. reduced ferredoxin. This change in metabolic activity finally led to differences in co-factor distribution and energy conservation. In this study, net  $CO_2$  fixation was achieved in gases at  $CO/H_2$  ratios of 0.18 to 0.62. Based on the  $CO_2$  uptake rates for these ratios, linear extrapolation would indicate that net  $CO_2$  fixation would be possible up to a  $CO/H_2$  ratio of 0.92 (linear regression of  $CO_2UR$  and  $CO/H_2$  ratio). If the goal was to utilize  $CO_2$  only via the methyl and CO only via the carbonyl branch and the flux through CO dehydrogenase linearly correlated with CO partial pressures, the BFG would have to be blended with 44%  $H_2$  (linear regression of CODH flux and  $pCO$ ). In other words, blending industrial BFG with  $H_2$  as an energy source can be used to control individual gas uptake rates and shift *A. woodii* metabolism



**Fig. 6.** Metabolic flux distributions of *A. woodii* chemostat cultivations depend on gas composition. Flux distributions for a, idealized gas without CO with low  $H_2$  (30%) (light blue) and high  $H_2$  (60%) (dark blue) and b, idealized gas with CO with low  $H_2$  (30%) (light green) and high  $H_2$  (60%) (dark green). Experimentally determined specific rates were used as input data and constraints for FBA simulations with maximization of the ATP maintenance pseudo reaction (NGAM) as the objective function. All fluxes are shown in  $\text{mmol g}^{-1} \text{ h}^{-1}$  (Biomass box: upper values in  $\text{Cmmol g}^{-1} \text{ h}^{-1}$ , lower values in  $\text{h}^{-1}$ ) and represent means of biological duplicates. Upper values for uptake and production rates represent experimental data, lower values represent data used for FBA simulation. Model input values may slightly deviate from measured experimental data because stoichiometric inconsistencies were corrected (see Methods). NGAM = non growth-associated ATP maintenance, HDCR = hydrogen dependent  $CO_2$  reductase, CODH = CO dehydrogenase, Hyd = electron-bifurcating hydrogenase, Rnf = *Rhodospirillum rubrum* nitrogen fixation complex,  $Fd_{red}$  = reduced ferredoxin,  $Fd_{ox}$  = oxidized ferredoxin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

towards a desired direction (ATP and biomass versus acetate formation). This strategy could also be applied to other gas fermenting organisms to control product spectra and shift production towards a desired target compound.

#### 4. Conclusion

In this study, the effect of different gas compositions and mass transfer rates on CO<sub>2</sub> utilization, growth, and acetate production were characterized in batch and continuous cultures of *A. woodii*. Blending industrial BFG with H<sub>2</sub> enabled co-utilization of CO, CO<sub>2</sub> and H<sub>2</sub> and net CO<sub>2</sub> fixation at high CO and H<sub>2</sub> partial pressures. This strategy allowed to control CO<sub>2</sub> fixation, gas uptake and metabolic fluxes in continuous cultures. Its flexibility with respect to the utilization of gases at varying compositions makes *A. woodii* a very promising host for the reduction of industrial CO<sub>2</sub> emissions, even in CO-containing gases.

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#### CRediT authorship contribution statement

**Katharina Novak:** Investigation, Data curation, Formal analysis, Conceptualization, Writing - original draft, Visualization. **Christian Neuendorf:** Investigation. **Irmela Kofler:** Resources, Investigation. **Nina Kieberger:** Resources, Investigation. **Steffen Klamt:** Formal analysis, Resources, Software. **Stefan Pflügl:** Conceptualization, Formal analysis, Resources, Writing - original draft, Supervision, Project administration, Funding acquisition.

#### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nina Kieberger is an employee of voestalpine Stahl GmbH. Voestalpine Stahl GmbH has interest to reduce its CO<sub>2</sub> emissions by recycling the carbon via gas fermentation for further use in products. All other authors declare that they have no competing interests.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2020.124573>.

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