Online monitoring of gas transfer rates during CO and CO/H₂ gas fermentation in quasi-continuously ventilated shake flasks

Marcel Mann | Aline Hüser | Benjamin Schick | Robert Dinger | Katharina Miebach | Jochen Büchs

RWTH Aachen University, AVT—Biochemical Engineering, Aachen, Germany

Correspondence
Jochen Büchs, RWTH Aachen University, AVT—Biochemical Engineering, Forckenbeckstraße 51, 52074 Aachen, Germany. Email: jochen.buechs@avt.rwth-aachen.de

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Abstract
Syngas fermentation is a potential player for future emission reduction. The first demonstration and commercial plants have been successfully established. However, due to its novelty, development of syngas fermentation processes is still in its infancy, and the need to systematically unravel and understand further phenomena, such as substrate toxicity as well as gas transfer and uptake rates, still persists. This study describes a new online monitoring device based on the respiration activity monitoring system for cultivation of syngas fermenting microorganisms with gaseous substrates. The new device is designed to online monitor the carbon dioxide transfer rate (CO₂TR) and the gross gas transfer rate during cultivation. Online measured data are used for the calculation of the carbon monoxide transfer rate (COTR) and hydrogen transfer rate (H₂TR). In cultivation on pure CO and CO + H₂, CO was continuously limiting, whereas hydrogen, when present, was sufficiently available. The maximum COTR measured was approximately 5 mmol/L/h for pure CO cultivation, and approximately 6 mmol/L/h for cultivation with additional H₂ in the gas supply. Additionally, calculation of the ratio of evolved carbon dioxide to consumed monoxide, similar to the respiratory quotient for aerobic fermentation, allows the prediction of whether acetate or ethanol is predominantly produced. Clostridium ljungdahlii, a model acetogen for syngas fermentation, was cultivated using only CO, and CO in combination with H₂. Online monitoring of the mentioned parameters revealed a metabolic shift in fermentation with sole CO, depending on COTR. The device presented herein allows fast process development, because crucial parameters for scale-up can be measured online in small-scale gas fermentation.

Keywords
Clostridium ljungdahlii, gas fermentation, gas transfer rate, small-scale online monitoring, syngas fermentation

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1 | INTRODUCTION

Syngas fermentation is a potential sustainable technology applicable for a zero-waste economy in the future. The combination of feedstock flexibility and an increasing variety of different products make syngas fermentation a research field of high interest (De Tissera et al., 2017; Sun et al., 2019). Process development is well advanced, and both, pilot plants and the first commercial plants are in operation (Lanza-Tech, 2018). Although some aspects of syngas fermentation are well understood, it is a relatively new technology, and various physiological aspects of the cultured microorganism are still unknown.

In aerobic liquid fermentations with dissolved solid substrates (e.g., sugars such as glucose or fructose), the availability of oxygen is a crucial parameter. Hence, a detailed understanding of oxygen transfer is crucial for process characterization and scale-up (Garcia-Ochoa & Gomez, 2009). The same applies for gaseous substrates in syngas fermentation. The availability of gaseous substrates for microbial cultivation has been mentioned in various reports (Bredwell et al., 1999; Keryanti et al., 2019; Siebler et al., 2019; Takors et al., 2018; Wan et al., 2017). However, to the best of our knowledge, online measurement of the gas transfer rates in syngas fermentation has been possible only at the fermenter scale.

In small-scale fermentation, predominantly in serum bottle experiments, various different gas ratios and differences in the partial pressure of individual gas components have been tested. These variations result in changes in the gas transfer rate over time, and thereby, the availability of gaseous substrates (Hurst & Lewis, 2010; Jack et al., 2019; Mohammadi et al., 2014; Najafpour & Younesi, 2006). It has been shown that higher carbon monoxide concentrations delay initial growth and enhance alcohol production (Hurst & Lewis, 2010; Jack et al., 2019). Increasing the availability of gaseous substrates further using a pressurized fermentation vessel reduces the metabolic activity and fewer products are detected (Oswald et al., 2018; Stoll et al., 2019). Accordingly, the effects of variations in gas transfer have been reported in the literature. However, online measurement of the crucial parameter in an easy-to-handle experimental setup has not been possible so far.

Industrial process development starts off using small-scale cultivation devices for strain screening and medium optimization, and to gain a fundamental understanding of the process. The development of online monitoring tools has accelerated process development and reduced expensive, laborious sampling experiments, used in traditional offline methods using microplates and shake flasks. For aerobic cultivation, a broad variety of online monitoring tools are available, which have been extensively used for process development (Anderlei & Büchs, 2001; Anderlei et al., 2004; Klein et al., 2013; Richter et al., 2007; Samorski et al., 2005; Schneider et al., 2010). For anaerobic cultivation, a limited number of devices are available, and small-scale online monitoring devices for syngas fermentation are currently unavailable. For aerobic processes, the respiration activity monitoring system (RAMOS) has been used in numerous applications (Anderlei & Büchs, 2001; Anderlei et al., 2004; Heyman et al., 2020; Kauffmann et al., 2020; Meier et al., 2013; Müller et al., 2018; Schilling et al., 2015). Semi-continuous online monitoring of oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR), and calculation of the respiratory quotient (RQ) enables the identification of effects important for cultivation, such as oxygen transfer limitation, pH effects, media limitations, and diauxic growth (Anderlei & Büchs, 2001; Anderlei et al., 2004). Furthermore, the determined RQ allows the online prediction of product formation (Heyman et al., 2020). Recently, the RAMOS device was modified and successfully applied for anaerobic cultivation (anaRAMOS) (Munch et al., 2019).

In anaerobic applications, CTR and the total gas transfer rate (TGTR) were used to identify diauxic growth, and to predict the hydrogen production by *Clostridium pasteurianum* (Munch et al., 2019). The anaRAMOS was also described as a potent tool for online monitoring of the cultivation of acetogenic *Clostridium* on dissolved solid substrates, such as fructose (Mann et al., 2020). Cultivation using a continuous supply of gaseous carbon and energy substrates has not been possible so far. Supplementation of a dissolved solid substrate and continuous addition of a gaseous carbon and energy source resulted in vast differences in the cultivation process, as described in detail previously (Geinitz et al., 2020). Differences in the mode of operation and the resulting changes in microbial cultivation between serum bottles, used for cultivation on gaseous carbon and energy sources; the anaRAMOS, used for cultivation on dissolved solid substrates such as fructose; and the syngas RAMOS (synRAMOS), used for cultivation on gaseous carbon and energy substrates such as CO and H₂; have been explained in Figure S1. anaRAMOS is constructed for classic batch fermentations, where all substrates are supplied right from the beginning. Conversely, synRAMOS is constructed for supplying gaseous carbon and energy sources semi-continuously. This is in contrast to serum bottles, where the gas composition, and thereby the gas transfer, constantly change over the course of the fermentation (Figure S1).

In this study, the development of a new synRAMOS device, that allows cultivation in eight parallel shake flasks with a flexibly mixed gas composition of up to three different gases, is described. Online measurements of CO₂TR and gross gas transfer rate (GGTR) for the cultivation of *Clostridium ljungdahlii* on pure carbon monoxide were used to calculate the carbon monoxide transfer rate (COTR). Furthermore, a new carbon dioxide to monoxide quotient (CORQ) was calculated, allowing online prediction of the main product. Additionally, cultivation on carbon monoxide and hydrogen was performed using the newly developed device.

2 | MATERIAL AND METHODS

2.1 | Description of the synRAMOS and experimental settings

The synRAMOS was constructed with some modifications based on the RAMOS used for aerobic cultivation (Anderlei & Büchs, 2001; Anderlei et al., 2004) and anaRAMOS (Munch et al., 2019) for anaerobic cultivation, both described in detail previously.
The schematic setup and a detailed sketch of a single measurement position are illustrated in Figure 1a. To enable a flexible composition of up to three gases (CO, H₂, and N₂), three mass flow controllers (MFCs; EL-Flow Select, Bronckhorst) were installed. Each MFC was used to control the flow of one of the mentioned gases. Behind the MFCs, the flows of the individual gases were combined and fed to the measurement platform (Figure 1a) at a total flow rate of 40 ml/min. Each of the eight individual measurement positions on the measurement platform were equipped with a carbon dioxide sensor (measurement range of 0–4% CO₂) (MSH-P-CO₂; Dynamex) and a type 26PCA differential pressure sensor (Honeywell Inc.) for online measurement, as shown in Figure 1a. The carbon dioxide sensor was installed in a measurement loop that was continuously ventilated at a flow rate of 20 ml/min with gas from the headspace of the cultivation flask using a microfluidic piezomembrane pump (Bartels Mikrotechnik). The measurement cycle consisted of two phases: the flow phase and the measurement phase. During the flow phase, the inlet and outlet valves were open, and the cultivation flasks were continuously ventilated. During the measurement phase, the inlet and outlet valves were closed, and the changing pressure and carbon dioxide partial pressure were monitored (Figure 1b,c). The change in total pressure and carbon dioxide partial pressure were used to calculate GGTR, named as the TGTR by Munch et al. (2019) and the carbon dioxide transfer rate (CO₂TR) according to Munch et al. (2019).

For all experiments, a gas flow rate of 5 ml/min was applied for each flask. Two different filling volumes, 40 and 60 ml, were used, resulting in a ventilation rate of 0.125 and 0.083 vvm, respectively. The measurement phase was set to 40 min, while the flow phase was set to 120 min.

2.2 | Organism and cultivation medium

C. ljungdahlii (DSM13528), kindly provided by Fraunhofer IME Aachen, was used for all experiments. C. ljungdahlii was stored in cryogenic cultures at −80°C, containing 90% (v/v) culture from an actively growing culture on initially 5 g/L fructose and 10% v/v DMSO. For each experiment, two sequential precultures were grown.

(Figure 1) (a) Schematic setup of the synRAMOS for gaseous carbon sources. The magnification shows a single flask setup with details of gas flows, and valve and sensor positioning. (1) Three individual mass flow controllers for up to three different gases (N₂, CO, H₂). Total flow rate of 40 ml/min resulting in 5 ml/min per flask. (2) Computer for gas flow and valve control, as well as data acquisition. (3) Temperature control and shaker unit holding up to eight RAMOS flasks. (4) A single RAMOS flask containing microbial culture. (5) Inlet valve, open during flow phase, closed during measurement phase. (6) Differential pressure sensor for a continuous monitoring of pressure difference between the flask and the environment. (7) Carbon dioxide sensor for online monitoring of carbon dioxide partial pressure. (8) Circulation pump, conveying gas from the headspace of the RAMOS flask to the carbon dioxide sensor (7) and back to the flask at a flow rate of 20 ml/min. (9) Outlet valve, open during flow phase, closed during measurement phase. (b) Typical progression of CO₂ partial pressure, total pressure, and carbon monoxide partial pressure during cultivation using pure carbon monoxide. (c) Typical progression of CO₂ partial pressure, total pressure, carbon monoxide partial pressure, and hydrogen partial pressure during cultivation using mixtures of carbon monoxide and hydrogen. (I) Flow phase (120 min) with open inlet and outlet valves for gas flow through the headspace of the RAMOS flask with specifically controlled gas mixture. (II) Measurement phase (40 min) with closed inlet and outlet valves [Color figure can be viewed at wileyonlinelibrary.com].
in serum bottles. The first preculture was inoculated with 10% (v/v) cryogenic culture and cultivated for 48 h. The second preculture was inoculated using 10% (v/v) from the first preculture, and grown to an OD600 between 0.6 and 0.8 before inoculating the experimental culture. Precultures were grown on the same media used for the experiments, and 5 g/L of fructose was added as the only carbon source.

The cultivation medium (ATCC 1754 medium with some modifications) was composed of the following stock solutions: salt stock solution containing 40 g/L NH₄Cl, 0.8 g/L CaCl₂-2H₂O, 8 g/L MgSO₄-7H₂O, 4 g/L KCl, 4 g/L KH₂PO₄, and 32 g/L NaCl. Wolfe’s vitamin solution contained 10 mg/L α-lipoic acid, 4 mg/L biotin, 0.1 mg/L cyanocobalamin, 10 mg/L α-panthothenic acid calcium salt hydrate, 4 mg/L folic acid, 10 mg/L nicotinic acid, 10 mg/L p-aminobenzoic acid, 20 mg/L pyridoxine hydrochloride, 10 mg/L riboflavin, and 10 mg/L thiamine hydrochloride. The trace elements solution contained 0.8 g/L (NH₄)_2Fe(SO₄)₂-6H₂O, 0.2 g/L CoCl₂-6H₂O, 0.02 g/L CuCl₂-2H₂O, 1 g/L MnSO₄·H₂O, 0.02 g/L NiCl₂-6H₂O, 2 g/L nitroacetic acid, 0.02 g/L Na₂MoO₄·2H₂O, 0.02 g/L Na₂- SeO₃·5H₂O, 0.02 g/L Na₂WO₄·2H₂O, and 0.0002 g/L ZnSO₄·7H₂O. L-Cysteine solution contained 75 g/L l-cysteine, and was stored under anaerobic conditions. Bis-Tris buffer solution was prepared in 0.8 M stock solution (16× concentrated), and the pH was adjusted to 6. Fructose stock solution was prepared with 100 g/L fructose (20-times concentrated). Salt stock solution, fructose stock solution, and Bis-Tris stock solution were autoclaved. Wolfe’s vitamin solution, trace element solution, and l-cysteine stock solution were sterile-filtered using a 0.2 µm PES sterile syringe filter (VWR). Yeast extract (1 g) (Oxoid), 25 ml salt stock solution, and 25 ml Bis-Tris solution were mixed and made up to a total volume of 975 ml using DI water. The solution was autoclaved for 20 min at 121°C. After cooling, 10 ml trace elements solution and 5 ml vitamin solution were added. After the medium was made anoxic (described subsequently), 10 ml of l-cysteine stock solution was added, and a final volume of 1 L was attained.

2.3 | Experimental procedure

The desired volume of medium without l-cysteine was added to the cultivation flask (36 or 54 ml for a final filling volume of 40 or 60 ml, respectively). To obtain anaerobic conditions, the filled flasks were purged with ultrahigh purity nitrogen (5.0; Praxair) for a minimum of 2 h before adding the l-cysteine solution. Two hours before inoculation, the temperature was maintained at 37°C. Six hours after inoculation, the gas supply was changed from 100% N₂ to the desired gas composition, that is, CO in N₂, or CO and H₂ in N₂. During the experiment, the concentrations of CO and H₂ were increased stepwise (details given in each figure). Offline samples were taken on a regular basis using a 1-ml syringe with a needle (0.8 mm × 120 mm), from flasks not used for online monitoring. The optical density (OD600) was measured (dilution using deionized water when OD exceeded 0.3) at a wavelength of 600 nm using a spectrophotometer (Genesys 20; Thermo Fisher Scientific). Subsequently, the remaining sample was centrifuged at 18,000 g for 5 min (Rotina 35R; Hettich Zentrifugen). The supernatant was transferred into a new vial, and the pH was measured using a pH meter (HI 2111pH/ORP Meter; Hanna instruments). The sample was then stored at ~20°C for further analysis. For high-performance liquid chromatography (HPLC), samples were defrosted and filtered using a 0.2 µm cellulose acetate membrane (VWR). HPLC (Prominence HPLC; Shimadzu) analysis was performed with an organic acid column (ROA-Organic Acid H⁺; Phenomenex Inc.) at 60°C, with a flow rate 0.8 ml/min of 5 mM H₂SO₄. For detection, a refracting index detector (RID-10A; Shimadzu) was used. The osmolarity was measured using an Osmomat 3000 basic freezing point osmometer (Gonotec).

2.4 | OD to cell dry weight correlation

For accurate mass balancing, the carbon contained in the biomass had to be calculated. Therefore, the OD600 to cell dry weight (CDW) correlation (Figure S2) and Equation (S1) were used. As per the elemental composition, the empirical formula CH₁.₆₆₆NO₀.₂₃O₀.₂₇ was used for biomass estimation, with a molecular weight of 20.7 g/mol for the calculation of the contained carbon (Atkinson & Mavittuna, 1982).

3 | RESULTS AND DISCUSSION

C. ljungdahlii was cultivated in the synRAMOS using either CO in N₂ or CO + H₂ in N₂ as the sole source of carbon and energy. Each gas composition was tested using filling volumes of 40 and 60 ml. Cultivation on CO was performed using five different CO concentrations, and shaking frequencies of 100/min or 200/min. Cultivation on CO + H₂ was performed at a fixed CO to H₂ ratio of 1:2, using two different concentrations and shaking frequencies of 100/min or 200/min. In total, this translates to 18 different cultivation conditions.

3.1 | Cultivation on carbon monoxide

C. ljungdahlii was cultivated in seven parallel shake flasks (four online shake flasks mounted in the synRAMOS, and three offline shake flasks for sampling purposes) with a stepwise increase in CO concentration from 10% to 50% CO in N₂, and a subsequent increase in the shaking frequency from 100 to 200/min (Figure 2a). Online results from the four synRAMOS flasks (2 × 40 ml filling volume, and 2 × 60 ml filling volume) and offline results from the 3 additional flasks (1 × 40 ml filling volume, and 2 × 60 ml filling volume) are shown in Figure 2d.e.

The CO₂TR, plotted in Figure 2b, is a result of the microbial conversion of CO into soluble products (ethanol and acetate) and CO₂ (Equations 1 and 2).
CO₂ is the most oxidized carbon compound and cannot be further utilized, as no additional energy source is present (Drake et al., 2008). Therefore, it is transferred from the liquid phase into the gas phase. Hence, CO₂TR is a direct measure of metabolic activity. CO₂TR increased after each increment in CO concentration in the gas supply (Figure 2a), and a steady state plateau was formed (Figure 2b). In addition to the increasing CO₂TR with increasing CO concentration, an increasing CO₂TR was observed upon decreasing the filling volume from 60 ml to 40 ml. The same correlation between filling volume and gas transfer in aerobic processes has been described previously (Maier & Büchs, 2001).

In the anaRAMOS described previously, pressure sensors were used to measure TGTR (Munch et al., 2019). The study used glycerol as the substrate. Thus, gases (except N₂) were only transferred from the liquid phase into the gas phase (as produced from sugar conversion). In the synRAMOS, pressure sensors are used to measure the GGTR. GGTR (Figure 2c) is a sum signal of gas transfer into (in this case, CO) and out of (in this case, CO₂) the liquid phase, and is

\[
\begin{align*}
4\text{CO} + 2\text{H}_2\text{O} & \rightarrow \text{CH}_3\text{COOH} + 2\text{CO}_2, \\
6\text{CO} + 3\text{H}_2\text{O} & \rightarrow \text{C}_2\text{H}_5\text{OH} + 4\text{CO}_2.
\end{align*}
\]

FIGURE 2  Cultivation of Clostridium ljungdahlii (DSM 13528) at 10–50% carbon monoxide in the gas supply in 250-ml shake flasks with 40- and 60-ml filling volumes. (a) Overview of sequential cultivation phases with varying carbon monoxide concentrations in the gas supply and changing shaking frequencies. (b) Duplicates of online measured carbon dioxide transfer rate (CO₂TR), gray box indicating CO₂ sensor saturation and, therefore, unavailability of data. For a better overview, every 10th data point is plotted. (c) Duplicates of online measured GGTR. For a better overview, every 10th data point is plotted. (d) Optical density and pH values measured offline. Error bars indicate the maximum and minimum values measured. (e) Acetate and ethanol concentration measured offline. Error bars indicate the maximum and minimum values measured. Experimental conditions: Inoculation density OD₀ = 0.06, inoculation from actively growing preculture in a serum bottle on ATCC medium and an initial fructose concentration of 5 g/L, temperature T = 37°C, pH₀ 6, shaking frequency n = 100/min–200/min, shaking diameter d = 50 mm, culture volume Vₗ = 40 or 60 ml, gas supply = 10–50% carbon monoxide in nitrogen, flow rate qᵢᵣ = 5 ml/min per flask. GGTR, gross gas transfer rate [Color figure can be viewed at wileyonlinelibrary.com]
fundamentally different from the TGTR (Figure 1b,c). GGTR (- Figure 2c) shows the same plateau formation as the CO2TR, following the stepwise increase of the CO concentration in the gas supply as a function of the filling volume. With an increasing CO concentration in the gas supply and a decrease in the filling volume, the GGTR increases. Additionally, GGTR shows the influence of an increase in shaking frequency. When the shaking frequency was increased from 100 to 200 rpm after 192 h, a sharp increase in GGTR was observed (Figure 2c). CO2TR would show the same behavior. However, the CO2 concentration in the headspace exceeded the measurement range (0–4% CO2) of the applied CO2 sensors (indicated by the gray box in Figure 2b).

With each of the initial increases in the carbon monoxide supply (increase in the CO concentration in the gas supply, or increase in the shaking frequency), the biomass (OD), as well as the acetate and the ethanol production rate increased (Figure S3). The pH (Figure 2d) correlated well with the acetate concentration (Figure 2d). Unfortunately, due to an experimental error, no samples were taken from the 60 ml cultivation after 264 h. As the acetate concentration increased, the pH decreased. The changes in metabolic activity, from now on referred to as a metabolic shift, were observed after 168 and 192 h for the 40 and 60 ml cultivation, respectively. Instead of acetate, ethanol production increased, and the acetate concentration decreased. For both filling volumes, this metabolic shift was observed at a GGTR of approximately 3 mmol/L/h (Figure 2c). Nutrient limitation and a resulting increase in intracellular undissociated acetic acid were shown to result in a shift toward solventogenic metabolism (Richter et al., 2016). At this point, reducing equivalents are shifted from acetate conversion to alcohols. These findings match our results very well, as the metabolic activity represented by the gas transfer rate comes to a halt once acetate is depleted. Metabolic shift also occurs at high biomass concentrations (Valgepea et al., 2017). The data in Figure 2d shows that for both filling volumes, the OD is approximately 1.7 when the metabolic activity changes. Furthermore, in agreement with other data from the literature concerning the influence of CO on alcohol production, alcohol production increases with increasing CO supply. In serum bottle experiments, higher CO availability results in increased ethanol production (Hurst & Lewis, 2010).

The online measured GGTR was validated using the results from offline samples. GGTR (Figures 2b and 3b) represents all the carbon that is transferred from the gas phase into the liquid phase in the form of CO, and is not emitted in the form of CO2 (Figure 1b). From offline acetate, ethanol, and biomass concentration measurements, the total carbon assimilation rate (TCAR) can be calculated using Equations (3)–(6). The TCAR represents the total amount of carbon that is fixed in the form of dissolved products (acetate, ethanol, and biomass) and is not emitted in the form of CO2. As shown in Figure 3b, the online measured GGTR matches very well with the calculated TCAR. Hence, the measured GGTR is a reliable online indicator of metabolic activity. As discussed before, GGTR increases with increasing substrate availability (increase in CO concentration in the gas supply, or shaking frequency). The TCAR increases similarly. Hence, GGTR can be used as a highly accurate measure for carbon fixation in CO gas cultivation.

\[
TCAR = CAR_{\text{Acetate}} + CAR_{\text{Ethanol}} + CAR_{\text{Biomass}} \left( \frac{\text{mmolC}}{L \times h} \right) .
\]

\[
CAR_{\text{Acetate}} = \frac{\Delta C_{\text{Acetate}}}{M_{\text{Acetate}} \times \Delta t_{\text{samples}}} \left( \frac{\text{mmolC}}{L \times h} \right) .
\]

\[
CAR_{\text{Ethanol}} = \frac{\Delta C_{\text{Ethanol}}}{M_{\text{Ethanol}} \Delta t_{\text{samples}}} \left( \frac{\text{mmolC}}{L \times h} \right) .
\]
The online measured CO₂TR and GGTR can be used to calculate COTR (Figure 1b). The calculation was performed according to Equation (7). The results are presented in Figure 4.

\[
\text{COTR} = \text{GGTR} + \text{CO₂TR} \quad \text{[mmol L⁻¹ h⁻¹]} \tag{7}
\]

COTR shows a similar plateau formation as GGTR and CO₂TR. To validate the COTR calculated from the CO₂TR and GGTR measured online, an estimation based on the calculation of the maximum oxygen transfer capacity (OTR_max) was used to calculate the maximum carbon monoxide transfer capacity of the cultivation system (Meier et al., 2016). The calculation of OTR_max was performed for 10%, 20%, and 30% oxygen in the gas phase. For each oxygen concentration in the gas phase, the calculation was performed for filling volumes of 40 and 60 ml, and shaking frequencies of 100 and 200 rpm at a shaking diameter of 50 mm (Figures 5 and 8).

\[
\text{OTR}_{\text{max}} = 3.72 \times 10^{-7} \times \text{osmol}^{0.05} \times n \times \left(1.18 + \frac{\text{osmol}}{1000}\right) \times V_L^{0.74} \\
\times d_0^{0.33} \times d^{1.88} \times \rho_k \times Y_{o_2} \quad \text{[mmol L⁻¹ h⁻¹]} \tag{8}
\]

The osmolality measured at the beginning of a cultivation was 0.17 Osm/kg, and was used for the OTR_max calculation in Equation (8).

OTR can be calculated according to the following equation:

\[
\text{OTR} = k_L \alpha_{O_2} \times L_{O_2} \times \rho_{\text{abs}} \times \left(Y_{O_2} - Y_{O_2, L}\right) \quad \text{[mmol L⁻¹ h⁻¹]} \tag{9}
\]

In analogy, COTR can be calculated according to the following equation:

\[
\text{COTR} = k_L \alpha_{CO} \times L_{CO} \times \rho_{\text{abs}} \times \left(Y_{CO} - Y_{CO, L}\right) \quad \text{[mmol L⁻¹ h⁻¹]} \tag{10}
\]

Therefore, the difference between COTR and OTR depends on the solubility (L_{O_2} and L_{CO}) as well as the k_L a (k_L a_{O_2} and k_L a_{CO}). The k_L a term is defined in the following equation:

\[
k_L a_{O_2/CO} = \frac{D_{O_2/CO}}{\delta} \times \frac{1}{h} \tag{11}
\]

The thickness of the liquid boundary layers (δ) and the volume specific mass transfer area (a) are not influenced by the applied gas (O_2 or CO). Hence, the difference in the k_L a term is solely based on the difference in the diffusion coefficient (D) of the different gases (O_2 and CO). Therefore, COTR_max can be calculated from OTR_max by simply considering the difference in the solubility and the diffusion coefficient, as shown in Equation (12). Values of 0.92 mmol/L and 1.18 mmol/L (303.15 K and 1 atm) were used as the solubility of carbon monoxide and oxygen, respectively (Geventman, 1999). The diffusion coefficients 2.03 × 10⁻⁵ cm²/s and 2.10 × 10⁻⁵ cm²/s were used for carbon monoxide and oxygen, respectively (Cussler, 2009).

\[
\text{COTR}_{\text{max}} = \text{OTR}_{\text{max}} \times \frac{L_{CO}}{L_{O_2}} \times \frac{D_{CO}}{D_{O_2}} \times \frac{\text{mmol L⁻¹ h⁻¹}}{L \times h} \tag{12}
\]

As shown in Figure 5, the calculated COTR_max fits well with the experimental results of COTR_max for the 40 and 60 ml filling volumes as well as CO concentrations of 10%, 20%, and 30%. Hence, the plateau formation observed in CO₂TR, GGTR, and COTR results from a COTR limitation. The cultivation has therefore been CO-limited for most of the time.

COTR is a direct measure of substrate conversion. Therefore, the information provided by COTR can be compared to the information provided by the OTR, which is well known for aerobic fermentation with dissolved solid substrates. As previously shown, the plateau formation of COTR can be interpreted as a COTR limitation, analogous to plateau formation of the OTR in aerobic solid substrate fermentation (Anderlei & Büchs, 2001). Gas-liquid mass transfer limitation has been considered as a major issue in gas...
fermentation (Bredwell et al., 1999; Vega et al., 1989). To overcome this, the application of pressure has been investigated (Oswald et al., 2018; Stoll et al., 2019; Van Hecke et al., 2019). Elevated pressure has been reported to change the spectrum of products and result in unstable fermentation conditions (Oswald et al., 2018; Stoll et al., 2019). These effects are explained by elevation in the concentrations of dissolved toxic gases, resulting from transfer rates exceeding the uptake rate of the microbial culture. Scale-up procedures for gas fermentation processes can now be streamlined by a detailed understanding of the process gained in small-scale experiments, as is frequently done for aerobic fermentation (Gamboa-Susavart et al., 2018; Lattermann & Büchs, 2016; Seletzky et al., 2007; Zimmermann et al., 2006). Calculation of COTR can also be used to evaluate effects in a large-scale fermenter.

For aerobic processes, online measurement of RQ can be used to distinguish between different cultivation phases, substrates, and products (Heyman et al., 2020). RQ is the ratio of the CO₂ exhausted and O₂ uptake. Similarly, CORQ can be defined for CO fermentations, as shown in Equation (13). As shown in Equations (1) and (2), the theoretical CORQ for the production of acetate and ethanol is 0.5 and 0.66, respectively. The measured CORQ (Equation 13) and the theoretical CORQ from Equations (1) and (2) are shown in Figure 6.

\[
\text{CORQ} = \frac{\text{CO}_2 \text{TR}}{\text{COTR}} \text{ [\text{m}]}. \tag{13}
\]

Additionally, CORQ was calculated and plotted in Figure 6 based on offline samples (offline CORQ) using the following equations:

\[
\text{normCAR}_{\text{acetate}} = \frac{\text{CAR}_{\text{acetate}}}{\text{CAR}_{\text{acetate}} + \text{CAR}_{\text{ethanol}}} \times \frac{\text{mmolC}}{1 \text{ h}}. \tag{14}
\]

\[
\text{offlineCORQ} = \text{normCAR}_{\text{acetate}} \times \left(1 - \frac{1}{2} \times \left(1 - \text{normCAR}_{\text{acetate}}\right) \right) \times \frac{2}{3} \text{ [\text{m}]}. \tag{15}
\]

**FIGURE 5** Calculation of the maximum carbon monoxide transfer capacity (COTR<sub>max</sub>) based on the calculation of the OTR<sub>max</sub> by Meier et al. (2016) for different oxygen and carbon monoxide concentrations in the gas supply. Measured COTR is the average of the COTR plotted in Figure 4. (a, b) Calculated OTR<sub>max</sub> (Meier et al., 2016), COTR<sub>max</sub> calculated using Equation (10), and average of the calculated experimental COTR (Figure 4) for 40 ml (a) and 60 ml (b) filling volumes. COTR, carbon monoxide transfer rate; COTR<sub>max</sub>, maximum carbon monoxide transfer capacity; OTR<sub>max</sub>, maximum oxygen transfer capacity [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 6** Carbon dioxide to carbon monoxide coefficient (CORQ) for the cultivation of *Clostridium ljungdahlii* (DSM 13528) shown in Figure 2, grown on carbon monoxide with 40- and 60-ml filling volumes. (a) Carbon monoxide concentrations in the gas supply. (b) CORQ calculated from online data (for a better overview, every 10th data point is plotted) (Equation 11), CORQ calculated from offline measured data (Equations 12 and 13), and theoretical CORQ calculated from Equations (1) and (2) [Color figure can be viewed at wileyonlinelibrary.com]
The online and offline CORQ measurements matched, and were within the expected range between acetate and ethanol production. As shown in Figure 2e, the acetate concentrations in the 40-ml and 60-ml cultures were the same until 144 h, while more ethanol was produced in the 40-ml culture. This is proven by the online CORQ measurement. The CORQ of the 40-ml culture was closer to the expected CORQ for ethanol production in comparison to the 60-ml culture. Hence, the CORQ shows a change in product formation depending on the availability of carbon monoxide. This agrees with the reported effect of higher ethanol production with increasing availability of carbon monoxide (Hurst & Lewis, 2010).

3.2 Cultivation using a combination of carbon monoxide and hydrogen

Besides sole carbon monoxide, C. ljungdahlii can utilize mixtures of carbon monoxide and hydrogen (Tanner et al., 1993). The addition of hydrogen has the potential to reduce carbon dioxide emissions; thus, more carbon can be fixed in valuable products such as acetate and ethanol (cf. Equations 16 and 17).

\[
2CO + 2H_2 \rightarrow CH_3COOH. \quad (16)
\]

\[
2CO + 4H_2 \rightarrow CH_3CHOH. \quad (17)
\]

C. ljungdahlii was cultivated in six parallel shake flasks (three online shake flasks mounted in the synRAMOS and three offline shake flasks for sampling purposes) with a constant CO to H₂ ratio of 1:2. The gas supply was initially set to 10% CO and 20% H₂, which was doubled after 48 h (Figure 7a). After 88 h, the shaking frequency was increased from 100/min to 200/min. Online results from the three synRAMOS flasks (1 × 40 ml filling volume, and 2 × 60 ml filling volume) and offline results from the three additional flasks (1 × 40 ml filling volume and 2 × 60 ml filling volume) are shown in Figure 7d,e.

For the first two settings (0–48 h and 48–90 h), CO₂TR decreased slightly within each phase (Figure 7b), with a concurrent increase in GGTR (Figure 7c). This phenomenon likely resulted from the initial consumption of pure CO as the substrate with the highest energy content. Subsequently, hydrogen consumption increased, leading to a decrease in CO₂TR (less CO₂ is produced) and an increase in GGTR. CO is toxic to hydrogenases. Thus, hydrogen consumption is only possible when the dissolved CO concentration is below a critical level (Menon & Ragsdale, 1996). Hence, after an increase in the CO concentration, an adaption period is necessary until the dissolved CO tension is low enough for hydrogenase activity.

The offline data (Figure 7d,e) correlate well with the online measured metabolic activity. The optical density increased, whereas the pH decreased slightly. Due to the lower acetate production in comparison to the experiment shown in Figure 2, the pH decreased less during the cultivation shown in Figure 7. During the first phase, acetate is primarily produced, while during the second phase, ethanol production increases.

When the shaking frequency was increased after 90 h to 200/min, CO₂TR (Figure 7b) and GGTR (Figure 7c) increased. No plateau was formed, and the experiments with the two different filling volumes progressed differently. For the 40-ml culture, CO₂TR increases for approximately 80 h, before declining slowly. The corresponding GGTR increases sharply and decreases after approximately 30 h, while CO₂TR further increases. Hence, less CO₂ is initially exhausted, which indicates the additional consumption of hydrogen. By altering the cultivation conditions due to product formation, pH shift, and medium component consumption, the hydrogen uptake decreases. The resulting rise in CO₂ production is represented by an increase in CO₂TR and a decrease in GGTR. After a total cultivation time of 170 h, CO₂TR starts to decrease, while GGTR remains at a low level. As the OD₆₀₀ did not increase further at this point, and the pH started increasing slightly, a secondary substrate limitation might have possibly caused the decrease in metabolic activity. CO₂TR of the 60-ml culture remained at around 1 mmol/L after 88 h. Only a slight decrease followed by a slow increase was observed. GGTR initially increased to a maximum value of 10 mmol/L/h after 120 h, before steadily decreasing for the rest of the cultivation. This can be explained by the ratio of CO and H₂ consumed, as outlined in detail for the 40-ml culture. The offline parameters plotted in Figure 7d,e show similar results for both filling volumes. For the 60-ml culture, the optical density and pH were slightly lower. Toward the end of the cultivation, more ethanol was produced in the 60-ml culture compared to the 40-ml culture.

The results in Figure 7 show a predominant production of ethanol when a H₂ to CO ratio of 2:1 was provided. The acetate-to-ethanol ratio was investigated by other groups before, and revealed that a H₂ to CO ratio of 3:1 increased ethanol production using Clostridium autoethanogenum in continuous stirred tank reactor experiments (Valgepea et al., 2018). Batch serum bottle experiments have been reported to result in an increased acetate concentration at high H₂ to CO ratios (Jack et al., 2019). This difference might arise from different ventilation conditions of serum bottles and continuously ventilated shake flasks, and, thus, gas availability. As discussed before, gas availability has a major impact on cultivation performance.

SynRAMOS measures CO₂TR and GGTR. For the experiment with pure CO, determination of CO₂TR and GGTR could be used to calculate the COTR (Equation 7). The addition of hydrogen to the gas supply results in changes in the CO₂, CO, and H₂ partial pressures during the measurement phase. Therefore, three parameters change, whereas only two values, CO₂TR and GGTR, were measured. Thus, COTR and H₂TR cannot be calculated solely from online data measurements. To further analyze the values measured online, COTR and H₂TR were calculated with the help of offline data according to Equations (18)–(20). For the H₂TRmax calculation, an H₂ solubility of 0.76 mmol/L (303.15 K and 1 atm) (Geventman, 1999) and a diffusion coefficient of 4.5 × 10⁻⁵ cm²/s (Cussler, 2009) was used.
COTR plotted in Figure 8b shows that the 40- and 60-ml cultures reached COTR$_{\text{max}}$ for the first 88 h. After 88 h, the 60-ml culture is likely to remain CO transfer limited, while the 40-ml culture is not. COTR of the 40-ml culture increased until 160 h, and reached the theoretical COTR$_{\text{max}}$ for approximately 10 h before continuously decreasing for the rest of the experiment. The H$_2$TR plotted in Figure 8c did not reach the theoretical H$_2$TR$_{\text{max}}$ at any point. Thus, sufficient H$_2$ was supplied throughout the experiment. If additional CO$_2$ would have been supplied, no significant difference would be expected. As shown in Figure 7a, CO$_2$ was exhausted from the culture throughout cultivation. However, the addition of CO$_2$ could be beneficial when changing the ratio of CO to H$_2$ by increasing the share of H$_2$, as CO$_2$ in combination with H$_2$ could be utilized as an additional carbon source.
CONCLUSION

Cultivation of *C. ljungdahlii* as a model acetogen was successfully demonstrated in the newly developed synRAMOS. Online monitoring of syngas cultivation shows great potential for application in process characterization and development. Online measured values are clearly superior to offline measurements, and can be used to determine critical operating conditions leading to a change in the metabolic activity. Online monitoring of the cultivation performed using a combination CO and H₂ allowed us to distinguish between metabolic activities based solely on CO consumption and on combined consumption of CO and H₂.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Marcel Mann designed the study, conducted the experiments, analyzed and interpreted the data, and drafted the manuscript. Aline Hüser conducted the experiments, participated in data interpretation, and critically revised the manuscript. Benjamin Schick conducted the experiments and analyzed the data. Robert Dinger supported with the construction of the synRAMOS. Katharina Miebach conducted the experiments and analyzed the data. Jochen Büchs initiated and supervised the study, participated in data interpretation, and critically revised the manuscript.

NOMENCLATURE

\[ D_{O_2} \]  
\[ D_{CO} \]  
\[ D_{H_2} \]  
\[ L_{CO} \]  
\[ L_{H_2} \]  
\[ L_{O_2} \]  
\[ \Delta C_{\text{Acetate}} \]  
\[ \Delta C_{\text{Biomass}} \]  
\[ \Delta C_{\text{Ethanol}} \] 

- oxygen diffusion coefficient (m²/s)
- carbon monoxide diffusion coefficient (m²/s)
- hydrogen diffusion coefficient (m²/s)
- solubility of carbon monoxide (mol/L)
- solubility of hydrogen (mol/L)
- solubility of carbon monoxide (mol/L)
- acetate concentration difference between two samples (g/L)
- biomass concentration difference between two samples (g/L)
- ethanol concentration difference between two samples (g/L)
Δt_{sample}  
duration between two samples (h)

CAR_{acetate}  
carbon assimilation rate into acetate (mmolC/L/h)

CAR_{biomass}  
carbon assimilation rate into biomass (mmolC/L/h)

CAR_{ethanol}  
carbon assimilation rate into ethanol (mmolC/L/h)

M_{acetate}  
molar mass of acetate (g/mol)

M_{biomass}  
molar mass of biomass (g/mol)

M_{ethanol}  
molar mass of ethanol (g/mol)

normCAR_{acetate}  
normalized ratio between carbon assimilation rate into acetate and carbon assimilation rate into ethanol

CO2TR  
carbon dioxide transfer rate (mmol/L/h)

CORQ  
carbon dioxide to monoxide quotient

COTR  
carbon monoxide transfer rate (mmol/L/h)

COTR_{max}  
maximum carbon monoxide transfer capacity (mmol/L/h)

GGTR  
gross gas transfer rate (mmol/L/h)

H2TR  
hydrogen transfer rate (mmol/L/h)

H2TR_{max}  
maximum hydrogen transfer capacity (mmol/L/h)

k_i  
mass transfer coefficient (m/s)

k_{i,a}  
volumetric mass transfer coefficient (1/h)

OD  
optical density

OTR_{max}  
maximum oxygen transfer capacity (mmol/L/h)

p_{abs}  
absolute pressure in the system (bar)

RAMOS  
respiration activity monitoring system

rcf  
relative centrifugal force g

RQ  
respiratory quotient

TCAR  
total carbon assimilation rate (mmol/L/h)

TCCR  
total carbon capture rate (mmol/L/h)

TGTR  
total gas transfer rate (mmol/L/h)

y^*  
gas mole fraction in the liquid at the phase boundary (mol/L)

y_L  
gas mole fraction that is equivalent to the dissolved concentration (mol/L)

δ  
liquid boundary thickness around a gas bubble (mc)

ORCID

Marcel Mann  
http://orcid.org/0000-0002-2819-0358

Robert Dinger  
http://orcid.org/0000-0002-6721-0868

Katharina Miebach  
https://orcid.org/0000-0001-5495-3761

Jochen Büchs  
https://orcid.org/0000-0002-2012-3476

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