Fermentation of H_2 and CO_2 with *Clostridium ljungdahlii* at Elevated Process Pressure – First Experimental Results

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Fermentation of synthesis gas mixtures (H_2, CO and CO_2) with anaerobic bacteria acting as a biocatalyst is a promising process for the production of fuels and chemicals with first large-scale applications. A known bottleneck in gas fermentation is gas-liquid mass transfer of low soluble gas components like H_2 and CO. One possibility to overcome these limitations could be the elevation of process pressure. *Clostridium ljungdahlii* is a known acetogenic bacterium for the production of acetic acid and ethanol, well investigated for the fermentation of synthesis gas at ambient pressure. Aim of the current experiments was to study the fermentation of a H_2/CO_2-mixture (without CO) at elevated process pressure. The 90 h-experiments were performed in a high-pressure experimental set-up, using a 4 L-semi-batch stirred tank reactor. Fermentation temperature was 37 °C and pH was constantly kept at 5.9. Total process pressure was set up to 7 bar absolute. As pressure increased, reactor cell concentrations at the end of the experiments were lower than at atmospheric conditions. Product distribution changed with pressure elevation. The concentration of the expected fermentation products, ethanol and especially acetate, decreased. Instead, the production of formate with a mean concentration up to 98 mmol L^{-1} was observed. At a pressure of 7 bar, formate became the main product of H_2/CO_2-fermentation.

1. Introduction

Acetogenic bacteria can be used as a biocatalyst for the microbial conversion of H_2, CO_2 and CO into biofuels and chemicals. Products of gas fermentation range from C_2-compounds, acetate and ethanol, to butanol, butandiol and butyrate (Neumann et al., 2016). Progress in genetic engineering offers the possibility of producing chemicals of higher value (Schiel-Bengelsdorf and Dürre, 2012), from a gaseous substrate that can be delivered as waste material, like steel mill off-gas. Other possible substrate sources are reformed biogas or gasification of wastes, residues from agriculture (Muslim et al., 2017) and forrestry. An alternative approach for gas supply is water and/or CO_2 electrolysis, using overload renewable electricity (Liew et al., 2016).
Acetogens like *Clostridium ljungdahlii* are able to grow autotrophically, from inorganic carbon sources like CO_2 and CO. Acetic acid and ethanol are produced via the Wood-Ljungdahl pathway, an ancient metabolic pathway that is described in literature (Schuchmann and Müller, 2014). The reaction stoichiometry from H_2 and CO_2 is given by Eq (1) and (2) (Phillips et al., 2017):

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \Delta G°' = -74.3 \text{ kJ mol}^{-1} \qquad (1)$$

$$6H_2 + 2CO_2 \rightarrow CH_3CH_2OH + 3H_2O \qquad \Delta G°' = -97.0 \text{ kJ mol}^{-1} \qquad (2)$$

The prevailing opinion in literature is that syngas fermentation efficiency is limited due to low solubility of the gaseous substrate in the liquid fermentation medium, which results in poor gas-liquid mass transfer (Acharya et al., 2014, Bredwell et al., 1999, Munasinghe and Khanal, 2010). Accordingly, it can be assumed that the availability of gas molecules inside the cell, where product formation takes place, is restricted. A bioreactor with a suspended cell culture and gas entry represents a multiphase system in which different limitations can affect gas mass transport. To get to the substrate compounds inside the microbial cell, the

substrate gas has to be transferred from the bulk gas bubble across the liquid film at the gas-liquid interface, through the bulk liquid, across the liquid-cell interface and the cell membrane. Major transport limitation is often reduced to the liquid film at the gas-liquid interface around the gas bubble (Bailey and Ollis, 1986, Klasson et al., 1992). Here, the driving force for gas mass transfer is the concentration gradient between the dissolved gas (c) in the bulk liquid and the equilibrium concentration (c^*) at the interface. The mass transfer rate also depends on the volumetric gas-liquid mass transfer coefficient (k_La) and is determined by Eq. (3) (Kadic and Heindel, 2014).

$$\frac{dc}{dt} = k_La(c^* - c) \tag{3}$$

According to Henry's Law, the concentration of a gas component i in the liquid phase is proportional to its partial pressure (p_i) in the gas phase (Eq (4))

$$c_i = \frac{p_i}{K_{H,i}} = \frac{x_i \cdot p_{total}}{K_{H,i}} \tag{4}$$

Where the constant of proportionality is represented by the Henry volatility $K_{H,i}$ (Sander, 2015) and the partial pressure is the product of the total pressure p_{total} and the mole fraction x_i of the respective gas component. The connection between these relations, mass transfer rate and Henry's Law, is presented in a review by Phillips et al. (2017) (Eq (5)).

$$\frac{1}{V_L}\frac{dn_i}{dt} = \frac{k_La}{V_L}(c_i^* - c_i) = \frac{k_La}{V_L}\frac{1}{K_{H,i}}(p_i - p_i^*) \tag{5}$$

Driving force for substrate transfer is therefore the gradient between the partial pressure in the gas phase p_i and the partial pressure of the substrate in the liquid phase p_i^*, and can be enhanced by increasing the total pressure inside the reactor.

Partial pressure changes of the substrate gas components may also have an effect on biochemical cell activities. Since enzymes like hydrogenase or formate dehydrogenase directly use H_2 and CO_2 as substrate, their metabolic efficiency can be influenced by the substrate gas partial pressure (Skidmore et al., 2013). The aim of the current study is to investigate the effect of pressure elevation on the fermentation performance of *Clostridium ljungdahlii*, using a mixture of H_2 and CO_2 as substrate gas.

2. Materials and Methods

2.1 Microorganism and cultivation

The organism used in this study was *Clostridium ljungdahlii* DSM13528. The culture medium is based on a formulation by Tanner (2007), the definite composition was described before by Oswald et al. (2016). Preculture medium was anaerobized with a gas mixture of 20 % CO_2 and 80 % N_2. The medium bottles were autoclaved at 121 °C for 20 minutes. Afterwards, cysteine-HCl • H_2O (1 g L^{-1}) was added. For precultivation, fructose (5 g L^{-1}) was used. The initial preculture (50 mL) was inoculated one week before the experiment and was carried out over two additional preculture stages (50 mL and 250 mL). For inoculation, 10 vol-% of seed culture were used, respectively. Precultures were grown at 37 °C for 48 h. (Oswald et al., 2016)

2.2 Experimental system and bioreactor operation

The experimental set-up is shown in Figure 1. Experiments were performed in a stainless steel stirred tank reactor with a total volume of 4 L and maximum operating pressure of 130 bar. The agitator shaft is equipped with two six blade impellers (Büchi AG) and four baffles are attached to the reactor lid. A pH electrode (Corr Instruments) allows steady pH-control, base (KOH, c = 4 mol L^{-1}) and acid (H_3PO_4, c = 4 mol L^{-1}) can be pumped into the reactor vessel with a dosing pump (Bischoff). The fermentor is also equipped with a foam probe, a temperature probe and an ORP electrode (Corr Instruments). A heating jacket ensures that the fermentation medium is kept at process temperature. Gas flow is regulated by a flow meter (Bronkhorst) and is dispersed in the reactor over a metal filter (pore size: 50 μm) at the outlet of a ¼'' tube. A pressure sensor (Bronkhorst) and a pressure control valve (Bronkhorst) are installed to maintain the given process pressure. The reactor exhaust gas is led to a gas chromatograph (Inficon). Process parameters are measured, logged and automatically controlled by a control software (STEP 7, Siemens). (Oswald et al., 2018)

Prior to fermentation, the reactor was sterilized with a steam cleaner. The heating jacket was used for additional heating of the vessel. Since the pH electrode was not temperature-resistant at 121 °C, it was cleaned with isopropanol and installed afterwards. Experiments were performed in semi-batch mode with continuous gas flow, while no liquid medium was fed or removed. The reactor was charged with 2.26 L medium and anaerobized with process gas, a mixture of H_2 (53.3 %), CO_2 (26.7 %) and N_2 (20 %). Temperature was set at 37 °C and agitator speed was 757 rpm. The fermentor was inoculated with 250 mL preculture.
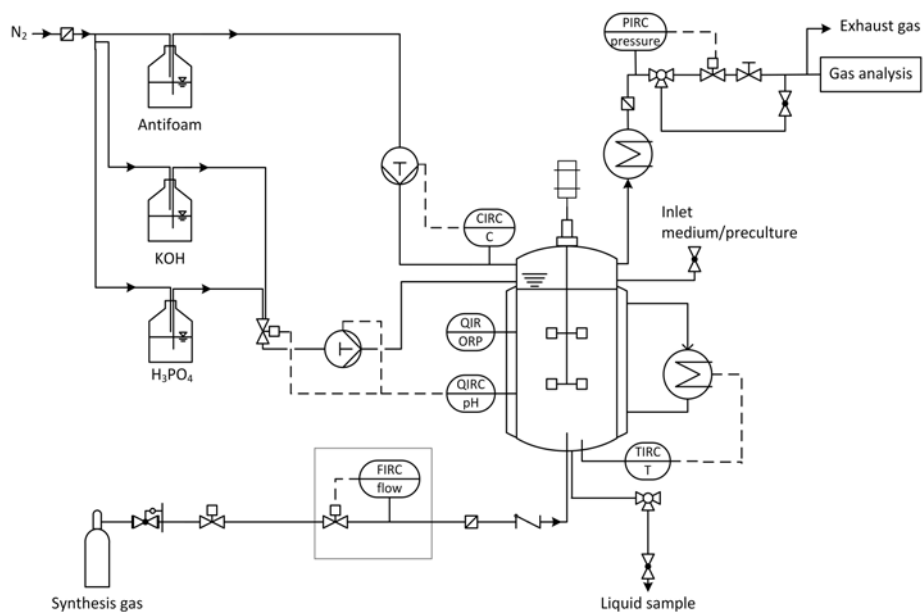
Figure 1: Schematic description of the experimental fermentation system. F: flow, Q: quality, T: temperature, P: pressure, C (first letter): current, I: indicate, R: record, C: control.

After inoculation, the experimental system was pressurized. Gas flowrate was set at 72 mL min^{-1} and process pH was adjusted to 5.9. Experiments were performed in triplicates at atmospheric pressure and at 7 bar, and in duplicate at 4 bar pressure. Experiment runtime was 90 h. Liquid samples were taken twice a day. (Oswald et al., 2018)

2.3 Analytical methods

The optical density (OD) of the liquid samples, which is proportional to the total biomass amount, was measured at 600 nm using a photometer (Shimadzu). If the OD exceeded a value of 0.4, samples were diluted with saline solution (c_{NaCl} = 9 g L^{-1}). Samples were centrifuged at 5480 x g for 10 min and the OD of the supernatant was measured because of possible changes during fermentation runtime. The supernatant was then analysed for acetic acid, ethanol and formic acid with HPLC (Hitachi), using an Aminex HPX-87H column with 4 mmol L^{-1} H$_2$SO$_4$ as eluent and refractive index detection. Ethanol concentrations were additionally determined by GC analysis, measurements were performed as described before by Oswald et al. (2016). The composition of the exhaust gas was determined using a micro gas chromatograph (Inficon) with a thermal conductivity detector. The two-channel instrument contains a Molsieve column (isotherm, 80 °C) with argon as carrier gas, for the measurement of H$_2$, O$_2$, N$_2$ and CO concentration, and a PoraPLOT U column (isotherm, 60 °C) with helium as carrier gas, where CO$_2$ concentrations are determined. Total measurement run time is 2.5 minutes.

3. Results and discussion

OD measurements revealed first effects of pressurization. For fermentations at atmospheric pressure, OD keeps increasing for more than 80 % of process runtime (75 h) to a maximum of approximately 0.5 (Figure 2A). At a total pressure of 4 bar and 7 bar, OD values are significantly lower and almost constant after 20 h runtime. Maximum values are 0.25 and 0.22, respectively, which indicates that less cell biomass is formed at higher pressure. In the 1990s, the University of Arkansas (1993) investigated the production of ethanol and acetate with *C. ljungdahlii* at pressures up to 11 bar. Substrate gas in this study was a mixture of H$_2$ and CO, without CO$_2$. It was reported that with increasing pressure, cell concentration in the reactor decreases and ethanol production is reduced, approaching zero. Despite the differences in substrate gas composition, these results are in good agreement with those shown below (Figure 2A & 2B). The authors suggested that a proven fermentation medium at atmospheric conditions is not necessarily suitable for fermentation at elevated pressure, assuming that nutrient limitation resulted in low productivity.

The influence of process pressure on product formation is shown in Figure 2B – 2D. Expected products of *C. ljungdahlii*, acetate and ethanol, were produced at a mean ratio of 10:1 at atmospheric conditions, with a highest concentration of 101 mmol L^{-1} and 13 mmol L^{-1}, respectively. The amount of these C$_2$ compounds

decreased significantly with increasing process pressure, while an augmented formation of formate was observed. At a total pressure of 7 bar and process time of >65 h, formate concentrations up to 116 mmol L^{-1} and 119 mmol L^{-1} were measured.

Results of liquid analysis differed within the respective experiment replicates, which is illustrated by error bars (standard deviation). Despite these differences in absolute values, the product ratios of the individual experiments are well comparable and show a clear trend with increasing process pressure. Figure 3 shows the ratio of the maximum concentration measured c_i (i = formate; acetate; ethanol) in relation to the total maximum product concentration $c_{product}$ ($c_{product}$ = $\sum c_i$). As can be seen, the main fermentation product shifts from acetate to formate with increasing process pressure. At atmospheric conditions, the mean concentration ratio of acetate is 0.89 mol mol^{-1}, whereas at a total pressure of 7 bar, $c_{acetate}/c_{product}$ is only 0.14 mol mol^{-1}. The concentration ratio of formate is inversely proportional and increases from 0.02 to 0.85 mol mol^{-1}. Ethanol only plays a minor role in the range of products with a highest concentration ratio of 0.09 mol mol^{-1} at atmospheric pressure.

Kantzow and Weuster-Botz (2016), who investigated the influence of H_2 partial pressure (p_{H2}) on growth and product formation of *Acetobacterium woodii* in a 2 L stirred-tank reactor, found similar results. An increase in formate production from 4.2 g L^{-1} to 7.3 g L^{-1} was observed with variation of p_{H2} from 1.4 bar to 2.1 bar at a total process pressure of 3.5 bar. Almost no formate was detected at atmospheric conditions (p_{H2} = 0.4 bar). Acetate formation decreased by almost 50 % as p_{H2} increased. In the current study, H_2 partial pressures were 0.54 bar, 2.13 bar and 3.73 bar. On average, formate formation increased from 2 mmol L^{-1} to 30 mmol L^{-1} and 97 mmol L^{-1}, respectively, while the mean acetate concentration decreased from 91 mmol L^{-1} to 38 mmol L^{-1} and 13 mmol L^{-1}. A study of Peters et al. (1999) discussed the production of formate by anaerobic bacteria growing on H_2 and CO_2. For acetogenic organisms, the results showed a proportionality between the maximum formate concentration and the initial H_2 partial pressure. These reports along with the results of the current experiments indicate a major role of p_{H2} concerning metabolic processes.
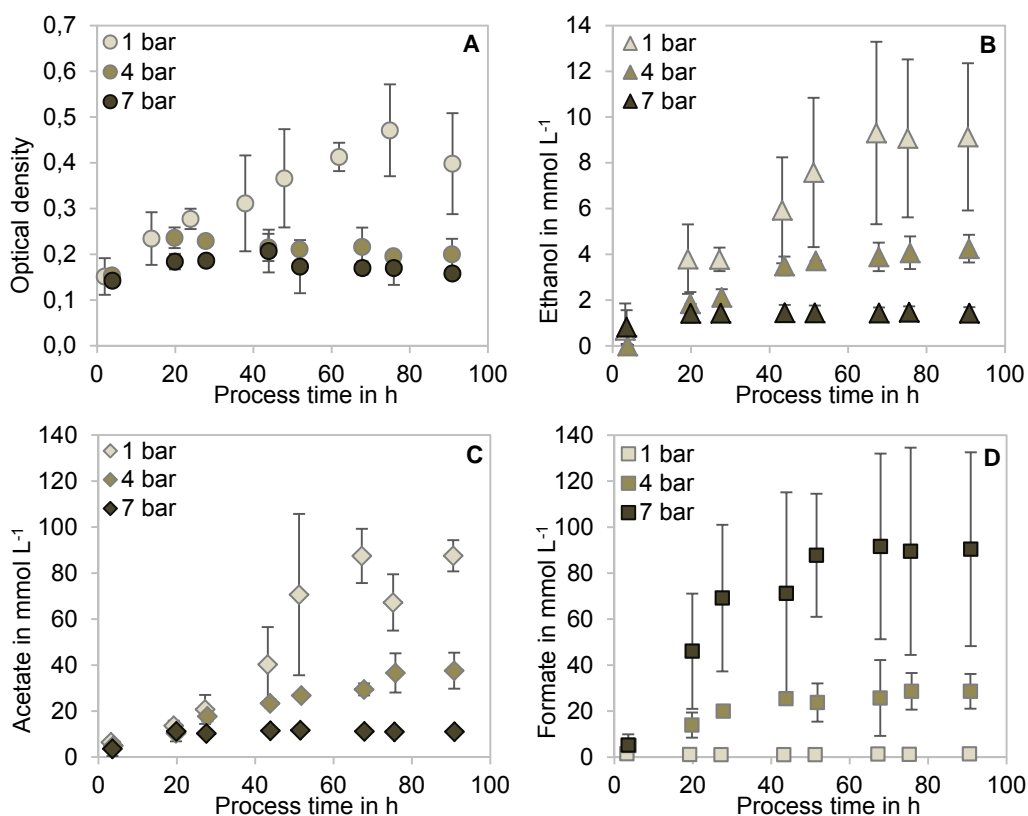

Figure 2: Analytical results as a function of process time. A) Optical density (OD); B) – D) ethanol, acetate and formate concentration in the fermentation liquid. Error bars indicate standard deviation, results are given as the mean of three (1 / 7 bar) or two replicates (4 bar).
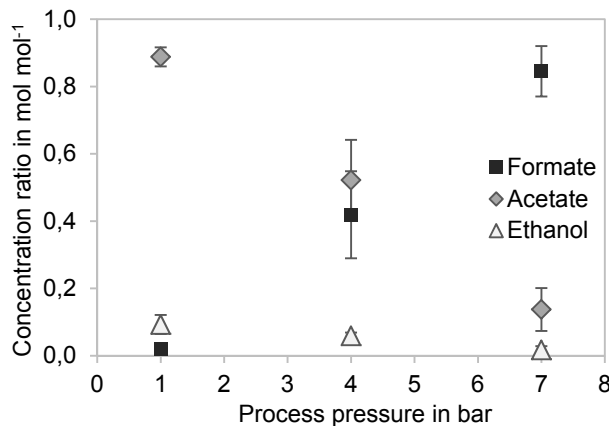
Figure 3: Concentration ratio $\frac{c_i}{c_{product}}$ of formate, acetate and ethanol as a function of process pressure. c_i: maximum concentration of acetate, ethanol, formate, $c_{product}$: $\sum c_i$.

In acetogens like *C. ljungdahlii* and *A. woodii*, formate is formed in the first reaction step of the methyl branch, as an intermediate of the Wood-Ljungdahl pathway. The following step, conversion of formate to formyl-THF, requires the cofactor THF as well as energy (ATP) (Schuchmann and Müller, 2014). The changes in the fermentation system induced by pressure elevation seems to have an influence on metabolic processes after formate formation. The observed shift of gas fermentation end product is likely linked to the increase of substrate gas partial pressure, which was also found by Schmidt and Cooney (1986). They designed their fermentation experiments in a way that total pressure was increased, but partial pressures of H_2 and CO_2 remained the same, using different gas mixtures. They stated that the elevation of hydrostatic pressure with constant partial pressures did not affect fermentation performance of *Clostridium sp.* ATCC 29797, but with increasing p_{H2} and p_{CO2}, specific productivity and acetate concentration decreased. The production of formate was not considered in this study.

It seems evident, that elevated partial pressures are a crucial factor in high-pressure gas fermentation and it is therefore necessary to couple physical theory of gas solubility with biochemical metabolic processes of the overall fermentation system. An important factor to be considered is the formation of cell mass, which is apparently reduced at higher pressure. Cell concentration is linked to overall productivity and therefore, it would be advantageous to achieve a higher amount of cell mass (Grethlein et al., 1990). The production of formate is an interesting aspect of gas fermentation at elevated pressure. It shows that it is possible to influence product distribution of acetogens by varying pressure and enables the biocatalytic synthesis of formate as a base chemical. Schuchmann and Müller (2013) also proposed to use the bacterial conversion of CO_2 to formate as a way of intermediate storage for H_2, since problems with economical transportation and storage is a major downside of its use as energy carrier.

4. Conclusion

The first results with a newly built high-pressure gas fermentation system demonstrated a significant effect of pressure elevation on product formation of *C. ljungdahlii*. At atmospheric conditions, the main product of H_2/CO_2 fermentation was acetate, with a mean maximum concentration of 91 mmol L^{-1}. With increasing pressure up to 7 bar, acetate concentration decreased by approximately 85 %. Conversely, the mean concentration of formate increased from 2 mmol L^{-1} to 98 mmol L^{-1}. Only a small amount of ethanol was formed throughout the experiments, with c_{max} = 13 mmol L^{-1} at ambient conditions. Decreasing OD values of the fermentation liquid indicate that cell concentration in the reactor fell with elevation of process pressure. The obtained results along with comparable reports in literature indicate that substrate gas partial pressures, especially p_{H2}, have a strong impact on fermentation performance. Pressurization changed the product distribution of gas fermentation and enabled the biological conversion of H_2 and CO_2 to formate as main product.

Further investigations to high-pressure gas fermentation are essential, with regard to cell mass formation and productivity as well as general considerations to reactor design concerning overall process efficiency. A correlation between enhanced gas solubility at higher pressure and enzyme-dependent metabolic processes has to be established.

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