Diploma Thesis

Optimization of process performance and mixotrophic cultivation of *Clostridium carboxidivorans* for the production of biofuel alcohols

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Declaration:

I declare that I have authored this thesis independently and that I did not use any other than the mentioned remedies. I have explicitly marked all material that has been quoted either literally or by content from the used sources.

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ABSTRACT

In times of limited energy resources, and increasingly significant negative climate and environmental changes, the focus is on renewable energy sources. Biofuels have proven to be a very good alternative to currently used fossil fuels. Biowaste and residual materials can be sensibly reused.

Industrial bioethanol production from CO_2/CO-rich waste gases (e.g. steel mills industry) is an effective way for the production of valuable biofuels. Acetogenic clostridia have the ability to use organic and inorganic (gaseous) substrates simultaneously, which is referred to as anaerobic, non-photosynthetic mixotrophy in which alcohols are produced by syngas fermentation under optimized conditions. The main syngas components, carbon monoxide, carbon dioxide, and hydrogen, are converted into alcohols.

Clostridium carboxidivorans was only investigated using autotrophic substrates, which led to low biomass formation and low productivity. In order to solve this problem, *C. carboxidivorans* was tested for the use of heterotrophic and mixotrophic substrates and their conversion into alcohols such as ethanol, butanol, but also hexanol.

In the first experiment, *C. carboxidivorans* was cultivated in serum bottles with different sugar concentrations in order to additionally test the influence of the sugar concentration on the effect of the mixotrophic conversion. The study has shown that mixotrophy performed successfully and a lower sugar concentration with added carbon monoxide leads to higher alcohol production, achieving 0.53 g/L butanol, 2.07 g/L ethanol, and 0.12 g/L hexanol.

In recent years, attempts have been made to achieve high productivity of the continuous process in order to achieve high butanol, ethanol and hexanol yields.

In the second experiment *C. carboxidivorans* was cultivated in a continuous process with mixotrophic substrates (glucose and various gas mixtures). Heterotrophic and mixotrophic substrates were compared in the formation of products. The setpoint with added carbon monoxide (60.12 % H_2, 9.54 % CO_2, 10.57 % CO, 19.77 % N_2) achieved the highest results. *C. carboxidivorans* can use carbon monoxide to form alcohols, which makes mixotrophy

successful. Alcohol concentrations in steady-state conditions reached values of 0.37 g/L butanol, 4.89 g/L ethanol, and 0.75 g/L hexanol.

The study also showed that lowering the pH in the chemostat was not possible, even from pH 6 to 5.5. Biomass or OD dropped sharply overtime at pH 5.5. Low pH led to a low growth rate.

With this work, a first continuous fermentation with *C. carboxidivorans* using mixotrophic substrates was carried out with the first evidence of mixotrophy and the effect of different gas mixtures.

ZUSAMMENFASSUNG

In Zeiten knapper Energieressourcen und immer stärker werdenden negativen Klima- und Umweltveränderungen liegt der Fokus auf erneuerbaren Energien. Biokraftstoffe haben sich als sehr gute Alternative zur natürlichen Energiegewinnung erwiesen. Bioabfälle und Reststoffe können sinnvoll wiederverwendet werden.

Die industrielle Bioethanolproduktion aus CO_2/CO-reichen Abgasen (z. B. Stahlwerksindustrie) ist ein effektiver Weg zur Herstellung wertvoller Biokraftstoffe. Acetogene Clostridien haben die Fähigkeit, organische und anorganische (gasförmige) Substrate gleichzeitig zu nutzen, was als anaerobe, nicht-photosynthetische Mixotrophie bezeichnet wird, bei der Alkohole durch Syngas-Fermentation unter optimierten Bedingungen hergestellt werden. Die Hauptkomponenten des Syngases, Kohlenmonoxid, Kohlendioxid und Wasserstoff, werden in Alkohole umgewandelt.

C. carboxidivorans wurde nur mit autotrophen Substraten untersucht, was zu geringer Biomassebildung und geringer Produktivität führte. Um dieses Problem zu lösen, wurde *C. carboxidivorans* auf den Einsatz heterotropher und mixotropher Substrate und deren Umwandlung in Alkohole wie Ethanol, Butanol, aber auch Hexanol getestet.

Im ersten Versuch wurde *C. carboxidivorans* in Serumflaschen mit unterschiedlichen Zuckerkonzentrationen kultiviert, um zusätzlich den Einfluss der Zuckerkonzentration auf die Wirkung der mixotrophen Umwandlung zu testen. Die Studie hat gezeigt, dass die Mixotrophie erfolgreich durchgeführt wurde und eine niedrigere Zuckerkonzentration mit zugesetztem Kohlenmonoxid zu einer höheren Alkoholproduktion führt, wobei 0,53 g/l Butanol, 2,07 g/l Ethanol und 0,12 g/l Hexanol erreicht werden.

In den letzten Jahren wurde versucht, eine hohe Produktivität des kontinuierlichen Verfahrens zu erreichen, um hohe Butanol-, Ethanol- und Hexanolausbeuten zu erzielen.

Im zweiten Experiment wurde *C. carboxidivorans* in einem kontinuierlichen Prozess mit mixotrophen Substraten (Glucose und verschiedene Gasmischungen) kultiviert. Heterotrophe und mixotrophe Substrate wurden bei der Bildung von Produkten verglichen. Der Sollwert mit zugesetztem Kohlenmonoxid (60,12 % H_2, 9,54 % CO_2, 10,57 % CO, 19,77 % N_2) erzielte die

höchsten Ergebnisse. *C. carboxidivorans* kann Kohlenmonoxid verwenden, um Alkohole zu bilden, was die Mixotrophie erfolgreich macht. Die Alkoholkonzentrationen unter Steady-State-Bedingungen erreichten Werte von 0,37 g/L Butanol, 4,89 g/L Ethanol und 0,75 g/L Hexanol.

Die Studie zeigte auch, dass eine Senkung des pH-Werts im Chemostat nicht möglich war, nicht einmal von pH 6 auf 5,5. Biomasse oder OD fiel im Laufe der Zeit bei pH 5,5 stark ab. Ein niedriger pH-Wert führte zu einer niedrigen Wachstumsrate.Mit dieser Arbeit wurde eine erste kontinuierliche Fermentation mit *C. carboxidivorans* unter Verwendung von mixotrophen Substraten mit ersten Anzeichen von Mixotrophie und der Wirkung verschiedener Gasgemische durchgeführt.

1 INTRODUCTION

To solve the energy and environmental crises, a huge problem of today and the main topic of current research is the generation of energy from renewable, environmentally friendly raw materials for the purpose of replacing petroleum fuels (Robak and Balcerek 2020; Abubackar et al. 2018). Today's bioethanol, according to 2020 data, is produced 96 % from first-generation raw materials such as sugar cane and corn, and the product itself has led to conflicts with food and feed supply (Robak and Balcerek 2020). The choice of raw materials also has a great impact on the price of the final product, as well as on greenhouse gas emissions. According to the European Parliament waste in the EU amounted to about 88 million tons every year (*Food waste: the problem in the EU in numbers [infographic]*, 2017). Globally, food waste has led to the production of 3.3 Gt of carbon dioxide per year (Carpio-Aguilar et al. 2019). Food waste made up of sugar and starch, as well as agricultural residues and plant-based biomass, are one of the alternative raw materials for generating energy and the so-called second-generation fuels (Vees, Neuendorf and Pflügl, 2020). Second-generation biofuels could replace petroleum fuel in the near future (Robak and Balcerek 2020).

According to the IPCC, the global temperature rose 1 °C above pre-industrial levels in 2017 and at the current rate of growth, an increase of 2 °C is expected, which would create catastrophe risks for human society and the planet. The main causes are greenhouse gases, CO_2, and methane, so the reduction of their emissions must be accelerated (Djalante 2019; IPCC et al. 2018; IPCC et al. 2018). In 2016, the Paris Agreement embraced plans to reduce CO_2 emissions by using "above carbon" feedstocks for fuel and chemical production (Köpke and Simpson, 2020). Switching from fossil fuel utilization to renewable energy sources would result in zero net carbon emissions (Köpke and Simpson, 2020).

Acetogenic clostridia have a special metabolism in that they can use both heterotrophic substrates (e.g. sugar) via glycolysis and autotrophic substrates (CO_2 and CO; C1 gases) via the Wood-Ljungdahl Pathway. For mixotrophic substrates (sugar + gas), acetogens use the WLP in which additional acetyl-CoA is produced from two CO_2 (also CO) molecules, whereby two converging branches of CO_2 molecules combine to form one acetyl-CoA molecule. In this way, sustainable biofuel such as butanol, ethanol, or/and hexanol could be generated and carbon

could also be fixed from industrial waste gases. Among the acetogenic clostridia, *C. carboxidivorans* is exciting because it can produce not only ethanol but also butanol and hexanol (Vees, Neuendorf and Pflügl, 2020).

1.1 Problem formulation

Large-scale formation of higher alcohols from synthesis gas has not yielded acceptable results to date. So far, *C. carboxidivorans* has only been investigated autotrophically where batch tests and continuous experiments have been carried out. Due to the low biomass concentration these processes suffer from low alcohol productivity. Autotrophy is not as profitable because the cells have less energy and therefore they grow poorly (low yields). In addition, the mass transfer from gas to liquid is usually poor. Also, a restriction of the growth of the biomass and metabolic activity of *C. carboxidivorans* was observed by bacterial agglomeration or was inhibited by end products (Shen et al. 2020; Fernández-Naveira et al. 2017). The effects of trace metal composition, culture temperature, and gas-feed flow rates have shown a major influence on the growth and product formation (Shen et al. 2017; Fernández-Naveira et al. 2017). The low pH values, which lead to alcohol production, also cause a decrease in growth rate and therefore the dilution rate. Published studies have also shown that a very low alcohol concentration was produced in relation to the total product and that the product formation was highly depending on the growth terms (Shen *et al.*, 2020).

For increased energy availability, heterotrophic cultivation would be the solution, on the other hand, *C. carboxidivorans* requires electron donors to fix CO_2. Mixotrophy could solve the problem by adding electron donors through H_2 and CO. The hypothesis of this research is that the mixotrophic cultivation of *C. carboxidivorans* improves the yield of alcohols because more energy is available.

In order to continue to promote industrial use, a continuous process would be much more efficient, requiring less preparation and less downtime, the continuous use of continuously accumulating waste streams, and the continuous generation of products as well as steady downstream processing. Therefore, the main elements in process optimization would be the reactor type and operating strategy (Vees, Neuendorf and Pflügl, 2020).

1.2 Aim of this work

The aim of this work was a feasibility study to prove whether *C. carboxidivorans* can grow mixotrophically. Two questions had to be answered. The first was whether mixotrophic conversion of *C. carboxidivorans* on CO with added sugar is possible and the second was how sugar concentration affects mixotrophic conversion. Carbon monoxide was used here because it is both, an electron donor and a carbon donor. To test the influence of the organic part in mixotrophy, different sugar concentrations were used. This experiment was compared to the same experiments using *C. ljungdahlii* as there is already a lot of research available with this strain where the mixotrophy has already been proven. So, the goal of the study was to increase the C-efficiency and thus the product yield. The experiment proved the mixotrophic growth with *C. carboxidivorans* on fructose and CO and by using a lower concentration of fructose in the mixotrophic conversion a higher concentration of alcohols was achieved.

The second aim of this work was to optimize the process performance of a continuous anaerobic fermentation for biofuel production with *C. carboxidivorans*. A continuous process would be efficient for industrial use and heterotrophic cultivation brings energy but lacks electron donors to fix CO_2. Mixotrophy could solve the problem by adding electron donors (H_2 and CO), and so could improve the yield of alcohols. The questions to be answered were whether the mixotrophic conversion is possible in the continuous reactor, which gas mixture under the mixotrophic conversion has a positive effect on ethanol production, and whether pH lowering is possible. Mixotrophic conversion with glucose and CO, CO_2, H_2 - gas mix proved to be successful. This gas mixture was advantageous for the formation of ethanol. Lowering the pH in the chemostat was not possible.

2 BACKGROUND

2.1 History of bioethanol and current status

The first biofuels were produced in the late 19th century. In the early days of production, biofuels were considered a potential transport fuel, but falling fossil fuel prices slowed their further development. Due to the oil crisis and the sharp rise in the price of fossil fuels, interest in the production of biofuels for transport reappeared in the 1970s (Balat and Balat, 2009), when Brazil and United States started with the production of bioethanol from corn and sugarcane (Chin and H´ng 2013). The most frequently used microorganisms for alcohol production by far are yeasts, especially *Saccharomyces cerevisiae* due to their robustness and good tolerance (Tse et al. 2021).

Solventogenic clostridia have been used in the Weizmann process for hundreds of years, producing solvents such as ethanol, butanol, and acetone. Hence, they are very well known and widely researched in the biotechnology industry. *C. acetobutylicum has* been used for ABE fermentation (Vees, Neuendorf and Pflügl, 2020).

US and EU biofuel policies stimulate the production of second-generation biofuels from cheap waste and therefore second-generation bioethanol is expected to increase its contribution to the world market. Global biofuel production in 2020 represents 96 % of first-generation biofuels. In 2018 the global production of bioethanol was around 110 billion liters, and the increase in production is expected to reach 140 billion liters in 2022. The main producers are the USA with 56 %, Brazil with 28 %, the European Union with 5 %, China with 4 %, and finally Canada with the lowest production of 2 % (Robak and Balcerek 2020).

In the production of lignocellulosic ethanol, lignin (up to 40 % of the biomass) is not converted. Plant biomass can be gasified into CO, CO_2, H_2, and N_2 (synthesis gas), and thus almost all of the carbon can be converted into fuels (Kleinsteuber *et al.*, 2016).

Three companies operating pilot gas fermentation plants around the world are Coskata Inc., Synata Bio, and LanzaTech. LanzaTech (based in Illinois, USA) is operating a process dealing with the conversion of H_2, CO_2, and CO-containing industrial waste gases, primarily originating

from steel mills, and is the only one of these three companies which have implemented its technology in commercial plants on a large scale (Vees et al. 2020; Stoll et al. 2020).

The autotrophic production of ethanol and butanol from syngas with *C. carboxidivorans* has been demonstrated, but only in a batch process where a low product titer was achieved (Cheng *et al.*, 2019).

2.2 Clostridia for bioethanol production

Clostridia are gram-positive rod-shaped obligate anaerobes that do not grow in the presence of oxygen, but there are also species that are aerotolerant. Thanks to their extracellular enzymes, they can use a wide variety of carbohydrates, including substrates like cellulose, hemicellulose, and starch (Tracy *et al.*, 2012).

Over the last century, solventogenic clostridia were used for industrial solvent production. *C. acetobutylicum* was the main organism for production of these solvents via the acetone–butanol–ethanol (ABE) fermentation. Other solventogenic clostridia determined for high butanol production are *C. saccharoperbutylacetonicum*, *C. beijerinckii* and *C. saccharobutylicum* (Vees, Neuendorf and Pflügl, 2020).

The synthesis of acetate from hydrogen and carbon dioxide was discovered by Fischer in 1932. The conversion required very little energy ($\Delta G^{0'} = -95$ kJ/mol), and in 1936 acetogenic bacteria were isolated in pure culture for the first time (Schuchmann and Müller, 2016).

Since the isolation of acetogenic *C. ljungdahlii* and the discovery of the possibility of using the greenhouse gases CO and CO_2 as a carbon substrate for industrial solvent production, acetogenic clostridia have become the center of interest. They can grow heterotrophically on different carbon substrates as well as autotrophically on gas mixtures that contain CO, CO_2, and H_2 (Vees et al. 2020; Schuchmann and Müller 2016). Other acetogens used for biofuel production by gases are *C. autoethanogenum*, *C.carboxidivorans*, and *C. ragsdalei* (Abubackar, Veiga and Kennes, 2018).

Acetogens have the Wood-Ljungdahl pathway (WLP), a reduction module that uses reduction equivalents to fix CO_2 and create acetyl-CoA. These reduction equivalents are produced from oxidative modules Embden-Meyerhof-Parnas (EMP) and Pentose Phosphate Pathways (PPP)

or from CO or H_2 oxidation (see Chapter **Error! Reference source not found.**) (Vees, Neuendorf and Pflügl, 2020). During cultivation, solventogenic and acetogenic clostridia grow in two phases: the acidogenic and the solventogenic phase. In the first phase of growth, called acidogenesis, acetyl-CoA is converted into acids and in the second phase of growth, solventogenesis, these acids are converted into alcohols via reductive modules.

C. carboxidivorans is of great interest due to its broad substrate spectrum (C6, C5 sugars) and its ability to produce not only ethanol and butanol, but also the C6 products hexanoat and hexanol, and is mainly used for the conversion of CO-abundant exhaust gases into alcohols. (Fernández-Naveira et al. 2016a; Fernández-Naveira et al. 2016b; Vees et al. 2020).

2.2.1 Acetogenic metabolism

Depending on the milieu, acetogenic clostridia can adapt their metabolism to produce acids or alcohols (Abubackar, Veiga and Kennes, 2018). In syngas fermentation, pH plays a significant role. Low pH values lead to the formation of alcohols from organic acids, a metabolic shift from the acidic phase to the solvent phase (Doll 2018a). The acidity and the degree of reduction of the substrate are two parameters that can also be controlled in order to influence the shift to the solvent, as these parameters have an influence on the metabolism of the acetogens and thus on the product formation (Vees, Neuendorf and Pflügl, 2020).

There are two ways of taking up the substrates: heterotrophic via Embden-Meyerhof-Parnas (EMP) and pentose phosphate pathways (PPP), and autotrophic via Wood-Ljungdahl Pathway (WLP).

The breakdown of hexose and pentose sugars takes place via oxidative metabolic modules, EMP and PPP, via which pyruvate is formed, and finally converted into acetyl-CoA through the release of CO_2 (Vees, Neuendorf and Pflügl, 2020).

For mixotrophic or heterotrophic substrates, acetogens use the Wood-Ljungdahl Pathway (WLP) in which reduction equivalents, generated by oxidative modules EMP and PPP or by CO and H_2 oxidation, are fixed to form acetyl-CoA. The WLP produces acetyl-CoA from two CO_2 (also CO) molecules, whereby two converging branches of CO_2 molecules combine to form one acetyl-CoA molecule. In the methyl branch, CO_2 is reduced to formate by formate dehydrogenase, which is then bound to the cofactor tetrahydrofolate (THF), and then further

reduced to a methyl group linked to an iron-sulfur protein in the following step. In the carbonyl branch, CO_2 is reduced by CO dehydrogenase, and finally, these two intermediate products of different branches are bound together with CoA to form a molecule of acetyl CoA. Acetyl-CoA derived from WLP and EMP pathway is further converted in the metabolism for the production of either acids or solvents. Typically, acids are produced in the first growth phase and in the second they are converted into alcohols by reductive modules. It is also important to note that produced acetic acid is a product of the incomplete oxidation of ethanol (Vees et al. 2020; Schuchmann and Müller 2016). Depending on the product, more reduction equivalents are required. Products that are more highly reduced (here ethanol, butanol, hexanol) require more reduction equivalents than acids. In addition, the respective acids are produced from an ATP phosphorylation of acetyl-P / butyryl-P / hexanoyl-P. In order to produce more alcohol, more reduction equivalents must be available so that the cell can process the acids into alcohols.

The WLP works on the energy limit and serves as an "electron sink" so that it is only profitable through the energy mechanisms (energy conservation) that also take place in the cell. Chemiosmosis is coupled to the WLP via the accumulation of Fd^{2-} (reduced ferredoxin). The oxidation of reduced ferredoxin by the Rnf complex creates a chemiosmotic gradient that is used to generate energy via ATPase (Schuchmann and Müller, 2014; Vees, Neuendorf and Pflügl, 2020).

2.2.2 Anaerobic, non-photosynthetic (ANP) mixotrophy

Syngas is a gas mixture that mainly consists of CO_2, CO, and H_2. It is produced by gasifying biomass or is found in an industrial waste stream as an exhaust gas of steel mills and the oil industry (Vees et al. 2020; Devarapalli and Atiyeh 2015). Industrial CO-rich waste gases can be directly used for fermentation (Shen *et al.*, 2020). Its origin affects the composition, so various impurities such as ammonia and nitrogen oxides, as well as enzyme inhibitors such as ethane, acetylene, ethylene, and oxygen, can appear. Acetogens convert CO, CO_2, and H_2 (only as an electron donor) into organic acids and alcohols (Vees et al. 2020; Devarapalli and Atiyeh 2015).

Mixotrophy is the ability of bacteria to simultaneously use both organic and inorganic substrates (CO, CO_2, H_2, methanol, etc.) for their metabolism and growth (Fast *et al.*, 2015). The advantage of mixotrophy is an additional supply of carbon sources and also electron sources.

The main use of mixotrophy is to fix the CO_2 released during glycolysis to generate additional acetyl-CoA (Fast et al. 2015). Additional carbon sources can be delivered by adding syngas (Vees et al. 2020; Devarapalli and Atiyeh 2015). This enhanced mixotrophy will increase the amount of re-capture CO_2. Additional H_2 acts here as an electron donor and thus as an energy supplier for the cell. Acetogenic bacteria use the Wood-Ljungdahl pathway for carbon fixation and can stoichiometrically convert 1 mol of glucose into 3 mol of acetate (Fast *et al.*, 2015).

The advantage of mixotrophy is the higher carbon efficiency and higher biomass formation. With the mixotrophy sugar-containing wastewater can be used as a heterotrophic carbon source and, at the same time, carbon-containing waste gases can be used as an autotrophic carbon source. This could then increase productivity greatly.

2.2.3 Process development (Chemostat)

The basic aim of this work is to adapt the process to the requirements in the industry. The process should be easy to control, efficient, and should lead to high productivity and high yield. The three types of fermentation processes are batch, fed-batch, and continuous process (e.g. chemostat and retentostat).

The batch process is an excellent and simple method for achieving a high solvent yield, but the disadvantage of batch use in the industry are long reactor preparations and lower productivity due to longer lag phases (Vees, Neuendorf and Pflügl, 2020). Fed batch reaches higher ethanol formation but has the disadvantage of increased costs and longer downtimes between batches (Tse et al. 2021). The goals of this work can best be achieved with a continuous process, which in contrast to batch and fed-batch has the advantages of improved capabilities of process design, steady-state conditions, faster production rate, growth control, less work in the pure culture preparation, continuous substrate conversion, continuous downstream, lower costs and increased productivity (Vees et al. 2020; Yang and Ma 2019;Tse et al. 2021).

Due to the advantages of continuous processes, it is beneficial to implement a chemostat process for the *C. carboxidivorans*.

Optimal temperature and pH values are of great importance for process optimization. They affect cell growth, enzyme stability and function, the solubility of gases, and therefore alcohol production. The optimal temperature of *C. ljungdahlii* and *C. carboxidivorans* was found to be between 37 and 40 °C, and the external pH for cell growth varies between 5.5 and 6.5 during

synthesis gas fermentation and between 4.5 and 4.8 for solvents production (Devarapalli and Atiyeh, 2015). In order to catalyze biochemical reactions, vitamins, minerals, and metals are necessary for the medium, and yeast extract is used for the purpose of obtaining amino acids and nitrogen compounds necessary for cell synthesis (Devarapalli and Atiyeh, 2015).

Special attention in the continuous process, in relation to the batch, is directed to the creation of strict anaerobic conditions and sterile work (Vees, Neuendorf and Pflügl, 2020). The application of chemostat cultivation is challenging due to cell degeneration, and reaching steady state is also difficult (Vees, Neuendorf and Pflügl, 2020).

3 MATERIALS AND METHODS

3.1 Strains

The strain of the acetogenic bacteria, *C. carboxidivorans* P7 (DSM 15243), was used in both chemostat experiments and serum bottle experiments. *C. ljungdahlii* was also used in serum bottle experiments as well investigated reference strain. Both strains were obtained from 'Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)' and stored in glycerol at -80 °C.

3.2 Pre-culture

For preculture preparation, the components shown in Table 1 were weighed and dissolved in deionized water. The medium was then sparged with 100 % N_2 for 45 minutes, afterwards 0.5 g/L L-cysteine-HCl·H_2O was added. The pH was adjusted to 6 using 5 M KOH. 38.8 mL of the medium was dispensed into each 100 mL serum bottle and was sparged with 100 % N2 for 5 minutes, sealed airtight, and autoclaved. Further preparation of the medium was continued in an anaerobic chamber using 70 % isopropanol to achieve sterile conditions. After the medium had cooled, a sterile anoxic fructose stock solution (see Chapter 3.3.1) for serum bottle experiments and a sterile glucose stock solution were added, both at a final concentration of 5 g/L. Thereafter, 10 ml/L of the sterile vitamin stock solution was added (see Chapter 3.3.5). The bottles are shaken and 2 drops of 100 g/L L-Cysteine-HCl solution were added. After 30 minutes, the media was inoculated with a 1.7 ml cryo culture, shaken, and incubated at 37 °C and 230 rpm.

***Table 1**: Components and corresponding concentrations of DSM135YE+ for pre-culture media*

Component	Concentration
NH_4Cl	1 g/L
KH_2PO_4	11.73 g/L
K_2HPO_4	2.406 g/L
Yeast extract	2.00 g/L
$MgSO_4$ * $7H_2O$	0.10 g/L
Trace element solution	20 ml/L
Na-resazurin solution (0.2 % w/v)	0.25 ml/L
$FeSO_4$*$7H_2O$	0.026 g/L
Na_2SO_4*$5H_2O$ Stock (0.0194 g/mL) Na_2WO_4*$2H_2O$ Stock (0.0192 g/mL)	10 µL/L

3.3 Media

3.3.1 Media of serum bottle experiments

Medium from serum bottle experiments was prepared in the same way as a medium from preculture of the same experiment, but with different fructose concentrations with or without added CO, according to the corresponding setpoint (see Chapter 3.4.1).

3.3.2 Media of chemostat experiments

To produce batch media DSM135 YE + (Acetobacter medium modified +Fe +Se +W) for the chemostat experiment the components shown in Table 2 are weighed and dissolved in deionized water. The batch medium was poured into the reactor and autoclaved at 121 °C for 20 minutes. To be anoxic, the media was afterwards sparged with 100 % N_2 for 45-60 minutes. After cooling of batch media, 10 ml/L media sterile vitamin solution (see Chapter 3.3.5) and sterile glucose solution (see Chapter 3.3.3) were injected through the septum with a syringe. Afterwards, 0.5 g of L-cysteine-HCl · H_2O in form of a 100 g/100 mL solution were added via the septum and the pH was adjusted to 6 with 5 M KOH. The pH was checked and adjusted with 5 M NH_4OH if it was needed.

For the chemostat feed media DSM135 YE+, the components in Table 2 are weighed and dissolved in deionized water. The medium was poured into a 10-20 L bottle and the biosystem was added. After the feed media was sparged, 0.5 g L-cysteine-HCl · H_2O was added and the pH was adjusted to 6 using 5 M KOH. The feed medium was then autoclaved at 121 °C for 20 minutes. After cooling, 10 mL/L of sterile vitamin solution (see Chapter 3.3.5) via a sterile filter and sterile anoxic glucose solution (see Chapter 3.3.3) were added into feed media, sparged with 1 sL/min N_2 for 60 minutes, and finally, an N_2-filled balloon was added to the ingas filter.

Trace element solution and vitamin solution were prepared as described in Chapters 3.3.4 and 3.3.5.

***Table 2**: Components and corresponding concentrations of DSM135YE+*

Component	Concentration
NH_4Cl	1 g/L
KH_2PO_4	0.587 g/L
K_2HPO_4	0.12 g/L
Yeast extract	2.00 g/L
$MgSO_4$ * $7H_2O$	0.10 g/L
Trace element solution	20 ml/L
Na-resazurin solution (0.2 % w/v)	0.20 ml/L
$FeSO_4$*$7H_2O$	0.026 g/L
Na_2SO_4*$5H_2O$ Stock (0.0194 g/mL) Na_2WO_4*$2H_2O$ Stock (0.0192 g/mL)	10 µL/L

3.3.3 Sterile anoxic fructose/glucose solution

For the fructose stock solution used in the serum bottle experiments, 250 g of fructose was weighed and dissolved in 1 liter of deionized water, flushed with N_2 for about 45 minutes, and autoclaved at 121 °C for 20 minutes. For experiments with 5 g/L fructose, 800 µL was added to each serum bottle and 400 µL for 2.5 g/L.

For a glucose concentration of 10 g/L in the batch media and feed media, 11.1 g of glucose monohydrate was weighed for 1 L of the medium. The amount of calculated glucose was weighed and dissolved in deionized water (50 mL/1 L batch media, 900 mL/ 10 L feed media) and sparged with N_2 for about 45 minutes. Finally, the glucose solution was autoclaved at 121 °C for 20 minutes and stored at room temperature until usage.

3.3.4 Trace element solution

The trace element solution was prepared earlier and stored at 4 °C until use. First, nitrilotriacetic acid was dissolved and the pH adjusted to 6.5 with 5 M KOH. Afterwards the components were added as 100-1000-fold concentrated stock solutions to reach the final concentrations given in Table 3. Finally, the pH was adjusted to 7.0 with 5 M KOH.

***Table 3**: Components and corresponding concentrations of Trace element solution*

Component	Concentration
Nitrilotriacetic acid	1.50 g/L
$MgSO_4$ x $7H_2O$	3.00 g/L
$MnSO_4$ x H_2O	0.50 g/L
NaCl	1.00 g/L
$FeSO_4$ x 7 H_2O	0.10 g/L
$CoSO_4$ x 7 H_2O	0.18 g/L
$CaCl_2$ x 2 H_2O	0.10 g/L
$ZnSO_4$ x 7 H_2O	0.18 g/L
$CuSO_4$ x 5 H_2O	0.01 g/L
$KAl(SO_4)_2$ x 12 H_2O	0.02 g/L
H_3BO_3	0.01 g/L
Na_2MoO_4 x 2 H_2O	0.01 g/L
$NiCl_2$ x 6 H_2O	0.03 g/L
Na_2SeO_3 x 5 H_2O	0.30 mg/L
Na_2WO_4 x 2 H_2O	0.40 mg/L

3.3.5 Vitamin solution

The vitamin solution was prepared by dissolving ingredients according to Table 4. The solution is then bottled up in a large serum bottle and sparged with 1 sL/min 100 % N_2 for 30 minutes. The bottle was sealed and stored in the dark at 4 °C. Before each use, the mixture was left to stand for some time at room temperature while stirring.

***Table 4**: Components and corresponding concentrations of Vitamin solution*

Component	Concentration
Biotin	2 mg/L
Folic acid	2 mg/L
Pyridoxine-HCl	10 mg/L
Thiamine-HCl	5 mg/L
Riboflavin	5 mg/L
Nicotinic acid	5 mg/L
D-Ca-pantothenate	5 mg/L
Vitamin B12	0.10 mg/L
p-Aminobenzoic acid	5 mg/L
Lipoic acid	5 mg/L

3.4 Serum bottles

In these experiments, the feasibility of mixotrophy and the influence of the sugar concentration on the mixotrophic conversion with *C. carboxidivorans* was tested. The product spectrum and productivity were determined depending on the proportion of heterotrophic or autotrophic carbon sources. The experiments were compared to the reference experiments with *C. ljungdahlii.*

3.4.1 Setpoints of serum bottle experiments

The experimental setpoints were carried out in triplicate. Both strains were performed in DSM135YE+ media using two fructose concentrations with/without flushing the bottle headspace with pure carbon monoxide as shown in Table 5.

***Table 5**: Setpoints of the serum bottle experiments*

Bottle Code	C-Source
A	**5 g/L fructose**
B	**2.5 g/L fructose**
C	**5 g/L fructose + CO**
D	**2.5 g/L fructose + CO**

3.4.2 Preparation of serum bottles with varying sugar concentration

After the media, prepared according to a recipe in Chapter 3.3.1, had cooled, 0.8 mL of fructose stock solution (250 g/L) was added to serum bottles for experiments with a fructose concentration of 5 g/L and 0.4 mL for serum bottles of experiments with 2.5 g/L fructose. Thereafter, 0.4 mL of vitamin solution was added to all serum bottles. The bottles were shaken and 2 drops of sterile, anoxic 100 g/L L-cysteine HCl solution were added and waited for about 30 minutes. For the experiments with (additional) CO source, the headspace of each serum bottle was finally flushed with pure CO (Linde, carbon monoxide 2.0, UN1016) for 20 seconds. After 24 hours of growth, the pressure of CO was adjusted to 1.5 bar overpressure.

3.4.3 Inoculation of *C. carboxidivorans* and *C. ljungdahlii*

The inoculation of the serum bottles was carried out in an anaerobic chamber. The goal was to achieve an initial OD_{600} of 0.1. The inoculation volume was calculated using Equation 1 (see Chapter 3.5.3). Immediately after the inoculation, 1 mL sample was taken, the pH and the OD_{600} was measured and the sample was prepared for the HPLC measurement. The serum bottles were incubated at 37 °C without shaking and after 24 hours set to 200 rpm. Serum

bottles were checked every 24 hours and pH adjusted to 5.5-6.0 with a few drops of 5 M NH_4OH.

3.5 Reactor

In a chemostat experiment, anaerobic cultivation was carried out with *C. carboxidivorans* for alcohol production. The strain was tested for its ability to mixotrophic growth on glucose in co-utilization with various gas mixtures, and its growth at different pH values.

3.5.1 Process setup

Chemostat cultivation was carried out in a 1 liter stirred tank glass bioreactor (Applikon Biotechnology, Delft, The Netherlands) in which a pH electrode (Mettler Toledo, 405-DPAS-SC-K8S/225), and a temperature sensor (Pt100) were installed.

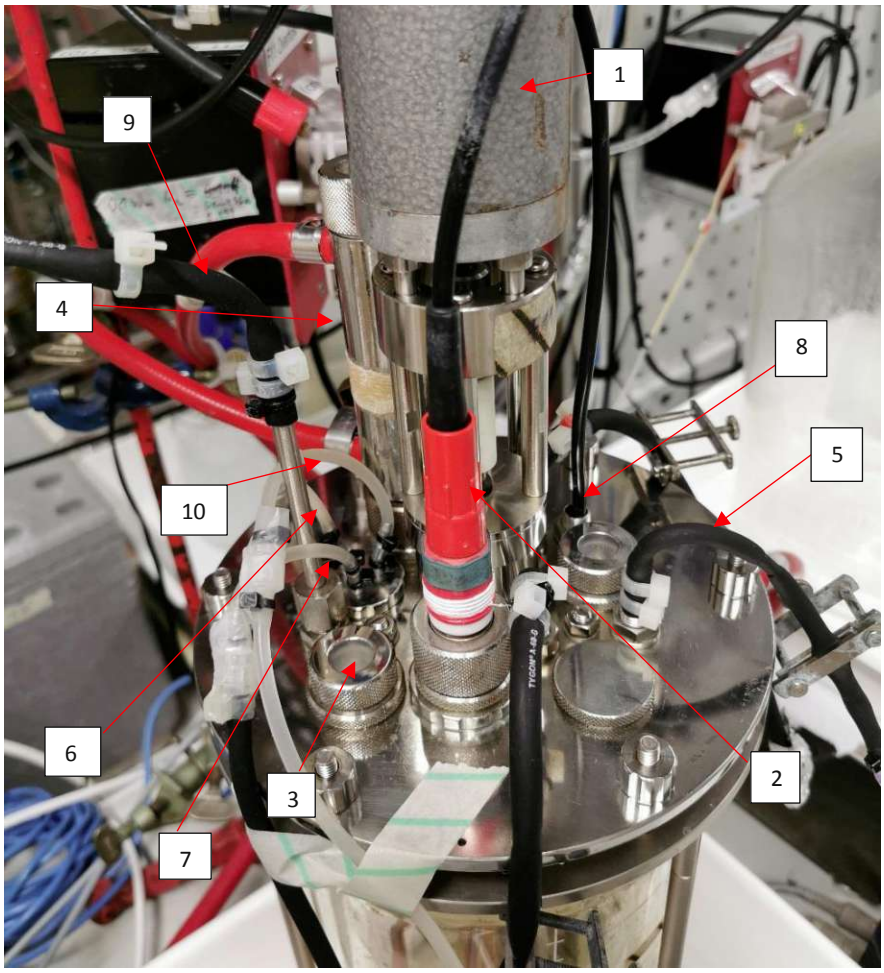

***Figure 1**: Inlets on the lid of the bioreactor: stirrer motor (1), pH electrode (2), septum (3), off-gas cooler (4), sample port (5), base port (6), feed port (7), temperature probe (8), inlet gas (9), antifoam port (10)*

Temperature was maintained via a water bath connected to the double jacket. Mass flow controllers (4800 Series, Brooks) were used for in-gas control of nitrogen (Messer, Stickstoff 5.0 with 99.999 % purity) and hydrogen (Air Liquide, Alphagaz 1, H_2 with 99.999 % purity). Needle valves were used for the mixed gases. The off-gas was cooled at the outlet of the bioreactor with an off-gas cooler. Since the solvents produced are volatile the off-gas was additionally passaged through an off-gas wash-bottle which was stored in fresh ice. The off-gas wash-bottle contained an acetate solution of approximately 10 g/L which was previously autoclaved at 121 °C for 20 minutes. The now water-free off-gas was afterward measured with an in-line H_2 and CO_2 sensor (BlueSens gas sensor, Herten Germany) and afterward led into the gas chromatograph (Thermo Fisher Scientific S.p.A K8880604, Milan, Italy) (see Chapter 3.6.6) for the exact off-gas measurement.

The feed bottle and harvest bottle were connected to the bioreactor for continuous operation. The feed bottle was constantly stirred with a magnetic stirrer so that constant conditions of the feed composition were achieved. A peristaltic pump (Lambda Preciflow, Zürich, Schweiz) was used for feed flow regulation. Feed flow was set to 50 mL/h for 1 L working volume of the reactor, which corresponds to a dilution rate of 0.05 h^{-1}. For Harvest, we used a digital pump that was connected to a dip tube. The level in the reactor was kept constant by drawing off the liquid continuously. The bioreactor setup is shown in Figure 2. Process parameters were controlled and monitored via the Lucullus PIMS software.

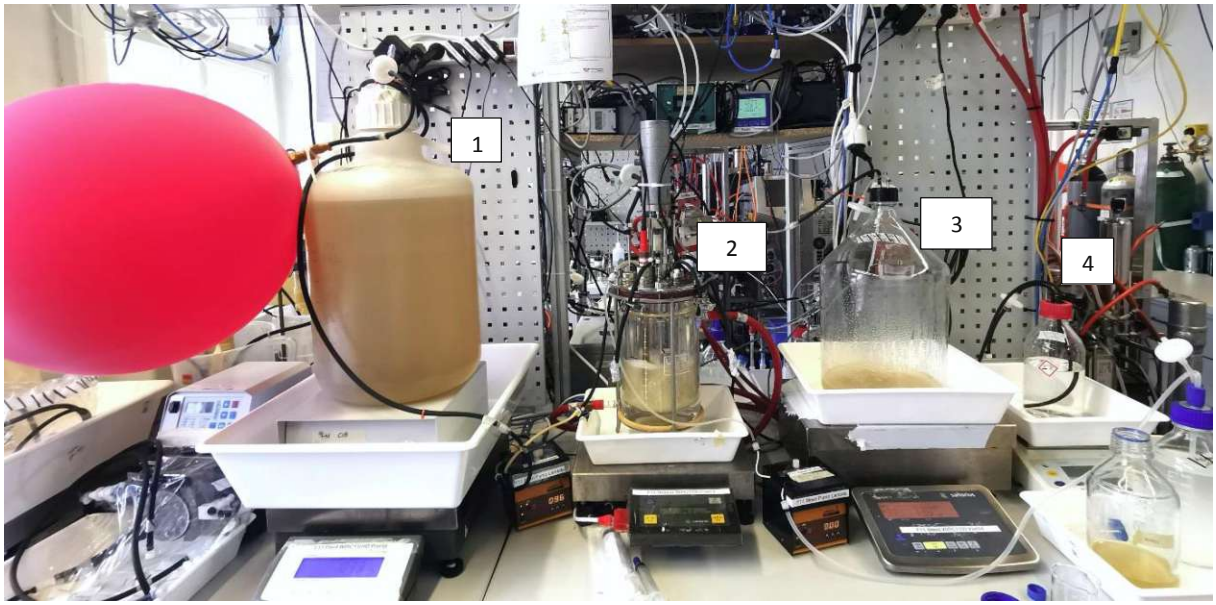

***Figure 2**: Bioreactor setup: feed bottle (1), bioreactor (2), harvest bottle (3), base bottle (4).*

3.5.2 Calibration of sensors

The pH was calibrated directly on the Siemens control module (SIMATIC Multi Panel). The zero points for the CO_2 and H_2 off-gas sensors were measured directly at the IP output of the sensors via the respective IP in Telnet. Afterwards, the data was entered in Lucullus PIMS for each of the two measuring points.

3.5.3 Inoculation

The volume of the inoculum was calculated (see Equation 1) so that the starting OD_{600} in the bioreactor would be approximately 0.2. The volume of the inoculum was removed from the initial volume of the medium in the bioreactor. The inoculum was added through the septum in the lid of the bioreactor with sterile syringes. From this point, the cultivation time was started and a starting sample for measuring OD_{600}, cell dry weight, and HPLC was taken.

$$V_{start} * OD_{600start} = V_{Inoculum} * OD_{600Inoculum}$$

Equation 1: *Calculation of inoculation volume*

V.....Volume
OD_{600}.....Optical density at a wavelength of 600 nm

3.5.4 Setpoints

The experiments were carried out at pH 6 and pH 5.5 with different gas mixing ratio settings as shown in Table 6.

Table 6: *Setpoints of the chemostat experiments*

Process Setup			
10 g/L Glucose, D=0.05, 0.25 vvm	**pH 6**	**100 %N_2**	Heterotrophic setpoint
		60 % H_2, 40 % N_2	H_2 - gas mix
		60.03 % H_2, 9.54 % CO_2, 30.43 % N_2	CO_2, H_2 - gas mix
		60.12 % H_2, 9.54 % CO_2, 10.57 % CO, 19.77 % N_2	CO, CO_2, H_2 - gas mix
	pH 5.5	**100 % N_2**	Heterotrophic setpoint, 5.5
		60 % H_2, 40 % N_2	H_2 - gas mix, 5.5

3.6 Analytics

3.6.1 Sampling

During the bioreactor cultivation, samples were taken sterile using 10 mL syringes. The sample port and syringe were sterilized using 70 % isopropanol. First, 10 mL was removed from the sample tube and discarded to avoid the influence of dead volume, and then 10 mL was removed for OD_{600}, DCW, and HPLC analysis. A 2 mL sample was taken from the off-gas wash bottle and used for the HPLC analysis of the stripped alcohols.

Samples from serum bottle experiments were taken every 24 hours for several days to monitor both bacterial growth and product formation. About 2 mL of the sample was taken from the serum bottles with a sterile syringe and a sterile needle for further measurements. The samples were taken sterile by spraying 70 % isopropanol on the lids and flamed. For the experiments with CO, no flame was used. Sampling took place in the fume hood, where the lids were sprayed with 70 % isopropanol, left to dry, and the sample was taken.

3.6.2 pH measurements

The SenTix® 81 precision electrode (WTW, Weilheim, Germany) was used to measure the pH value. The two-point calibration was performed using pH 7.0 and pH 4.0 buffer solutions. Pre-washed with deionized water and dried, the electrode was immersed in a test tube and the value was noted.

3.6.3 Optical density (OD)

A Thermo Scientific ™ GENESYS ™ 20 visible spectrophotometer (Thermo Fischer Scientific, Waltham, US) was used for measuring the optical density of the samples. Measurements were made at 600 nm of wavelength in the linear range of 0.1-0.6, and samples exceeding this linear range were appropriately diluted with deionized water. For the serum bottle samples, a factor of 0.4 is taken for the calculation of the biomass concentration in g/L.

3.6.4 Cell dry weight (CDW)

For the biomass analysis, 2 mL of fermentation broth were pipetted into three weighed-out test tubes and centrifuged for 12 minutes at 4 °C and 4800 rpm (Sigma Laborzentrifugen GmbH, 3-18KS, Osterode am Harz, Germany). The supernatant was transferred into Eppendorf tubes for further analysis, and the pellet was washed with 2 mL of deionized water and centrifuged again with the same settings.

Finally, the supernatant was removed, and the test tubes were dried at 105 °C for at least 24 h. After drying and cooling in a desiccator, the test tubes were weighed. The cell dry weight was calculated (see $CDW = \frac{m_{glass\ tube+sample} + m_{glass\ tube}}{V_{sample}}$ **Equation 2**) and the average of the triplicates including standard deviations was determined.

$$CDW = \frac{m_{glass\ tube+sample} + m_{glass\ tube}}{V_{sample}}$$

***Equation 2**: Calculation of Cell Dry Weight*

CDW……cell dry weight (g/L)

m…… mass (g)

V……..volume (L)

3.6.5 High-performance liquid chromatography (HPLC) analysis

HPLC measurements were performed on Thermo Scientific UltiMate 3000 (Thermo Fischer Scientific, Waltham, US) with an Aminex® HPX-87H column. The separation was done by using 4 mM H_2SO_4 as a mobile phase, with a flow rate of 0.6 mL/min, at a temperature of 60 °C and a pressure of 48-52 bar. The samples were measured for 90 minutes. For peak detection two detectors were used, a refractive index (RI) detector, ERC RefractoMax 520 (DataApex, Prague, Czech Republic), and a UV/VIS detector, DAD-3000RS (Thermo Fischer Scientific, Waltham, US), at a wavelength of 210 nm. Analysis of chromatograms was done with Chromeleon 7

software and evaluated with calibration standards. Standard solutions of the expected substances (glucose/fructose, lactic acid, formic acid, acetic acid, butyric acid, ethanol, butanol, hexanoic acid, and hexanol) with concentrations between 0.1 g/L and 50 g/L have already been prepared.

Retention times of the acids and alcohols with RI were 12.95 min for lactic acid, 13.93 min for formic acid, 15.11 min for acetic acid, 21.19 min for butyric acid, 21.98 min for ethanol, 36.34 min for butanol, 40.66 min for hexanoic acid, and finally hexanol with a retention time of 83.78 min.

With overlapping ethanol and butyric acid (mix peak) in the RI, the ethanol concentration was calculated using butyric acid concentration determined via the UV, using the following formulas:

$$Area(Ethanol\ RI) = Area(double\ peak\ RI) - Area(Butyric\ acid\ RI)$$

$$Amount(Ethanol\ RI) = Slope * Area(Ethnol\ RI) + intercept$$

$$Amount(But.\ acid\ RI) = Amount(But.\ acid\ UV)$$

Equation 3: *Formulas for calculating the concentration of ethanol in RI with mix peak of ethanol and butyric acid in RI and butyric acid in UV*

Area RI…….[µRIU*min]

Area UV……..[mAU*min]

Amount……[g/L]

Only acids are detected with UV. The retention time of the butyric acid measured with UV was 20.91 min.

The HPLC sample was prepared by using the supernatant of the sample, which was obtained during dry cell weight determination described in Chapter 3.6.4. An HPLC eluent stock solution with 40 mM was diluted 1:10 with the supernatant, by mixing 450 µL of the supernatant with 50 µL. Diluted samples were vortexed and centrifuged for 10 minutes at 4 °C and 14000 rpm

(Sigma Laborzentrifugen GmbH, 3-18KS, Osterode am Harz, Germany). Approximately 0.3 mL of sample (supernatant) was pipetted into HPLC vials.

3.6.6 Gas chromatography (GC)

The GC (Thermo Fisher Scientific S.p.A K8880604, Milan, Italy) with a ShinCarbon ST 100/120 column and thermal conductivity detector (TCD) was used to quantify inorganic gases (hydrogen, nitrogen, carbon dioxide, and carbon monoxide) from the reactor, as well as calibration gases. The flow of 2 mL/min and a split-flow of 10 mL/min (factor 5 split) were used. Argon was used as the carrier gas and the samples were measured for 21 minutes. The temperature was held at 30 °C for up to 6.5 minutes, then the temperature was increased at a rate of 16 °C/minute until the retention time of 19.625 min and 240 °C was reached. The temperature of 240 °C was kept constant until the retention time of 21 minutes was reached. The retention time of hydrogen was 1.97 min, of nitrogen 4.51 min, of carbon monoxide 5.78 min and at a retention time of 14.43 min, carbon dioxide occurred.

3.7 Key formulas

Serum bottle experiment		
Parameter	Calculation	Description
Product yield	$Y = \frac{C_{metabolite}}{C_{substrate}}$	Y.....product yield [Cmol/Cmol] $C_{metabolite}$..conc. metabolite [Cmol/L] $C_{substrate}$ ...conc. substrate [Cmol/L]
Amount of carbon atoms in substance	$C = \Delta c * z * M^{-1}$	C..... equivalent to the amount of a component that contains 1 mol of carbon Amount of carbon atoms in substance [Cmol] ΔC...change in concentration of a substance [g/L] z.....number of carbon atoms per molecule [-] M...molar mass of component [g/mol]
Number of carbon atoms in biomass	$c_{biomass} = \Delta OD * 0.4 * 0.4705^{1} * M^{-1}$	$C_{biomass}$...number of carbon atoms in biomass [mol/L] ΔOD_{600}...change in optical density [-] $(OD_{end} - OD_{start})$
Amount of carbon in the serum bottle headspace	$n = \frac{p*V}{R*T} * \%_{CO,CO_2}$	n.....amount of carbon in the headspace [mol] p.... pressure in the headspace [Pa] V.....gas volume in the headspace, V=5*10^{-5} m^3 R...ideal gas constant, R=8.314 J/mol/K T....temperature, T = 293 K

[1] Biomass-carbon content measurement: The carbon content of the biomass was determined as ± 0.80% (w/w) using an elemental analyzer (Vario EL III, Elementar Analysen systeme GmbH, Hanau, Germany) (Shen *et al.*, 2020)

		$\%_{CO,CO_2}$....ratio of CO and CO_2 in the gas mixture
Maximum theoretical yield	$Y_{th} = \frac{n(C)_{gas}+n(C)_{glucose}}{n(C)_{glucose}}$	Y_{th}....maximum theoretical yield [Cmol/Cmol] $n(C)_{gas}$...amount of carbon atoms in the gas [Cmol] $n(C)_{glucose}$....amount of carbon atoms in glucose [Cmol]
Batch/Serum bottle experiments		
Parameter	Calculation	Description
Growth rate	$\mu = \frac{\ln(OD_{t(2)}/OD_{t(1)})}{t_2 - t_1}$	µ.....growth rate [1/h] OD_t....optical density at time point t [-] t_x....runtime at timepoint [h]
Product formation	$r_p = \frac{c_{p,2}-c_{p,1}}{t_2-t_1}$	r_p...volumetric productivity [g/L/h], [Cmol/L/h] $c_{p,t}$...product concentration [g/L], [Cmol/L]
Substrate consumption	$r_s = \frac{c_{s,2}-c_{s,1}}{t_2-t_1}$	r_s...volumetric substrate uptake [g/L/h], [Cmol/ L/h] $c_{s,t}$...substrate concentration [g/L], [Cmol/L]
Yields	$Y_{x/S} = \frac{c_{x,2} - c_{x,1}}{c_{s,2} - t_{s,1}}$ $Y_{P/s} = \frac{c_{p,2} - c_{p,1}}{c_{s,2} - c_{s,1}}$	$Y_{X/S}$...biomass yield [g/g] $Y_{P/S}$...product yield [g/g] $c_{x,t}$....biomass concentration [g/L]

Chemostat experiments		
Parameter	Calculation	Description
Dilution rate in chemostat	$D = \frac{\dot{V}_{feed} + \dot{V}_{base}}{V_{reactor}}$ $D = \mu$	D.....dilution rate [1/h] $\dot{V}_{feed}$.....feed flow rate [mL/h] $\dot{V}_{base}$.....base flow rate [mL/h] $V_{reactor}$...reactor volume [mL]
Growth rate	$\mu = \frac{\ln(OD_{t(2)}/OD_{t(1)})}{t_2 - t_1}$	µ.....growth rate [1/h] OD_t....optical density at time point t [-] t_x....timepoint [h]
Product formation	$r_p = (c_{p,reactor(t)} - c_{p,feed}) * D$	r_p...volumetric productivity [g/L/h], [Cmol/ L/h] $c_{p,reactor\ (t)}$...product concentration in reactor at timepoint t [g/L], [Cmol/L] $c_{p,feed}$..product concentration in feed [g/L], [Cmol/L] D.....dilution rate [1/h]
Substrate consumption	$r_s = (c_{s,reactor(t)} - c_{s,feed}) * D$	r_s.....volumetric substrate uptake [g/L/h], [Cmol/L/h] $c_{s,reactor(t)}$...product concentration in reactor at timepoint t [g/L], [Cmol/L] $c_{s,feed}$.....substrate concentration in feed [g/L], [Cmol/L]
Specific productivity	$q_P = \frac{r_p}{c_s}$	q_P... specific productivity [g/g/h] r_p.....volumetric productivity [g/L/h] c_s..... substrate concentration [g/L]

Specific substrate uptake	$q_s = \frac{r_s}{c_x}$	q_s... specific substrate uptake [g/g/h] r_s... volumetric substrate uptake [g/L/h] c_x..... biomass concentration [g/L]
Yields	$Y_{P/S} = \frac{r_p}{r_s}$	$Y_{P/S}$...product from a substrate [g/g] r_p.....volumetric productivity [g/L/h] r_s.....volumetric substrate uptake [g/L/h]

4 RESULTS AND DISCUSSION

4.1 Serum bottle experiments

Mixotrophic growth and product formation was shown for *C. autoethanogenum*, *C. ljungdahlii*, and *A. woodii* (Jones et al. 2016; Maru et al. 2018; Peters, Janssen, and Conrad 1998). The general aim of this experiment was to prove mixotrophy in *C. carboxidivorans*. In addition to this, the influence of the sugar concentration was tested.

The tests were carried out at two sugar concentrations, each with/without 1.5 bar CO, and autotrophic blank only with CO. Parallel experiments were carried out with *C. ljungdahlii* as previous studies have shown that *C. ljungdahlii* can grow mixotrophically (Köpke *et al.*, 2011a). Since *C. ljungdahlii* cannot metabolize glucose (B. Maru *et al.*, 2018), fructose was used as a substrate in this experiment.

4.1.1 Changes in OD_{600}, pH, and growth rate during sugar experiments

During the cultivation of *C. carboxidivorans* and *C. ljungdahlii* in fructose with/without CO changes in fructose concentration, pH, OD_{600}, and growth rate were monitored.

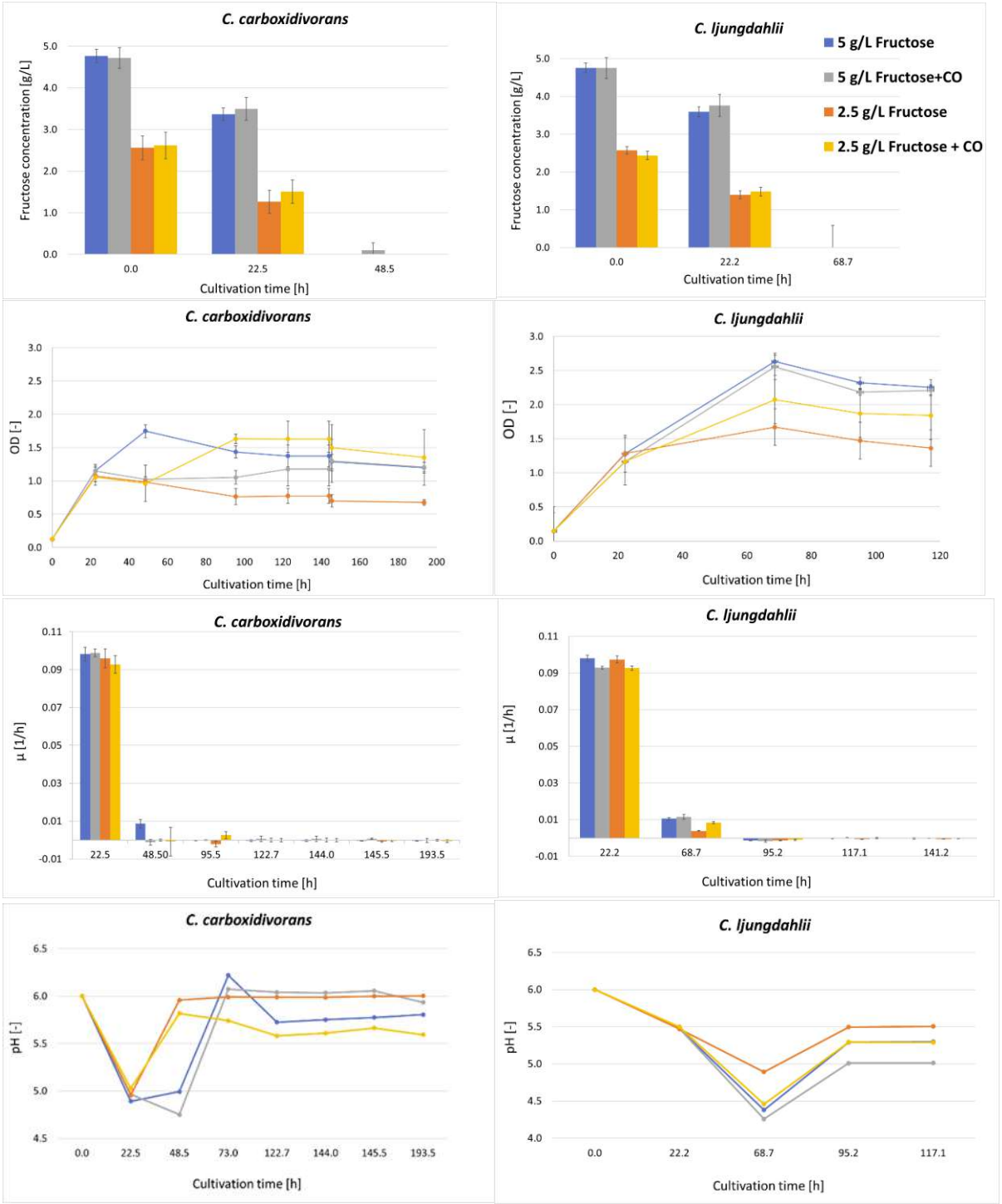

***Figure 3**: Measurements of fructose concentration, optical density (OD_{600}), pH, and calculated growth rate (μ) for the cultivation of C. ljungdahlii and C. carboxidivorans in serum bottles grown with heterotrophic and mixotrophic substrates*

Figure 3 shows that in the experiments with the same fructose concentration without CO and with CO, fructose was consumed at about the same rate up to a certain concentration.

A key factor in product formation is the pH value. Low pH values lead to the formation of alcohols from organic acids, a metabolic shift from the acidic phase to the solvent phase (Doll 2018a). A drop in the pH value indicates acid formation. The lowest pH values of experiments with *C. carboxidivorans* were reached in a shorter time than those with *C. ljungdahlii*. The lowest pH reached with *C. carboxidivorans* was 4.7, reached after 48.5 h with a substrate combination of 5 g/L fructose + CO. *C. ljungdahlii* reached the lowest pH value of 4.3 at 68.7 h of cultivation.

The minimum pH appears to be strain-dependent (Doll 2018a). Doll (2018a) also discussed this observation in their experiments with *C. carboxidivorans*, in which the pH was lowered from 6 to 4.5 and then increased to 5.4.

The maximal ethanol concentration of *C. ljungdahlii* was measured at a pH of 5.3, and of *C. carboxidivorans* at a pH of 5.9, which does not match the findings in the literature. Cotter et al. (2009) reported on reaching a 110 % higher ethanol concentration at pH 6.8 than at pH 5.5, and Fernández-Naveira et al. (2017) reached the maximum ethanol and butanol concentration of *C. carboxidivorans* at pH 5.75.

The optical density and thus the biomass formation of *C. carboxidivorans* shows some fluctuations, but it can be clearly seen how added CO leads to higher OD_{600} values. The highest OD_{600} values of 1.75 and 2.63 were achieved with *C. carboxidivorans* and *C. ljungdahlii*, respectively.

4.1.2 Products and feasibility of mixotrophy

Figure 4 shows the concentration differences of the resulting products in the experiments. Both strains were also tested for autotrophic use of CO only.

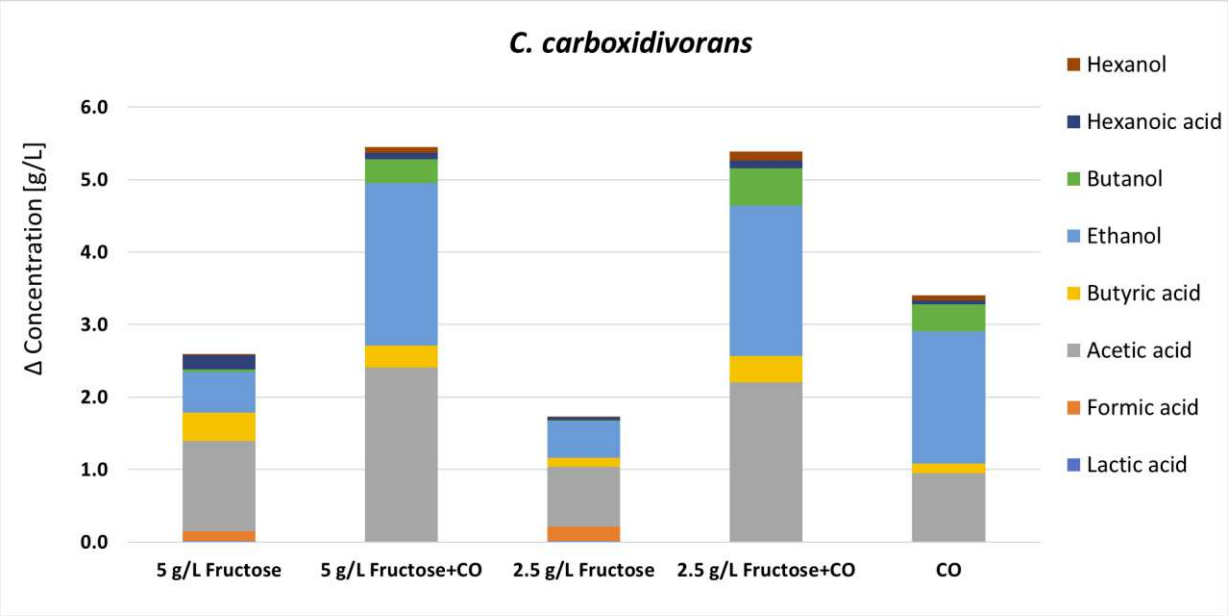

***Figure 4**: Δ Concentration [g/L] of serum bottle experiments with C. carboxidivorans at 193.5 h grown with heterotrophic and mixotrophic substrates*

Carbon monoxide increases the concentrations of the desired products butanol, ethanol, hexanol, and acetic acid (see Figure 4). Both sugar concentrations combined with CO showed a 4-fold increased ethanol titer compared to the bottles where no CO was added. The butanol and hexanol titer increased by 8.5 times and 3.9 times, respectively, with 5 g/L fructose and by up to 29 times and 17 times, respectively, with a fructose concentration of 2.5 g/L.

Combined with 5 g/L fructose the CO increased acetic acid concentration by 1.9 times and with 2.5 g/L fructose by 2.7 times. In combination with CO, 2.5 g/L fructose increased the biomass concentration by 2.2 times, while 5 g/L fructose showed no further increase.

Ethanol, butanol, and hexanol titers for experiment with 2.5 g/L fructose with added CO reached values of 0.53 g/L, 2.07 g/L, and 0.12 g/L hexanol, respectively.

Table 7 summarizes all product concentrations at 193.5 hours of cultivation of all experiments with *C. carboxidivorans*. According to measurements fructose was at 48 hours completely consumed.

Table 7: *Δ Product concentrations and biomass from serum bottle experiments with C. carboxidivorans at 193.5 h grown with heterotrophic and mixotrophic substrates*

Parameter	5 g/L Fructose [g/L]	5 g/L Fructose + CO [g/L]	2.5 g/L Fructose [g/L]	2.5 g/L Fructose + CO [g/L]	CO [g/L]
Butanol	0.04	0.33	0.02	0.53	0.37
Ethanol	0.56	2.24	0.51	2.07	1.82
Hexanol	0.02	0.08	0.01	0.12	0.07
Acetic acid	1.25	2.41	0.82	2.21	0.95
Butyric acid	0.39	0.30	0.13	0.36	0.14
Hexanoic acid	0.19	0.09	0.04	0.11	0.06
Formic acid	0.13	0.00	0.20	0.00	0.00
Lactic acid	0.02	0.00	0.02	0.00	0.00
Biomass	1.07	1.08	0.55	1.22	1.00

In contrast to *C. carboxidivorans*, *C. ljungdahlii* is more sensitive to CO, so that they no longer grow at a CO partial pressure of 0.743 bar (Doll 2018a). As described in Chapters **Error! Reference source not found.** and 2.2.2, CO is required to generate reduction equivalents that would be used for CO_2 fixation via the Wood–Ljungdahl pathway.

The reference bottles with only CO as a carbon source showed autotrophic growth of *C. carboxidivorans* with solely CO. Moreover, experiments with CO alone achieved higher concentrations of butanol, ethanol, and hexanol than experiments with fructose alone (see Table 7).

Previous research has shown that autotrophic cultivation of *C. carboxidivorans* in serum bottles with solely CO hardly resulted in any growth. Growth and product formation were obtained using mixtures of gases, such as $CO/H_2/CO_2$, CO/CO_2, and H_2/CO (Phillips et al., 2015;

Doll, 2018a). Yeast extract in the medium is required for autotrophic growth of *C. carboxidivorans* (Doll, 2018a).

Since it was not possible to measure the amount of CO, the true yield ($C_{metabolites}/C_{substrate}$) values of experiments with added CO do not show the exact value of the product yields as the amount is up to 3. The product yields sum up to the carbon balance. The yields were calculated in such a way that the product was based only on the utilized fructose, so that the ratios of products with the same fructose concentration with and without the addition of CO can be clearly seen in Figure 5. The resulting higher concentrations of the products in the experiments with added CO are clearly due to increased carbon uptake via carbon monoxide co-utilization.

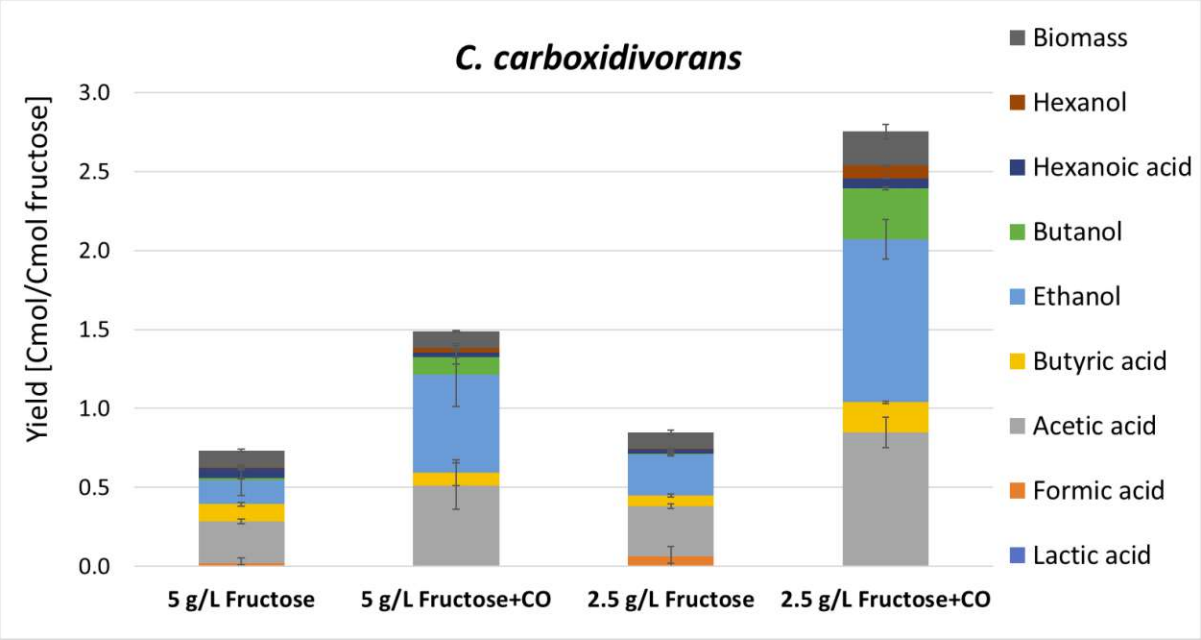

***Figure 5**: Mean metabolite and biomass yields of the batch serum bottle experiment with C. carboxidivorans at 193.5 h grown with heterotrophic and mixotrophic substrates. The sum of the yields gives the carbon balance without CO_2.*

It is important to emphasize that the yield in the bottles only with fructose is lower than 1. A possible cause is the formed carbon dioxide, which could not be measured and was therefore not included in the carbon balance.

Based on the yields of *C. carboxidivorans* after 193.5 hours shown in Figure 5, it can be seen which experiments had added CO: much more product of ethanol, butanol, hexanol, and

acetic acid was produced in these experiments. Mixotrophy with *C. carboxidivorans* at both sugar concentrations can therefore be confirmed.

A carbon balance over 100 % (see Figure 5) of the executed experiments confirm the findings of previous studies, showing that *C. carboxidivorans* and *C. ljungdahlii* can use CO on their own, and mixotrophy for *C. ljungdahlii* was also confirmed (Shen et al. 2020; Fernández-Naveira et al. 2017; Phillips et al. 2015; Ramió-Pujol et al. 2015; Lanzillo et al. 2020; Jones et al. 2016; Doll 2018a). Mixotrophy for *C. carboxidivorans* is proven with this experiment for the first time.

Product yields and carbon balance of experiment with sole CO as a substrate were not calculated because CO could not be measured, although higher solvent concentrations were achieved compared to the experiments with fructose alone (see Figure 4).

The yields presented in Figure 5 show that co-feeding of CO and fructose leads to significantly higher product yields of *C. carboxidivorans* than experiments with fructose alone.

Table ***8*** summarizes all molar yields at 193.5 hours.

Table 8*: Yields of metabolites and biomass with carbon balance from experiments with C. carboxidivorans; The standard deviation for three replicates is listed*

Parameter	5 g/L Fructose [Cmol/Cmol$_{fructose}$]	5 g/L Fructose + CO [Cmol/Cmol$_{fructose}$]	2.5 g/L Fructose [Cmol/Cmol$_{fructose}$]	2.5 g/L Fructose + CO [Cmol/Cmol$_{fructose}$]
Butanol	0.01 ± 0.00	0.11 ± 0.04	0.01 ± 0.01	0.32 ± 0.01
Ethanol	0.21 ± 0.00	0.62 ± 0.20	0.26 ± 0.01	1.03 ± 0.13
Hexanol	0.01 ± 0.01	0.03 ± 0.02	0.01 ± 0.00	0.08 ± 0.01
Acetic acid	0.26 ± 0.02	0.51 ± 0.15	0.32 ± 0.02	0.85 ± 0.10
Butyric acid	0.11 ± 0.01	0.08 ± 0.08	0.07 ± 0.01	0.19 ± 0.01
Hexanoic acid	0.06 ± 0.01	0.03 ± 0.03	0.03 ± 0.01	0.07 ± 0.00
Formic acid	0.02 ± 0.03	0.00	0.06 ± 0.06	0.00

Hexanoic acid	0.06 ± 0.01	0.03 ± 0.03	0.03 ± 0.01	0.07 ± 0.00
Biomass	0.11 ± 0.01	0.11 ± 0.00	0.10 ± 0.01	0.22 ± 0.05
C-balance	0.72	1.49	0.85	2.75

The butanol, ethanol, and hexanol yields at 2.5 g/L fructose with CO increased by 53, 4, and 16-fold, respectively, compared to experiments without CO.

A lower fructose concentration with added carbon monoxide resulted in a higher product yield. Experiments with 2.5 g/L fructose + CO led to 2.8 times higher butanol yield, 1.6 times higher ethanol yield, and 2.7 times higher hexanol compared to experiments with 5 g/L fructose + CO. Acetic acid was also increased up to 1.7 times. Ethanol, butanol, and hexanol yields from the 2.5 g/L + CO experiment reached values of 1.03 Cmol/Cmol, 0.32 Cmol/Cmol, and 0.08 Cmol/Cmol, respectively.

The carbon balance in the experiments with 2.5 g/L + CO increased by 1.8 times compared to the experiments with 5 g/L fructose + CO. It can be concluded that lower sugar concentrations, have a positive effect on the product formation during mixotrophic growth with *C. carboxidivorans*. At lower sugar concentration, CO uptake was presumably enhanced and the Wood-Ljungdahl route mainly produced ethanol and acetic acid, but also other products. It is possible that the sugar leads to a "carbon catabolite repression (CCR)", which means that the sugar is given priority and the gases are neglected.

*C. carboxidivoran*s uses a part of the CO to turn it into CO_2, producing Fd^{2-}, which is the reduction equivalent with the highest energy. If there is glucose or fructose in the medium, *C. carboxidivorans* generates CO_2 during glycolysis. Acids are then formed from glucose/fructose. The cell needs reduction equivalents to form alcohols from acids. In the conversion of pyruvate to acetyl-CoA reduction equivalent Fd^{2-} is generated. Additional Fd^{2-} is produced from the CO and H_2 oxidation, meaning more acid can also be converted to the associated alcohol.

The same experiments were carried out with *C. ljungdahlii* as reference to *C. carboxidivorans*. There is a clear difference in the product range between *C. ljungdahlii* and *C. carboxidivorans*. This is because *C. ljungdahlii* typically only able to produce acetic acid, ethanol, and lactic acid (Köpke et al., 2011a; Tanner et al. 1993).

The results with *C. ljungdahlii* were observed at 117.08 h, since the maximum ethanol concentration had been reached at this point in time. According to measurements fructose was completely consumed after 68 hours.

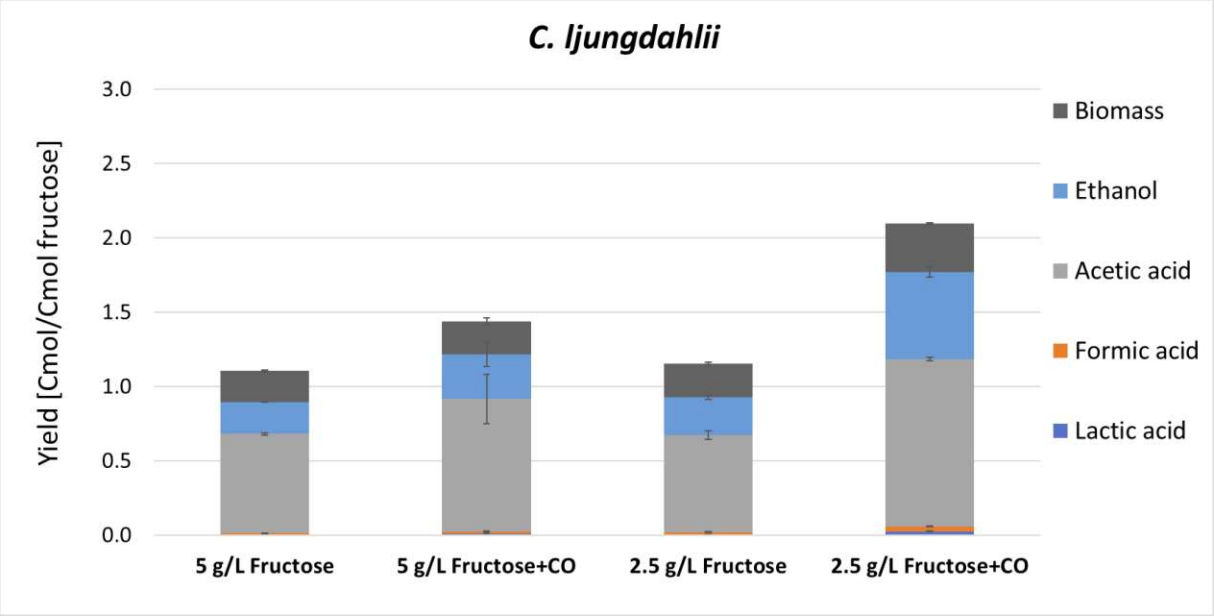

***Figure 6**: Mean metabolite and biomass yields of the batch serum bottle experiment with C. ljungdahlii at 117.08 h grown with heterotrophic and mixotrophic substrates. The sum of the yields gives the carbon balance without CO_2.*

In Figure 6, the carbon balance only with fructose was in sum higher than 1, for an unclear reason. Some errors may have occurred during the measurement. From Figure 6 it is also concluded that the lower fructose concentration in combination with CO leads to higher product yields, as in the experiments with *C. carboxidivorans*.

In experiments with *C. carboxidivorans* and *C. ljungdahlii* it is possible that the cells are more likely to emit CO_2 with 5 g/L, which has not yet been resumed. At 2.5 g/L, the CO_2 from the glycolysis could be further converted at this point with the energy from CO, which is why the yield of acetates and ethanol increases. As described above, it is also possible that the sugar leads to a "carbon catabolite repression (CCR)".

Jones et al. (2016) achieved a carbon balance (sum of all molar carbon yields, Cmetabolite/Csubstrate) of 195 % in their experiments with *C. ljungdahlii* and synthesis gas mixture $CO/CO_2/H_2/N_2$ = 55/10/20/15. The previous fermentation experiments with the

C. ljungdahlii also showed that the use of CO as an energy source mainly produces alcohols and the use of H_2/CO_2 creates acetates (Zhu *et al.*, 2020).

In Table 9, all yields of *C. ljungdahlii* are given for 117.08 hours at which the highest ethanol yield was achieved.

***Table 9**: Carbon molar yields of metabolites and biomass with carbon balance from experiments with C. ljungdahlii. The standard deviation for three replicates is listed.*

Parameter	5 g/L Fructose [Cmol/Cmol$_{fructose}$]	5 g/L Fructose + CO [Cmol/Cmol$_{fructose}$]	2.5 g/L Fructose [Cmol/Cmol$_{fructose}$]	2.5 g/L Fructose + CO [Cmol/Cmol$_{fructose}$]
Ethanol	0.22 ± 0.00	0.30 ± 0.08	0.26 ± 0.02	0.59 ± 0.03
Acetic acid	0.67 ± 0.01	0.89 ± 0.17	0.65 ± 0.03	1.13 ± 0.01
Formic acid	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
Lactic acid	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.00
Biomass	0.21 ± 0.01	0.22 ± 0.02	0.22 ± 0.01	0.33 ± 0.00
C-balance	1.10	1.44	1.15	2.10

Butanol and hexanol, as well as butyric acid and hexanoic acid, were only produced during the fermentation with *C. carboxidivorans*, which was also shown in earlier studies(Köpke et al., 2011a; Tanner et al., 1993; Fernández-Naveira, Veiga, et al., 2017; Liou et al., 2005).

Compared to *C. ljungdahlii*, *C. carboxidivorans* showed faster decrease to the lowest pH value (see Figure 3), and also higher concentrations of produced ethanol were reached.

The maximum ethanol yield (based on fructose) of 0.59 Cmol/Cmol at 117.08 hours was achieved in experiments with *C. ljungdahlii* compared to the experiments with *C. carboxidivorans* where ethanol gave 1.03 Cmol/Cmol at 193.5 hours.

The experiments with *C. carboxidivorans* were calculated for 193.5 hours, but it is important to note that the experiments were stopped at this point and that better results may be possible if the experiment was continued.

From this experiment it is concluded that *C. carboxidivorans* can grow mixotrophically. Sugar concentration has an impact on mixotrophic conversion. With a lower sugar concentration,

the CO uptake was presumably increased and a higher concentration of ethanol and acetic acid, but also of other products, was achieved.

4.2 Continuous fermentation experiments

The purpose of the chemostat cultivation experiments was to test *C. carboxidivorans* for its ability to utilize mixotrophy of glucose and various gas mixtures and its growth at different pH values in order to optimize the process performance of a continuous anaerobic fermentation for biofuel production. Cheng et al. 2019 only discussed the metabolic potential of *C. carboxidivorans* for industrial mixotrophic biofuel production.

Lowering the pH was tried because its lowering shifts the cells towards alcohol formation, due to the switch to solventogenesis.

The tests were carried out under anaerobic conditions in a stirred tank glass bioreactor as described in Chapter 3.5.

4.2.1 Variation of different gas mix

C. carboxidivorans was tested for its ability to mixotrophic utilization in a continuous reactor and the effect of varying gas mixes (100 % N_2; 60 % H_2; 60.03 % H_2, 9.54 % CO_2; 60.2 % H_2, 9.54 % CO_2, 10.57 % CO) on alcohol production, mainly ethanol, was observed.

To investigate the influence of varying gas mixtures, all reactor runs were carried out at the same parameters with pH 6, 500 rpm stirrer speed, 0.25 vvm in-gas rate, 10 g/L glucose, and a dilution rate of 0.05 h^{-1}.

As described in Chapter 3.5.4, the heterotrophic reference setpoint was carried out with glucose as substrate, using 100 % N_2 as in-gas. This setpoint was executed in quadruplicates and measurements were taken at chemostat steady-state.

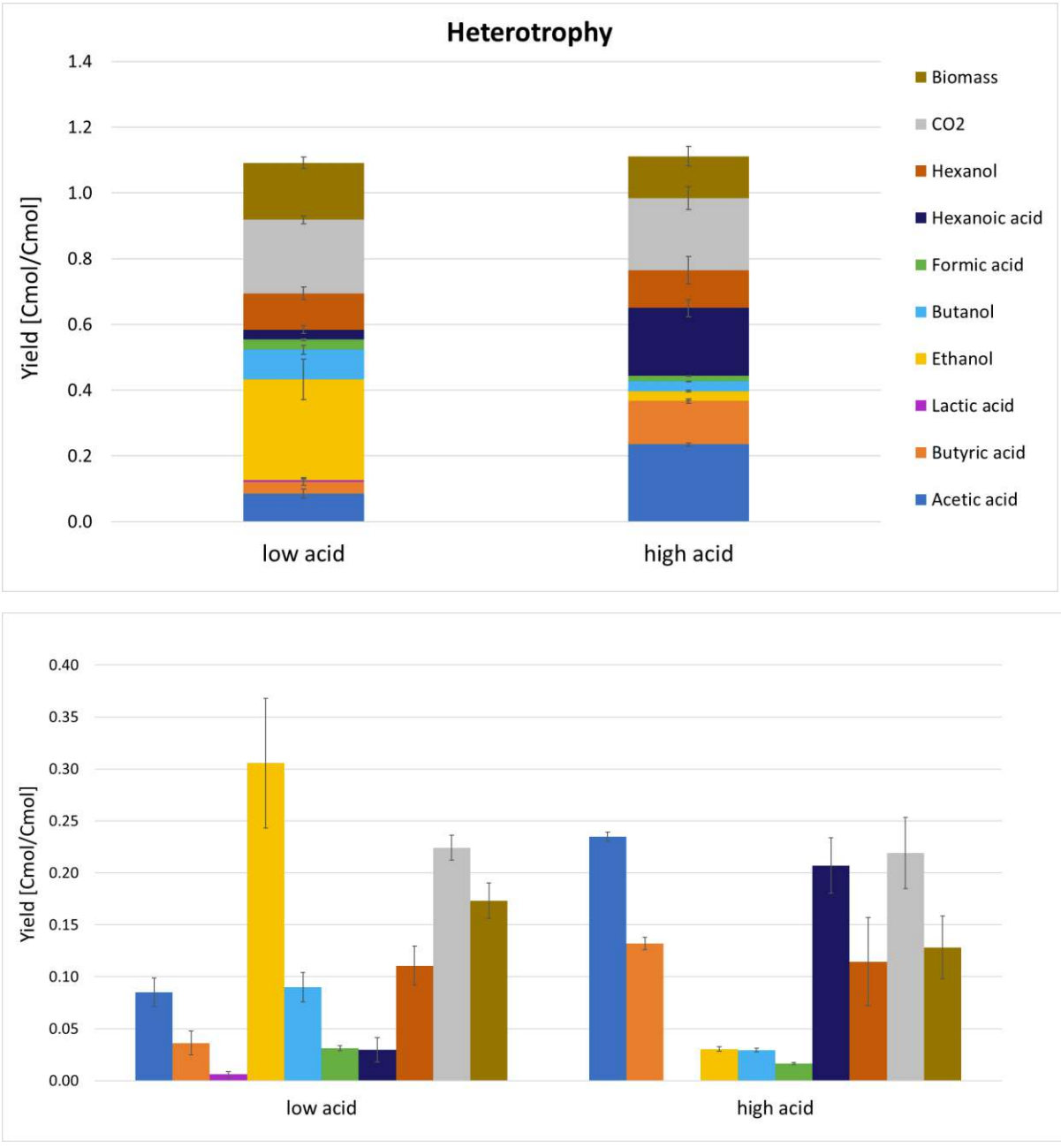

Figure 7: *Mean metabolite and biomass yields during steady-state of heterotrophic chemostat experiments with C. carboxidivorans using 10 g/L glucose and 100 % N_2 as in-gas. The sum of all yields results in the carbon balance. The legend is valid for both diagrams.*

Two of the replicas showed similar behavior in product formation, achieving the high ethanol yield and low hexanoic acid (low state). The other two replicas showed opposite behavior in which the low ethanol and high hexanoic acid content were formed (high state). In Figure 7 the mean values of the biomass and metabolite yields of each state (low acid state, high acid state) are shown.

Hexanoic acid yield in the high state is 6.9 times higher than in the low state. Ethanol and butanol are also conspicuous for the low hexanoic acid and high ethanol state since their yields are 10 and 3.1 times higher than the high state.

The replicates in the high state were performed after a longer time from the beginning of the process. Therefore, the reason for the two states could be a metabolic change in the cells that occurred after a longer runtime. The ethanol concentration was about 10 times lower in a high state compared to a low state (see Table 10 and Table 11).

In most fermentations, over a third of the sugar carbon is lost by CO_2 emission. Mixotrophy can lead to an increase in product yield and a reduction in CO_2 emissions (Jones *et al.*, 2016).

All four reduction equivalents which can arise from glucose are needed for the fixation of two CO_2 molecules in one molecule of acetyl-CoA. Since reduction equivalents are also required for biomass production, there is a lack of reduction energy and thus complete carbon fixation is not feasible (Maru et al. 2018; Jones et al. 2016). This problem was overcome by introducing, for example, H_2 or CO as an additional reducing agent

To investigate the effect of H_2 and CO, the following reactor runs were co-fed with either 60 % H_2 (H_2 – gas mix), a gas mix containing 10 % CO_2 and 60 % H_2 (CO_2, H_2 - gas mix), or a gas mix with 10 % CO_2, 60 % H_2, and 10 % CO (CO, CO_2, H_2 - gas mix). All metabolite yields were determined during steady-state and are displayed in Figure 8.

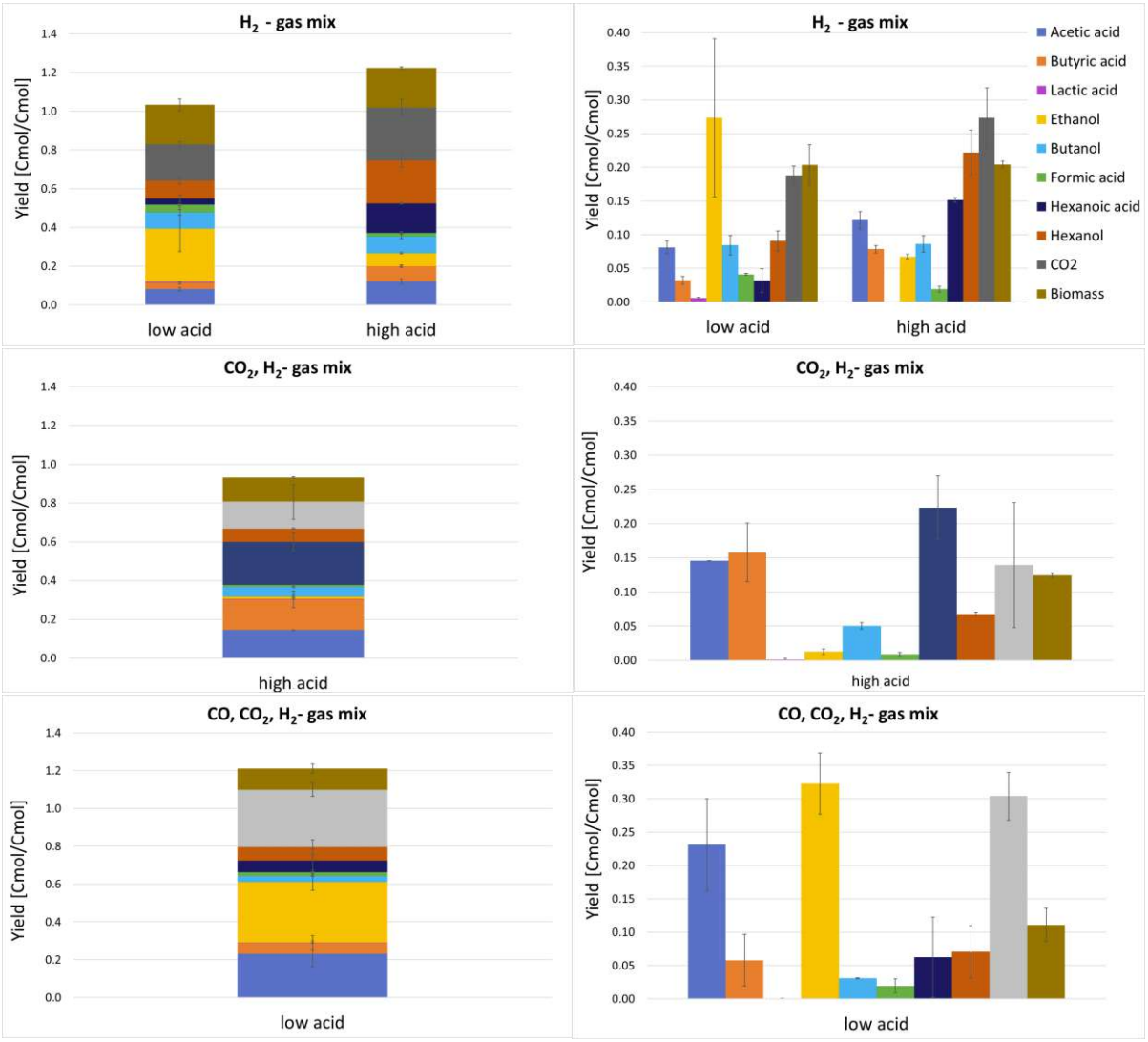

Figure 8: *Yields from mixotrophic (glucose + gas mix) chemostat experiments with C. carboxidivorans. H_2-gas mix: 60 % H_2; CO_2, H_2 -gas mix: 60 % H_2 and 10 % CO_2; CO, CO_2, H_2-gas mix: 60 % H_2, 10 % CO_2, and 10 % CO. Left: adjustment to 100 %; Right: no adjustment to 100 %. Right and Left: H_2-gas mix: low acid state (2 replicas), high acid state (2 replicas); CO_2, H_2-gas mix: high acid state (2 replicas); CO, CO_2, H_2-gas mix: low acid state (2 replicas). The legend is valid for all diagrams.*

As described for the heterotrophic reference setpoint, the setpoint with 60 % H_2 also results in two different states: low ethanol and high hexanoic acid content or high ethanol and low hexanoic acid content. Since our research is focused on alcohol production, only the high ethanol and low hexanoic acid state is used in the interpretation.

The achieved yield of ethanol with CO_2, H_2 - gas mix was 0.013 Cmol/Cmol, compared to the setpoint with 60 % H_2 at the state of high ethanol content, where ethanol reached 0.27 Cmol/Cmol, and the setpoint with the CO, CO_2, H_2 - gas mix led to 0.32 Cmol/Cmol.

Carbon monoxide of the CO, CO_2, H_2 – gas mix setpoint increased the ethanol yield by almost 25 times compared to the gas mix only with H_2 and CO_2. The molar carbon yield from butanol and hexanol in H_2- gas mix (high ethanol and low hexanoic acid content) reached values of 0.085 Cmol/Cmol and 0.032 Cmol/Cmol. Of all three setpoints, butanol had the highest and hexanol the lowest production at this setpoint. Hexanol achieved two-fold higher yields with CO_2, H_2-gas mix, and CO, CO_2, H_2-gas mix, and butanol was 1.7 times reduced in CO_2, H_2-gas mix with a value of 0.050 Cmol/Cmol and 2.7 times using CO_2, CO, H_2-gas mix with a value of 0.03 Cmol/Cmol (all compared to the setpoint using H_2-gas mix, state of high ethanol and low hexanoic acid content).

The highest acetic acid yield of 0.23 Cmol/Cmol was achieved at the setpoint with the added carbon monoxide (CO, CO_2, H_2 - gas mix), which was 1.6 times higher than the acetic acid yield reached in the setpoint with CO_2, H_2 - gas mix and 2.9 times higher than the yield at setpoint with H_2 - gas mix

With CO, CO_2, H_2 - gas mix, it has been proven that CO leads to an increased C-recovery of 121.03 %. The setpoint with CO_2, H_2 - gas mix, achieved a lower C-recovery of 93.14 %. It is obvious that CO leads to more product formation.

In earlier research experiments in which a gas mixture $CO/CO_2/H_2/Ar$ = 56/20/9/15 (exhaust gas ratio from a steel industry) was used, CO was shown to be the main energy carrier. After the CO was exhausted, the consumption of H_2 and CO_2 began (Shen *et al.*, 2020). The energy content of CO is higher than that of H_2 (Hu, Bowen and Lewis, 2011). In this experiment, H_2 uptake rate was low and CO_2 was not utilized at all.

In the literature, most of the alcohol produced in syngas fermentations was ethanol, followed by butanol, and finally hexanol (Fernández-Naveira *et al.*, 2017). In this study, the setpoint with CO, CO_2, H_2 – gas mix achieved hexanol concentration 2-fold higher than those of butanol (see Table 10 and Table 11).

Table 10: *Mean concentration of metabolites and biomass in chemostat experiments with C. carboxidivorans with heterotrophic and mixotrophic substrates (glucose + gas mix) at low acid state (all setpoints in duplicates). Standard deviations are also listed.*

	Heterotrophic setpoint	H_2 - gas mix	CO, CO_2, H_2 - gas mix
Acetic acid [g/L]	0.88 ± 0.15	0.84 ± 0.11	4.42 ± 1.05
Butyric acid [g/L]	0.28 ± 0.09	0.25 ± 0.04	0.87 ± 0.61
Lactic acid [g/L]	0.06 ± 0.03	0.06 ± 0.01	0.01 ± 0.01
Formic acid [g/L]	0.49 ± 0.03	0.65 ± 0.01	0.56 ± 0.28
Hexanoic acid [g/L]	0.20 ± 0.08	0.208 ± 0.12	0.84 ± 0.81
Ethanol [g/L]	2.40 ± 0.46	1.88 ± 0.67	4.89 ± 1.02
Butanol [g/L]	0.57 ± 0.08	0.45 ± 0.02	0.37 ± 0.02
Hexanol [g/L]	0.64 ± 0.10	0.45 ± 0.16	0.75 ± 0.38
CO_2 (metabolite) [g/L]	3.38 ± 0.23	2.84 ± 0.25	8.64 ± 0.44
Biomass [g/L]	1.42 ± 0.12	1.67 ± 0.22	1.71 ± 0.27

Setpoint with CO, CO_2, H_2 – gas mix reached 0.37 g/L butanol, 4.89 g/L ethanol, 0.75 g/L hexanol, and 4.42 g/L acetic acid, which is compared to setpoint with CO_2, H_2 – gas mix almost 49 times higher in ethanol, 1.2 times higher in butanol, 1.9 times higher in hexanol, and 3 times higher in acetic acid content. In earlier research experiments Shen et al. (2017) managed 7.0 g/L ethanol in syngas fermentation using a continuous, horizontally rotating packed bed

reactor. Doll et al. (2018b) achieved values of steady-state concentrations of 0.7 g/L butanol, 6.1 g/L ethanol, and 0.1 g/L hexanol in the autotrophic production test with *C. carboxidivorans* using continuously operated two stirred tank bioreactors. Fernández-Naveira et al. (2016b) reached alcohol concentrations in bioreactors with *C. carboxidivorans* values of 2.66 g/L butanol and 5.55 g/L ethanol in a batch process.

Table 11: *Mean concentration of metabolites and biomass in chemostat experiments with C. carboxidivorans with heterotrophic and mixotrophic substrates (glucose + gas mix) at high acid state (all setpoints in duplicates). Standard deviations are also listed.*

	Heterotrophic setpoint	**H_2 - gas mix**	**CO_2, H_2 - gas mix**
Acetic acid [g/L]	2.32 ± 0.13	1.20 ± 0.08	1.46 ± 0.06
Butyric acid [g/L]	0.96 ± 0.01	0.57 ± 0.02	1.14 ± 0.21
Lactic acid [g/L]	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
Formic acid [g/L]	0.25 ± 0.01	0.28 ± 0.05	0.13 ± 0.03
Hexanoic acid [g/L]	1.31 ± 0.12	0.97 ± 0.06	1.43 ± 0.19
Ethanol [g/L]	0.23 ± 0.01	0.51 ± 0.01	0.10 ± 0.03
Butanol [g/L]	0.18 ± 0.00	0.52 ± 0.05	0.31 ± 0.01
Hexanol [g/L]	0.65 ± 0.26	1.24 ± 0.14	0.39 ± 0.03
CO_2 (metabolite) [g/L]	4.74 ± 0.33	3.98 ± 0.79	2.12 ± 1.18
Biomass [g/L]	1.53 ± 0.29	1.61 ± 0.02	1.00 ± 0.06

With *C. carboxidivorans*, reproducibility was difficult. It looked like the cells were changing over time in the continuous runs. Setpoints established initially resulted in a low acid state and setpoints set after a long period of cultivation resulted in a high acid state, as shown in Figure 7 and Figure 8. The heterotrophic setpoint and the H_2 - gas mix setpoint resulted in both a high and low acid state. CO_2, H_2 - gas mix resulted in a low acid state, and CO, CO_2, H_2- gas mix resulted in a high acid state

The maxima of the volumetric ethanol productivity and the specific productivity of 0.28 g/L/h and 0.14 g/g/h were reached at the setpoint with CO, CO_2, H_2 – gas mix (see Figure 9), which is compared to the setpoint with CO_2, H_2 – gas mix (see Figure 10) 56 times higher in production rate and 1.3 times higher in the specific productivity.

The unexpected challenge was forming biofilms (pellets) during the cultivation of *C. carboxidivorans*. According to the literature, agglomerates were formed in the late cultivation phase at 37 °C and the two-stage temperatures from 25 °C to 37 °C resulted in higher alcohol production rather than the use of anti-agglomerate surfactants (Shen et al. 2020).

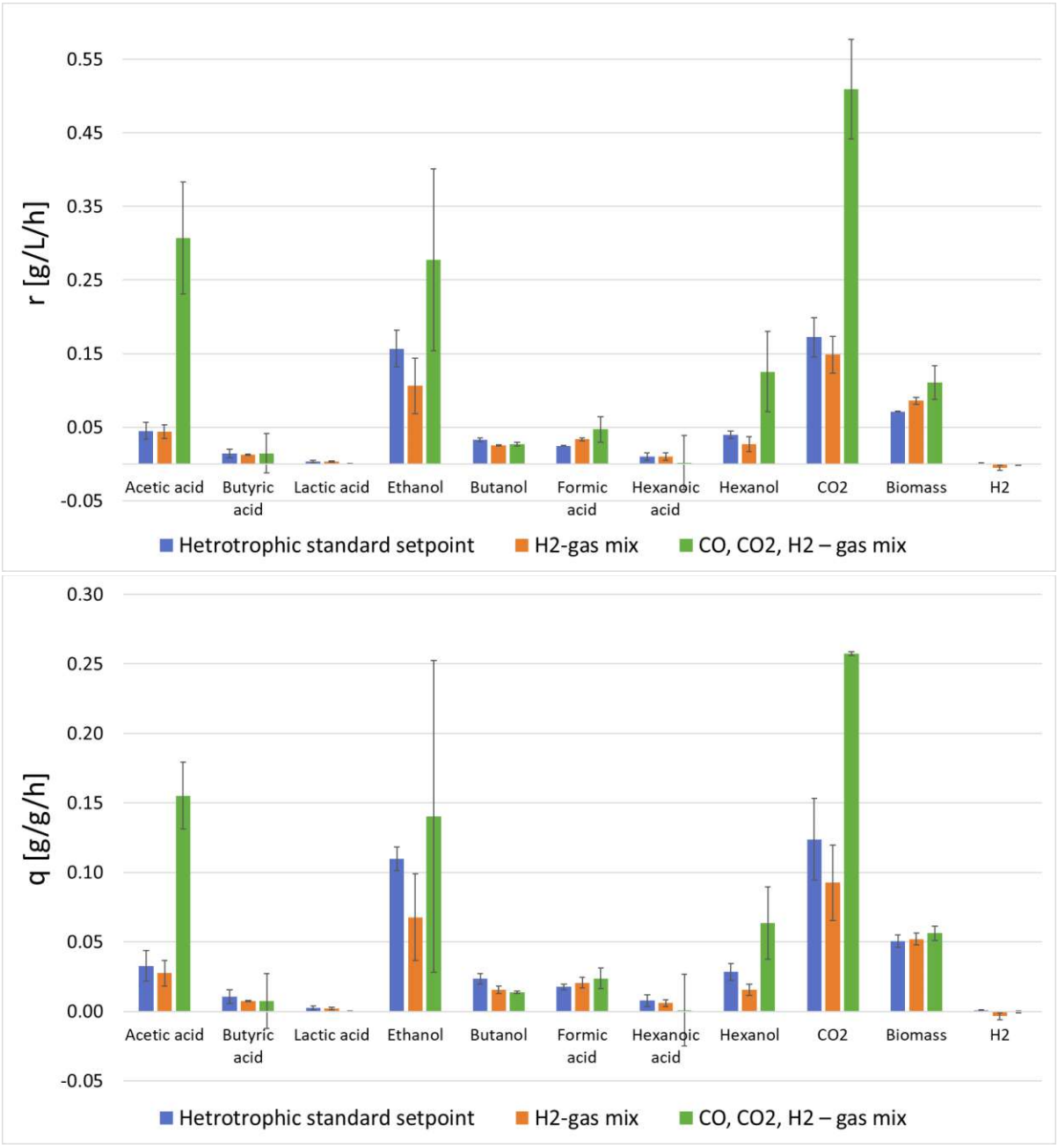

Figure 9: *Volumetric rate r and specific rate q for solvent production in continuous fermentation experiments at heterotrophic and mixotrophic (glucose + gas mix) setpoints: heterotrophic standard setpoint = 100 % N_2, H_2-gas mix = 60 % H_2, CO, CO_2, H_2-gas mix: 60 % H_2, 10 % CO_2, and 10 % CO. All for the low acid state.*

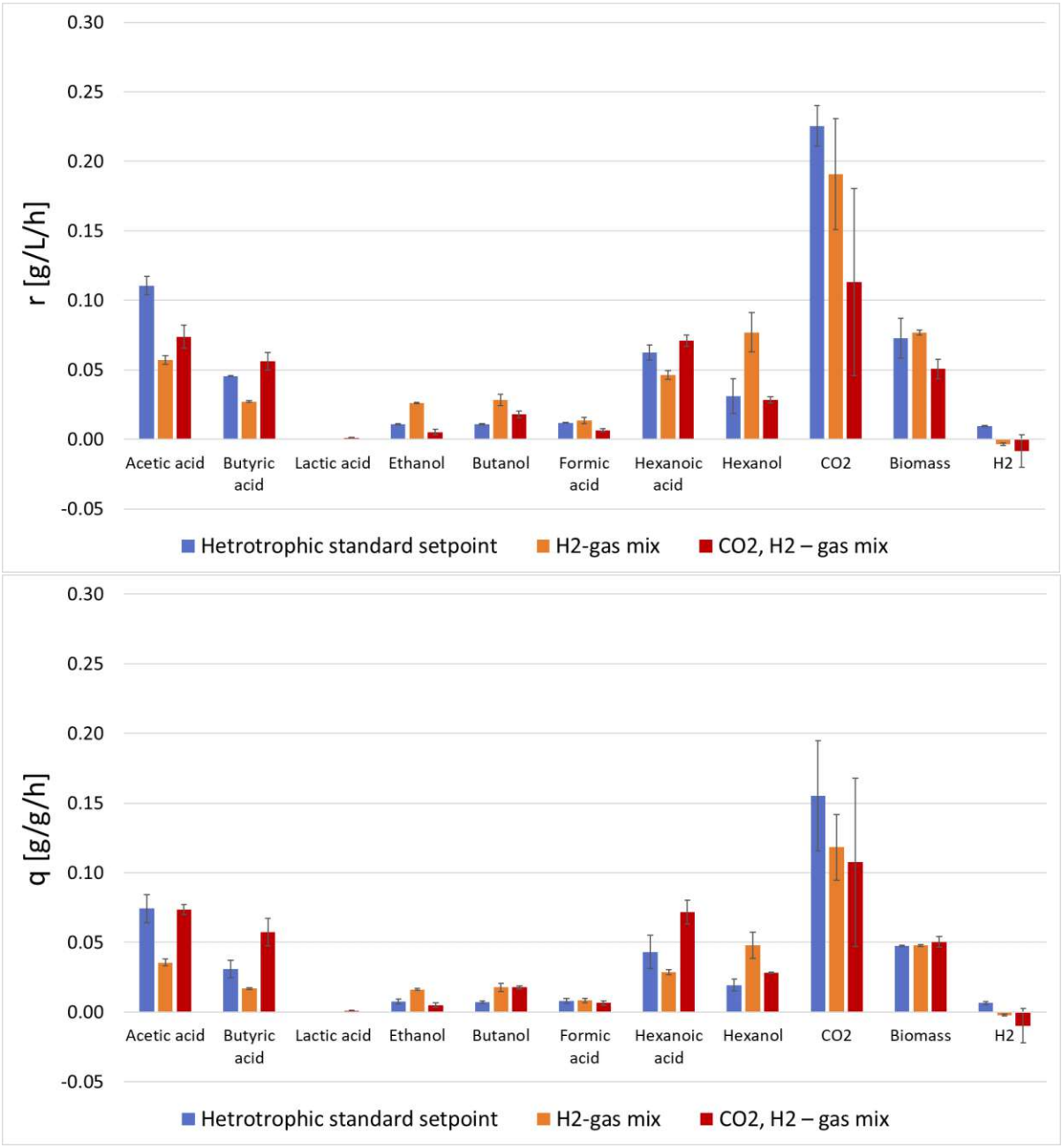

Figure 10: *Volumetric rate r and specific rate q for solvent production in continuous fermentation experiments at heterotrophic and mixotrophic (glucose + gas mix) setpoints: heterotrophic standard setpoint = 100 % N_2, H_2-gas mix = 60 % H_2, CO_2, H_2 -gas mix: 60 % H_2 and 10 % CO_2. All for the high acid state.*

In the setpoint with CO, CO_2, H_2 – gas mix ethanol achieved volumetric and specific productivity values of 0.28 g/L/h and 0.14 g/g/h, respectively, which is 28 and 14 times higher compared to the setpoint with CO_2, H_2 – gas mix.

Butanol reached values of 0.03 g/L/h and 0.01 g/g/h in setpoint with CO, CO_2, H_2 – gas mix, which is compared to setpoint with CO_2, H_2 – gas mix 1.5 times higher in production rate and 15 times lower in the specific productivity.

In literature, the specific rate of ethanol and butanol reached 0.16 g/g/h and 0.07 g/g/h at a lower pH of 4.75 (Fernández-Naveira *et al.*, 2016b). A lower pH value showed increased alcohol production (switch from an acidogenic phase to a solventogenic phase) but also harmed biomass formation (Fernández-Naveira *et al.*, 2016b).

Hexanol reached values of 0.13 g/L/h and 0.06 g/g/h in setpoint with CO, CO_2, H_2 – gas mix, which is compared to setpoint with CO_2, H_2 – gas mix, 4.5 times higher in production rate and 3 times lower in the specific productivity.

At the setpoint with CO, CO_2, H_2 – gas mix acetic acid achieved volumetric and specific productivity of 0.31 g/L/h and 0.16 g/g/h, respectively, which is almost 7 and 5 times higher than in heterotrophic reference setpoint (low acid state) and also 7 and 5.5-fold higher than a setpoint with H_2 - gas mix (low acid state). Mixotrophy with CO resulted also in 0.01 g/g/h and 0.02 g/L/h butyric acid, with slightly lower specific productivity than at heterotrophic reference setpoint (low acid state). According to previous studies acetic acid and butyric acid reached values of 0.13 g/g/h and 0.03 g/g/h at pH 4.75 (Fernández-Naveira *et al.*, 2016b).

The carbon monoxide content significantly increases the formation rate and specific productivity of the ethanol. The energy content of CO is higher than that of H_2. At this setpoint (CO, CO_2, H_2 – gas mix), very little H_2 was consumed and CO_2 was not consumed at all (see Figure 11). The transition to the liquid phase can be worse with H_2 than with CO_2 and CO. In the case of hydrogen, the calculation error was also large.

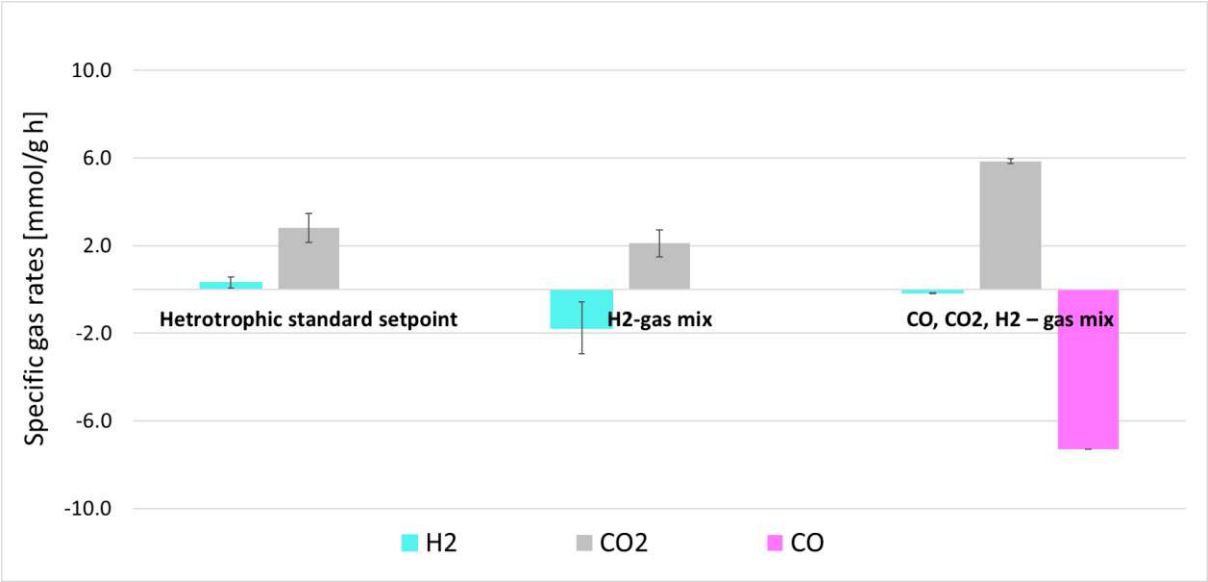

***Figure 11**: Specific gas rates for solvent production in continuous fermentation experiments at heterotrophic and mixotrophic (glucose + gas mix) setpoints: heterotrophic standard setpoint = 100 % N_2, H_2-gas mix = 60 % H_2, CO, CO_2, H_2-gas mix: 60 % H_2, 10 % CO_2, and 10 % CO. All for the low acid state. Negative specific gas rate = gas utilized, positive specific gas rate = gas released*

C. carboxidivorans is able to utilize mixotrophy of glucose and various gas mixtures. The gas mixture with added CO achieved the highest alcohol yields.

4.2.2 Variation of pH

Other authors showed that the decrease of pH can increase the solvent production of *C. carboxidivorans,* but also showed a negative effect on the growth of the cells(Fernández-Naveira et al. 2017a).

To investigate if a pH decrease is possible in a chemostat and to determine the influence of different pH values, two experiments were carried out with the same parameters as the reactor runs in Chapter 4.2.1 (500 rpm stirrer speed, 0.25 vvm gas rate, 10 g/L glucose, and dilution rate of 0.05 h^{-1}) but at a lower pH value of 5.5. One setpoint was performed with 100 % N_2 (heterotrophic standard setpoint) and the other with 60 % H_2 and 40 % N_2 (H_2 enhanced mixotrophy).

Figure 12 shows that the OD_{600} at pH 5.5 drops sharply. Steady-state conditions at pH 5.5 were never settled.

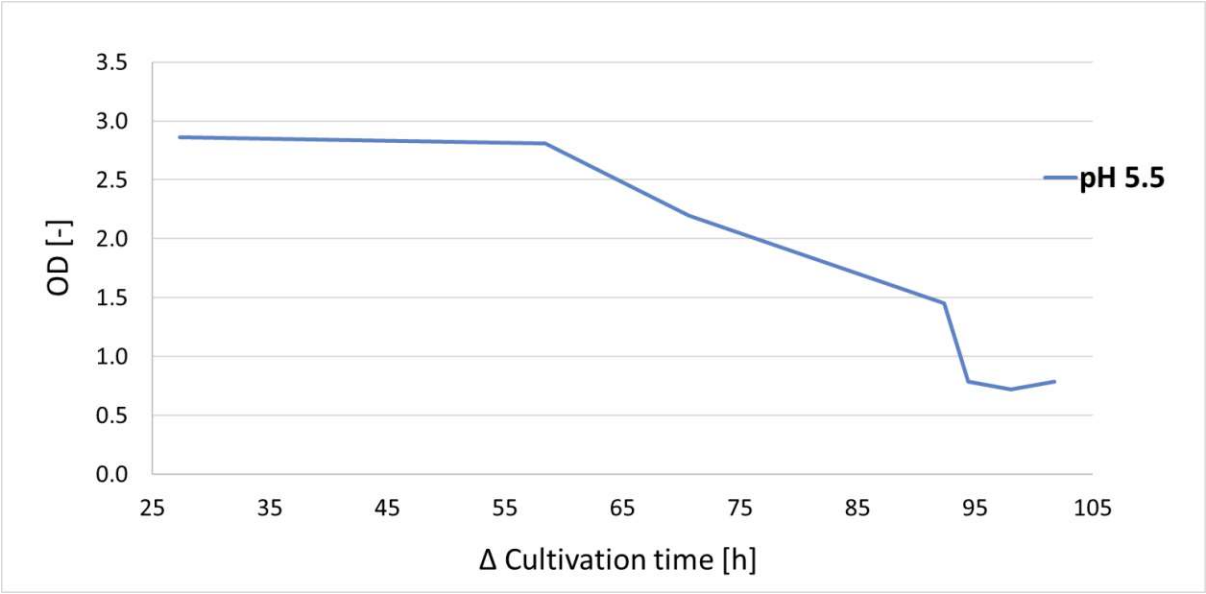

***Figure 12**: Optical densities of continuous fermentation experiments by C. carboxidivorans of heterotrophic standard setpoint (100 % N_2) at pH 5.5.*

Lowering the pH in both experiments in the chemostat was not possible, even from pH 6 to 5.5. The cells were in a stable state until it was adjusted at pH 5.5, the cells began to wash out. Biomass or OD drops sharply overtime at pH 5.5. Low pH leads to a low growth rate or hardly any growth.

One solution of lowering the pH would be a two-stage continuous fermentation process, one with pH 6 and one with pH < 5.5 as shown by Doll et al. (2018a). In the first bioreactor with pH 6 acids were formed, and then in the second bioreactor at pH 5 the acids were converted into alcohols. As reported in research, the use of carbon monoxide as the sole substrate also led to increased alcohol formation (ethanol, butanol, hexanol) in a two-stage process (pH 6 and pH 5). The steady-state alcohol concentrations in the second reactor reached values of 6.1 g/L ethanol, 0.7 g/L butanol, and 0.1 g/L hexanol (Doll et al. 2018a). According to Abubackar et al. (2018) a two-stage continuous system with pH 6 in the first bioreactor and pH 5 in the second bioreactor with a flow rate of 22 mL/h and syngas ($CO/CO_2/H_2/N_2$=30/10/20/40) led to an alcohol/acid ratio of 0.32 and a total alcohols concentration of 1.51 g/L (did not have the 3-4 volume change and thus never reached a steady-state).

Cell retention could be the other solution to lowering the pH, but since we have seen *C. carboxidivorans* formed biofilms and pellets in chemostat experiments, getting good results in lab-scale experiments could also be a challenge.

Lowering the temperature could solve problems with agglomeration. Shen et al. (2020) addressed cell pellets by using a TST culture (two-step temperature), 37–25 and 37–29 °C.

C. carboxidivorans is an interesting microorganism for gas fermentation due to a wide range of products, in particular ethanol, butanol, and hexanol, which are of industrial relevance. However, if individual products are to be produced in a targeted manner, other products are undesirable. A corresponding genetic modification of the strain could lead to an increased flow of carbon in the direction of the desired target product via the regulation or deactivation of certain enzymes.

5 CONCLUSION

The first aim of this work was to investigate the feasibility of mixotrophic growth on glucose and gases (e.g. CO) and its effect on the production of ethanol, butanol, and hexanol in serum bottles using higher and lower sugar concentrations with *C. carboxidivorans*.

The experiment proved the mixotrophic growth with *C. carboxidivorans* on fructose and CO. Using a lower concentration of fructose in the mixotrophic conversion, a higher concentration of alcohols was achieved. The fructose-based carbon balance in the experiments with 2.5 g/L + CO increased by 1.8 times compared to the experiments with 5 g/L fructose + CO, which showed that the sugar concentration probably has an influence on how much CO_2 can be fixed and thus on the product formation. The ethanol, butanol, and hexanol titers reached values of 0.528 g/L, 2.070 g/L, and 0.119 g/L.

The second aim of this work was to optimize the process performance of a continuous anaerobic fermentation for enhanced biofuel production with *C. carboxidivorans*. The strain was tested for its ability to grow mixotrophic on glucose and various gas mixes, and also tested for its growth at different pH values.

With *C. carboxidivorans*, reproducibility proved to be difficult. It appeared that cell morphology changed over time. Heterotrophic fermentation resulted in a shift from low hexanoic acid and high ethanol content to high hexanoic acid and low ethanol formation state. After a longer period of operation, the cells switched over and produced a higher concentration of acids and a lower concentration of alcohols. The reason for this switch is not known.

Mixotrophic conversion with glucose and CO, CO_2, H_2 - gas mix proved to be successful. This gas mixture was advantageous for the formation of ethanol. Only CO was absorbed. H_2 and CO_2 were not absorbed, so they have no effect on product formation. Carbon monoxide increased the ethanol yield of 0.323 Cmol/Cmol by almost 25 times compared to a setpoint with H_2, CO_2 - gas mix. Mixotrophic conversion with H_2, CO_2 - gas mix, where only little H_2 was absorbed, was beneficial for butanol formation. Butanol yielded 0.050 Cmol/Cmol, which was 1.6 times higher than with CO, CO_2, H_2 - gas mix. Hexanol showed the same yields in both experiments with the value of 0.070 Cmol/Cmol.

Lowering the pH in the chemostat was not possible, even from pH 6 to 5.5. The cells began to wash out. Low pH leads to a low growth rate or hardly any growth.

Cell retention could be a solution to lowering the pH, but since we have seen *C. carboxidivorans* formed biofilms and pellets in chemostat experiments, getting good results could also be a challenge. Lowering the temperature could possibly solve problems with agglomeration. The other solution of lowering the pH would be a two-stage continuous fermentation process, one with pH 6 and one with pH < 5.5.

The advantage of mixotrophy demonstrated by this work is the use of various residues as substrates of *C. carboxidivorans*, e.g. SSL + synthesis gas, in order to increase the biomass. *C. carboxidivorans* could be an exciting strain for the mixotrophic production of ethanol, butanol, and even hexanol in the future and thus contribute to a sustainable and resource-saving industry.

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FIGURES

TABLES