MSc Thesis Biobased Chemistry and Technology

Influence of H_2 partial pressure on the acetate production by extremophiles and *in situ* conversion to ethanol using a heterogeneous ruthenium catalyst

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02. July 2018

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Name course	:	MSc Thesis Biobased Chemistry and Technology
Number	:	BCT-80436
Study load	:	36 ECTS
Date	:	January – July 2018

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Study programme	:	MBT
Report number	:	102BCT

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Abstract

Currently, there is a demand for more sustainable products and production processes. Therefore, a novel system was proposed which combines syngas fermentation with *in situ* hydrogenation to obtain bio-based platform chemicals. To evaluate the feasibility of this proof of concept, a model system was investigated. The extremophile *Carboxydothermus hydrogenoformans* was used to convert syngas into acetate, which was hydrogenated to ethanol by a ruthenium-based catalyst, supported on activated carbon. First, systems were assessed individually to determine temperature, pH and H_2 partial pressure of the biochemically combined system. Also, it had to be determined when *C. hydrogenoformans* switched from H_2 generation to the acetate producing Wood Ljungdahl pathway. For this, it was observed that high CO levels inhibit the Wood Ljungdahl pathway. Hence, acetate production occurred only at low levels of dissolved CO. A higher H_2 partial pressure resulted in higher acetate production. While 0.11 $mmol_{acetate}$ day^{-1} were found for 4 bar supplied CO, 4 bar CO with 6 bar H_2 yielded 0.18 $mmol_{acetate}$ day^{-1}. Catalytic conversion was tested at the organism's optimal growth temperature, 70 °C. Showing production rates of 0.31 $mmol_{ethanol}$ day^{-1}, operation at 70 °C was concluded to be feasible. However, hydrogenation was only observed from acetic acid and not acetate. Catalytic conversion therefore required an acetic pH while the organism must grow at neutral pH. Lowering the H_2 partial pressure from 50 to 30 bar H_2 did lower the ethanol yield. Lastly, the combined system was examined, for which no activity could be noted. Next to pH, medium components were determined as bottlenecks of the biochemically combined system, as they deactivate the catalyst. Even though promising results were found for the individual systems, an *in situ* combination was not feasible yet. Further experiments should address the pH and medium limitations.

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1 Introduction

1.1 Background

In the past decades, there have been vast developments towards more sustainable production processes. This is due to increasing greenhouse gas emissions, resource depletion and a growing demand in energy, food and chemicals [1, 2]. In line with this, numerous novel production strategies for fuels and bulk chemicals have been proposed, to move away from the petrochemical industry. However, by substituting fossil resources for biomass as a substrate, new challenges arise. On the one hand, high-quality feedstocks, like sugarcane, corn or soybean, are suitable for fermentation into bulk chemicals, but raise the question whether arable land cannot better be used for food-crop cultivation [3]. On the other hand, widely available agricultural and municipal residual biomass is of poorer quality and its building blocks have a low accessibility, resulting in a lower yield fermentation process [4]. This is especially critical for lignocellulosic biomass, where tight bonds between lignin, cellulose and hemicellulose prevent direct fermentation of sugars. Therefore, residual biomass must be exposed to harsh and expensive pre-treatments [5], making the process economically less favourable and less environmentally friendly.

An alternative approach, resolving all these disadvantages, is to gasify the highly available but complex feedstocks to synthesis gas, also known as syngas [4]. By gasifying the biomass to a mixture of mainly carbon dioxide (CO_2), carbon monoxide (CO) and hydrogen (H_2), the entire biomass is made available for subsequent conversion into bulk chemicals. By-products of this conversion are methane, sulfides and tars [5]. Subsequent syngas conversion to organic acids and alcohols can either occur thermo-chemically or biologically.

Thermochemical conversions use a heterogeneous metal catalyst, which operates under high pressure and temperature [6]. An example is the Fischer-Tropsch process. Even though the chemical conversion has been optimised intensively during the last decades, there are still major disadvantages. Firstly, high pressure and temperature make it a costly process [7]. Secondly, the metal catalysts are prone to inactivation or poisoning by contaminants, mainly sulfides, which are by-products of the gasification. Therefore, expensive clean-up of the syngas is required [7]. Furthermore, distinct H_2:CO ratios are necessary for the catalytic conversion, which differ from the common composition of syngas. Removal of CO to adjust the ratio is achieved through the costly water-gas shift reaction (WGS) [5].

Alternatively, biological syngas fermentation does not only offer a higher product specificity than chemical catalysis, but also overcomes other drawbacks, while maintaining the advantage of increased feedstock utilisation. Due to the high robustness and flexibility of the microorganisms, less gas clean-up, and neither the H_2/CO ratio adjustment, nor the harsh process conditions are necessary [6]. However, limitations can still be found in the low solubility of the gaseous substrate, making the process mass transfer limited, as well as in product separation through energy-intensive downstream processing [3, 8]. Currently, improvements and novel concepts are being proposed to overcome these drawbacks.

1.2 Introduction to proposed concept

Accordingly, a novel concept was proposed for this thesis. This combines biological syngas fermentation with *in situ* catalytic hydrogenation of metabolites to yield desired bio-based platform chemicals from a low-quality feedstock. Compared to current approaches the proposed concept offers a number of advantages. Especially regarding production costs and time by performing both conversions in the same reactor. Firstly, there will be a reduction in equipment, resulting in lower investment and maintenance costs. Secondly, no purification of intermediate products is required, preventing the need for additional expensive downstream processing. Most importantly however, there will be a reduction in overall conversion steps. Purely chemical routes from biomass require multiple separate conversion steps, whereas a single organism facilitates all necessary conversions through enzymatic catalysis. This advantage is also employed in syngas fermentation. At this moment however, complete decrease in steps by *in situ* conversion of a metabolite to the desired platform chemical is not feasible yet.

The main challenge of the proposed system is the temperature difference between biological and chemical systems, prohibiting operation in the same reactor. While hydrogenation occurs at high temperatures and pressures, organisms require much milder conditions. Normal microbial growth temperatures range between 30-40 °C [6]. The proposed concept approaches this gap by employing extremophiles for the syngas conversion, with optimal growth temperatures of 60-70 °C [9]. For *in situ* conversion of the resulting metabolite however, the heterogeneous catalysis must occur at lower temperatures, lowering its conversion rate [10]. To evaluate whether this reduction will be too severe, by making it the rate limiting step of the overall conversion, the biological and the chemical conversion rates were compared relative to one another for the chosen temperature. Generally, however, chemical catalysis occurs at much higher rates than biological catalysis, which suggested that operating at lower temperatures might still be feasible.

To the best of our knowledge, no research has yet been conducted on the combination of syngas fermenting extremophiles and heterogeneous catalysis. This project is therefore seen as a proof of concept, to research its potential, limits and challenges. A relatively simple system was chosen for this proof of principle, namely the production of ethanol from syngas. Here, the extremophile *Carboxydothermus hydrogenoformans* was used to produce acetate from syngas, which was then converted into ethanol via hydrogenation on a ruthenium-based catalyst (Figure 1). Next, each system will be introduced separately.

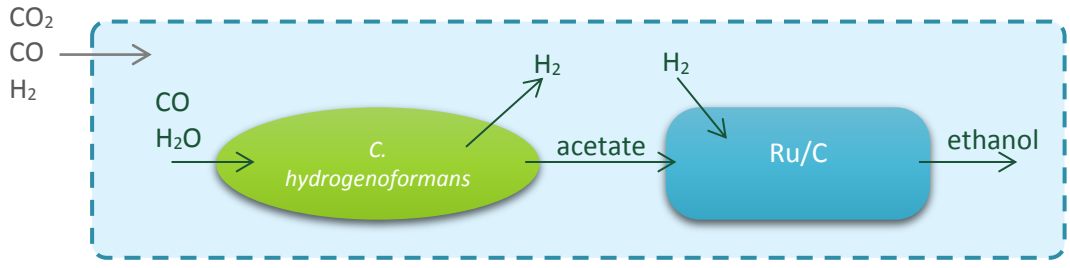

Figure 1 Schematic overview of proposed model system: Carboxydothermus hydrogenoformans converts syngas to the metabolite acetate, which the ruthenium catalyst on activated carbon (Ru/C) converts to the platform chemical ethanol. Dashed lines indicate that both reactions are performed in the same compartment.

1.2.1 Metabolism of Carboxydothermus hydrogenoformans

Carboxydothermus hydrogenoformans is a thermophilic bacterium with an optimal growth temperature of 70 °C. This anaerobic extremophile is able to solely grow on CO by generating energy through catalysing the water gas-shift reaction [11]. Here, CO_2 and H_2 are produced from CO and H_2O (Figure 2). More specifically, a monofunctional carbon monoxide dehydrogenase (CODH) first oxidises CO to CO_2. Resulting protons are reduced to molecular hydrogen in an energy converting hydrogenase. Simultaneous proton translocation results in a proton gradient across the membrane, which fuels ATP synthase [1]. Even though this reaction has a relatively low standard Gibbs free energy of -20 $kJ.mol^{-1}$, the organism shows a very high growth rate [12]. This is explained by the presence of five different, uniquely-specialised CODH complexes, with a turnover frequency of up to 39000 s^{-1} [13].

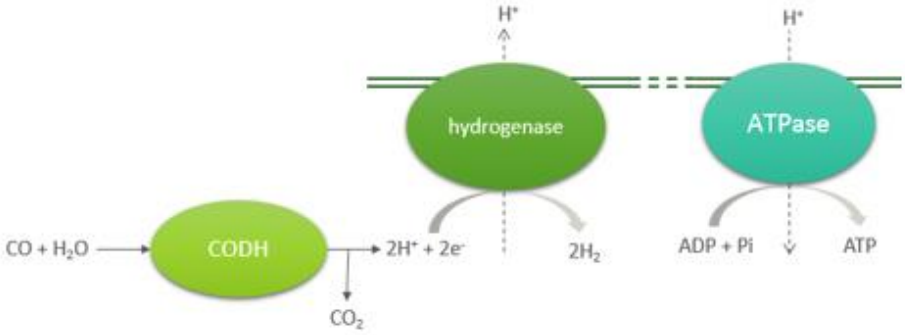

Figure 2 Schematic representation of water gas-shift reaction: ATP generation through H_2 production from CO in C. hydrogenoformans. CODH: carbon monoxide dehydrogenase

Another major advantage of *C. hydrogenoformans* is its robustness, regarding relevant parameters for this study. Firstly, it can tolerate high gas pressures. Previous research on this organism has shown growth for up to 20 bar [14], while higher pressures are expected to be tolerated. Secondly, *C. hydrogenoformans* shows growth on a wide range of CO concentrations. Although a high CO affinity of CODH allows growth at trace levels [13], no toxicity is noted at elevated concentrations [15, 16]. Together, its fast growth and robustness make this organism suitable for this study.

Under certain conditions, which are yet unknown, the metabolism of *C. hydrogenoformans* shifts away from using the water gas-shift reaction to the Wood-Ljungdahl pathway, used for acetogenic growth [4]. Instead of creating an ion gradient, acetate is now being produced to facilitate energy generation through substrate level phosphorylation. The Wood-Ljungdahl pathway, or reductive acetyl-CoA pathway, consists of two branches, the methyl and the carbonyl branch, as can be seen in Figure 3.

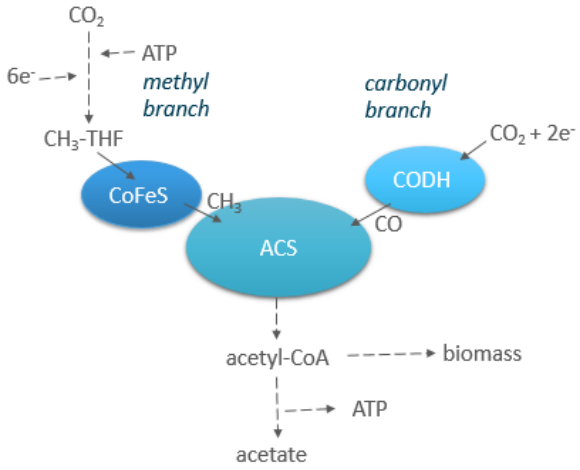

Figure 3 Schematic representation of acetate production in C. hydrogenoformans using the Wood-Ljungdahl pathway. ACS: Acetyl-CoA synthase. CODH: carbon monoxide dehydrogenase. CoFeS: corrinoid iron-sulfur-containing protein. Dashed lines indicate that intermediate reactions are involved.

In the methyl branch, CO_2 undergoes stepwise reduction to methyl-tetrahydrofolate at the expanse of one ATP and six reduction equivalents [17]. At the last step of the methyl branch, the methyl group is transferred to a corrinoid iron-sulfur-containing protein, from where it is transported to the acetyl-CoA synthase (ACS). The ACS is coupled to a carbon fixating CODH, forming a bifunctional complex [18], which reduces a second molecule of CO_2 to CO in the carbonyl branch requiring two additional reduction equivalents. Together with coenzyme A, the methyl group and CO are combined in the ACS/CODH complex to yield the central metabolite acetyl-CoA [5]. Subsequently, the enzymes phosphotransacetylase and acetate kinase convert acetyl-CoA to acetate under the production of one ATP [4, 6]. However, as CO_2 reduction in the methyl branch requires one ATP, there is no net production of ATP. Instead, a simultaneously generated transmembrane ion gradient drives ATP synthesis, creating a net gain of energy when converting the gaseous feedstock to acetate [9].

Note that a fraction of acetyl-CoA will also be used for carbon assimilation, limiting the maximum possible yield of acetate on CO_2. Furthermore, the pathway can also be used with CO as sole substrate instead of CO_2. In that case, the CO_2 needed for the methyl branch will first be generated through oxidation of CO in the monofunctional CODH, whereas reduction of CO_2 through the bifunctional CODH in the carbonyl branch is not necessary anymore [4].

As the requirements and environmental conditions for this metabolic shift between the water gas-shift and acetogenic metabolism were not yet known, they were investigated during this project. So far, it was merely theorised that the shift occurs at a certain H_2/CO ratio. At this threshold, CO levels will be low enough to not sustain growth anymore, while a high concentration of H_2 makes further conversion trough the WGS thermodynamically unfavourable. This demands for carbon utilisation through the Wood-Ljungdahl pathway. However, no correlations between acetate production and H_2, CO concentrations or total pressure were found yet.

1.2.2 Metal catalyst hydrogenation

The final conversion step of the proposed system, from the metabolite to the desired platform chemical, will be performed through hydrogenation. Hydrogenation is widely applied in industry,

where it finds applications in the pharmaceutical, petrochemical and food industry [19]. In hydrogenation, H_2 is used to reduce an organic compound in the presence of a catalyst to yield the desired product. The final product composition, as well as the efficiency and reaction rate are determined by the specific activity and selectivity of the applied catalyst towards the desired product and the reaction conditions [20]. It is therefore crucial, to find the right catalyst and conditions for each conversion.

Olcay et al. (2010) studied the catalytic activity and ethanol selectivity of different transition metals at a range of temperatures, using acetic acid as substrate. In this study, the best performance was achieved with a ruthenium catalyst bound to activated carbon (Ru/C), showing an ethanol selectivity of 80 %. Due to its promising ethanol specificity from acetic acid, the heterogeneous 5 wt. % Ru/C catalyst was chosen for this study. Respective experiments from Olcay et al. (2010) were conducted at temperatures between 100-225 °C and at pH 2.3. In a combined system however, the conditions have to be in a viable range for the organism. In literature, these were found to be between 40-70 °C and a pH of 6.4-7.7 [12]. Consequently, performance of the selected catalyst at desired conditions for the organism must be researched.

When comparing the catalytic performance from Olcay et al. (2010) for Ru/C on acetic acid at the given range of temperatures, several predictions for the performance at the desired conditions can be made. Firstly, it can be seen that at lower temperatures, the carbon conversion and turnover frequency decrease exponentially, while the ethanol selectivity increases (Appendix II. Expected Ru/C conversion for different temperatures). Conversion at 70 °C is thus expected to be feasible, even though it will occur much slower. Moreover, the main by-product at lower temperatures is ethyl acetate, while methane can be found majorly at higher temperatures. Furthermore, possible conversion routes of acetic acid towards ethanol are proposed for the heterogeneous ruthenium catalyst. Even though conversion through acetate is one possible path, it involves initial dehydrogenation which is unlikely to occur at an already hydrogen-rich environment. When performing the same reaction at a neutral pH under the presence of the organism however, acetic acid is already present as acetate and does not require infeasible initial conversions. Hence, it was assumed that at neutral pH conversion to ethanol from acetate is also possible.

1.3 Research objective and approach

The aim of this thesis was to get more insight into the potential of combining biological and chemical systems for the production of platform chemicals from syngas. As little knowledge is available on this concept, a model system for ethanol production was chosen, which was researched based on the following question:

What is the influence of H_2 partial pressure on the acetate production by extremophiles and in situ conversion to ethanol using a heterogeneous ruthenium catalyst?

This was done by assessing each system individually. In the first part, the influence of environmental conditions causing the metabolism to switch from hydrogenogenic to acetogenic production was explored. For this, the effect of different H_2 partial pressures and total pressure were investigated.

Furthermore, feasible growth temperatures were determined for *C. hydrogenoformans*, to determine possible ranges for the combined system, away from the organism's optimum.

In the second part, the performance of a ruthenium-based catalyst on the ethanol production from acetate was examined, under conditions which are suitable for the organism. These conditions include temperature, pH and H_2 partial pressure. The catalytic performance was evaluated by looking at the activity, ethanol production, by-product formation and conversion speed.

By comparing these results, conditions for the combined system were defined. Lastly, the combined system was tested for the chosen conditions and evaluated based on its performance. An additional scientific challenge for this project results from combining the fields of biotechnology, microbiology and chemistry, which are all relevant to fully research and understand the system.

2 Materials and Methods

To gain more insight into the potential of combining biological and chemical systems to produce platform chemicals from syngas, the chosen model system was explored in three separate steps. Firstly, the conditions causing the extremophile *Carboxydothermus hydrogenoformans* to produce acetate from CO and H_2 were examined. Secondly, a ruthenium-based catalyst was researched for its potential to hydrogenate acetate to ethanol at conditions which are suitable for the organism. Finally, both the organism and the catalyst were tested in a combined system. Following this division, the next chapters are subdivided into these three parts.

2.1 Acetate production through Carboxydothermus hydrogenoformans

2.1.1 Organism and Medium

For this study, the thermophilic anaerobic bacterium *Carboxydothermus hydrogenoformans* (DSM 6008), obtained from the German Culture Collection (DSMZ, Germany), was used.

Cultivation occurred a bicarbonate-buffered medium described by Stams et al. (1993). This standard medium is used by the Microbiology department for anaerobic growth. A detailed description of the compounds and the preparation method can be found in Appendix III. CP Medium composition and preparation (MIB protocol).

2.1.2 Inoculation and conservation of an active cell culture

Prior to inoculation, substrate was added to the 117 mL anaerobic medium flasks. Growth occurs on carbon monoxide (CO) as the sole substrate, under strictly anaerobic conditions. Hence, a syringe was used to exchange 35 mL of the N_2/CO_2 headspace for 35 mL of pure CO, causing the headspace to be enriched with 50 vol. % substrate. For the inoculation, 2 mL of the existing active culture were added to 51.5 mL of medium. For growth, the culture was kept in a shaking incubator (innova®44, New Brunswick, Germany) at 65 °C and 150 rpm. Growth was examined by analysing the gas phase for an increase in H_2 and decrease in CO. Details on applied analysis techniques are given in section 2.4.

During the entire period of this project, an active cell culture was maintained. Inoculated cell cultures were checked daily for their CO availability and replenished when necessary to increase the cell density. Once per week, 2 mL of this densely grown cell culture was used to inoculate a new, freshly prepared medium flask for maintaining an active culture. While transferring the active cell culture into fresh medium for new growth at 65 °C, the used inoculum was stored at room temperature (RT) as backup culture. When biomass was needed for experiments, additional medium flaks were inoculated three days prior to the experiment, to transfer the culture in an active and dense state.

2.1.3 Low pressure microbial experiments

Initial experiments for getting insight into the metabolism were performed in 117 or 250 mL anaerobic glass flasks. Even though they allow quicker processing due to a simple set-up, they can only be operated until 3 and 2 bars, respectively.

Unless noted otherwise, the medium flasks for the low-pressure microbial experiments were prepared according to the method described in section 2.1.2. Prior to inoculation, the headspace was adjusted to the desired composition using a gas exchanger and/ or a 50 mL syringe. After inoculation, cells were incubated at 65 °C and 150 rpm (innova®44, New Brunswick™, Germany), for the duration of the experiment. At respective time intervals, 0.2 mL gas and/or 1.0 mL liquid samples were taken with 1.0 mL syringes and analysed immediately.

For evaluation of the results, the CO consumption (mmol), as well as H_2, CO_2 and acetate production (mmol) were investigated. These were determined by the difference between the initial and the final concentration. From this, the CO_2/H_2 ratio from the produced gases was determined:

$$ratio_{CO_2/H_2} = \frac{produced\ CO_2\ (mmol)}{produced\ H_2\ (mmol)} \quad (1)$$

Additionally, the acetate yield from converted CO was considered:

$$yield_{ac/CO}(\ mol\ \%) = \frac{acetate\ (mmol)}{converted\ CO\ (mmol)} * 100\ \% \quad (2)$$

Lastly, the carbon recovery (%) was investigated by comparing initial and final carbon presence (mmol):

$$carbon\ recovery\ (mol\ \%) = \frac{CO_{final}+CO_{2,final}+2*aceate_{final}\ (mmol)}{CO_{initial}+CO_{2,initial}\ (mmol)} * 100\ \% \quad (3)$$

2.1.3.1 Effect of temperature

The performance of *C. hydrogenoformans* was tested for different temperatures, namely 70, 80 and 90 °C. For this, cells were kept in non-shaking temperature chambers (Hettich Benelux, The Netherlands). To balance the resulting mass transfer limitation, cells were grown in 250 mL flasks and stored horizontally [16]. Initial CO concentration was 27 vol. % (70 mL). The initial pressure was 1.3 bar at RT.

2.1.3.2 Effect of H_2 partial pressure

The effect of H_2 partial pressure was studied through applying different initial H_2 to CO ratios. This was studied twice. Firstly, 4 distinct CO to H_2 ratios were tested, which are presented in Table 1. To investigate the actual effect of H_2 partial pressure, the same conditions were tested with N_2 instead of H_2. In all cases, the headspace was enriched with 10 vol. % of CO_2 for the carbonate buffer. 250 mL anaerobic flasks were used and the initial pressure was 1.26 bar. Flasks with adjusted headspace and pure medium were placed in a 65 °C incubator the night before inoculation. Pre-heated medium was inoculated with 4 mL of an active culture, and growth was followed by taking gas and liquid samples every 1-3 hours.

Table 1 Overview of applied gas ratios to investigate the effect of H_2 partial pressure on acetate production. Entry points denote the respective CO/H_2 or CO/N_2 ratio in vol. %, not considering CO_2.

entry point	**CO (mmol)**	**H_2 (mmol)**	**N_2 (mmol)**	**CO_2 (mmol)**
(A) 5:95	0.53	10.04		1.06
(B) 10:90	1.06	9.51		1.06
(C) 20:80	2.11	8.45		1.06
(D) 30:70	3.17	7.40		1.06
(E) 5:95	0.53		10.04	1.06
(F) 10:90	1.06		9.51	1.06
(G) 20:80	2.11		8.45	1.06
(H) 30:70	3.17		7.40	1.06

For the second experiment, the same conditions were used, except that a different inoculation strategy was employed. This time, merely 0.5 mL of inoculum was added and the medium was heated to 65 °C only after inoculation occurred. Now, solely B_r, D_r, F_r and H_r were investigated and tested in triplicate. $_r$ indicates repeated experiment.

2.1.3.3 Substrate requirement for acetate production

The substrate requirements for acetate production were investigated by incubating the cells with a CO_2 to H_2 ratio of 2:4 and no CO [21]. Hence, 80 mL CO_2 and 160 mL H_2 were supplied. While a background triplicate was incubated with additional 50 mL of CO, the actual experiment was enriched with 50 mL to yield the same initial pressure of 1.45 bar.

2.1.4 High pressure microbial experiments

To get a better understanding of the impact different pressures and H_2 to CO ratios have on the acetate production in *C. hydrogenoformans*, growth experiments were also carried out at higher pressures. For these high-pressure microbial experiments, a Parr Series 5000 Multiple Reactor System (Parr Instrument Company, USA) was employed, consisting of six individually controllable 75 mL reactors. More specifically, temperature, pressure and agitation speed could be regulated for each reactor. Online output data for the temperature and pressure were obtained every 10 seconds for each reactor separately. For the presented pressure profiles, an average of each 15 min was used. Glass liners (Parr Instrument Company, USA) were used. Using identical stirrers ensured equal mass transfer rates.

2.1.4.1 Experimental set-up

Reactor preparation and assembly occurred in an anaerobic tent. Here, 15 mL of fresh medium, as well as 15 mL of an active cell culture were pipetted in each liner. For increased heat transfer between the walls of the reactor and the glass liners, 6 mL of fresh medium were additionally pipetted into this gap. Afterwards, each headspace was exchanged with 1 bar of N_2/CO (80:20). Finally, the respective amounts of CO, H_2 and N_2 were added.

Each reactor was connected to a temperature and pressure sensor, and inserted in a heating pocket. Reactors were heated to 70 °C over four 4 h, after which they were kept at 70 °C until the pressure remained constant. An agitation speed of 200 rpm was chosen. Experiments were stopped by decreasing the temperature set-point and letting reactors cool down.

For analysis, 10 mL gas samples were taken from each reactor through the gas outlet before their disassembly. The liquid from each reactor was pooled and kept for analysis. All experiments were performed in triplicate. Experiments were evaluated based on the obtained online pressure profile, as well as the parameters described in section 2.1.3.

2.1.4.2 Experimental design

Firstly, the effect of increased total initial pressure was investigated. For this, cell growth was observed for initial pressures of 1.0, 2.5 and 4.0 bar, with a background of 1 bar of N_2/CO.

Secondly, the effect of an increased H_2 to CO ratio was evaluated. For this, cell growth was overserved for the following ratios of CO/H_2 (bar): 1:9 and 4:6. All had a background of 1 bar N_2/CO.

2.2 Ethanol production through the ruthenium-based catalyst

2.2.1 Chemicals

The following chemicals were purchased from Sigma-Aldrich (Germany): acetic acid (glacial), sodium acetate, a 5 wt. % ruthenium catalyst on activated, deuterium oxide (99.9 atom % D) and acetaldehyde. Ethanol (EMSURE®), ethyl acetate (LiChrosolv®) and sulfuric acid (98%, EMSURE ®) were obtained from Merck (Germany). Acetone (RE) was acquired from Actu-All Chemicals (The Netherlands).

2.2.2 Experimental set-up

All hydrogenation experiments were performed in the Parr Series 5000 Multiple Reactor System (Parr Instrument Company, USA), described in section 2.1.4. To reduce impurities within the system, PTFE liners (Parr Instrument Company, USA) were used. Employing identical stirrers ensured equal mass transfer rates. An agitation speed of 200 rpm was chosen.

30 mL of either a 10 wt. % acetic acid solution or a 10 wt. % acetate solution was used as substrate. To this, 0.2 g of a heterogeneous 5 wt.% ruthenium catalyst supported on activated carbon (Ru/C) was added.

2.2.3 Experimental design

For the start-up of an experiment, each reactor was connected to a temperature and pressure sensor, and inserted in a heating pocket. By filling the reactors with the desired pressure of H_2 and specifying the desired temperature, an experiment was initiated. It was stopped by decreasing the temperature set point and letting them cool down.

10 mL gas samples were taken from each reactor through the gas outlet before their disassembly. Afterwards, 5 mL liquid samples were taken, where the catalyst was removed from the sample by filtration through 0.2 µm filter (Whatman™, General Electric, USA). The liquid weight, as well as the pH, were determined before and after an experiment.

For the analysis, the duration of each experiment is defined as the time between which the desired temperature was first reached in the reactors and the time at which the reactors were taken out of the heating pockets. Results are expressed in final yield of ethanol per day (mmol day^{-1}). All experiments were conducted in triplicate.

2.2.3.1 Effect of low and neutral pH

The impact of using either acetic acid or acetate as substrate for the catalytic conversion to ethanol was investigated. A substrate of pH 6.5 was obtained by mixing a 10 wt. % acetate and a 10 wt. % acetic acid solution, which was compared to a 10 wt. % acetic acid solution of pH 2.2. Catalysis was performed at 50 bar hydrogen pressure and 70 °C.

2.2.3.2 Effect of temperature on catalytic activity

The effect of temperature on the final yield of ethanol was determined by performing the catalytic conversion at a hydrogen pressure of 50 bar, at 70, 80, 90, 100 and 150 °C, respectively. Acetic acid was used as a substrate. A reduced catalyst was employed.

2.2.3.3 Effect of H_2 partial pressure on catalytic activity

To test the influence of hydrogen partial pressure, the temperature was kept at 70 °C, while the hydrogen pressure was set to 30, 40 and 50 bar, respectively. Acetic acid was used as a substrate, and the catalyst was reduced.

2.2.3.4 Effect of reducing the catalyst

To ensure no major deactivation of the catalyst had occurred during storage, the effect of reducing the catalyst prior to the experiment was investigated. For the reduction, the catalyst was heated with a temperature ramp of 300 °C per hour to 150 °C, and keeping it at 150 °C for one hour. Reduction occurred under a constant flow of 30 mL min^{-1} N_2 and 30 mL min^{-1} H_2. For subsequent cooling of 30 minutes, 30 mL min^{-1} N_2 was present in the gas flow.

To evaluate the effect of reduction, both reduced and non-reduced catalyst were tested. Hydrogenation occurred at 70 °C, 50 bar hydrogen pressure and acetic acid as the substrate.

2.2.3.5 Nuclear magnetic resonance (NMR)

^{1}H Nuclear magnetic resonance (^{1}H NMR) was performed to detect which by-products formed during the hydrogenation reaction of the Ru/C at lower temperature. Therefore, a 10 wt. % acetic acid solution was prepared in deuterium oxide. 30 mL of this substrate were used in the PARR system. Reduced catalyst was added. The reaction occurred at 70 °C with 50 bar hydrogen pressure. After the experiment, liquid samples were analysed with ^{1}H NMR, as explained in 2.4.5.

2.3 Combined system

The combined performance of the extremophile and the catalyst was evaluated in a PARR multiple reactor system, described in section 2.1.4. Glass liners were used, as well as identical stirrers to ensure equal mass transfer rates. To minimise inactivation of the catalyst, no sulfides were added to the system [22]. Furthermore, the effect of adding yeast extract to the system was investigated by performing one experiment with, and one experiment without, adding yeast extract.

The liners were filled with 20 mL of the respective medium while 6 mL were filled in the gap between reactors and liners. Furthermore, each liner was supplemented with 0.2 g of the Ru/C catalyst. The gas phase consisted of 1 bar N_2/CO_2, 4 bar CO and 6 bar H_2. The temperature was set to 70 °C with a heating over 4 hours. Set-up and stopping of the experiment occurred as described in 2.1.4.1. Liquid samples were filtered first to remove the catalyst, as explained in 2.2.3.

2.4 Analytical methods

2.4.1 High Performance Liquid Chromatography (HPLC)

Liquid samples were analysed by using high performance liquid chromatography (HPLC). The HPLC analysis was conducted at two different chair groups.

2.4.1.1 Microbial experiments

Liquid samples from the microbiological experiments were analysed with a TSD-HPLC (Thermo Scientific, Waltham, USA) on a MetaCarb 67H column (Agilent Technologies, Santa Clara, CA). It was operated at 45 °C for 20 min, at a liquid flow of 0.9 mL min^{-1}. The eluent was 0.01 M H_2SO_4. Both a RI and UV detector were employed.

To remove any cell debris form the liquid samples, they were first centrifuged at 13000 g for three minutes. 10 mM DMSO was used as internal standard by adding it to the sample in a ratio of 6:4. Calibration curves for acetate, formate and ethanol were constructed for concentrations of a 0.125, 1.250, 2.500, 5.000 and 10.000 mM, respectively.

2.4.1.2 Catalytic experiments

Liquid samples from the hydrogenation were investigated with a Dionex UltiMate™ 3000 RS autosampler HPLC (Thermo Fischer Scientific, Germany), equipped with an Aminex HPX87H, 300x7.8 mm (BioRad 125-0140) column. It was operated at 50 °C for 30 min, at a liquid flow of 0.5 mL min^{-1}. 5 mM H_2SO_4 was used as eluent. Detection occurred through both a RI and UV detector.

For quantification of the substrate (acetic acid or acetate) and the products (ethanol and ethyl acetate), calibration curves were employed. Concentrations of 5, 10, 50, 75 and 100 g L^{-1} were used for the substrate, and 0.5, 1.0, 5.0 and 10.0 g L^{-1} for the products.

2.4.1.3 Combined system

For the liquid samples of the combined system, the HPLC at the Microbiology department was used with the conditions described in 2.4.1.1.

2.4.2 Gas Chromatography (GC) for gas samples

All gas samples were measured through gas chromatography (GC). For this, 0.2 mL of the sample was analysed in a Compact GC 4.0 (Global Analyser Solutions, The Netherlands), equipped with a molsieve 5A column, coupled to a Carboxen 1010 pre-column. Detection occurred at 100 °C through a thermal conductivity detector.

To quantify the gas content of the sample, 0.2 mL of 100 % CO, 100 % methane, 100 % ethane, and 80 % H_2 with 20 % CO_2 respectively were used for calibration.

2.4.3 Gas Chromatography for liquid samples

For the hydrogenation experiments, volatile products could be detected by HPLC. Hence, a GC-2010 with an AOC-20i autoinjector and an AOC-20S autosampler (Shimadzu, Japan) was used, too.

Acetone was used as internal standard for the analysis by adding it to the filtered liquid sample in a 1:1 ratio. The same concentrations as for the HPLC were used to create the respective calibration curves.

2.4.4 GC/MS

For determination of unknown compounds, a GC/MS equipped with a TRACE™ column, a DSQ detector and a quadrupole analyser (Thermo Fischer Scientific, Germany) was applied. It was scanned for a mass range of 10-50 kDa with a temperature ramp from 40 to 150 °C in 6 min.

2.4.5 Nuclear Magnetic Resonance (NMR)

NMR was determined with a 500 MHz Bruker NMR machine (Bruker, MA, United States), using a ^{1}H NMR spectrum. For the analysis, 1 mL of undiluted sample was filled in respective tubes. Calibration of the spectrum was based on the known peak positions of water at 4.7 ppm and acetic acid at 2 ppm [23, 24].

2.4.6 Dry weight determination

The dry weight of biomass was determined at the end of each microbial experiment. This was done by separating the cells from the medium through centrifugation at 4600 g for 10 min, and the salts through 2 washing steps with demineralised water at 13000 g for 4 min. The final pellet was transferred to pre-weighted aluminium cups and dried overnight at 46 °C, after which it was weighted again.

3 Results and Discussion

The aim of this project was to get more insight into the potential of combining biological and chemical systems for the production of platform chemicals from syngas. As little knowledge was present on such combined systems, a simple model system was chosen for this project. This model system combined an extremophile facilitating acetate production from CO with an *in situ* conversion using heterogeneous ruthenium-based catalyst to produce ethanol (Figure 1). This chapter will be subdivided into the individual results of both the biological and the chemical part, as well as the results of the combined system.

3.1 Acetate production through Carboxydothermus hydrogenoformans

The first part of this project investigated the metabolism of the extremophile *Carboxydothermus hydrogenoformans.* More specifically, it was assessed at which environmental conditions the organism would switch from hydrogen to acetate production for energy generation. This was expected to occur at certain H_2 to CO ratios, at which acetogenesis becomes thermodynamically more favourable.

3.1.1 Effect of temperature on growth

The effect of temperature on growth and acetate production for *C. hydrogenoformans* were investigated, in order to determine the feasible temperature range for the proposed combined system. Literature stated that its optimal growth temperature is 72 °C [12]. Consequently, cells were incubated at 70, 80 and 90 °C for 7 days. Growth was indicated by an increase in H_2 concentration, as the organism generates energy by converting one mol CO into one mol H_2 plus one mol CO_2, during the water gas-shift (WGS) reaction [16]. CO_2 was regarded as a less reliable indicator, due to the carbonate-buffered medium used for the experiments.

The H_2 concentration profile for the examined temperatures is depicted in Figure 4. It can be seen, that the H_2 concentration at 70 °C first increases exponentially, after moving to a constant value. Only limited H_2 was detected at 80 °C. No H_2 increase was observed for 90 °C.

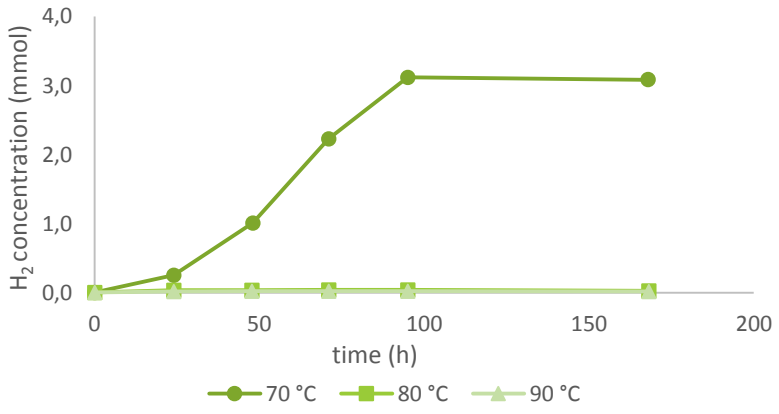

Figure 4 H_2 concentration profile for growth at 70, 80 and 90 °C over time. Average of triplicate experiments.

Comparing the final H_2 concentration with the amount of added substrate, both around 3 mmol, full growth can be concluded for 70 °C. On the other hand, hardly any substrate was converted at 80 °C, and none at 90 °C.

While the growth curve for 70 °C followed the expected profile, with a short lag and an exponential phase, its duration was much longer than anticipated. In previous activation of the organism, where equal inoculum, medium and gas volumes were used, 3 mmol CO was fully consumed in about 2 days.[1] Now, it took twice as long. This may be explained by the lack of shaking during growth, as the shaking incubators could maximally operate at 65 °C. To decrease resulting mass transfer limitation, large bottles were employed, which were stored horizontally. However, the slow conversion suggests that growth was mass transfer limited, and horizontal storage was not sufficient. The requirement of shaking during incubation was also overserved by Henstra (2006), who concluded that growth is gas/liquid mass transfer limited without employing shaking. As this experiment merely aimed for a comparison among the proposed temperatures, operating at equally non-ideal mass transfer rates was not regarded as a problem. However, following experiments were performed with equal agitation rates to prevent mass transfer limitation and provide similar gas availability in the liquid.

Moreover, final acetate concentrations and biomass weights were determined which are presented in Table 2. It can be noted, that 0.010 and 0.011 mmol acetate was present for 80 and 90 °C, where no cell activity was observed. Background checks revealed that no acetate was detected in pure medium, although 0.007 mmol were added through the inoculum. Hence, small concentrations of acetate were detected, even though no growth was observed. The missing 0.003 mmol can result from measurement errors, as detection occurred around detection limits, as well as from low initial cell activities. For 70 °C, 0.062 mmol was measured, which equals an acetate yield from converted CO of 2.1 mol %. As hardly any CO was converted for 80 and 90 °C, respective yields were not considered.

Looking at the dry biomass weight, the results seem questionable. While no activity was observed above 70 °C, similar or even higher amounts of biomass were measured for 80 and 90 °C, compared to 70 °C. More likely, samples were not washed thoroughly enough to remove salts. The issue of accurately determining biomass yield through dry weight measurement at low cell concentrations has been described in literature [25]. Due to the lack of accuracy, it was decided to not determine the dry weight biomass for further experiments.

Table 2 Final acetate and biomass concentrations from incubating cells at 70, 80 or 90 °C for 168 h. Average of triplicate experiments.

temperature (°C)	**produced acetate (mmol)**	**dry biomass weight (mg)**
70	0.062 ± 0.002	2.97 ± 0.90
80	0.011 ± 0.004	2.17 ± 0.68
90	0.010 ± 0.001	4.55 ± 0.35

For the proposed biochemically combined system, the operating temperature is of great importance as it determines respective conversion rates. As the chosen catalyst has higher conversion rates at increased temperature, with favourable conversions around 150 °C [26], the maximum growth temperature of the organism should determine the operating temperature. The determined maximum

[1] The quick CO consumption during growth in shaking incubators was based on observations from maintaining an active culture. The active culture always fully consumed the supplied CO within less than two day, when being incubated at 65 °C.

growth temperature of 70 °C is in line with literature. There 40-78 °C were found to be the feasible growth range for *C. hydrogenoformans*, with 70-72 °C being the optimum [12]. As hardly any growth and acetate production could be overserved above 70 °C, this was chosen as the optimum temperature of the combined system.

3.1.2 Substrate requirement for acetate production

Little knowledge was present on the metabolism of *C. hydrogenoformans* during acetate production [11, 14]. To get more insight into the substrate requirements for acetate production, cells were observed during CO depletion. More specifically, they were observed at the presence of H_2 and CO_2 to simulate the end of the WGS reaction. In one scenario, an initial amount of 2.20 mmol CO was added to ensure initial biomass activity, while no CO was supplied in a second scenario. In both cases, H_2 and CO_2 were added to anaerobic bottles in a 2:1 ratio, 7.05 and 3.52 mmol, as this is required for acetate production [21].

As Figure 5 shows, acetate was detected in both scenarios. For the first 112 h, an increase in acetate production can be seen for both cases, moving to different values. For growth with initially supplied substrate, the final acetate concentration was 0.126 ± 0.001 mmol. Without CO, 0.066 ± 0.001 mmol acetate was detected. After 112 h, the acetate concentration remained largely constant until the end of the experiment, after 450 h. The acetate yield from converted CO was 5.7 mol %.

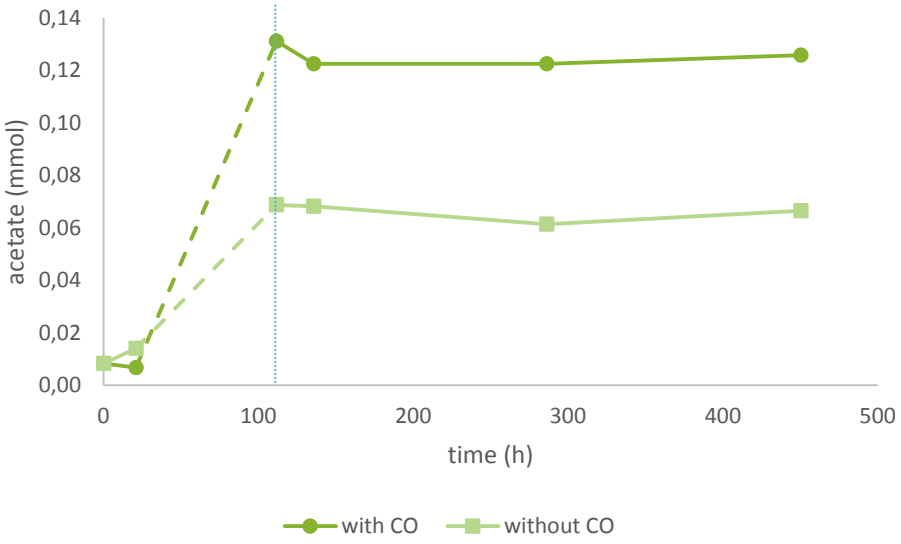

Figure 5 Acetate production over time for growth at H_2 and CO_2, either with or without CO availability. Average of duplicate experiments. Dashed line between 21 and 112 h indicates that the exact slope of exponential growth was not known. The vertical line indicates CO depletion.

Even though the initial concentration of CO, being the substrate of the WGS, was lower than that of the products, the supplied CO was fully consumed. Hence, the WGS was able to proceed until detection limits of CO were reached, despite high product concentrations. Due to a low accuracy of the GC at high H_2 levels, no conclusions on growth based on the H_2 concentration profile could be made for either scenario. Furthermore, one of the triplicates failed in each scenario, which were not considered for analysis.

Furthermore, acetate production was observed for incubation with and without CO. In each case, an initial amount of 0.008 mmol was introduced through the inoculum. Compared to the supplied gas concentrations, final acetate concentrations were low. Therefore, little of the substrates were used for acetate production. Moreover, the final concentrations were already reached between 21 and 112 h. No specific time could be determined as the slope during this period was unknown. This was indicated by dashed lines in between 21 and 112 h (Figure 5). For cultures with supplied CO, the substrate was fully converted at that point, as indicated by the vertical line. This suggests, that CO is required for acetate production and cell growth. Even at CO depletion, energy could theoretically be generated through the Wood Ljungdahl pathway (WLP). For the WLP, H_2 and CO_2 are taken as substrates for energy generation through acetate production [4]. More specifically, 4 mol H_2, and 2 mol CO_2 produce 1 mol acetate and 2 mol water [21]. However, this did not occur although high amounts of H_2 and CO_2 were available, and cells were incubated long enough for switches to occur.

3.1.2.1 Theory for metabolism under CO depletion

Possibly, CO is necessary for *C. hydrogenoformans* to produce sufficient high potential energy carriers, e.g. ferredoxin. Normally, these are produced when catalysing the CO to CO_2 conversion through the WGS by the monofunctional CODH complex [16]. However, using H_2 and CO_2 as substrate, merely generates low potential electrons (NADH and NADPH). To overcome this, certain anaerobic bacteria possess bifurcating enzyme complexes [27]. These use two intermediate pairs of electrons to generate one higher potential (ferredoxin) and one lower potential (NADH) energy carrier. *A. woodii* and multiple *Clostridium* species were found to possess these complexes [28, 29]. As this process has only been discovered and described recently, it was not known, whether *C. hydrogenoformans* possesses such enzymes. Not showing growth at CO depletion, however, suggests that the respective enzymes are not present in *C. hydrogenoformans*.

Considering that no activity could be observed upon CO depletion where CO was supplied, it was unexpected to detect acetate production when no CO was added. Especially, as the production was equal to that of the 70 °C temperature experiment, where 3 mmol CO was added. The most likely explanation for this was that the detected 0.06 mmol acetate resulted from background activities. Instead of using gaseous substrates through the Wood Ljungdahl pathway, acetate could be produced by breaking down amino acids or other substrates present in yeast extract, like pyruvate [30, 31].

3.1.2.2 Theory on acetate production

As explained in section 1.2.1, it was theorised, that a metabolic switch from H_2 to acetate production occurred upon exceeding a distinct CO/H_2 ratio. At that ratio, the concentration of the WGS product H_2 would be too high for the WGS to be thermodynamically feasible. Instead, acetate would be production through the WLP. Now, under elevated H_2 and CO_2 concentrations at the presence of CO, increased acetate levels were noted. More specifically, around twice as much acetate was detected as for the scenario without CO, as well as the previous 70 °C experiment without initially added H_2. Therefore, the theory of increased acetate production at increased H_2 partial pressure was supported by the result of this experiment. However, nothing could yet be said about the switch.

Summing up, limited conclusions could be drawn on substrate requirement during acetate production at ambient pressure. Some acetate resulted from background activities. H_2 partial pressure seemed to influence the final acetate production. Furthermore, it was concluded that no switch from WGS to WLP occurred, and that CO was required for cell growth and activity.

3.1.3 Effect of H_2 partial pressure on acetate production– low pressure

Next, the effect of H_2 partial pressure on the acetate production was studied. For this, different initial H_2 to CO ratios were applied in two experiments. Experiments with N_2 instead of H_2 served as control. Both scenarios were tested in anaerobic bottles.

3.1.3.1 Results and discussion of effect of H_2 partial pressure on acetate production – part 1

The first experiment was used for orientation purposes of the metabolic activities, and was thus performed in duplicate. Initially applied gas ratios of the first experiment were summarised in Table 1. Results are depicted in Figure 6. When growth occurred with high background concentrations of H_2, final acetate concentrations were 0.061 ± 0.001, 0.062 ± 0.001, 0.069 ± 0.003 and 0.071 ± 0.028 mmol, for 5, 10, 20 and 30 % initial CO. With N_2, 0.054 ± 0.001, 0.055 ± 0.006, 0.063 ± 0.015 and 0.078 ± 0.007 mmol acetate were produced. The large error bar of 30 % CO with H_2 is caused by a low activity of one of the duplicates. In all cases, 0.007 mmol acetate was introduced through the inoculum.

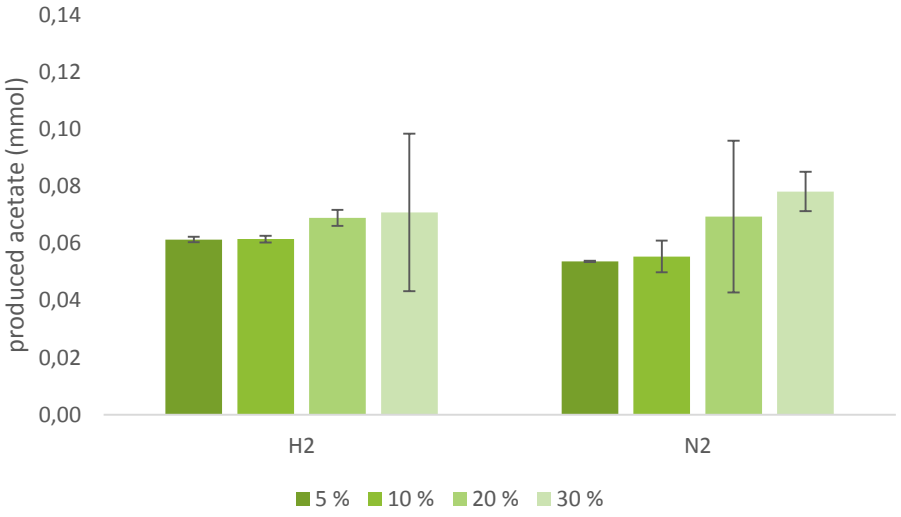

Figure 6 The effect of H_2 partial pressure on acetate production. 5, 10, 20 or 30 % CO were supplied with either H_2 or N_2 to reach 100 %. 5% equals 0.53, 10 % equals 1.06, 20 % equals 2.11 and 30 % CO equals 3.17 mmol CO. Averages and standard deviations of duplicate experiments are shown.

Acetate production was very similar amongst the applied H_2 partial pressures. The small increase in acetate production for lower initial H_2 pressures was expected. As explained previously, it was assumed that cells always produce small amounts of acetate. Having an enlarged CO availability more cells can grow which produce acetate. Based on previous acetate productions, around 0.06 mmol acetate was expected to be caused by background activities with the yeast extract. On top of that, the acetate production was slightly higher for growth at H_2, compared to N_2 environments. This supports the initial theory of an increased acetate production at lower CO/H_2 ratio. However, the differences are very small and therefore not considered as confirmation.

For more insight into the metabolic activities involved in acetate production, gas concentrations and product ratios were examined, which are summarised in Table 3. When comparing the amount of converted CO with the initial concentrations, it can be seen that CO was nearly fully converted. This is the case for all initially applied concentrations, disregarding of adding H_2 or N_2. H_2 production showed deviant results for H_2 or N_2 incubated growth. For the former, productions were 1.60 ± 0.39, 3.00 ± 1.23, 2.60 ± 1.49 and 4.37 ± 1.11 mmol H_2 for 5, 10, 20 and 30 % CO. For the latter, respective productions were 0.56 ± 0.03, 1.05 ± 0.04, 2.08 ± 0.07 and 2.80 ± 0.34 mmol. The produced amounts of CO_2 were similar to the CO consumption. Generally, slightly higher CO_2 concentrations were detected for H_2 incubated cells, compared to their N_2 counterparts. Moreover, CO_2 to H_2 ratio of the produced gases was assessed. At H_2 growth, the ratios varied between 0.38 ± 0.10 and 0.78 ± 0.09, while ratios between 0.64 ± 0.33 and 0.96 ± 0.05 were found for growth with N_2. Next, the acetate yield from converted CO was determined. This decreased with increasing initial amounts of CO. Finally, the carbon balance was examined. Full carbon recovery was observed in nearly all scenarios, with yields slightly higher than 100 % for scenario A, B, D, E and F. Letters refer to entry points of Table 3.

Table 3 Summary of gas conversions, acetate production and product ratios for different H_2 partial pressures. Concentrations of entry points refer to initially added amount of CO. Either H_2 (A-D) or N_2 (E-F) were added for total concentration of 11.57 mmol. In all cases, 1.06 mmol CO_2 were initially added.
Produced H_2 and CO_2 were based on final minus initial concentration. Acetate yield from CO refers to converted CO. Average and standard deviations are based on duplicate experiments.
[a] 100 % conversion of initial CO

entry point (mmol CO)	**converted CO (mmol)**	**produced H_2 (mmol)**	**produced CO_2 (mmol)**	**CO_2/H_2 ratio**	**acetate/CO (mol %)**	**carbon recovery (mol %)**
A (0.53)	0.53 [a]	1.60 ± 0.39	0.58 ± 0.01	0.38 ± 0.10	11.5 ± 0.2	57.8 ± 0.4
B (1.06)	1.06 [a]	3.00 ± 1.23	1.15 ± 0.20	0.40 ± 0.10	5.8 ± 0.1	71.1 ± 6.1
C (2.11)	2.01 ± 0.05	2.60 ± 1.49	1.82 ± 0.11	0.78 ± 0.09	3.3 ± 0.2	75.1 ± 3.7
D (3.17)	2.85 ± 0.34	4.37 ± 1.11	2.92 ± 0.74	0.67 ± 0.02	2.4 ± 0.7	88.8 ± 10.8
E (0.53)	0.53 [a]	0.56 ± 0.03	0.47 ± 0.12	0.85 ± 0.54	10.2 ± 0.1	55.1 ± 2.5
F (1.06)	1.05 ± 0.01	1.05 ± 0.04	1.00 ± 0.02	0.96 ± 0.05	5.3 ± 0.5	66.3 ± 0.6
G (2.11)	2.06 ± 0.08	2.08 ± 0.07	1.33 ± 0.64	0.64 ± 0.33	3.0 ± 0.6	62.5 ± 14.6
H (3.17)	3.11 ± 0.08	2.80 ± 0.34	2.41 ± 0.36	0.86 ± 0.03	2.5 ± 0.3	76.1 ± 8.6

From the data, several observations could be made. Firstly, *C. hydrogenoformans* was able to convert CO until concentrations below the detection limit, irrespective of the initial concentration. Secondly, great deviations were visible for H_2 production during growth at high H_2 concentrations, both internally and in comparison to their N_2 counterparts. This was explained by high initial presence of H_2, for which large measurement errors were found. Hence, detections at high concentrations occurred with low accuracy. Consequently, the CO_2/H_2 ratios with background H_2 were not reliable and not considered for analysis. On the contrary, growth with N_2 yielded H_2 concentrations which were similar to the CO conversions. This indicates that the majority of CO was converted through the WGS reaction. This observation was largely confirmed by CO_2 production. Especially at growth with H_2, CO_2 production was merely slightly larger than CO consumption. For growth with N_2, lower CO_2 production was observed. CO_2/H_2 ratios for N_2 were close to 1 for all tested substrate concentrations. Again, this indicated that the predominant cell activity was the WGS reaction. Thirdly, it was indicated that the

acetate yield from converted CO decreased for increasing initial CO. More specifically, at high presence of H_2, the decrease was proportional to the CO increase, suggesting that the yield of acetate is indifferent to the amount of supplied CO. This supports the theory of having a background acetate production independent of the applied conditions. For N_2, the same general trend could be seen. Due to the slightly lower acetate production, however, the yield on CO was also slightly lower for lower H_2 partial pressures. Lastly, the carbon balance was used to assess how much of the converted substrate could be traced back. Yields varied between 55-88 mol %. A part of the carbon loss was explained by formation of biomass. As biomass could not be determined accurately however, this was not included in the carbon balance. A higher carbon recovery was found for higher initial concentrations of CO. This was assumed to result from measurement errors, where small deviations at lower concentrations have a bigger impact than small deviations at higher concentrations.

The gas and acetate productions were compared for more information on the metabolic activities concerning acetate production. For this, entry points E-H were assessed (Table 3), due to more reliable data on the H_2 production. As mentioned earlier, the amount of produced H_2 and CO_2 nearly equalled the amount of converted CO. This indicates that WGS predominantly occurred, and little subsequent conversions occurred. At the same time, 0.054-0.078 mmol acetate was produced. It was assumed that during WLP, 4 mol H_2 and 2 mol CO_2 are required for 1 mol acetate. Consequently, 0.216-0.312 mmol should have been converted to produce the amount of detected acetate. As this was not the case, the theory of background acetate production from yeast extract derived compounds was strengthened.

Summing up, several conclusions were drawn from these results. Firstly, CO was fully converted, disregarding the CO/H_2 ratio. Secondly, WGS reaction was found to be the major cell activity. Lastly, no conclusions could yet be drawn regarding acetate production. On the one hand a slight indication was given, that acetate production is more beneficial at increased CO/H_2 ratios. On the other hand, acetate partially resulted from other substrates than H_2 and CO_2, like yeast extract.

3.1.3.2 Results and discussion of effect of H_2 partial pressure on acetate production – part 2

Aim of the first experiment was to collect data during the exponential growth phase. As this did not succeed the experiment was conducted again, but with a different inoculation strategy. Moreover, it was only looked at initial concentrations of 10 and 30 % CO, thus 1.06 and 3.17 mmol CO, with 9.51 and 7.40 mmol H_2. Results of the acetate production are presented in Figure 7. With growth in H_2 conditions, final acetate concentrations were 0.35 ± 0.08 and 0.67 ± 0.05 mmol for 10 and 30 % initial CO. When N_2 was used in addition to the initially supplied 10 and 30 % CO, 0.33 ± 0.02 and 0.62 ± 0.04 mmol acetate were produced. 0.002 mmol acetate was added through the inoculum for each scenario.

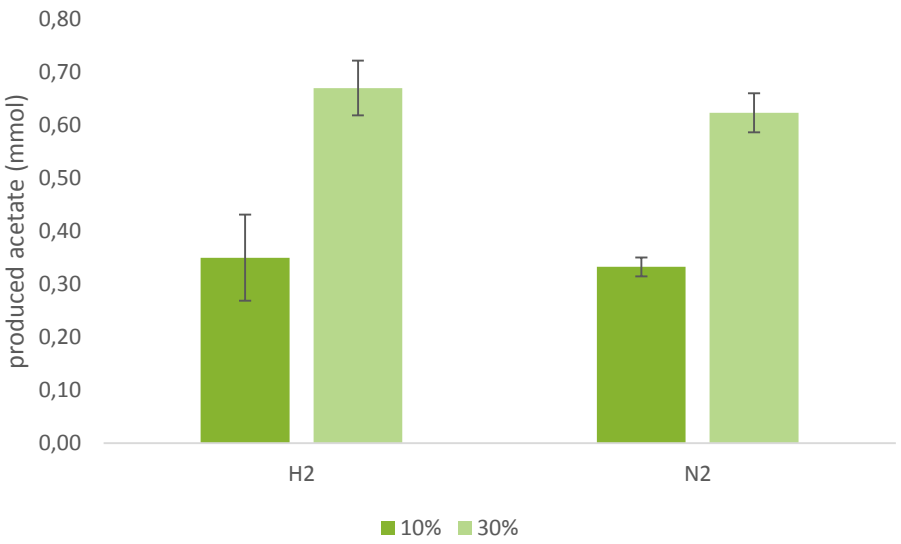

Figure 7 The effect H_2 partial pressure on acetate production. 10 or 30 % CO were supplied with either H_2 or N_2 to reach 100 % 10 % equals 1.06 and 30 % equals 3.17 mmol CO. Averages and standard deviations of triplicate experiments are shown.

Acetate production doubled from 10 to 30 % substrate in H_2, as well as N_2 growth. Unlike the previous experiment, this suggested that the acetate yield depends on the amount of initially supplied CO. Furthermore, similar average acetate productions were observed for equal substrate concentrations in H_2 or N_2 environments. However, considering that one of the triplicates from scenario B_r (Table 4) showed limited growth, the average of growth at 10 % CO and H_2 was expected to be higher, namely around 0.41 mmol. This indicated that acetate production increased at higher CO/H_2 ratios. Strikingly, final concentrations were five to ten times higher than in the previous experiments under low total pressure.

A summary of the gas concentrations and product ratios for insight on the metabolism can be seen in Table 4. It can be seen, that for all repeated scenarios the substrate was nearly fully converted with CO conversions of above 95 %. H_2 production showed deviant results for growth under H_2 or N_2. 1.87 ± 1.13 and 2.63 ± 0.54 mmol acetate were produced for 10 and 30 % CO at H_2, while 0.93 ± 0.84 and 2.51 ± 2.51 mmol were found for N_2. In all cases, H_2 production is higher than CO conversion. For CO_2, comparable amounts were produced for H_2 and N_2 conditions, with 1.5 mmol at 10 % and 3.0-3.2 mmol at 30 % substrate. Next, the CO_2/H_2 ratio is largely similar across the H_2 partial pressures. Acetate yield on converted CO was 72.5 ± 11.3 and 30.6 ± 2.8 mol % for 10 and 30 % initial CO and H_2. Respective yields at N_2 were 63.5 ± 4.2 and 23.7 ± 11.8 mol %. Finally, the carbon recovery was assessed, which showed values between 106.1 ± 5.2 and 130.3 ± 2.3 mol %.

Table 4 Summary of gas conversions, acetate production and product ratios for different H_2 partial pressures. Concentrations of entry points refer to initially added amount of CO. Either H_2 (A-B) or N_2 (C-D) were added for a total concentration of 11.57 mmol. In all cases, 1.06 mmol CO_2 were initially added on top of that. $_r$ stands for repeated.
Acetate yield from CO refers to converted CO. Average and standard deviations are based on triplicate experiments.

entry point (mmol CO)	**converted CO (mmol)**	**produced H_2 (mmol)**	**produced CO_2 (mmol)**	**CO_2/H_2 ratio**	**acetate/CO (mol %)**	**carbon recovery (mol %)**
B_r (1.06)	0.51 ± 0.01	1.87 ± 1.13	1.49 ± 0.04	1.25 ± 1.12	72.6 ± 11.3	108.5 ± 1.7
D_r (3.17)	2.01 ± 0.04	2.63 ± 0.54	3.19 ± 0.13	1.25 ± 0.30	30.6 ± 2.8	130.3 ± 2.3
F_r (1.06)	0.52 ± 0.01	0.93 ± 0.84	1.49 ± 0.14	1.59 ± 0.04	63.5 ± 4.2	106.1 ± 5.2
H_r (3.17)	1.98 ± 0.11	2.51 ± 0.21	3.00 ± 0.17	1.20 ± 0.04	23.7 ± 11.8	121.1 ± 10.9

Looking at the data, several observations can be made. Firstly, the CO consumption was high in all scenarios, but did not reach 100 %. In hindsight, cells were still active once the experiment was stopped. Based on the 100 % conversion rates from previous experiments, it was expected that at longer incubation, full CO conversion would have been achieved, too. Secondly, the H_2 and CO_2 productions exceed the CO conversion in all scenarios. Assuming, that H_2 and CO_2 solely result from WGS reaction this was unexpected. High H_2 productions for growth at background H_2 could be explained by inaccurate GC measurements. No explanation was found for a high H_2 production during growth with background N_2. In contrast, three explanations were considered for the high CO_2 production. First, it could have been caused by measurement errors. Due to the consistency in data, however, this was not regarded as likely. Next, CO_2 could have been a by-product of additional metabolic activities, which were undetected so far. Lastly, CO_2 could have dissociated from the liquid to the gas phase, to balance the pH at increasing acetate production. Based on the available data, this could not be further invested. Consequently, the CO_2/H_2 ratios were not considered reliable and not included in analysis. Thirdly, the acetate yield from converted CO was examined. It can clearly be seen that the yield increased at low CO concentrations, and higher H_2 partial pressures, supporting initial theories. However, no distinct values or ratios for an increased acetate production could be deduced from the available data. Lastly, the high carbon recovery was striking. Partially, this was caused by the inexplicably high CO_2 production. However, when recalculating the carbon recovery for a CO_2 production equal to CO consumption, the recovery still reached 80-110 %.

3.1.3.3 Evaluation of effect of H_2 partial pressure on acetate production at ambient pressure

Comparing the results of the two experiments, large inequalities were observed. Next, these were assessed and compared to find explanations, and to draw general conclusions on the influence of H_2 partial pressure on acetate production.

Firstly, the overall acetate production was five to ten times higher for the repeated experiment. Secondly, while the acetate production across the tested CO concentrations was similar in the first experiment, a doubled production occurred for the repeated one. Considering that the experiments were equal, except for the inoculation strategy, similar results were anticipated. As this was not true, the different inoculation strategies were suspected to influence the metabolism. The experiment was repeated to follow the exponential growth phase at different times. Hence, a large inoculum was used in the morning for the first trial, while little inoculum was used in the evening for the second one. For

full conversion, experiments were observed at least 24 h. Consequently, duration of the experiment and cell activity were longer for the second trial, with 44 compared to 27 h. When comparing the acetate productions over time, summarised in Appendix IV. Acetate production profiles, it can be seen that acetate production was still linear during the end of the first experiments, while it levelled off for the repeated ones. Therefore, cell activities might not have been terminated upon stopping the experiment, and more acetate would still be produced. However, previous experiments showed that upon CO depletion, which was nearly reached, cell activity stopped. Furthermore, acetate production largely occurred during the exponential phase of the repeated experiment. Consequently, no major increase was expected for the first experiment, explaining the large deviation.

No conclusions could be drawn on the impact of CO addition, due to the ambiguous results of the repeated experiments. While acetate production was similar for supplying CO in a range of 0.53 and 3.17 mmol during the first trial, a doubled production was observed when increasing CO threefold from 1.06 to 3.17 mmol. No explanation was found for the highly deviating acetate production. Overall, a slight trend is visible that at higher CO/H_2 ratios and higher H_2 partial pressures, acetate production increased. Due to the uncertain acetate generation, however, this could not be regarded as conclusion.

Summing up, the acetate productions greatly varied between the two trials at equal conditions. This could not be explained. Nevertheless, full CO conversion could be noted for all tested initial amounts and H_2 partial pressure in the anaerobic bottles. Therefore, high H_2 partial pressures were concluded to not limit CO conversion through the WGS reaction. Overall, a trend could be seen that acetate production increased slightly for higher CO_2/H_2 ratios.

3.1.4 Effect of increased initial CO pressure on acetate production

Next, the effect of increased initial CO pressures on the metabolism was investigated. For this, *C. hydrogenoformans* was grown in high-pressure batch reactors in 1.0 (A), 2.5 (B) and 4.0 (C) bar CO initially, with 1 bar N_2/CO_2 each. Note, that the used pressure sensor expresses pressure in overpressure, where 0 bar equals atmospheric pressure.

3.1.4.1 Results and discussion of growth at 1 bar CO

Figure 8 presents the pressure curves from 1 bar initial CO pressure. All results of the triplicate measurement are shown, which followed a similar profile. A steep increase from 1.2 to 1.6 bar can be seen during the first 4 h. Until 14, 15 and 23 h, respectively, the pressure increased very little. Afterwards, the pressure rose rapidly, first describing an exponential curve, then gradually transitioning towards a saturation curve. A constant pressure of 2.2 bar was reached after 30-45 h.

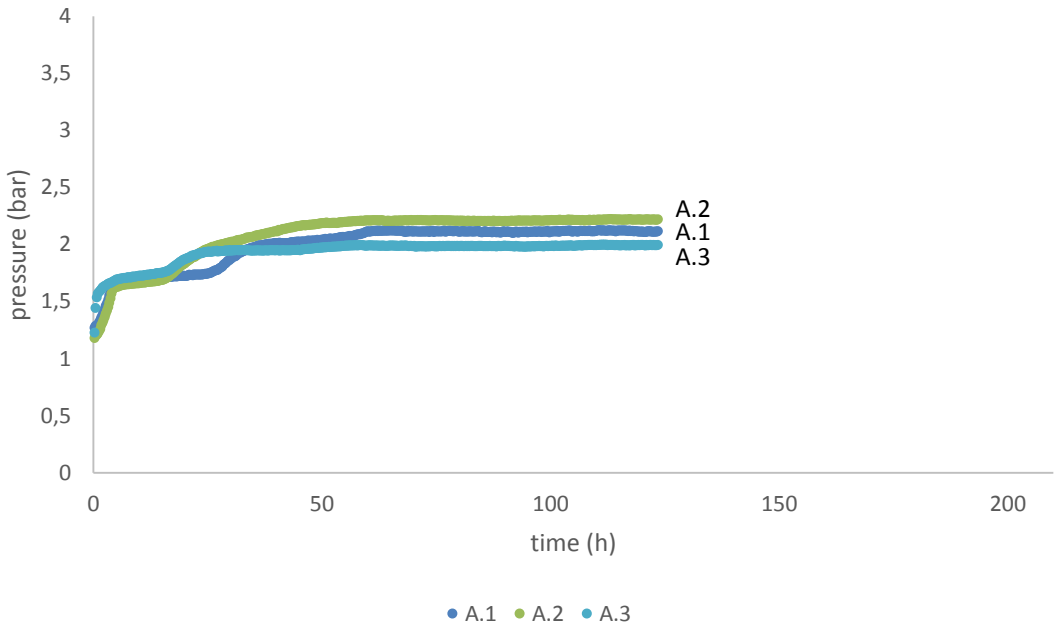

Figure 8 Pressure profiles of growth under 1 bar initial CO and 70 °C, which refers to A. A.1, A.2 and A.3 describe the individual triplicates.

This pattern can be explained through temperature changes and metabolic activities. As cells require higher temperatures, a ramp was introduced during the first 4 h, heating the system from ambient temperatures to the desired 70 °C. Following the ideal gas law, pressure increases upon increasing the temperature, causing the initial pressure rise. As explained previously, the organism generates energy through catalysing the WGS, which means 1 mol CO is converted into 1 mol of H_2 and 1 mol of CO_2 [16]. In other words, 1 mol gas is converted to 2 moles gas, resulting in a doubling of pressure. Seeing an initially slow increase in pressure, therefore resembles the lag phase, in which only limited cell activity occurred. Moreover, the exponential pressure increase indicates the exponential growth phase, which decreases towards a constant pressure at which cell activity stopped. During growth, the pressure increased from 1.6 with 0.6 bar, which equals 38 %. Assuming that this increase was merely caused by H_2 and CO_2 production, 38 % of the initial CO, namely 0.63 mmol, should have been converted.

For more insight into the metabolic activities, final gas and acetate concentrations were considered. Results are shown in Table 5. On average, 1.26 ± 0.17 mmol of the initially added 1.65 mmol CO was converted, which equals a substrate conversion of 76.6 ± 12.0 mol %. From this, 1.03 ± 0.05 mmol H_2 and 0.79 ± 0.09 mmol CO_2 were produced, where production entails final minus initial concentration. This translates to a CO_2 to H_2 ratio of 0.77 ± 0.07. Measured liquid products were 0.15 ± 0.03 mmol acetate, as well as 0.020 ± 0.002 mmol formate. The acetate yield from CO equals 11.9 ± 4.4 mol %, and the carbon balance completeness was 92.0 ± 8.4 mol %. Note, that the carbon balance did not include biomass production.

Table 5 Summary of gas conversions, acetate production and product ratios for 1 bar initial CO. Produced H_2 and CO_2 were based on final minus initial concentration. Acetate yield from CO refers to converted CO. Average and standard deviations are based on triplicate experiments.

	converted CO (mmol)	produced H_2 (mmol)	produced CO_2 (mmol)	final acetate (mmol)	CO_2/H_2 ratio	acetate/CO (mol %)
1 bar CO	1.26 ± 0.17	1.03 ± 0.05	0.79 ± 0.09	0.15 ± 0.03	0.77 ± 0.07	11.9 ± 4.4

From these results, several hypotheses over metabolic activity of *C. hydrogenoformans* were made. Firstly, CO conversion could not entirely be explained by WGS, as 1.26 mmol CO were converted, while merely 1.03 ± 0.05 and 0.79 ± 0.09 mmol H_2 and CO_2 were produced. On top of that, a conversion of merely 0.63 mmol CO was predicted based on the pressure increase. While part of this was expected to be caused by direct biomass production from CO, further conversions from H_2 and/ or CO_2 were suggested. Looking at the CO_2/H_2 production ratio of 0.77 ± 0.07, it can be concluded that either more H_2 was produced, or more CO_2 consumed. Considering that WGS is the main metabolic activity of *C. hydrogenoformans*, which would yield a ratio of 1:1, the latter option is regarded as more likely [16]. Assuming that CO was majorly converted through WGS, 1.26 mmol of both H_2 and CO_2 should have been formed. In that case, 0.23 and 0.47 mmol of H_2 and CO_2, respectively, were used for solvent production through the WLP. In all cases, acetate and formate were detected. The latter occurred at such small quantities, that they were not further considered. For acetate, the expected 4:2:1 ratio between H_2, CO_2 and acetate was assessed. Hence, 0.6 and 0.3 mmol H_2 and CO_2 should have been produced for the detected 0.15 mmol acetate. As a maximum H_2 conversion of 0.23 mmol was determined, only 0.05 mmol of the produced acetate can be explained by the WLP. Assuming that the previously found background amount of around 0.06 mmol acetate is always present, all acetate could be accounted for.

3.1.4.2 Proposed theory on metabolic activities and feasibility of acetate production

Based on these conclusions, a hypothesis was built regarding acetate production. Namely, that acetate production through the WLP was triggered, after the WGS reaction generated a certain $CO/H_2/CO_2$ ratio. At that point, the two pathways operated simultaneously, together determining the overall conversion rate. Looking at the slope of the exponential growth phase, shown in Figure 8, such a trend can be observed. While the pressure increased exponentially during initial growth, the slope quickly levelled off. This indicated that initially only the WGS reaction occurred. After the WLP was triggered, the produced gases were further used for solvent production, lowering the net pressure increase. After a while, the pressure reached a constant value, at which it remained until the experiment was terminated. Taking previous findings into account, this was most likely be caused by CO depletion. Furthermore, the WLP trigger was hypothesised to involve gas/liquid mass transfer rates and pathway limitations. In literature it was found, that at low cell densities, biomass growth is the limiting factor [16]. Only after a certain cell density is reached, CO diffusion into the liquid becomes rate limiting. Due to the high turnover number of the CODH complex, the effective CO concentration in the liquid gets close to zero during mass transfer limitation. On the other hand, CO is toxic for many organisms that employ the WLP, as CO inhibits necessary enzymes [32], [9]. From the moment that growth gets diffusion limited and the effective liquid CO concentration gets zero, the WLP is not inhibited anymore,

allowing acetate production. A schematic representation of the proposed acetate trigger can be found in Figure 9.

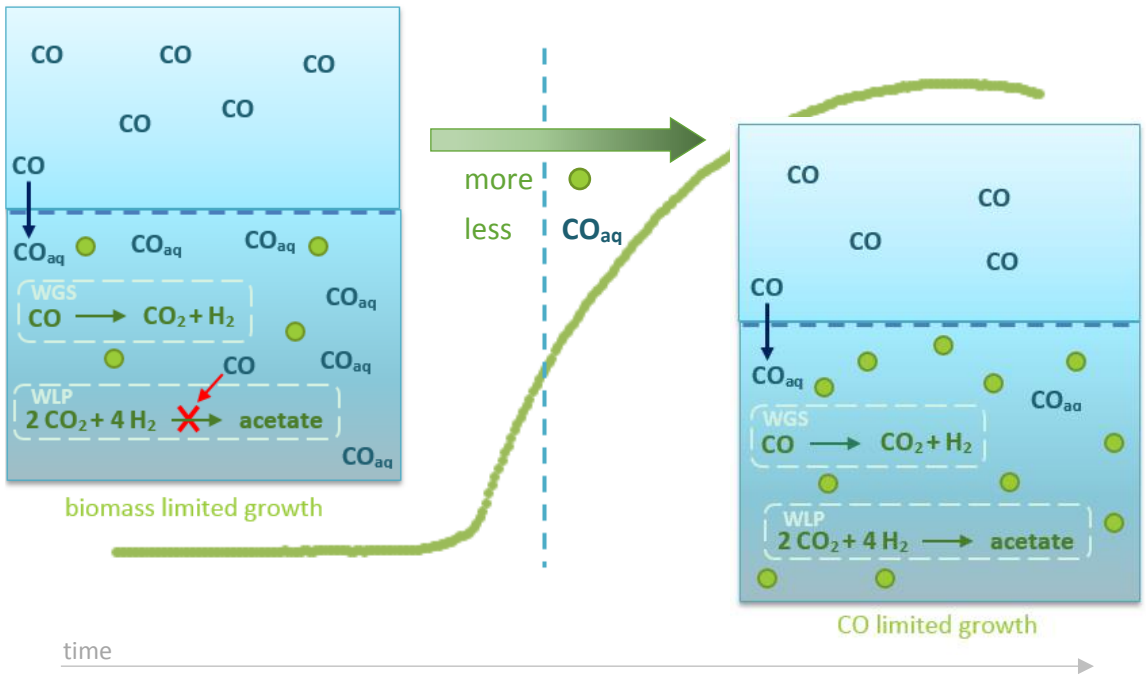

Figure 9 Schematic representation of proposed theory on acetate production trigger in C. hydrogenoformans. The graph indicates the change in pressure over time during high pressure incubation. Initially (left box), growth is biomass limited which results in WLP inhibition by dissolved CO. With increasing biomass (green dots), more dissolved CO is being converted, effectively lowering the concentration of dissolved CO. When this concentration is low enough to not inhibit the WLP anymore, acetate production becomes feasible (right box). Note, that the transition from biomass to CO limitation occurs gradually, meaning that the CO inhibition also decreases gradually. The dashed line merely indicates a point in time after which acetate production becomes feasible.

Summing up, several requirements for acetate production in *C. hydrogenoformans* were proposed. Firstly, liquid CO concentrations must be low enough to not cause inhibition of the WLP. Secondly, some CO must be present, as cell activity stops upon its depletion. Thirdly, a certain CO/H_2 ratio must be reached for the WLP to be feasible. Lastly, it was concluded that some acetate was always produced independent of the WLP. Most likely, this resulted from the yeast extract.

3.1.4.3 Results and discussion of growth at 2.5 bar CO

The pressure profiles for 2.5 bar initial CO are shown in Figure 10. Just like for the previous profiles, a steep pressure increase during the first 4 h can be seen, namely from 2.7 to 3.1 bar. After this increase, the pressures remained constant. Unlike for 1 bar, the triplicates of 2.5 bar show different results. The first triplicate, changed to an exponential increase after 26 h. Like the previous profiles, the slope levelled-off reaching a maximum pressure of 3.8 bar. Instead of remaining at the maximum values, the pressure declined again, nearly reaching the starting level of 3.1 bar. The resulting curve had a steeper increase than decrease. While the second triplicate did not show any change, the third one followed the same shape as the first one. As the pressure increase merely started after 37 h, it reached the plateau value just at the end of the experiment.

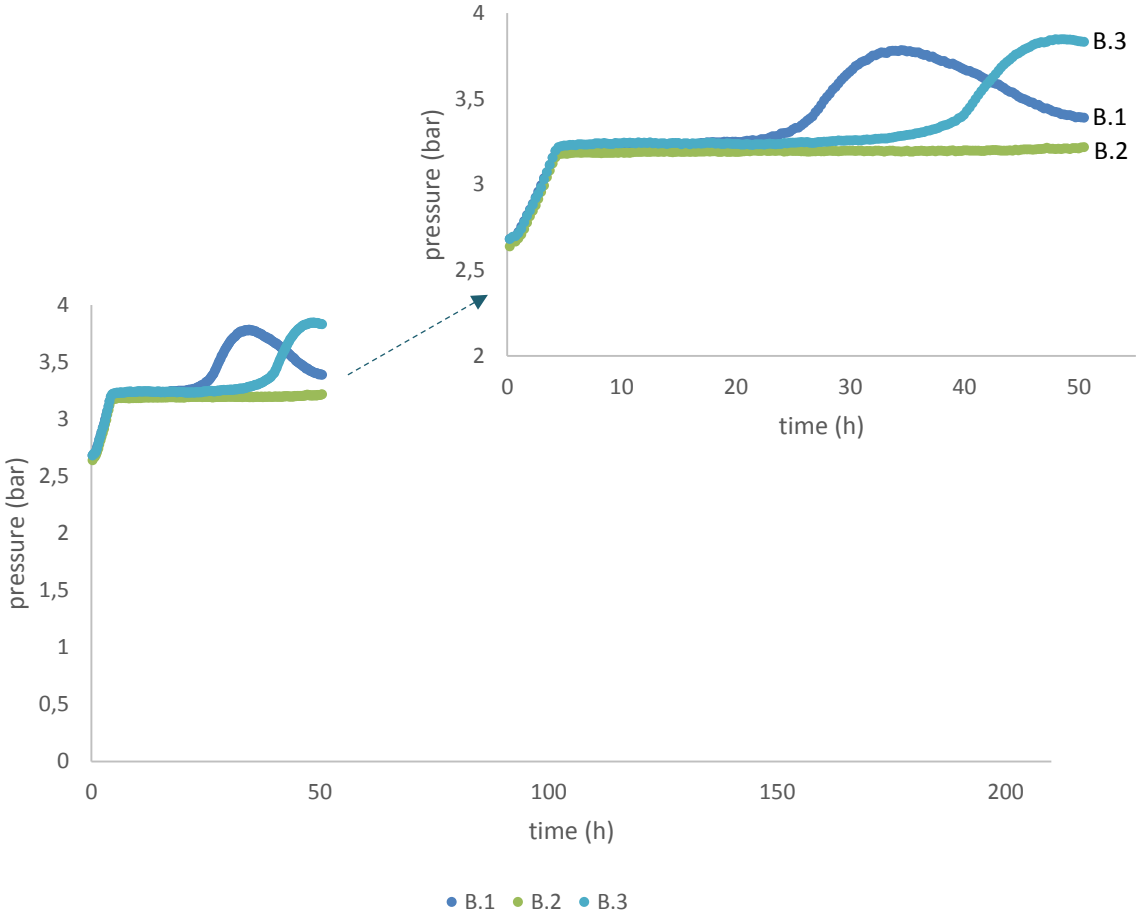

Figure 10 Pressure profiles of growth under 2.5 bar initial CO and 70 °C, which refers to B. B.1, B.2 and B.3 describe the individual triplicates. Upper graph shows the same results with adjusted axes.

Based on the theories explained for the 1 bar experiment, the 2.5 bar CO results were evaluated. The steep increase from 2.7 to 3.1 bar resulted from the temperature increase from 20 to 70 °C. The constant pressure from triplicate two, indicates non-active biomass. After variable lag-phases, cells grew exponentially in the remaining triplicates, following the same trend as for 1 bar CO. Surprisingly, the pressure did not remain constant, but decreased again for triplicate one, indicating that the WLP continued for a longer time. The pressure increased with a steeper slope than it declined, which is expected to be caused by the different rates of WGS and WLP over time. As the exponential phase of the third triplicate started later, cell growth could only be followed until the plateau was reached. Due to time constraints, the experiment could not be continued. However, it was believed that at longer incubation, the same pressure decline could have been overserved.

Again, final gas and acetate concentrations were investigated for more insight into metabolic activities. Due to the different activities among the triplicates, no average results were taken but data were considered individually (Table 6). In the first triplicate, 3.75 of the 4.2 mmol CO were converted, thus 89.1 %. Simultaneously, 1.33 and 1.91 mmol H_2 and CO_2 were produced, which results in a CO_2/H_2 ratio of 1.44. 0.51 mmol acetate were measured, with an acetate yield from CO of 13.5 mol %. The second triplicate merely converted 18.9 % CO, thus 0.77 mmol. For the last triplicate, a CO conversion of 2.69 mmol or 66.4 was measured. A CO_2/H_2 ratio of 0.93 was achieved with productions of 1.34 and

1.14 mmol. For all triplicates, around 0.017 ± 0.007 mmol formate was detected. The carbon balances were closed for 82.3, 90.1 and 80.2 mol %, respectively.

Table 6 Summary of gas conversions, acetate production and product ratios for 2.5 bar initial CO. Data from each triplicate are presented, where B.1, B.2 and B.3 refer to the individual triplicates. Acetate yield from CO refers to converted CO.
[a] Triplicate did not show any activity
[b] Triplicate was stopped at peak pressure

triplicate	converted CO (mmol)	produced H_2 (mmol)	produced CO_2 (mmol)	final acetate (mmol)	CO_2/H_2 ratio	acetate/CO (mol %)
B.1	3.75	1.33	1.91	0.51	1.44	13.5
B.2 [a]	0.77	0.07	0.13	0.10	1.76	13.0
B.3 [b]	2.69	1.44	1.34	0.23	0.93	8.7

These data support the previous assumptions about different metabolic activities and their interaction. While triplicate one was almost finished, hardly any activity could be seen in two. Cells were at the peak pressure in the last triplicate. Results from triplicate two were not included in this discussion as the experiment failed. Similar to the 1 bar CO metabolism, it was suggested that both WGS and WLP occurred during growth at 2.5 bar CO. For triplicate one, 3.75 mmol CO was converted, while a net production of 1.33 mmol H_2 and 1.91 mmol CO_2 was determined. Consequently, 2.43 and 1.84 mmol of H_2 and CO_2 were maximally converted through the WLP, which would account for 0.61 mmol acetate. The actual acetate production was 0.51, of which 0.06 were expected to result from background activities. A final acetate amount of 75 % of the maximum value was explained by biomass formation. This was in line with biomass yields on CO of 20-30 %, which were found in other experiments [14],[33]. CO was not fully converted for triplicate one. This can be seen back in the pressure profile of Figure 10, as it was not constant yet. The ratio of produced CO_2 and H_2 was 1.44. Expecting a ratio of 1 from the WGS reaction and a ratio of 2 from the WLP, the hypothesis of simultaneous operation was supported. Upon stopping the experiment, triplicate three just started to show a net gas reduction. This suggested, the rate of the WLP exceeded the rate of the WGS reaction. At that point, the CO consumption was equal to 2.69 mmol, whereas netto 1.44 and 1.34 mmol of H_2 and CO_2 were formed, respectively. Considering maximum possible H_2 and CO_2 productions of 1.25 and 1.35 mmol, this would translate to 0.31 mmol acetate. Consequently, the actual amount of 0.3 mmol acetate were accounted for. Moreover, the CO_2/H_2 ratio was close to 1, suggesting that the WGS was the overall more dominant pathway.

Summing up, the data support previously introduced hypotheses on acetate production. Additionally, it can be concluded, that the fraction between WGS reaction and WLP change over time, creating different patterns at different CO/H_2 ratios.

3.1.4.4 Results and discussion of growth at 4 bar CO

Finally, Figure 11 depicts the pressure profiles for 4 bar initial CO. Triplicates showed similar curves, indicating good reproducibility. After a steep increase in pressure from 4.1 to 4.8 bar during the first 4 h, the pressure remains constant until 50 h. Then, the pressure increases logarithmically to 7.3 bar at 100 h, after which it gradually decreases again. The decline occurs at a lower rate than the increase, reaching a final value of 4.7 bar after 210 h.

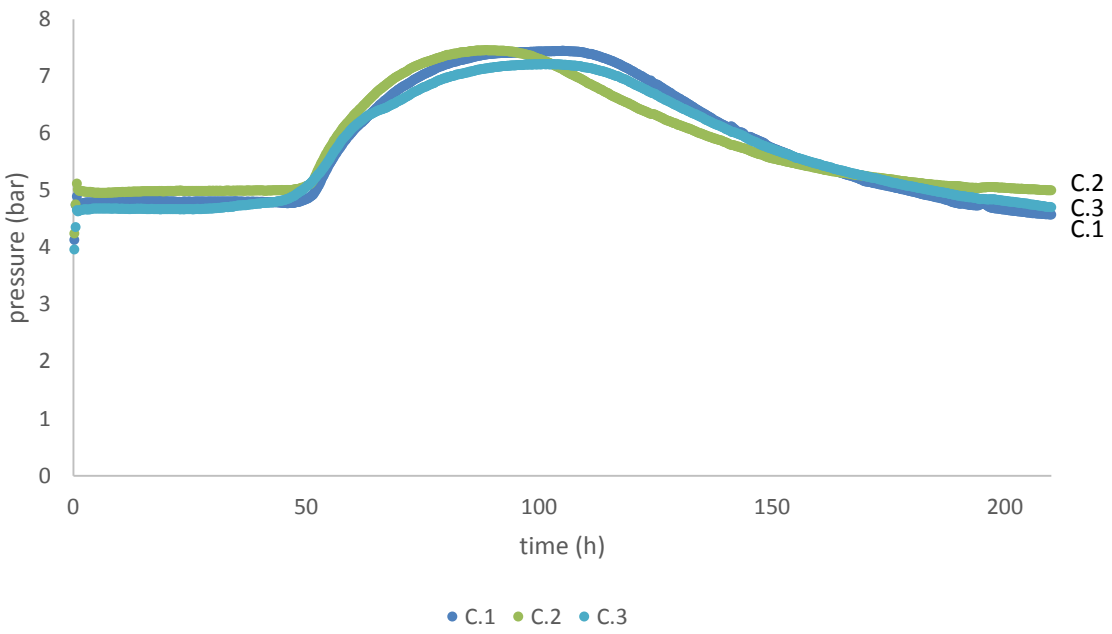

Figure 11 Pressure profiles of growth under 4 bar initial CO and 70 °C, which refers to C. C.1, C.2 and C.3 describe the individual triplicates.

In line with previous experiments, the initial increase resulted from heating the reactors to 70 °C. Afterwards, the same pattern could be observed for all triplicates. A lag-phase during which little cell activity was observed, followed by the growth phase. As observed for 2.5 bar CO, the pressure did not remain at the peak value, but declined again, suggesting formation of soluble products. Again, the decline occurred at a lower rate than the increase. Generally, slopes around the peak value were flatter at 4 bar, compared to 1 and 2.5 bar, indicating a more gradual transition between a net gas production and a net gas reduction. Together, this favours the theory that even through gases are being used for solvent production, the WGS still occurs.

Average gas and acetate concentrations from initially 4 bar CO are given in Table 7. 99.9 % CO was converted, with 6.58 ± 0.24 from the initially present 6.59 mmol. Also, a net H_2 production of 2.07 ± 0.08 mmol was measured, and a net CO_2 production of 3.32 ± 0.23 mmol, resulting in a CO_2/H_2 ratio of 1.60 ± 0.11. The acetate yield from CO was 14.7 ± 0.8 mol % as 0.97 ± 0.02 mmol acetate were present.

Table 7 Summary of gas conversions, acetate production and product ratios for 4 bar initial CO. Acetate yield from CO refers to converted CO. Average and standard deviations are based on triplicate experiments.

	converted CO (mmol)	**produced H_2 (mmol)**	**produced CO_2 (mmol)**	**final acetate (mmol)**	**CO_2/H_2 ratio**	**acetate/CO (mol %)**
4 bar CO	6.58 ± 0.24	2.07 ± 0.08	3.32 ± 0.23	0.97 ± 0.02	1.60 ± 0.11	14.7 ± 0.8

For all triplicates, full CO conversion was found. Considering that all CO was converted to H_2 and CO_2, maximum amounts of 4.51 mmol H_2 and 3.26 mmol CO_2 were used for acetate production in the WLP. Therefore, the maximum acetate production would be 1.13 mmol, which is close to the measured 0.97 ± 0.02 mmol. The CO_2/H_2 ratio was the highest one found, up to now, with 1.60 ± 0.11. This indicates that the WLP, relatively, occurred for a longer period than it did at lower substrate concentrations. This, together with the flattening pressure curves, supports the CO requirement of

C. hydrogenoformans. Furthermore, the acetate yield on converted CO was the highest one found until now. Therefore, it was suggested that more substrate was recovered in acetate at higher initial substrate concentrations. Note, that the maximum possible yield of acetate on CO is 25 % for the assumed reaction. Compared to the pressure profile of 2.5 bar CO, the slope at 4 bar CO changes more gradually, having much flatter slopes around the peak value. Possibly, this results from the higher substrate availability, at which the simultaneously occurring WGS reaction and WLP can interact with and complement each other.

3.1.4.5 Evaluation of growth at elevated CO partial pressures

From the high-pressure growth experiments with 1.0, 2.5 and 4.0 bar CO, several hypotheses could be made for the effect of substrate partial pressure on acetate production in *C. hydrogenoformans*. Besides the background production, acetate production through the WLP seemed to require a distinct CO range. Above this range, CO becomes toxic by inhibiting required enzymes, while at absence of CO, cell activity stops. Moreover, it was indicated that certain CO/H_2 ratios must be met for the WLP to be feasible. The assumed ratio of 4 mol H_2 and 2 mol CO_2 per mol acetate closely describes the observed gas conversions. Lastly, the acetate on CO yield increased for higher substrate availability.

3.1.5 Effect of H_2 partial pressure on acetate production– high pressure

Finally, the effect of different initial CO to H_2 ratios at high pressure was studied for their effect on the metabolism. For this, 1 and 9, as well as 4 and 6 bar of CO and H_2 respectively were used as substrate, with 1 bar N_2/CO_2 each. High pressure vessels were employed.

3.1.5.1 Results and discussion of growth at 1 bar CO with 9 bar H_2

The pressure profiles from initial gas concentrations of 1 bar CO and 9 bar H_2 can be seen in Figure 12. A steep increase in pressure during the first 4 hours could be observed. For the first triplicate, the pressure increased from 10.0 to 11.0 bar. For the other two, it rose from 10.7 to 11.6 bar. Afterwards, small differences could be noted. The first triplicate showed a slight increase in pressure until 28 h, after which pressure decreased first. Then, it increased more rapidly again until a maximum value of 11.5 bar at 51 h. Thereafter, pressure decreased at a slightly higher rate, to a final pressure of 10.9 bar after 91 h. For the second triplicate, a subtler increase was measured, with a peak pressure of 11.8 bar at 50 h. The increase was staggered. Afterwards, the pressure remained largely constant, with a very small decrease towards the end. The profile from the third triplicate followed a straight line.

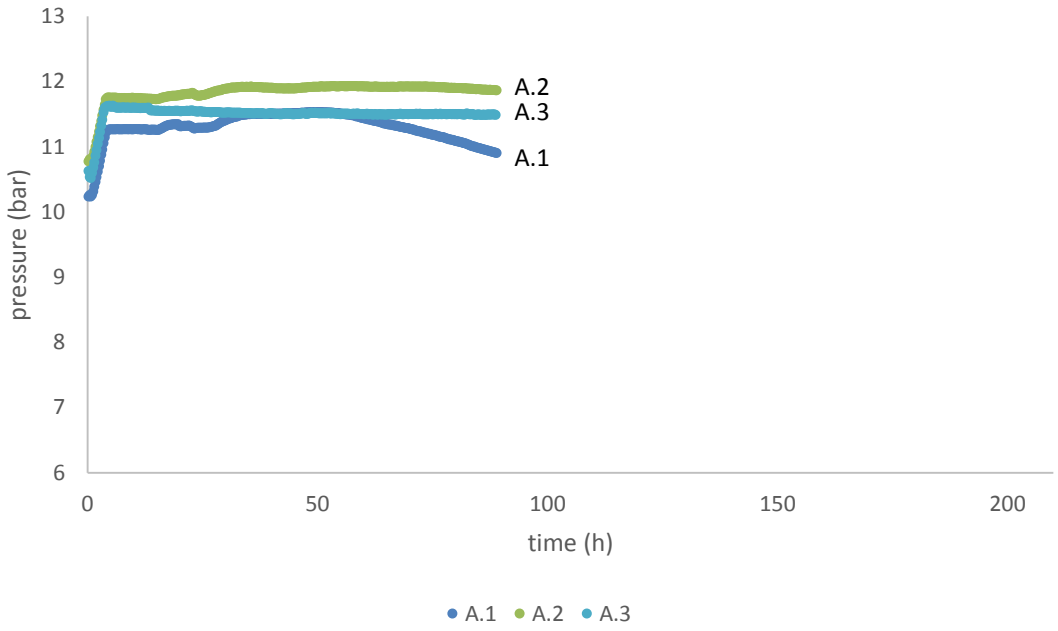

Figure 12 Pressure profiles of growth under initial pressures of 1 bar CO and 9 bar H_2 and 70 °C, which refers to A. A.1, A.2 and A.3 describe the individual triplicates. Note that A.3 failed.

As described in section 3.1.4.1, the steep increase during the first 4 hours was explained by ideal gas behaviour upon heating the reactors to 70 °C. For triplicate three, merely a straight line was seen, which was expected to come from non-active cells. Respective results were not considered in the discussion. For triplicate one and two, a small lag phase could be observed, before the pressure increased, indicating growth through the WGS reaction. However, the increase was not smooth. This could indicate that WGS did not occur at a constant rate, but interacted with the WLP. Based on the pressure decrease after 51 h in triplicate one, an active WLP was suggested. The final pressure was lower than the initial one, indicating that the final gas concentration was lower than the initial one. As the final slope was not zero yet, it was not possible to predict the final value. From the pressure profile of triplicate two, it could not be concluded whether the WLP was triggered.

For more information, final gas and product concentrations were investigated, which can be seen in Table 8. Due to slightly deviating results, it was chosen to present them individually and not to take an average. Either 1.59 or 1.65 mmol CO were converted, which equalled 100 % conversion. Net H_2 productions of 3.24 and 3.53 mmol were detected. For triplicate one, 1.03 mmol CO_2 was produced, resulting in a CO_2/H_2 ratio of 0.32, while respective values for triplicate two were 0.46 mmol and 0.13. The final acetate concentrations varied between 0.30 mmol for triplicate one and 0.17 mol for triplicate two. Consequently, acetate yields from CO were 18.7 and 10.2 mol %.

Table 8 Summary of gas conversions, acetate production and product ratios for 1 bar CO and 9 bar H_2. A.1 and A.2 refer to the individual triplicates. A.3 failed and was therefore not considered. Acetate yield from CO refers to converted CO.

triplicate	**converted CO (mmol)**	**converted H_2 (mmol)**	**produced CO_2 (mmol)**	**final acetate (mmol)**	**CO_2/H_2 ratio**	**acetate/CO (mol %)**
A.1	1.59	3.24	1.03	0.30	0.32	18.7
A.2	1.65	3.53	0.46	0.17	0.13	10.2

Comparing the results from Table 8, differences can be seen which are in line with the different pressure profiles from Figure 12. While the pressure remained constant for triplicate two, less acetate was produced in comparison to triplicate one, where a pressure decrease was observed. This suggests that the WLP was triggered to a higher extend in triplicate one. Moreover, high amounts of H_2 were converted in both triplicates, resulting in very low CO_2/H_2 ratios. As an equal conversion was found for triplicate three, where no activity was detected, and no explanation was found for such high conversions, it was assumed to result from experimental errors. Possible, H_2 partially vanished from the sampling bottles, as they were stored for a while before being analysed [34]. Even though little CO was initially present, compared to the amount of supplied H_2, CO was fully converted in both cases. This is in line with the substrate experiments from section 3.1.2. There it was shown, that CO could fully be converted through the WGS reaction, even though reactants were initially present at high amounts. Furthermore, as H_2 was initially present at high concentrations, favourable CO/H_2 ratios for WLP to occur were reached quicker than when only CO as initially present. CO_2 largely still had to be produced from the WGS reaction. Working at the border of whether a reaction was feasible and sufficient substrate was present or not, could explain the staggered pressure profile from Figure 12. Also, this might explain why one triplicate clearly showed activity of the WLP while the other did not. Small changes in gas transfer rates could have a big impact on the feasibility of one pathway over the other. Lastly, the acetate production was compared for 1 bar CO with and without adding H_2. While the former resulted in 0.15 ± 0.03 mmol acetate, the latter obtained an average production of 0.23 ± 0.09 mmol. This strengthens the hypothesis of an increased acetate production at higher H_2 partial pressures.

Summing up, growth at initially high H_2 pressure results in different growth profiles compared to supplying only CO. It seems that necessary CO/H_2 ratios for the WLP to occur were reached much quicker. Consequently, transitions between WGS reaction and the WLP are less smooth and a higher influence on each other was expected. Lastly, it could be concluded that even at high initial amounts of H_2, CO could fully be converted.

3.1.5.2 Results and discussion of growth at 4 bar CO with 6 bar H_2

In Figure 13 the pressure profiles of 4 bar initial CO with 6 bar H_2 are depicted. All triplicates follow a similar pattern. A steep increase from 10.5 to 11.4 bar was measured during the first 4 hours. During the entire experiment, the pressure of triplicate two was slightly lower than that of the remaining two. Between 4 and 21 h, the pressure kept constant. Then, a fast exponential increase until 12.7 bar at 41 h occurred, after which the pressure decreased slowly again. At 55 h, a pressure of 12.4 bar was reached, before the pressure started to increase again. Now, the increase happened much slower and followed a saturation curve. For triplicate one and three, a maximum value of 12.9 bar was reached after 94 h, and a steep decrease was observed from 113 h onwards. For triplicate two, the peak pressure was reached after 75 h, while a rapid decease started after 90 h. Towards the end, all triplicates levelled off to a pressure of 9.1 bar after 191 h.

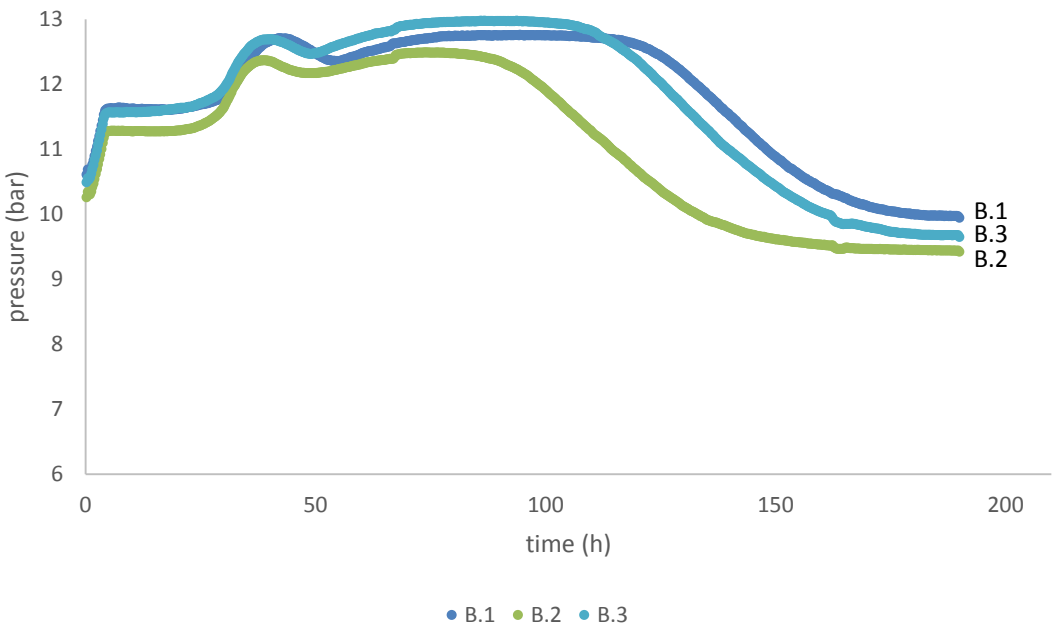

Figure 13 Pressure profiles of growth under initial pressures of 4 bar CO and 6 bar H_2 and 70 °C, which refers to B. B.1, B.2 and B.3 describe the individual triplicates.

From the patterns described in Figure 13, previously formulated theories on metabolic activities of *C. hydrogenoformans* during high pressure growth, were supported. The lower pressure of triplicate two was explained by a deviant sensor output, as the pressure at time zero should be the same and no leakages were observed. Again, the steep initial increase was explained by heating of the reactors. The pressure then remained constant as cells were adjusting during the lag phase. Transition towards the exponential phase occurred simultaneously for all triplicates. After the exponential growth, the pressure quickly reached a first peak value. While an indication for this peak value was given at the 1 bar CO with 9 bar H_2 experiment, it could now clearly be seen. Afterward, the triplicates followed the trend which was described for triplicate one under 1 bar CO and 9 bar H_2 conditions. For 4 bar CO and 6 bar H_2, the slopes were much smoother. Consequently, clear switches between the WGS reaction and the WLP could be indicated. Unlike experiments without background H_2, multiple shifts were observed.

Table 9 summarises the gas conversions and final product concentrations. 6.58 ± 0.14 mmol CO was converted, which equalled 100 %. Moreover, a net conversion of 1.90 ± 0.67 mmol H_2 and a net production of 4.10 ± 0.44 mmol CO_2 were measured. This resulted in a CO_2/H_2 ratio of 2.30 ± 0.86. A final amount of 1.43 ± 0.02 mmol acetate was determined, which leads to an acetate yield on CO of 21.5 ± 0.89 mol %.

Table 9 Summary of gas conversions, acetate production and product ratios for 4 bar CO and 6 bar H_2. Acetate yield from CO refers to converted CO. Average and standard deviations are based on triplicate experiments.

CO, H_2 (bar)	**converted CO (mmol)**	**converted H_2 (mmol)**	**produced CO_2 (mmol)**	**final acetate (mmol)**	**CO_2/H_2 ratio**	**acetate/CO (mol %)**
4,6	6.58 ± 0.14	1.90 ± 0.67	4.10 ± 0.44	1.43 ± 0.02	2.30 ± 0.86	21.5 ± 0.9

In all scenarios, CO was converted for 100 %. The standard deviation was caused from slightly variable starting concentrations. It becomes apparent that while high CO partial pressure experiments showed one transition from just the WGS reaction to a cooperation of the WGS reaction and the WLP, multiple transitions occurred when high H_2 partial pressures were added on top of the substrate. This indicates that multiple factors determine the feasibility of the WLP. Also, it seems that the organism operated close to the boundary where the WLP becomes feasible. The pattern can be explained based on the theory proposed in section 3.1.4.2. While during the initial exponential growth biomass density was limiting, gas/liquid mass transfer of CO became the rate limiting step once a certain biomass density was exceeded. Due to the high CO selectivity of the CODH complex, hardly any CO was dissolved in the liquid. Consequently, CO did not inhibit the WLP any longer and acetate production through the WLP could occur. Comparing the slope of the first peak with that of the 4 bar CO experiment (3.1.4.4), it was now much steeper and the plateau value was reached much quicker. This was explained by the high initial amount of H_2. While in previous cases H_2 first had to be produced through the WGS reaction and was present at lower partial pressures, it was now already abundantly present at the beginning. Hence, the initial rate of the WLP was much higher than what was noted previously. After 55 h, however, the pressure rose again, indicating that WLP had stopped and that WGS reaction occurred predominantly. This was explained by CO_2 limitation. Whereas H_2 was abundantly available upon WLP activation, much smaller amounts of CO_2 were supplied. Once these initial amounts were converted, the activity of the WLP fully dependent on the CO_2 production of the WGS reaction. This also explained the presence of a second peak. After initial amounts of CO_2 were depleted, CO_2/H_2 ratios were not sufficient anymore for the WLP. Instead, CO_2 first had to be generated again by the WGS reaction to exceed the required threshold. At the noted second pressure decrease in Figure 13, this was reached. Note that the decrease occurred much earlier in triplicate two. This was explained by working very close to the feasibility boundary of the WLP, where small deviations regarding gas concentration and mass transfer rates had a large impact. Towards the end of the experiment, all triplicates moved towards the same constant pressure. As CO was fully converted, this was explained by CO depletion. The highest acetate production among all experiments of this project was determined, with 1.43 ± 0.02 mmol. This corresponded to a yield of 21.5 ± 0.89 mol %. Compared to the 1 bar CO with 9 bar H_2 experiment, equal total amounts of H_2 were present. Showing a six times higher acetate production at 4 bar CO with 6 bar H_2 indicated that CO_2 availability also impacts the acetate production.

3.1.5.3 Evaluation of effect of H_2 partial pressure on metabolism at high pressures

From the high-pressure growth experiments at 1 bar CO with 9 bar H_2, and 4 bar CO with 6 bar H_2, previous theories on metabolism in *C. hydrogenoformans* could be supported and elaborated further. Firstly, full CO conversion through the WGS reaction was noted, even at initially high product concentrations. Secondly, metabolic activities stopped upon CO depletion. Furthermore, an increased availability of H_2 caused higher acetate production, however also resulted in CO_2 limitation. Hence, multiple transitions between the WGS reaction and the WLP were observed at high initial H_2 partial pressures.

For clarification on CO_2 and/ or CO limitation, it was proposed to investigate growth at initially high concentrations of both CO_2 and H_2. Moreover, it was planned to investigate growth at 2.5 bar CO with 7.5 bar CO for a complete data set. Due to time constraints, however, this was not possible.

3.1.6 Summary of microbial experiments

From the obtained data, several conclusions could be drawn on the acetate production through *C. hydrogenoformans.* Firstly, a good reproducibility was achieved in comparison to previous studies [14]. Secondly, a full CO conversion could be observed across all experiments, showing the effectiveness of the CODH complexes. Concerning acetate production, several impacts could be determined. The CO availability seemed to trigger the activity of the WLP, facilitating acetate production, as indicated in Figure 9. Only upon gas transfer limited growth of the organism, CO levels were low enough in the medium to not inhibit the WLP. However, once all CO was consumed through the WGS reaction, all activities, including the WLP stopped. This was expected to result from a lack of bifurcation enzyme complexes, which can generate high potential electrons without converting CO. Therefore, acetate production was only possible at a certain CO range. As acetate and CO data were only available from the end of the experiment, no quantification of such a ratio was possible. Furthermore, higher H_2 partial pressures were found to increase acetate yield on converted CO. This was explained by requiring 4 moles of H_2 per mol of acetate. At lower H_2 conditions, all H_2 must be produced through the WGS reaction, which happens at a 1:1 ratio with CO. Therefore, a maximum yield of 25 % acetate on CO can be achieved without initial H_2. Once H_2 was added, more H_2 was available for the WLP. When H_2 was added abundantly, CO_2 or CO became the limiting factors, as it could be seen in 3.5.1.2. Lastly, acetate was always produced in small background quantities. This was expected to result from conversion of yeast extract. Likely, this also caused the constant low quantity of formate.

This conclusion was merely partially in line with the initial theory, which was derived in section 1.2.1. On the one hand, a higher H_2 partial pressure resulted in an increased acetate yield as predicted. On the other hand, acetate production was not triggered by certain gas ratios, making acetate production thermodynamic more favourable than H_2 production. Instead, the trigger was expected to result from resolving CO inhibition of WLP enzymes. Consequently, it does not seem likely that the metabolism switches from H_2 to acetate production, as initially suspected. Instead, WGS and WLP seem to operate in parallel. More specifically the WGS is always active despite the present gas ratios, while the WLP first must be enabled.

3.1.6.1 Comparison of theory on acetate production with literature

As acetate production through *C. hydrogenoformans* was only observed recently, little literature was available for comparison of the proposed theories[11, 14]. In literature, *C. hydrogenoformans* was mainly described for obtaining pure H_2 gas from CO, or for gaining more insight into the fast and selective CODH complexes [31, 35, 36].

Henstra & Stams (2011) compared H_2 and acetate production in *C. hydrogenoformans* over time with and without a CO_2 trap. While elevated levels could be found without CO_2 removal, no acetate was detected during CO_2 removal. This supports the CO_2 requirement for acetate production. After 55 h

and nearly full conversion of initially added 17 mmol CO, 0.76 mmol acetate were found. This equals an acetate on CO yield of 4.5 %, which is in line with yields of 2-6 % for initial low-pressure experiments. Compared to high-pressure experiments, with yields between 13 and 21 %, the 4.5 % from literature was low. Hofstede (2017), investigated the effect of CO partial pressure and H_2/CO ratios on the metabolism of *C. hydrogenoformans.* Here, it was observed that H_2 and CO_2 were removed from the gas phase during batch incubation. This was explained by activation of the WLP, resulting in acetate production. Consequently, acetate was detected in several scenarios. However, no clear correlation between experimental conditions and acetate production could be determined. Additionally, the variability among duplicate experiments was very high. This could have been caused by the lack of stirring. As shown in the temperature experiment (3.1.1), not employing stirring largely impacts the gas transfer rates and thus metabolism. Due to the high variability and uncertainty, final yields were not directly compared. Hofstede explained the transition towards acetate production by low levels of CO, which were not sufficient for energy generation anymore. With the findings from this project, it was concluded that this transition was not likely to be caused by thermodynamic but by inhibitory reasons.

3.1.6.2 Conclusions for combined system

From the microbial experiments, several conclusions could be made for the combined system. Firstly, the maximum growth temperature was determined to be 70 °C. Therefore, the combined system must not be operated above this temperature. Secondly, the CO concentration determined the feasibility of acetate production. Consequently, CO should always be present in the combined system, but never above inhibitory values. As this is difficult to realise in a batch system, a continuous system should be investigated. Lastly, sufficient H_2 must be available for acetate production. When H_2 majorly results from CO conversion by the WGS reaction, and CO levels must be low, it might be necessary to supply H_2 externally.

3.2 Ethanol production through the ruthenium-based catalyst

For this section, the hydrogenation of acetate and acetic acid to ethanol was investigated using a 5 wt. % ruthenium catalyst supported on activated carbon (Ru/C). Experiments and predictions were based on findings from Olcay et al. (2010). They showed, that with a high activity and ethanol selectivity, the heterogeneous Ru/C catalyst had the highest potential for acetic acid hydrogenation to ethanol amongst tested transition metal catalysts. However, these results were based on reaction temperatures between 100-225 °C, a pH of 2.3 and H_2 pressure of 50 bar. In the proposed combined system, however, hydrogenation needs to be adapted to conditions which are suitable for *C. hydrogenoformans*. Thus, at temperatures around 70 °C, neutral pH and possibly lower pressures. Therefore, the catalytic performance of Ru/C at milder conditions was evaluated. The results are presented in this section.

3.2.1 The effect of using acetate vs. acetic acid as substrate

It is known, that a Ru/C catalyst converts acetic acid to ethanol [26], [37]. However, it has not been shown yet how its performance alters when operating at a higher pH, using acetate as a substrate. This was investigated by comparing hydrogenation at an initial pH of 2.2 and 6.5, for the respective product formation.

At the end of the hydrogenation, the ethanol, acetic acid and acetate contents were determined through HPLC. From this an indication about the catalytic activity was given. Results are summarised in Table 10. For pH 2.2 an acetic acid conversion of 1.65 ± 0.26 mmol was measured, with an ethanol production of 0.78 ± 0.09 mmol. Consequently, the ethanol selectivity was 47.4 %. Ethanol selectivity referred to ethanol production from converted substrate. Values refer to an operation time of 93 h. At pH 6.5, the substrate consisted of an acetate and acetic acid mixture. Of this substrate, 1.43 ± 1.27 mml were shown to be converted, while 0.11 ± 0.06 mmol ethanol were produced. This results in an ethanol selectivity of 7.4 %

Table 10 Amount of converted substrate, referring to acetic acid and acetate respectively, and produced ethanol, as well as the ethanol selectivity for hydrogenation at 70 °C. Experiment was run for 93 h. Average and standard deviation from triplicate experiments.

	converted substrate (mmol)	**produced ethanol (mmol)**	**ethanol selectivity (%)**
pH 2.2	1.65 ± 0.26	0.78 ± 0.09	47.4
pH 6.5	1.43 ± 1.27	0.11 ± 0.06	7.4

Consequently, ethanol production at low pH was nearly 8 times higher than at pH 6.5.

3.2.1.1 Closing the carbon balance of hydrogenation experiments

Table 10 shows that at similar conversions of substrate the ethanol selectivity was low. Having detected ethanol as the only product, the carbon balance was not closed, indicating a loss. Partially, this can be explained by the low accuracy at which the final substrate concentration could be determined. Therefore the exact amount of converted substrate was unknown. Beyond that, it indicated that other products were formed.

Based on literature, acetaldehyde, ethyl acetate, methane, ethane and CO_2 were likely by-products [26, 38, 39]. Especially ethyl acetate was expected to be found at lower conversion temperatures. Consequently, final gas and liquid samples were also analysed with gas chromatography. While no additional products could be detected in the gas phase, small amounts of the volatile ethyl acetate were found in the liquid. This, however, was not enough to explain the carbon loss. Finally, ^{1}H NMR was applied as a method to surely detect all present compounds. Instead of identifying additional products, the analysis could merely be used to confirm the presence of ethanol and ethyl acetate. Detailed results are given in Appendix V. NMR spectrum of hydrogenation products.

Having shown that the unaccounted carbon did not result from unidentified by-products, internal loss due to evaporation was regarded as the most likely explanation. More specifically, after evaporating at 70 °C, the reactor's cooling causes ethanol and ethyl acetate to condensate again, where part of the products flows into the small gap between the reactors and the applied liners. This phenomenon was previously described by Spekreijse (2016).

Neither being able to close the carbon balance, nor to measure the substrate concentration accurately, it was chosen to express the ethanol and ethyl acetate concentration in mmol normalised per day (mmol day^{-1}), instead of selectivity, for comparison of the results. Furthermore, it was concluded to analyse liquid samples with both HPLC and GC. Firstly, they showed a higher accuracy for either acetic acid or ethyl acetate. Secondly, it served as a double control for ethanol analysis.

3.2.1.2 Evaluating the effect of acetate and acetic acid on ethanol production

The adjusted ethanol and ethyl acetate results for pH 2.2 and pH 6.5 are presented in Figure 14. This shows an ethanol production of 0.19 ± 0.02 and 0.03 ± 0.02 mmol ethanol for either pH 2.2 or pH 6.5. Ethyl acetate was merely detected at pH 2.2 with 0.022 ± 0.001 mmol. In line with previous ratios, the ethanol conversion at the lower pH was seven times higher than at neutral pH. The ethanol to ethyl acetate ratio at low pH was 8:1.

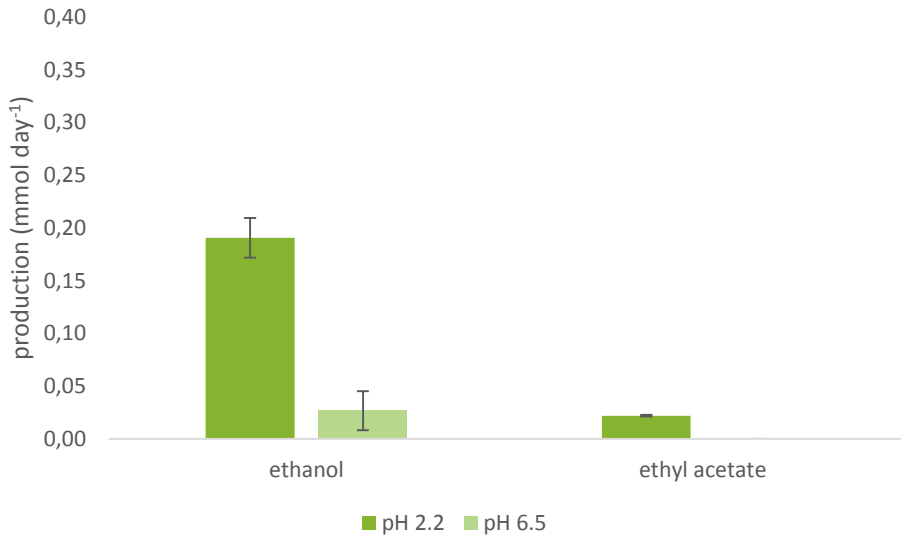

Figure 14 Ethanol and ethyl acetate production in mmol day^{-1} at 70 °C and an initial pH of 2.2 or 6.5. Catalyst was not reduced. Average and standard deviation of triplicate experiments.

The findings are only partially in line with the predictions. Firstly, the presence of ethanol and ethyl acetate for the conversion at lower temperatures was expected. Also, the partitioning between the products, with a clear preference for ethanol, was expected. The low conversion at a higher pH, however, was not anticipated. This assumption was based on results from Olcay et al. (2010), who support their findings with a density function theory study, exploring possible conversion routes from acetic acid to ethanol. There, acetate is mentioned as a possible first intermediate, involving initial dehydration. Working at a low pH, however, this would have to occur in a hydrogen-rich environment, which was regarded as unlikely. When operating at a higher pH, no infeasible initial conversion from acetic acid to acetate would be necessary, allowing the conversion from acetate to ethanol.

Instead, the catalyst shows limited activity at pH 6.5. Two possible explanations were proposed. Firstly, the ruthenium only reacts with the protonated acid form, such that the low conversion is caused by a low substrate concentration. Secondly, the affinity for the salt form is much lower, which means the reaction does occurs, but much slower. This was examined by the acid and salt partitioning at pH 2.2 and 6.5, which was determined according to Appendix VI. Calculating acetic acid – acetate partitioning at pH 6.5. Accordingly, merely 2 % of the substrate was present in the protonated form at pH 6.5, which equals 1.01 mmol. In total, 0.11 ± 0.06 mmol ethanol were produced, which is 10 % of the amount of present acetic acid. It is therefore likely to believe, that the ruthenium catalyst solely shows affinity for the acid form. This would be in line with Zhang et al. [41], who found that hydrogenation from lactate salts through a heterogeneous 5 wt. % Ru/C catalyst was not feasible. Instead, protonated lactic acid was required. Furthermore, no literature on hydrogenation of carboxylic acid salts through Ru/C could be found, supporting the conclusion that the catalyst merely binds the acid form.

Contrary to previous assumptions, hydrogenation was therefore concluded to solely result from low pH carboxylic acids, and not their salt form. Consequently, further experiments were performed with a 10 wt. % acetic acid solution, operating at pH 2.2.

3.2.2 Effect of temperature on hydrogenation

So far, the effect of temperature on ethanol production from acetic acid through Ru/C has merely been described for temperatures between 100-225 °C. As the feasible range of *C. hydrogenoformans* is around 70 °C, catalytic performance was evaluated for 70, 80 and 90 °C. To enable comparison of general productivity between literature and the obtained data, 100 and 150 °C were also tested.

As shown in Figure 15, the catalytic activity generally increased with increasing temperatures. While the production increased rapidly between 70 and 90 °C, with 0.31 ± 0.02, 0.69 ± 0.14 and 1.43 ± 0.11 mmol day^{-1} respectively, it merely increased to 1.63 ± 0.08 mmol day^{-1} at 100 °C. For 150 °C, production even decreased to 0.14 ± 0.02 mmol day^{-1}. A similar trend can be observed for ethyl acetate, with decreased production rates from 100 °C onwards. Ethanol to ethyl acetate ratios were equal between 70 and 90 °C, with 10:1, while they increased to 17:1 and 41:1 for 100 and 150 °C, respectively. In contrast to this, the amount of converted acetic acid greatly increased, as presented in Table 11, suggesting different catalytic activities for higher temperatures.

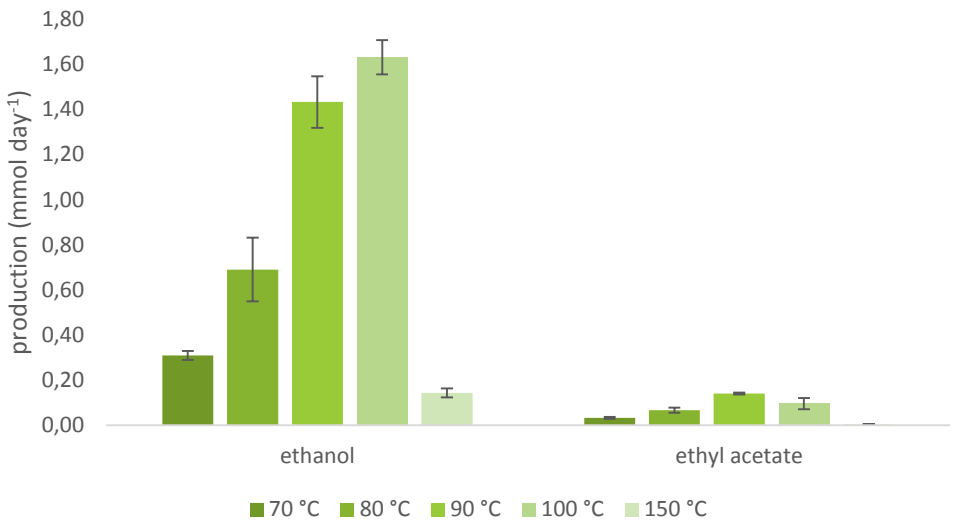

Figure 15 Ethanol and ethyl acetate production in mmol day^{-1} for the tested temperature range of 70, 80, 90,100 and 150 °C. Average and standard deviation of triplicate experiments.

On top of that, HPLC analysis showed a clear unknown peak at a retention time of 28.9 min of the RI spectrum with different areas at different temperatures. While nothing was detected at 70 °C, average peak areas of 1.35 ± 0.24, 1.49 ± 0.15, 0.78 ± 0.99 and 0.37 ± 0.42 $\mu_{RIU.}$ min was seen for 80, 90, 100 and 150 °C, respectively. Based on the expected production of short-chain carbon molecules, GC-MS was applied to identify the unknown peak. Next to ethanol and ethyl acetate, acetaldehyde was detected by this analysis. Being the intermediate from acetic acid to ethanol, small amounts of this compound were not surprising. While, acetaldehyde indeed could be found during HPLC analysis, it had a different retention time and thus did not cause the peak. Moreover, it was examined which compound would be expected at 28.9 min on the applied column. According to a document from BCT, summarising all retention times ever measured at 50 °C with the used BioRad column, 2 propanol would correspond to 28.9 min. However, no mechanism could be proposed, converting a C2 to a C3 molecule with the given substrates [42]. Lastly, acid catalyst aldol reactions could have caused additional by-product formation. However, due to time constraints, it was neither possible to investigate this option nor to identify the unknown compound.

Next to the liquid products, the gas composition was evaluated. All results, including carbon recovery are depicted in Table 11. It can be seen, that once ethanol and ethyl acetate production decreased, formation of gaseous products increased. Methane is first detected at 90 °C in a 3 times lower amount than ethanol, namely 0.53 mmol day^{-1}. For 100 °C, two out of three experiments did not show any methane, whereas one detected 0.44 mmol day^{-1}. Considering the trend amongst the temperatures of the remaining products, seeing no product was estimated to result from errors during sampling. Consequently, it was speculated tentatively to continue with the single result of 0.44 mmol day^{-1}, instead of taking an average. At 150 °C, 17.03 and 3.01 mmol day^{-1} of methane and ethane were produced, explaining the reduced ethanol and ethyl acetate production. Moreover, with increased gas production the carbon recovery increases, to nearly full accountability at 150 °C.

Table 11 Substrate conversion, and product production rates (mmol day^{-1}) for the tested temperature range, as well as degree of C balance closure. Average and standard deviation of triplicate experiments.
** instead of average, merely one value was taken*

temperature (°C)	**converted acetic acid (mmol day^{-1})**	**produced ethanol (mmol day^{-1})**	**produced ethyl acetate (mmol day^{-1})**	**produced methane (mmol day^{-1})**	**produced ethane (mmol day^{-1})**	**C balance closure (%)**
70	1.39 ± 0.78	0.31 ± 0.02	0.032 ± 0.004	0.00	0.00	26.9
80	1.37 ± 0.21	0.69 ± 0.14	0.066 ± 0.012	0.00	0.00	59.9
90	2.74 ± 0.12	1.43 ± 0.11	0.140 ± 0.004	0.53 ± 0.03	0.00	72.3
100	2.99 ± 0.68	1.63 ± 0.08	0.096 ± 0.024	0.44*	0.00	63.4
150	10.47 ± 0.04	0.14 ± 0.02	0.003 ± 0.002	17.03 ± 0.66	3.01 ± 0.17	97.3

These findings are not in line with results from Olcay et al. (2010). At 150 °C, they described an ethanol and alkane selectivity of 80 and 9 %. In this study, reversed selectivities of 1 and 96 % were found for 150 °C. This discrepancy is most likely caused by applying different systems. Whereas Olcay et al. (2010) used a system which immediately stripped ethanol from the system, this study operated in batch mode, where ethanol was further reduced to alkanes. Wan et al. (2012) assessed the effect of temperature on aqueous phase hydrogenation of acetic acid with Ru/C in batch reactors. At 48 bar H_2 and 150 °C, they obtained an ethanol selectivity of 45 %, with a methane and ethane selectivity of each 12 %. At 300 °C, high amounts of methane and CO_2, as well as trace amounts of ethane were found, while no ethanol was detected anymore. Even though these results are more in line with those obtained during this project, there are still some deviations. A possible explanation could be the difference in reaction times, as Wan et al. (2012) merely operated for 1 h, while this experiment was run for 50-64 h. A sufficiently short enough reaction time is thus expected to have a similar effect as stripping the ethanol. Therefore, it can be concluded, that choosing a suitable system and operation mode for the type of products one wants to obtain, is very important.

Summing up, hydrogenation of acetic acid to ethanol with Ru/C at lower temperatures was in line with predictions. Even though the conversion rate was lower than at temperatures around 100 °C, resulting in a lower ethanol production, the selectivity was high. Showing only little gas production, with reasonable ethanol yields, an operating temperature of 90 °C would be favoured. However, as the organism did not grow at that temperature, it was chosen to perform catalytic experiments at 70 °C.

3.2.3 The effect of H_2 partial pressure on hydrogenation

Varying the initial H_2 partial pressure, and thus the total pressure of the reaction was investigated next. For this, the initial pressure was decreased from 50 to 40 and 30 bar H_2 at 70 °C.

Results are depicted in Figure 16. For 30, 40 and 50 bar, ethanol productions of 0.31 ± 0.02, 0.24 ± 0.02 and 0.31 ± 0.02 mmol day^{-1} were measured. Respective ethyl acetate productions of 0.037 ± 0.002, 0.015 ± 0.003 and 0.032 ± 0.004 mmol day^{-1} were achieved. Accordingly, the ethanol to ethyl acetate ratios were 8.4:1.0, 16.3:1.0 and 9.7:1.0. For neither of the H_2 partial pressures, gaseous products were detected. However, the in section 3.2.2 introduced unidentified peak at 28.9 min emerged for 40 and 30 bar with average peak areas of 0.19 ± 0.2 and 3.06 ± 0.64 $\mu_{RIU.}$ min.

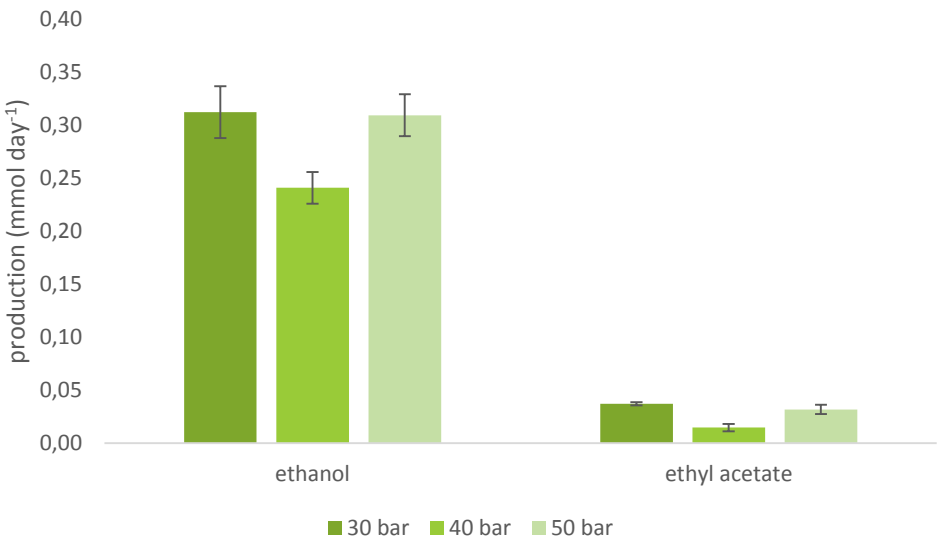

Figure 16 Ethanol and ethyl acetate production at 70 °C for the tested pressure range. Average and standard deviation of triplicate experiments.

While production slightly decreased at 40 bar H_2, compared to 50 bar, similar production rates were observed at 30 bar. This result was not expected. Following Henry's law, where less dissolved H_2 is present for catalysis at lower partial H_2 pressures, it was expected to observe decreased production rates at lower H_2 pressures [44]. Furthermore, literature indicated that hydrogenation using Ru/C at 200 °C with 30, 48 and 89 bar H_2 pressure, product selectivies remained constant [43]. As the results were based on triplicate experiments and two different analysis, deviations were assumed to not result from experimental errors. A possible explanation for higher conversion rates at 30 compared to 40 bar could be a higher availability of active ruthenium sites. In other words, at a high partial pressure of H_2, too much gas is dissolved in the liquid phase which blocks the catalytic sites and thus lowers conversion rates [37]. It has been shown, that the applied partial pressure influences the production formation, showing a lower ethanol selectivity at lower H_2 pressures [45]. This theory could be supported by the appearance of the unidentified compound, showing higher surface areas at lower partial pressures.

Even though the causes of the results could not fully be explained, it can be concluded that conversion from acetic acid to ethanol with the chosen Ru/C catalyst at 70 °C is feasible at lower pressures. Based on the available results, it was suggested to also evaluate performance for 10 and 20 bar H_2. However, due to time constraints this was not possible. Instead, this is proposed to be done in future research.

For the combined system, lower pressures are beneficial as they enable easier operation and lower operational costs. As production at the lowest tested pressure yielded similar production rates, a partial pressure of 30 bar H_2 was proposed for the combined system.

3.2.4 Investigation of conversion rate

The duration among experiments slightly differed. Therefore, results are compared based on daily production, assuming the conversion rate does not change significantly over time. This assumption was tested by comparing total and daily ethanol production of three experiments which were performed at the same conditions, but had different durations.

All experiments were operated at 70 °C, with acetic acid as the substrate and unreduced catalyst. While 0.19 mmol ethanol were produced after 21.1 h, 0.67 mmol ethanol were detected after 63.7 h, and 0.78 mmol ethanol after 93.0 h (Figure 17). This equals acetate production rates of 0.22 ± 0.03, 0.25 ± 0.05 and 0.20 ± 0.02 mmol day^{-1}.

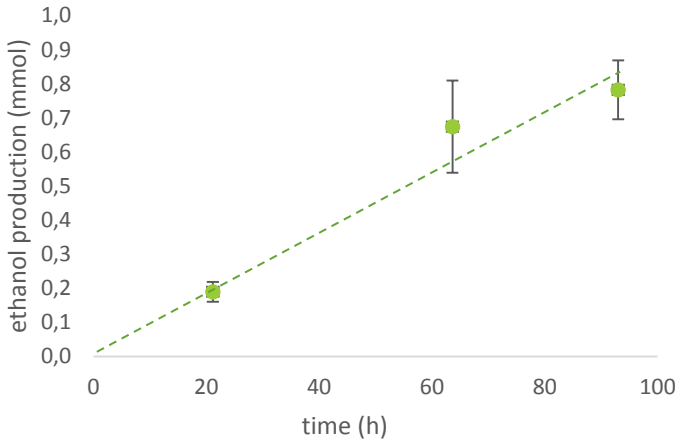

Figure 17 Final ethanol concentration from experiments with different durations, all operated at 70 °C. Average and standard deviation of triplicate experiments. Dashed line approximates linear ratio between produced ethanol and time.

Taking into account that the larger production after 63.7 h results from one deviant measurement, the assumption of a constant conversion rate over time was expected to be valid. Especially when operating below 100 °C where the substrate conversion remains below 20 %. Observing a constant conversion rate is in line with literature. Chen et al. (2007) found linear conversion rates over time for the hydrogenation of lactic propionic acid through Ru/C. They operated at 130 °C and 70 bar H_2 pressure. Consequently, normalising the obtained conversion rates per day was concluded to be valid.

3.2.5 Effect of catalyst reduction prior to hydrogenation

The employed catalyst had been exposed to surrounding air for a while. This may have caused oxidation of the ruthenium and thus partial inactivation of catalytic sites [47]. To investigate the degree of inactivation, the catalytic performance was compared for reduced and unreduced Ru/C.

As shown in Figure 18, the reduced catalyst yielded 0.31 ± 0.02 mmol day^{-1} ethanol and 0.032 ± 0.004 mmol day^{-1} ethyl acetate. Respective productions through the non-reduced catalyst were 0.25 ± 0.05 and 0.026 ± 0.04 mmol day^{-1}, respectively. In both cases, the ethanol to ethyl acetate ratio was 10:1.

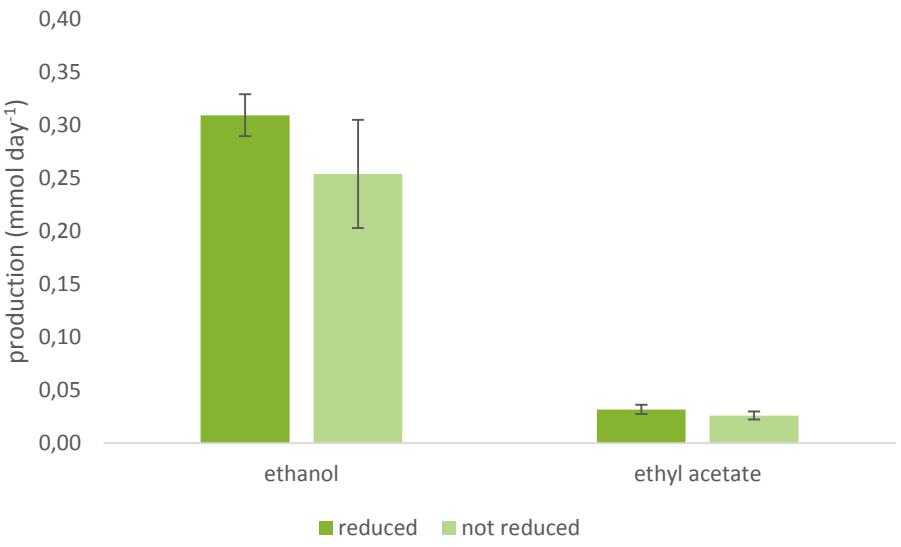

Figure 18 Ethanol and ethyl acetate production for reduced and unreduced Ru/C catalyst. Average and standard deviation of triplicate experiments.

From Figure 18 it can be seen that product formation slightly increased and greatly stabilised upon reduction of the catalyst. While the average ethanol production increased with around 20 %, the standard deviation decreased from 20.1 to 6.4 % after reduction. The partitioning between ethanol and ethyl acetate remained constant. This was explained by activation of deactivated catalytic sites during reduction. By removing deactivating oxygen groups, the amount of active sites was uniformed, stabilising the amount of possible conversions.

As little oxidation of the metal catalyst was expected during storage, a 20 % increase in activity after reduction was higher than anticipated [48]. Nevertheless, reduction of the Ru/C catalyst prior to hydrogenation of carboxylic acids largely occurred in literature [41, 49]. Due to the stabilisation of active catalytic sites and the resulting stabilised ethanol production it was chosen to always reduce the catalyst.

3.2.6 Evaluation of the chosen catalyst for the proposed system

In order to assess the catalyst choice for the proposed system, the combined data were first compared to literature, and then evaluated for the microbial requirements. Generally, ruthenium was found to yield the highest selectivity and activity for aqueous-phase hydrogenation of carboxylic acids to respective alcohols [37]. However, experiments were mainly performed at elevated pressures equal to or higher than 40 bar H_2, as well as elevated temperatures between 150 and 300 °C [43, 49]. Overall, the conclusions are in line with the ones obtained in this project. Firstly, hydrogenation occurs from carboxylic acids and not their salt counterparts. Secondly, at lower temperatures the conversion rates are lower, but the ethanol specificity increases. Thirdly, reducing the catalyst is preferred. However, the effect of H_2 partial pressure shows deviating results. Therefore, further investigation of this was proposed.

Next to Ru/C, different metal catalysts were proposed in literature for the hydrogenation from acetic acid towards ethanol. Just like Ru/C, they involve high temperatures and pressures. Zhang et al. (2013)

investigated Pt-Sn/alumnia catalysts between 195-275 °C and 10-50 bar H_2. Rachmady & Vannice (2000) worked with Pt catalysts between 149-192 °C and Wang et al. (2014) examined Ir catalysts at 100 °C and 60 bar H_2. Generally, similar effects were observed across the applied catalysts. Higher temperatures resulted in increased acetic acid conversion but lower ethanol selectivity. Higher pressures also increase the conversion.

Based on the catalytic results, the following conclusions were made for conditions of the combined system. Firstly, hydrogenation required acetic conditions, which contradicts with the neutral conditions necessary for the organism. Secondly, the catalytic temperature was lowered to the organism's optimum of 70 °C. This resulted in a decreased overall activity but increased ethanol specificity. Lastly, hydrogenation was shown to be feasible at lower pressures, up to 30 bar H_2. However, optimal H_2 pressure should be further investigated.

3.3 Evaluation of combined biochemically system

As a last part of this project, the proposed biochemically combined system was investigated. However, no data could be obtained as no cell activities were visible during an incubation of >100 h. Several observations, explanations and bottlenecks could be determined which will be explained next.

3.3.1 Evaluation of failure of combined system

The most likely explanation for not having observed cell activity in the combined system, was the lack of sulfides. Previous experiments by Torres (2018) indicated that growth of *C. hydrogenoformans* was possible without the presence of sulfides, provided that no O_2 entered the system. Due to the coloured pink medium, O_2 was expected to have entered the system however. As the reducing agent for O_2 removal lacked, undesired aerobic conditions were present for the strictly anaerobic organism [11]. Inhibitory effects on metabolic activities of *C. hydrogenoformans* by the Ru/C catalyst were not expected, based on experiments performed by Torres (2018).

On the other hand, deactivation of the Ru/C catalyst was expected to be caused by medium components [22]. As sulfides were found to majorly deactivate ruthenium catalysts, the combined system was operated without sulfides [52]. The effect of other medium compounds on hydrogenation by Ru/C could not be determined, as no acetate was produced in the first place. Therefore, it could not be concluded whether the lack of catalytic activity was caused by a lack of substrate, deactivation or too low H_2 partial pressures.

3.3.2 Summary of bottlenecks of the combined system

Even though little conclusions could be drawn from the actual combined system, information on its limitations was obtained from the individual experiments. Firstly, the initially described bottleneck was the temperature gap between microbial and catalytic conversions. To evaluate this, the individual production rates ($mmol_{product}$ day^{-1}) were compared for 70 °C. For the organism, two scenarios were considered. In case of an initial amount of 4 bar CO, 0.11 mmol acetate were produced per day. When 6 bar H_2 were added to the 4 bar CO, the acetate production was 0.18 mmol day^{-1}. Operating at 70 °C, pH 2.2 and a reduced catalyst, the ethanol conversion from acetate was equal to 0.31 mmol day^{-1}. Consequently, the conversion rates were in the same order of magnitude. Lowering the catalytic temperature to 70 °C, was thus not regarded as a bottleneck of the combined system.

More importantly, the pH difference between acetate and acetic acid was discovered to be one of the major bottlenecks of operating in one compartment. While the organism required neutral pH conditions for growth, the catalyst was shown to only bind to the acid form [12]. A catalytic rate of merely 0.03 mmol day^{-1} for Ru/C at 70 °C, pH 6.5 and unreduced catalyst was found, which is much lower than the microbial conversion rate. Furthermore, the catalyst was prone to deactivation by medium components [53]. While severe deactivation of sulfides was known, the impact of individual compounds was not yet investigated. For final conclusions of the impact of the growth medium described in Appendix III. CP Medium composition and preparation (MIB protocol), it is referred to studies from Torres (2018). Finally, the influence of pressure was investigated. Hydrogenation was shown to be feasible at H_2 partial pressures of 30 bar. Microbial activity was tested at a maximum pressure of 10 bar, with 4 bar CO. Based on previous studies, growth at higher pressures was expected

to be feasible [14]. It was aimed to grow *C. hydrogenoformans* at pressure up to 50 bar, with at least 30 bar H_2. However, this was not feasible due to time restrictions. However, obtained results from variable H_2 partial pressures suggest that high H_2 amounts do not inhibit growth or acetate production, but evoke limitation of other gases.

4 Conclusion

A novel system was proposed which combines fermentation with *in situ* heterogeneous catalysis, to produce bio-based platform chemicals from syngas. For the chosen model system, the extremophile *Carboxydothermus hydrogenoformans* was employed to convert syngas to acetate, which was then hydrogenated to ethanol by a heterogeneous ruthenium-based catalyst, supported on activated carbon. Feasibility was assessed by first examining each part separately, to determine suitable operating ranges for the combined system. While the influence of H_2 partial pressure was the main parameter of interest, impacts of pH, temperature and total pressure were also considered. Finally, the combined system was tested experimentally.

While little prior knowledge was available on acetate production through *C. hydrogenoformans*, it was found that its production was determined by the combined effect of H_2 partial pressure and availability of soluble CO. At liquid CO rates above diffusion limitation the acetate-yielding Wood Ljungdahl pathway was inhibited and thus inactive. On the other hand, metabolic activity stopped entirely upon CO depletion. This was suspected to result from the lack of bifurcating enzyme complexes. Moreover, higher H_2 partial pressures were found to increase the acetate yield on CO. A yield of 14.7 ± 0.8 % was found for an initial amount of 4 bar CO, while 21.5 ± 0.89 % were found when supplying 4 bar CO with 6 bar H_2. Thus, with an initial amount of 6.58 ± 0.19 mmol CO, 0.97 ± 0.02 and 1.43 ± 0.02 mmol acetate were produced respectively.

Catalytic activity and ethanol selectivity were analysed for conditions that are suitable for the extremophile. While the organism produced acetate at neutral pH, the catalyst was found to merely show selectivity for acetic acid, indicating that a low pH environment is required. Although production occurred at low conversion rates, high ethanol selectivities were observed at the proposed 70 °C. With a rate of 0.037 ± 0.002 mmol day^{-1} ethanol at 30 bar H_2, the product yield was similar to the 0.032 ± 0.004 mmol day^{-1} at 50 bar H_2. No activity was detected for the combined system, which was concluded to mainly be limited by deviant pH requirements and deactivation by medium components.

Functioning as a proof of principle, this project showed that microbial and catalytic conversion could occur in similar temperature and pressure ranges. pH and medium compounds were determined as major bottlenecks. For further research on the proposed combined system, it was therefore suggested to investigate system designs that approach these limitations.

5 Recommendations

Lastly, several recommendations were proposed for a possible continuation of this project. This entails further investigation of the individual parts, especially the microbial part. While this project merely showed that acetate production occurs at a certain $H_2/CO/CO_2$ ratio, methods were suggested to quantify this ratio for an optimal acetate production. Firstly, it was suggested to investigate the effect of an initially high concentration of both H_2 and CO_2 in the high pressure batch reactors. This could further specify the substrate requirement and respective bottlenecks. By including pH measurements, the final CO_2 concentrations can be determined more accurately. Secondly, current microbial experiments were performed in batch reactors without the possibility of intermediate gas or liquid sampling. Consequently, variable $H_2/CO/CO_2$ ratios existed throughout the entire experiment, which could not be determined. When operating in a continuous system, distinct $H_2/CO/CO_2$ ratios can be investigated for their effect on acetate production. A continuous system was therefore suggested to quantify the $H_2/CO/CO_2$ ratio. Additionally, a continuous system can be used to further investigate the CO requirements. By maintaining CO at constant desired levels for a longer period of time, the minimal and maximum CO values can be quantified for acetate production. Thirdly, it is proposed to examine microbial activity for higher total and partial pressures. More specifically, pressure requirements of the catalyst should be investigated, thus around 30-50 bar H_2. Furthermore, the growth medium should be explored. The medium contains many compounds which can inhibit or deactivate the Ru/C catalyst. To reduce this impact, it is suggested to test how much of the medium components can be removed without affecting growth. Lastly, it was proposed to perform a proteomics study. This could be used to gain more insight into the exact metabolic activities and rates during different growth phases. Consequently, proteomics can assess the proposed theory on acetate production.

Furthermore, some recommendations were suggested for the hydrogenation. Firstly, it should be investigated which compounds of the medium inhibit or deactivate the catalysis. By comparing these to the essential compounds for the organism, the bottleneck of the combined system can be defined and addressed closely. Secondly, the effect of H_2 partial pressure should be further investigated. While it was shown that conversion at 30 bar H_2 is feasible, results were ambiguous in comparison to literature. It was suggested to repeat the 40 and 30 bar H_2 experiment, as well as investigating 20 and 10 bar H_2. The latter one could determine the minimally required H_2 partial pressure required for the catalyst and thus the combined system.

Next to improvements of the individual systems, recommendations for the combined system were also given. Ultimately it is desired to operate the system at optimal gas ratios for acetate production with constant *in situ* hydrogenation to ethanol. In line with the microbial part, it was therefore advised to assess the combined system in a continuous reactor allowing intermediate gas and liquid sampling. Secondly, distinct systems should be designed and investigated, which address the identified pH and medium bottlenecks. Moreover, the investigated acetate and ethanol producing system was merely a simple model system for this proof of principle study. Ultimately, it is desired to produce higher-quality or longer-chain platform chemicals. Lastly, it was proposed to further investigate the performance of *C. hydrogenoformans* regarding CO clean-up of syngas. Having the potential to produce highly pure H_2 gas opens interesting possibilities in the H_2 fuel cell industry.

6 Acknowledgement

I would like to express my sincere gratitude to all the people who contributed to this thesis. Firstly, to Elinor Scott and Diana Machado de Sousa for coming up with and realising this project. Your input, guidance and feedback were very much appreciated and helped me immensely during the course of this thesis. Secondly, I would like to acknowledge Martijn Diender and Tomas van Haasterecht for their practical supervision, as well as their dedication and highly valued feedback. Thanks to my partner in crime, Timon Torres, for the effective brainstorm sessions and company during long lab days. Special thanks to all technicians and people from the Biobased and Chemical Technology, and the Microbiology department for their assistance. Lastly, I like to acknowledge the help of Marlene, Mirka and the thesis ring for their support and advices during this thesis. I am grateful for the opportunity that was given to me and the things I was able to learn.

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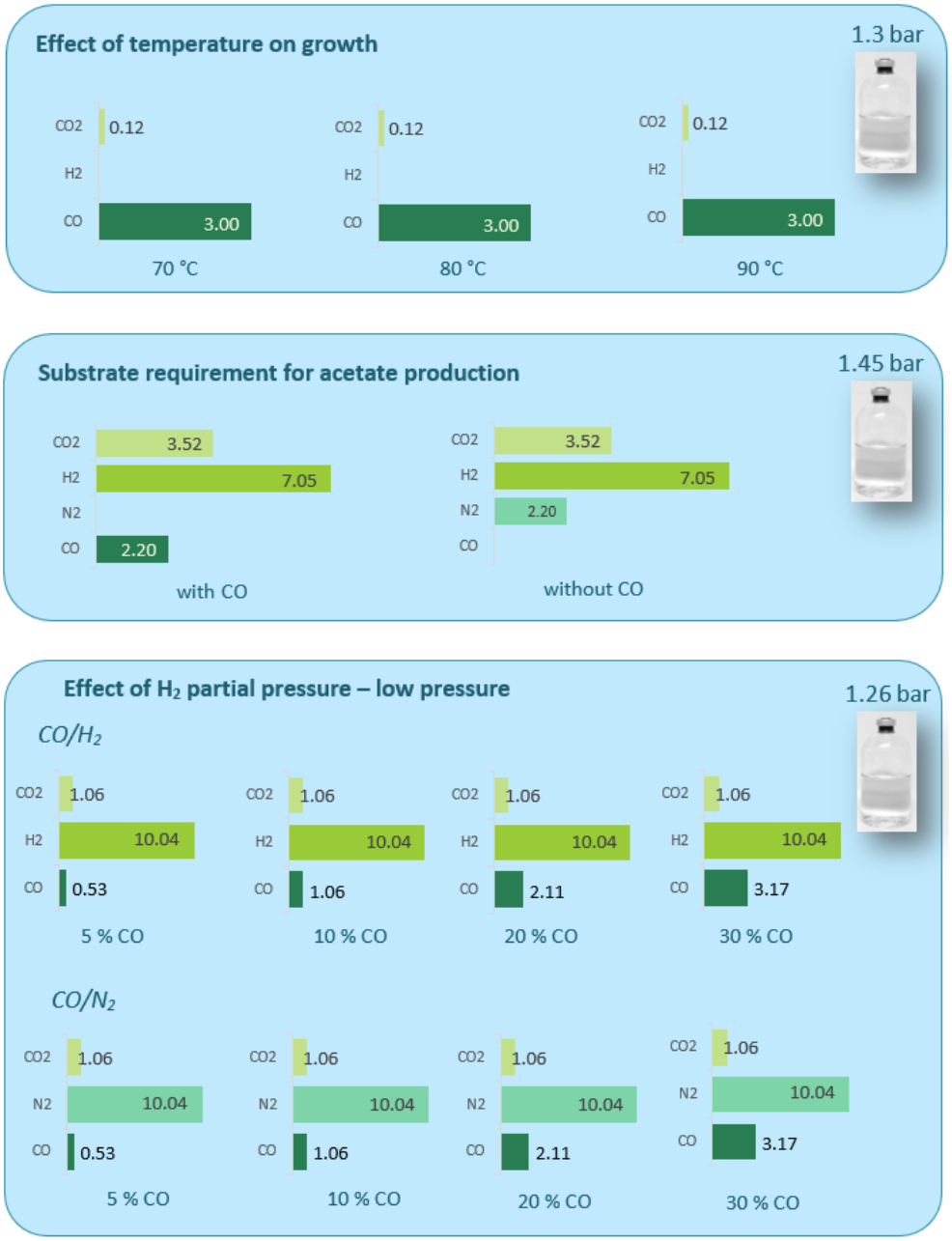
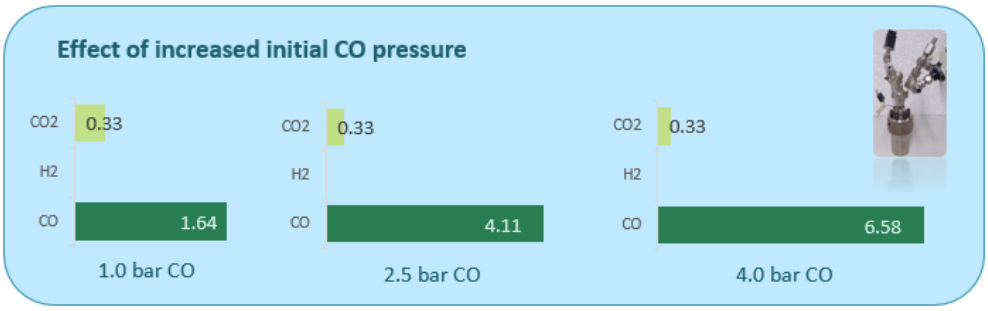
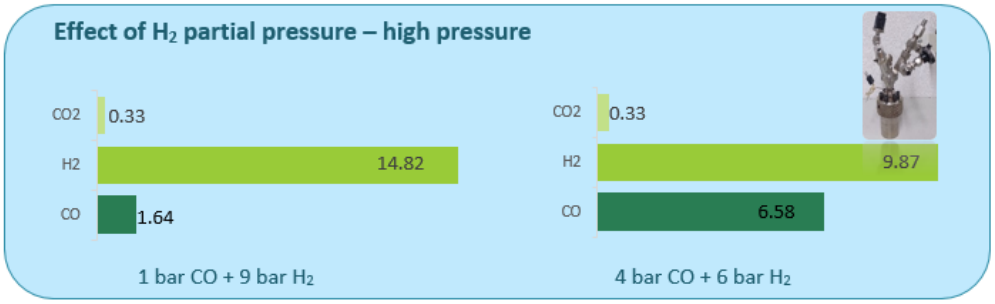
Appendix

Appendix I. Overview of microbial experiments

A schematic overview of the initially supplied amounts of gas (mmol) for the microbial experiments. Furthermore, the initial pressure and the chosen system are denoted.




Effect of increased initial CO pressure
CO2
H2
CO
0.33
1.64
1.0 bar CO
CO2
H2
CO
0.33
4.11
2.5 bar CO
CO2
H2
CO
0.33
6.58
4.0 bar CO


Effect of H_2 partial pressure – high pressure
CO2
H2
CO
0.33
14.82
1.64
1 bar CO + 9 bar H_2
CO2
H2
CO
0.33
9.87
6.58
4 bar CO + 6 bar H_2

Appendix II. Expected Ru/C conversion for different temperatures

Experiments from Olcay et al. (2010), were performed with a heterogeneous 5 wt.% ruthenium catalyst on activated carbon (Ru/C), for the conversion of acetic acid towards ethanol. Based on their results, the following graphs were made to predict the catalytic performance at 70 °C.

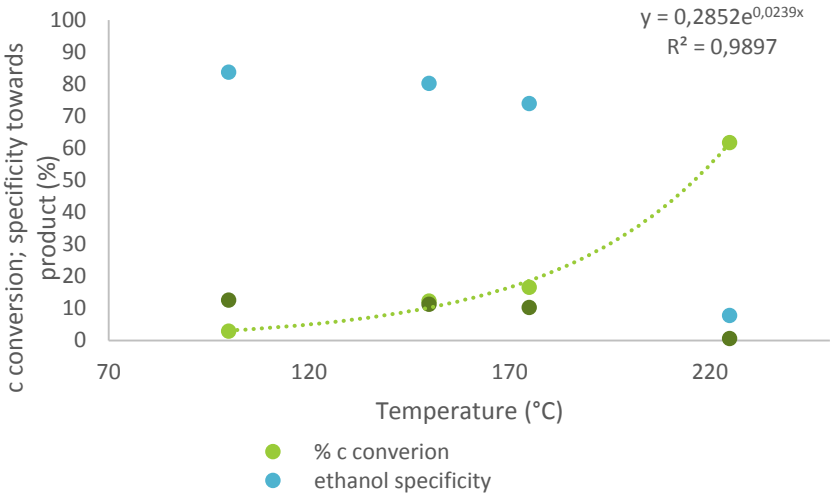


Figure 19 Results from Olcay et al. (2010), showing the carbon conversion (%) as well as ethanol and ethyl acetate specificity of Ru/C from acetic acid for the tested temperature range. Curve fitting was applied to predict conversion at 70 °C.

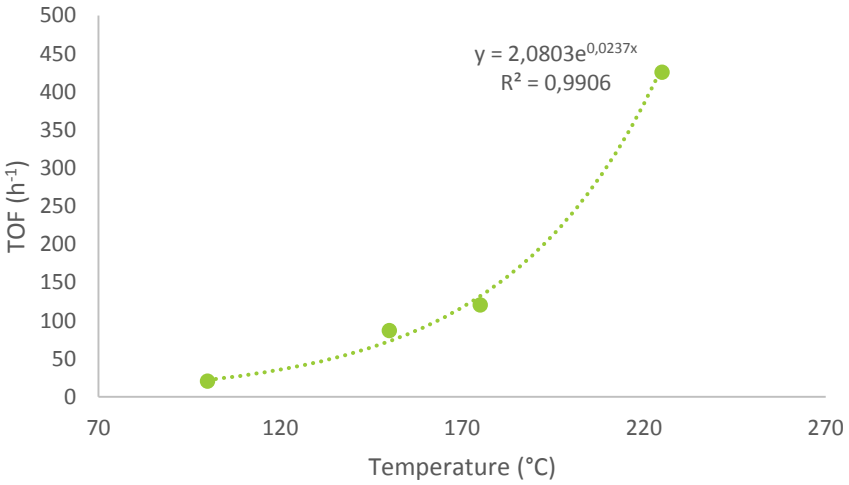


Figure 20 Results from Olcay et al. (2010), showing the turnover frequency (TOF) per hour of Ru/C for the tested temperature range. Curve fitting was applied to predict conversion at 70 °C.

Figure 19 and Figure 20 show, that the carbon conversion and turnover frequency (TOF) decrease exponentially with temperature. However, while catalytic activity decreases for lower temperatures, the ethanol specificity increases exponentially to 80 % at 100 °C. The main by-product, especially at lower temperatures, is ethyl acetate with a 13% specificity at 100 °C.

Based on the curve fitting applied to the available data, a carbon conversion of 1.5 % and a TOF of 10.9 h^{-1} was predicted for 70 °C, with an ethanol selectivity of above 85 %. In other words, it was expected that conversion from acetic acid to ethanol is possible at 70 °C, occurring with a high specificity but a low rate.

Appendix III. CP Medium composition and preparation (MIB protocol)

Stock solutions to be used to "compose" all the media:

All concentrations are given in grams per litre.

Solution 1: 27.2 g KH_2PO_4

Solution 2: 35.6 g $Na_2HPO_4.2H_2O$

Solution 3A: 24 g NH_4Cl; 24 g NaCl; 8 g $MgCl_2.6H_2O$

Solution 3B: 11 g $CaCl_2.2H_2O$

Solution 3C: 300 g $MgCl_2.6H_2O$

Solution 4: 80 g $NaHCO_3$

Trace elements: Acid Stock Solution (I)

50 mM HCl	1.8 g
1 mM H_3BO_3	61.8 mg
0.5 mM $MnCl_2$	61.25 mg
7.5 mM $FeCl_2$	943.5 mg
0.5 mM $CoCl_2$	64.5 mg
0.1 mM $NiCl_2$	12.86 mg
0.5 mM $ZnCl_2$	67.7 mg
0.1 mM $CuCl_2$	13.35 mg

Alkaline Stock solution (II)

10 mM NaOH	400 mg
0.1 mM Na_2SeO_3	17.3 mg
0.1 mM Na_2WO_4	29.4 mg
0.1 mM Na_2MoO_4	20.5 mg

Vitamins:

Biotin	20 mg
Nicotinamid	200 mg
p-Aminobenzoic acid	100 mg
Thiamin (Vit B1)	200 mg
Panthotenic acid	100 mg
Pyridoxamine	500 mg
Cyanocobalamine (Vit B12)	100 mg
Riboflavine	100 mg

Solution 7: 240.2 g $Na_2S.9\ H_2O$ Store under N_2 and in the dark

Na_2S is not stable in water due to chemical reactions. Make a fresh stock every month

Solution 8: 0.5 g Resazurin

PREPARATION OF REDUCED MEDIA FOR THE CULTIVATION OF ANAEROBES

Composition per litre medium.

MINERAL SALTS MEDIUM, bicarbonate buffered (Code: **BM**)

Add before autoclaving (use an Erlenmeyer):

500 ml dH_2O
1 ml solution 8
15 ml solution 1
15 ml solution 2
12.5 ml solution 3A
1 ml solution trace I
1 ml solution trace II

-make up to 950 ml with dH_2O

-Bring to a full boil to remove O_2 (do not boil longer than 20 sec. to prevent evaporation)

-Cool down under an oxygen-free N_2 flow, to prevent oxygen diffusion into the medium

-Disperse into serum bottles (always leave at least 50% of the bottle volume as gas phase!)

-Stopper and seal the bottles

-Change to the desired gas phase (N_2/CO_2 or H_2/CO_2 → 80/20 mixture). Pressure in the bottles 1.5 – 1.8 atm.

Important: When other mixtures of gases are used, the pH should be recalculated with the new CO_2 concentrations.

*Autoclave 20' 121^oC

Add after autoclaving from ***sterile*** stock solutions:

-1% v/v of a filter sterilised calcium/vitamin-solution; make up per litre medium:

10 ml sol 3B + 1 ml vitamin solution.

(so: in 50 ml medium you add 0.5 ml of the sterilized Ca/Vit mixture)

-Add the carbon source.

Finally, to completely reduce the medium add:

- 5% v/v of a sterilized reducing solution; make up per litre medium:

50 ml sol.4 + 1 ml sol.7 + 0.5 gram cystein

(so: in 50 ml medium you add 2.5 ml of the bicarb/sulf/cyst mixture)

Appendix IV. Acetate production profiles

In section 3.2.1.1 the effect of H_2 partial pressure on acetate production at low pressures was investigated twice. The final acetate concentrations largely varied, which was expected to result from different inoculation strategies. The acetate production over time for the first (Figure 21) and the second (Figure 22) experiment are shown. It can be seen that for the first experiment, acetate production was still increasing exponentially, while it started to decline in the second one. For more information, more sampling points would be required.

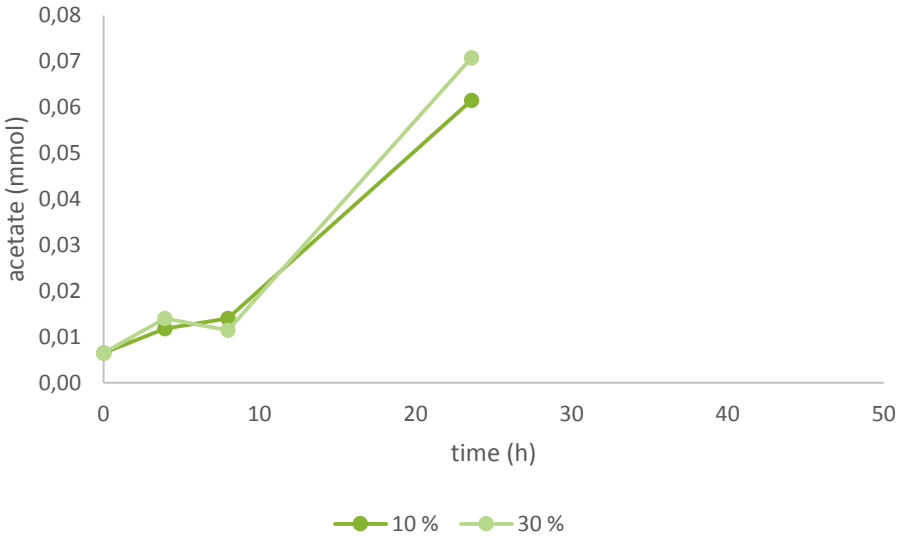


Figure 21 Acetate production over time for initial experiment about effect of H_2 partial pressure on the acetate production. 10 and 30 % CO refer to 1.06 and 3.17 mmol, respectively. Average data from duplicate experiments.

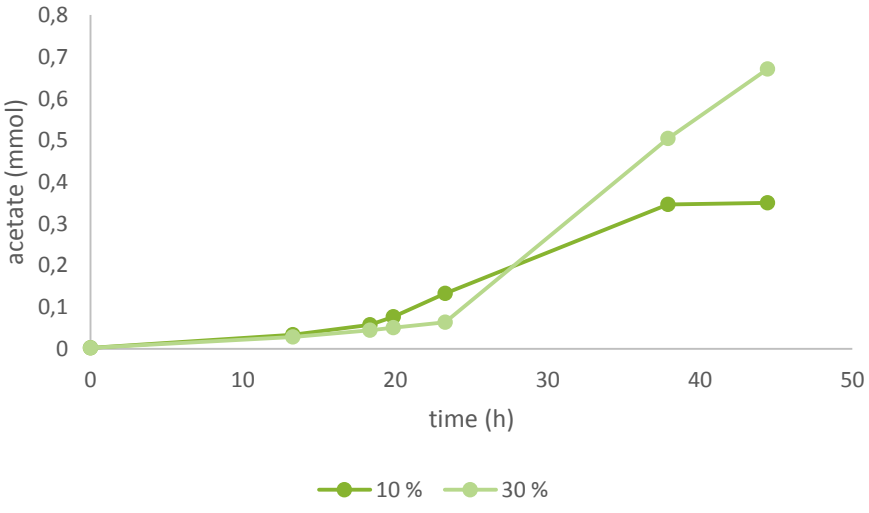


Figure 22 Acetate production over time for second experiment about effect of H_2 partial pressure on the acetate production. 10 and 30 % CO refer to 1.06 and 3.17 mmol, respectively. Average data from triplicate experiments.

Appendix V. NMR spectrum of hydrogenation products

During evaluation of the catalytic hydrogenation experiments, the carbon balance could not be closed. To investigate whether this loss of carbon resulted from so far undetected by-products, NMR was performed. For this, 10 wt. % acetic acid was prepared in D_2O, and catalysis was performed at 70 °C. The obtained spectrum is shown in Figure 23 and Figure 24, where the former one shows the full spectrum and the latter an enlargement of the detected peaks.

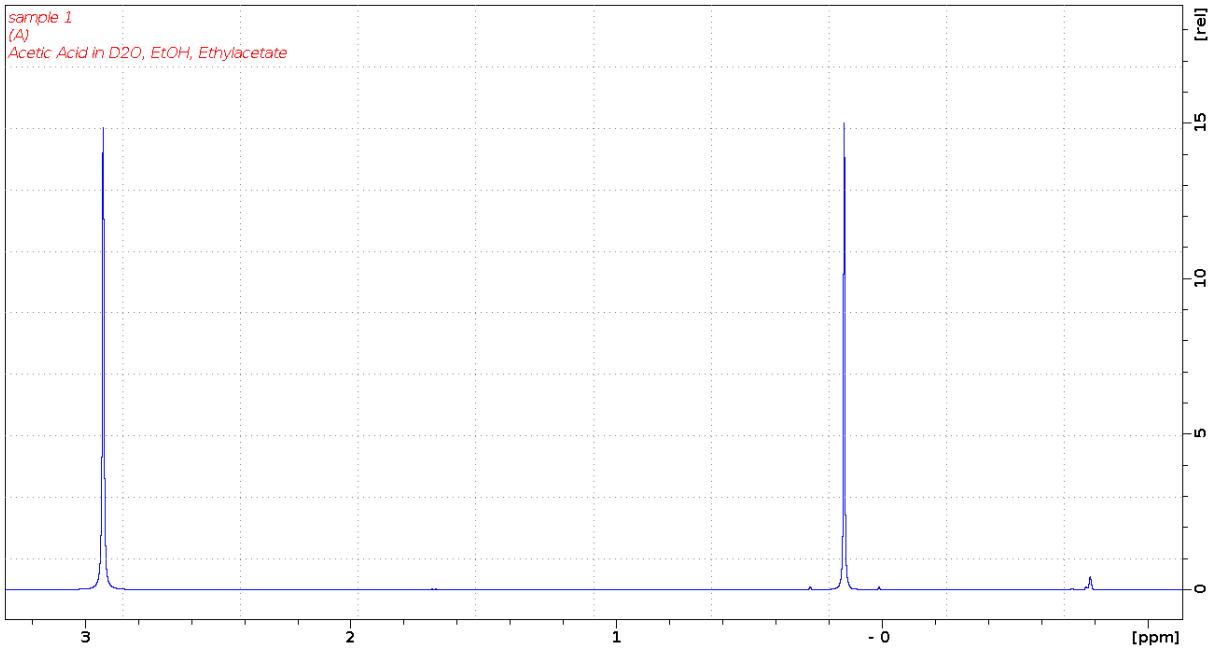


Figure 23 NMR spectrum of the liquid composition at the end of a hydrogenation reaction, which was performed at 70 °C with 10 wt. % acetic acid prepared in D_2O. Note that the chemical shift was adjusted manually after the picture was taken. Therefore, the large peak around 0 ppm should be at 2 ppm, whereas the large peak at 3 ppm should be at 4.7 ppm.

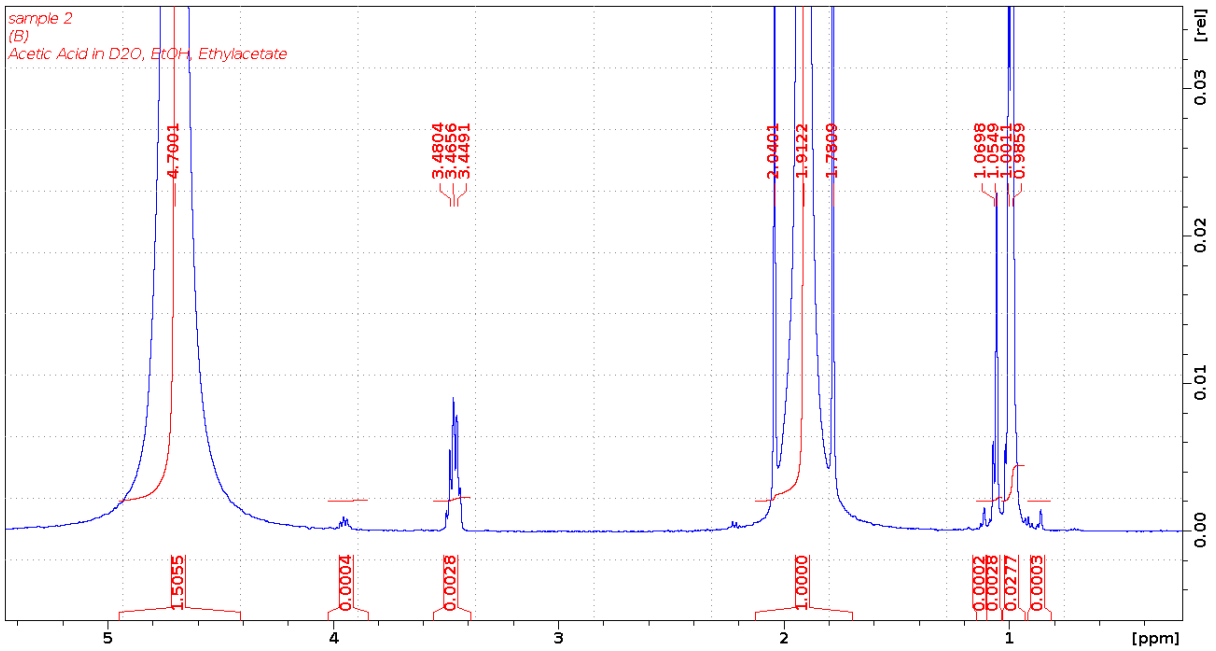


Figure 24 Enlarged NMR spectrum of the liquid composition at the end of a hydrogenation reaction, which was performed at 70 °C with 10 wt. % acetic acid prepared in D_2O. Here, the chemical shift was adjusted and peaks were integrated for quantification.

The two mayor peaks at 2.0 and 4.7 ppm correspond to acetic acid and D_2O, respectively. Knowing its concentration, the integral of acetic acid was set to 1 as the reference. Moreover, the triplet at 1.0 ppm and the quartet at 3.5 ppm correspond to ethanol, both having an integral of 0.0028. The small smaller triplet at 0.9 ppm, the singlet at 1.1 ppm, as well as the quartet at 4.0 ppm, with an integral of 0.0004 indicate the presence of ethyl acetate.

The spectral looked identical for all triplicates, showing small peaks for the expected products ethanol and ethyl acetate, next to large peaks from D_2O and the substrate acetic acid. It was thus concluded, that no additional by-products were formed which could explain the carbon loss.

Appendix VI. Calculating acetic acid – acetate partitioning at pH 6.5

In section 3.2.1, the effect of using acetate instead of acetic acid as substrate for hydrogenation to ethanol through a Ru/C catalyst was examined. For this, a 10 wt. % acetic acid solution with pH 2.2 was compared with a 10 wt. % mix of acetic acid and acetate with pH 6.5. Results showed limited conversion at neutral compared to acidic conditions. To get an indication whether the catalyst operates with low conversion rates or merely reacts with acetic acid, the partitioning between acid and salt form at pH 6.5 was determined.

The reaction occurred at 70 °C. The closed value that could be obtained from literature was for 60 °C. As the purpose was to get an indication about the partitioning, this was regarded as accurate enough. The dissociation constant (K_a) for acetic acid at 60 °C was found to be $1.54*10^{-5}$ [54].

$$pK_a = -\log(K_a) \tag{1}$$

Based on equation (1), this equals a pK_a of 4.81 for 60 °C.

Equation (2) describes the ratio between acetic acid (AH) and acetate (A), both in mol L^{-1}, as a function of the pK_a and the pH. Equation (3) indicates that the total molarity equals 1.667 mol L^{-1}. This value is based on the chosen 10 wt. % acetic acid solution with a molecular weight of 60.05 g mol^{-1}.

$$pK_a = pH + \log\left(\frac{AH}{A}\right) \tag{2}$$

$$AH + A = 1.667\ M \tag{3}$$

Substituting (3) in (2) and solving for A results in equation (4).

$$A = \frac{1.667}{10^{(pK_a - pH)} + 1} \tag{4}$$

Solving equation (4) with a pK_a of 4.81, and pH 2.2 or 6.5, results in the concentrations shown in Table 12.

Table 12 Calculated dissociation between acetate and acetic acid for pH 2.2 and 6.5 at 60 °C.

	acetate		acetic acid		acetic acid dissociation (%)
	(mol L^{-1})	(mmol)	(mol L^{-1})	(mmol)	
pH 2.2	1.663	49.89	0.004	0.12	99.8
pH 6.5	0.021	1.01	1.633	49.00	2.01