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MICROBIAL RECOVERY OF HYDROGEN AND CARBON DIOXIDE FOR BIO-BASED FUEL PRODUCTION

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Kurzfassung

Das größte Potential von erneuerbaren Energien wird in Solar- und Windenergie gesehen, welche allerdings keine konstante und wirtschaftliche Energieversorgung garantieren können. Um kurzfristige Stromspitzen entgegen dem Strombedarf zu nutzen, können entsprechende Kapazitäten an Überschussstrom auf dem Weg der Elektrolyse von Wasser in Wasserstoff (H_2) konvertiert werden. Jedoch sind H_2-Nutzungspfade nach ökonomischen und energetischen Gesichtspunkten nicht realistisch. Die Biokonversion von H_2 und Kohlendioxid (CO_2) in das Intermediat Acetat, welches in einer zweiten Stufe mikrobiell in flüssige Energieträger konvertiert werden kann, stellt eine vielversprechende Alternative dar. Im ersten Teil dieser Arbeit wurden zwei homoacetogene Clostridienstämme, *Morella thermoacetica* und *Acetobacterium woodii*, erfolgreich an ein Minimalmedium adaptiert, um eine chemolithoautotrophe Produktion von Acetat aus H_2 und CO_2 zu induzieren. Mit einer Konzentration von 27 g/L Acetat in Batchfermentationen entpuppte sich *A. woodii* als der produktivere Organismus. Des Weiteren wurde gezeigt, dass durch die Immobilisierung von *M. thermoacetica*-Zellen Endproduktkonzentrationen gesteigert werden konnten. Im zweiten Teil wurde das Potential der Umwandlung von Acetat in Aceton, Butanol und Ethanol (ABE) über den Weg der ABE-Fermentation untersucht. Als Produktionsorganismus wurde der Clostridienstamm *Clostridium beijerinckii* gewählt. Obwohl in ersten Vorversuchen noch keine direkte Umsetzung gezeigt werden konnte, wird das Potential dieses Teilprozesses als vielversprechend angesehen.

Abstract

Solar and wind power have considerable potential to satisfy future renewable energy needs but efficient and scalable methods for storing the intermittent electricity they produce are required. Current power-to-fuel storage strategies are based on electrolytic splitting of water to produce hydrogen (H_2), which in principle is an attractive fuel. However, H_2 encounters several limitations from a liquid fuel based infrastructure. The microbial conversion of H_2 and carbon dioxide (CO_2) into the storable intermediate acetate, which can be further converted into liquid fuels, is a potential technology to overcome these restrictions. In the first part of this work *Morella thermoacetica* and *Acetobacterium woodii*, homoacetogenic Clostridia, were successfully adapted to minimal media for the chemolithoautotrophic production of acetate from H_2 and CO_2. *Acetobacterium woodii* was found to be the more productive organism in batch fermentations, reaching final concentrations of 27 g/L acetic acid. However, product concentrations stated in literature could not be reached. Acetate concentrations were successfully increased by immobilization of cells in pre-experiments. In the second part the feasibility of a further acetate conversion into acetone, butanol and ethanol via ABE-fermentation was assessed using the strain *Clostridium beijerinckii*. Although a direct transformation of acetic acid was not accomplished in pre-experiments, this innovative subprocess bears a highly promising potential.

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1 Introduction

1.1 Renewable energy sources

Traditional energy sources on the basis of natural gas, oil and coal are established as effective economical driving forces, but are at the same time highly damaging to the environment and to human health. While the price for gas and oil fluctuates, a transition to renewable energy sources has proceeded in the past years due to dropping costs for solar, wind and other alternative energy systems. The fact that these systems can in principle meet the world's energy demands many times (Herzog et al., 2001) and that these technologies minimize environmental impacts by minimal secondary waste production (Panwar et al., 2011) confers them a promising future. Most widely spread green energy technologies comprise energy generation from hydro, geothermal, wind and solar power (Herzog et al., 2001).

1.2 Storage problem of peak currents

Wind and solar energy are considered the most promising technologies in Austria to achieve the EU's target of higher use of renewable energy sources. However, because of the stochastic nature of wind, electric power that is generated by wind nozzles is very inconsistent (Díaz-González et al., 2012). This fact and the intermittent characteristic of solar energy (Garg et al., 1985) make it impossible to satisfy the need of demand driven response because an easily adjustable and regular supply cannot be provided (Ibrahim et al., 2008). Harnessed peak power outputs may not correlate with power requirements. This has an adverse effect on the economic feasibility of these technologies and on the electricity grid. To overcome this drawback, energy storage is a crucial factor for these green energies to be considered as primary energy sources. Todays need for energy storage in a larger scale is greater than ever before (Hadjipaschalis et al., 2009). Many technologies are already available and in use, such as pumped hydro storage, thermal energy storage, compressed air energy storage, and chemical energy storage in form of batteries (Ibrahim et al., 2008). The choice of storage technique depends greatly on the application and required storage amount (Ibrahim et al., 2008). Some technologies may be more suitable for smoothing out annual fluctuations, while others would satisfy short peak power requirements more effectively (Hadjipaschalis et al., 2009). Electrolytic hydrogen production and subsequent conversion to methanol (Olah, 2005) or methane (Sterner et al., 2001) provide alternatives for long term storage of renewable energy, specifically from wind and solar power (Agbossou et al., 2004).

Different methods for hydrogen production from renewable energies like thermochemical cracking, photovoltaic electrolysis of water or hydrogen production from biomass (Bartels et

al., 2010) have already been developed. However, the only one currently practical is the conventional hydrolysis of water. Hydrogen production capacities up to 1500 kg/day (NREL Technical Report, 2009) and efficiencies of more than 70 % are attained with this mature technology (Barbir, 2005). Nevertheless, hydrogen as a storage medium for peak currents is still fraught with problems.

Most of the technologies, that could be used for large scale and long-term storage of hydrogen are still in stages of research and face a number of challenges. For example, storage as compressed hydrogen depends on mechanical stability of used materials and their permeability to hydrogen. Taking into account that 1 kg hydrogen has a volume of 11 m^3 (at ambient standard conditions of 298.15 K and 100 kPa) this kind of storage results in a relatively low ratio of stored hydrogen per unit weight at pressures of 200 - 250 bar. For the purpose of higher storage capabilities pressures of up to 700 bar would be required which would be associated with high-energy requirements and high costs for the compression. Hydrogen can be compiled also in its liquid state at -253°C. Liquid hydrogen storage over a longer period of time is currently limited by the enormous amount of energy needed for liquefaction and the costs for the container material (Hadjipaschalis et al., 2009; Züttel, 2003). Another issue that has to be considered for high hydrogen capacities is the challenge of product transmission. (Mazloomi and Gomes, 2012) came to the conclusion that both, small scale delivery of H_2 gas via tube trailers or liquid transportation via road, and large scale transportation via pipeline, are associated either with high energy inefficiency or extremely high investment costs (Mazloomi and Gomes, 2012).

Despite the progress in H_2 utilization, distribution and storage infrastructure is currently lacking. Due to this reason, hydrogen is not yet extensively used for electricity storage or as transportation fuel (Torella et al., 2015). This leads to the investigation of potential alternative energy carriers with increased safety characteristics and distribution potential. The task is to investigate approaches for the transformation of hydrogen into easier to handle and more cost effective alternatives as sophisticated infrastructure for storage and distribution of liquid fuels like underground tanks and containers, pipe lines and road tankers already exist (Torella et al., 2015).

1.3 Alternative liquid energy carriers

In the last decade a large range of sustainable energy carriers like bio-diesel, bio-ethers bio-alcohols etc. have been extensively discussed in literature as alternative fuels to fossil-based energy carriers (Festel, 2007; Latif et al., 2014; Liu et al., 2013; Luque and Clark, 2010; Nigam and Singh, 2011). Some of the most prominent fusel alcohols based on microbial production are ethanol, iso-propanol and butanol, which can be produced by microbial fermentation are shortly characterized in the following:

Ethanol is a renewable fuel based on biomass, which is mainly produced by alcoholic fermentation of sugars from corn, sugar cane, and barley as well as from agricultural residues. Ethanol has excellent fuel properties due to its high octane number and is used as fuel additive, as it is highly miscible with diesel. Nevertheless, ethanol has also several disadvantages as a diesel supplement. Its low viscosity can reduce lubricity of the fuel and thereby cause problems in fuel pumps. Its high vapor formation requires additional precautionary measures (Savage et al., 2008). Furthermore its high corrosivity towards ferrous materials makes distribution via traditional gas lines challenging (Grousseau et al., 2014).

Isopropanol is a higher fusel alcohol, which is already used as a diesel and gasoline additive. Its mainly used as a solvent in the chemical industry and as an additive in household products like inks and paints (Grousseau et al., 2014).

Butanol is like ethanol a renewable biomass based alcohol that can be obtained by fermentation of crude glycerol, a by-product from bio-diesel production. (Rakopoulos et al., 2010). This primary alcohol has advantages over ethanol when it is used as an alternative fuel for combustion engines. Many of its fuel properties are close to fossil based fuels as it can be blended with diesel without phase separation. It provides more energy per unit and is less evaporative than ethanol. The low auto ignition temperature enhances its ignition when burnt in diesel engines (Doğan, 2011). In addition, low corrosivity and hygroscopicity confer great potential to butanol as an alternative energy carrier (Fortman et al., 2008).

Another very important substance that will play a key role in the following process is acetic acid or acetate. Acetate itself does not serve as a biofuel but it can be used as an energy storage form. It is a common anion in biology and states no hazards like toxicity and can easily be stored as aqueous solution. A great advantage of this substance is that it can serve as a substrate for further microbial conversion into alternative energy carriers as listed above.

1.4 Transformation of Hydrogen

Peak currents from renewable energy sources can be stored by electrolytic splitting of water into its elements H_2 and O_2. However, H_2 has failed to assert itself as a reliable and wide spread energy source, since the world's infrastructure is not set up to handle H_2.

So are there possibilities, to convert this easy to produce but difficult to store and distribute hydrogen gas?

One possibility is the application of the Sabatier process (Behr, 1987), in which H_2 and CO_2 are catalytically transformed to methane (CH_4) at temperatures around 400 °C and pressures of ca. 10 bar. However, there are several drawbacks of this technology: First, the reaction depends on catalysts like nickel and ruthenium, which are very sensitive to heavy metals, sulfur and traces of carbon monoxide (Abu Bakar et al., 2012). Secondly, the energy demand due to the required high temperatures is associated wisth high operating costs. By the use of a microbiological system these drawbacks would be eliminated.

Torella et al. (2015) claim to have invented an integrated bio-electrochemical system, in which CO_2 together with H_2 and O_2 produced by water splitting is converted into fusel alcohols using the bacterium *Ralstonia eutropha*. A drawback of this technology may be its lack of flexibility, as it is confined to the production of iso-propanol. An alternative approach is the biological H_2 conversion into liquid energy carriers with an intermediate step via acetate that can potentially be transformed into different energy carriers like butanol, ethanol, acetone, iso-propanol or methane depending on the choice of used microorganisms.

In the following the focus will be on a sustainable microbiological conversion of hydrogen gas into acetate as liquid storage intermediate and the subsequent conversion into acetone, butanol and ethanol. The process cascade consists of two steps. In the first step, homoacetogenic Clostridia are used to convert H_2 and CO_2 into acetate, the storable intermediate. The second step comprises the investigation of the microbial conversion of acetate to liquid fuels via ABE-fermentation using a Clostridium strain.

1.4.1 Wood-Ljungdahl pathway - from H_2 and CO_2 to acetate

The key component of the first step, the conversion of H_2 and CO_2 into acetate, is the so-called Wood-Ljungdahl or acetyl-CoA pathway. This metabolic pathway enables acetogens to generate energy. Additionally and for this process by far the most crucial property is chemolithoautotrophic fixation of exogenous CO_2 with H_2 as an electron donor (Drake and Harold L., 1994). At the end of the process stands acetate as the sole metabolic product. In the following the acetyl-CoA pathway will be enlightened from historical, physiological and enzymatic perspectives:

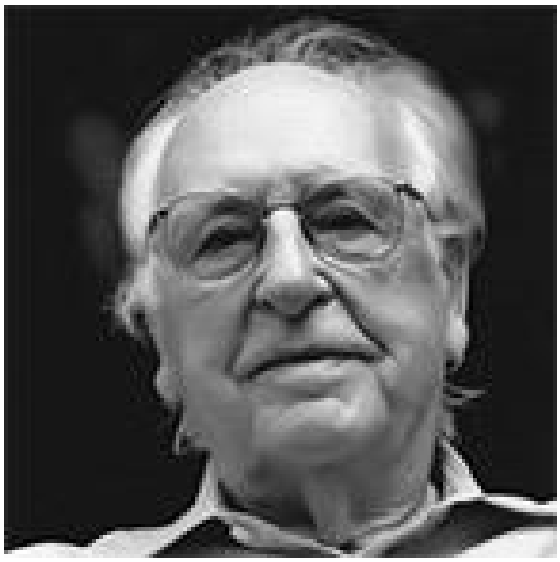

Figure 1: Lars G. Ljungdahl, ("University of Georgia," 2016)

Figure 2: Harland G. Wood ("Nationalmedals.org" 2016)

From 1958 on Harland G. Wood and his at first graduate student Lars G. Ljungdahl dedicated 33 years of research to resolve the mechanisms of acetate synthesis from CO_2. Lars focused on determining the methyl group formation of acetate (also called Eastern branch), while Harland tried to figure out how the carboxyl group of acetate was formed (Western branch) (Ragsdale, 1997). In 1991 the biochemical puzzle was collectively assembled, and the framework of the acetyl-CoA pathway was resolved, which is now referred to as Wood-Ljungdahl-pathway.

The Wood-Ljungdahl-pathway provides three main cell functions (Drake and Harold L., 1994):

- Terminal electron acceptation
- Energy conservation
- Autotrophic assimilation of carbon

Depending on a cells growth condition the purpose of this pathway can alternate.

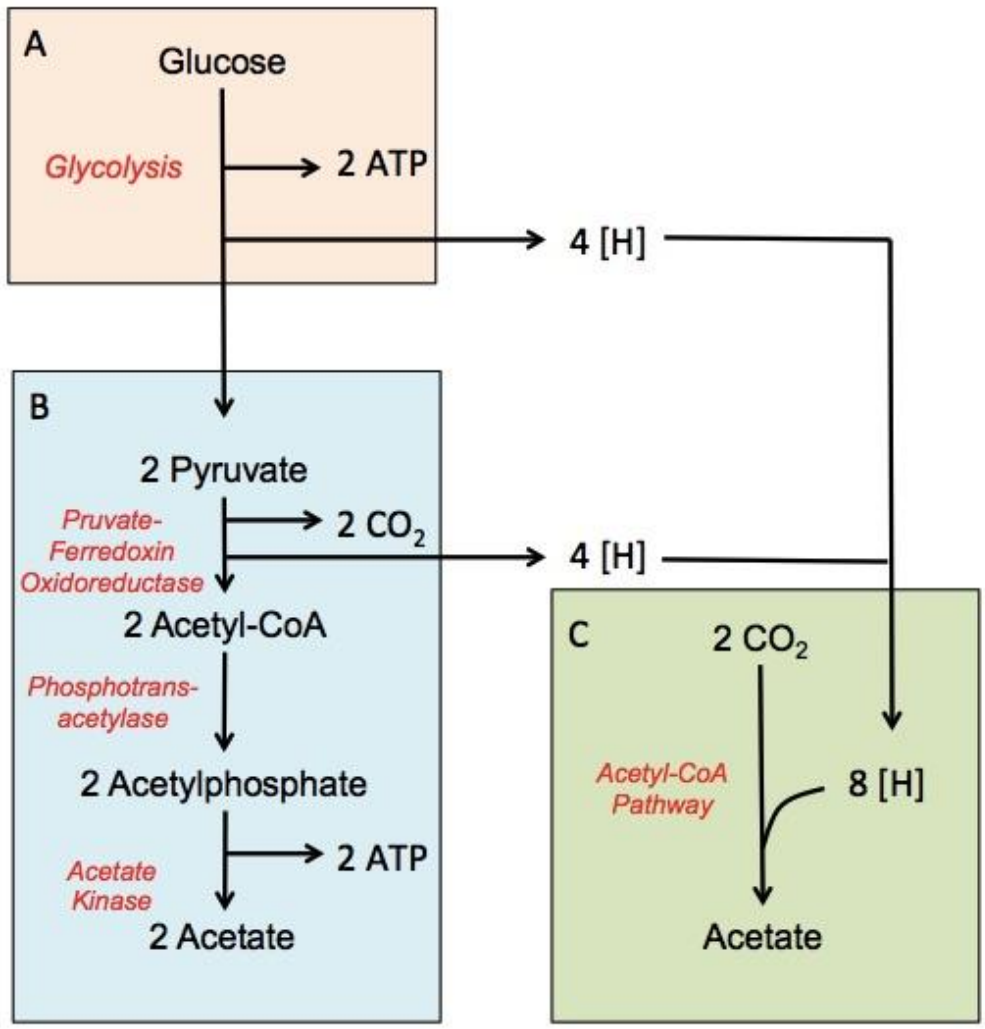

Figure 3: Overview of main processes in acetogens. (Dürre, 2005) adapted

Figure 3 shows an overview of the three main processes that yield three molecules of acetate per molecule glucose. Box A represents the degradation of glucose to 2 pyruvates during glycolysis. Box B shows the decarboxylation of pyruvate to acetyl-CoA and the subsequent phosphorylation to acetate. Box C represents the actual acetyl-CoA or Wood-Ljungdahl pathway in which two molecules of CO_2 are fixed and the third molecule of acetate is formed (Drake and Harold L., 1994).

Wood-Ljungdahl-pathway as a reduction sink

As acetogens are anaerobes, other terminal electron acceptors than oxygen are required. When the acetyl-CoA pathway is pursued CO_2 serves this purpose. During glucose-dependent heterotrophic growth for example, the pathway acts mainly as an electron sink as the cell has access to ATP, acetyl-CoA and other metabolic intermediates via the breakdown of sugars. Crucial chemolithoautotrophic functions like energy conversion or acetyl-CoA production are less remarkable. Here the main function focuses on re-oxidation of reduced electron carriers like ferredoxin and NAD (Drake and Harold L., 1994).

Formation of acetate and conservation of energy

As a second function the conservation of energy has to be recorded. ATP is formed by phosphorylations during the oxidative degradation of glucose in the process of glycolysis. But during the reductive synthesis of acetate from CO_2 no ATP is conserved. In fact one ATP is used for activation of formate, which then is regained via acetate-kinase. Additionally the carbonyl branch requires energy for the reduction of CO_2 to CO as well.

Generally 10 g of cell dry weight are obtained from 1 mole ATP. As in glycolysis 4 moles of ATP are produced, 40 g of cell dry weight per mole glucose are expected. In reality, acetogens grow to a cell dry weight of 50-70 g per mole glucose, which led to the apprehension, that acetogens conserve energy by chemiosmotic mechanisms. Experiments showed, that gradient-driven phosphorylation occurs in acetogens (Müller, 2003). Details on the mechanisms engaged during energy conservation through the acetyl-CoA pathways are not yet uncovered. However, it is known, that different acetogens use different mechanisms for energy conservation on a non-substrate level (Drake and Harold L., 1994). Two groups of acetogens can be described from a bioenergetic point of view. On the one hand there are H^+ dependent acetogens like *Morella thermoacetica* and on the other hand there are Na^+ de-

pendent ones like *Acetobacterium woodii.* The latter ones couple primary and electrogenic translocation of Na^+ to the acetyl-CoA pathway using membrane bound corrinoids, while the former group comprises cytochromes and a H^+ motive electron transport chain. A H^+ or a Na^+ translocating ATP synthase is taking advantage of established ion gradients (Müller, 2003).

Chemolithoautotrophic CO_2 fixation

This third fundamental function of the pathway is crucial for biosynthesis and the autotrophic growth. Acetyl-CoA that has been formed in the pathway must be utilized as the main building block in cellular growth. In this step, pyruvate:acceptor oxidoreductase reductively carboxylates acetyl-CoA to pyruvate (Müller, 2003), which is then transformed into phosphoenolpyruvate an assimilated via gluconeogenesis and an incomplete TCA-cylce. This assimilatory function is tightly regulated (Drake and Harold L., 1994; Eden and Fuchs, 1983, 1982).

The two branches

The Wood-Ljungdahl pathway can be divided into two major parts. On the one side, there is the methyl or Eastern branch and on the other side the carbonyl or Western branch. The Eastern branch is a folate-dependent one-carbon pathway that is present in bacteria as well as in humans. The Western branch is special to acetogens and some other groups of bacteria (methanogens, sulfate reducers). When acetogens us H_2-CO_2 as a substrate, carbon enters the pathway in the form of CO_2 and H_2 is used as an electron donor (Ragsdale, 2008). An overview of the two branches is presented in **Figure 4**.

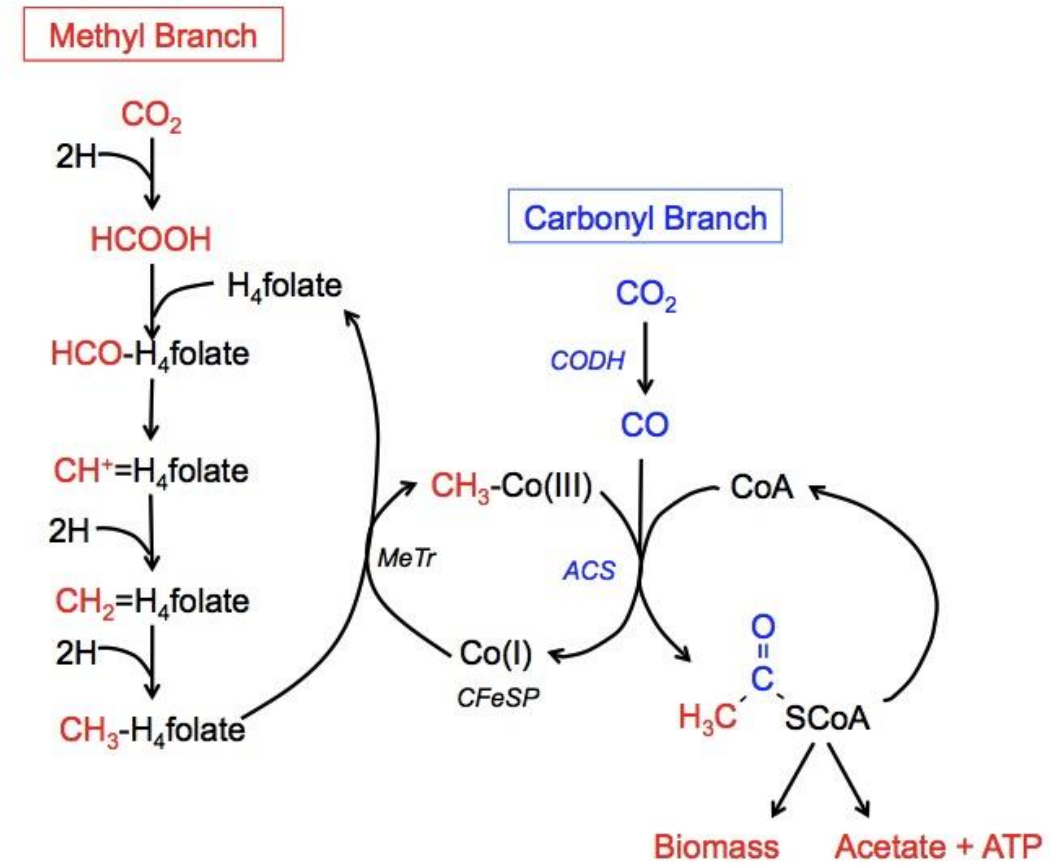

Figure 4: Wood-Ljungdahl pathway, (Ragsdale and Pierce, 2008) adapted

The Eastern branch: CO_2 is reduced via formate and H_4-formate to methyltetrahydrofolate (CH_3-H_4 folate), which serves as a precursor for the methyl group of acetate (Pezacka and Wood, 1988, see **Figure 4**).

The Western branch: In the second part another molecule of CO_2 is converted to C_1 by the CO-dehydrogenase (CODH). H_2 provides electrons via the hydrogenase for the reduction of CO_2 to CO. The transfer of the CH_3-group from CH_3-H_4-folate to the corrinoid-enzyme (Co) is catalyzed by the methyltransferase (MeTr). The methyl group is then transferred to the CODH. The condensation of CH_3, CO and CoA groups is synthesized by ACS (acetyl-CoA synthase) and thereby facilitates the last step in the synthesis of acetyl-CoA. The corrinoid-iron-sulphur protein (CFeSP) acts as a methyl-transfer agent between the ACS and the MeTr (Dobbek, n.d.; Pezacka and Wood, 1988).

1.4.2 Acetogenic Prokaryotes

A short overview of history, physiology, ecology and biochemistry of acetogens will be presented in this chapter.

Since the term "acetogens" is afflicted with inconsistency in literature, confusion can be caused. Any organism that produces acidic acid as a final metabolic product could be referred to as acetogen. In this thesis, to avoid misunderstandings, the following definition, proposed by Drake and Harold, (1994) will be applied:

"Acetogens are obligate anaerobic bacteria that can use the acetyl-CoA pathway as their predominant (i) mechanism for the reductive synthesis of acetyl-CoA from CO_2, (ii) terminal electron accepting, energy conserving process, and (iii) mechanism for the synthesis of cell carbon from CO_2."

Acetogens are differentiated from organisms that synthesize acetate by other pathways (Müller, 2003). Acetogenic clostridia may be called "homoacetogens" if acetic acid or acetate is secreted as a single product, synthesized from CO_2 via the reductive acetyl-CoA pathway (Ragsdale and Pierce, 2008).

The domain of bacteria is the only one that acetogenic prokaryotes originated from (Drake et al., 2006).

Discovery

More than 80 years ago, in 1932, the first organisms, that could convert CO_2 and H_2 into acetate, were discovered (Ragsdale and Pierce, 2008). Only four years later the Dutch microbiologist Wieringa was able to isolate the first acetogen. This spore forming mesophilic rod was called *Clostridium aceticum* and used the following net reaction for energy generation.

$$2\mathrm{CO_2} + 4H_2 \rightarrow \mathrm{CH_2COOH} + 2\mathrm{H_2O}$$

It was observed for the first time, that an organism grows at the expense of this reaction. Only a couple of years later, *Clostridium thermoaceticum*, later renamed and known under *Morella thermoacetica* was isolated. Unfortunately *C. aceticum* was "lost" for 40 years, until G. Gottschalk found an old testing tube filled with spores of this acetogen, while visiting Barker's laboratory. Due to this fact *M. thermoacetica* was the only acetogen cultivated *in vitro* and available for laboratory studies, on which the chemolithoautotrophic acetyl-CoA pathway was elucidated. For many years it was classified as an obligate heterotroph and was observed to convert one mole of glucose stoichiometrically into 3 moles of acetate. Until this moment there was no known metabolic process that could account for the formation of more than 2 moles acetate per consumed mole glucose. That hinted, that *Morella* could produce CO_2 via oxidation and subsequently reduce it to acetate.

Barker formulated a direct correlation of heterotrophic and autotrophic systems:

Oxidation

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 8H$$

Reduction

$$8H + 2CO_2 \rightarrow CH_3COOH + 2H_2O$$

Net reaction

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH$$

In 1952, Wood repeated ^{14}C-experiments firstly conducted by Barker and Kamen, and confirmed, that acetate synthesis from two moles of CO_2 by an unknown CO_2 fixing process in *M. thermoacetica*. In 1990 the enzymology of the "autotrophic" pathway for the organism was securely proved and the initially heterotrophic bacterium was finally found autotrophic (Drake and Harold L., 1994; Drake et al., 2006). Table 1 gives information about important milestones in the discovery of acetogens and the acetyl-CoA pathway.

Table 1: History of discovery of acetogens (Drake et al., 2008)

Year	Event
1932	H_2-dependent conversion of CO_2 to acetate in sewage sludge
1936	Discovery of first acetogen, *Clostridium aceticum*; total synthesis of acetate from H_2-CO_2
1942	Discovery of second acetogen, *Clostridium thermoaceticum*; conversion of glucose to 3 acetate
1944	Acetogenic conversion of pyruvate to acetate
1945-52	Synthesis of acetate from $^{14}CO_2$
1955	Formate as a methyl-group precursor
1964	Methylcobalamin as methyl-group precursor
1965	Autotrophic synthesis of cell-carbon precursors from CO_2
1966-69	Proposal of one-carbon pathway for the tetrahydrofolate/corrinoid-mediated synthesis of acetate from CO_2
1973-76	Discovery that tungsten is a biologically active metal in formate dehydrogenase
1973-86	Resolution of the tetrahydrofolate pathway
1978-80	Discovery of CO dehydrogenase as a nickel-containing enzyme
1981	Resolution of enzymes required for synthesis of acetyl-CoA from pyruvate and methyltetrahydrofolate
1981-82	Demonstration that CO replaces the carboxyl-group of pyruvate and undergoes an exchange reaction with acetyl-CoA
1982	Discovery of hydrogenase
1982	Purification of CO dehydrogenase
1982	Use of H_2 and CO under organotrophic conditions
1984	Resolution of nutritional requirements
1984	Enzyme system for H_2-dependent synthesis of acetyl-CoA
1984-86	CO dehydrogenase is acetyl-CoA synthase and CO is the carbonyl precursor ion the acetyl-CoA pathway under growth conditions
1985-91	Catalytic mechanism of acetyl-CoA synthase
1986-90	H_2- and CO-dependent electron-transport system coupled to the synthesis of ATP

1990	Chemolithoautotrophic growth on H_2-CO_2 and CO-CO_2
1991	Integrated model for catabolic, anabolic, and bioenergetic features of the acetyl-CoA Wood-Ljungdahl pathway

Diversity and habitats of acetogens

In the past decades extensive effort was put into the identification and description of acetogens. The number of known acetogens increased to over a hundred different species that can be assigned to 22 different genera (Drake et al., 2008). They not only differ in their morphology, but also in their physiological and cytological properties. Most of identified acetogens are represented by the genera Clostridium and Acetobacterium. Acetogens inhabit almost all anoxic environments (Drake et al., 2006) and 1 trillion tons of acetic acid per year are produced at their expense. They play essential roles in the microbiology of soils, as well as in gastrointestinal systems of mammals like cows, sheep and human (Ragsdale, 1997). Although most of the isolates known today are mesophilic some thermophilic and psychrotolerant species, which are originally settled in extreme environments, are known as well (Drake et al., 2006). Isolates were attained for example from deep subsurface sediments (Krumholz et al., 1999; Liu and Suflita, 1993). *Acetohalobium arabaticum* e.g. was discovered in a saline lagoon, while others were detected in termite guts, coal mine pond sediments, sewage sludge, rumen fluids and even oil fields (Drake et al., 2008).

Morella thermoacetica

Morella thermoacetica is the most studied acetogen, as the enzymology of the Wood-Ljungdahl pathways was resolved with it (Drake et al., 2006). It is a common bacterium in soils and was initially isolated from horse manure. At first it was described as *Clostridium thermoaceticum* (Fontaine et al., 1942) but due to phylogenetic analysis of 16S rRNA it was reclassified as *M. thermoacetica* (Collins et al., 1994). The organism is classified as a gram-positive, spore forming obligate anaerobe that grows to sizes of 1,8 by 0,4 µm. Peritrichous flagella enable the bacterium to move in liquid cultures. Optimum cultivation temperatures range from 55 to 50 °C. In order to survive unfavorable conditions, spores are formed. During sporulation of vegitative cells a slight swelling can be observed (Fontaine et al., 1942). Very diverse metabolical capabilities allow growth on different substrates like methanol, pentoses, hexoses, formate, methoxylated benzoic acids, many two carbon-compounds like oxalate or glycolate and CO or CO_2 and H_2.

Acetobacterium woodii

Acetobacterium woodii was isolated from black sediment of a marine mouth of a river and enriched with H_2 and CO_2 as a substrate. These gram-positive cells are oval shaped rods that grow to a size of 1,0 by 2,0 µm. One or two sub terminal flagella enable them to move around in liquid substrates. Optimal growth occurs at temperatures of 30 °C and a pH of 6,8. However, at a pH of 5 cells stay viable for weeks. *Acetobacterium woodii* does not form endospores (Balch et al., 1977). A broad range of substrates is available to this organism including 2,3-butandiol, ethylene glycol, glycerol, sugars, acetoin, methosylated aromatic acids, formate, CO and H_2-CO_2. Mixotrophic growth, meaning the simultaneous utilization of H_2-CO_2 and organic compounds, is possible as well (Drake et al., 2006). Formation of acetate, growth and motility is highly dependent on sodium ions (Heise et al., 1989).

Despite very slow doubling times of 9 to 25 h (Daniel et al., 1990; Sakai et al., 2005) *Morella thermoacetica* and *Acetobacterium woodii* were chosen as potential production organisms, because they are considered model organisms for acetogens.

In order to determine and compare specific acetate productivities from H_2 and CO_2 as a sole substrate of *M. thermoacetica* and *A. woodii* in batch fermentations, both organisms first had to be adapted to a defined minimal medium without additional carbon sources like glucose to stimulate chemolithoautotrphic growth. Growth kinetics of both organisms and the influence of yeast extract (YE) on the cultivation *of M. thermoacetica* were determined in pH-controlled lab scale batch fermentations.

Besides low growth rates another limiting factor is the end product inhibition of growth by acetate (Wang and Wang, 1984). By using immobilized cells this issue may be overcome and higher efficiencies of the process may be achieved. Margaritis and Kilonzo (2005) stated, that microbial ethanol production was more effective when bacterial or yeast cells were immobilized in a bioreactor system. Furthermore, retaining immobilized cells in the bioreactor could enable a continuous production system with simultaneous acetate production and recovery and improve cell stability (Dolejš et al., 2014). The elimination of the lag phase and an efficient continuous operation without repeated inoculation are two more advantages of immobilizing Clostridia in this process (Jiang et al., 2009). A range of different immobilization matrices for a adsorption based attachment type process has been described in literature, including activated carbon, glass beads, pumice and ceramic stone as well as lignocellulosic biomass (Kumar et al., 2016). Further prominent carrier materials are polysulfone (PSU), nylon, celite, and polypropylene. Their porosities with pore sizes ranging from 0.22 to 500 µm and physico-chemical characteristics promote the adherence of microorganisms (Chauhan and Ogram, 2005). Chauhan and Ogram (2005) found that anaerobic consortia adhere better

to hydrophobic than to hydrophilic support materials. So generally speaking, hydrophobicity is a crucial factor for the selection of immobilization support materials.

Besides adsorption induced immobilization, other state of the art methods based on encapsulation, polymer and nano-materials are described in literature (Kumar et al., 2016) and may be worth to test.

The immobilization potential based on cell adsorption of A. woodii and M. thermoacetica were tested in experiments using a range of different carrier materials and synthetic membranes as carrier materials. Effects of biofilm formation on acetate formation and end product inhibition will be discussed.

1.4.3 Biotechnological applications

Biotechnological applications of acetogens have been investigated many times. Yet, there is no application available at industrial-scale (Drake et al., 2006).

Production of acetic acid from sugars

As acetogens possess the ability of converting sugars stoichiometrically into acetate, many studies tried to evaluate this potential for commercial applications. The microbiological state-of-the-art process for acetic acid production comprises the two-stage vinegar process, in which hexoses are converted into alcohols and subsequently into two moles of acetate by yeast. The production of acetic acid by acetogens could be feasible in a single stage process. Still, no commercialization has been realized due to two problems: First, acetogens are inhibited by high product concentrations, and secondly, they halt growth in acidic conditions (Wang and Wang, 1984). Therefore a commercially feasible acid production cannot be established, as at least 50 g/L acidic acid are required for such a process (Drake et al., 2006).

Bioremediation

The contribution of acetogens to the turnover of organic matter in bioreactors and landfills is enormous. Some organisms are able to degrade aromatic rings, although this feature is not very widespread. *Morella thermoacetica* showed the ability of sequestering the heavy metal cadmium and other certain acetogens were reported to de-halogenate anthropogenic compounds. In *situ* activities of these organisms are still poorly explored, since acetogenic processes are very complex in such systems. Therefore the potential for bioremediation of acetogens is still subject to research. The question, if acetogens can be used in remediation of environments or materials contaminated with anthropogenic substances, remains open (Drake et al., 2006; Dürre, 2005).

Bioconversion of synthesis gas to liquid chemicals

Acetogenic bacteria are able to convert synthesis gas, which mainly consist of CO, CO_2 and H_2, to acetate. Some strains, like *Clostridium ljungdahlii* or *Butyribacterium methylotrophicum* are able to convert these components to ethanol or butyrate (Buschhorn et al., 1989). A process for the electrochemical conversion of CO_2 to acetate using enzymes of *M. thermoaceticа* is known as well. (Drake et al., 2006)

However, the microbial conversion of H_2 and CO_2 to acetate could be a useful combination of an improved storage strategy of hydrogen and peak currents and the sensible utilization of industrial off gas CO_2.

1.5 From acetate to butanol

1.5.1 The ABE-fermentation

The second part of the process comprises the microbial conversion of acetate to liquid biofuels. The underlying metabolic mechanism for this transformation is the anaerobic ABE-fermentation. In the following a physiological description of strictly anaerobic Clostridia that are able to carry out ABE-fermentation is delivered.

A bi-phasic fermentation is a typical characteristic of the clostridial solvent production. At the beginning of the fermentation substrates like hexoses or pentoses are taken up. Hexoses are degraded via glycolysis and pentoses via the pentose phosphate pathway (Schiel-Bengelsdorf et al., 2013). Figure 5 represents an overview of the ABE-pathway. During the initial growth phase, the acidogenic phase, acid-forming pathways are activated and CO_2, H_2, acetate and butyrate are formed as main products. Lactic and succinic acid may also be produced to some extent (Dürre, 2005). During the second phase, the solventogenic phase, which occurs during stationary growth, the formerly secreted acids are re-assimilated and transformed into butanol, ethanol and acetone (Jones and Woods, 1986). The transition from acetogenesis to solventogenisis results from a severe change in gene expression (Lee et al., 2008). Typically, a carbohydrate substrate is converted to a mixture of acetone, butanol and ethanol in the ratio of 3:6:1. (Gutierrez et al., 1998). Typically natural solventogenic strains can produce up to 20 g/L ABE (Qureshi and Blaschek, 2001) with 10.5 (Richmond et al., 2012) to 13 g/L butanol, which is considered the upper limit for natural strains (García et al., 2011; Qureshi and Blaschek, 2001)

Shift from acetogenesis to solventogenesis

Due to the accumulation of acids and the incapacity of cells to maintain a constant pH, the internal (usually ca. 1 unit higher than the external) and the external pH reach the lowest points during fermentation (Lee et al., 2008; Schiel-Bengelsdorf et al., 2013). At a pH of 4,5 most of acetic and butyric acids are un-dissociated and able to cross the cytoplasmic membrane. The acids dissociate inside of the cell, as the internal pH is about 5 ($pKa_{acetic\ acid}$ = 4,75; $pKa_{butyric\ acid}$ = 4,82). Thereby, the existing proton gradient over the cytoplasmic membrane collapses due to the released protons and results in cell death. As a countervailing measure cells converts these acids to solvents resulting in an increase of the surrounding pH (Kumar et al., 2013) and a prevention of acidification (Schiel-Bengelsdorf et al., 2013). The

low pH acts as a trigger for the induction of solventogenesis. The solventogenic phase is closely linked to sporulation (Liu et al., 2013) to assure a long-term survival of cells as high concentrations of butanol are toxic as well. From this point of view, solventogenisis is just a cellular tactic to gain time for the completion of sporulation (Schiel-Bengelsdorf et al., 2013). The exact mechanism and significance of triggers for this shift are still not well understood. Most likely a combinatorial integration of a great number of cellular stimuli is responsible for this transition (Kumar et al., 2013). Studies suggest that the impact pH and phosphate and nitrogen limitation contribute a great deal to this process (Bahl et al., 1982; Roos et al., 1985).

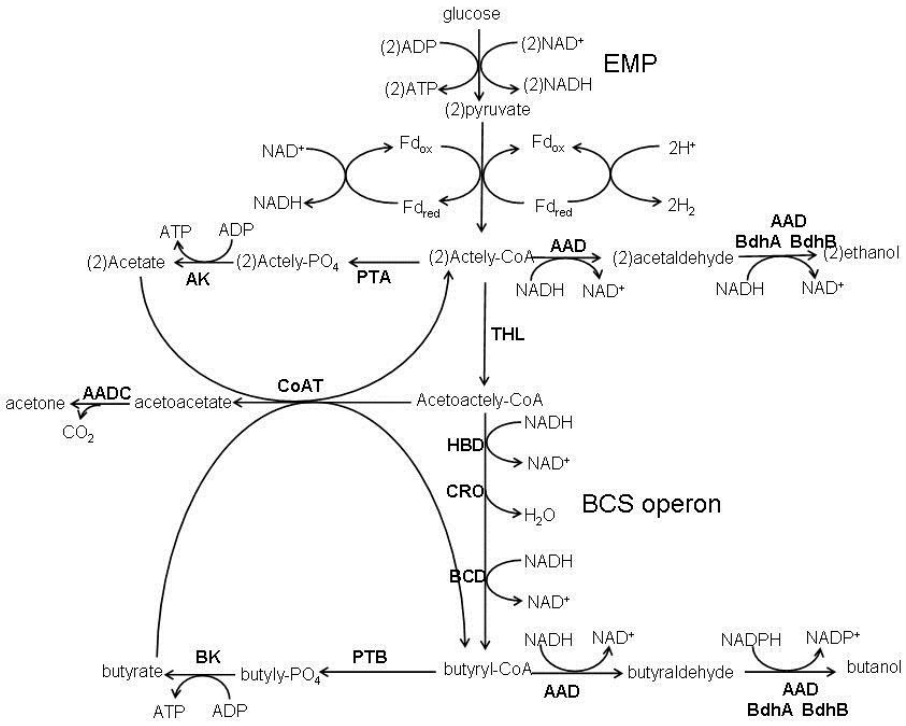

Figure 5: ABE-fermentation pathway (Liu et al., 2013)

By using the solventogenesis as an access point with acetate as a substrate a new way in ABE-fermentation is opened. The solventogenic phase will be triggered by manually lowering the external pH. Reduction equivalents for the conversion of organic acids to solvents will be supplied in the form of H_2 gas in the headspace of fermentation vessels. Experiments for the evaluation of the potential of *C. beijerinckii* to grow on and directly convert acetate into solvents were conducted. By the usage of microorganisms with high acetate utilization high efficiencies in acetate conversion could be achieved.

History of ABE-fermentation

Louis Pasteur observed butanol-producing bacteria as early as in 1861. 51 years later, a bacterium called *Clostridium acetobutylicum*, which was able to ferment starch to acetone, butanol and ethanol was discovered by the so-called "father of ABE-fermentation" (Dürre et al., 1992), Chaim Weizmann. The focus of this fermentation process shifted in the advent of World War I from the production of butanol to the production of acetone, which could be used in manufacturing cordite, a component in explosives (Zverlov et al., 2006). Due to its extraordinary product formation, Weizmann's *C. acetobutylicum* was chosen for acetone production in the former British Empire and the US. This steady supply of acetone was a crucial factor in winning the war. Weizmann declined any financial or official acknowledgements by the government. This and his positive attitude towards a Jewish homeland in Palestine, affected the Balfour declaration of 1917 and led to the foundation of the State of Israel, with him becoming its first president (Dürre, 2008). As Weizmann's patent expired, fermentation plants were built across the US and ABE-fermentation became the second major industrial fermentation process after yeast-based ethanol production. After a plunge in usage due to unfavorable economical conditions (Zverlov et al., 2006), interest in this technology reestablished years later. In the 90s tremendous effort was put into the development of strains with improved fermentation characteristics (Ezeji et al., 2004; Jones and Woods, 1986).

Figure 6: Chaim Weizmann, ("Wikipedia," 2016)

1.5.2 ABE-Clostridia

For commercial butanol production the classical solventogenic clostridiae are: *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum, C. saccharoperbutylacetonicum.* Those organisms are known as model organisms for ABE fermentation, with *C. acetobutylicum* as the most prominent one. The biphasic metabolism is well understood in its details. Gene expression as well as enzymes involved in solvent production are well characterized (Schiel-Bengelsdorf et al., 2013).

Isolation of these solvent producing and spore forming bacteria is relatively easy, as they have quite simple growth requirements. Usually these organisms are associated with living

plant material. Roots of nitrogen-fixing legumes, potatoes and other root crops have been found to deliver optimal growth conditions. Additional habitats are agricultural soil, cereal crops, and fruits like gooseberries (Jones and Woods, 1986).

Clostridium beijerinckii

Due to the fact, that a viable cell line was already established at the laboratory of IFA-Tulln, experiments on ABE-fermentation were conducted with *C. beijerinckii* as a production organism. Therefore only this organism will be described in the following.

Clostridium beijerinckii species are commonly isolated from soil samples, as they are omnipresent in nature. The wild-type *C. beijerinckii NCIMB 8052* is a strictly anaerobic, mesophilic, rod-shaped bacteria with sub-terminal, oval spores. Motility is enabled by peritrichous flagella. The potential for the production of acetone and butanol and its broad substrate range including pentoses, hexoses, starch and others make *C. beijerinckii* biotechnologically interesting ("Clostridium beijerincki NCIMB 8052," 2016). Further fermentation products include lactate, butyrate, acetate, H_2, CO_2, acetoin, and acetyl methyl carbonyl. Cell morphology changes over the growth cycle. During exponential growth, cells are lone, filamentous and very motile. As the stationary phase, which corresponds to solventogenisis, is approached, cells show a lower level of motility as they shorten and become plumb (Chen and Blaschek, 1999). Furthermore this bacteria is responsive to genetic improvement and highly solvent producing strains have been engineered (Annous and Blaschek, 1991).

2 Scope

The scope of this thesis focuses on the microbial conversion of hydrogen and carbon dioxide into the storable intermediate acetate and liquid fuels.

It is divided into two sections:

First the microbial conversation of H_2/CO_2 into acetate, called homoacetogenesis; Secondly, the biological transformation of acetate into the liquid energy carriers acetone, butanol and ethanol (ABE). This process is referred to as ABE-fermentation.

The first part of this work was devoted to the adaptation of two homoacetogenic strains, *Morella thermoacetica* DSMZ 2955 and *Acetobacterium woodii* DSMZ 1030, to a defined minimal medium using only H_2 and CO_2 as a substrate. This established the basis for CO_2 to serve as the sole carbon source as well as for establishing mass balances. Subsequently pH-controlled batch fermentations were conducted for the determination of productivities and maximum product concentrations. Subsequently it was aimed to show a decrease in lag-phases may be reached by whether adding YE to the fermentation or glucose to pre-cultures. As it is mentioned in literature that immobilizes systems provide higher productivities and in preparation for a continuous fermentation system subsequent immobilization experiments had to be conducted. Besides different carrier materials hollow fibers were to be tested for their immobilization capacity.

Last part of the thesis was the investigation of direct conversion of exogenous acetate into ABE using *Clostridium beijerinckii* DSMZ 1739 as a production strain. It shall be shown whether instead of the generally applied two-step procedure a single step solventogenisis can be achieved.

3 Materials and Methods

Lists of used laboratory equipment and chemicals can be found in Annex.

3.1 Media

3.1.1 Complex Medium (CM)

Table 2: Composition of CM

Medium Component	Concentration
Trypticase Peptone	5.00 g/L
Peptone from meat	5.00 g/L
Yeast extract	10.00 g/L
Salt solution 1	40.00 mL/L
L-Cysteine-HCl*H_2O	0.50 g/L
D-Glucose	5.0 g/L
Distilled Water	1000 mL

The composition of CM (Table 2) is based on the medium 104b provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

All ingredients except glucose and L-Cysteine were dissolved in double distilled water (ddH_2O), the pH was adjusted to 6.8 and the medium autoclaved at standard autoclaving conditions (20 minutes at 121 °C and 1 bar overpressure). Thereafter, the solution was sparged with 100 % N2 for 30 minutes and glucose was added from a sterile anoxic stock solution (400 g/L). L-Cystein was dissolved in 1 mL of ddH2O and added under sterile conditions.

Table 3: Composition of salt solution 1

Component	Concentration
$CaCl_2$*$2H_2O$	0.25 g/L
$MgSO_4$*$7H_2O$	0.50 g/L
K_2HPO_4	1.00 g/L
KH_2PO_4	1.00 g/L
$NaHCO_3$	10.00 g/L
NaCl	2.00 g/L

All components were added together in the corresponding amounts displayed in Table 3 and dissolved in ddH_2O by stirring at a temperature of 40 °C for 30 min.

3.1.2 Minimal Media

Morella thermoacetica

Table 4: Minimal Medium 1 (MM1)

Medium Component	Concentration
KH_2PO_4	2.00 g/L
K_2HPO_4	3.00 g/L
NH_4Cl	2.00 g/L
Salt solution 2 100x	10.00 mL/L
L-Cystein-hydrochloride-monohydrate	0.50 g/L
Sodiumsulfide-nonahydrate	0.20 g/L
Vitamine solution 100x	10.00 mL/L

The composition of this medium (Table 4) is based on a mixture of literature sources (Dabrock et al., 1992; Hatch and Finneran, 2008; Lund et al., 1984). L-Cystein-hydrochloride-monohydrate and Sodiumsulfide-nonahydrate were used as reducing agents. KH_2PO_4, K_2HPO_4, NH_4Cl were weight out, added together with the salt solution to ddH_2O, dissolved and autoclaved at standard autoclaving conditions (20 minutes at 121 °C and 1 bar over-pressure). Subsequently the medium was cooled to 50 °C, purged with N_2 for 20 minutes in order to create anaerobic conditions and then completed with L-Cystein-hydrochloride-monohydrate, sodiumsulfide-nonahydrate and vitamine solution, which were added through a Swanlock® - valve using a disposable sterile filter. Gassing and addition of components was done using a 2 L bottle with a three-port-lid.

Two autoclavable membrane filters and one Swanlock® - valve were connected with the lid by hoses. The gas was led through one sterile membrane filter into the liquid, while the exiting gas was led out through a hose only reaching into the headspace. The third (sampling-) port was closed using a metal clamp. This setup was used for preparation of anaerobic flasks, also referred to as serum bottles. Before each usage, the medium was cooled to the respective culturing temperature and flushed with N_2 to ensure anaerobic conditions.

Table 5: Composition of the vitamin solution 100x

Component	Concentration
Kao & Michayluk solution	1000 mL
Niacin	200.00 mg/L
Haemin	500.00 mg/L
Vitamin K1	50.00 mg/L
Lipoic acid	200.00 mg/L

The vitamin solution was prepared according to Lund et. al. Niacin, haemin, vitamin K1 and lipoic acid were weight out into 1.5 mL reaction tubes. Subsequently Niacin was dissolved in 1 mL ddH_2O, haemin in 1 mL NaOH 1M and vitamin K1 and lipoic acid in 1mL 97% ethanol. All four substances were added to Kao & Michayluk vitamin solution. Remaining haemin crystals were resolved by adding 3 mL NaOH 2 M to a total volume of 100 mL of vitamin solution, which then was aliquoted and stored at -20 °C. The corresponding composition is shown in **Table 5**.

Table 6: Composition of the salt solution 2 100x

Component	Concentration
NaCl	50.00 g/L
$MgCl_2$*6H_2O	25.00 g/L
$CaCl_2$*2H_2O	5.00 g/L
Na_2EDTA*2H_2O	640.00 mg/L
$FeCl_2$*4H_2O	500.00 mg/L
Na_2SeO_4	130.00 mg/L
$CoCl_2$*6H_2O	170.00 mg/L
Na_2WO_4*2H_2O	100.00 mg/L
$NiCl_2$*6H_2O	250.00 mg/L
$MnCl_2$*4H_2O	290.00 mg/L
$ZnCl_2$	50.00 mg/L
$NaMoO_4$*2H_2O	50.00 mg/L
$CuSO_4$*5H_2O	25.00 mg/L
H_3BO_3	15.00 mg/L
$AlCl_3$*6H_2O	5.00 mg/L

All components were added together in the corresponding amounts displayed in Table 6 and dissolved in ddH_2O by stirring at a temperature of 40 °C for 30 min.

Clostridium beijerinckii

Minimal medium adapted

For cultivation of *C. beijerinckii* MM1 was mixed with glucose (5 g/L).

Acetobacterium woodii

Minimal medium 2 (MM2)

Table 7: Composition of MM2

Component	Concentration
Yeast extract	0.50 g/L
NH_4Cl	0.50 g/L
K_2HPO_4	1.00 g/L
KH_2PO_4	0.45 g/L
$MgSO_4$*/H_2O	0.10 g/L
$NaHCO_3$	10.00 g/L
Na_2SO_3	0.50 g/L
L-Cystein-hydrochloride-monohydrate	0.50 g/L
Resazurin	1.00 mg/L
Salt solution 2 100x	20.00 mL/L
Vitamin solution 100x	10.00 mL/L

This medium was prepared according to a mixture of literature sources (Atlas, 2010; Rothe and Thomm, 2000). A 1000 mL volumetric flask was filled up to approximately 500 mL with ddH_2O. Yeast extract, NH_4Cl, K_2HPO_4, KH_2PO_4, $MgSO_4$ and salt solution were added and filled up to 1000 mL. Final concentrations of ingredients are shown in Table 7. The content was transferred into a large media bottle with a sparging setup, including a sparging stone. The bottle was placed into a 70 °C water bath and sparged with N_2 for at least 15 minutes for oxygen removal. $NaHCO_3$, Na_2SO_3, L-Cystein-hydrochloride-monohydrate and resazurin, were added during sparging. Resazurin is a redox indicator, which indicates fully (colorless) or partially (slightly pink) anoxic conditions. Anaerobic flasks were purged with N_2. The medi-

um was then distributed into serum bottles using a pipette (40 mL medium per bottle). The serum bottles were sealed, the headspace sparged with N_2 and autoclaved at standard conditions. The media were colorless after autoclaving. They turned pink during inoculation or sparging with N_2 or H_2, due to small amounts of O_2 present in the gas line. After some hours the liquid turned colorless again. The initial pH of MM2 was 9.2, but changed to 6,8 upon CO_2 equilibration (substrate gas).

Table 8: Composition of salt solution 2 100x

Component	Concentration
$MgSO_4$*7H_2O	3.00 g/L
Nitrilo-triacetic acid	1.50 g/L
NaCl	1.00 g/L
$MnSO_4$*2H_2O	500.00 mg/L
$CoSO_4$*7H_2O	180.00 mg/L
$ZnSO_4$*7H_2O	180.00 mg/L
$CaCl_2$*2H_2O	100 mg/L
$FeSO_4$*7H_2O	100.00 mg/L
$NiCl_2$*6H_2O	25.00 mg/L
$KAl(SO_4)_2$*12H_2O	20 mg/L
H_3BO_3	10 mg/L
Na_2MoO_4*4H_2O	10 mg/L
$CuSO_4$*5H_2O	10 mg/L
Na_2SeO_3*5H_2O	0.30 mg/L

Nitrilo-triacetic acid was added to 500 mL ddH_2O, dissolved by adjusting the pH to 6,5 with KOH. The remaining components were added, the solution was filled up to 1000 mL with ddH_2O and thoroughly mixed. The exact composition of the salt solution is shown in Table 8.

Vitamin solution

See media preparation *M. thermoacetica*.

3.2 Anaerobic cultivation of Clostridia

3.2.1 Preparation of anaerobic flasks

For small-scale batch cultivation with uncontrolled conditions 100 mL anaerobic serum bottles were used. Flasks used for stirred cultures were equipped with a small magnetic stirrer while bottles for shaking cultures were left empty. All bottles were closed with butyl stoppers and sealed with metal crimps. Subsequently the flasks were flushed with N_2 (100 %) at a flow rate of 60 mL/min for 15 minutes, so that the gas volume was at least exchanged 10 times. In order to establish a constant gas flow, two needles were plugged into the butyl stopper. One of them was connected to the gas line. The other one was used as a gas exit. Figure 6 gives a visual representation of this setup. Subsequently, the bottles were autoclaved at standard conditions and stored at room temperature.

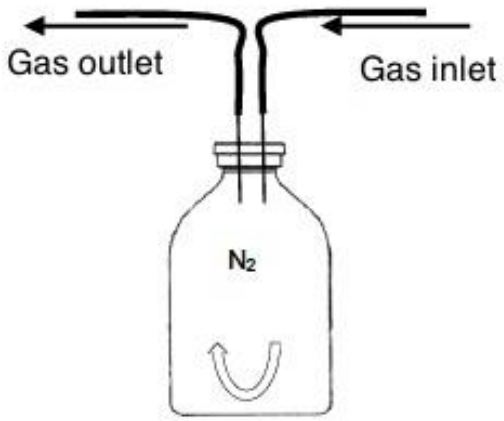

Figure 6: Gassing setup for serum bottles (Morris, 2015)

3.2.2 Implementation of a working cell bank

To guarantee a sufficient amount of inoculation material for further experiments, a working cell bank of each relevant organism was established. For that reason an exponentially growing cell culture was mixed with glycerol (70 %) in a ratio of 1:1 and gently mixed. The resulting solution was portioned into cryo-vials using a disposable syringe and aliquots of 2 mL were kept at -80 °C. All manipulation steps were conducted under anoxic conditions in an anaerobic tent.

3.2.3 Inoculation of active cultures

For the re-cultivation of frozen cell cultures, two cryo-vials were slowly thawed at room temperature. Approximately 10 mL of the corresponding medium were put into a sterile serum bottle. Then the cell solution was carefully poured into the flask, which then was sealed and purged with either substrate gas or N_2 for 5 minutes at a gas flow rate of 600 mL/min. The gassing design was basically the same as shown in Figure 6. A sterile filter was added between gas line and inlet needle, to maintain sterile conditions. *Morella thermoacetica* and *A. woodii* cultures were pressurized to approximately 1000 mbar with substrate gas. In the case of *C. beijcherinckii* glucose was added as substrate. The culture was incubated according to specific cultivation conditions. As soon as substrate consumption was monitored further substrate was periodically added until a working volume of 50 mL was reached. From then on, the cultures were passaged as described in section 3.2.4.

3.2.4 Cultivation conditions, sampling and passaging

Acetobacterium woodii and *M. thermoacetica* were grown in shaking cultures, whereas *C. beijerinckii* was continuously stirred. In all three cases the culture volume was 50 mL. Cultivation conditions for all three bacteria are listed in Table 9.

Table 9: Flask cultivation conditions for *A. woodii, M. Thermoacetica and C. beijerinckii.*

Organism	*A. woodii*	*M. thermoacetica*	*C. beijerinckii*
Temperature	30 °C	55 °C	35 °C
RPM	150	150	280
Substrate	H_2/CO_2 (80/20 %)	H_2/CO_2 (80/20 %)	Glucose, c = 5 [g/L]
Initial pressure	1000 mbar	1000 mbar	6.8
pH	6.8	6.8	N_2
Redox-potential	-160 - -210 mV	-160 - -210 mV	-160 - -210 mV

Initially, growth kinetics were determined. Substrate consumption, product concentrations and pH-value were monitored on a regular basis. For that reason samples were taken of each active cell culture according to following procedure:

The cultures were taken out of the incubator and allowed to cool down to room temperature. This guaranteed constant conditions for pressure determination. The pressure was measured by puncturing the butyl stopper with a needle connected to a pressure gauge. For sampling, the culturing vessels, sterile needles, syringes and 1.5 mL reaction vials were wiped

with EtOH 70 % and transferred into the sterile workbench. The cultures were shaken and 1 mL samples were withdrawn using syringes with needles and transferred into reaction vials.

From these samples optical density, concentrations of volatile fatty acids and in case of *C. beijerinckii* also the pH and glucose concentration were determined. The sample preparation for HPLC determination of these analytes is described in section 3.6.

Passaging

Cell cultures were passaged, as soon as substrate consumption dropped to zero and indicator parameters, including acetate concentration, optical density and pH reached a certain threshold.

Cultures of *M. thermoacetica* and *C. beijerinckii* were passaged in the sterile workbench, whilst *A. woodii* was manipulated in the anaerobic tent. Using sterile 50 mL syringes 35 mL of a *M. thermoacetica* or 25 mL of a *C. beijerinckii* culture were replaced by fresh medium. *Acetobacterium woodii* cultures were passaged by transferring 10 mL cell suspension into a serum bottle containing fresh medium (see 3.1.2). *Morella thermoacetica* and *A. woodii* cultures were then pressurized; *Clostridium beijerinckii* cultures were enriched with glucose and incubated at the corresponding culturing conditions.

3.3 Cultivation of homoacetogenic clostridia

3.3.1 Media adaption

It was known from previous experiments that *M. thermoacetica, A. woodii* and *C. beijerinckii* could not directly be passaged from a complex medium to a minimal medium. Hence, a progressive adaption according to pattern shown in Figure 7 was conducted:

Figure 7: Adaption scheme of *M. thermoacetica, A. woodii* and *C. beijerinckii;* All strains were re-cultivated in PYX medium. At each step of the adaption process, the cultures were passaged 3 times. Before incubation, serum bottles were pressurized with substrate gas in the headspace. After four steps of passaging an adaption to 100 % minimal medium was achieved.

Acetobacterium woodii

To test the growth behavior and product formation in a pure minimal medium, a gradual adaption of *A. woodii* on minimal medium without YE was conducted. Figure 8 represents the underlying adaption scheme. After three steps of passaging an adaption to 0 g/L YE was achieved.

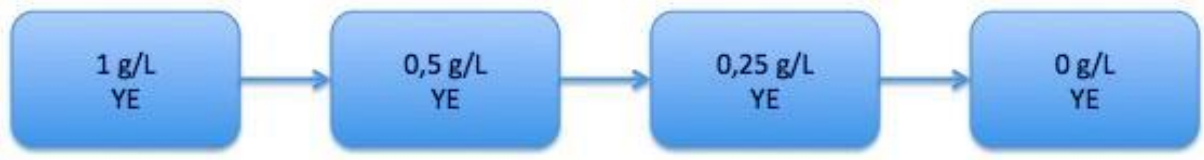

Figure 8: Adaption scheme of *A. woodii*

3.3.2 Batch Fermentation

Controlled batch fermentations of *A. woodii* and *M.* thermoacetica were conducted in 1 L lab scale bioreactors (INFORS-HT). The working volume was 600 mL. Controlled parameters were T, pH and stirrer speed.

Preparation of the fermentation system

Before fermentations could be started, preparatory steps had to be taken. The calibration of the pH-probe was carried out for two points - one at pH=7.00 and one at pH=4.02. A total volume of 1200 mL of minimal medium (MM1 or MM2 for *M. thermoacetica* and A. woodii, respectively) were prepared and equally divided over 2 fermenters. All connection tubes, valves and sealing plugs etc. were attached to the bioreactors. The fully assembled fermentation unit was placed in the autoclave and sterilized for 20 min. at 121 °C and 1 bar over pressure. Substrate gas was continuously introduced at a flow rate of 0.07 L/min. Cooling water was connected and the medium was heated to the corresponding operation temperature. Reducing agents and vitamin solution were aseptically added to the sterile medium using sterile filters and syringes. After that, a one-point calibration of the pO_2 probe was done.

Inoculum preparation

A number of pre cultures was chosen in a way that the fermenters could be inoculated with an OD_{600} of 0.1. Glucose was added to a concentration of 5 g/L to inoculation cultures of *M. thermoacetica.* Depending on the desired organisms 2 - 5 pre cultures per reactor were transferred into 50 mL centrifugation tubes and centrifuged at 3000 rpm for 5 minutes. Cell pellets were pooled and re-suspended in 10 mL medium. This suspension was equally distributed over two fermenters (see preparation of the fermentation system). The handling of cell cultures was done under anoxic conditions in an anaerobic tent.

Process start

After all preparations were completed, process parameters (Table 10) were set and a process was started in the control software IRIS 6 (provided by INFORS HT). Samples were taken prior to and directly after inoculation.

Table 10: Process parameters for batch fermentations of *M. thermoacetica* and A. woodii in 1 L bioreactors (INFORS-HT)

Parameter	Setpoint
T	55/33 °C
Stirrer speed	400 rpm
pH	6.8
Gas flow rate	0.07 mL/min

Fermentation

Constant on-line monitoring of crucial operational parameters (Table 11) allowed a controlled process. A pH drop due to growth associated acetic acid production was compensated by steady addition of 3 M NaOH by the control unit.

Samples were taken at intervals of approx. 24 h in the stationary phase. As soon as exponential growth was detected, samples were taken two to three times a day.

Table 11: Fermentation parameters

On-line parameters	Off-line parameters
pH	OD_{600}
pO_2	VFA concentrations
T	Ammonium concentration
rpm	
Gas flow rate	

Batch fermentations of *M. thermoacetica* were performed using the media MM1 and MM1 + YE (2 g/L) and *A. woodii* was cultured in MM2.

Comparison of different for growth determination methods

Three different methods for cell growth determination were applied in the first fermentation of *M. thermoacetica*:

Cell count

The cell concentration of a fermentation suspension was determined by cell counting in a Thoma chamber. Using the smallest magnification (100x) the microscope was focused and the distribution of cells was visually checked for homogeneity. Cells were then counted in 4 large squares at a magnification of 400x. Dilutions of cell suspensions were chosen in a way that the number of cells in one large chamber was between 50 and 150. The cell concentration was calculated from the counting chamber volume, the number of counted cells and dilution factors. Four large squares were enumerated for each sample.

$$V = a^2 * d = 0.05^2\, mm * 0.1\, mm = 0.00025\, mm^3 \mathrel{\widehat{=}} 2.5 * 10^{-7}\, mL$$

V = Volume of a large square

a = side of the square

d = depth of counting chamber

$$N = \frac{Sum\ of\ cell\ counts\ per\ large\ square}{nr.\ of\ larg\ square}$$

N = mean cell count per large square

$$n = \frac{N}{V} * f$$

n = cell concentration [cells/mL]
f = dilution factor; e.g.: Dilution of 1:2 ➔ f = 2

Optical density

The optical density was measured using a two-beam-photometer at a wavelength of 600 nm. Since a change in absorption over time of pure medium was monitored, roH_2O was used as a blank. Linearity of absorption is assumed in a range of 0.1 - 0.8, which assures direct proportionality between absorption and cell density. For this reason samples of densely growing cultures were diluted so that their absorption values shifted to the appropriate range. Measured OD_{600} values had to be corrected by the dilution factor.

$$OD_{600} = OD_{600,measured} * dilution\ factor$$

Dry weight

Another method for evaluating cell growth was the determination of the cell dry weight. Therefor a membrane filter was left in a desiccator for at least 12 h, weighed (tare weight [mg]) and applied on a vacuum filtration flask with a water suction pump attached to it. An 8 mL fermentation sample was pipetted onto and then sucked through the filter, which retained the biomass. The filter then was carefully removed with tweezers, placed into a petri dish and dehumidified in a drying chamber (105 °C) for at least 12 h. It was then placed into a desiccator for another 12 hours to cool and finally weighed. The weight of the biomass was determined (gross weight [mg]) by subtracting the weight of the unloaded filter before filtration from the weight of the loaded one.

$$net\ weight = gross\ weight\ -\ tare\ weight$$

3.4 Immobilization of cells

Prior to cell immobilization in a 1 L batch fermentation pre - experiments were carried out in order to test different materials for their immobilization potentials. Each material was tested in duplicate.

3.4.1 Flask scale

Immobilization carrier:

- Media only (Negative control)
- Activated carbon
- Celite
- Cellulose Powder
- Cellulose Phosphate
- Chitin
- Clay
- Meth max
- Migusan
- Pipe cleaner
- Wood chips

Immobilization membranes

- Nylon (hydrophobic)
- PSU (hydrophilic)
- Mixed cellulose ester (hydrophobic)
- Glass fiber (hydrophobic)

Preparation of immobilization carrier materials

Approximately 0.1 g of each material was weight out separately into 100 mL serum bottles and covered with MM1. The liquid was flushed with N_2 for approx. 20 min to eliminate oxygen from the system. For equilibration the bottles were autoclaved at 121 °C for 25 min and left to cool down. The medium was removed by centrifugation and replaced by fresh one. Reducing agents were added to each flask, which were subsequently autoclaved again at 121 °C for

25 min and left for equilibration and cooling down for 13-14 h. Vitamins were added using syringes and sterile filters (0.22 µm). The flasks were again left to equilibrate for 24 h and finally inoculated.

Preparation of carrier membranes

Pieces of the same size (20 cm^2) were excised from membrane sheets and inserted into serum bottles, in a way that one part dipped into the liquid and the other stayed in contact with the gassed headspace. The bottles were sealed, gassed with N_2 and autoclaved. The already cooled down flasks, which did not show degradation of the carrier material were inoculated.

Inoculation

For inoculation, 16.6 % (v/v) cell culture with an OD_{600} of approximately 0.15 were added to prepared flasks. The headspace of each flask was purged with substrate gas (H_2/CO_2 80/20 %) for 10 min and pressurized to approx. 1000 mbar overpressure. Cells were incubated in a shaker with 150 rpm at 55 °C.

Every 3-4 days gas pressures in the flasks was measured and liquid samples were taken using sterile syringes. In case the pressure dropped below 300 mbar, flasks were repressurized. Acetate production was monitored using High performance liquid chromatography.

3.4.2 Batch fermentation with membrane module

In this experiment cells were immobilized in batch fermentation by recirculating the broth through a hollow fiber membrane module with substrate gas passing through fibers. The HFMB module consists of polypropylene (PP) fibers pasted into a polycarbonate- (PC) shell. The experimental design was inspired by Shen et al. (2014) who did experiments on optimization of gas and liquid flow rate on a similar system. Fermentation system used of this working group was eight times larger (working volume of reactor: 8 L, membrane surface: 1.4 m^2). A scale down was conducted to meet given laboratory prerequisites. Data sheets of the respective membrane modules can be found in the annex. Calculations were based flow velocity Shen et al. (2014) used in their experiments. Fiber specifications obtained from Shen et al. (2014).

Determination of liquid flow velocity (Shen et al., 2014):

d_i = 220 µm

r_i = 110 µm

n_F = 7400

Q = 0.0002 m^3/min

$$A = r_i^2 * \pi * n_F = 0.00028\, m^2$$

$$v = \frac{Q}{A} = 0{,}71\ {}^{m}/_{min}$$

Determination of flow rate Q' based on constant liquid flow velocity:

d_i' = 220 µm

r_i' = 110 µm

n_F' = 2300

v = 0.71 m/min

$$A = r_i^2 * \pi * n_F = 0.000087\, m^2$$

$$Q' = v * A = 0.00006\ {}^{m^3}/_{min}$$

This calculation revealed an optimal adapted flow rate of 60 mL/min. Taking less biomass, and the equipment limitation (maximum pump capacity not sufficient) into account, a flow rate of 19 mL/min was chosen.

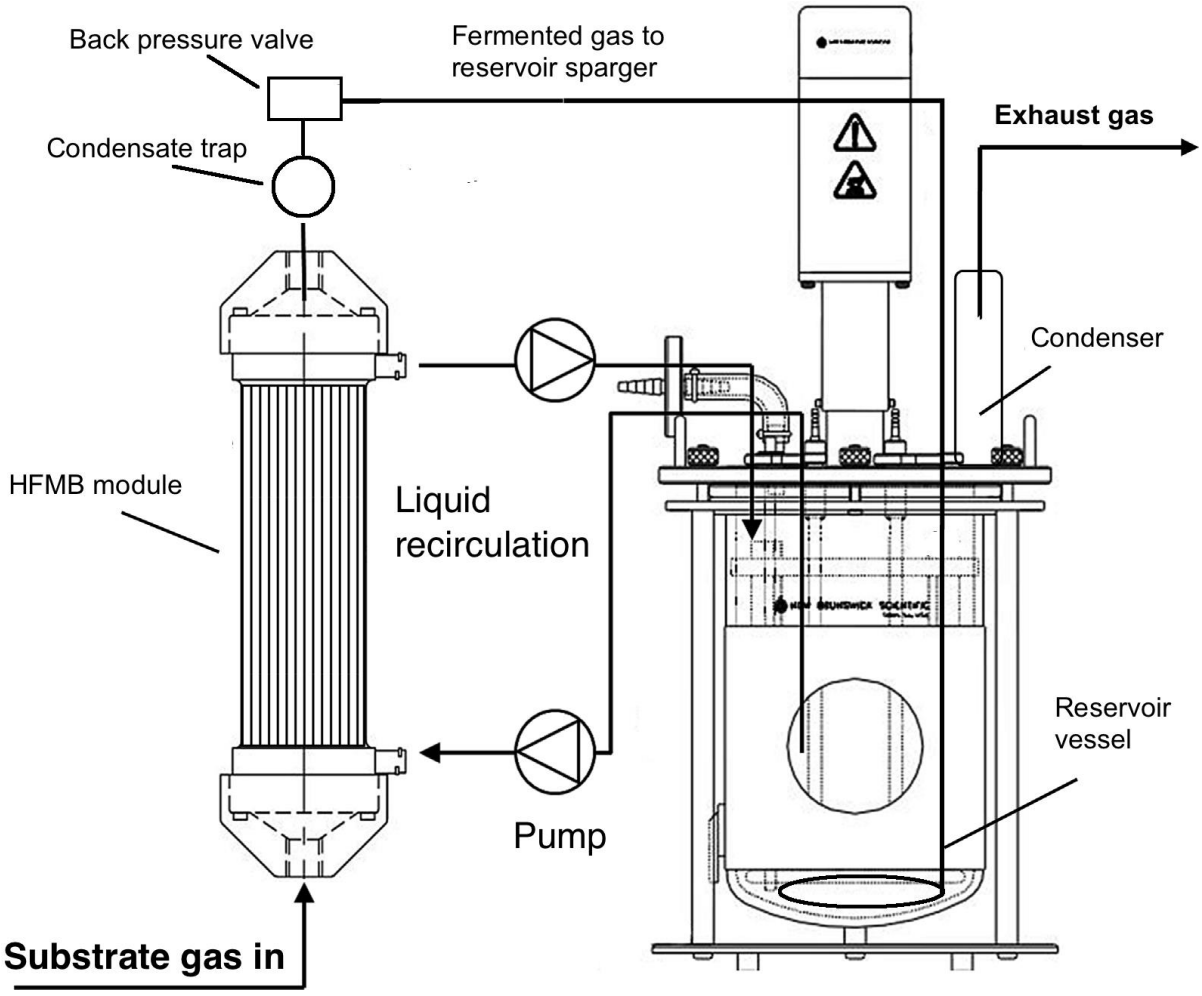

Figure 9: Schematic representation of recirculation design. (Shen et al., 2014, adapted)

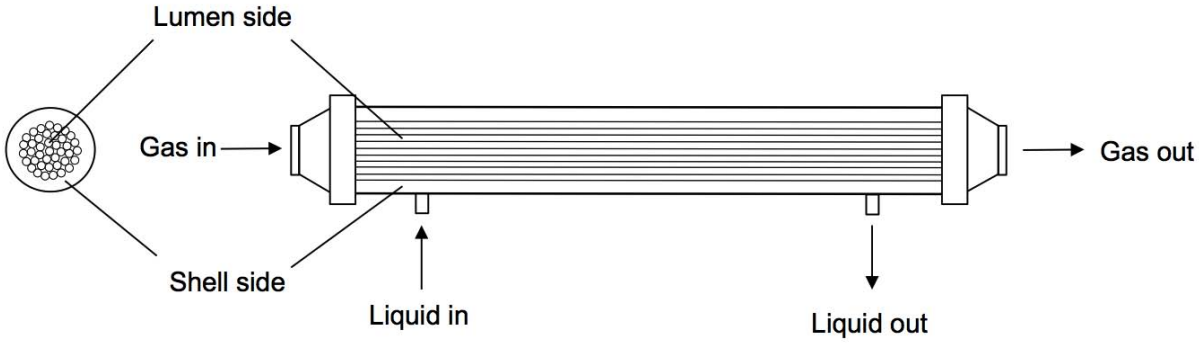

Figure 10: Schematic layout of a HFMB module

A batch fermentation was prepared and started as described above. The organism of interest was *M. thermoacetica*, and therefore all parameters were chosen accordingly. Before an immobilization via recirculation through the HFMB-module was started, the recirculation unit was sterilized and assembled as follows:

The shell side (Figure 10) of the module was filled and then rinsed with EtOH 70 % at a flow rate of 1 mL/min for 24 h. To ensure that no remains of alcohol were present in the module, it was washed with 2 L of sterile ddH_2O, while the lumen side (Figure 10) was flushed with N_2.

As soon as exponential growth of the fermentation culture was monitored, the module was connected to the fermenter. The broth was pumped out of the reactor into the module (shell side) through the sampling port and then returned into the fermenter through the inoculation port. A peristaltic pump established recirculation.

The gas line was directly attached to the lumen side of hollow membrane fibers. Sterile conditions were created by a membrane pore size of 0.04 µm. An overpressure of approx. 950 mbar was established in side of hollow fibers to ensure gas transfer through the membrane into the liquid. Possible vapor in the gas phase was condensed and dripped into a condensation trap attached between the membrane module and the backpressure valve. The gas was allowed to flow intermittently from the backpressure valve into the fermenter and then out via the off-gas line. The experimental setup is illustrated in Figure 9.

In order to reduce heat loss, the module was insulated with neoprene sheets and aluminum foil. Recirculation hoses were as well wrapped in aluminum foil.

Important parameters for immobilization are listed in Table 12.

Table 12: Recirculation parameters

Parameter	Setpoint
Gas flow rate	0.02 mL/min
Gas pressure in fibers	850 - 950 mbar
Recirculation flow rate	19 mL/min

3.5 Cultivation of ABE-Clostridia

As mentioned above *C. beijerinckii* was cultured in 100 mL serum bottles. Four active cultures were passaged on a regular basis and used as master seeds for upcoming experiments.

3.5.1 Fermentation on acetate

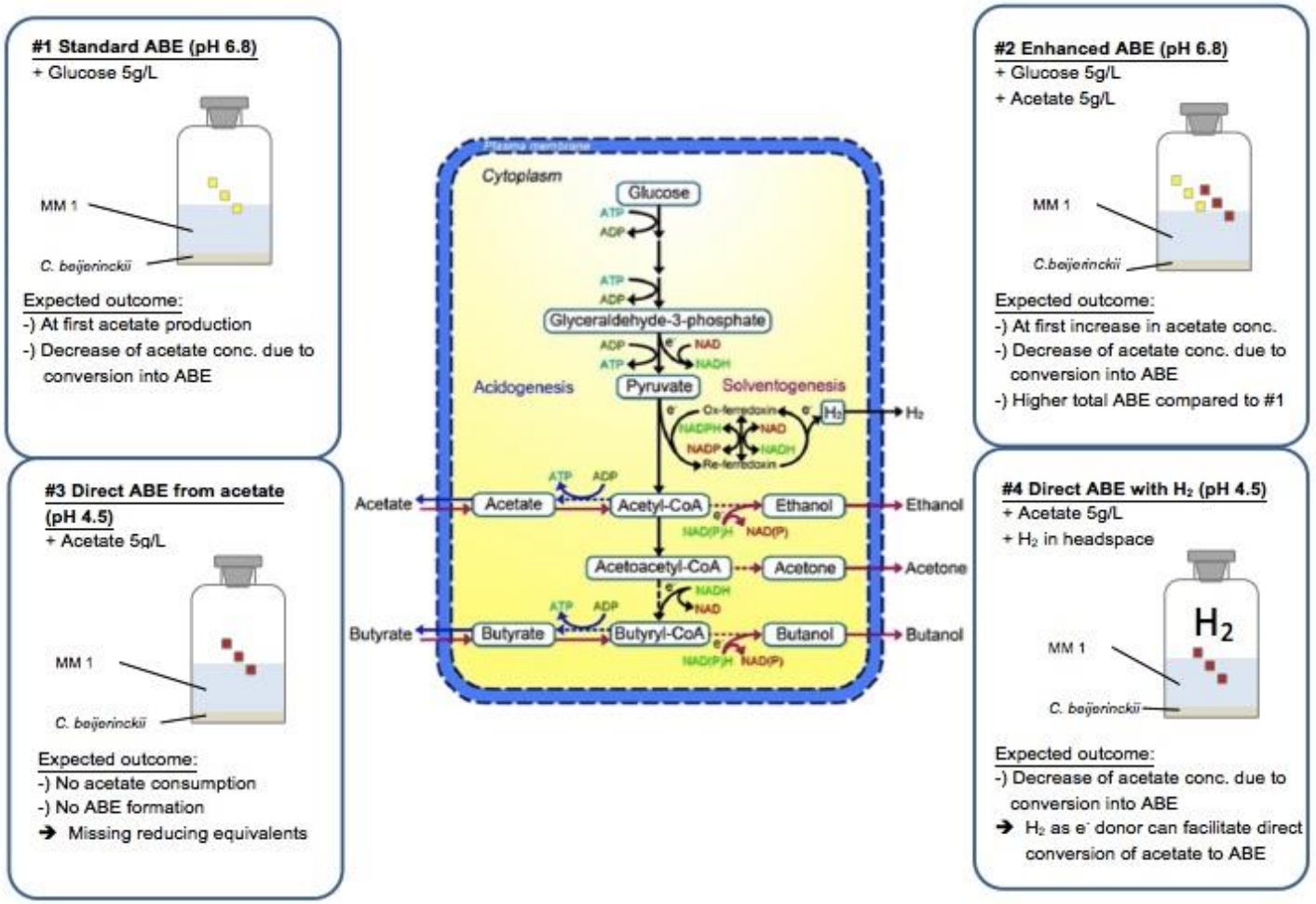

Figure 11: Experimental plan of ABE experiment

As shown in **Figure 11** four fermentation experiments with different culturing conditions were performed.

For that, sterile serum bottles were filled with 10 mL MM1, flushed with N_2 and completed with reducing agents and vitamin solution. The pH of trials #3 and #4 was adjusted to 4.5 by sterile addition of 1.2 mL HCl 1M. Stock solutions of sodium acetate (100 g/L) and glucose (100 g/L) were prepared and separately added to the corresponding trials #2, #3 and #4 (**Figure 11**). Prior to inoculation, active cultures were passaged (2x: 30 mL suspension + 20 mL fresh medium + 1.75 mL glucose stock solution (100 g/L)) and incubated for 20 h until culture reached exponential phase. These cultures were pooled and 10 mL were added to each preheated (35 °C) reaction mixture. Subsequently trials #1 and #2 were flushed with Nitrogen for 1 minute to remove potential oxygen. Trial #3 was flushed with N_2 and pressurized to 1 bar overpressure and trial #4 was flushed with H_2/CO_2 (80/20 %) and pressurized to 1 bar overpressure. All cultures were incubated at 35 °C. All four trials were prepared in duplicates. Samples were taken one to two times a day for six days.

3.6 Analytical methods

3.6.1 Volatile Fatty acids, glucose, acetone and alcohols

VFAs, glucose and ABE were analyzed by HPLC. Samples were centrifuged at 12,500 rpm for 10 minutes to remove cells from the suspension. Subsequently, 200 µL of the supernatant were added to 760 µL H_2SO_4 0.0025mM - pH was adjusted in the range of 4-6 - and vortexed thoroughly. Then 20 µL of C1 (sodium hexacyanoferrate-trihydrate 106.5 g/L) and 20 µL of C2 (zinc sulfate-heptahydrate 288 g/L) were added one by one. After each addition the solution was vortexed. The reaction mixture was left 10 minutes for incubation. After centrifugation (12,500 rpm, 15 min.) the supernatant was filtered into HPLC vials through 0.45 µm filters, sealed and stored at 4 °C until analyzed.

HPLC equipment and operating conditions

Chromatographic separation column	IC-Sep ION300
Temperature of column oven	65 °C
Mobile phase	0.01 N H_2SO_4
Flow rate of mobile phase	0.9 mL/min
Injection volume	40µL
Detector	Refractive Index detector

Chem Station B.01.03 Revision was used to process HPLC raw data. Analytes of interest comprised Acetate, Butyrate, Glucose, Acetone, Ethanol and Butanol.

3.6.2 Ammonium

Two grams of the samples were weight out into a Kjeldahl flask and inserted into the automated distillation unit (Büchi®). For distillation and titration NaOH 30 %, Boric acid 20 g/L and HCl 0,05 M were provided. The exact content of NH_4^+ was then calculated manually in consideration of a blank value. Measurements were conducted in duplicates. The manual calculation was done according to the following calculation:

$$\% - NH_4^+ = 1.4007 * M_t * \frac{(vs - vb)}{w}$$

M_t = Titer Molarity (HCl) [mol/L]

vs = Titer consumption for sample [mL]

vb = Titer consumption for blank [mL]

w = weight of sample [g]

4 Results

4.1 Cultivation experiments of homoacetogenic clostridia

4.1.1 Media adaption

In order to facilitate only chemolithoautotrophic growth of *M. thermoacetica* and *A. woodii* and thereby enable a direct conversion of H_2 and CO_2 into acetate both organisms had to be adapted to a chemically defined minimal medium without additional carbon sources.

Adaption of *Morella thermoacetica*

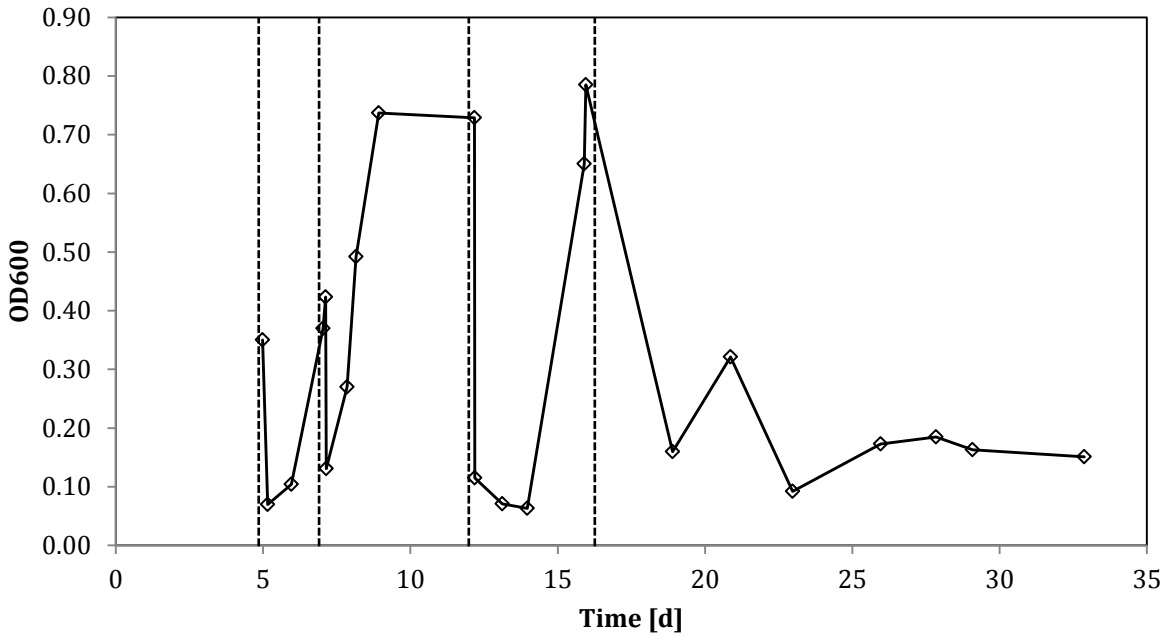

Figure 12: Adaption of *M. thermoacetica* to MM1. The adaption progress with its passages is shown at 25 %, 50 %, 75 % and 100 % MM1 after 5, 7, 12 and 16 days respectively.

Figure 12 shows the adaption process of *M. thermoacetica* to MM1. It is easy to see, that as long as CM and therefore glucose is present in the flask, ODs between 0.7 and 0.8 are reached. As soon as cells grow in 100 % MM1, average ODs reach values between 0.1 and 0.15 The average acetate production leveled off at 1 to 1.6 g/L (experimental data not shown).

Adaption of *Acetobacterium woodii*

Adaption to MM1

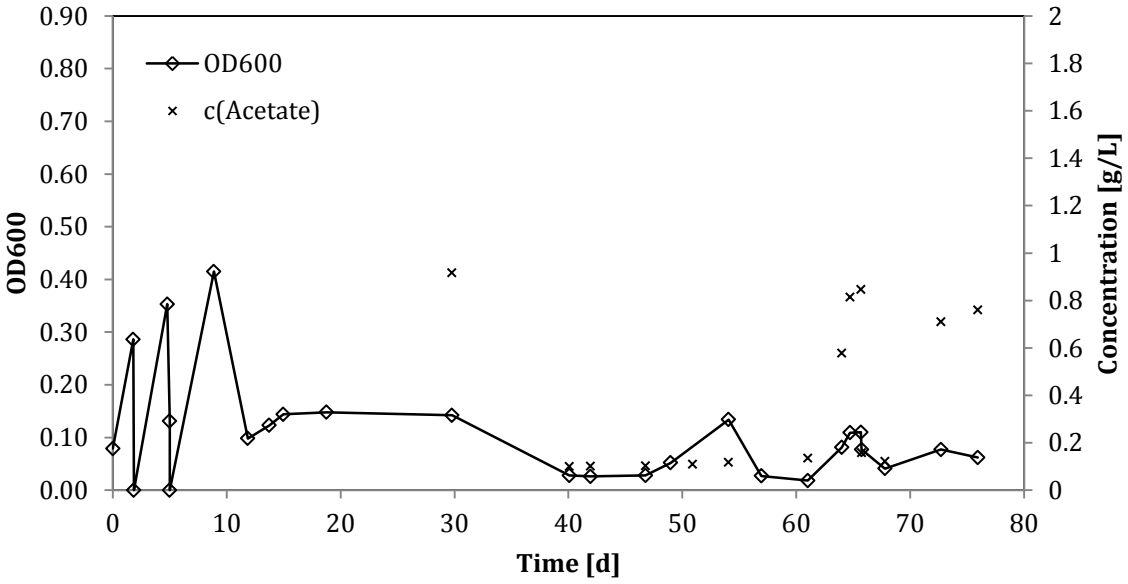

Figure 13: Adaption of *A. woodii* to MM1 and progress of long-term storage. Passages and corresponding ratios of MM1 are indicated.

Figure 13 shows the progress of the cell density of a shaking culture of *A. woodii* adapting to MM1 from the CM. Each of the first three peaks at the beginning of the adaption represents a passage. The culture was inoculated at an OD of 0.79 in 50 % MM1 and 50 % CM and proliferated to an OD of 0.286. The suspension was passaged in 75 % MM1, which caused a drop of the OD. Since no measurement is available for this point, a symbolic OD of 0 was chosen, to indicate a dilution. The following two peaks show passages in 100 % MM1. After the second passage in 100 % MM1 a decrease in OD to 0.098 and a following upswing to 0.148 is observed. After another passage on day 30, the culture shows only sparse activity. Acetate concentrations, which have been determined from day 30 on, reached a maximum value of 0.85 g/L on day 66. Slow growth and the fact, that product concentrations were only half of those from *M. thermoacetica*, led to the measure of modifying the medium. The adaption of *A. woodii* to this new medium, MM2, is shown in Figure 14.

Adaption to MM2 without YE

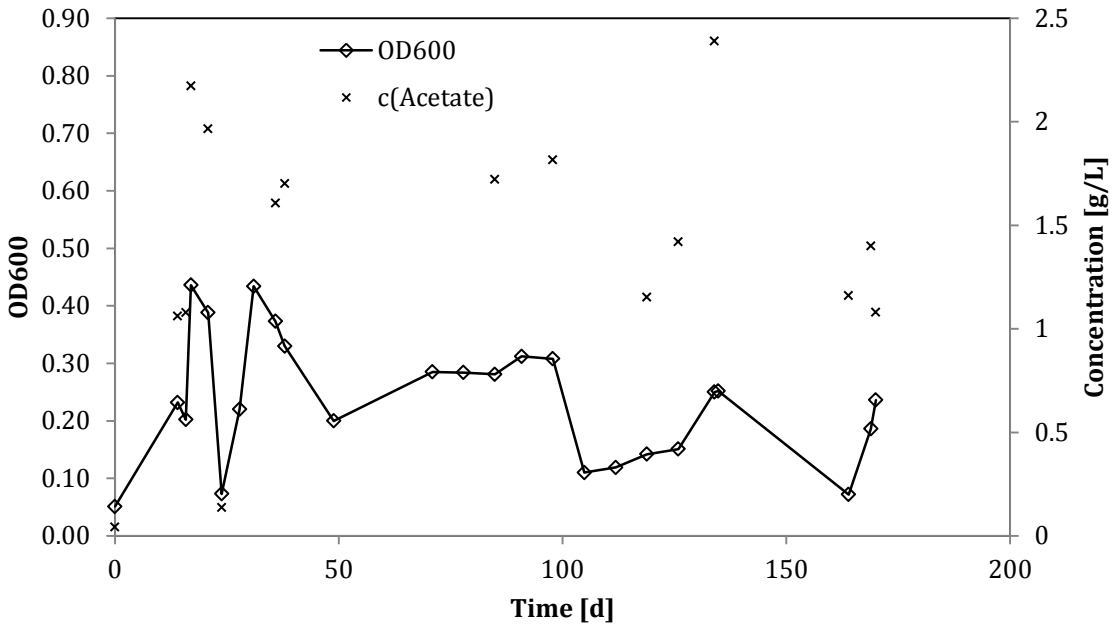

Figure 14: Adaption of *A. woodii* to MM2 followed by adaption to growth without YE.

Figure 14 represents the adaptation of *A. woodii* from MM 1 to MM 2, which contains YE. (1 g/L) In succession the influence of YE depletion on cell growth is displayed. The culture was inoculated in 50 % MM 1 and 50 % MM 2 at an OD of 0.051. Within the first two weeks, no samples were taken. Cultures were only visually checked for changes in turbidity. After two weeks, on day 15 an OD of 0.232 was measured, indicating cell growth. On day 17 the cell density doubled, and the culture was passaged in 20 % MM 1 and 80 % MM 2. The OD was calculated from the used dilution factor 2.3. The resulting OD was 0.186. From day 17 to 21 OD reached a new local maximum of 0.388 and the culture was finally passaged into 100 % MM 2. On day 31 the cell density reached the maximum of 0.434, the culture was passaged again, and leveled off at an OD between 0.2 and 0.285. Between days 49 and 71 cell densities were not determined photometrically, but only visually. It is for that reason that no data points appear in this period of time. On day 78, YE concentration was decreased from 1 g/L to 0.5 g/L. From this point on, YE concentration was periodically lowered and reached a concentration of 0.0 g/L on day 98. After another passage very slow growth is indicated by a linear increase in OD over 30 days. Acetate concentration after the adaption to MM2 reached maxima between 1.6 and 2.2 g/L. After depletion of YE the acetic acid production tended to decrease with final concentrations between 1.1 and 1.4, with an exception of 2.4 g/L on day 133.

4.1.2 Influence of glucose on cell density

In order to show that a higher final OD_{600} can be reached when *M. thermoacetica* is grown heterotrophically, glucose was supplemented to flask cultures grown on MM 1.

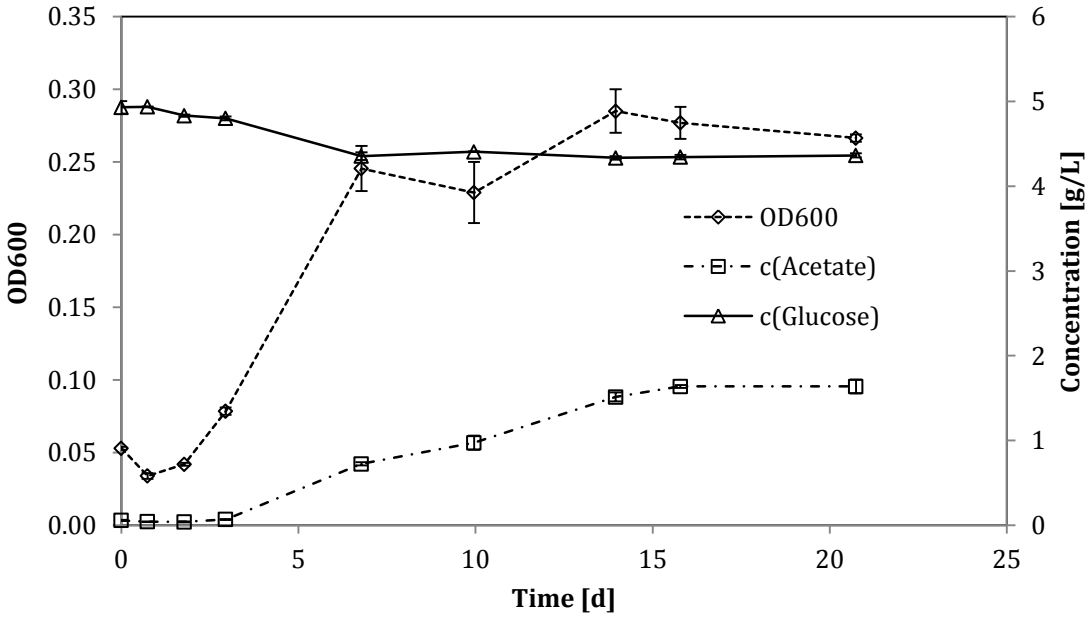

Figure 15: Progress of a glucose supplemented flask culture of *M. thermoacetica.*

Figure 15 shows the progress of a culture of *M. thermoacetica* grown on MM1 in presence of an initial glucose concentration of 5 g/L. As cells start to grow, reaching a maximum OD of 0.28, glucose concentration drops to a final concentration of 4 g/L on day 7. A final acetate concentration of 1.8 g/L was obtained. Error bars indicate the standard deviation between duplicates.

4.1.3 Batch - Fermentation

Comparison of methods for cell density determination

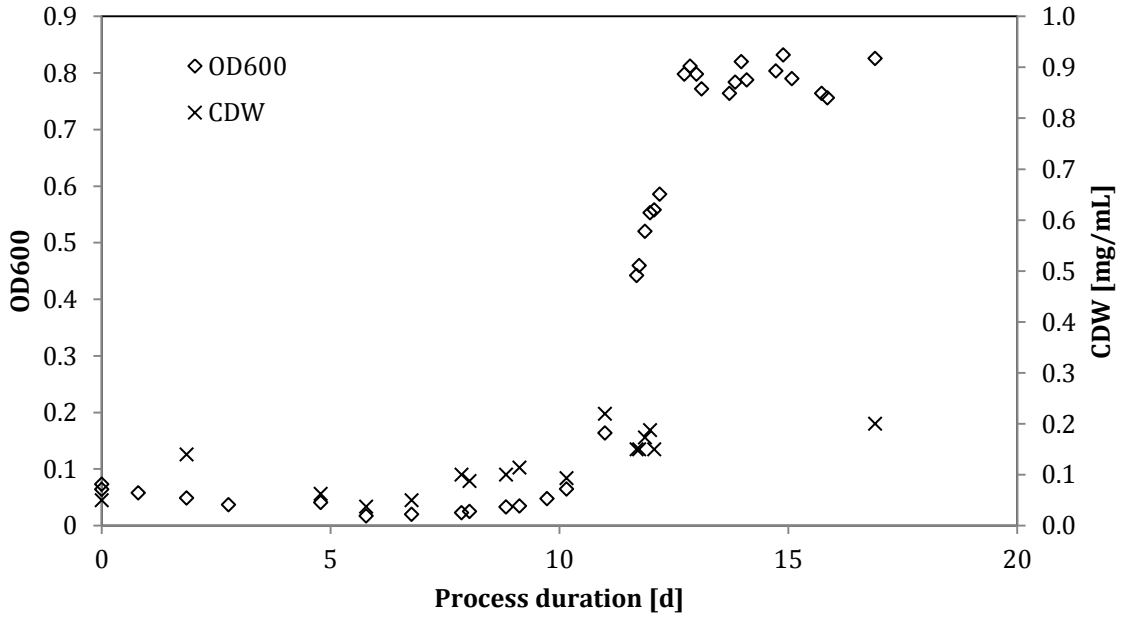

Figure 16: Comparison of OD_{600} - with cell dry weight (CDW) - measurements.

The correlation of CDW measurement with OD_{600}-readings is plotted in Figure 16. Results demonstrate that dry cell weight cannot be related to OD measurements. A limited sample amount makes a gravimetric evaluation of growth curves impossible. Therefore CDW was rejected as method for cell quantification.

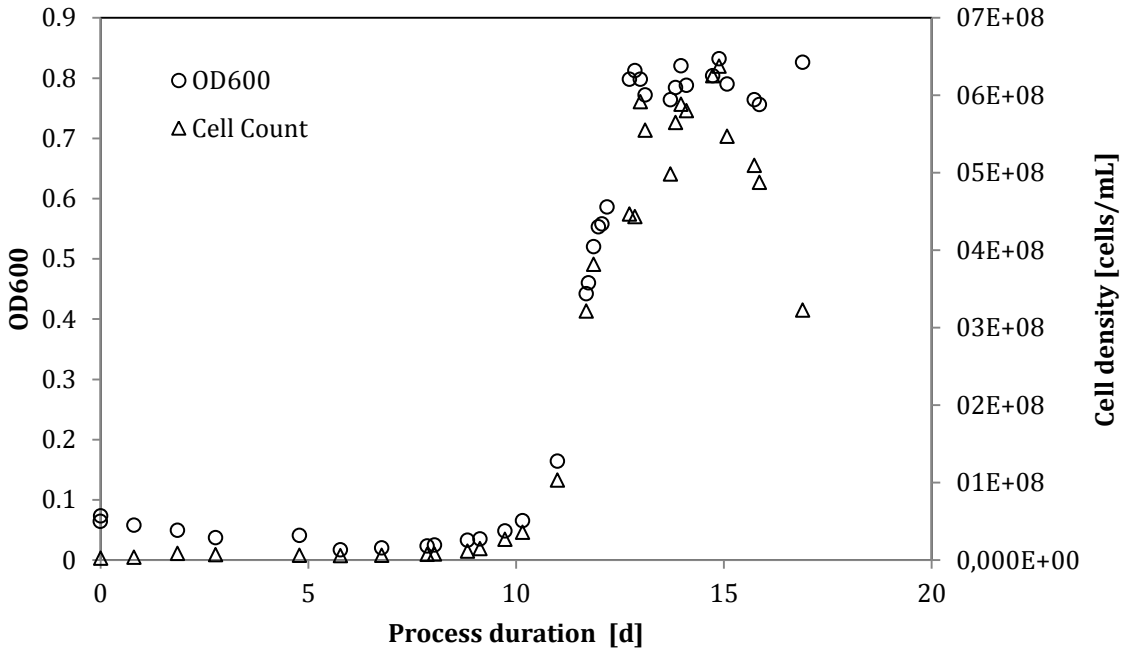

Figure 17: Comparison of OD_{600} - measurements with cell counts.

The correlation of OD_{600} measurements with the counted number of cells, displayed in Figure 17, shows a thorough compliance until the stationary phase was reached. Variations in cell density at higher OD_{600} readings result from too low dilutions for cell counts in the Thoma counting chamber. Due to the simple facts, that cell counting is a very time consuming and cumbersome method, and measuring points at high cell concentrations are wide spread, OD_{600} measurement was chosen as a standard cell density determination method.

Morella thermoacetica

- **Growth without yeast extract**

As visually represented in Figure 18 after a lag-phase of about 10 days, cells had finally adopted themselves to fermentation conditions and started to proliferate.

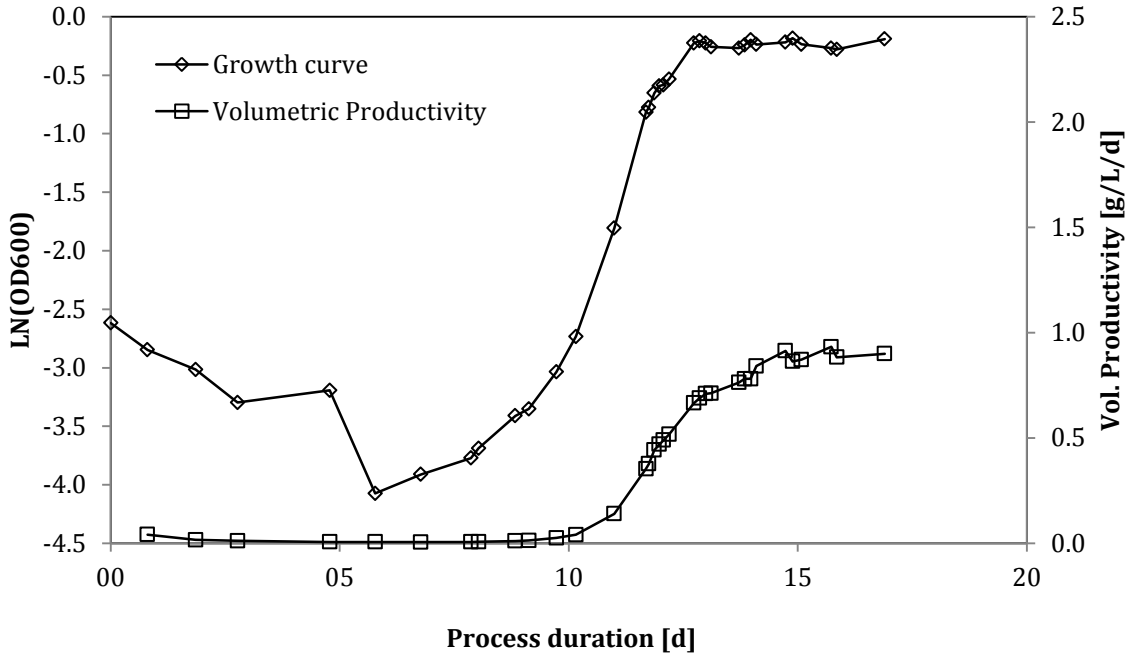

Figure 18: Correlation of the growth curve and volumetric productivity of a batch fermentation of *M. thermoacetica*.

Almost simultaneously an increase in vol. productivity could be monitored. According to the growth curve the growth rate decreased during lag-phase. OD readings deteriorate because the media is clearing at the beginning of fermentation. The exponential growth phase lasted for about 2.5 days. After about 12 days the batch culture reached the stationary phase. The fermentation run had to be abolished prematurely due to consumption of the substrate gas stock. Acetate production comes along with cell growth. Volumetric productivity commences to increase on day ten and levels off at around 0.9 g/L/d on day 14.

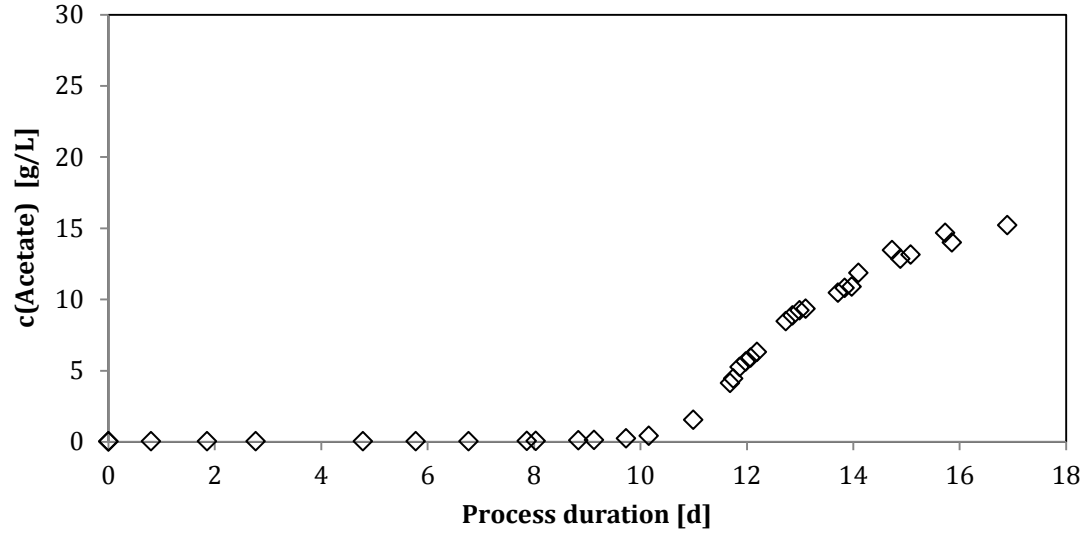

Figure 19: Progress of acetic acid concentrations in a batch fermentation of *M. thermoacetica.*

As expected, acetate concentration, as shown in Figure 19, increases on day 10, as exponential growth was detected (Figure 18). Once the cells reached the stationary phase a slight decrease in acetate production rate was monitored. Although, as mentioned above, the fermentation could not be completed in terms of passing through all phases of microbial growth, it seems, that the maximum acetate concentration was iterated (15.2 g/L) at the end of the process.

- **Growth with yeast extract**

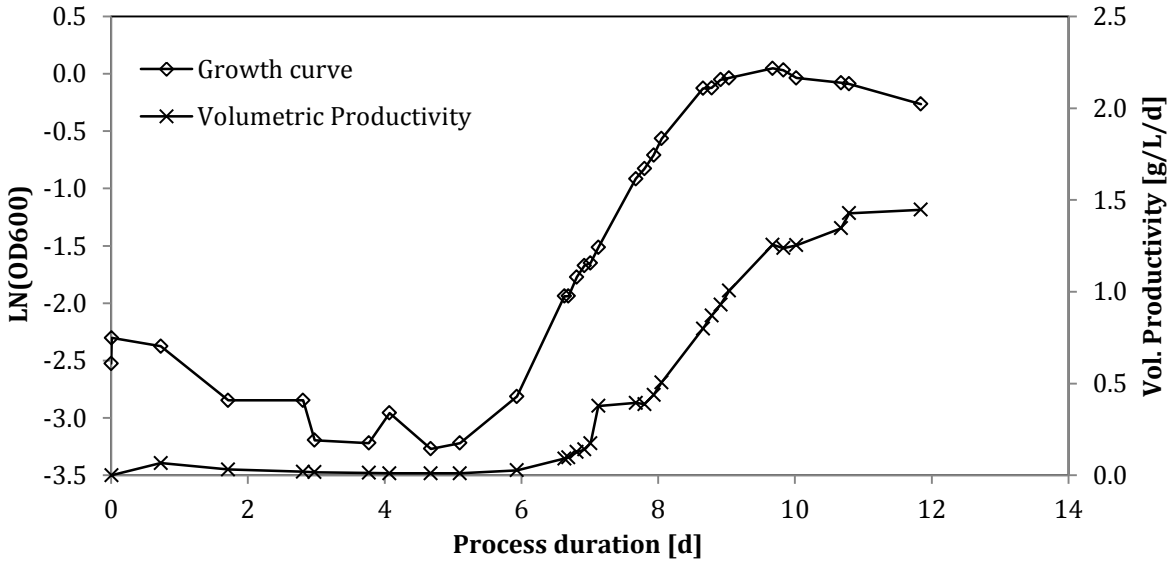

Figure 20: Correlation of the growth curve and volumetric productivity of a YE supplemented batch fermentation of *M. thermoacetica*.

This fermentation was supplemented with YE (2 g/L). Again a decrease in OD readings can be noticed in Figure 20. On day 5, cell growth commenced. Exponential growth lasted for 4 days, until stationary growth was reached. Volumetric productivity reached its maximum on day 10 and flattened off around 1.45 g/L/d.

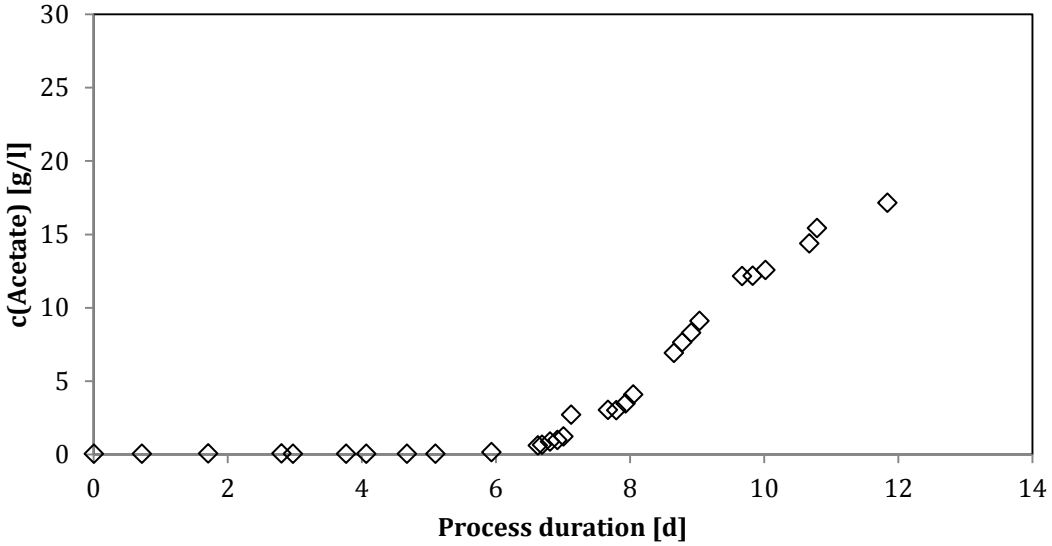

Figure 21: Progress of acetic acid concentrations in a YE supplemented batch fermentation of *M. thermoacetica.*

As shown in Figure 21, within the first 6 day of the fermentation acetate concentration stagnates at 0 g/L. As cells start to grow between day 6 and 7 product concentration increases to a maximum value of 17.1 g/L on day 12. Fermentation over a longer period may have resulted in slightly higher acetate concentration. But again a flattening of the curve implements maximum product concentration has almost been reached.

Acetobacterium woodii

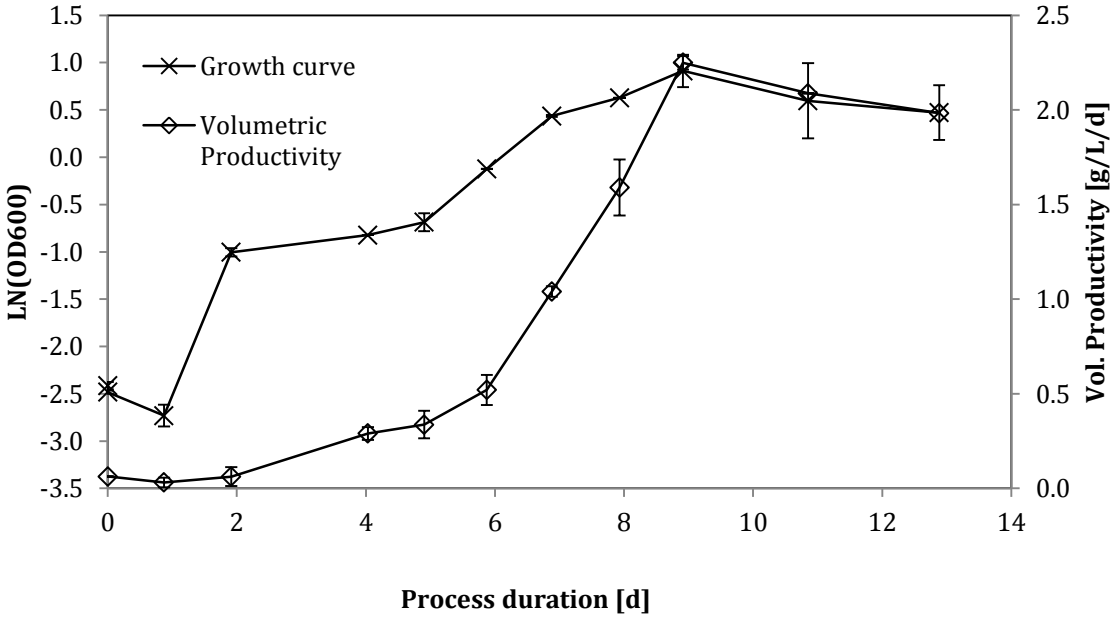

Figure 22: Correlation of the growth curve and volumetric productivity of a batch fermentation of *A. woodii*.

Cell growth and volumetric productivity displayed in Figure 22, show the same tendencies as previous fermentations of *M. thermoacetica* (Figure 18, Figure 20). OD readings deteriorate at the beginning of the process. Exponential cell proliferation was established between day 2 and day 4 after inoculation. Maximum cell concentration aswell as maximum volumetric productivity (2.25 g/L/d) were reached on day 9 during stationary phase. Errorbars indicate standard deviations between dual approaches.

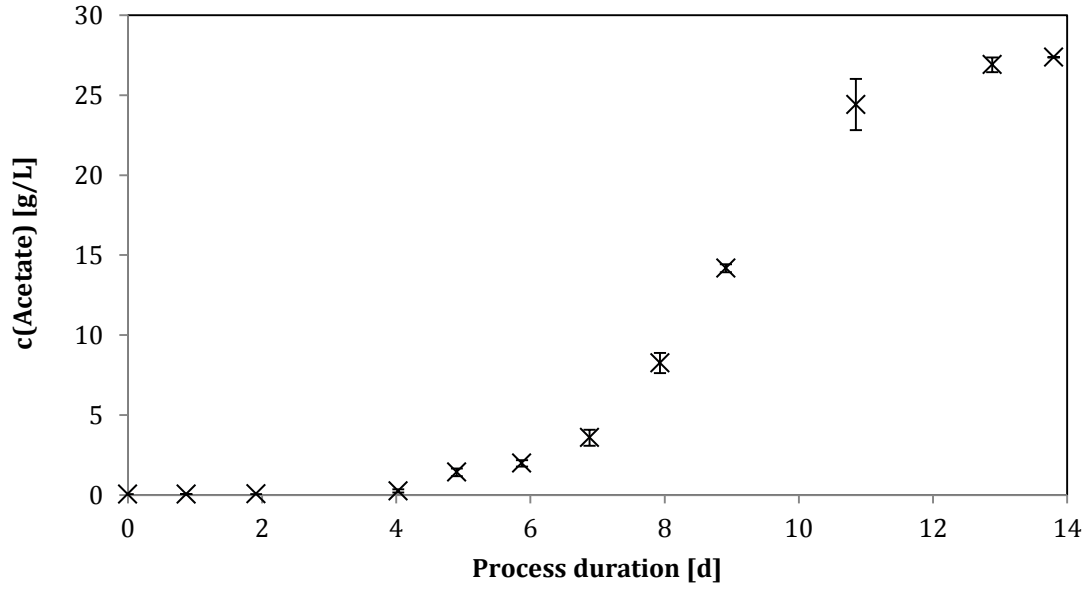

Figure 23: Progress of acetic acid concentrations in batch fermentation of *A. woodii.*

Figure 23 shows the increase in acetate concentration during a batch fermentation of *A. woodii*. In accordance with Figure 22, acid secretion starts at day 4 and reaches a maximum of 27.4 g/L after 14 days of cultivation. The displayed values represent the average of two parallel fermentations.

4.1.4 Immobilization

Flask

- **Immobilization on carrier materials**

Comparative evaluation of different immobilization matrices with respect to final acetate concentrations reveals only view materials that facilitate a significantly higher product formation (Figure 24).

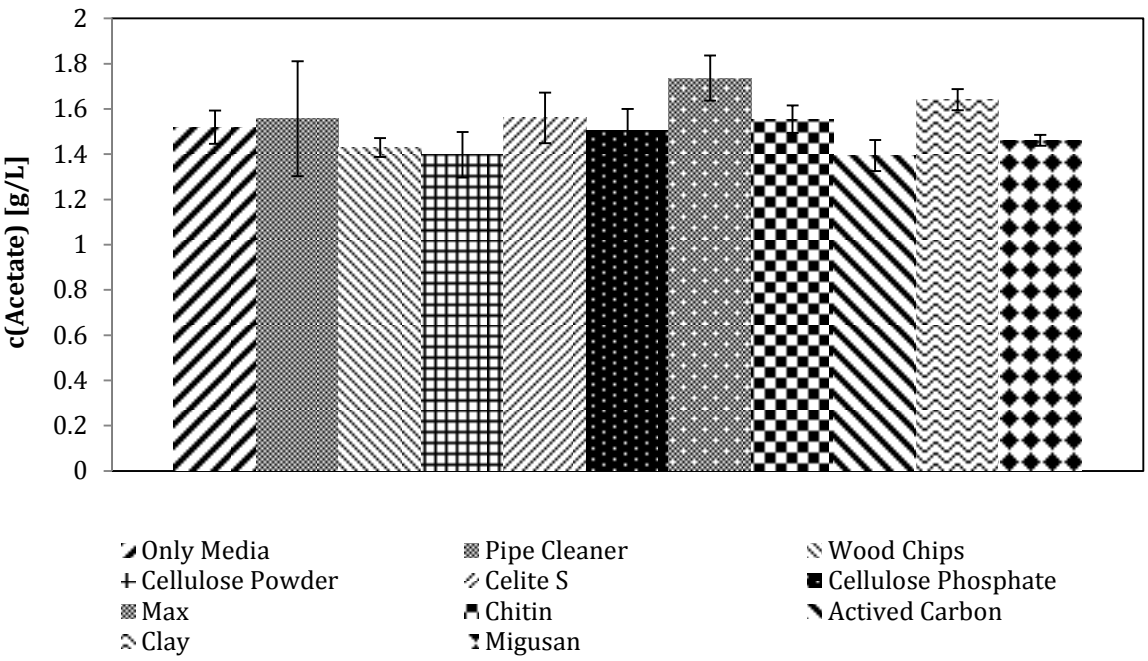

Figure 24: Final product concentrations of flask cultures with different carrier materials.

Error bars represent the standard deviation of experimental batches cultivated in duplicates. When cells were grown in media only 1.52 g/L acetate were produced, and when Meth Max or Clay was appended, median final product concentrations of 1.73 g/L and 1.64 g/L were obtained, before product inhibition fulminated. Pipe cleaner facilitated on one hand outstanding product secretion to a concentration of 1.81 g/L and on the other hand below average acidic acid concentration of 1.30 g/L. Other materials did not contribute any benefits to cell growth or acetic acid production. Cultures immobilized on Cellulose powder, Celite S, Clay, Migusan, Chitin and activated carbon show staged curves of acetate concentrations (Figure 25).

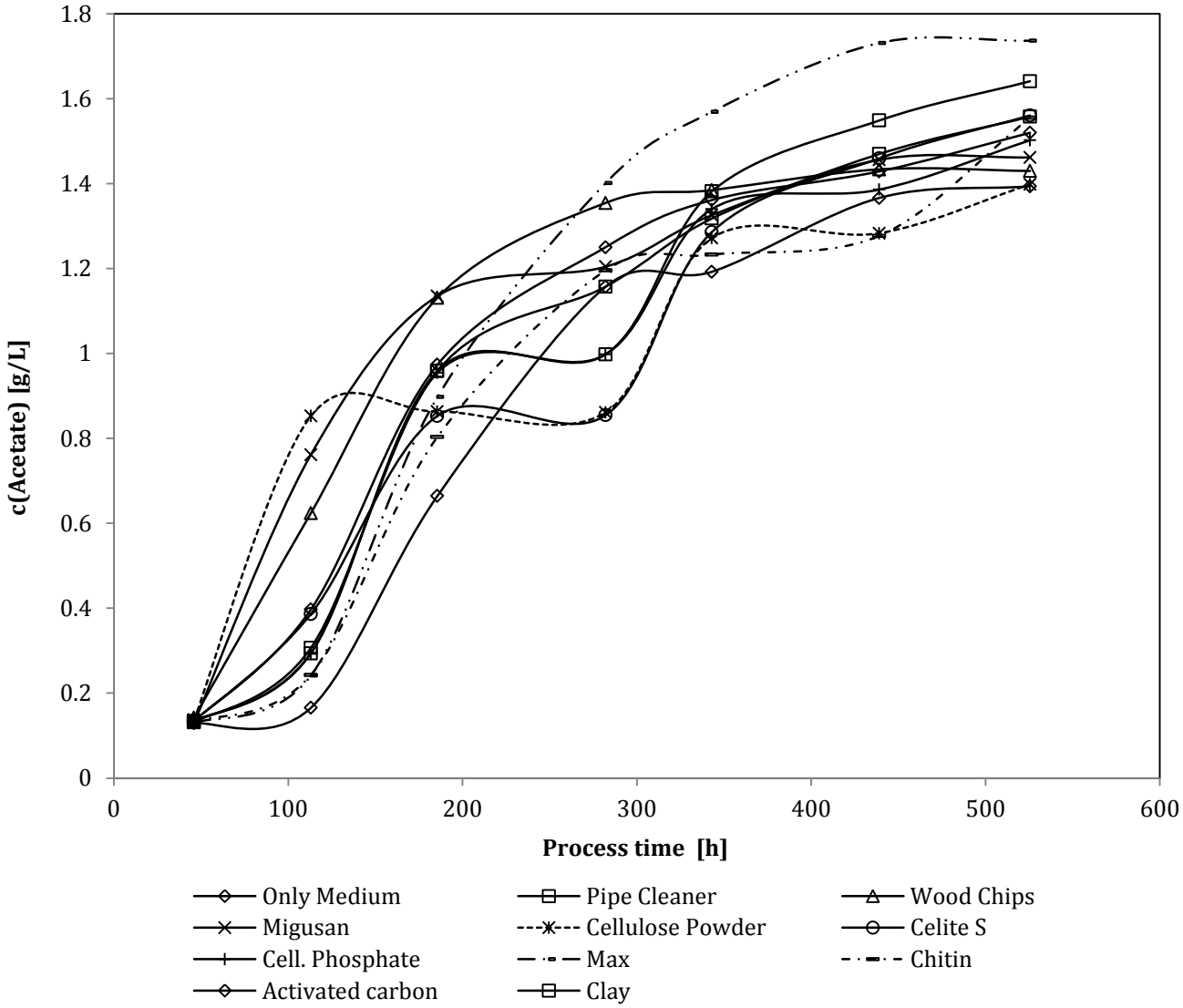

Figure 25: Acetate concentrations of cultures with immobilization carrier over time.

- **Immobilization on membranes**

Figure 26 shows a quantitative comparison of flask cultures with *M. thermoacetica* immobilized on different membrane materials.

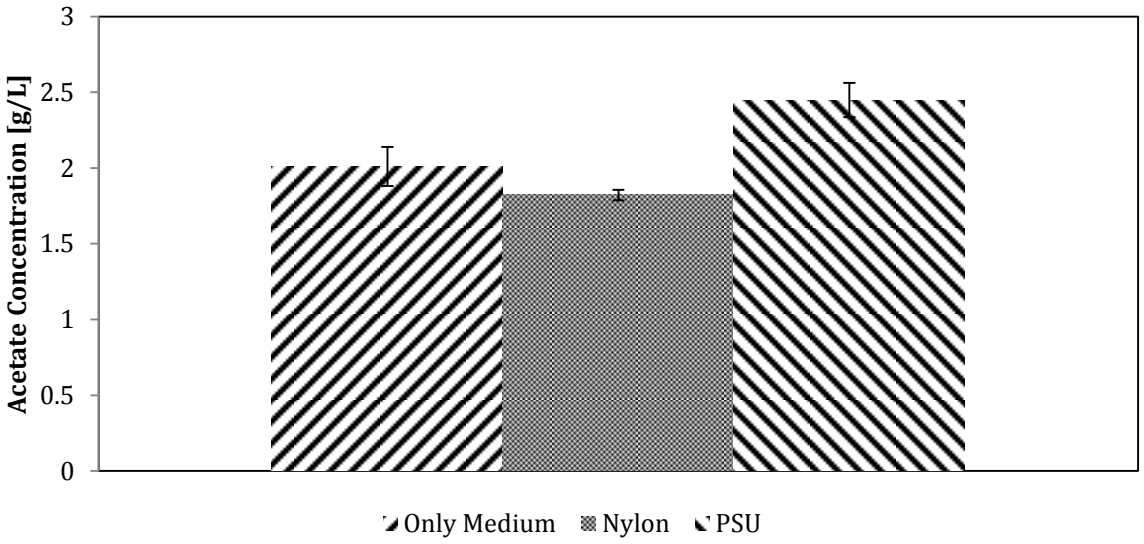

Figure 26: Final product concentrations of membrane immobilized flask cultures.

The blank culture and the one grown with nylon as a support matrix show almost the same final acetate concentrations of 1.7 and 1.65 g/L. Bacteria grown with PSU as a carrier material produced acetate to a final concentration of 2.5 g/L.

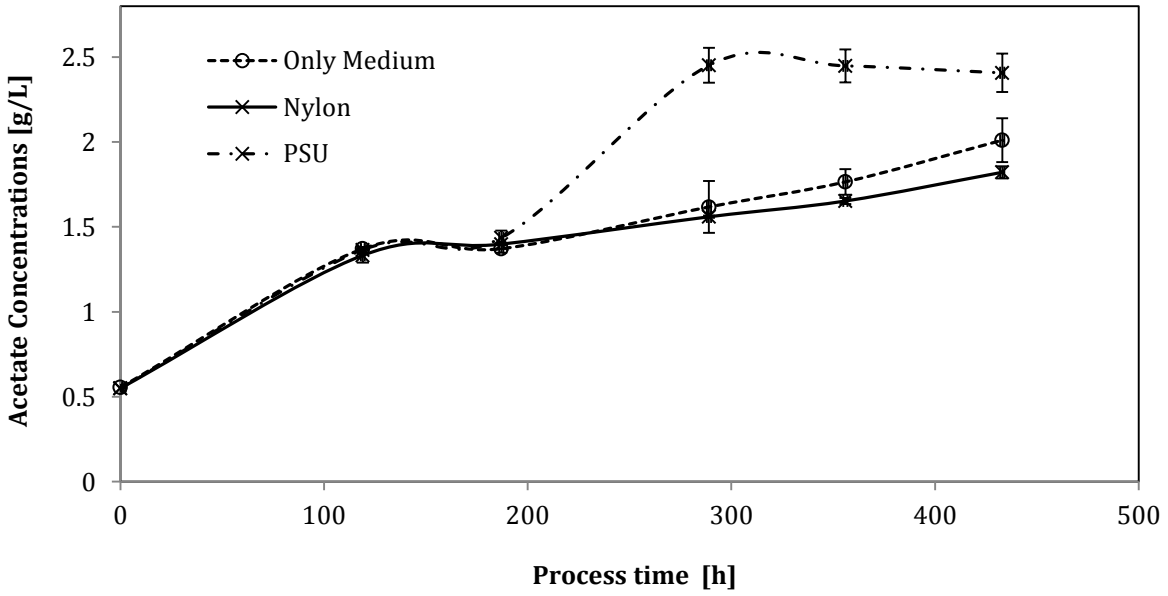

Figure 27: Acetate concentrations of membrane immobilization cultures over time.

In Figure 27 one can see, that the acetate production in all three cultures are congruent. After approx. 190 h in culture, product formation is ceased in the blank and the nylon culture. The PSU culture proceeds growth and acetate production until it reaches its maximum concentration of 2.4 on day 190.

Batch recirculation through hollow fiber membrane module

The immobilization potential and its effect on productivities and final product concentrations in a pH-controlled environment were tested by recirculating the broth of a batch fermentation through a micro-porous HFMB-module.

Gas supply at atmospheric pressure

Figure 28 demonstrates the anticipated drop of OD readings during lag phase, which lasted for two days. A subsequent increase in cell density implicates exponential growth. On day nine, recirculation of the fermentation broth through the HFMB-module was initiated.

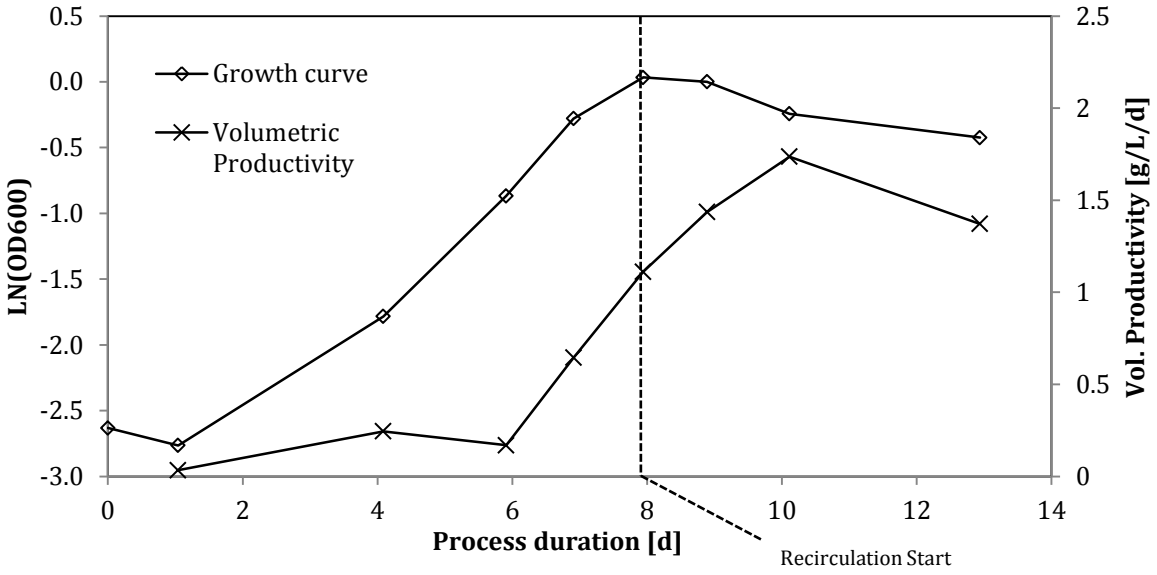

Figure 28: Illustration of microbial growth (*M. thermoacetica*) and volumetric productivity in a batch process with recirculation through an HFMB-module.

It can be observed, that cell density decreases subsequently. Volumetric productivity reaches its maximum of 17.4 g/L/d on day 10. For recirculation parameters see Table 12.

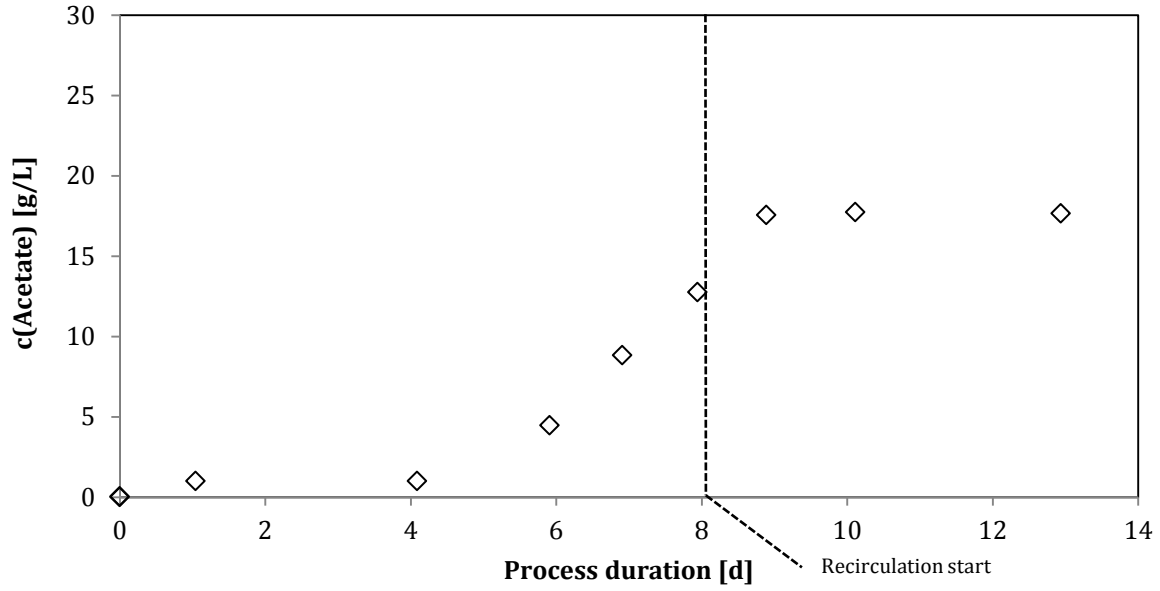

Figure 29: Progress of acetic acid concentrations in batch fermentation with recirculation through an HFMB-module of *M. thermoacetica.*

Figure 29 shows, that the secreted product concentration attains its maximum of 17.6 g/L on day nine and levels off at this value.

Gas supplied at over pressure

In order to increase the mass transfer into the fermentation broth and thereby increase the nutrient supply for bacteria, an overpressure was applied to the gas phase inside the HFMB-module.

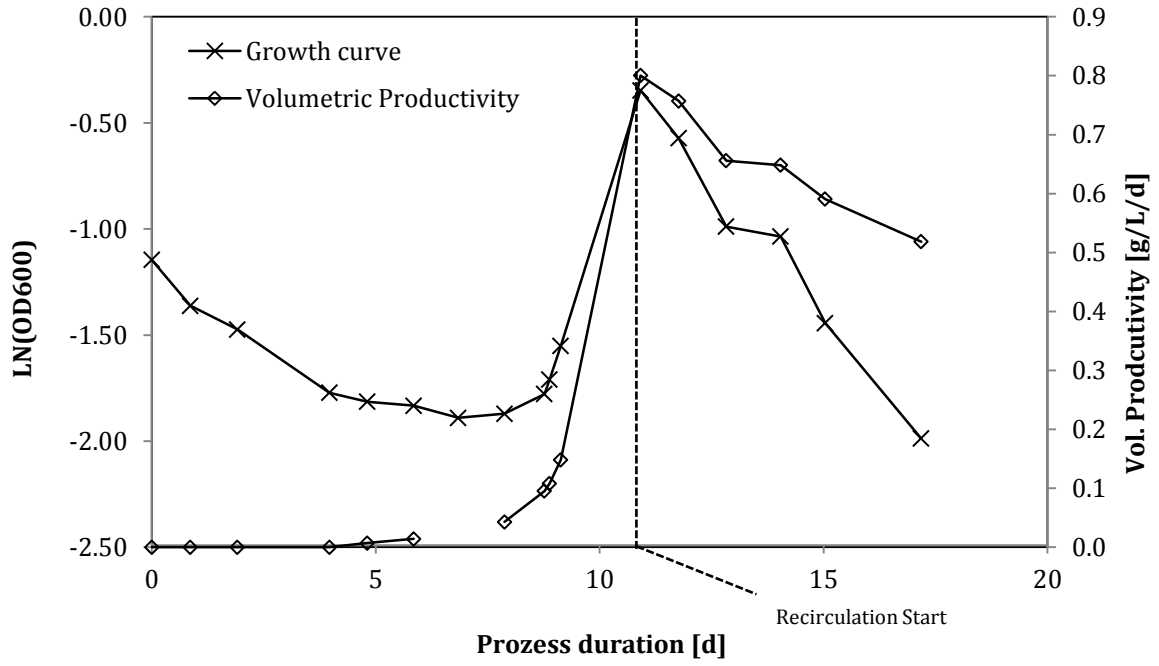

Figure 30: Illustration of microbial growth (*M. thermoacetica*) and volumetric productivity in a batch process with recirculation through a pressurized HFMB-module.

Figure 30 illustrates a batch process with subsequent immobilization of *M. thermoacetica* in a pressurized HFMB-module. A drop of OD-readings indicates a lag phase of eight days. A subsequent rise in cell density and productivity suggests that cells adapted to cultivation conditions. As soon as exponential growth was reached, here indicated by a linear increase in the logarithmic representation of cell density, recirculation of the fermentation broth was started on day elven. As a consequence, cell density and volumetric productivity diminished. Product formation set in accordingly on day 8 (shown in **Figure 31**) and reached its maximum as cell immobilization was initiated, and stagnated at a concentration of approximately 8.9 g/L.

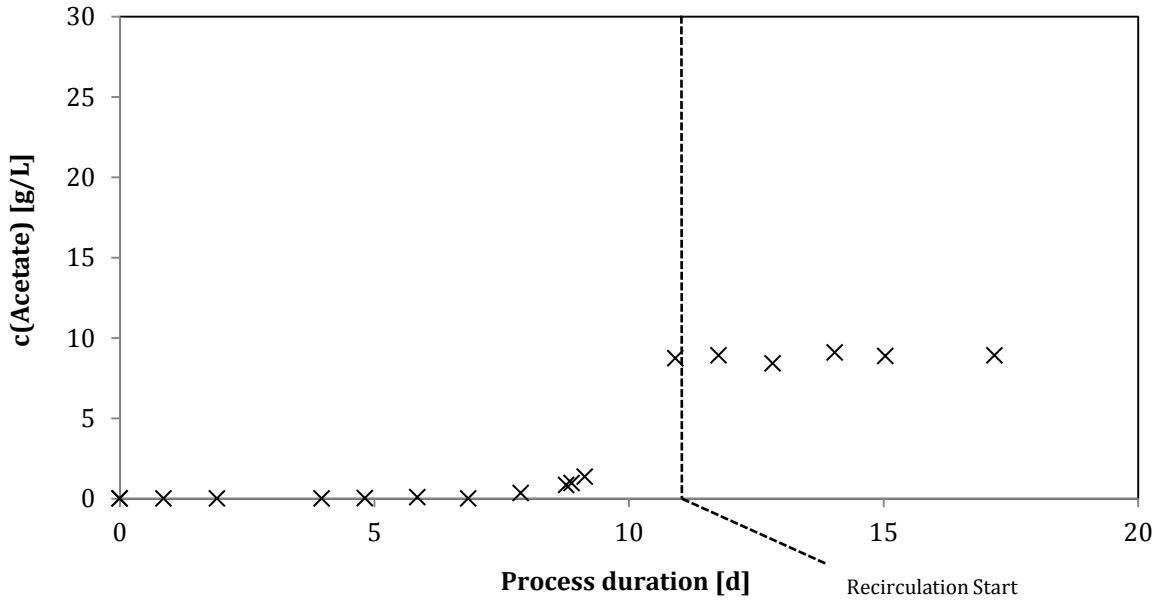

Figure 31: Progress of acetic acid concentrations in batch fermentation with recirculation through a pressurized HFMB-module of *M. thermoacetica*.

4.2 Experiments on ABE-fermentation

All experiments in this part were conducted in duplicates with standard deviations given as error bars. All four experimental set ups were inoculated and sampled simultaneously. The following plots give the progress of substrate- and product concentrations as well as pH and OD values over time. A decrease of initial concentrations from approximately 9.00 g/L to 7.20 g/L glucose and a decrease of approximately 3.00 g/L acetate are inherent in all four cases. This is due to during inoculation appearing dilution of the medium. Presence of glucose in pre-cultural cell suspension leads to a lower dilution of sugar than of acetic acid, which was present at lower concentrations in the inoculum. In case one, a slight increase in acetate concentration may be observed. The same applies for butyrate concentrations in all four cases. A pH drop from 6.8 to 6.8 in the first two approaches was caused by already used up buffer capacities in the inoculum. As neither acetone nor butanol was produced in any approach, visual representation was renounced.

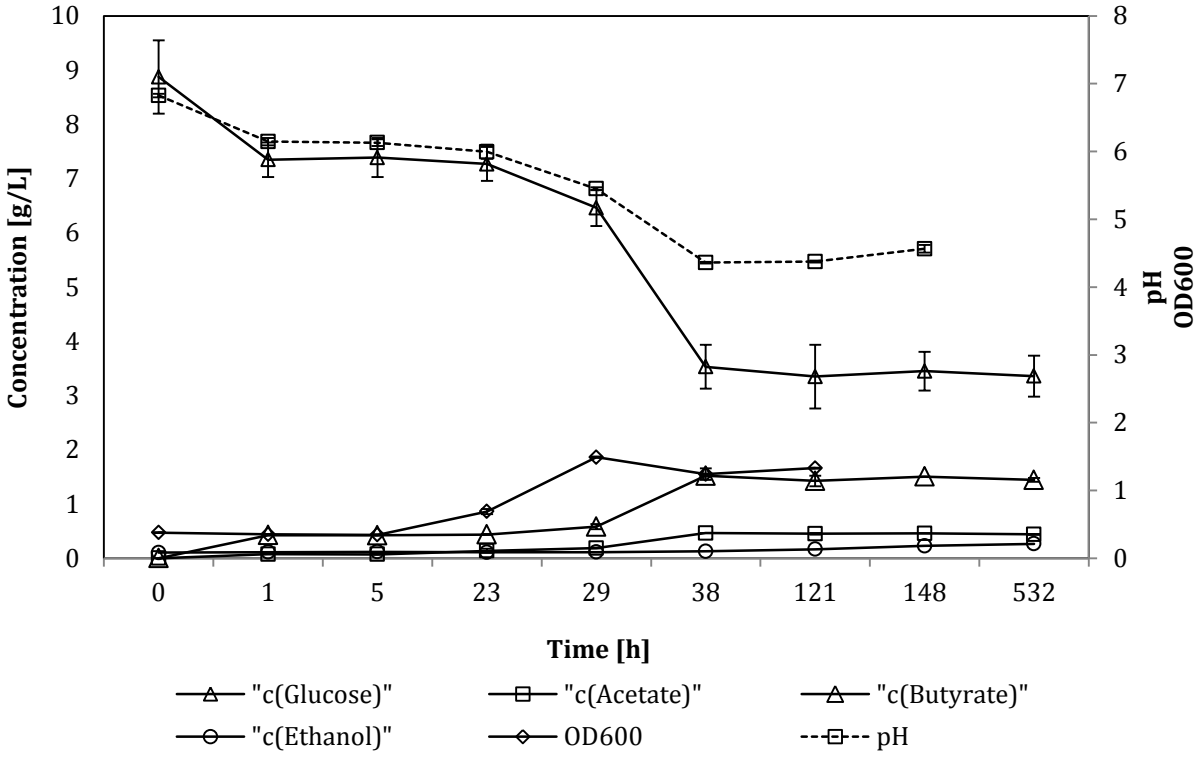

Figure 32: Overview of the progress of glucose, acetate, butyrate, ethanol concentrations, OD_{600} and pH over the time of incubation of trial #1.

Figure 32 shows that glucose concentration and pH drop from 8.87 to 7.34 g/L and 6.8 to 6.8 respectively, whereas the concentration of butyric acid increased from 0.00 to 4.25 g/L. After a lag phase of almost one day, cells started to consume glucose for half a day, until glucose concentration reached a minimum of 3.53 g/L. During this growth phase, pH dropped to a minimum of 4.3 due to the production of 1.52 g/L butyrate and 4.64 g/L acetate. A subsequent increase of the pH and ethanol concentration to 4.50 and 0.22 g/L respectively can be identified. Related to this the amount of butyrate decreased to a minimum of 1.44 g/L.

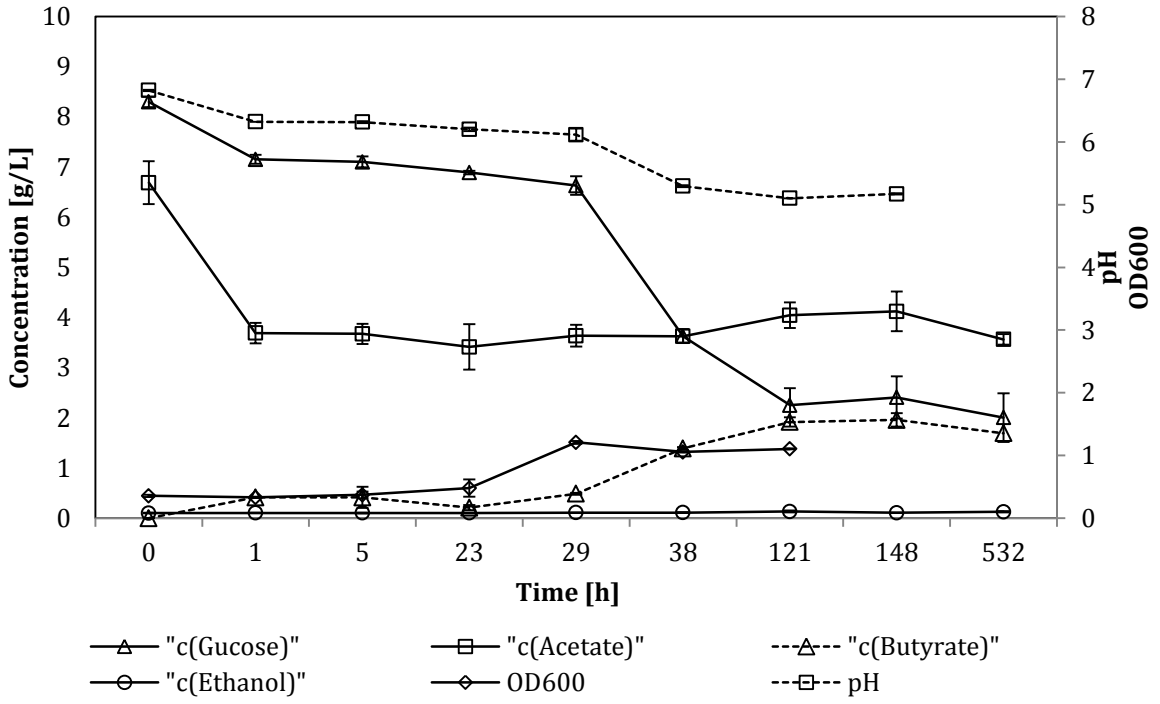

Figure 33: Overview of the progress of glucose, acetate, butyrate, ethanol concentrations, OD_{600} and pH over the time of incubation of trial #2.

After inoculation initial glucose and acetate concentration reach a concentration of 7.15 and 3.69 g/L, as shown in Figure 33. The pH drops from 6.8 to 6.3 and butyrate concentration rises to 4.14 g/L. 1 day later, cell growth was initiated, glucose concentration dropped to a concentration of 2.25 g/L, the pH to 5.1 whilst butyrate and acetate concentrations increased to 1.91 and 4.0 g/L respectively after 121 h. Ethanol concentration stagnates at 0.120 g/L over the whole course of the experiment.

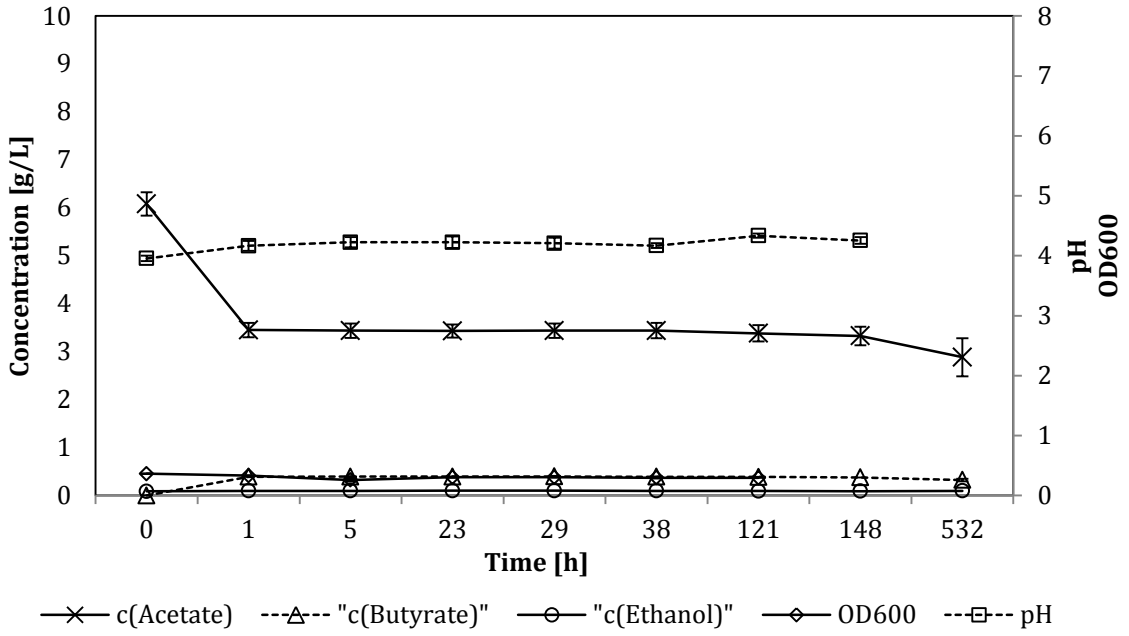

Figure 34: Overview of the progress of acetate, butyrate, ethanol concentrations, OD_{600} and pH over the time of incubation of trial #3.

This experimental approach (Figure 34) again starts with an initial drop in acetic acid and butyric acid concentration from 6.07 to 3.45 g/L. After inoculation no alteration of monitored parameters were observed. A final decrease of acetic acid to a concentration of 2.88 g/L was attained after 532 h.

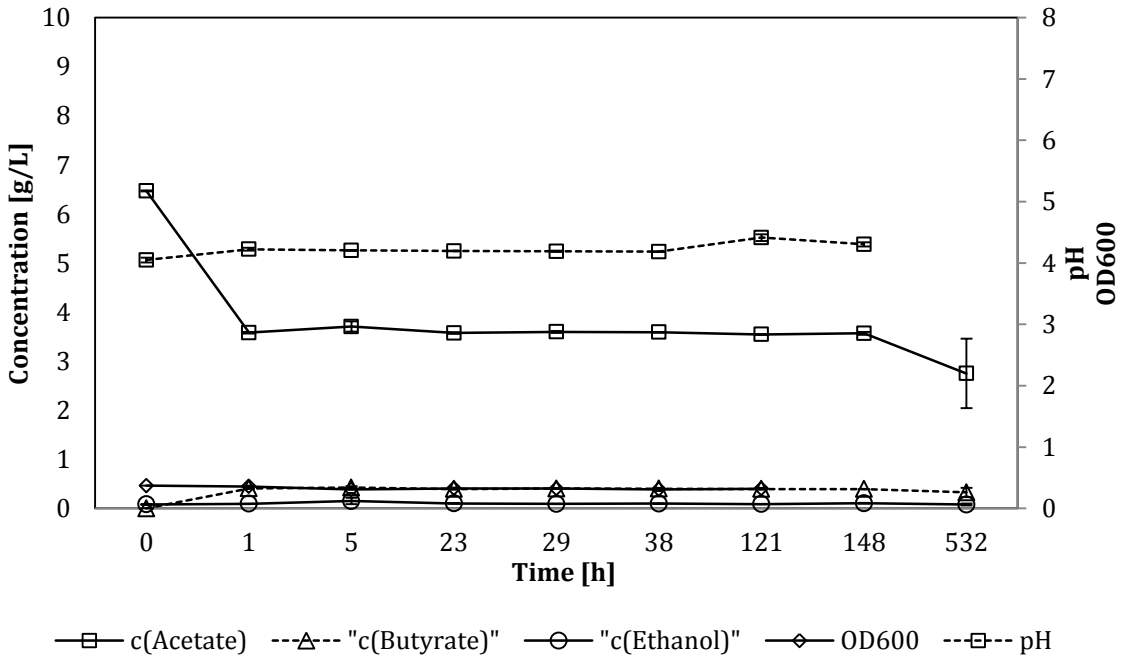

Figure 35: Overview of the progress of acetate, butyrate, ethanol concentrations, OD_{600} and pH over the time of incubation trial #4.

In approach number four (Figure 35) acetate concentration changes from 6.47-to 3.58 g/L and butyrate concentration from 0.00 to 0.410 g/L after inoculation. After 148 h of fermentation no cell activity was indicated by measured parameters. After 532 h a decrease in acetate to 2.75 g/L was measured.

5 Discussion

5.1 Cultivation experiments of homoacetogenic clostridia

5.1.1 Media adaption

It should be noted beforehand, that the aim of flask cultures was for bacteria to adapt to new conditions and to enable long-term cultivation of bacteria. These experiments were not intended for the determination of maximum possible product concentrations or cell densities, because cultivation conditions could not be controlled. However, the adaption to a chemically defined medium is also crucial to enable a future mass-balance calculation.

Product concentrations are not representative for bacterial product formation potential. Acetate concentrations only depend on the buffer capacity of the medium, which has been chosen arbitrarily for all experiments with acetogens. For that relative maximum cell densities and product concentrations were determined. The only controlled parameter was temperature and the pH was allowed to fluctuate freely. Growth rates may also differ from optima, due to varying substrate gas partial pressure. Controlled constant conditions are established in batch fermentations.

Morella thermoacetica

The adaption of *M. thermoacetica* from a medium containing glucose to a minimal medium was conducted successfully. A drop of maximum cell density and growth rate was expected, as energy generation from glucose is much more efficient than in autotrophic growth. Acetate concentrations leveled off at approx. 1.6 g/L at the end of a passage. A decrease of maximum acetate concentrations was observed from day 210 on. This could have been due to the very long period bacteria have been in culture. Microbial contamination that could have inhibited growth and productivity of *M. thermoacetica* is very unlikely, as no microscopic evidence was delivered. Intermediate analysis by HPLC gave no hint of by-product formation by a potential contamination. However, 70 days later, on day 280 bacteria resumed growth as described before. This may indicate, that the phase of low productivity and slow proliferation may have been caused by introduction of small amounts of oxygen during passaging or sampling. *Morella thermoacetica* needs a redox potential of at least -290 mV (Drake et al., 2006) for chemolithoautotrophic growth and responds very sensitive to such fluctuations. Another, more likely possibility could be a leak in the medium storage bottle, so that oxygen entered the medium, and the redox potential increased. This would be reasonable, considering the fact that the medium was stored over some weeks, and impermeability for the sur-

rounding air could not have been guaranteed. For future experiments the storage of the media bottle in N_2 atmosphere in a pressurized container would be advised.

Acetobacterium woodii

A successful adaption to MM1 was accomplished on first sight but growth and acetate formation were not as efficient compared to *M. thermoacetica*. This led to the idea of changing the medium. The reducing agent Na_2S was substituted with Na_2SO_3, which brings several advantages compared to Na_2S. First, its redox potential is lower; second, it does not cause precipitations of trace element cations in contrast to Na_2S; and third it is not volatile at acidic pH-values (Rothe and Thomm, 2000). However, the main difference between MM 1 and MM 2 and probably the most decisive change was the addition of YE. Growth commenced immediately after re-cultivation in MM 2, without a decrease in productivity. Another fact, that points towards the addition of YE being the crucial factor for the chemolithoautotrophic growth of *A. woodii*, is that as soon, as an a concentration of 0.0 g/L YE in MM 2 was reached, growth and the resulting acetate formation slowed down tremendously. Still final acetate concentrations of approx. 1.6 g/L were accomplished without supplementary YE. This fact is verified by a work of Leclerc that states that YE is not obligatory for growth but very supportive (Leclerc et al., 1998), in contrast to findings of Demler (2012), who stated that growth of A. woodii without YE could not be achieved (Demler, 2012), probably due to a lack in vitamins or trace elements. In a work of Drake and Harold L. (1994) it is also claimed that YE is only mandatory for chemolithoautotrophic of some selected acetogens like *Clostridium magnum*. For others, YE just acts as a stimulator of growth on H_2/CO_2. This effect is probably owing to present fermentable organic compounds like amino acids and glucose in YE (Leclerc et al., 1998) and a complex mixture of partly undefined vitamins. However, as my results underline a concentration of 0.5 g/L YE is advisable to achieve stable growth.

5.1.2 Glucose

As the cell density in pre-cultures could be doubled by addition of glucose (5 g/L) an initial OD_{600} of 0.1 of batch fermentations could easily be reached. Results compare to findings of Liu et al., (2015), which state, that biomass yield heterotrophic growth is much higher than from autotrophic (Liu et al., 2015). Increasing the inoculation cell density of fermentations increases the amount of vivid cells present, as one fraction of inoculated cells is dividing properly, while another fraction is dying or inactive (Tsao, 1976). Therefore, by the simple measure of adding glucose to a pre-culture, the lag phase of batch fermentations might be

reduced. This issue is discussed later in chapter 5.1.3 (Influence of yeast extract addition on *Morella thermoacetica)*.

5.1.3 Batch - Fermentation

Preliminary it has to be mentioned, that acetate concentrations from controlled fermentations stated in this work are not the exact true values. This nuisance is due to a defective recording of volume addition by the pH correction. For that reasons final acetate concentrations may even be higher, as approximately 150 mL of base were added during the course of fermentation.

Additionally it has to be stated, that it was not possible to compile a carbon mass balance. This circumstance is due to the experimental set-up and limited off gas analytics. As a consequence, it was not possible to calculate product and biomass yields.

Furthermore, due to the very limited amount of inoculation biomass, very long cell doubling times and lag-phases as well as a shortage of substrate gas at times, fermentations could not be repeated as often as necessary to generate a sufficient amount of data to confirm significance in differences.

Influence of yeast extract addition on *Morella thermoacetica*

First it has to be stated, that fermentations were not started simultaneously and inocula did not come from the same pre-cultures. Therefore cells could have undergone different stages of the cell cycle. So their physiological and metabolically states could have diverged. This could have played an important role in the progress of fermentations - a point that is returned to later. However, displayed trends are very likely to be actual effects, as they correlate with data found in literature.

The effect of supplementary YE on the progress of a controlled fermentation of *M. thermoacetica* was investigated and led to the following conclusions:

When fermentations of *M. thermoacetica* - one YE supplemented and the other one without supplementation - are compared, it is easy to see, that the lag-phase of the supplemented trial is significantly shorter. Furthermore, cells grown on the supplemented medium produce a slightly higher concentration of acetic acid (approx. 17.5 g/L) than those grown without YE (15 g/L). The latter effect is, as also described in literature (Drake and Harold L., 1994; Witjitra et al., 1996), thanks to traces of usable present organic compounds like glucose.

However, the shortened lag phase is not necessarily an effect of YE. There are two more factors that influence cell growth within the fermenter drastically. First, as mentioned above,

the physiological state of cells plays a crucial role in their adaption to fermentation conditions. Cells of the pre-culture have to be in exponential growth phase at the time of inoculation of the fermenter. Secondly, the initial cell density in the fermenter has to be as high as possible. Fulfillment of those two points enables dramatically accelerated cell propagation and the saving of time and resources. This fact is underpinned by the progress of the fermentation shown in Figure 28, which has been inoculated with glucose-supplemented pre-cultures and without the use of YE. However, the addition of glucose to pre-cultures has no influence on final acetate concentrations of the batch culture.

Final acetate concentrations of 15 g/L and 17.5 g/L respectively are a little lower than results found in literature. Sakai et al. (2005) were able to produce approx. 20 g/L acetic acid in batch fermentation (Sakai et al., 2005).

So YE addition is not the only decisive factor for an immediate start of growth in bioreactor, but certainly a supportive measure. What can be stated is that the reproducible cultivation of *M. thermoacetica* is a very delicate and difficult thing to accomplish.

Comparison of *Morella thermoacetica* and *Acetobacterium woodii*

As stated above, a statistical guarantee of significance of these results cannot be provided, as the obtained data lack statistical analysis. However, the observed trends are in compliance with trends found in literature.

When comparing batch fermentations of M. thermoacetica (YE supplemented) and A. woodii, which was always cultivated YE supplemented for reasons of substantial time saving due to a short lag phase, it becomes obvious that A. woodii was adapting faster to the cultivation environment than *M. thermoacetica* (Figure 36). From Figure 37 it emerges that *A. woodii* was not only faster growing, but also the more efficient organism in terms of productivity and final product concentration.

The final acetate concentration of 27.5 g/L produced by *A. woodii* is lower than values found in literature (44 g/L) (Demler and Weuster-Botz, 2011). This might be due to a slightly different experimental set-up, as Demler and Weuster-Botz (2011) used a pressurized fermentation system with a hydrogen partial pressure of 1700 mbar. The higher pressure results in increased mass transfer of hydrogen into the liquid phase and therefore better substrate availability for bacteria. Such pressurized process was not feasible due to technical limitations of the available fermentation system.

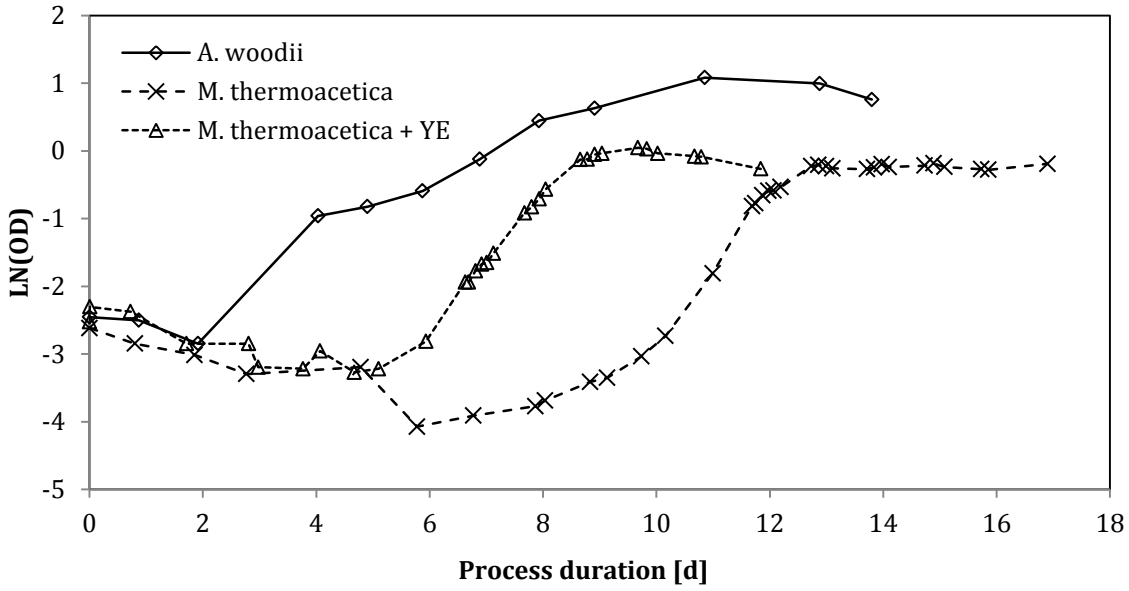

Figure 36: Comparison of growth curves of *A. woodii, M. thermoacetica, M. thermoacetica* with YE supplement.

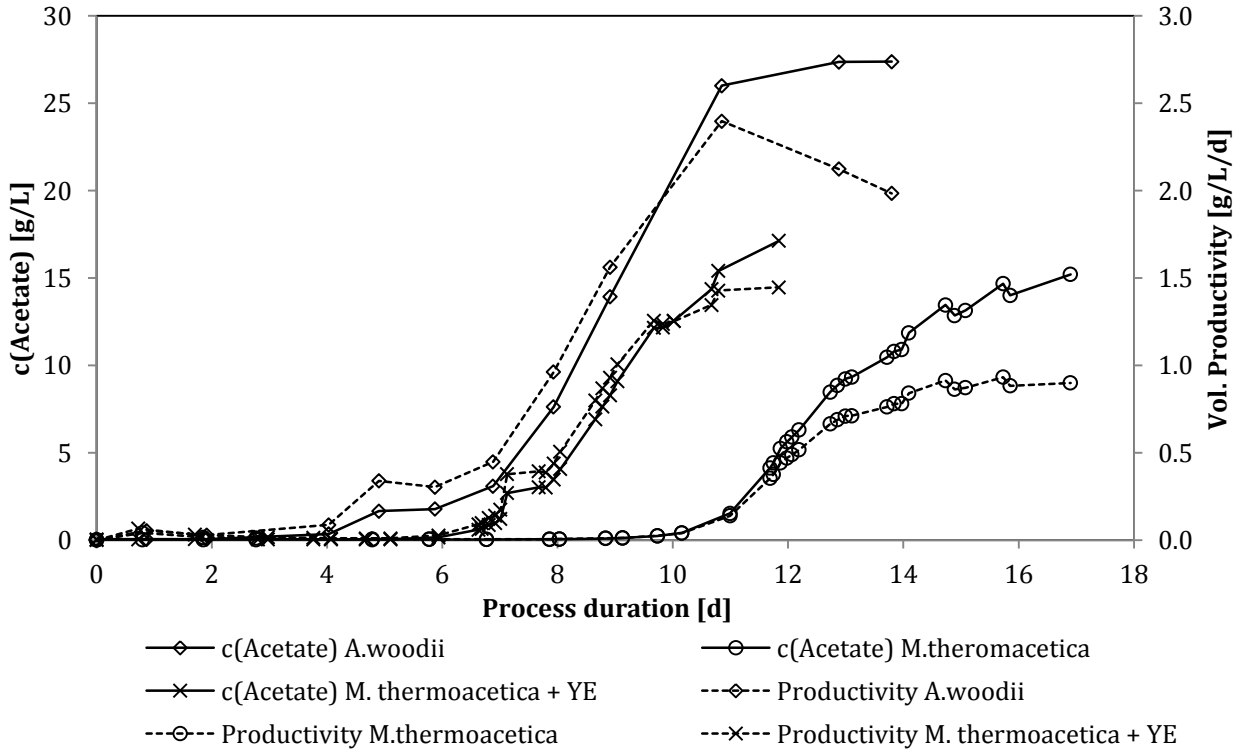

Figure 37: Comparison final acetic acid concentrations and volumetric productivities of *A. woodii, M. thermoacetica, M. thermoacetica* with YE supplement.

5.1.4 Immobilization

Flask

A screening of 14 materials was performed to evaluate the suitability of a wide variety of different carrier materials and membranes for immobilizing acetogens. Focus was put on enhanced acetate formation of adherent cells.

Immobilization with carrier materials

As visualized in Figure 25 the best results regarding consistency of acetate concentrations over duplicates were achieved with Meth Max. The second highest value in product concentration was achieved with pipe cleaner used as a carrier material, although a high variation between doublets occurred. In order to assure representative result with a significantly higher product formation, the experiment should be repeated. The third carrier material displaying high immobilization potential is clay. Chatterjee et al. (1996) did experiments for the evaluation of immobilization potential of different support matrices and found that best results were obtained with celite as a carrier material (Chatterjee et al., 1996). This result could not be confirmed by this work, as "celite-cultures" provided average acetate concentrations in this experiment. The same is also true for wood chips. Different affinities to support substrates of different bacteria could be responsible for this fact, as Chatterjee used *Butyribacterium methylotrophicum* in his setup.

The enhanced production of metabolites can be explained by the following. In cultures without carrier matrices *M. thermoacetica* showed signs of spontaneous flocculation, as well as of the spontaneous formation of a biofilm at the interface of liquid and gas on the wall of serum bottles. It is thought, that these self-aggregating cells gain benefits of higher tolerance to acetate by being densely packed together. Furthermore, immobilization addresses the key limitation of slowly growing organisms, which are easily washed out in continuous processes, and enables a stable long-term product formation. Granule formation also increases tolerance to metabolic stress like pH and temperature (Kumar et al., 2016). It seems, that, certain materials induce the aggregation of cells by providing an appealing surface for cells to attach to.

The observed stepwise acetate production suggests a stepwise growth behavior as product formation is coupled to cell growth. This phenomenon might be caused by the adsorption of media components by the carrier material. The different growth patterns may be a result of a combination of the adsorption of nutrients, cell and products.

A possible disadvantage of the used carrier materials may be their high specific density, that lets particles submerge and sink to the bottom. This may impair the availability of substrate gas to cells, as the gas-liquid mass transfer of H_2 low. To overcome this drawback of immobilization in bottles a different approach is required to imitate the conditions of the naturally occurring biofilm on the wall of serum flasks. Given that this pre-experiment just serves the evaluation of affinity of certain acetogens to immobilization matrices, no further investigations concerning this problem were done. Implementation of this system in a bubbled and stirred reactor would prevent such issues but give rise to a different set of challenges, like higher sheer forces, higher viscosity and therefore a higher required energy input (Sánchez Pérez et al., 2006).

Immobilization with membranes

As described in Figure 26 only 3 of 5 immobilization trials (including the blank) provided evaluable quantitative data of acetate concentrations. Trials comprising mixed cellulose ester and glass fiber membranes could not be analyzed due to the disintegration of the membranes. Shear forces and the relatively high temperature of 55 °C led to a brown coloring of mixed cellulose ester and the disruption of both materials. OD_{600} measurements were not conclusive, because of disintegration to a certain extent of membranes. However, the best overall result, also taking carrier materials into account, was obtained with PSU. This finding is contradicting those of Chauhan et al. (2005). According to his experiments on the evaluation of immobilization potentials of different carrier matrices including nylon, PSU, teflon and PP, PSU showed the least satisfactory result. A high hydrophobicity facilitates cell to cell interactions of bacteria and thereby enhancing cell aggregation (Krasowska and Sigler, 2014). PSU is a hydrophilic material and therefor should not promote the formation of aggregates (Chauhan and Ogram, 2005). However, in this case best results were achieved. Further experiments should be conducted, in order to prove this finding and evaluate the potentially higher immobilization capacity of different other support materials.

However, when comparing cultures immobilized on carrier materials and membranes it has to be considered that they do not derive from the same pre-culture. This may have an influence on the results, as cells experienced different physiological conditions at the time of inoculation.

Batch recirculation through hollow fiber membrane module

As described above, the immobilization was implemented into a regular fully controlled batch fermentation. Although cells were cultivated in absence of YE, the lag phase only lasted for two days. This peculiarity is discussed in chapter 5.1.3 (Influence of yeast extract addition on Morella thermoacetica).

Gas supplied at atmospheric pressure

The steep ascent of the growth curve during exponential growth indicates the well being of cells. As soon as recirculation was started, a drop in OD was expected, as cells started to attach to the membrane and cell density of the fermentation broth decreases. This kink in the growth curve is clearly recognizable on day eight. Unfortunately another kink followed by a flattening of the acetate accumulation curve can be observed on day nine. This stop in product formation indicates an inhibition of cell growth as a response to the unfavorable cultivation conditions in the membrane module. This effect could have been caused by two different circumstances. First, the low room temperature in the laboratory (approx. 20 °C) led to massive heat loss especially through recirculation hoses resulting in a module temperature of 35-40°C whereas the optimum cultivation temperature would have been at 50°C. Secondly, limited substrate availability due to slow diffusion of H_2 through the fiber membrane into the liquid could have hindered cells from proliferating. As a consequence, pressure was applied on the "gas-side" of the module, to force substrate gas through the micro pores into the circulating broth. This "hotspot" of substrate availability should attract cells and induce their attachment to the membrane.

Gas supplied at overpressure

Immobilization, using a pressurized HFMB system almost lead to the same results as described in the section above (Gas supplied at atmospheric pressure). Therefore it can be concluded, that the main influence causing growth inhibition is the low temperature in the membrane module. However, immobilization by recirculation through a HFMB module could be a feasible option for organisms that require lower temperatures like *A. woodii*. In order to maximize the mass transfer of H_2 into the liquid phase its optimal volumetric gas transfer coefficient (kL_a) should be determined by varying liquid and gas flow rates, as it was done by Shen et al. (Shen et al., 2014). Furthermore the optimal lumen gas pressure should be determined for maximum gas transfer.

For the immobilization of *M. thermoacetica* in batch fermentation, a different approach has to be chosen. Using an external membrane module for this purpose is suboptimal. Although heat loss could be minimized by insulation of hoses and the module itself, a reasonable temperature within the immobilization module could not be kept. An external heating unit for the HFMB-module could solve this issue. However, the installation of such a temperature-control unit would involve considerable financial and labor-intensive effort.

An intrinsic approach for immobilization of *M. thermoacetica* should be followed. One possibility would be the use of a mesh or a membrane that could be inserted into the fermenter in a cylindrical manner around the stirring unit.

5.2 Experiments on ABE-fermentation

5.2.1 Directed ABE production in flasks

It is known from literature, that *C. beijerinckii* typically produces ABE in a ratio of 3:6:1 (Jones and Woods, 1986). In none of the four trials ABE was produced which is very peculiar, because cell growth was observed in trials #1 and #2. The produced intermediates acetate and butyrate should have been converted to ethanol and butanol. No butanol was produced at all. However the concentration of ethanol increased minimally, indicating an initiation and a premature termination of solventogenisis. Cause of this preliminary cessation of solvent production could have been an acid crash. Acid crashes occur, when acids are produced in excess and solventogenisis is not switched on significantly (Maddox et al., 2000). As Maddox states, this occurrence is not unlikely for *C. beijerinckii* cultivated in an uncontrolled environment. Solventogenisis is induced, when concentration of un-dissociated acids (acetic acid and butyric acid) exceeds a threshold of about 20 mM and the pH reaches values around 5. However, if acids are produced too quick and their concentration increases over 60 mM, acid crashes happen (Evans et al., 1998). The acid production rate and pH influence the concentration of un-dissociated acids. Product formation is closely related to glucose uptake rate. Hence, slowing down the metabolism by reducing the incubation temperature can lower the acid production rate. In order to ensure productivity the temperature can be increased, as soon as solventogenisis was initiated. Another approach of preventing an acid crash is to control the pH. At a pH of 5, most of the acids will be in the dissociated form. However, a very high concentration of dissociated acids may be left in the broth and thereby impairing the productivity of a fermentation (Maddox et al., 2000).

However, in trial #1 a total acid concentration of 24.7 mM was reached, so an acid crash can be excluded as a cause for cessation of solventogenisis. It is possible, that phosphate contained in the medium influences the metabolic behavior of *C. beijerinckii*. Also a possible

degeneration of *C. beijerinckii* due to a very long time of cultivation as an active culture could have led to an impaired ability of solventogenisis. Wooley and Morris (1990) found a drift of solventogenic towards acetogenic cultures of *C. beijerinckii* when cultivated over a long period of time (Woolley and Morris, 1990).

An enhanced solvent production was expected from acetate enriched trial #2, as it was achieved by Chen and Blaschek (1999) (Chen and Blaschek, 1999). In order to prevent initial acetic acid concentrations of 61 mM with an inhibiting effect on solventogenesis , the culture should be enriched with acetate when solventogenisis is already reached.

The progress of trial #3 met expectations. No growth, acid or solvent production was monitored as to a lack in reducing equivalents since no H_2 was supplied in the headspace. Nor was any glucose provided as substrate.

Trial #4 unfortunately led to the same results as #3. An initiation of solventogenisis by a low pH and high acid production was not achieved. Experiments should be conducted following the strategy of inducing solventogenisis by lowering the pH to 5 and providing acetic acid at concentrations of around 20-30 mM.

In order to increase solventogenic efficiency, the use of high ABE-producing mutants, that is not that sensitive to acid crash like *C. beijerinckii* BA101 (Qureshi and Blaschek, 1999) should be investigated.

6 Conclusions

Within the present study experiments on the microbial conversion of H_2 and CO_2 into acetate were carried out. A first glimpse on the further metabolism into the solvents acetone, butanol and ethanol was also gathered by testing the possibility of direct entrance into the solventogenic stage of ABE fermentation. Thereby the acetogens *Morella thermoacetica* and *Acetobacterium woodii* were successfully adapted to minimal media to facilitate chemolithoautotrophic growth on H_2 and CO_2, paving the way for a future economical transformation of H_2 into the intermediate acetate. Furthermore both organisms were compared in their metabolic performances. Productivities were determined in laboratory scale bioreactors. It was found, that *A. woodii* produces almost twice as much acetic acid as *M. thermoacetica* under optimal cultivation conditions. Moreover, the effect of yeast extract on the growth behavior and production rates of *M. thermoacetica* was determined. Adding YE to the growth medium or glucose to the pre-culture significantly decreased the typically long lag-phase for chemolithoautotrophic growth of M. thermoacetica. *Acetobacterium woodii* could successfully be cultivated without YE in serum bottles, but not in batch fermentations.

No significant effect of YE supplementation on final product concentrations was described.

The evaluation of different immobilization carrier materials for *M. thermoacetica* led to the conclusion that final product concentrations could be increased by the cell attachment to meth max, clay and a pipe cleaner. However, immobilization attempts in an HFMB-module during batch fermentation did not enhance productivities or product tolerance, as cells did not form a protective biofilm on membrane fibers due to a too low culturing temperature within the HFMB-module.

Experiments for the direct conversion of acetate into solvents via ABE-fermentation could not deliver the expected results. Degeneration of *C. beijerinckii* cells due to long-term cultivation led to the loss of their solventogenic ability. Experiments should be repeated with freshly revived cells from the working cell bank or a different organism like *Clostridium acetobutylicum*. Furthermore the medium should be altered in order to prevent strain degeneration, which is enhanced by the present phosphate (Bahl et al., 1982; Chen and Blaschek, 1999). Once this process is established, immobilization attempts should be conducted to increase its productivity.

To sum up, the reproducible conversion of H_2 and CO_2 into acetate by acetogens is a difficult task due to their high sensitivity to traces of oxygen and their slow metabolism. However, with both organisms satisfactory results were accomplished to build upon. The direct conversion of acetic acid is affiliated with plenty of challenges due to its innovative character, ranging from strain degeneration to the prevention of acid crashes during manually induced solventogenisis. Nevertheless, acetate as a hydrogen storage medium with the subsequent

conversion into liquid fuels is still a promising method to overcome the problem of peak currents of wind and solar electricity generation in the future. However, the investigated approach is just at its beginning and a significant research effort is required until this process becomes economically feasible and a realistic alternative for the conservation of wind and solar power.

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8 Annex

List of used laboratory equipment

1 L Suction bottle, water suction pump)	
1 x 5.5 MiniModule®	
1000 µL pipette	Gibson, Eppendorf
200 µL pipette	Gibson, Eppendorf
250 mL Kjeldahl flask, Auto Kjeldahl	
5 mL pipette	Eppendorf
Aluminum foil	
Anaerobic tent,	Cole-Parmer
Analysis scale, Scaltec SBA 41	Denver Instruments
Autoclavable sterile filters (0.2 µm)	Merck Millipore
Autoclave, Dampfsterilisator Typ 500	VARIOKLAV
Backpressure valve, JUMO iTron 08	
Beakers of different sizes	VWR Collection
Büche, Vapodistillation unit,	C. Gerhardt GmbH
Büchi, Kjeldahltherm-Digestion System,	C. Gerhardt GmbH
Butyl stopper (Ø 20 mm)	VWR
Captair 2200ANM Pyramid Glove Bag	
Centrifuge, 5450D	Eppendorf
Crimp top vials (3 mL)	VWR
Crimper (Ø 20 mm)	VWR
Desiccator	VWR
Digital pressure indicator, DPI 705	
Dispette	Eppendorf
Filtration unit (stainless steel frit,	VRW Collection
Freezer, (-80 °C)	PMI Labortechnik
Funnel (glass)	VWR Collection
Gas hoses, Festo	VWR
High Performance Liquid Chromatograph	Agilent Technologies
Hollow fiber membrane module,	Liqui-Cel

Hose clamps, metal	VWR
Incubator, Multitron	INFORS-HT
LaminAir HB 2472	
Laminar flow working bench	
Lids for Schott bottles (GL-45, 3 Ports)	DURAN Group
Light microscope, Eclipse E200	Nikon
Luer connectors	Cole Parmer
Magnetic stirrer, IKAMAG RCT	IKA-Werke GmbH & Co.
Membrane filter, pore size 0,45 µm	Ahlstrom
Microscope slide	VWR
Multifors 2 fermentation system	INFORS-HT
Pasteur pipette (glass)	VWR Collection
Peristaltic pump, Heidolph Pumpdrive 5201	
Petri dish (glass)	VWR Collection
pH-probe, WTW3110, Sentix 81	Mettler-Toledo
precision scale, CPA 1003P	Sartorius
Reaction tubes (1.5 mL)	VWR
Return condenser	INFORS-HT
Rotilabo-CME syringe filters	Carl Roth
Schott bottles of different sizes	DURAN Group
Semi micro cuvettes	VWR
Serum bottles (100 mL)	Sigma Aldrich
Silicone hoses	VWR
Swanlock-valves	CODAN
Thoma counting chamber, cover slip	VWR
Two beam photometer, Lambda 2S	Perkin
Tygon hoses	Saint-Gobain
Unit K 370	
Variostat Control Unit	
Volumetric flasks of different sizes	VWR Collection
Volumetric pipetts of different sizes	VWR collection
Vortex, VORTEX-Genie 2	USA scientific
Water bath, GFL 1002	Laborgeräte München

List of used Chemicals and Substances

Aluminium chloride hexahydrate	Merck KGaA
Ammonium Chloride	Sigma Aldrich
Boric acid, ≥ 99.5 %	Sigma Aldrich
Calcium chloride dihydrate	VWR Chemicals
Cobalt (II) Chloride Hexahydrate	Merck KGaA
Cobalt (II) Sulfate Heptahydrate	Merck KGaA
Cobalt (II) Sulfate Pentahydrate	Merck KGaA
Glucose	Sigma Aldrich
Hydrochloric acid	VWR Chemicals
Iron (II) Chloride TetrahydrateMerck	Merck KGaA
Iron (II) Sulfate Heptahydrate	Sigma Aldrich
Magnesium Chloride Hexahydrate	Sigma Aldrich
Magnesium Sulfate Heptahydrate	Sigma Aldrich
Manganese (II) Chloride Tetrahydrate	Sigma Aldrich
Manganese (II) Sulfate Dihydrate	Merck KGaA
Nickel (II) Chloride Hexahydrate	Merck KGaA
Peptone from meat	
Potassium Dihydrogen Phosphate	Sigma Aldrich
Potassium Hydrogen Phosphate	Sigma Aldrich
Sodium Chloride	Sigma Aldrich
Sodium EDTA Dihydrate	Merck KGaA
Sodium Hydrogen Carbonate	Fluka Analytical
Sodium Molybdate Dihydrate	Merck KGaA
Sodium Molybdate Tetrahydrate	Merck KGaA
Sodium Selenate	Sigma Aldrich
Sodium Selenite Pentahydrate	Merck KGaA
Sodium Sulfite	Merck KGaA
Sodium Tungstate Dihydrate	Merck KGaA
Sulfuric acid	Sigma Aldrich
Trypticase Peptone	Oxoid
Yeast extract	Oxoid
Zinc (II) Chloride	Sigma Aldrich
Zinc Sulfate Heptahydrate	Sigma Aldrich

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Chemicals

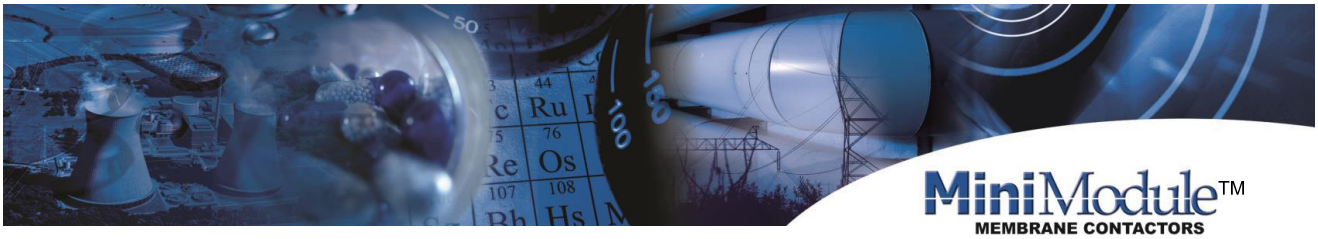
$AlCl_3*6\ H_2O$	Aluminium chloride hexahydrate
$CaCl_2*2\ H_2O$	Calcium chloride dihydrate
$CoCl_2*6\ H_2O$	Cobalt (II) Chloride Hexahydrate
$CoSO_4*7\ H_2O$	Cobalt (II) Sulfate Heptahydrate
$CuSO_4*5\ H_2O$	Cobalt (II) Sulfate Pentahydrate
$FeCl_2*4\ H_2O$	Iron (II) Chloride Tetrahydrate
$FeSO_4*7\ H_2O$	Iron (II) Sulfate Heptahydrate
H_2SO_4	Sulfuric acid
H_3BO_3	Boric acid
K_2HPO_4	Potassium Hydrogen Phosphate
$KAl(SO_4)_2*12\ H_2O$	Potassium Hydrogen Phosphate
KH_2PO_4	Potassium Dihydrogen Phosphate
$MgCl_2*6\ H_2O$	Magnesium Chloride Hexahydrate
$MgSO_4*7\ H_2O$	Magnesium Sulfate Heptahydrate
$MnCl_2*4H_2O$	Manganese (II) Chloride Tetrahydrate
$MnSO_4*2\ H_2O$	Manganese (II) Sulfate Dihydrate
$Na_2EDTA*2H_2O$	Sodium EDTA Dihydrate
$Na_2MoO_4*4\ H_2O$	Sodium Molybdate Tetrahydrate
$Na_2SeO_3*5\ H_2O$	Sodium Selenite Pentahydrate
Na_2SeO_4	Sodium Selenate
Na_2SO_3	Sodium Sulfite
$Na_2WO_4*2H_2O$	Sodium Tungstate Dihydrate
$NaCl$	Sodium Chloride
$NaHCO_3$	Sodium Hydrogen Carbonate
$NaMoO_4*2H_2O$	Sodium Molybdate Dihydrate
NH^{4+}	Ammonium
NH_4Cl	Ammonium Chloride
$NiCl_2*6\ H_2O$	Nickel (II) Chloride Hexahydrate
$ZnCl_2$	Zinc Chloride
$ZnSO_4*7\ H_2O$	Zinc Sulfate Heptahydrate HCl Hydrochloric acid

List of abbreviations

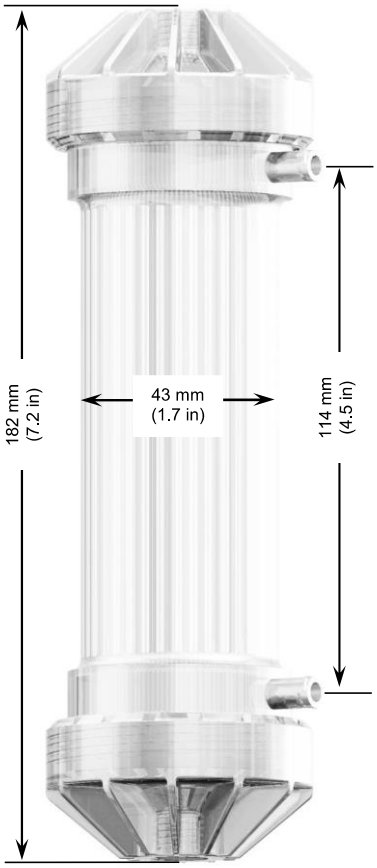
A. woodii	*Acetobacterium woodii*
ABE	Acetone-Butanol-Ethanol
C. beijerinckii	*Clostridium beijerinckii*
C1	Carrez reagent 1 (Sodiumhexacyanoferrate-trihydrate)
C2	Carrez reagent 2 (Zinksulfate-heptahydrate)
CBM	Citrate buffer medium
CDW	Cell dry weight
CH_4	Methane
CM	Complex medium
CO2	Carbon dioxide
CoA	Coenzyme A
CODH	CO-dehydrogenase
d	Days
ddH_2O	Double destilled water
d_i	Inner diameter of hollow fiber (Shen)
d_i'	Inner diameter of hollow fiber
DSMZ	Deutsch Sammlung von Mikroorganismen und Zellkulturen
EtOH	Ethanol
g	Gram
h	Hours
H2	Hydrogen
HFMB	Hollow fiber membrane
HPLC	High performance liquid chromatography
K	Kelvin
kPa	Kilo Pascal
M. thermoacetica	*Morella thermoacetica*

mg	Milligram
min	Minute
mL	Milliliter
MM 1	Minimal Medium 1
MM 2	Minimal Medium 2
n_F	Number of fibers
n_F'	Number of fibers
OD_{600}	Optical density at 600nm
PC	Polycarbonate
PP	Polypropylene
PSU	Polysulfone
Q	Volumetric flow rate
r_i	Inner radius of hollow fiber (Shen)
r_i'	Inner radius of hollow fiber
RID	Refractive index detector
v	Liquid flow velocity
VFA	Volatile fatty acids
vol.	volumetric
WCB	Working cell bank
YE	Yeast extract

HFMB-module Data sheet



1.7 x 5.5 MiniModule™ PRODUCT DATA SHEET



Membrane Characteristics	
Cartridge Configuration	Parallel Flow. Lumenside Liquid Flow.
Liquid Flow Guidelines	<2500 ml/min
Membrane Type	X50 Fiber
Membrane/Potting Material	Polypropylene/Polyurethane
Typical Membrane Surface Area	0.5 m^2 Calculated based on inner diameter of hollow fiber
Priming Volume (approximate)	
Shellside	78 ml
Lumenside	53 ml
Pressure Guidelines*	
Maximum Lumenside LIQUID Working Temperature/ Pressure	5-20° C, 4.1 barg (41-68° F, 60 psig) 40° C, 2.1 barg (104° F, 30 psig)
* Note: Liquid pressure should always exceed gas pressure.	
Housing Options and Characteristics	
Material	Polycarbonate
Flange Connections	
Shellside (gas/vacuum)	Standard Female Luer Lock *Supplied with two ¼ inch Hosebarb adaptors which mate to ¼ inch ID tubing*
Lumenside (wetted surface)	1/4 inch FNPT
Seal Options	
Material	**Applications**
EPDM	All Purpose
Weight (approximate)	
Dry	142 grams
Shipping weight (max)	151 grams
Regulatory	
Complies with the limits as set by RoHS Directive 2011/65/EU Annex II; recasting 2002/95/EC. Constructed of FDA CFR title 21 compliant materials for wetted parts only at ambient temperatures.	

Note: All dimensions are nominal values. Refer to liqui-cel.com for detailed housing drawings.