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„Ecological and biotechnological aspects of mono- and multispecies hydrogen production systems“

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Abstract (Deutsch)

Biologisch hergestellter molekularer Wasserstoff (H_2) hat in den letzten Jahrzehnten als vielversprechende Alternative zu fossilen Energieträgern viel Aufmerksamkeit erregt. Untersuchungen haben gezeigt, dass Bakterien, welche die „dark fermentation“ nutzen, ein hohes Potenzial zeigen, H_2 effizient und in großen Mengen zu produzieren. Diese H_2-Produzenten metabolisieren organische Verbindungen unter anschließender Produktion von H_2 und gelösten Metaboliten. Zu den dark-fermentation nutzenden Organismen gehören *Clostridium acetobutylicum* und *Enterobacter aerogenes* als vielversprechendste mesophilen Kandidaten für die Optimierung und Steigerung der industriellen Biowasserstoffproduktion.
Diese Organismen wurden in dieser Studie verwendet, um ein neu entwickeltes Co-Kultur-H_2-Produktionssystem zu untersuchen. Die Ergebnisse wurden mit dem Wachstum, der Substrataufnahme und der Produktion der entsprechenden Reinkulturen verglichen.
Während und nach den Experimenten wurden die produzierten Gaszusammensetzungen mittels Gaschromatographie analysiert. Die H_2-Entwicklungsraten (HERs) von 26,3 mmol / L / h (C-molar) und 1,7 mmol / L / h (C-molar) und die Ausbeuten (H_2 / S) von 0,91 mol / C-mol und 0,43 mol / C- Mol wurden jeweils mit *E. aerogenes* bzw. *C. acetobutylicum* im spezifischen Medium gefunden. Das Verhältnis von H_2 zu CO_2 erreichte während der Exponentialphase bis zu 1,9: 1,2. Dies zeigt, dass die gewählten Organismen eine erhöhte H_2-Produktion unter bestimmten Bedingungen erreichen können. Die gelösten Metaboliten wurden mittels HPLC analysiert und die Ergebnisse zeigten ein breites Spektrum an Verbindungen wie Ethanol, Ameisensäure, Butandiol, Essigsäure und Milchsäure.
Zur Quantifizierung der Wachstumskinetik wurden verschiedene Instrumente verglichen, einschließlich der optischen Dichte, der quantitativen Polymerase-Kettenreaktion (qPCR) und der Zellzahlen. qPCR zeigte als einzige Methode korrekte Zellzahlen auch während der Biofilmbildung. Zur Unterscheidung der beiden Organismen in Co-Kultur wurde eine Visualisierungsmethode auf Einzelzellbasis mittels fluoreszierender In-situ-Hybridisierung (FISH) gezeigt. Gruppenbasierte FISH-Sonden waren zum Färben von *E. aerogenes*-Zellen geeignet. *C. acetobutylicum* war hingen resistent gegen spezifische Färbung aufgrund von Sporenbildung und konnte nur mit einem unspezifischen DNA-Farbstoff gefärbt werden.
Um die H_2-Produktion zu verbessern, wurde ein neu entwickeltes Medium für die Co-Kultur dieser beiden Bakterien entwickelt und getestet. In diesem „DOE E-Medium“ konnten beide Bakterien wie im spezifischen Medium in Serumflaschen wachsen. Bei unkontrollierten pH-Bedingungen im Bioreaktor konnte die Co-Kultur die Kohlenstoffquelle vollständig nutzen. In den Experimenten mit den Reinkulturen als auch mit der Co-Kultur im DOE E-Medium war allerdings fast keine Produktion von H_2 nachweisbar. Die Kultivierung des strengen anaeroben *C. acetobutylicum* als Reinkultur in Bioreaktoren mit DOE E-Medium war bisher nicht möglich. Zusätzliche methodische Verbesserungen der Kultivierung strikter Anaerobier sind erforderlich, um ein ideales Co-Kultur-H_2-Produktionssystem zu entwickeln. Es müssen mehr Untersuchungen zur Kultivierung dieser Mikroorganismen im Bioreaktor durchgeführt werden, um das bislang gehemmte Wachstum und die eingeschränkte Aktivität zu überwinden.

Zusammenfassend lässt sich festhalten, dass die in dieser Arbeit gezeigten Ergebnisse als Ausgangspunkt für das Design eines Biowasserstoff-Co-Kultur-Systems im Hinblick auf die Herausforderungen dienen. Besondere Beachtung ergibt sich aus den unterschiedlichen Physiologien und Morphologien von *C. acetobutylicum* und *E. aerogenes,* und ihrer unterschiedlichen Kultivierung, Eigenschaften und Anforderungen. In Zukunft könnte ein solcher Ansatz für die kombinierte Kultivierung möglicherweise die biologische H_2-Produktion zu einem hocheffektiven biotechnologischen Prozess machen.

Abstract (English)

Biologically produced molecular hydrogen (H_2) has gathered attention in the last decades as a promising alternative to fossil energy carriers. Recent investigations have revealed the high potential of dark fermentative bacteria to produce H_2. Dark fermentative H_2 producers metabolize organic compounds with the subsequent production of H_2 and dissolved metabolic end products. Among the dark fermentative H_2 producing organisms, *Clostridium acetobutylicum* and *Enterobacter aerogenes* are promising mesophilic candidates for examining, optimizing and scaling-up industrial biohydrogen production.
Therefore, in this study, *C. acetobutylicum* and *E. aerogenes* were used for establishing and investigating a co-culture H_2 production system in closed batch cultivation mode and comparing the results to their growth, substrate uptake and production in monocultures. During and after H_2 production experiments, the headspace gas compositions were analysed via gas chromatography. H_2 evolution rates (HERs) of 26.3 mmol/L/h (C-molar) and 1.7 mmol/L/h (C-molar) and yields $_{(H2/S)}$ of 0.91 mol/C-mol and 0.43 mol/C-mol were found for *E. aerogenes* and *C. acetobutylicum* species-specific media, respectively.
The relative ratio of H_2 partition to CO_2 reached up to 1.9:1.2 during the exponential phases, underlining that these organisms can be suitable for enhanced H_2 production. Dissolved metabolites were analysed via HPLC and revealed the production of a wide spectrum of compounds such as ethanol, formic acid, butanediol, acetic acid and lactic acid.
For quantification of growth kinetics, different tools, including optical density, quantitative polymerase chain reaction (qPCR), and cell counts, were compared. qPCR was the only suitable method when evaluating cell numbers during biofilm formation. To distinguish the two organisms in co-culture, a visualisation method on a single cell basis was established via fluorescent *in situ* hybridisation (FISH). Group-based FISH probes were suitable for staining cells of *E. aerogenes*. Conversely, *C. acetobutylicum* was resistant to specific staining due to spore formation and could only be stained using a nonspecific DNA dye.
To enhance H_2 production, a newly designed medium for the co-culture of these two bacteria was designed and tested. In this “DOE E medium”, both bacteria could grow as well as in their own specific medium in closed batch. At uncontrolled pH conditions in batch, the co-culture was able to fully use the carbon source. However, almost no H_2 production was detectable from the mono-cultures or the co-culture in the DOE E medium. So far, cultivation of the strict anaerobe *C. acetobutylicum* was not possible as mono-culture in the DOE E medium grown in batch mode in bioreactors. Additional methodical improvements in cultivation of strict anaerobes are needed to establish a co-culture H_2 production system. More intensive research on cultivation of these microorganisms in batch must be done to overcome inhibited growth and activity so far.

In conclusion, the results shown in this thesis can be used as a starting point to design a biohydrogen co-culture system with respect to known challenges arising from the different physiologies and morphologies of *C. acetobutylicum* and *E. aerogenes* and their different cultivation characteristics and nutritional demands. In the future, such an approach for parallel medium and co-culture design could possibly render biological H_2 production an effective biotechnological process.

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1 Introduction

1.1 Biofuels

After almost 200 years of fossil energy carrier-depletion, intensive research on alternatives has become one of the most relevant topics in our daily life. The fossil energy carriers have been contributing to our societal and technological status until today. The negative facets such as to greenhouse gas emissions and related climate change (Jiang et al., 2010) were ignored for most of the time. It has come to a point of inevitable need for a change to new energy sources, to be able to save future human generations and a vast amount of other species.

Currently, oil, coal and natural gas as fossil-based energy carriers supply the worldwide energy requirement. When categorizing the economic sectors (reviewed in the report of IPCC 2014), "Electricity and heat production" due to burning of fossil fuels is the biggest part with 25% of global greenhouse gas emissions. "Agriculture, forestry and other land use" contribute with 24% to the global emissions, mostly from cultivation of crops, livestock and deforestation. "Industry", with the burning of fossil fuels for energy production, contributes with 21% to the global greenhouse gas emissions. The sector of "Transportation" is the 4th biggest contributor to global emissions with 14%, concerning all transportation methods based on fossil fuels worldwide.
Negative environmental and health impacts as well as the unstable price of fossil fuel already brings science and politics together, to improve alternative techniques and trigger the implementation in energy supplying with an annual increase rate of 2.5% (EIA 2013; Bundhoo and Mohee, 2016).

With respect to transportation fuels, one million electric vehicles were sold promoting a clean and environmentally friendly and the first commercial alternative to internal combustion engines in 2018 (The Global EV Outlook 2018; IEA analysis, 2018). At the same time, this alternative is not as green as it is supposed to be when focusing on greenhouse gas emissions related to manufacturing and usage of electric vehicles (IVL Swedish Environmental Research Institute, 2017). At this point, the environmental costs of disposal of batteries are not even included, leading to truly question if this is a long-term alternative to fossil fuels.

The Intergovernmental Panel on Climate Change (IPCC) (2019) claims, that renewable energy sources and energy carriers must replace fossil fuel usage of industry, production of electricity and transportation to keep global warming beyond the critical marker of 1.5 °C above pre-industrial levels. Apart from fossil energy carriers, biofuels were verified to be a promising energy alternative for industry, production of electricity and transportation *(*Porqueras et al., 2013), since, biofuels can be generated with minimum amount of greenhouse gases and without any fossil energy source (Momirlan and Veziroglu, 2004). Moreover, industrial waste materials can be recycled as substrate for biofuel-production (Kleerebenzem, 2007). Methane (CH_4), ethanol, molecular hydrogen (H_2) and many other biofuels are promising alternatives or additives to fossil fuels, formed by plants and organisms which can be easily cultivated *(*Porqueras et al., 2013). Depending on the production and the main components, these biofuels can be categorized in 5 generations.

The 1st generation considers the fuels that have been derived from sources like corn or sugarcane, animal fats or vegetable oil. These resources are converted to bioethanol and biodiesel (Dhaman and Roy, 2013). This generation is already widely commercialized.

The 2nd generation is based on lignocellulosic part of plants, which circumvents the use of edible foods for forming biofuels. The yields of the 2nd generation of biofuels tend to be low, which inhibited this generation from large commercialization so far (Dhaman and Roy, 2013).

As an algal-based biofuel, the 3rd generation-biofuels have been promising due to the high land efficiency, high bio-oil production of algae (compared to e.g. soy beans), and the ability of these plants to use freshwater, saltwater and even wastewater (Potts et al., 2012) as a water source. The fuels are mostly based on triglycerides, which are extracted from algae (Pabbi et al., 2011). This generation is still on a demo scale and not commercialized yet.

As a further improvement of the 3rd generation, the 4th generation-biofuels imply metabolic engineering of algae to get higher yields over advanced biochemistry and petroleum-like hydro processing (Dhaman and Roy, 2013). Biofuels of this generation are based on photofermentation and usage of CO_2, making them a “solar converter” (Kagan, 2010). Research on this generation of biofuels is still in progress and no commercialization has been done yet.

The 5th generation biofuels are summarized as the conversion of acetate, methylated compounds and/or carbon dioxide (CO_2) and molecular H_2 to methane (CH_4) and water (H_2O) (Porqueras et al., 2014) under strict anaerobe conditions. This generation is supposed to be ready for commercial application within the next years (Porqueras et al., 2014).

1.2. Hydrogen

Today, one of the most focused biological energy carriers is H_2. H_2 contains a high level of energy (142 MJ/kg) compared to CH_4 (55.5 MJ/kg) and petrol (47.3 MJ/kg) (Singh and Wahid, 2015). In general, H_2 could be used for a variety of settings: for producing electricity, as a fuel for transport and for industrial procedures. The product of combustion of H_2 is H_2O (Momirlan and Veziroglu, 2004), concluding that no greenhouse gases like CO_2, CH_4, nitrogen dioxide (NO_2) and other pollutants are produced to contribute to global warming and climate change (Singh et al., 2015; Navarro-Díaz et al., 2016). H_2 can be produced based on fossil fuels and electrolysis, or alternatively by microorganisms from biomass, sugars, and organic acids, as well as from H_2O and carbon monoxide via the water gas shift reaction (Ratnasamy et al., 2009). H_2 as a non-bound molecule is rare in nature. It must be produced by a variety of industrial or biotechnological pathways to enrich in higher and purer amounts.

Today, about 98% of artificially formed H_2 is based on fossil fuels (Kalinci et al., 2009). About 40% are formed from natural gas or steam reformation of CH_4, 30% from oil-gasification, 18% from coal-gasification, and about 4% by electrolysis of water (Singh and Wahid, 2015; Sinha and Pandey, 2011).
All these processes can be performed with comparatively low effort but demand a lot of energy over fossil energy carriers, and the overall reactions are very uneconomical (Salvi et al., 2013). Alternatively, biological processes can be used to produce H_2. These are less energy intensive and can be operated at mesophilic temperatures (Das and Veziroğlu, 2001).

Producing H_2 from organic compounds biologically is an efficient way to reduce the costs of production, the dependency of fossil resources and consequently the impact on the environment. Additionally, microorganisms which produce H_2 can be rapidly grown under certain conditions in bioreactors (Rittmann et al., 2015). These organisms can use a variety of substrate like industrial waste (Kleerebenzem, 2007), monosaccharides, disaccharides, complex polysaccharides (e.g. starch) (Liu et al., 2008), and even cellulose (Lynd et al., 1989) in an efficient way, offering a way of recycling of waste and the use of a comparatively cheap substrate.

With reflect on the introduced generations of biofuels, the production of biohydrogen is a coupling process of 1st, 2nd and 4th generation of biofuel (Porqueras et al., 2014), depending on the process of production. The current drawbacks of biological H_2 production are the costs which limit the common usage as an upcoming energy source (Liu et al., 2008). Therefore it is important intensify research in this field as well as to improve biotechnological applications to get a higher efficiency/yield of biohydrogen production ($Y_{(H2/S)}$), H_2 produced per substrate consumed ($Y_{(H2/S)}$ [mol/mol], substrate conversion efficiency), H_2 evolution rate (HER [mmol/L/h], volumetric productivity) and the specific H_2 production rate (qH_2 [mmol/g/h], biological production capacity) under certain physiochemical parameters (e.g. pH, temperatures and substrates) (Rittmann and Herwig, 2012).

To get comparable H_2 production values, it is necessary to base the study on a C-molar basis of substrate and avoid using complex media, especially for the ability of comparing studies. C-molar based $Y_{(H2/S)}$ and HER are important to relate results of dark fermentation processes (Herwig et al., 2001; Rittmann and Herwig, 2012).

1.3 Biological H_2 production processes

Currently, four different processes are known for biohydrogen production: photofermentation, (photofermentative bacteria), direct and indirect biophotolysis (cyanobacteria and green algae) and dark fermentation (fermentative bacteria and archaea).

1.3.1 Photofermentation

Photofermentation is an anaerobic process and depended on a light source (in difference to dark fermentation). H_2-production occurs via nitrogenases, which is driven by reduced ferredoxin and ATP provided by substrates like reduced sulphur compounds or organics like lactic, succinic, butyric acid, or alcohols, and is done by photosynthetic non-sulphur bacteria (Karthic and Shiny, 2012). These organisms can use organic waste as substrate, which can be converted completely to H_2 and CO_2. The major drawback of this process is the low photochemical efficiency (3-10%) and the specific photobioreactors for achieving optimal light penetration into the medium (Hallenbeck and Ghosh, 2009). As one example, *Rhodobacter sphaeroides* is one of the most promising species with a qH_2 of 94 mmol/L/h based on lactate as substrate in continuous culture (Ting et al., 2004).

1.3.2 Biophotolysis

Biophotolysis is an oxygenic process. Cyanobacteria and green microalgae can use chlorophyll and other pigments in PSI and PSII to harvest light energy in the form of electrons and to concomitantly perform oxygenic photosynthesis (Yu and Takahashi, 2007). Under light irritation, a part of the excess electrons is stored directly in H_2 over a hydrogenase in the heterocysts, especially under nitrogen limiting conditions to store energy directly derived from energy-rich carbohydrates (direct biophotolysis) (Yu and Takahashi, 2007). This reaction done in these hydrogenases is reversible (Yu and Takahashi, 2007).
If organisms store the produced carbohydrates and use them in a second step, it is called indirect biophotolysis. These pathways use an abundant substrate (H_2O) and have simple products (H_2 and O_2). It is performed by a few cyanobacteria and microalgae.
Drawbacks are the O_2 sensitivity, expensive photobioreactors (Hallenbeck and Ghosh, 2009), the low energy productivity of 0.38 kJ/L/h (Yu and Takahashi, 2007) and relatively small solar energy conversion efficiency (< 10%) (Karthic and Shiny, 2012). As ways of producing biological H_2, direct and indirect biophotolysis still need an intensive research.

1.3.3 Dark Fermentation

Dark fermentation is an anaerobic process performed by H_2-producing bacteria and archaea. Various carbohydrates, organic molecules, polymers and even industrial waste can be used as substrate for dark fermentation (Kleerebenzem, 2007). Different metabolic pathways can be used for producing biological H_2 and organic metabolites.
The production of biological H_2 over dark fermentation is independent from a light source. The energy requirement of the process is comparatively low. Especially when it is performed at mesophilic conditions, it requires less energy and a comparatively simple reactor technology (Hallenbeck and Ghosh, 2009).
The organisms produce valuable metabolites like volatile fatty acids (VFAs), which can be directly extracted for industrial processes, or the whole system gets combined with other biofuel production processes, such as CH_4 production, microbial fuel cells (Kumar et al., 2016) or photofermentation (Das and Veziroğlu, 2001) to further enhance the $Y_{(H2/S)}$.
It has been reported that dark fermentation using organisms can reach high values for HER (reaching up to 228 mmol/L/h in a mixed culture in continuous setting (Eroglu et al., 2006)) compared to other biohydrogen production processes, which led to a very strong focus on dark fermentative H_2 production process in the last decades. However, one major drawback of dark fermentation is a lower $Y_{(H2/S)}$ compared to other biohydrogen production systems. This is mostly because of the formation of a wide range of side products, which causes the final value of $Y_{(H2/S)}$

(Ramirez et al., 2015). This value varies and is strongly dependent on the organism's metabolic pathway-portfolio.

The metabolic by-products of dark fermentation are VFAs, alcohols, and a gas mixture consisting of H_2 and CO_2. If acetic acid is the product, a theoretical maximum of 4 moles H_2 per monosaccharide can be gained (1), and if butyrate is the product, a maximum of 2 moles H_2 per mole monosaccharide (2) is possible (Levin et al., 2004):

(1) $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + \mathbf{4H_2} + 2CO_2$

(2) $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + \mathbf{2H_2} + 2CO_2$

This limitation of 4 and 2 moles of H_2, respectively, is well known as the "Thauer Limit" (Thauer et al., 1977; Kim et al., 2018).Reaching values close to this limitation can be reached by a well-chosen consortium of organisms and settings, because these factors can be crucial to get higher $Y_{(H2/S)}$. The best way to increase dark fermentative H_2 production must focus the strong impact of environmental conditions, as well as the fundamental metabolic functions of the organisms (Ergal et al., 2018). One of the most important factors to regulate biohydrogen production is the temperature (Mnatsakanyan et al., 2004).

1.4 Dark fermentative organisms

Dark fermentative organisms provide a natural conversion of biomass to H_2. These organisms are heterotrophs and degrade organic substrates like carbohydrates via oxidation to provide energy in an anoxic environment. H_2 is mainly produced as a side product because of the excess electrons within the cell or is taken up by H_2 consumers in the ecosystem (Khanna and Das, 2013). Dark fermentative organisms can operate at a wide range starting from mesophilic conditions (25-44°C), over thermophilic conditions (45-79°C) and up to hyperthermophilic conditions (>80°C) (Stetter, 2006). H_2 producing organisms can be categorized in mesophilic and thermophilic organisms depending on the preferred temperature.

Most of the known thermophilic H_2 producers belong to the genus of *Thermoanaerobacterium* (Ahn et al., 2005). Additionally, Thermotogaceae with a $Y_{(H2/S)}$ of 0.47 mol/C-mol and Thermoanaerobacteriales with a $Y_{(H2/S)}$ of 0.44 mol/C-mol belong to the highest mean values measured in closed batch (Ergal et al., 2018). The highest reported values (C-molar) for the archaeon *Thermococcus onnurineus* reached a $Y_{(H2/S)}$ of 1.25 mol/C-mol, a HER of 233 mmol/L/h and a qH_2 of 404 mmol/g/h based on formate as substrate (Lee et al., 2012; Lim et al., 2012). For reaching these values, a generation of formate for utilization is fundamental. So far, the formation over CO or electricity would be two possible options (Ceccaldi et al., 2017).

With focus on mean values of $Y_{(H2/S)}$ and with excluded values for formate utilising organisms, thermophiles have potential because of slightly higher $Y_{(H2/S)}$, more favourable conditions for H_2 production, because H_2 is less soluble at high temperatures, advanced resistance of the system to contamination of mesophiles and consumption of substrates (Khanna and Das, 2013). Moreover, thermophiles can utilize substrates like formate to achieve better results (Ergal et al., 2018), and complex sugars like cellulose, whereas mesophiles need addition of exogenous cellulose enzymes (Elsharnouby et al., 2013). The growth rate and utilization of substrates of thermophilic cultures are higher compared to mesophilic conditions. However, it has been reported that the difference between mesophilic and thermophilic $Y_{(H2/S)}$ is low (Kanai et al., 2005), which was disproven by Ergal *et al*. 2018, who showed that the physiological constraints can be associated to phylogenetic contraints.

Mesophilic H_2 production is well known for facultative anaerobes of the gram-negative genera Enterobactericeae, the gram-positive obligate anaerobic spore-forming Clostridiaceae and for some genera of Bacillus (Jo et al., 2007). These genera are intensely used for mesophilic H_2 and metabolite production in the last two decades.

A big advantage of mesophilic conditions for biohydrogen production is the less expensive tem-

perature regulation. A negative energy balance can be achieved by running reactors at high temperatures (Lee et al., 2011), making mesophilic conditions depending on growth conditions more favourable in total.

Ergal et al. (2018) analysed the data of 117 years of dark fermentative H_2 production in pure cultures. In this review, mesophilic cultures of Enterobacteriaceae showed to have more than 2-folds higher values for qH_2 than thermophiles in batch. In closed batch, Enterobacteriaceae and Clostridiaceae showed slightly higher qH_2 values compared to thermophiles. This qH_2 shows the capacity of H_2 production, and it is related to physiological and metabolic potential, which cannot be extended to produce higher levels of H_2 even with the perfect environment.

Additionally, a continuous H_2 removal with a “milking” system (Ananyev et al., 2012) can operate only at mesophilic temperatures due to the fact, that the electrochemical cells are developed only for mesophilic conditions so far. This system can be used to selectively remove the produced H_2. Moreover, a combination of mesophilic H_2 producing bacteria with electro active organisms such as *Geobacter sp.* and hydrogenotrophic methanogens such as *Methanospirillum* sp. or *Methanolinea* sp. for further methane production can only be operated at mesophilic conditions so far (Lee et al., 2016).

In a comparison of organisms and their highest values of $Y_{(H2/S)}$, qH_2 and HER (Ergal et al., 2018) with focus on mesophilic organisms, *Enterobacter asburidae* with a $Y_{(H2/S)}$ of 1.1 mol/C-mol (Shin et al., 2010) and *Clostridium sp.* with a $Y_{(H2/S)}$ of 0.74 mol/C-mol (Taguchi et al., 1996) belong to the top organisms concerning $Y_{(H2/S)}$. Regarding HER, *E. aerogenes* was reaching values up to 26.67 mmol/L /h (C-molar) (Ito et al., 2005). The highest qH_2 values of *E. aerogenes* with 70.70 mmol/g/h (C-molar) (Seol et al., 2008) belongs to the highest production rates of H_2 ever measured. However, qH_2 showed as theoretical potential the most promising values for Enterobatericeae and Clostridiaceae in closed batch and batch cultivation (Ergal et al., 2018). These values might outdo results of thermophiles when putting respect on the more energy consuming cultivation method. Due to the already produced data and the fact, that Enterobatericeae and Clostridiaceae are two very active and well described mesophilic H_2 producing families, these families were chosen for further investigation.

1.4.1 Enterobactericeae

The family Enterobactericeae contains a variety of gram-negative, non-spore-forming facultative anaerobe bacteria including genera of *Salmonella*, *Shigella*, *Enterobacter* and many others. These bacteria are commonly used for fermentation of monosaccharides such as glucose to produce lactic acid and reduce nitrate to nitrite in an anoxic environment (Donnerberg, 2015). Under oxic conditions many of them switch to aerobic respiration as a preferred pathway (Donnerberg, 2015).

Several strains were sampled in soil and human gut, then characterized and investigated, like *Enterobacter aerogenes* (Rachman et al., 1997; Tanisho and Ishiwata, 1997) and *E. cloacea* (Kumar and Das, 2000). The $Y_{(H2/S)}$ of pure cultures of Enterobacteriaceae ranged from 0.18 to 0.63 mol/C-mol (Fabiano and Perego, 2002; Kumar and Das, 2000; Palazzi et al., 2000; Rachman et al., 1997; Kumar et al., 2001; Nath and Das, 2004; reviewed in Lee et al., 2011) depending on the substrate type.

The microorganism *E. aerogenes* is a facultative anaerobe with high growth rate and the potential to grow even in presence of oxygen (Zhang et al., 2011). The theoretical $Y_{(H2/S)}$ of 1.67 mol/C-mol (Tanisho, 2000) as well as the potential to contribute beneficially to a higher production of H_2 in co-cultures containing *E. aerogenes* and Clostridiaceae (Yokoi et al., 1998) showed good reasons for further research in this study.

1.4.2 Clostridiaceae

The family of Clostridiaceae contains different genera of Firmicutes, which are generally spore-forming, gram-positive, obligate anaerobe bacteria.

The members of Clostridiaceae family have been widely investigated due to the high production potential of different metabolites. Especially *C. acetobutylicum* ATCC 824 is well known as "Weizmann Organism" and was used for ABE (acetone–butanol-ethanol) fermentation processes, where it produces butanol, acetone and ethanol in a mass ratio of 6:3:1 (Jones and Woods, 1986) from various carbohydrates. Additional strains like *C. butyricum*, *C. pasteurianum*, *C. thermosuccinogenes*, *C. bifermentans* have been examined. The $Y_{(H2/S)}$ for these organisms are between 0.18 - 0.43 mol/C-mol (Lee et al., 2011).

Some members of Clostridiaceae family have high resistance against the unfavourable environmental conditions (e.g. high temperature, pH, level of oxygen, chemical toxicity or lack of substrate) due to formation of spores (Sung et al., 2002).

Experiments with Clostridiaceae showed that even if these organisms were not the most abundant species in a mixed-species bioreactor, they had the highest influence on H_2 production in co-cultures due to the variety of metabolic pathways (Chojnacka et al., 2011). Clostridiaceae showed to deal with a wide range of pH, changing substrate types or physiological parameters. They can produce H_2 especially at lower pH levels by switching metabolic pathways.

For example*, C. acetobutylicum* can switch metabolism between solventogenesis and acidogenesis (Cabrol et al., 2017). If this microorganism is confronted with low pH conditions, acetone and butanol, but only small levels of H_2 are produced (solventogenesis) (Lütke-Eversloh and Bahl, 2011). However, acidogenesis as the main pathway for H_2 production (over acetate and butyrate) is dominant in higher pH-levels and can be provided at defined conditions (Cabrol et al., 2017).

1.5 Physiology of dark fermentative biohydrogen producers

Dark fermentative H_2 producers break down sugar to H_2, CO_2 and other fermentation products including acetate, butyrate, butanol, acetone, and many more VFAs (Hallenbeck et al., 2012). Glucose, as initial substrate, is broken down to pyruvate, generating ATP and NADH, which are both used as energy carriers. From pyruvate, a wide variety of metabolites and VFAs can be formed, depending on the fundamental enzymes and pathways (Ferry, 2011; Ergal et al., 2018).

This conversion of pyruvate to acetyl-CoA can be done over two main reactions, which are linked to the main H_2 producing pathways in microorganisms (Ramírez-Morales et al., 2015):
The pyruvate ferredoxin oxidoreductase (**PFOR**) pathway and the pyruvate formatelyase (**PFL**) pathway.

The PFOR pathway is most commonly found in obligate anaerobes. During the PFOR-pathway, the PFOR reduces a ferredoxin or NADH by pyruvate, which further gives electrons to a hydrogenase to produce H_2. During this reaction, acetyl-CoA is formed.
Besides, a wide range of side products can be formed. Depending on physiological conditions, a switch during the PFOR pathway between acidogenesis and solventogenesis is possible (Lütke-Eversloh and Bahl, 2011). Acidogenesis (which can theoretically produce 4 mol H_2/ mol glucose) is the preferred pathway to produce H_2. During this route, VFAs like acetate, butyrate, propionate, lactate, formate and ethanol are produced, depending on physiological conditions (Wong et al., 2014). In terms of biotechnology, acidogenesis is the preferred pathway to produce H_2.

During solventogenesis, a variety of VFAs can be produced too (especially butyrate and acetate) but the ratio of butyrate to acetate and H_2 is higher than during acidogenesis (Ramírez-Morales et al., 2015).

The highest potential for further improvements of H_2 production is in acidogenesis, because of a higher production of H_2, and a wide range of pathways producing side products (Ramírez-Morales et al., 2015), which can be modulated, to additionally enhance H_2 production.

Controversially, the PFL pathway is found mostly in facultative anaerobes. PFL using organisms split pyruvate into lactate, acetyl-CoA or formate. Formate is degraded to H_2 and CO_2 via formate

hydrogen lyase (FHL) (Ramírez-Morales et al., 2015), which is the main H_2 producing reaction of this pathway. PFL using organism are limited to 2 mol H_2/ mol glucose, because they cannot access NADH for H_2 production (Hallenbeck et al., 2012).
Besides, ethanol, acetate and other VFAs are formed over acetyl-CoA.

It has been described (Hendrix et al., 2011) that a variety of genes encoding PFL pathway are also present in obligate anaerobes like Clostridiaceae and only one cluster contained PFOR linked genes, concluding that there is no clear separation in two enzyme-using groups based on genes. Additionally, it has been reported that *C. acetobutylicum* is using additional pathways over crontonyl-coA and butyryl-CoA to produce H_2 (Servinsky et al., 2014).

The PFOR and the PFL pathways are modular and the linked production of H_2 is depending on pH, temperatures, substrates and products (Angenent et al., 2004; Hallenbeck et al., 2012; Ramírez-Morales et al., 2015).

Especially the PFOR pathway is modulated under different physiological conditions. One example is described by Wong et al. (2014) for different genera of Clostridiaceae at an optimum range of pH 6.0-8.0. On the one hand this range exists because of the nutrient uptake of the cell membrane under certain pH-levels (Feng et al., 2010). On the other hand, it is because of the most important enzymes - the hydrogenases - which get inhibited at lower pH (Mnatsakanyan et al., 2004).

The systems pressure also plays a key role for modulating pathways. Khanna and Das (2013) describe the production of H_2 during PFOR over ferredoxin as more favourable than over NADH, which needs a low partial pressure in the environment. Increasing pressure leads to production of lactate, ethanol, acetone and butanol (Abo-Hashesh and Hallenbeck, 2012) and even the conversion of acetate to H_2 is correlated to the partial pressure (Classen et al., 1999). Problems with inhibition of H_2 production can be avoided with open systems (like a continuous batch system) where no gas is accumulating. This would also reflect natural systems, where no accumulation of gas happens because produced gas is often directly used by other functional groups in the community.

Temperature is also critical for the physiological activities of H_2 producing microorganisms. Many bacteria live under mesophilic conditions and their highest $Y_{(H2/S)}$ was obtained between 35-37°C (Wu et al., 2008). Other bacteria with specialised physiology for thermophilic and hyperthermophilic conditions use hydrogenases which function optimally at temperatures of 50-70°C (Hallenbeck and Benemann, 2002).

1.6 Co-cultures of dark fermentative organisms

In general, combinations of different pure cultures (co-cultures), lead to significant improvements of production and stabilities in biologically H_2 production processes.
Elsharnouby et al. (2013) compared ten different independent studies conducted with multiple microorganisms to see the effectiveness of co-cultures and concluded, that all studies had increased values in HER and $Y_{(H2/S)}$ compared to the results of mono-cultures. The addition of facultative anaerobes can take the function of an expensive reducing agent (like Na_2S or L-cysteine) for depleting low levels of oxygens in the system. Haruhiko et al. (1998) compared the growth and H_2 production of *E. aerogenes* and *Clostridium butyricum* and showed an enhanced production and growth of both organisms in co-culture compared to mono-culture. The authors also showed that obligate anaerobes are not inhibited in their growth due to oxygen and additionally, the lag phase of *C. butyricum* is reduced, because *E. aerogenes* seem to consume the residual oxygen.Seppälä et al. (2011) showed that a co-culture of *C. butyricum* and *E. coli* had a higher glucose conversion efficiency $Y_{(H2/S)}$.

Another advantage of a co-culture system is a higher resistance of the whole biological system against contaminations and invasions of e.g. viruses as well as fluctuations of temperature, pressure, pH or substrate concentrations. A combination of aciduric microorganisms like *Candida maltosa* HY-35, which still can produce H_2 at a pH of 1.3 and *E. aerogenes* as a H_2 producer, which produces acids as metabolite, can offer a wide range of pH, where the system is still creating H_2 (Lu et al., 2007).

Additionally, complex carbohydrates of industrial waste or plant material can be degraded by a consortium of cellulose degrading organisms and highly efficient H_2 producers, which then use simple sugars (Elsharnouby et al., 2013). An example would be the consortia of *Clostridium thermocellum* JN4 as a cellulose degrader and *Thermoanaerobacterium thermosaccharolyticum* GD17 as a H_2 producer. In co-culture, these bacteria doubled the $Y_{(H2/S)}$ to a level of 0.3 mol/C-mol (Liu et al., 2008). However, one of the most important points of cultivation a co-culture is to establish a common medium, where different organisms can co-exist and grow without competing. For this reason, it is crucial to identify all critical influences on growth in pre-experiments, to get several factors and to determine the relationship between them. For this purpose, the multivariate method design of experiments (DOE) is an effective tool to minimize the costs and time to develop a common medium for microbial co-cultures. In the same time, it is possible to identify the most important environmental triggers to increase growth and production of metabolites.

In this thesis, an artificial co-culture was established. A combination of *E. aerogenes* and *C. acetobutylicum* was combined and investigated.

Enterobacteriaceae (3.15 +/- 4.13 mmol/L/h [C-molar]) and Clostridiaceae (2.08 +/-1.58 mmol/L/h [C-molar]) showed to have high HER in batch culture system *(*Porqueras et al., 2013). Enterobacteriaceae reached highest mean qH_2 of 2.26 mmol/g/h (C-molar), represented by *E. aerogenes.* Due to the advantages of mesophilic cultivation technique, the oxygen reducing abilities of *E. aerogenes* and the fact and the values in HER and qH_2 of these families, it was decided to take *C. acetobutylicum* and *E. aerogenes* as optimal candidates for establishing a co-culture system.

Besides combination of two H_2 producers, there are also consortia engineering studies containing different functional groups in natural environments, like acetogenic bacteria, methanogenic archaea or sulphate-reducing bacteria, which can use the metabolites and gases of the H_2 producers to produce H_2 or CH_4 (Liu et al., 2008). This way can be promising for production of biohydrogen, methanogen and other valuable gases in a more efficient way.

2 Aims and Hypothesis of this study

2.1. Aims

This study focuses on cultivation of *E. aerogenes* ATCC 13048 and *C. acetobutylicum* ATCC 824 in mono- and co-culture systems. Both mesophilic bacteria are well investigated with a wide range of publications and reviews (e.g. Ito et al., 2005; Seol et al., 2008; Taguchi et al., 1996; Zhang et al., 2006; Ergal et al., 2018) and have a higher potential for biohydrogen production compared to other bacteria.

The first aim is to establish an optimized cell counting system by comparing three different methods to obtain cell numbers in a certain volume. Absorbance measurement via optical density at 600 nm (OD_{600}), microscope-based cell counts (cell counts) and quantitative polymerase chain reaction (qPCR), with designed primers specific to each microorganism, should be combined to find an optimal method for precise investigation of bacterial growth in closed batch and batch culture systems.

The second aim was to measure and improve H_2-production and productivity assessment by using pressure measurements and gas chromatography (GC). In further work, the total and relative cell numbers for the highest H_2-production of mono- and combined co-culture systems had to be determined.

Additionally, as a third aim, cells should be visualized with fluorescent in situ hybridization (FISH) to get information about spatial arrangements inside the cultures.

The ultimate goal was to establish a common medium for both organisms with DOE. The co-culture system had to be optimized concerning HER, qH_2 and $Y_{(H2/S)}$ and compared to the best mono-culture system based on quantification of productivity with GC, analysis of metabolites via HPLC and quantification with qPCR, $OD_{(600)}$ measurements and cell counts.

2.2. Hypotheses

This thesis trials the following hypothesis:

- Cell counting, OD_{600} and qPCR are effective methods for quantifying *E. aerogenes* and *C. acetobutylicum* in mono- and co-culture

- *E. aerogenes* and *C. acetobutylicum* are excellent biological H_2 producers with high values of HER, qH_2 and $Y_{(H2/S)}$ on chemically defined and modified medium under mesophilic conditions.

- *E. aerogenes* and *C. acetobutylicum* will produce a variety of metabolites, which can be used for further improvement of H_2 production.

- Co-cultivation of both strain in a newly developed medium will allow growth of the two strains and will permit a superior HER, qH_2 and $Y_{(H2/S)}$ as a co-culture system compared to mono-culture systems.

- Combining *E. aerogenes* and *C. acetobutylicum* strains will give a more stable system with no need of an oxygen reducing agent, especially higher $Y_{(H2/S)}$ and better growth compared to mono-culture system.

3. Material & Methods

3.1 Strains and media

Cryocultures of *C. acetobutylicum* ATCC 824 and *E. aerogenes* ATCC 13048 were obtained from the “Deutsche Stammsammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany)”.

In this study, three basic media were used. For experiments with *C. acetobutylicum*, a modified P2 medium described by Qureshi et al. (1999) was used. This medium was abbreviated as “Clostridium medium” during this thesis. For *E. aerogenes*, a modified version of medium described by Delisa et al. (1999) was used and abbreviated as “Delisa medium”.
The final common medium done with Design of Experiment analysis will be described as “DOE medium”.

The main components of all media used in this study are a combination of buffer, glucose solution, trace element solution, vitamin solution and, in case of the Delisa medium, an additional Thiamine- and EDTA-solution. The glucose concentration in the final medium was stated on 30g/L, made of a 500 g/L D-glucose stock solution.

3.1.1 Medium for *C. acetobutylicum* ATCC 824

For pure cultures of *C. acetobutylicum* Clostridium medium was used. The main parts in 50 mL medium for each serum bottle (after inoculation) were 45.0 mL buffer, 0.25 mL vitamin solution (200x), 3.0 mL glucose solution, and 0.5 mL trace element solution (100x). This composition was adjusted to 1500mL for the batch experiments.

The buffer contained of 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4 and 2.2 g/L NH_4CH_3COO. The pH was arranged with 10M NaOH to 6.8. Vitamin solution (200x) with additional thiamine contains: 0.9 g/L Thiamine, 0.002 g/L Biotin and 0.2 g/L 4-Aminobenzoid acid, filled up with Millipore water for 1L total volume. Trace element solution (100x) contained 20.0 g/L $MgSO_4 \cdot 7 H_2O$, 1.0 g/L $MnCl_2 \cdot 4 H_2O$, 1.0 g/L $FeSO_4 \cdot 7 H_2O$ and 1.0 g/L NaCl, filled up with Millipore water for 1L total volume.

3.1.2 Medium for *E. aerogenes* ATCC 13048

For pure cultures of *E. aerogenes* the modified Delisa medium was used.

The main parts of 50 mL medium (after inoculation) for each serum bottle were 45.5 mL Buffer, 0.5 mL Thiamine solution, 3.0 mL Glucose solution, 0.5 mL EDTA solution (100x) and 0.5 mL Trace element solution (100x). Buffer consisted of 20 g/L KH_2PO_4 and 8 g/L NH_4Cl. The pH was arranged with 10M NaOH to 6.8. Thiamine • HCL solution (100x) and made in a concentration of 4.5 g/L in Millipore water. EDTA solution (100x) had a concentration of 8,4 g/L. Trace element solution (100x) contained 62.0 g/L $MgSO_4 \cdot 7H_2O$, 0.25 g/L $CoCl_2 \cdot 6 H_2O$, 1.5 g/L $MnCl_2 \cdot 4 H_2O$, 0.124 g/L $CuCl_2 \cdot 2 H_2O$, 0.3 g/L H_3BO_3, 0.25 g/L $Na_2MoO_4 \cdot 2 H_2O$, 1.3 g/L $Zn(CH_3COO)_2 \cdot 2 H_2O$, and 10.793 g/L $Fe^{III}Cl_3 \cdot 6 H_2O$, filled up with Millipore water to 1L total volume.

3.1.3 Medium for co-culture

The DOE medium was similar to the Clostridium medium, except the phosphate buffer and the NH_4CH_3COO, which was separated in an ammonium source (NH_4Cl) and an acetate source ($NaCH_3COO$). The buffer was tested via DOE method. In this case, the concentrations of $NaCH_3COO$, NH_4Cl and phosphate buffers was changed along a gradient, to test in which

buffer both organisms can grow best.

For DOE pre-experiments nine different combinations of the buffer's ingredients were checked (**Tab.1**) to investigate buffer capacities. All buffers were initially arranged by titrating with 10M NaOH to a pH of 6.8.
The DOE experiments were done in triplicates with one negative control in total (contained no inoculum). The middle point (DOE E Buffer) was done in pentaplicates.

Tab.1 Ingredients used for DOE media

DOE Buffer	NH_4Cl (g/L)	$NaCH_3COO$ (g/L)	KH_2PO_4 (g/L)	K_2HPO_4 (g/L)
A	6.4189	0.2461	20.4129	10.4013
B	6.4189	0.2461	0.4083	0.2080
C	6.4189	2.4610	0.4083	0.2080
D	6.4189	2.4610	20.4129	10.4013
E	3.4769	1.3536	10.4106	5.3047
F	0.5349	2.4610	20.4129	10.4013
G	0.5349	0.2461	0.4083	0.2080
H	0.5349	2.4610	0.4083	0.2080
I	0.5349	0.2461	20.4129	10.4013

All buffers, trace element solutions and the glucose solution were autoclaved separately and stored in 4°C. The vitamin solutions were sterile filtered with 0.2 µm filters (Whatman FP 30/0.2 CA-S) under laminar hood and stored in 4°C.

For batch experiments, the medium was amended with 100µL/L antifoam (StruktolSB2023, Schill und Seilacher).

3.2 Cultivation

The long-time storage of the cryostock cultures was at -80°C and short-time storage (< 1 month) cultures were kept in serum bottles at 4°C.
Reactivation of spore-forming *C. acetobutylicum* after storage (>5 days) was done with a 24h incubation at 37°C. For *E. aerogenes* this step was skipped and 4°C cultures were used over a maximum of 14 days storage.

The cultivation systems described in Rittmann and Herwig (2012) were used for these experiments. A combination of closed batch system (serum bottles) and batch systems were used for different purposes.

3.2.1 Closed Batch

The cultures were grown in 120 mL serum bottles in 50 mL liquid medium with 70 mL headspace. The experiments were all done in quadruplicates combined with one negative control as a blank. Before inoculation, the serum bottles were vacuumed, and 2 minutes flushed with 0.4 Bar of pure N_2 (Alphagaz™, Air Liquide). For transferring medium, inoculum or other liquids in/out of bottles, 1 mL Syringes (Injekt® BRAUN) and needles (Ø = 0.60 mm x 30 mm;

Sterican® BRAUN) were used.

For inoculation, an active pre-culture with 50 mL was spun down 15 minutes with 4500 rpm at 4°C, the supernatant was discarded, and the pellet was washed with 50mL of the buffer used in the following experiment subsequently. This workflow was done two times before the pellet was dissolved in 4 mL of buffer and then transferred in 4 new serum bottles with anaerobe headspace with prepared medium afterwards. The starting OD_{600} of cultures was arranged to 0.1. Each flask was flushed with N_2 right after inoculation to minimize the level of oxygen inside the bottles.

Cultures were cultivated at 37°C with shaking (120rpm). Produced gas was released as soon as the pressure was above 1.0 bar.

3.2.2 Batch System

For batch experiments, the cultures were grown in an Eppendorf 2 L bioreactor system with a maximum initial medium volume of 1.5 L. All experiments were initially done in duplicates. To ensure anaerobic conditions inside the vessels, the bioreactors were flushed with N_2 with a rate of 1 L/h until the redox potential was below -200 mV, indicating that the level of O_2 is as low as possible in the system. After inoculation, flushing with 10 L/h N_2 proved to be an effective way to get rid of oxygen in the environment as fast as possible. Afterwards, changing it to 1 L/h was keeping it anaerobe during the experiment.

The rotation was arranged at 200 rpm in all bioreactors. Medium constellation was the same as in closed batch, except the addition of 1 mL/L of an Anti-foam liquid solution (StruktolSB2023, Schill und Seilacher). If pH-control was used, a 10M NaOH and 1M HCl solution for the acid-/base-control was added.

For GC sampling, the gas is first flushed threw the sampling serum flask (120 mL) and then collected in a plastic bag for 10 minutes. Afterwards, 100 mL of the gas is transferred from the bag to the serum flask and stored for further analysis via GC.

3.3 Quantification of cell growth

3.3.1 Absorbance / Optical density (OD_{600})

Quantity of cell density via optical density was done with a Beckman Coulter DU® 800 Spectrophotometer with an absorbance of 600 nm with 1 mL samples in Sarstedt Polystyrol cuvettes. Millipore water was used as a blank. If the OD_{600} was higher than 0.7, the sample was diluted from a 1:10 ratio initially, up to a 1:100 ratio as maximum with Millipore and recalculated after OD_{600} measurement. To avoid accumulation of cells, the cuvettes were mixed again with a syringe right before measuring.

3.3.2 Quantitative polymerase chain reaction (qPCR)

Primers for qPCR (**Tab.2**) were designed with different tools with help of Thornton and Basu (2011). Target genes were searched via the pathogenomics gene search website. The primers were designed with Primer Blast and controlled with Blast (NCBI), Snap Gene (GSL Biotech LLC) and Gene Runner. As targets DNA genes were chosen instead of 16S rRNA genes, which were unique for one of our two target organisms.

DNA extraction was done with 1 mL of samples with an OD_{600} >1.0 via Phenol/Chlorophorm/Isoamyl alcohol - extraction with beat beating for cell lysis. After the workflow, the purity and amount of DNA was checked with NanoDrop® (Spectrophotometer ND-1000).

The DNA samples were diluted 1:300 with DEPC H_2O. To avoid shifts in the DNA concentration, the dilutions were made in a three steps row: 1:10, 1:10 and 1:3 to get a total of 1:300 dilution.

For each sample, 10 µL SYBR Green labelled Luna Universal qPCR Master Mix (M3003L, New England Biolabs), 0.5 µL of forward- and 0.5 µL reverse-primer, 8 µL DEPC H_2O and 1 µL of diluted DNA were used. The samples were prepared in 8-well tubes (Biozyme EU 0.2 mL) with 8-cap stripes (Biozyme).

For qPCR an Eppendorf Mastercycler ep gradient S was used. The program consisted of a 2 minutes heating phase at 95°C, a 45x repeating replication circle with 15 seconds at 95°C, 30 seconds 60°C and 30 seconds 81°C, and an end phase of 15 seconds 95°C, 15 seconds 60°C and a 20 minutes melting step up to 95°C.

As standards samples from exponential phase of pure culture experiments with an already known cell density were used. The standards were always loaded in triplicates, ranging from a 1:10 dilution to a 1:1 000 000 dilution. For *E. aerogenes* the cell density of the 1:10 standard was $3.69x10^{10}$ and for *C. acetobutylicum* it was $5.61x10^{9}$.

Tab.2 Used primers (reverse and forward) for qPCR with position in genome, length of product and targeting genes

Primer Name	DNA Sequence (5'-3')	Position	Product Length (nt)	Targeting gene	
CloABC_FW	TGG CAC AGT CAG TCG GCT ACC	183850-183870	108	CDS:	AEI33449.1
CloABC_RV	GCG TGA TGC ACC TAA CCC AGC	183722-183742	108	Title:	ABC transporter (permease)
EntCDF_FW	GCG TTG TGG GGT TGC ACG AT	4223621-4223640	106	CDS:	AEG98846.1
EntCDF_RV	TGG CGC GCG AGC ACA TTT TC	4223496-4223515	106	Title:	Cation diffusion facilitator family transporter

3.3.3 Cell Counts

For cell counts, a Nikon Eclipse 50i microscope with a 40x and 60x magnification was used. 10 µL of bioreactor samples were transferred on a Neubauer counting chamber (Superior Marienfeld Germany). The fields had a deep of 0.1 mm and were used until the cell density was too high for counting (usually more than 20 cells per field) and then diluted with 1:10 (sample : Millipore water).

3.4 Analytic methods

3.4.1 GC

Pressure in serum flask was measured with a Manometer (Keller Leo 1) with 0.2 µm filters (Whatman FP 30/0.2 CA-S) and needles (Sterican® BRAUN) with Ø = 0.60 x 30 mm. The pressure was counted cumulatively and recalculated with blank bottles.

For qualification, GC was performed with the Agilent Gas Chromatograph (Agilent 78790A GC). The process was done as standard procedure already described by Reischl (2016), showed in **Fig.1**.

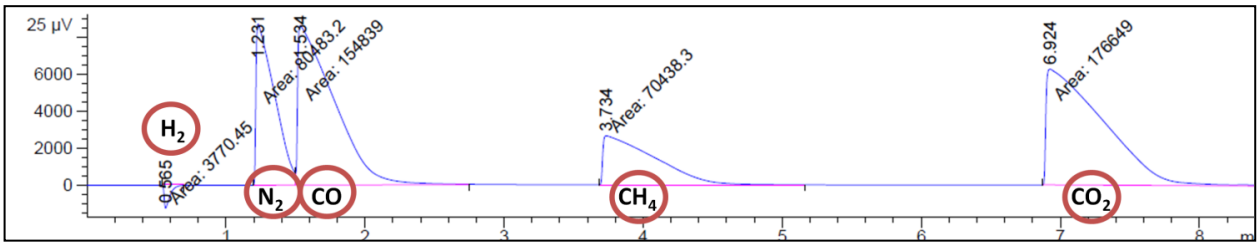

Fig.1 "Excerpt from gas chromatography analysis. Analysed gases H_2, N_2, CO, CH_4 and CO_2 are highlighted" (Reischl, 2016)

For this thesis, only the curves from H_2, N_2 and CO_2 were used. The areas of H_2, N_2 and CO_2 curves were calculated in perspective to the standard gases and further calculated to get the relative composition of H_2, N_2 and CO_2 of the gas samples took from the batch reactor.

3.4.2 HPLC

The determination of sugars, VFAs and alcohols was performed with an HPLC (Agilent 1100), consisting of a G1310A isocratic pump, a G1313A ALS Autosampler, a Transgenomic ICSep ICE-ION-300 column, a G1316A column thermostat set at 45°C, and a G1362A RID refractive index detector, measuring at 45°C – all modules from Agilent 1100. The measurement was performed with 0.005 M H_2SO_4 as solvent, with a flow rate of 0.325 mL/min and pressure of 48-49 bar. The injection volume was 40 µL.

3.5 Cell visualisation with FISH

For visualisation of *C. acetobutylicum* and *E. aerogenes* in mono- and co-cultures, a method for using Fluorescence in situ Hybridisation (FISH) with these organisms was tried to be established.
16S rRNA based probes (**Tab.3**) were used for targeting, including a general probe for Eubacteria (EUB338, Cy3-labelled) and Gammaproteobacteria including *E. aerogenes* (Gam42a, Fluos-labelled) and a probe specific for firmicutes (LGC354a, Fluos-labelled). As a general DNA targeting dye, DAPI was used.

All fixations were done via ethanol fixation standard procedure. After immobilization and dehydration of 1µl of the samples, the slides were hybridized with a 35% formamide containing buffer at 46°C over night, washed with a washing buffer for 10 minutes at 48°C water bath on the next day and then air-dried and stored for analysis with a "Nikon Optoteam Präzision" microscope. The pictures were taken with a 100x magnification and overworked with ImageJ.

Tab.3 Used probes for FISH with DNA sequence and type of fluorophore

Probe Name	DNA Sequence (5'-3')	Fluorophor
GAM42a	GCC TTC CCA CAT CGT TT	Fluos
LGC354a	TGG AAG ATT CCC TAC TGC	Fluos
EUB338	GCT GCC TCC CGT AGG AGT	Cy3

4 Results

All results shown in this section are based on the outcomes of the supplement tables. For further detailed information, all values are presented in the appendix.

4.1. Pre-Experiments

In the first trial experiments in closed batch system, the cultures showed growth in their microorganism-specific medium (Clostridium medium and Delisa medium). In the initial closed batch experiments, it was possible to reach a pressure of up to 5 bar inside the serum bottles overnight. The composition was not analysed, but this experiment demonstrated the production of high amounts of gas over short time. The gas inside the closed bottles was released frequently.

The pre-experiments **(Fig.2)** were conducted with microorganism-specific medium to examine the performance of pure culture systems, meaning that Clostridium medium and Delisa medium were used for *C. acetobutylicum* and *E. aerogenes*, respectively.

C. acetobutylicum was inoculated in Clostridium medium and showed a cumulative pressure of 6.7 bar after 64 h **(Fig.2 A)**. During the exponential phase, a maximum OD_{600} of 1.7 was reached at timepoint 40 h **(Fig.2 C)**. *E. aerogenes* in Delisa medium showed a cumulative pressure of 3.9 after 64 h **(Fig.2 B)**. The highest OD_{600} was measured at timepoint 40 h and reached 1.3 **(Fig.2 D)**.

To find a common medium for both microorganisms, the first step was to inoculate both organisms in the media vice versa. *C. acetobutylicum* was inoculated in serum bottles containing Delisa medium. Cultivation of *C. acetobutylicum* in this medium was not possible. The OD_{600} was stagnating, and no growth was observable. Inoculation of *E. aerogenes* in Clostridium medium showed a short growth, but after an OD_{600} of 0.4 **(Fig.2 D)** and only a small rise in pressure **(Fig.2 B)**, it was decided that this medium would also be not ideal for co-culture experiments. Consequently, it was necessary to construct a new common medium for co-culture experiments. The newly designed DOE medium will be compared to these results.

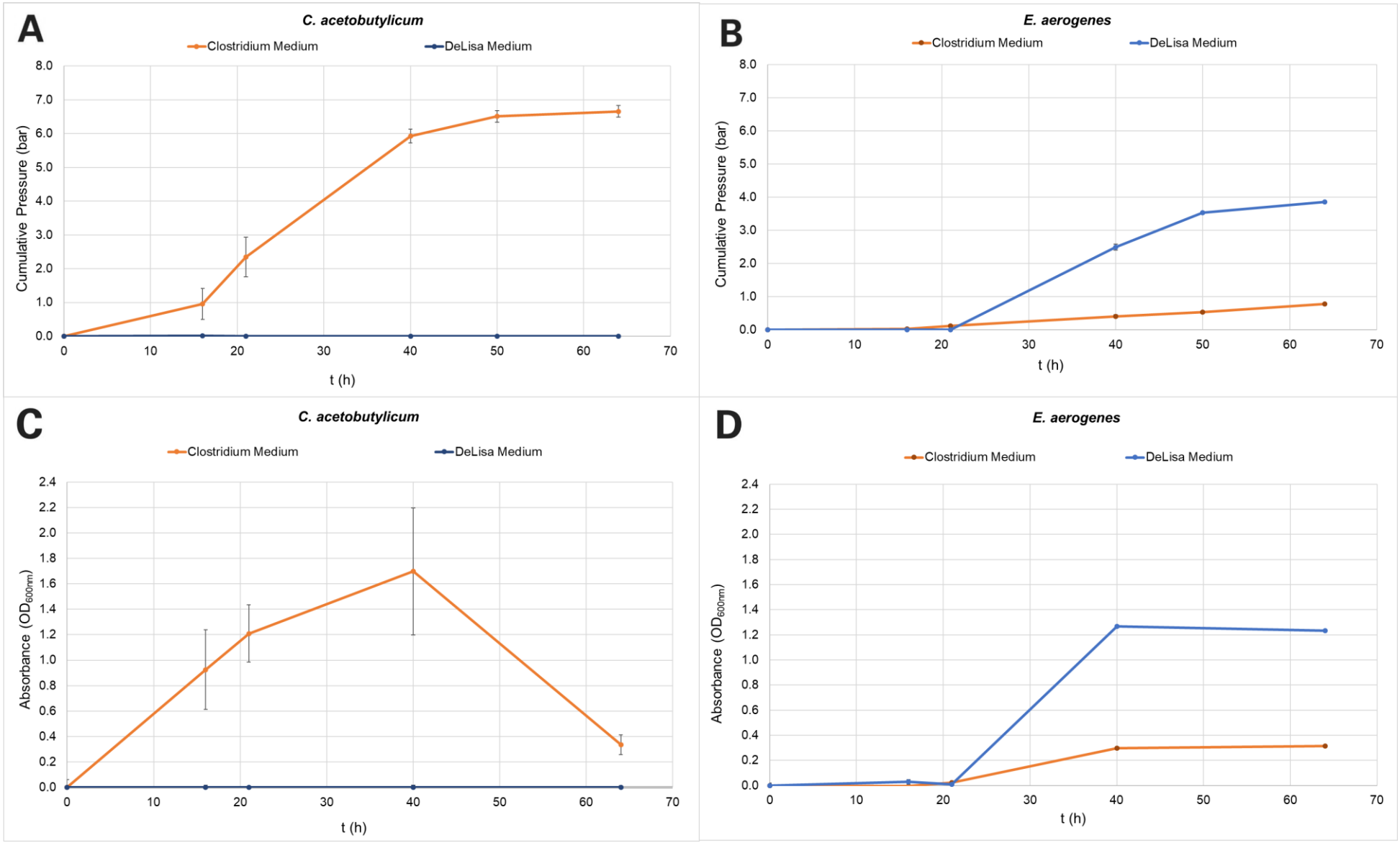

Fig.2 Pre-experiments in closed batch for comparison of cumulative pressure (**A**, **B**) and OD_{600} (**C**, **D**) over time of *C. acetobutylicum* (**A**, **C**) culture and *E. aerogenes* (**B**, **D**) in Clostridium medium and Delisa medium. Standard deviations are shown for the mean values.

4.2 Growth of *C. acetobutylicum* in Clostridium medium as pure culture

In closed batch cultivation mode, *C. acetobutylicum* grown in Clostridium medium showed a start of exponential phase after about 20 h of inoculation (**Fig.2**). To avoid the longer lag phase due to spore form of the microorganism, already medium-adapted pre-cultures were used for all the further experiments. Accumulated gas pressure in the headspace of the serum bottles was reaching values of up to 6.5 bar after 50 h. In total, the culture reached an OD_{600} of 1.7. Gas production stopped after 50 h. The culture showed a biofilm formation after exponential growth ended.

In batch system, exponential phase started after 25 h (**Fig. 3**) and stopped 7 h later. Highest number of cells were detected by cell counts and qPCR at the sample taken from time point 32 h, almost reaching a cell density of $2x10^8$ cells/mL. The production of flocks was observed, especially at the end of the exponential growth. The samples had to be taken carefully for dilutions to prevent a dilution bias.

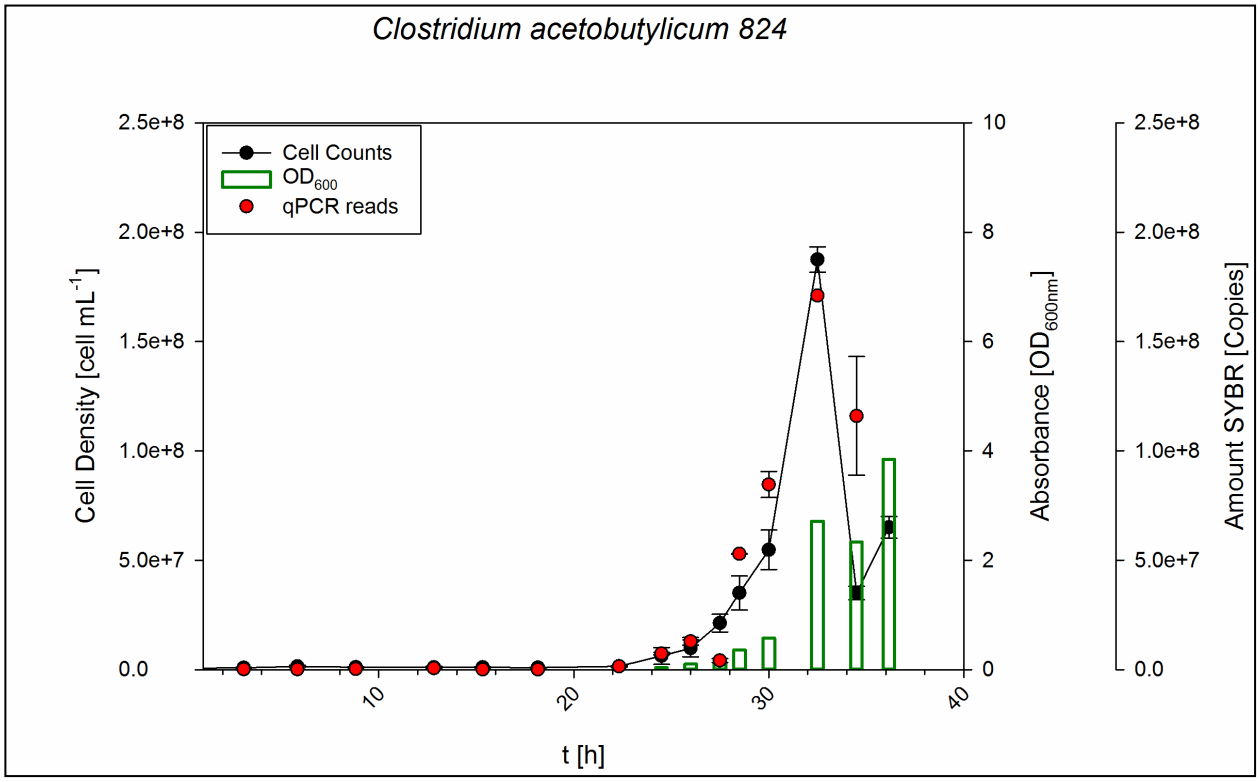

Fig.3 Measurement of growth of *C. acetobutylicum* 824 culture in Clostridium medium in batch with quantification via cell density, OD_{600} and qPCR. Standard deviations are shown for the mean values.

4.3 Growth of *E. aerogenes* in Delisa medium as pure culture

E. aerogenes grew well in Delisa medium (**Fig.2**). After a start of exponential phase at 20 h the highest OD_{600} of 1.3 was measured after 40 h in closed batch experiments. Cumulative pressure reached 3.9 Bar as its maximum after 65 h. For inoculation, cultures stored in 4°C (not older than 10 days) were used. Experiments in batch reactors (**Fig. 4**) showed a start of exponential phase

after about 30 h, reaching an OD_{600} of 6.7 after 48 h. Cell density at this point showed almost 10-folds higher cell number (almost reaching $2x10^9$ cells/ml) compared to the highest number of *C. acetobutylicum* in Clostridium medium (**Fig.3**). *E. aerogenes* also showed production of biofilms, especially in the late grow-phase.

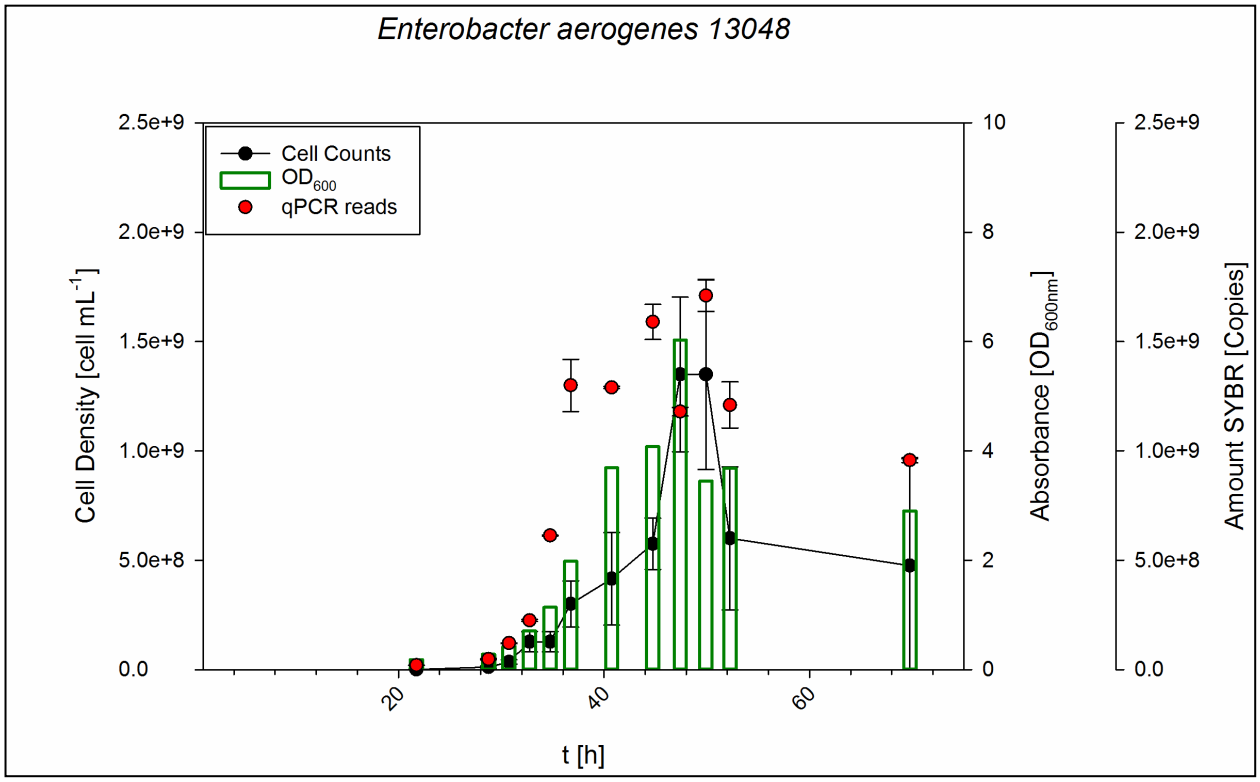

Fig.4 Measurement of growth of *E. aerogenes* 13048 culture in Delisa medium in batch with quantification via cell density, OD_{600} and qPCR. Standard deviations are shown for the mean values.

4.4 Growth of *C. acetobutylicum* and *E. aerogenes* in DOE medium as pure cultures

4.4.1 Growth in closed batch culture

To be able to find a common medium for both microorganisms, 9 different buffers were designed using DOE method, and these buffers were tested in closed batch system with both microorganisms (**Fig.5**).
C. acetobutylicum started with exponential growth after about 24 h and "Buffer E" as middle point buffer showed the best results in growth with the highest maximum OD_{600} of 3.4. After doing the DOE modelling of the measurements, it was seen that buffers with low buffer capacity tend to be better as substrate (**Fig.6**) and middle to low concentration of ammonium chloride and sodium acetate was preferred by *C. acetobutylicum*.

E. aerogenes showed an earlier start of exponential phase in under 10 h after inoculation. "Buffer E" also reached the highest maximum OD_{600} of 4.5 after 20 h. In general, high buffer capacities showed higher production and growth of *E. aerogenes* (**Fig. 6**). A middle concentration

of ammonium chloride and sodium acetate proved to work better than higher or lower concentrations. DOE “Buffer E” was chosen as common medium for both microorganisms due to results of pressure measurements and cell counts. All further DOE-experiments were conducted using DOE “Buffer E” and the medium is further described as “DOE E medium”.

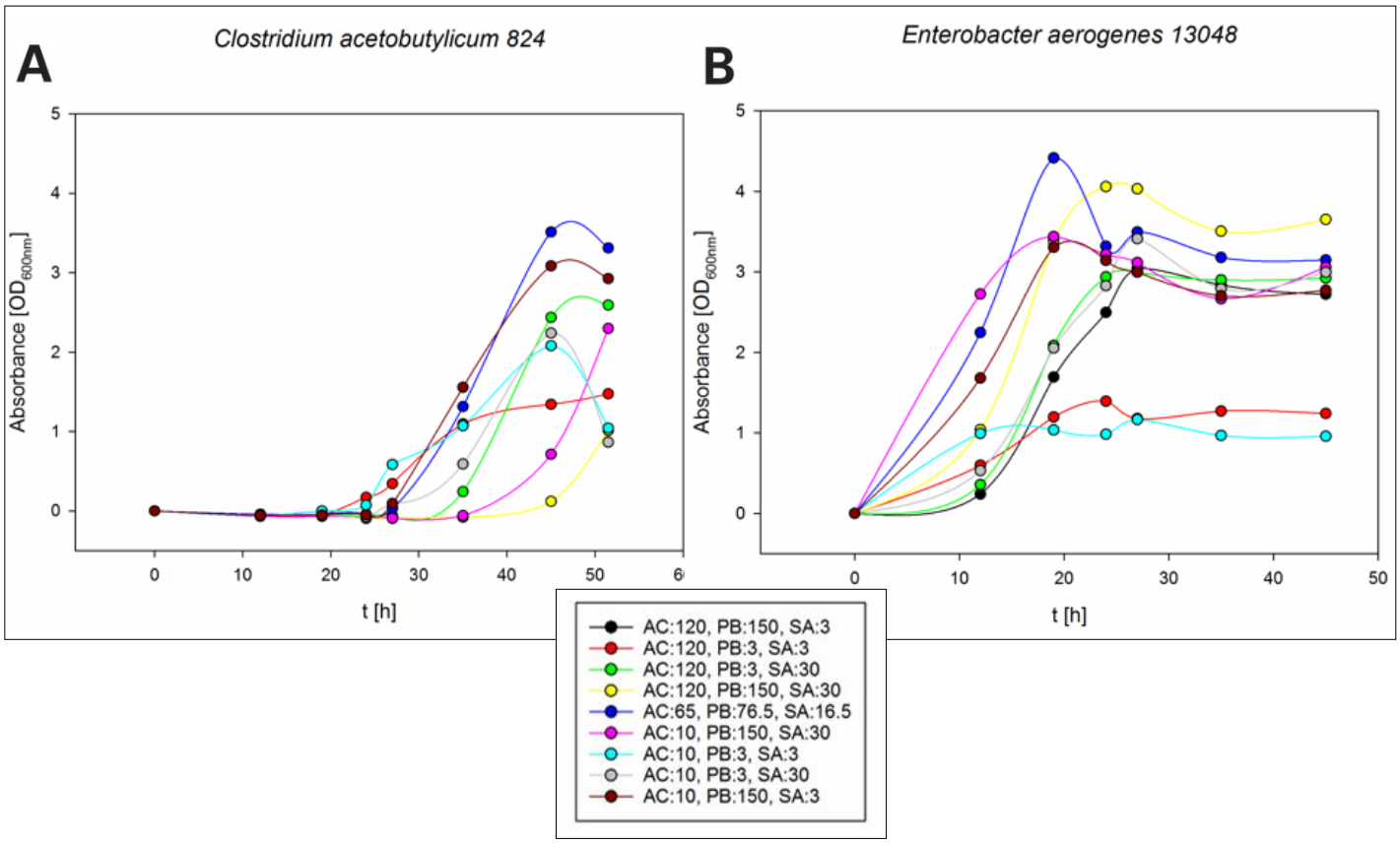

Fig.5 Growth-curve of *C. acetobutylicum* (**A**) and *E. aerogenes* (**B**) in closed batch mentioning OD_{600} in different DOE buffers. Chemicals of buffers are described in **Tab.1**, containing different concentrations (mmol/L) of ammonium chloride (AC), phosphate buffer (PB) and sodium acetate (SA).

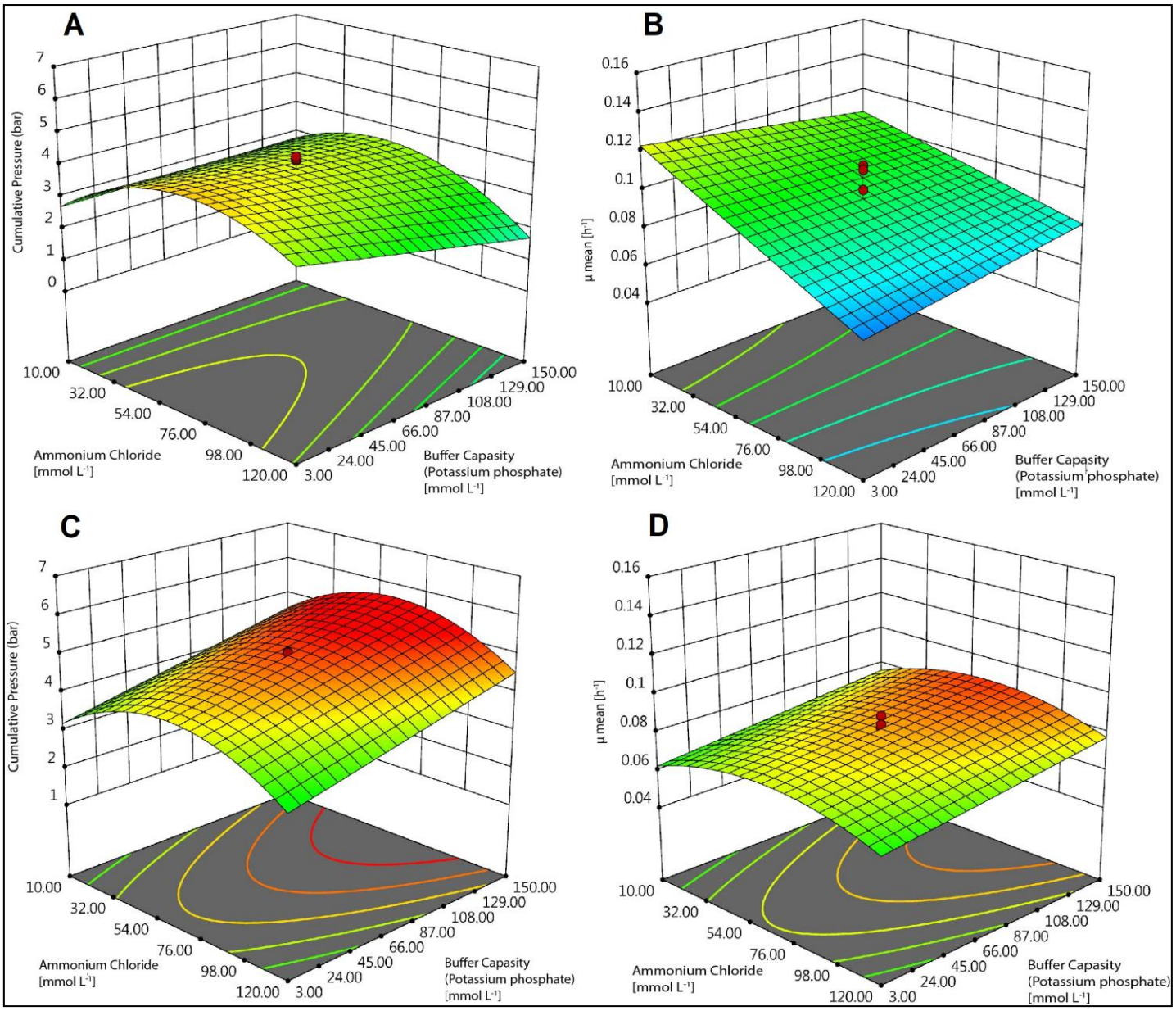

Fig. 6 Multivariate design of experiment graphs regarding cumulative pressure (left side) and growth (right side) at different ammonium chloride concentrations and buffer capacities for *C. acetobutylicum* (**A, B**) and *E. aerogenes* (**C, D**). Red dots represent the middle point concentration of ingredients (= Buffer E, **Tab. 1**)).

4.4.2 Growth in batch culture

In comparison to Delisa medium, *E. aerogenes* showed a very early start of exponential phase in batch experiments in DOE E medium before timepoint 10 h, and an end of growth after 34 h, shown via quantification with OD_{600} measurement (**Fig.7 A**). In these experiments, *E. aerogenes* reached a maximum OD_{600} of 4.4 under pH-controlled conditions. Under pH-uncontrolled experiments, a stop of growth after 15 h at a maximum OD_{600} of 1.5

was observed (**Fig.7 B**).

In comparison to Delisa medium, E. aerogenes showed a very early start of exponential phase in batch experiments in DOE E medium under 10 h and growth stopped after 34 h. Results of OD_{600} measurement and qPCR are shown via (**Fig.7 A**). In these experiments, E. aerogenes reached a maximum OD_{600} of 4.4 under pH-controlled conditions. Under pH-uncontrolled conditions, the growth stopped after 15 h at a maximum OD_{600} of 1.5. **(Fig.7 B)**.

Next, cultivation of *C. acetobutylicum* as pure culture in batch reactors in DOE E medium was performed. However, the cultivation was not showing growth. After several attempts, detection of growth and production of gas was not possible. Even after inoculation with higher amounts of DOE E medium adapted cells, growth could not be observed.

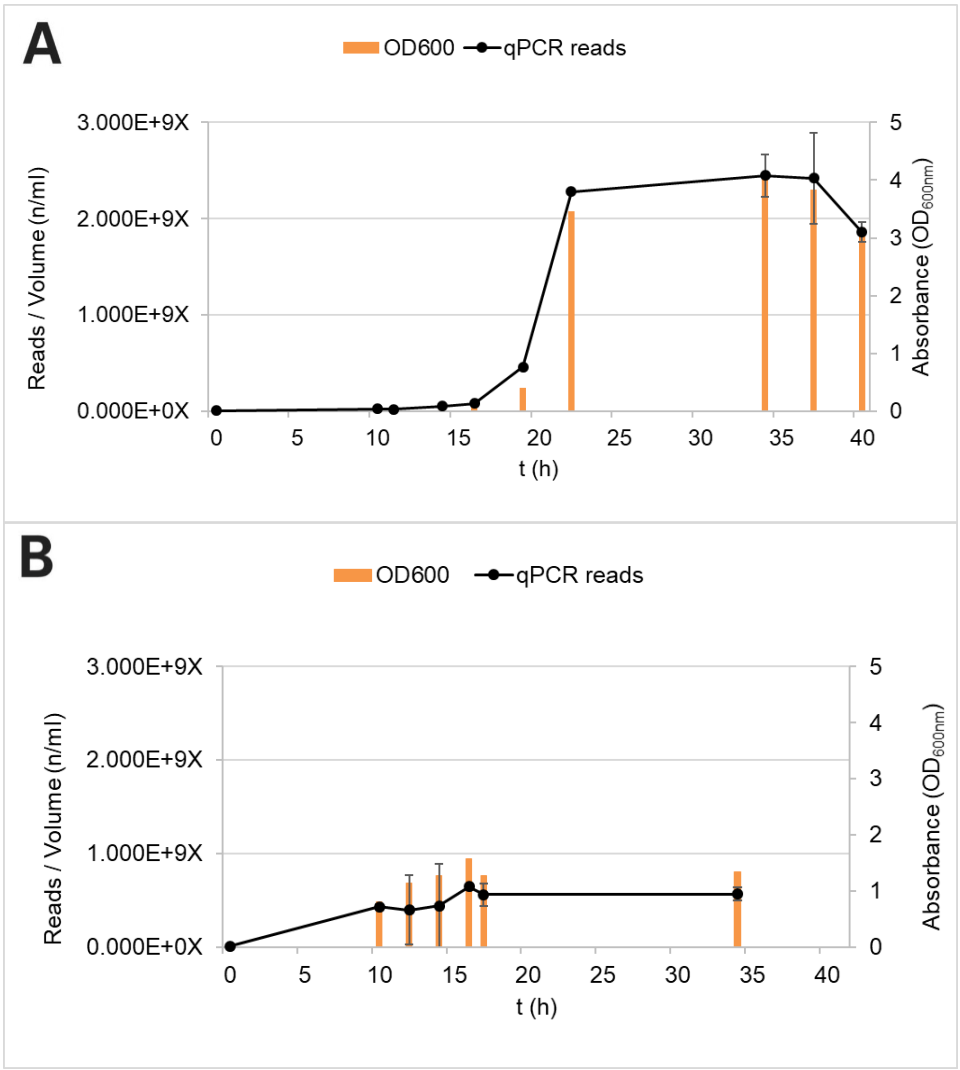

Fig.7 Quantification of *E. aerogenes* in DOE E medium under pH-controlled (**A**) and pH-uncontrolled (**B**) conditions, regarding OD_{600} (orange) and qPCR reads (black). Standard deviations are shown for the mean values.

4.5 Growth of *C. acetobutylicum* and *E. aerogenes* in DOE E medium as co-cultures

4.5.1 Growth in batch culture

In one experiment a co-culture containing *C. acetobutylicum* and *E. aerogenes* was grown in batch mode. This experiment represents the only results observed from *C. acetobutylicum* in DOE E medium in batch so far. In this co-culture, *E. aerogenes* was highly abundant and *C. acetobutylicum* was quantitatively under 0.1% of total cell-number most of the time (**Fig.8**) according to qPCR reads. Each experiment was made in duplicates. Under pH-controlled conditions, the final OD_{600} was higher (OD_{600} max. = 4.8) compared to conditions with uncontrolled pH (OD_{600} max. = 3.0).

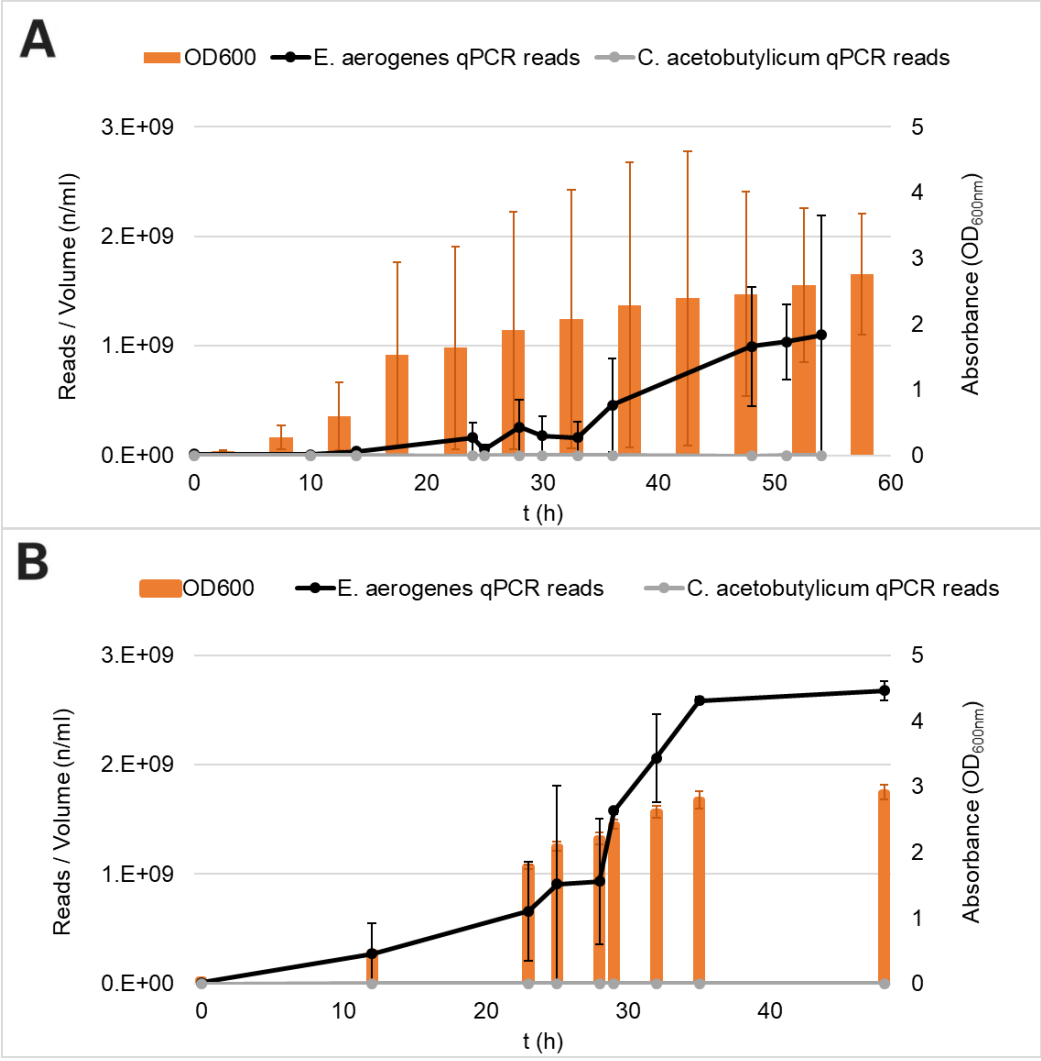

Fig.8 Quantification of co-culture in DOE E medium under pH-controlled (**A**) and pH-uncontrolled (**B**) conditions, regarding OD_{600} (orange) and qPCR reads of *C. acetobutylicum* (grey) and *E. aerogenes* (black). Standard deviations are shown for the mean values.

4.6 Analytic results via HPLC

First, the HPLC results for *E. aerogenes* are presented, followed by *C. acetobutylicum* and co-cultures. The results showing standard deviations are presented with the mean values of duplicates.

4.6.1 HPLC *E. aerogenes*

The analysis of batch samples of *E. aerogenes* in Delisa medium was done in duplicates under pH-controlled conditions (**Fig.9**). The results showed that concentration of glucose decreased after 22 h. After 40 h, glucose was fully used. At timepoint 48 h, OD_{600} of 6.0 was detected, which was the highest value so far. During the growth, lactic acid was produced as a first metabolite up to 42 mmol/L, following by ethanol with a maximum concentration of 61 mmol/L, formic acid with a maximum of 43 mmol/L and acetic acid with a maximum concentration of 43 mmol/L.

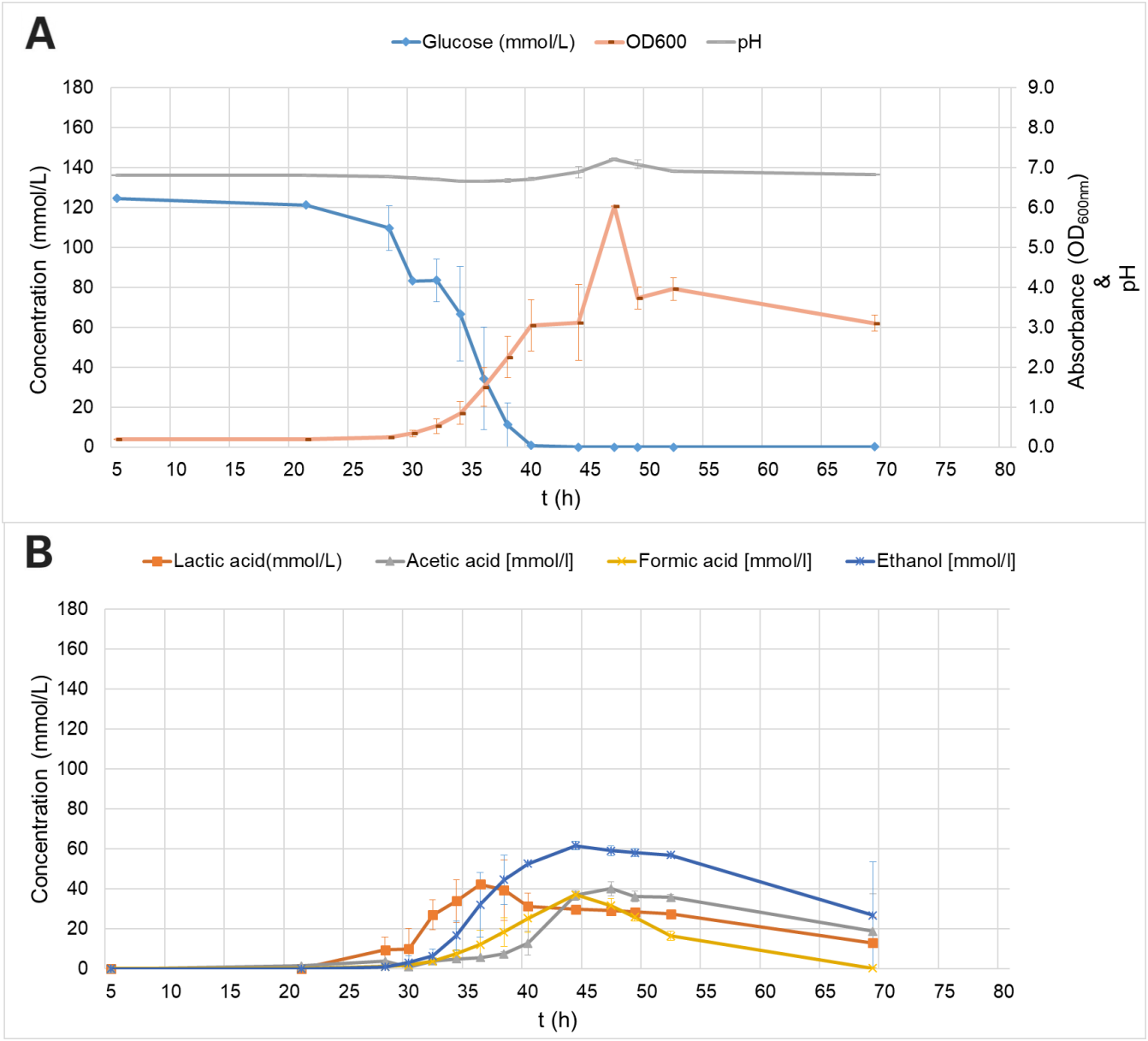

Fig.9 HPLC results of liquid metabolites in batch samples of *E. aerogenes* in Delisa medium under pH-controlled conditions, showing concentrations over time. **A** shows the glucose concentration combined with OD_{600} and pH-level of medium over time. **B** shows VFA concentrations of lactic acid, acetic acid, formic acid and ethanol over time. Standard deviations are shown for the mean values.

In DOE E medium under pH-controlled conditions (**Fig.10**), *E. aerogenes* showed an early consumption of glucose before timepoint 10 h, and it was completely consumed after 35 h with an OD_{600} of 3.5. Formic acid was produced as a main metabolite and reached a maximum concentration of 259 mmol/L. Ethanol concentration reached a maximum of 71 mmol/L and butanediol a maximum of 26 mmol/L. Little amounts of acetic acid of 10 mmol/L were detected.

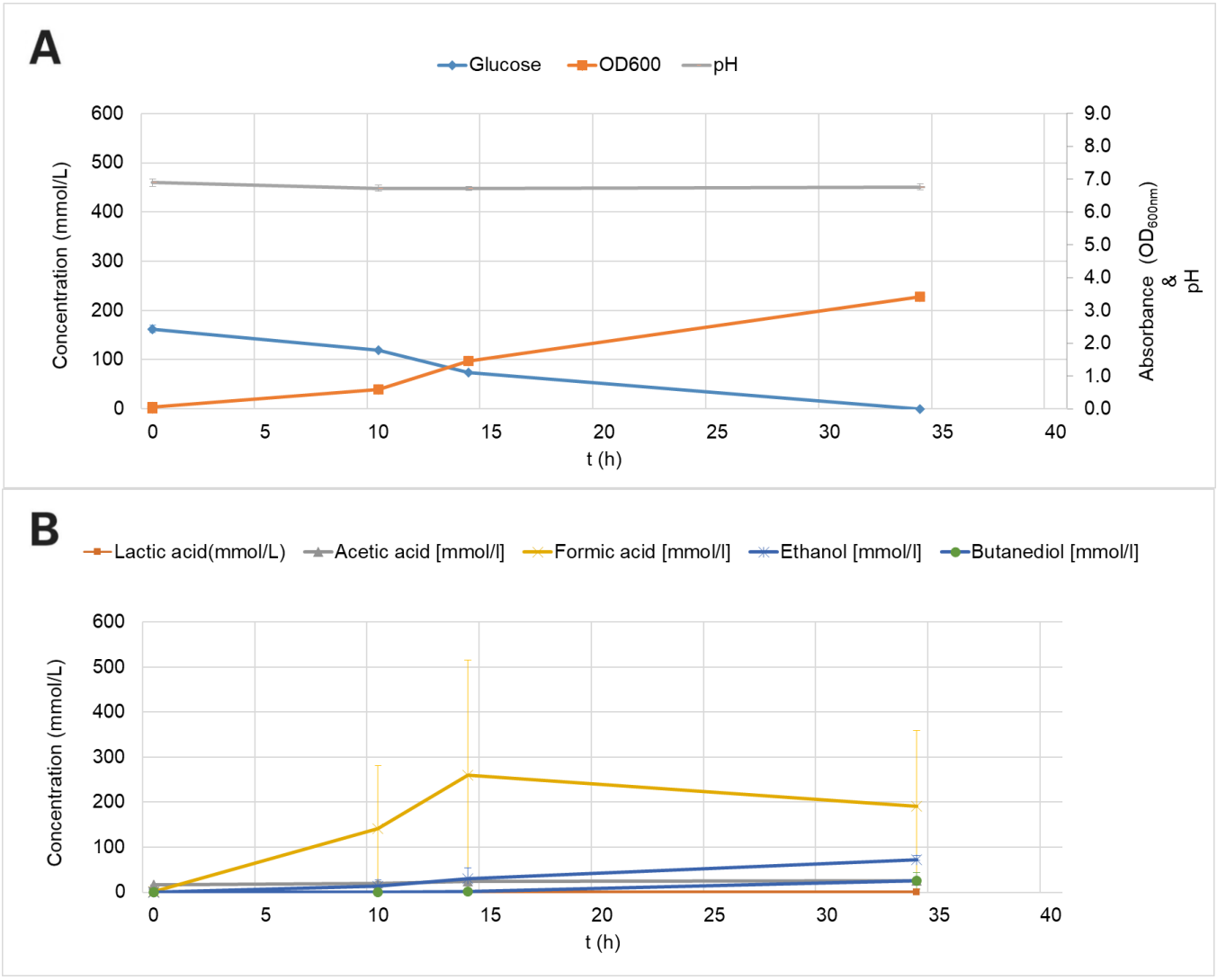

Fig.10 HPLC results of liquid metabolites in batch samples of *E. aerogenes* in DOE E medium under pH-controlled conditions, showing concentrations over time. **A** shows the glucose concentration combined with OD_{600} and pH-level of medium over time. **B** shows VFA concentrations of lactic acid, acetic acid, formic acid and ethanol over time. Additionally, concentration of butanediol was measured. Standard deviations are shown for the mean values.

To examine the pH changes and effect of the uncontrolled pH on cultures, the same experiments were done without an acid-/base-controlled system (**Fig.11**). In DOE E medium with uncontrolled pH, the growth of E. aerogenes started as the pH-controlled system. However, the growth stopped after 17 h with an OD_{600} of 1.5 and glucose was not consumed completely. A concentration of 90 mmol/L of glucose was still in the medium. Formic acid was produced as a main VFA up to 156 mmol/L in 10 h, next to ethanol with a maximum concentration of 33 mmol/L. The measured acetic acid concentration was 10 mmol/L. The pH dropped to a minimum of 4.8 after 35 h.

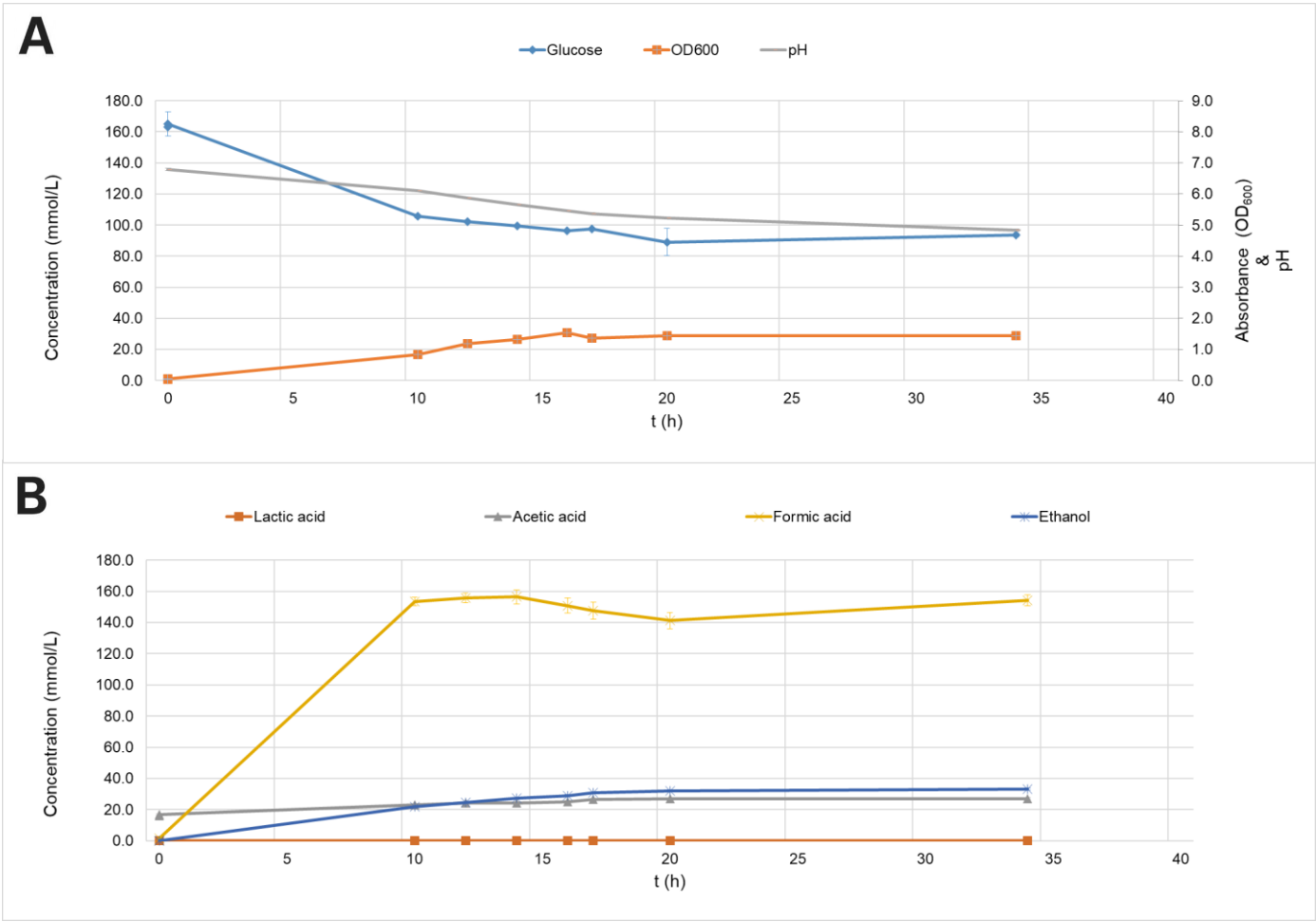

Fig.11 HPLC results of liquid metabolites in batch samples of *E. aerogenes* in DOE E medium under pH-uncontrolled conditions, showing concentrations over time. **A** shows the glucose concentration combined with OD_{600} and pH-level of medium over time. **B** shows VFA concentrations of lactic acid, acetic acid, formic acid and ethanol over time. Standard deviations are shown for the mean values.

4.6.2 HPLC: *C. acetobutylicum*

C. acetobutylicum in Clostridium medium with pH-controlled conditions (**Fig.12**) started to utilize glucose after 28 h and in approximately 40 h glucose was completely consumed. OD_{600} of 5.8 after 72 h. A comparatively high concentration of acetone of 100 mmol/L was measured in the end point sample. Also, butyric acid and acetic acid were produced to a maximum concentration of 37 mmol/L and 9 mmol/L, respectively.

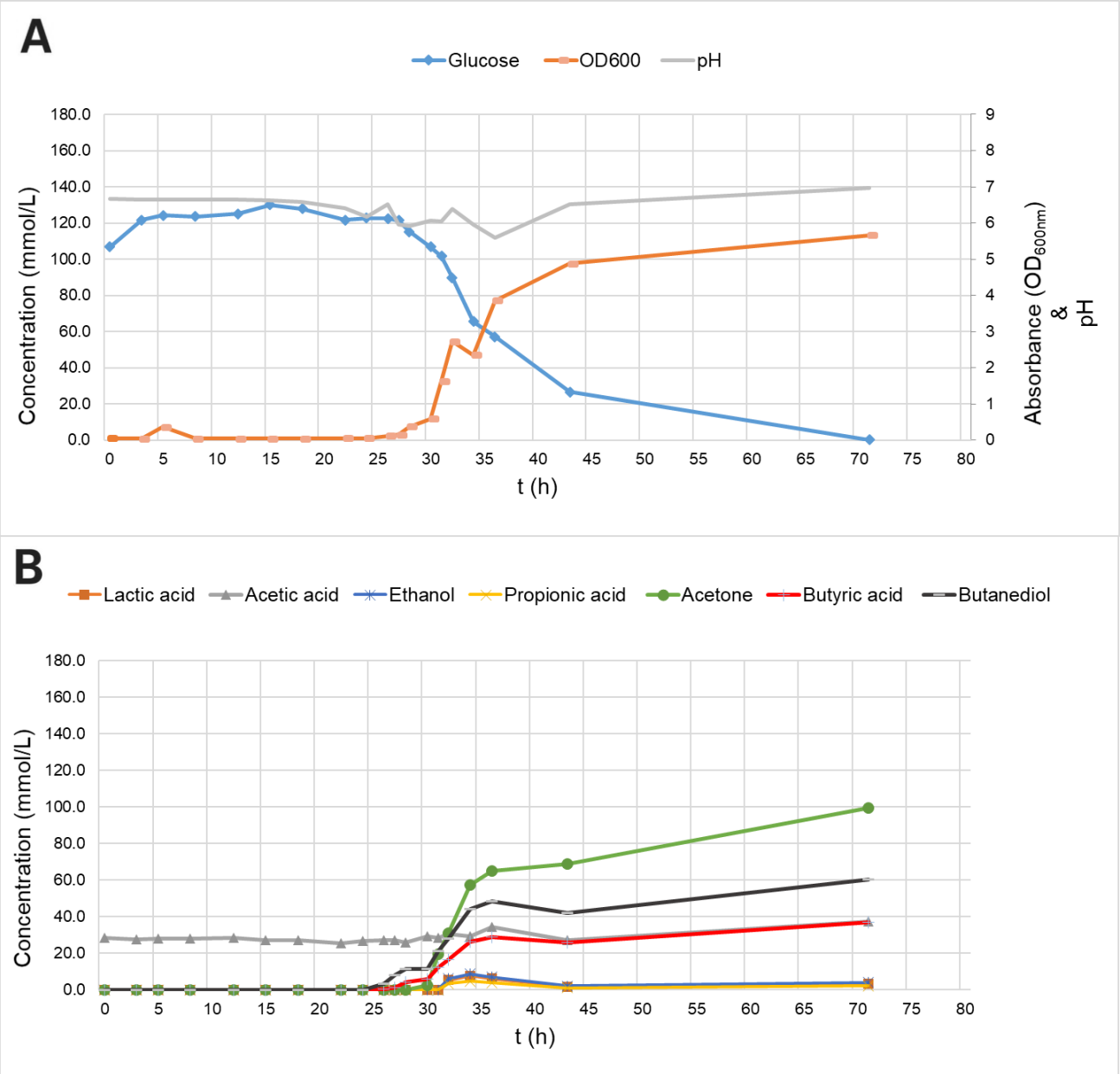

Fig.12 HPLC results of liquid metabolites in batch samples of *C. acetobutylicum* in Clostridium medium under pH-controlled conditions, showing concentrations (mmol/L) over time (h). **A** shows the glucose concentration combined with OD_{600} and pH-level of medium. **B** shows VFA concentrations of lactic acid, acetic acid, ethanol, propionic acid, acetone and butyric acid.

4.6.3 HPLC: Co-culture

The glucose consumption started after 35 h in the co-culture experiments (**Fig.13**). Glucose was completely depleted in 12 h until timepoint 47 h. OD_{600} started rising after 35 h, with a continuous growth until it reached the maximum value of 5.0 at timepoint 70 h, indicating that growth continued even after complete usage of glucose. Additionally, at these time period between the 35th and 70th h, ethanol, formic acid and butanediol were produced up to a concentration of 87 mmol/L, 48 mmol/L and 18 mmol/L, respectively.

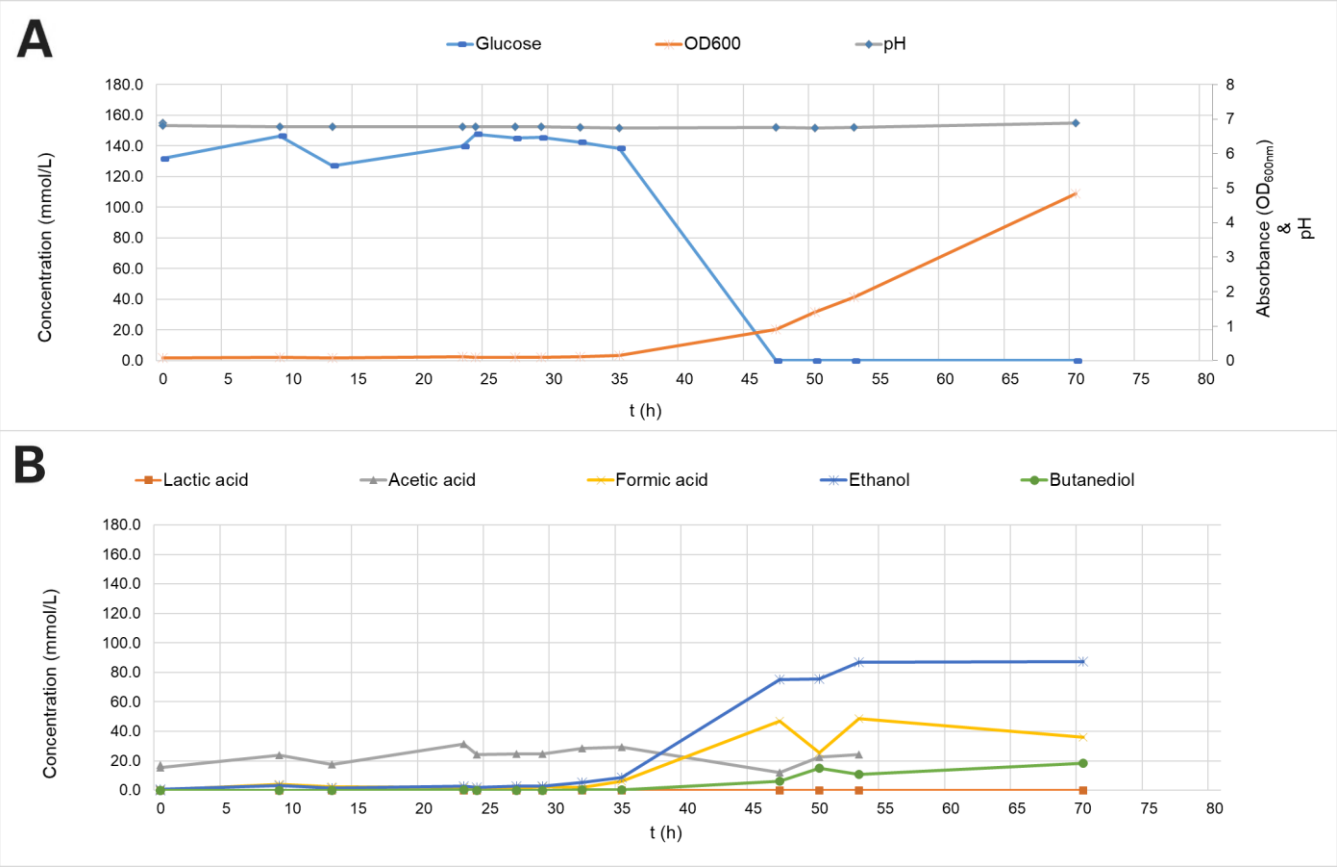

Fig.13 HPLC results of liquid metabolites in batch samples of co-cultured *C. acetobutylicum* and *E. aerogenes* in DOE E medium under pH-controlled conditions, showing concentrations (mmol/L) over time (h). **A** shows the glucose concentration combined with OD_{600} and pH-level of medium over time. **B** shows VFA concentrations of lactic acid, acetic acid, formic acid and ethanol over time. Additionally, concentration of butanediol was measured.

The glucose was completely consumed in 35 h by co-culture duing the uncontrolled pH experiments (**Fig.14**). After 35 h, the maximum OD_{600} was 3.0. Ethanol and lactic acid were produced to a maximum concentration of 100 mmol/L and 21 mmol/L, respectively. The pH value reached 5.3 after 48 h.

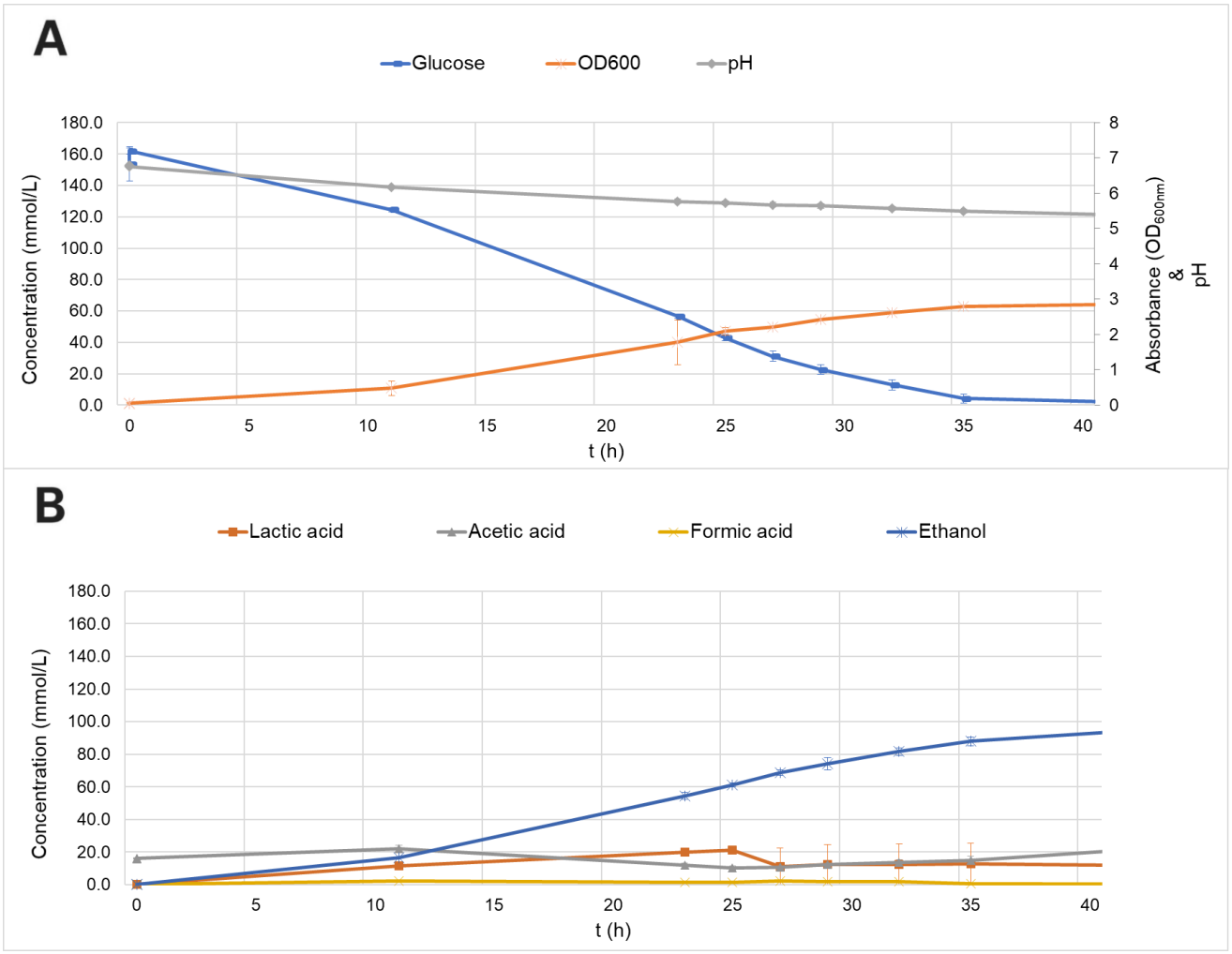

Fig.14 HPLC results of liquid metabolites in batch samples of co-cultured *C. acetobutylicum* and *E. aerogenes* in DOE E medium under pH-uncontrolled conditions, showing concentrations (mmol/L) over time (h). **A** shows the glucose concentration combined with OD_{600} and pH-level of medium over time. **B** shows VFA concentrations of lactic acid, acetic acid, formic acid and ethanol over time. Standard deviations are shown for the mean values.

4.7 Analytic results via GC

The results of GC analysis showed appearance of H_2 as well as CO_2 especially at the exponential phase. The *C. acetobutylicum* pure culture in clostridium medium (**Fig.15, A**) had high gas production especially in the period between the 25th and 40th h. The highest relative amount of H_2 was detected at 32 h sample with the ratio of 19:12:69 H_2:CO_2:N_2.

H_2 and CO_2 production *of E. aerogenes* in Delisa medium (**Fig.15, B**) reached the highest values between the 30th and 40th h. Especially in the time between 38th and 40th h an average of 17:34:49 for the H_2:CO_2:N_2 ratio was detected. This measurement was taken at a point, where the gas flow was increased from 1 sL/h to 10 sL/h N_2 to avoid H_2 accumulation.

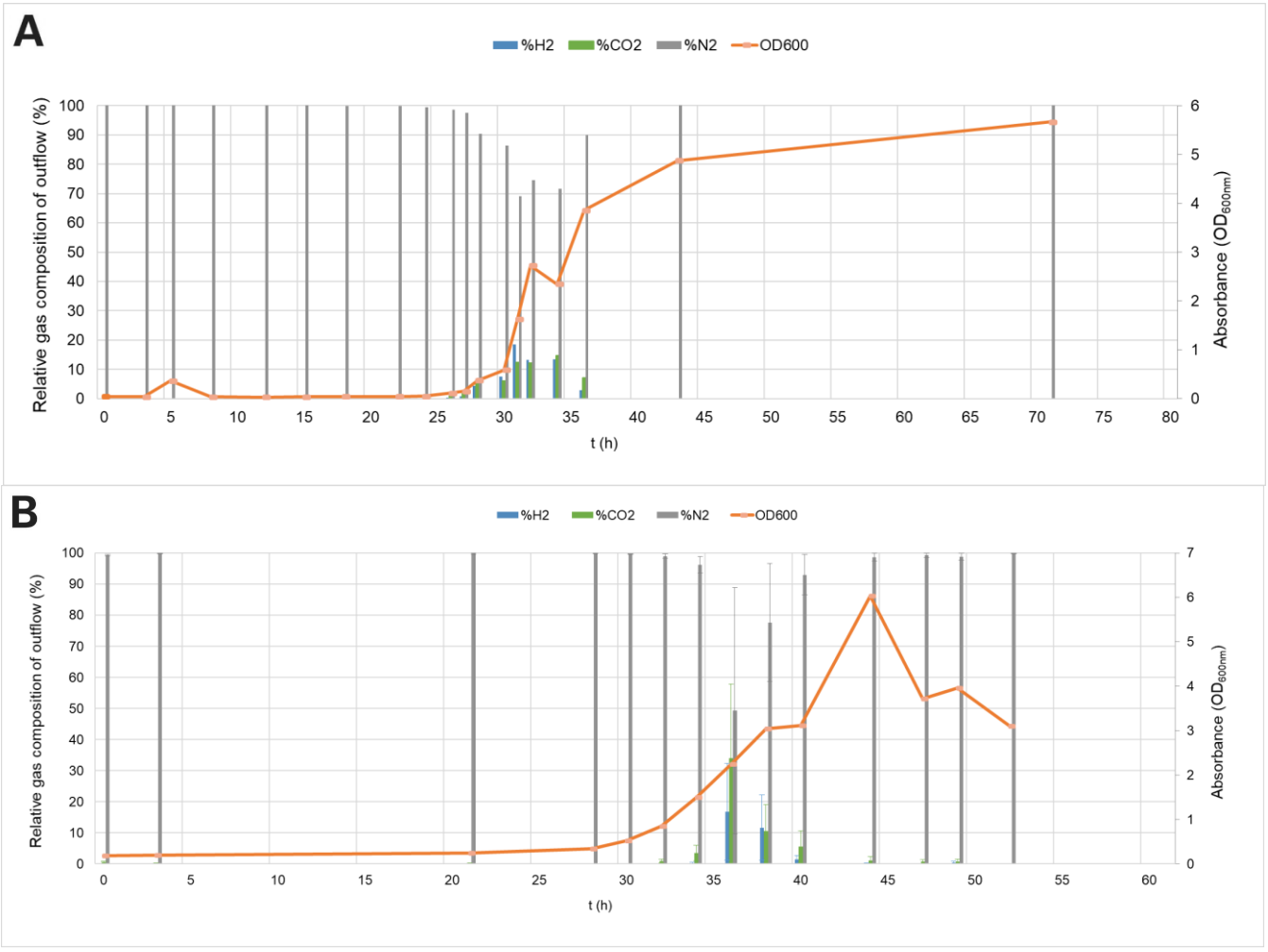

Fig. 15 GC results over time with relative ratio of H_2 (blue bars), CO_2 (green bars) and N_2 (grey bars) in combination with OD_{600} (orange) of *C. acetobutylicum* (Clostridium medium) (**A**) and *E. aerogenes* (Delisa medium) (**B**) under pH-controlled conditions. Standard deviations are shown for the mean values.

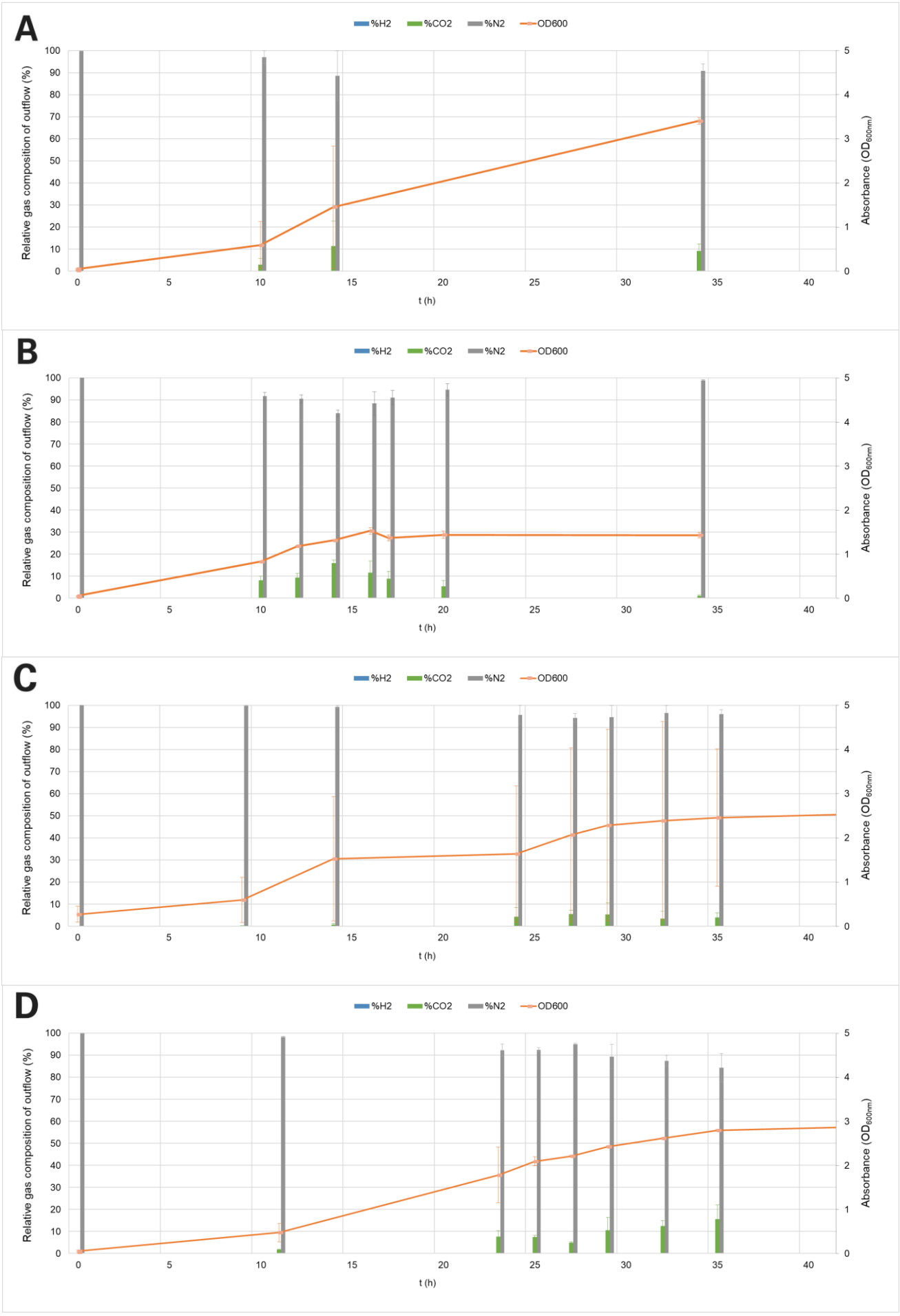

Fig. 16 GC results over time with relative ratio of H_2 (blue bars), CO_2 (green bars) and N_2 (grey bars) in combination with OD_{600} (orange) of *E. aerogenes* (DOE E medium) under pH-controlled conditions (**A**), *E. aerogenes* (DOE E medium) under pH-uncontrolled conditions (**B**), Co-culture (DOE E medium) under pH-controlled conditions (**C**) and Co-culture (DOE E medium) under pH-uncontrolled conditions (**D**). Standard deviations are shown for the mean values.

The GC results of DOE E medium (**Fig. 16**) showed that the relative gas composition was mostly containing N_2 and CO_2. The CO_2 level in *E. aerogenes* cultures in DOE E medium under pH-controlled conditions (**Fig**.**16 A, B**) reached the highest value (11%) at the time-point of 14 h. The relative amount of H_2 always kept under 1% of total gas composition, independent of pH conditions. Similar H_2 amounts were observed in the co-culture experiments (**Fig. 16 B, C**). The CO_2 production reached 6% and 16% of the total gas composition after 35 h under pH-controlled and pH-uncontrolled conditions, respectively.

The HER, CER, qH_2 and $Y_{(H_2/CO_2,\ H_2/S)}$ were calculated for each organism and the respective medium with additional calculation of C-molar level, represented with the maximum values in **Tab.4**.

C. acetobutylicum in Clostridium medium reached maximum values of HER with 1.72 mmol/L/h (C-molar), maximum CER with 4.18 mmol/L/h (C-molar), maximum qH_2 with $2.87x10^{-11}$ (C-molar) mmol/g/h and a maximum $Y_{(H2/S)}$ of 0.43 mol/C-mol.

E. aerogenes in Delisa medium reached average maximum values of HER with 14.22 mmol/L/h (C-molar), maximum CER with 26.95 mmol/L/h (C-molar), maximum qH_2 with $1.24x10^{-10}$ (C-molar) mmol/g/h and a maximum $Y_{(H2/S)}$ of 0.49 mol/C-mol.

Under pH-controlled conditions in DOE E medium*, E. aerogenes* reached average maximum values of HER with 0.003 mmol/L/h (C-molar), maximum CER with 1.29 mmol/L/h (C-molar), maximum qH_2 with $3.67x10^{-12}$ (C-molar) mmol/g/h and a maximum $Y_{(H2/S)}$ of 0.02 mol/C-mol.

Under pH-uncontrolled conditions in DOE E medium*, E. aerogenes* reached average maximum values of HER with 0.005 mmol/L/h (C-molar), maximum CER with 0.99 mmol/L/h (C-molar), maximum qH_2 with $3.22x10^{-10}$ (C-molar) mmol/g/h and a maximum $Y_{(H2/S)}$ of 0.0001 mol/C-mol.

The co-culture reached under pH-controlled conditions average maximum values of HER with 0.005 mmol/L/h (C-molar), maximum CER with 0.66mmol/L/h (C-molar), maximum qH_2 with $2.04x10^{-10}$ (C-molar) mmol/g/h and a maximum $Y_{(H2/S)}$ of 0.02 mol/C-mol.

Under pH-uncontrolled conditions in DOE E medium*,* the co-culture reached average maximum values of HER with 0.003 mmol/L/h (C-molar), maximum CER with 0.99 mmol/L/h (C-molar), maximum qH_2 with $6.35x10^{-13}$ (C-molar) mmol/g/h and a maximum $Y_{(H2/S)}$ of 0.0001 mol/C-mol.

More data is provided in the supplementary tables in the appendix, also showing calculated growth rate (μ), the C-balance and degree of reduction for comparing the results on a stoichiometrical meta-level.

Tab.4 Comparison of max. values of HER, CER, qH_2 and $Y_{(H2/CO2,\ H2/S)}$ on molar and C-molar level.

Run	HER (mmol/L/h)	HER (mmol/L/h [C-molar])	CER (mmol/L/h)	CER (mmol/L/h [C-molar])	qH_2 (mmol/g/h)	qH_2 (mmol/L/h [C-molar])	Y (H_2/CO_2)	Y (H_2/S)	Y (H_2/S [mol/C-mol])
***C. acetobutylicum* in Clost. Medium**									
pH-controlled conditions	10.31	1.72	25.07	4.18	1.72E-10	2.87E-11	1.50	2.60	0.43
***E. aerogenes* in DeLisa Medium**									
pH-controlled conditions ("Saruman")	157.82	26.30	281.90	46.98	1.43E-09	2.39E-10	0.56	0.35	0.06
pH-controlled conditions ("Legolas")	12.84	2.14	41.55	6.93	5.04E-11	8.39E-12	1.16	5.51	0.92
Co Culture in DOE E medium									
pH-controlled conditions (C1)	0.02	0.004	4.25	0.71	2.38E-09	3.97E-10	0.01	0.11	0.02
pH-controlled conditions (C2)	0.03	0.005	3.69	0.62	6.90E-11	1.15E-11	0.01	-	-
pH-uncontrolled conditions (C3)	0.02	0.004	7.85	1.31	3.07E-12	5.11E-13	0.003	0.0003	0.0001
pH-uncontrolled conditions (C4)	0.01	0.001	4.0778	0.68	4.55E-12	7.58E-13	0.01	0.0001	0.0000
***E. aerogenes* in DOE E medium**									
pH-controlled conditions (E1)	0.003	0.001	2.83	0.47	2.06E-13	3.44E-14	0.001	0.02	0.003
pH-controlled conditions (E2)	0.03	0.005	12.66	2.11	4.38E-11	7.30E-12	0.003	0.27	0.05
pH-uncontrolled conditions (E3)	0.02	0.003	6.56	1.09	3.18E-10	5.30E-11	0.003	0.0005	0.0001
pH-uncontrolled conditions (E4)	0.04	0.01	5.31	0.88	6.85E-11	1.14E-11	0.015	0.001	0.0001

4.8 Methods for quantifying culture growth

To establish an optimal cell quantification system, OD_{600} measurement, cell counts, and qPCR were compared. **Fig. 17** shows the correlation of OD_{600} and cell counts. The run of *C. acetobutylicum* in Clostridium medium and *E. aerogenes* in Delisa medium were chosen as demonstrative examples. The values after exponential phase were excluded, because OD_{600} seemed to be influenced by biofilm formation. Potential outliers were excluded, because the standard deviation was very high, most likely because of potential pipetting errors in the dilutions. The correlation of both factors showed to be <0.95 with these values.

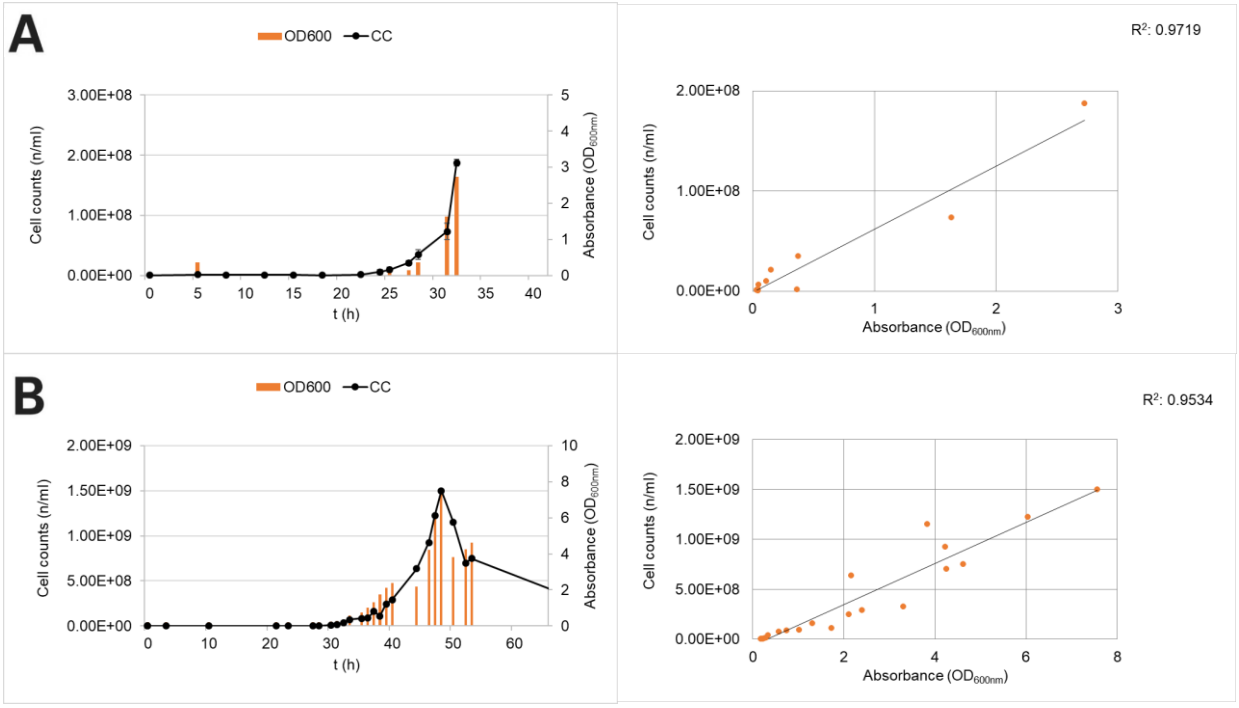

Fig. 17 Quantification of growth of *C. acetobutylicum* (Clostridium medium) (**A**) and *E. aerogenes* (Delisa medium) (**B**) via OD_{600} (orange) and cell counts (CC; black line), both under pH-controlled conditions. Additional regression curves analyzing the correlation of OD_{600} and cell count-results. Values with high standard deviations were excluded. Standard deviations are shown for the mean values.

Additionally, the correlation of OD_{600} and qPCR is shown in **Fig.18**. For visualisation again, the run of *C. acetobutylicum* in Clostridium medium and additionally, *E. aerogenes* in DOE E medium, both under pH-controlled conditions, were chosen. Once more, the correlation of values from OD_{600} and qPCR reached >0.95, if potential outliers with high standard deviations were excluded.

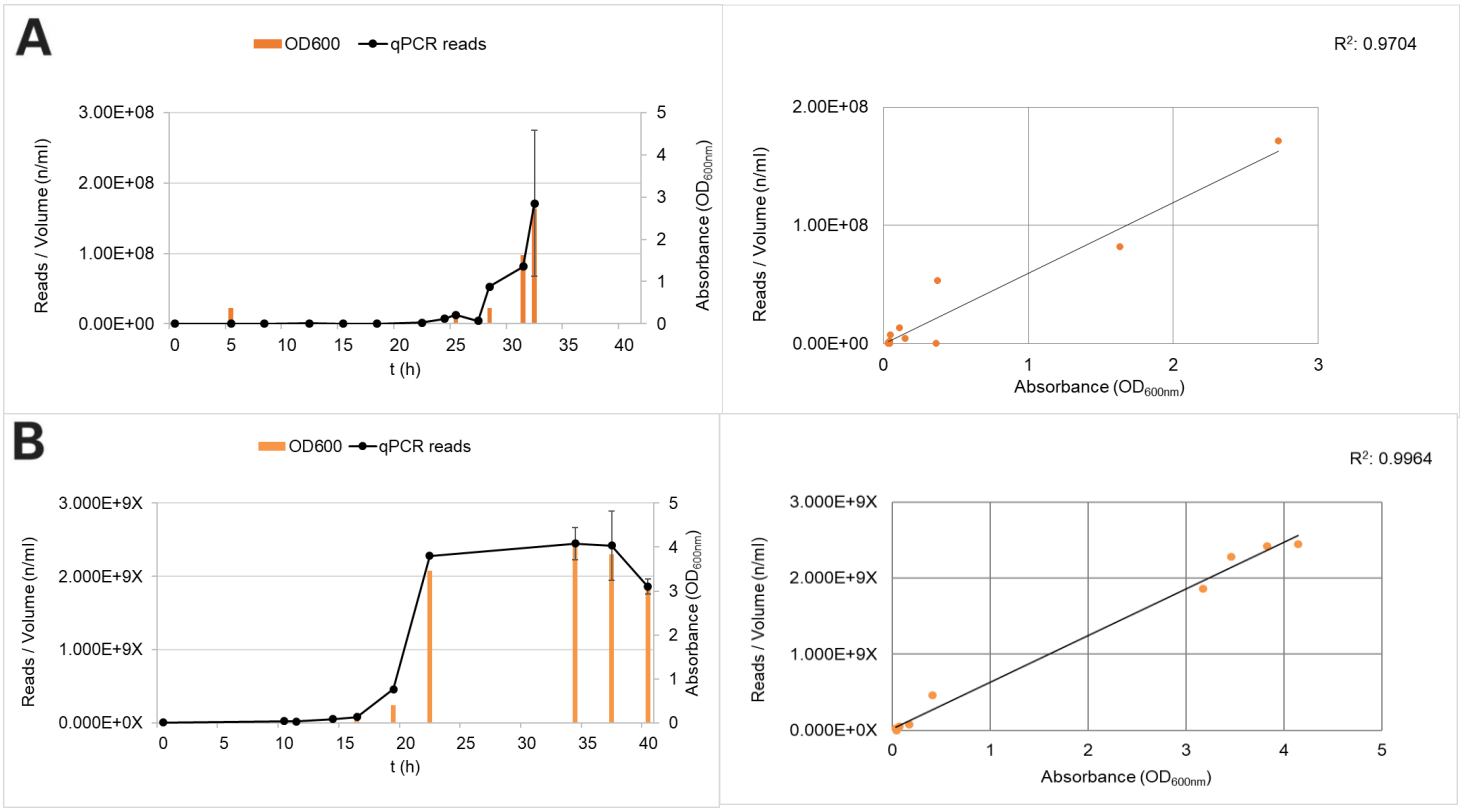

Fig. 18 Quantification of growth of *C. acetobutylicum* (Clostridium medium) (**A**) and *E. aerogenes* (DOE E medium) (**B**) via OD_{600} (orange) and qPCR (black line) under pH-controlled conditions. Additional regression curves analyzing the correlation of OD_{600} and qPCR-results (right side). Values with high standard deviations were excluded. Standard deviations are shown for the mean values.

4.9 Fluorescence *in situ* hybridisation

For visualization of cells and to determine the 3-dimensional arrangement of cells in co-cultures, FISH was chosen as a specific tool. First tests with different fluorophores showed that Cy3- and Fluos-labelled probes were the most suitable probes for detection of *E. aerogenes* and *C. acetobutylicum*. Cy5 was tested too, but no signal could not be detected. DAPI staining, which was used to stain all DNA containing cells, showed to be an efficient reagent to stain cells of *C. acetobutylicum* and *E. aerogenes* in mono- and co-cultures. Additionally, DAPI was used as a counter staining to see how efficient probes bonded.

Staining cells of *E. aerogenes* with DAPI and hybridisation with EUB338 and Gam42a was positive (**Fig.19**), showing positive fluorescent-signals under the microscope. All DNA containing cells which were labelled by DAPI were also covered by the 16S rRNA targeting probes, showing that EUB338 and Gam42a are suitable probes for targeting this organism.
Staining of *C. acetobutylicum* could not be performed by using 16S-rRNA targeting probes so far. EUB338 or LGC354a (general Firmicutes probe) were not hybridising, resulting in a negative signal under the fluorescent microscope. Only DAPI was able to stain the cells (**Fig.20 A**).

In a co-culture of *C. acetobutylicum* and *E. aerogenes*, a distinction of these two species would only be possible with Gam42a and EUB338 probes, which are binding to *E. aerogenes* but, so far, not binding to *C. acetobutylicum* (**Fig.20 A,B**). Hence, DAPI was used to stain *C. acetobutyli-*

cum (**Fig.20 A**). In an overlay with the specific-labelling of *E. aerogenes*, a distinction of these two bacteria was possible.

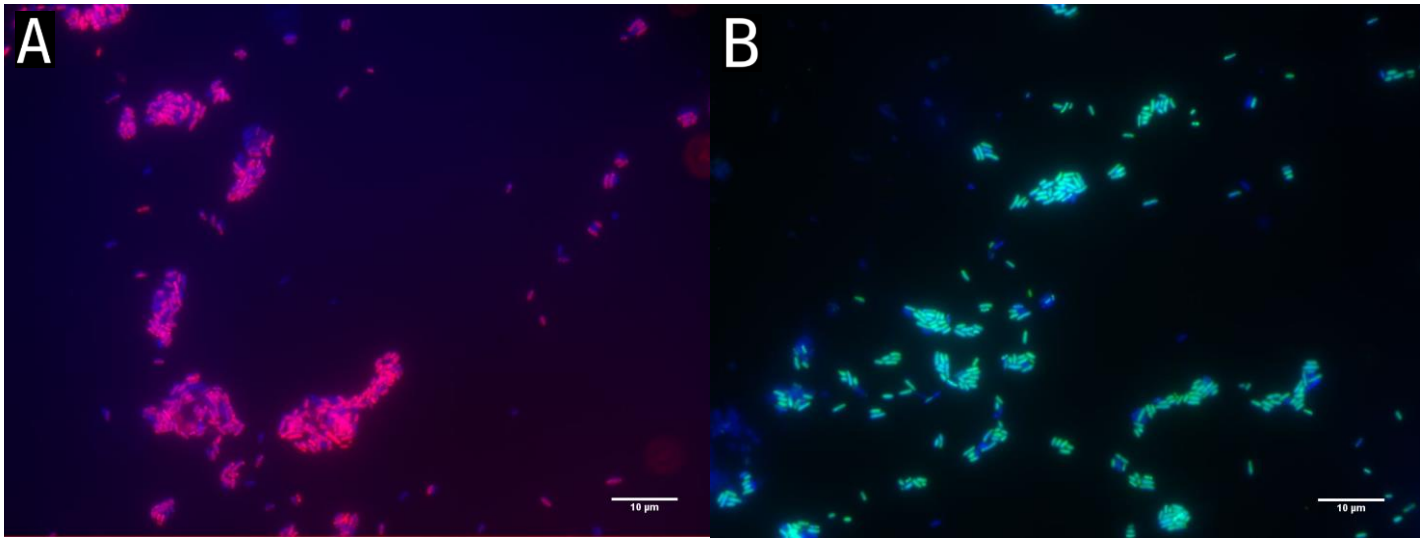

Fig.19 Pictures of two different samples at 100x magnification with fluorescent in situ hybridization of *E. aerogenes* pure cultures in Delisa medium with DAPI (blue) + EUB338 Cy3 (red) resulting in pink as overlay (**A**) and *E. aerogenes* pure cultures in Delisa medium stained with DAPI (blue) + Gam42a Fluos (green) resulting in light-green as overlay(**B**). Subsequent editing of colors with ImageJ. Scalebar represents 10 µm.

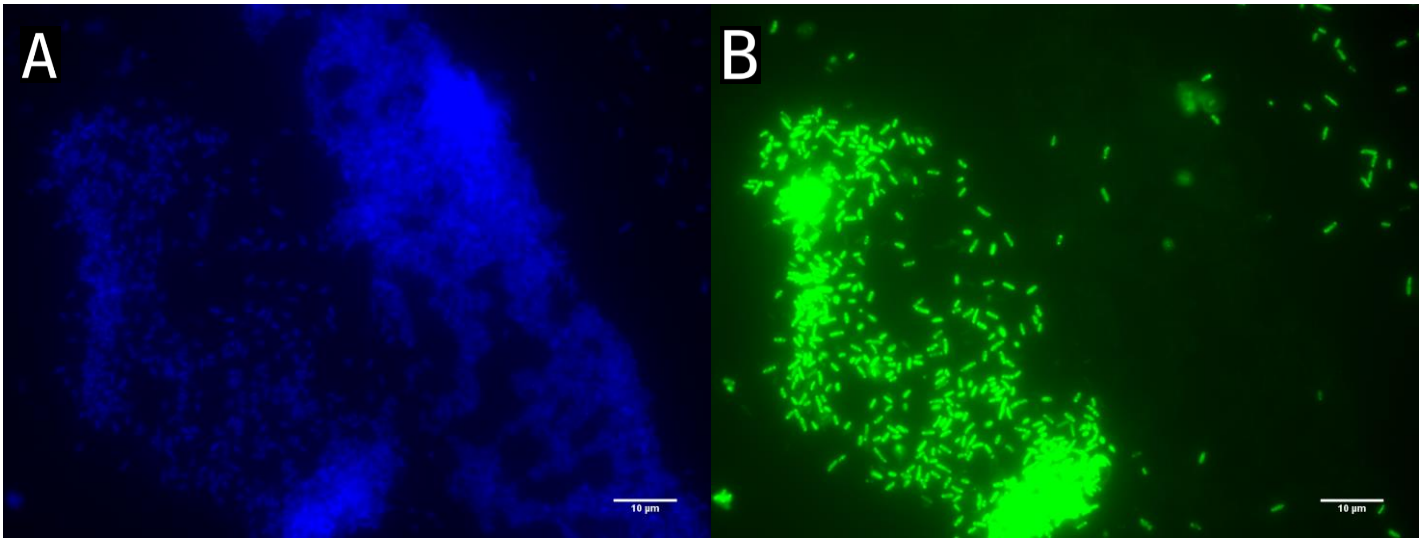

Fig.20 Pictures of a 50:50 combination of pure cultures of *E. aerogenes* and *C. acetobutylicum* at 100x magnification with DAPI, staining all DNA-containing cells (blue, **A**) and fluorescent in situ hybridization with Gam42a (Fluos) staining gamma proteobacteria (green, **B**). Subsequent editing of colors with ImageJ. Scalebar represents 10 µm.

5 Discussion

5.1 Growth and media

C. acetobutylicum and *E. aerogenes* were investigated for ecological, physiological and biotechnological aspects in mono- and co-culture systems. The benefits combining obligate anaerobes with facultative anaerobic dark fermentative biohydrogen producers were shown in previous studies (Elsharnouby et al., 2013; Haruhiko et al., 1998; Seppälä et al., 2011). In this study, it has been approved that the co-culture was reaching high cell numbers in the newly developed medium as well as the production of gas and metabolites.

C. acetobutylicum and *E. aerogenes* were able to grow in high cell densities under optimal conditions in the species-specific media, as well as in the common medium (DOE E medium).
C. acetobutylicum displayed fast and stable growth in Clostridium medium in closed batch (**Fig.2 A, C**). Even if this obligate anaerobe is more sensitive to oxygen than *E. aerogenes*, the serum bottles proved to be an efficient system for growing both organisms.
The batch system was providing more challenges in cultivating *C. acetobutylicum* (**Fig.3**) especially because of potential oxygen contaminations. The higher complexity of the vessel due to redox-,pH- and temperature-probes as well as valves for in- and outgas let to a higher susceptibility of minimal oxygen-contaminations in the environment.

Growth and H_2 production potential of *C. acetobutylicum* were examined in batch cultivation mode in Clostridium medium but reproducing the results strongly depended on a combination of the following factors.

A essential influence showed to be the time and the level of oxygen these obligate anaerobes were confronted with oxygen during the centrifuging process and the inoculation process itself. At the first experiments with *C. acetobutylicum*, growth showed to become more irregularly with increasing exposition to anaerobic conditions.

The inoculation was done anaerobically, and the medium was flushed with N_2. This step was included to avoid potential poisoning with oxygen and spore formation of *C. acetobutylicum* due to residual oxygen inside the liquid- and gas-phase of the medium.
Moreover, a phase of adaption to the new media showed positive effects on growth having adapted cells to the media when they are inoculated into higher scaled vessels.

Additionally, some other factors triggered initial growth of *C. acetobutylicum*. Cultivation in DOE E medium showed to be a suitable medium if adapted cultures of this organism are used for inoculation in closed batch. However, in batch it turned out to be hard to cultivate these organisms. Oxygen sensitivity was a crucial point in higher scaled reactors. The bigger setup and the higher potential of leakages was making cultivation experiments more challenging.

An additional reason why these obligate anaerobes were showing enhanced growth in closed batch could be the difference of agitation inside the batch reactor. In closed batch, serum flasks were shaken with 120 rpm, whereas batch system agitation was done with 200 rpm. *C. acetobutylicum* is forming biofilms and flocks, and if this is the requirement for growth of cells in the early beginning of exponential phase, it could be fatal to have a strongly shaking system or a stirrer. This potentially destroyed micro niches and protection in flocks and biofilms, where anaerobic conditions were provided for this organism.

However, regarding growth in DOE E medium, growth of *C. acetobutylicum* in DOE E medium as pure culture in batch system was not shown yet.

The cultivation of *E. aerogenes* needed comparatively low effort. Consequently, this organism has the potential to become an optimal candidate for higher H_2 production system. The best results in growth, gas- and metabolite production were reached in the species-specific medium.

This bacterium is not spore forming (Donnerberg, 2015), showing an advantage for growing cultures fast, even if small amounts of residual oxygen are present inside batch. *E. aerogenes*' growth showed to be stable and did not exhibit high deviations between the experiments (**Fig.2 B, D, Fig.4**).

The newly designed medium (DOE E medium) was verified as a suitable medium for this organism regarding growth and activity. Only one run in DOE E medium in bioreactors (**Fig.8**) showed to have comparatively high standard deviations in between the replicates. These deviations might be due to variances of cell numbers in the inoculum. A standardized inoculum regarding cell numbers could avoid this kind of biases in future experiments, as well as a higher number of replicates of the whole experiment (quadruplicates instead of duplicates).

On closer examination of the different media, the two species-specific media show variances in the buffer, the vitamin solution and in the trace element solution. Small changes in the trace elements and vitamin solution only had small effects on the growth of these organisms. The crucial switch was the difference in composition of the buffer.
Delisa medium contains no acetate, but high amounts of ammonia (**Tab.1**). This might be limiting and consequently inhibiting growth of *C. acetobutylicum* in this buffer (**Fig.2 A, C**). Further, if these results are compared to the DOE E medium results **(Fig.6)**, it seems that *C. acetobutylicum* needs a medium with a low buffer capacity. The starting pH of 6.8 seemed to be inhibiting until the pH dropped and exponential growth can start (**Fig.12 A**). For *E. aerogenes*, it appeared to be ideal to have a higher pH for a longer time favouring a higher buffer capacity, to get exponential growth as soon as possible (**Fig.10 A**, **Fig.11 A**). This organism prefers stabile and higher pH-levels. Experiments without pH-control (**Fig.11**) showed an inhibited growth under a pH of 5.5, and an instant stop of growth, production of VFAs and H_2 production beyond a pH of 5.0 (**Fig.16 B**).

In the DOE E medium experiments (**Fig.5**, **Fig.6**) it could be observed that both organisms showed highest values for OD_{600} and cumulative pressure in the middle point of buffer capacity with 76.5 mmol/L phosphate buffer and an acetate concentration of 16.5 mmol/L. *C. acetobutylicum* showed to be especially controlled by ammonium chloride, whereas *E. aerogenes* was triggered mostly by a combination of phosphate buffer and acetate (**Fig.5)**. These controls proved to be the main drivers for good growth and consequently finding an optimal common medium.

Ammonium has been reported (Tsai et al., 2014) to increase ABE fermentation of *C. acetobutylicum* with higher concentrations, as well as biohydrogen production, if the pH is controlled over 5.5.
For *E. aerogenes*, the phosphate buffer plays a key role. The higher buffer capacity provides longer period until the pH is dropping due to metabolite formation. Dropping pH is therefore inhibiting activity of *E. aerogenes (*Lu et al., 2007). Acetate concentration is a typical switch for pathways of Enterobacteriaceae. It has been reported (Contiero et al., 2000), that closely related organisms containing *E. coli* exhibit an acetate metabolism which is fundamental for cultivating these organisms to high cell densities.

5.2 Quantification methods

The newly developed and standardized quantification methods with OD_{600} measurement, cell counts and qPCR proved to be suitable methods for quantification of *C. acetobutylicum* and *E. aerogenes* (**Fig.3, Fig.4, Fig.7**).

Cell counts via microscope counting as a quantification method has been described (Hazan, 2012) to have high sensitivity and accuracy. The method is comparatively easy to operate and cheap for getting total cell numbers. It was possible to distinguish even between living and dead cells. The only drawback was the time-consuming workflow and the susceptibility of potential pipetting errors, especially if the cell density is high, flock-formation occurred, and a row of dilutions must be done.

Quantitative measurement via OD_{600} could be operated much faster as a high throughput method. Distinguishing between living and dead cells was not possible. In case of *C. acetobutylicum*, it showed to be challenging because of strong flock-forming and biofilm producing cultures, espe-

cially at the end of exponential phase. After diluting, pipetting and shaking, this biofilm was not always completely dissolved. This was probably the reason for higher deviations between OD_{600} and other quantification methods at the middle point and end of grow curves when the biofilm and flocks were becoming very slimy and dense, effecting the cloudiness of the liquid without an actual rise of cell number in the sample (**Fig.7**).

qPCR proved to be an efficient DNA based quantification method, which is independent of cell aggregation affected errors. This tool worked well for the established systems in this study. By using this method, total numbers of specific cells at different time points in multi-species-systems could be shown (**Fig.3, Fig.4, Fig.7, Fig.8**). Especially for co-cultures it has big advantages, because the quantification of the relative and total ratio of certain species is possible. qPCR is a specific tool and can be used as contamination-checking tool at the same time. This method is much more sensitive compared to OD_{600} measuring, because it can even detect cell numbers as low as 10 cells/mL (Hazan, 2012).

The limitations are the time-consuming, more complex and more expensive workflow. Additionally, live and dead cells cannot be distinguished.

However, all three methods proved to be suitable for the purposes of the presented experiments with *E. aerogenes* and *C. acetobutylicum* and can be recommended for quantification of these microorganisms. In case of strong biofilm formation, qPCR is not influenced at all. OD_{600} and cell counts can be influenced by the flock formations, and therefore influence the results during cell growth. At these conditions, it is recommended to use qPCR as quantification method.

5.3 Gas production

With focus on cumulative gas pressure, it was shown (**Fig.2 A, B**) that *C. acetobutylicum* can produce up to 6.7 bar and *E. aerogenes* up to 3.9 bar in closed batch. Both organisms produced H_2 and CO_2 in the species-specific medium (**Fig. 15, Fig. 16**) and showed high values of HER, CER and Y (**Tab.4**) compared to literature.

Already existing studies reported the highest values of HER for *E. aerogenes* with 26.67 mmol/L/h (C-molar) (Ito et al., 2005) and highest $Y_{(H2/S)}$ of 1.10 mol/C-mol (Shin et al., 2010). In this study (**Tab.4**), the results showed HER, and $Y_{(H2/S)}$ mol/C-mol for *E. aerogenes* in Delisa medium of 26.3 mmol/L/h and 0.91, as well as for *C. acetobutylicum* in Clostridium medium of 1.7 mmol/L/h and 0.43, respectively.
In the experiments of *C. acetobutylicum* in Clostridium medium (**Fig. 15 A**), the relative H_2 :CO_2 partition reached a ratio of 1.9:1.2 during the exponential phase, showing that these microorganisms switched to a H_2 producing pathway, which demonstrated the potential of this biological H_2 producing system.

Compared to the highest results of HER and $Y_{(H2/S)}$ in DOE E medium experiments, *E. aerogenes* as pure culture (0.0061 mmol/L/h and 0.27 mol/C-mol), and *C. acetobutylicum* together with *E. aerogenes* in co-culture (0.0051 mmol/L/h and 0.11 mol/C-mol) showed only low values. Additionally, the relative amount of H_2 kept under 1% in the off gas over the entire experiment (**Fig. 16**). This low amount of H_2 let to lower calculated values for HER, $Y_{(H2/S)}$ and qH_2. Even if the organisms were still able to produce very small amounts of H_2, HER and $Y_{(H2/S)}$, it would not reach an economical beneficial level of H_2 production at this point. Only higher levels of produced CO_2 (up to 16% of the relative off gas) were detectable.

The experiments of the co-culture of *C. acetobutylicum* together with *E. aerogenes* in DOE E medium could have been affected by potential H_2 losses during the gas-collecting step. The higher permeability due to the lower molecular weight of H_2 (2.016 g/mol) compared to N_2 (28.014 g/mol) and O_2 (31.999 g/mol [NCBI, 2019]) resulted in a higher potential risk of leaking threw the collecting vessels. GC samples were taken from plastic bags and transferred to serum flasks, which were stored for up to one week. H_2 could have leaked threw the cap. It would be necessary to test the permeability of the serum bottles for collecting the GC samples as well as enhance the speed of measurement by GC in general.

Moreover, it is possible that the main part of bacteria in co-culture, which was proved to be *E. aerogenes* via qPCR (**Fig. 8**), switched to aerobic respiration and produced CO_2 due to presence of O_2 in the system. Enterobacteriaceae as facultative anaerobes can switch from fermentation to aerobic respiration in the presence of oxygen, because these conditions are favourable due to the higher energy output compared to fermentation (Khanna and Das, 2013). Therefore, presence of oxygen in the system would be a possible explanation for the high level of CO_2 and the low level of produced H_2.

The DOE E medium itself, however, could inhibit H_2 production for the bacteria because of changes in the composition of phosphate buffer, ammonium and chloride. The chemicals concentration could inhibit certain pathways and hydrogenases, which are linked to the cell's H_2 production. It would be necessary to test the H_2 production with different buffers to see if certain ingredients are inhibiting the H_2 producing machinery.

At this point, however, it is important to take the higher standard deviations of HER and $Y_{(H2/S)}$ due to the experimental setup with DOE E medium in duplicates into account. Higher replicate numbers (e.g. quadruplicates) would allow a higher confidence about which biomolecules were consumed and produced.

Additionally, all experiments showed low values for qH_2, compared to already existing studies. The highest reported mean qH_2 in batch for Enterobacteriaceae showed 2.09 (C-molar) (Ergal et a., 2018) (C-molar). The maximum value for qH_2 in this study was 3.97×10^{-10} mmol/L/h (C-molar) for the co-culture at pH-controlled conditions. These qH_2 values are conducted due to the calculation based on the HER and cell numbers. Therefore, the best strategy to get a highly productive biohydrogen system in batch must involve improvement of these values.

5.4 Metabolisms

In case of *C. acetobutylicum*, it was shown that this organism can produce different VFAs like lactate, acetate, ethanol, butanol and other VFAs, depending on change of composition and pH of the medium. In Clostridium medium **(Fig.12)**, especially metabolites such as acetone, butanediol and butyric acid but only small amounts of ethanol were produced. The formation of these molecules is typical for ABE production, and has been already well described and used for industrial production of VFAs (Jones and Woods, 1986).

C. acetobutylicum has been reported (Brüggemann and Gottschalk, 2009) to change to solventogenesis (production of butanol and acetone) to prevent inhibition of acidic end products due to acetogenesis, allowing them to stay active in an environment with lower pH. This change in pathways is providing an ecological advantage under these conditions, because the organism can stay longer metabolically active. Moreover, the ability to start spore formation offers these organisms another ecological advantage after suboptimal conditions.

In Delisa medium, *E. aerogenes* **(Fig.9)** started with production of lactic acid and ethanol as first metabolites, followed by ethanol and smaller amounts of formic and acetic acid. This changed with a different medium. In DOE E medium, the metabolites switched **(Fig.10)**. Especially formic acid was produced in high amounts (up to a maximum concentration of 259 mmol/L), and an increased formation of ethanol could be observed. This accumulation of formic acid showed to drop the pH in this medium with relatively small buffer capacity quickly. Therefore, this formic acid proved to be the main inhibitor for growth of *E. aerogenes* under pH-uncontrolled conditions.

As described by Ramírez-Morales et al. (2015) and Ergal et al. (2018), *E. aerogenes* as a facultative anaerobe bacterium uses the PFL pathway during glycolysis with pyruvate formate lyase as a main enzyme for formate production and linked H_2 production from pyruvate.
Pyruvate can be converted to ethanol over a pyruvate decarboxylase and a alcohol dehydrogenase (Grossman et al., 2010). Additionally, pyruvate can be converted to lactate by a d-lactate dehydrogenase (Moroney et al., 1985). During formation of pyruvate to formate, formic acid can be formed by the pyruvate formate lyase (Grossman et al., 2010).It has been reported, that acetate can be formed over acetyl-coA via a phosphate acetyltransferase – acetate kinase pathway (Grossman et al., 2010).

These pathways could have been used by E. aerogenes to produce the formic acid, ethanol as well as the lactic acid/lactate in **Fig.9** and **Fig.10**.

The batch experiments with the co-culture underlined the study of Chojnacka et al. (2011), proving that members of the family Clostridiaceae can change the system dramatically even with a very low abundance. According to qPCR data **(Fig. 8)**, *E. aerogenes* overgrew *C. acetobutylicum* and the cell number of *C. acetobutylicum* kept most of the time below 1% of total cell numbers.

A facultative anaerobe like *E. aerogenes* showed to anaerobisize the medium, so that the obligate anaerobe *C. acetobutylicum* grow without starting to form spores. This was the only way so far, that *C. acetobutylicum* was able to grow in higher scaled DOE E medium volumes. The results of glucose usage of the co-cultures under pH-uncontrolled conditions (**Fig.14**), however, were different compared to mono-cultures (**Fig.11**). Under pH-uncontrolled conditions, glucose was completely used. Additionally, the level of formic acid was lower, concluding that the formation of formic acid due to pyruvate formate lyase was reduced du a maximum concentration of 2 mmol/L. At the same time, the level of ethanol raised to a maximum concentration of 100 mmol/L, showing a switch of pathways to produce ethanol over pyruvate via a pyruvate decarboxylase and an alcohol dehydrogenase.

It was shown, that the co-culture metabolised the residual glucose, even at lower pH-levels. At these pH-dropping conditions, ethanol was one of the highest produced side products. *E. aerogenes* could have produced this ethanol in DOE E medium as it was the case in Delisa medium. In co-cultures under pH-uncontrolled conditions, the comparatively small concentrations of formic acid (2.0 mmol/L, **Fig. 14**) which was found might lead to the theory, that *C. acetobutylicum* or *E. aerogenes* itself can eventually use and convert this formic acid in its metabolism. Especially, the results of pH-controlled DOE E medium co-cultures (**Fig.13**) underline this theory, where formic acid accumulated up to a concentration of 48 mmol/L. The pH stayed high and *E. aerogenes* produced formic acid again. At the same time, the pH was potentially too high, to allow *C. acetobutylicum* to become active and convert this formic acid. It has been reported, that *C. acetobutylicum* can re-assimilate acids during solventogenesis at low pH (Tsai et al., 2014). However, no enzyme for this conversion or metabolic pathway to convert this formic acid has been described in these two organisms so far.

It was also reported, that formic acid formation as a pathway is blocked under pH dropping conditions due to uncoupling (reviewed in Ergal et al., 2018). In this case, formic acid production would be inhibited if the pH stays at acidic conditions.

At this point, a co-culture of these two organisms demands a more intense research and an attention to the small parameters, which strongly influences activity of *C. acetobutylicum*. To get insights in the metabolisms, the production and use of this VFAs would be of essential interest, to see which organisms metabolize which products.
So far, the ratio of inoculum was not adjusted. To have a better controlled biological system, a defined initial cell number is needed, to minimize fluctuations and finally prevent overgrowing of one organism. This step could be challenging, because the number of active cells, which were already adapted to the DOE E medium, must be constant over all experiments to get comparable values.

5.6 FISH for understanding spatial arrangements

The results for FISH experiments with *E. aerogenes* confirmed a hybridisation with EUB338, Gam42a and a visualisation with DAPI (**Fig.19**). The probes can be used for specific targeting on class-level and demonstrated to hybridize with this organism efficiently. Staining of *C. acetobutylicum* was possible with general nucleic acid staining dyes like DAPI, but not with general domain-based bacterial probes (EUB338) or group-based probes (LGC35a), which are supposed to stain members of Firmicutes including Clostridiaceae. One option is, that the probe binding side in the 16S rRNA was not perfectly overlapping with the probe's nucleotide-sequence, preventing the binding on the intended position. Moreover, it could be difficult to penetrate the cell walls of spore-forming organism with EUB338 and LGC35a. Published FISH protocols (e.g. Ebeling et al., 1974) show procedures for staining spore-forming microorganisms with various chemicals and

enzymes for permeabilization treatments. For improved staining of *C. acetobutylicum*, an additional step with lysozymes or proteinases (e.g. Proteinase K) to increase permeability of spore coat layers would have been an opportunity (Filion,2009). This would offer the option to use FISH for spore-forming organism as a method for quantification as well. It could be used to see the spatial arrangement of co-cultures under certain environmental conditions (e.g. little amounts of oxygen). At this point, only a differentiation via counter staining was possible, which already offered the ability to visualize these two organisms at the same time (**Fig.20**). It was possible to see both organism in the same sample where *C. acetobutylicum* was just stained with DAPI as a contrast. To get more specific bindings on species level, additional probes could be designed and tested. Especially for a multi-species arrangement this improvement would deliver an opportunity to understand spatial arrangements of certain taxonomic and functional groups of bacteria.

6 Conclusion

Cultivation in closed batch and batch, the quantification of cells with three independent methods, visualisation of microorganisms via FISH and the analytic methods based on gas- and metabolite-analysis of the products have been tested with two already well studied biohydrogen producers. A newly developed medium was challenged to the already described species-specific media. The results proved the bacteria's potential to combine their biological framework with biotechnology and engineering, with the goal to enhance H_2 production.
The experimental trials for optimization of the media composition with focus on glucose concentration, buffer capacity, pH and contained gas showed to be the major switches for growth, metabolite- and gas-production.

C. acetobutylicum and *E. aerogenes* showed growth and the capability of producing H_2 and metabolites at defined media and described conditions. The experiments showed, that these organisms can adapt to small changes of media, community and environment. Both organisms showed comparatively high values of HER and $Y_{(H2/S)}$ their species-specific media. As side products, several metabolites were formed. The newly designed medium worked as basic medium for growth of the two studied organisms but showed limitations since no effective H_2-production was possible so far.

The study showed advantages and limitation of both organisms. The results of cultivation of co-cultures can be used for further improvement of cultivation in the DOE E medium as well as combine other functional groups of organisms, which can use the produced metabolites for a further improvement of especially HER and $Y_{(H2/S)}$.

At this point, more intensive research must be done for minimizing effects of outliers on the results due to the low replication number. Compared to literature, the results of the DOE E medium showed only small values for HER, CER, $Y_{(H2/S)}$ and qH_2 so far.

Focusing on growing conditions and medium, many challenges, including research on improving growth and enhancing production of gas and metabolites, must be solved with these organisms to reach their full potential. Especially establishing a procedure to cultivate *C. acetobutylicum* more stably even in higher scaled bioreactors would be essential. Reaching higher amounts of H_2 in the off gas and enhanced HER, qH_2 and $Y_{(H2/S)}$ should be of main interest. Additionally, an improvement of methods concerning GC measurement is necessary, to avoid potential H_2 losses. An improved workflow to prevent biofilm formation for OD_{600} measurement as well as for FISH staining would be of additional interest. Furthermore, a certain ratio of cells for inoculation must be evaluated to combine these two organisms in the best quantities.

Still, if both organisms become capable of biohydrogen production in a co-culture, it presents an opportunity to get a highly productive multi-species system with the potential to add even more species to produce biohydrogen as well as metabolites with simple carbon sources under mesophilic conditions. Higher complexity of ecological systems with higher number of species es promising to solve culture instability problems. Functional features of each organism can have positive effects inside the system which may lead to a higher fitness of the whole community, leading to even higher values of H_2 production and efficiency. Even more complex biotechnological cultivation systems considering higher scaled batches and even continuous cultures can be promising alternatives to reach the full

strength of these biological consortia. This field represents several exiting opportunities for launching a new era of commercial used energy carrier, with the help of engineering, physiology, biotechnology and microbiology. It demonstrates a promising way to reduce fossil energy carriers and therefore mitigate the negative effects of fossil fuel utilization by humans on Earth.

7 References

Software

Blast (NCBI) https://blast.ncbi.nlm.nih.gov/Blast.cgi

SnapGene (GSL Biotech LLC) https://www.snapgene.com

GeneRunner http://www.generunner.net

PathoGenomics http://www.pathogenomics.sfu.ca/ortholugedb/?page=paired-setup

NCBI Pubchem. https://pubchem.ncbi.nlm.nih.gov

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8 Appendix

Supplement Tab.1 Values from all time points measured of *C. acetobutylicum* in Clostridium medium showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	$Y(H_2/CO_2)$	$Y(H_2/S)$ (mol/C-mol))	% H_2	% CO_2	% N_2	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic acid (mmol/L)	Acetic acid (mmol/L)	Propionic acid (mmol/L)	Ethanol (mmol/L)	Acetone (mmol/L)	Butyric acid (mmol/L)	Butanediol (mmol/L)	C-balance	Degree of Reduction
0:00:00		0.000	0.000	0.0E+00	0.0E+00		0.00	0.00	100.00	6.673	0.044	106.851	0.000	28.310	0.000	0.000	0.000	0.000	0.000		
3:05:00	0.10	0.000	0.000	0.0E+00	0.0E+00	0.0E+00	0.00	0.00	100.00	6.641	0.038	121.624	0.000	27.644	0.000	0.000	0.000	0.000	0.000	0.00	0.00
5:50:00	0.21	0.000	0.000	0.0E+00	0.0E+00	0.0E+00	0.00	0.00	100.00	6.645	0.366	124.290	0.000	28.143	0.000	0.000	0.000	0.000	0.000	0.01	0.01
8:50:00	-0.10	0.000	0.000	0.0E+00	0.0E+00	0.0E+00	0.00	0.00	100.00	6.648	0.036	123.623	0.000	27.977	0.000	0.000	0.000	0.000	0.000	0.00	0.00
12:50:00	-0.01	0.000	0.002	0.0E+00	0.0E+00	0.0E+00	0.00	0.04	99.96	6.637	0.036	125.123	0.000	28.476	0.000	0.000	0.000	0.000	0.000	0.01	0.01
15:20:00	0.00	0.000	0.002	0.0E+00	0.0E+00	0.0E+00	0.00	0.04	99.96	6.618	0.040	130.065	0.000	27.311	0.000	0.000	0.000	0.000	0.000	0.02	0.02
18:10:31	-0.06	0.000	0.003	0.0E+00	0.0E+00	0.0E+00	0.00	0.06	99.94	6.573	0.045	127.899	0.000	27.144	0.000	0.000	0.000	0.000	0.000	0.06	0.06
22:31:31	0.13	0.000	0.012	0.0E+00	0.0E+00	0.0E+00	0.00	0.24	99.76	6.41	0.045	121.679	0.000	25.479	0.000	0.000	0.000	0.000	0.000	0.02	0.03
24:41:31	0.66	0.002	0.030	3.9E-13	8.2E-02	-5.0E-03	0.05	0.56	99.39	6.169	0.050	122.735	0.000	26.644	0.000	0.000	0.000	0.000	0.000	-0.04	0.00
26:11:31	0.30	0.021	0.055	2.1E-12	3.8E-01	9.4E-02	0.39	1.02	98.59	6.521	0.114	122.401	0.000	27.144	0.000	0.000	0.000	0.454	3.495	-0.13	-0.05
27:41:31	0.76	0.041	0.095	1.9E-12	4.3E-01	5.9E-02	0.77	1.77	97.47	5.96	0.152	121.346	0.000	27.144	0.000	0.000	0.000	1.362	7.988	-0.57	-0.34
28:41:31	0.21	0.262	0.307	7.5E-12	8.5E-01	4.2E-02	4.47	5.23	90.30	5.922	0.374	115.071	0.000	26.145	0.000	0.000	0.000	4.199	11.316	-1.60	-0.66
30:11:31	0.39	0.459	0.369	8.4E-12	1.2E+00	8.2E-02	7.55	6.08	86.37	6.06	0.590	106.685	0.000	29.142	0.000	0.000	2.596	6.129	11.316	3.79	2.04
31:41:31	0.10	1.444	0.962	2.0E-11	1.5E+00	4.3E-01	18.57	12.38	69.05	6.043	1.633	101.686	0.000	28.643	0.000	0.000	19.768	12.484	21.468	2.75	1.19
32:41:31	0.83	0.940	0.864	5.0E-12	1.1E+00	7.9E-02	13.25	12.18	74.57	6.393	2.728	89.746	5.440	30.641	3.470	6.252	30.950	16.570	28.124	1.28	0.74
34:41:31	-0.97	1.005	1.106	2.9E-11	9.1E-01	8.3E-02	13.47	14.82	71.71	5.948	2.348	65.477	7.660	29.309	4.887	8.804	57.308	26.331	44.101	1.31	0.66
36:21:31	0.10	1.719	4.178	2.6E-11	4.1E-01	3.4E-01	2.92	7.10	89.98	5.595	3.860	57.035	6.217	34.305	3.966	7.146	65.096	29.055	48.594	2.12	0.29
43:01:31	-0.03	0.000	0.000	0.0E+00	0.0E+00	0.0E+00	0.00	0.00	100.00	6.512	4.875	26.591	1.876	27.127	1.197	2.156	69.050	25.854	42.220	0.29	0.35
71:01:31	0.01	0.000	0.000	0.0E+00	0.0E+00	0.0E+00	0.00	0.00	100.00	6.978	5.665	0.072	3.541	37.452	2.259	4.070	99.661	37.090	60.509		

Supplement Tab.2 Values from all time points measured of *E. aerogenes* (first replicate "Saruman") in Delisa medium showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	$Y(H_2/CO_2)$	$Y(H_2/S)$ (mol/C-mol))	$\%H_2$	$\%CO_2$	$\%N_2$	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic acid(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Lactose (mmol/L)	Fructose (mmol/L)	C_balance	Degree of Reduction
0:00:00	0.00	0.000	0.000	0.0E+00	0.0E+00	0.0E+00														
5:48:11	0.00	0.000	0.022	0.0E+00	0.0E+00	0.0E+00	0.00	0.43	99.57	6.81	0.179	125.602	0.000	0.000	0.282	0.000	30.064	0.000		
21:48:11	0.03	0.000	0.014	0.0E+00	0.0E+00	0.0E+00	0.00	0.28	99.73	6.818	0.202	121.178	0.000	1.049	0.217	0.000	29.413	0.000		
28:48:11	0.09	0.000	0.018	0.0E+00	0.0E+00	0.0E+00	0.00	0.35	99.65	6.748	0.216	120.895	2.920	3.147	0.434	0.000	30.184	0.000	0.15	0.08
30:48:11	1.19	0.000	0.005	0.0E+00	0.0E+00	0.0E+00	0.00	0.10	99.90	6.719	0.260		0.000	0.000	0.000	0.000	0.000	0.000	0.68	0.56
32:48:11	0.69	0.000	0.014	0.0E+00	0.0E+00	0.0E+00	0.00	0.25	99.75	6.682	0.342	94.307	19.505	3.314	2.303	2.909	30.070	0.000	0.43	0.42
34:48:11	0.41	0.000	0.016	0.0E+00	0.0E+00	0.0E+00	0.00	0.30	99.71	6.641	0.564	90.510	23.346	3.847	5.214	9.290	31.662	0.000	0.62	0.64
36:48:11	0.03	0.000	0.057	0.0E+00	0.0E+00	0.0E+00	0.00	1.03	98.97	6.671	1.024	60.042	43.550	5.995	5.084	16.019	29.056	0.416	0.57	0.57
38:48:11	0.09	26.303	46.984	2.4E-10	5.6E-01	5.8E-02	32.43	57.93	9.64	6.736	1.732	22.036	54.440	6.261	11.384	32.320	29.556	0.000	2.28	0.51
40:48:11	0.48	0.689	1.308	2.4E-12	5.3E-01	2.8E-03	1.17	2.22	96.62	6.767	2.398	1.415	37.778	6.761	18.119	53.223	29.097	0.000	0.83	0.45
44:48:11	0.20	0.044	0.198	6.9E-14	2.2E-01	5.2E-03	0.08	0.36	99.56	7.027	2.166	0.000	30.062	39.167	38.562	63.773	29.100	0.000	0.58	0.57
47:28:11	0.25	0.003	0.028	2.7E-15	1.2E-01	0.0E+00	0.01	0.05	99.94	7.179	6.040	0.000	29.851	43.614	35.347	61.667	29.220	0.000	0.53	0.58
49:58:11	0.04	0.001	0.021	8.1E-16	5.3E-02	0.0E+00	0.00	0.04	99.96	6.972	4.000	0.000	28.830	38.818	28.134	60.082	27.841	0.000	0.49	0.55
52:18:11	-0.28	0.002	0.018	2.3E-15	8.8E-02	0.0E+00	0.00	0.03	99.96	6.873	4.250	0.000	26.587	37.302	14.230	56.175	26.325	0.000	0.44	0.50
69:48:11	-0.04	0.000	0.000	2.8E-17	5.6E-02	-4.5E-05	0.00	0.00	100.00	6.833	3.310	0.144	25.933	37.569	0.630	53.506	26.652	0.000		0.47

Supplement Tab.3 Values from all time points measured of *E. aerogenes* (second replicate "Legolas") in Delisa medium showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	$Y(H_2/CO_2)$	$Y(H_2/S)$ (mol/C-mol))	$\%H_2$	$\%CO_2$	$\%N_2$	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic ac-id(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Lactose (mmol/L)	Fructose (mmol/L)	C_bala nce	Degree of Reduction
0:00:00	0.00	0.000	0.000	0.0E+00	0.0E+00	0.0E+00														
3:18:53	0.00	0.000	0.466	0.0E+00	0.0E+00	0.0E+00	0.00	0.90	99.10	6.813	0.197	123.676	0.155	0.083	0.130	0.000	29.988	0.028		
21:48:53	0.00	0.000	0.006	0.0E+00	0.0E+00	0.0E+00	0.00	0.12	99.88	6.82	0.183	121.367	0.511	1.915	0.196	0.000	29.909	0.000	1.89	1.86
28:48:53	0.00	0.000	0.006	0.0E+00	0.0E+00	0.0E+00	0.00	0.12	99.88	6.804	0.281	98.587	15.997	4.380	1.977	2.040	30.029	0.000	0.81	0.81
30:48:53	2.32	0.000	0.006	0.0E+00	0.0E+00	0.0E+00	0.00	0.11	99.89	6.778	0.422	83.317	20.160	1.932	3.737	6.425	29.477	0.000	1.13	1.15
32:48:53	0.11	0.000	0.013	0.0E+00	0.0E+00	0.0E+00	0.00	0.24	99.76	6.74	0.706	72.776	34.458	4.713	5.518	10.028	29.217	0.172	1.09	1.13
34:48:53	0.64	0.009	0.081	5.4E-14	1.1E-01	2.4E-05	0.15	1.46	98.38	6.693	1.140	43.007	44.627	6.095	9.689	23.920	29.813	0.000	0.88	1.01
36:48:53	0.04	0.029	0.352	1.7E-13	8.2E-02	7.0E-05	0.49	5.98	93.54	6.663	1.983	8.687	41.197	5.412	19.161	48.296	29.477	0.000	0.77	0.79
38:48:53	0.17	0.069	0.617	2.9E-13	1.1E-01	6.8E-04	1.12	9.96	88.92	6.636	2.774	0.194	24.389	8.643	25.657	56.957	28.896	0.000	0.78	0.76
40:48:53	0.03	2.140	1.843	8.4E-12	1.2E+00	9.2E-01	22.20	19.12	58.68	6.674	3.690	0.000	24.745	18.834	32.479	52.160	27.245	0.000	1.22	0.98
44:48:53	0.20	1.789	6.925	3.1E-12	2.6E-01	0.0E+00	2.78	10.76	2.79	6.751	4.076	0.000	29.663	34.305	35.868	59.540	28.151	0.000	3.18	2.02
47:28:53	0.23	0.192	1.320	1.8E-13	1.5E-01	0.0E+00	0.34	2.35	97.31	7.254	6.030	0.000	28.364	36.686	28.112	56.610	27.748	0.000	1.15	1.12
49:58:53	0.12	0.000	0.771	0.0E+00	0.0E+00	0.0E+00	0.00	1.42	98.58	7.203	3.450	0.000	28.131	33.522	23.876	56.284	27.111	0.000	0.94	1.04
52:18:53	-0.09	0.482	0.804	4.1E-13	6.0E-01	0.0E+00	0.89	1.48	97.63	6.94	3.680	0.000	28.497	34.255	18.922	57.630	27.689	0.000	0.98	0.88
69:48:53	-0.05	0.000	0.009	0.0E+00	0.0E+00	0.0E+00	0.00	0.17	99.83	6.822		0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.900	

Supplement Tab.4 Values from all time points measured of *E. aerogenes* (first replicate "E1") in DOE E medium under pH-controlled conditions, showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	Y (H_2/CO_2)	Y (H_2/S) (mol/C-mol))	% H_2	%C O_2	%N_2	pH	Absorb-ance OD_{600}	Glucose (mmol/L)	Lactic ac-id(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Butanediol (mmol/L)	C_bala nce	Degree of Reduction
0:00:00	0.00	0.000	0.000	0.0E+00	0.0E+00	0.0E+00				7.01	0.021	159.001	0.000	15.670	0.739	0.000	0.000		
0:00:25	144.41	0.000	0.023	0.0E+00	0.0E+00	0.0E+00	0.00	0.05	99.96	7.01	0.051	165.079	0.000	16.553	1.173	0.000	0.000		-0.58
10:00:25	0.02	0.000	0.003	0.0E+00	0.0E+00	0.0E+00	0.00	0.07	99.93	6.821	0.058	159.551	0.000	16.453	1.173	0.499	0.000	-0.50	1.25
11:00:25	-0.05	0.000	0.004	0.0E+00	0.0E+00	0.0E+00	0.00	0.08	99.92	6.822	0.057	160.738	0.000	16.819	0.934	0.825	0.000	0.95	0.45
14:00:25	0.09	0.000	0.003	0.0E+00	0.0E+00	0.0E+00	0.00	0.07	99.93	6.77	0.065	146.556	0.000	19.384	4.041	5.947	0.216	0.32	0.57
22:00:25	0.59	0.000	0.072	3.4E-14	7.1E-04	1.2E-07	0.00	1.41	98.59	6.778	0.090	0.155	0.000	2.082	21.290	69.025	12.030	0.41	0.60
34:00:25	0.17	0.000	0.325	1.0E-14	3.4E-04	2.5E-03	0.00	5.94	94.06	6.671	3.481	0.133	0.000	6.661	20.965	81.680	43.577	0.51	0.51
37:00:25	0.10	0.000	0.472	3.1E-14	9.5E-04	0.0E+00	0.01	8.39	91.60	6.772	4.164	0.133	0.000	19.434	25.657	81.159	55.075	0.48	0.26
40:00:25	-0.04	0.000	0.239	8.3E-15	4.5E-04	0.0E+00	0.00	4.45	95.55	6.654	3.849	0.133	0.000	20.933	27.156	84.003	55.424	0.25	
57:00:25	-0.02	0.000	0.014	0.0E+00	0.0E+00	0.0E+00	0.00	0.28	99.73	6.927	3.197	0.128	0.000	18.768	22.572	76.666	37.704		

Supplement Tab.5 Values from all time points measured of *E. aerogenes* (second replicate "E2") in DOE E medium under pH-controlled conditions, showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	Y (H_2/CO_2)	Y (H_2/S) (mol/C-mol))	%H_2	%CO_2	%N_2	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic acid(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Butanediol (mmol/L)	C_balance	Degree of Reduction
0:00:00	0.00	0.000	0.000	0.0E+00	0.0E+00	0.0E+00				6.776	0.022	164.169	0.000	16.120	0.239	0.000	0.000	0.13	
0:00:37	108.96	0.000	0.002	0.0E+00	0.0E+00	0.0E+00	0.00	0.04	99.96	6.776	0.055	158.740	0.000	17.169	1.173	0.608	0.000	0.73	1.01
10:00:37	0.39	0.001	0.306	7.3E-12	1.9E-03	3.1E-06	0.01	5.74	94.25	6.63	1.124	79.942	0.000	24.130	281.469	27.089	0.150	0.73	0.96
12:00:37	0.87	0.002	0.693	3.5E-12	2.3E-03	3.5E-06	0.03	12.13	87.85	6.699	2.013	41.697	0.000	26.511	388.030	36.510	0.383	0.80	0.98
14:00:37	0.47	0.003	1.469	2.8E-12	2.3E-03	6.6E-06	0.05	22.64	77.31	6.66	2.842	0.155	0.000	30.008	515.512	54.743	1.830	0.80	0.95
16:00:37	0.24	0.003	1.419	1.6E-12	2.1E-03	4.5E-02	0.05	22.16	77.80	6.702	3.563	0.150	0.000	33.422	483.598	59.865	2.596	0.83	0.88
17:30:37	0.10	0.005	2.110	2.1E-12	2.2E-03	1.0E-02	0.07	29.78	70.15	6.703	3.891	0.122	0.000	36.453	425.679	62.362	3.394	0.84	0.85
20:00:37	0.07	0.004	1.893	1.6E-12	2.1E-03	2.5E-02	0.06	27.77	72.17	6.895	4.390	0.105	0.000	42.365	387.552	64.467	7.255	0.72	0.79
34:00:37	-0.03	0.002	0.665	1.4E-12	3.4E-03	0.0E+00	0.04	12.32	87.64	6.855	3.335	0.105	0.000	43.530	359.374	61.515	8.303		

Supplement Tab.6 Values from all time points measured of *E. aerogenes* (first replicate "E3") in DOE E medium under pH-uncontrolled conditions, showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	$Y(H_2/CO_2)$	$Y(H_2/S)$ (mol/C-mol))	% H_2	% CO_2	% N_2	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic acid(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Butanediol (mmol/L)	C_balance	Degree of Reduction
0:00:00	0.00	0.000	0.000	0.0E+00	0.0E+00	0.0E+00				6.753	0.034	163.542	0.000	16.087	0.196	0.000	0.000		
0:00:45	66.36	0.000	0.000	0.0E+00	0.0E+00	0.0E+00	0.00	0.00	100.00	6.753	0.059	157.308	0.000	16.370	1.434	0.000	0.000	0.05	
10:00:45	0.30	0.002	0.566	5.3E-11	3.4E-03	1.5E-05	0.03	10.00	89.97	6.104	0.823	104.648	0.000	23.447	156.116	22.205	0.000	0.70	0.86
12:00:45	0.49	0.002	0.655	1.7E-11	2.6E-03	4.9E-05	0.03	11.31	88.66	5.862	1.200	101.812	0.000	23.680	159.048	24.484	0.000	0.73	0.85
14:00:45	0.14	0.003	1.093	2.1E-11	2.5E-03	8.5E-05	0.04	17.42	82.54	5.652	1.326	99.109	0.000	24.113	160.960	27.545	0.000	0.84	0.84
16:00:45	0.14	0.003	1.060	1.9E-11	3.1E-03	8.4E-05	0.05	16.86	83.09	5.44	1.460	95.823	0.000	25.612	155.703	29.585	0.000	0.83	0.80
17:30:45	-0.03	0.002	0.726	1.3E-11	3.0E-03	-1.3E-04	0.04	12.17	87.79	5.342	1.438	96.910	0.000	26.561	152.944	31.235	0.000	0.78	0.81
20:00:45	0.07	0.001	0.463	4.4E-12	1.9E-03	5.3E-06	0.02	8.11	91.88	5.191	1.521	80.181	0.000	27.244	146.339	32.798	0.000	0.58	0.64
34:00:45	0.00	0.000	0.078	0.0E+00	0.0E+00	0.0E+00	0.00	1.47	98.54	4.799	1.497	92.536	0.000	27.560	157.897	34.013	0.000	0.62	0.80

Supplement Tab.7 Values from all time points measured of *E. aerogenes* (second replicate "E4") in DOE E medium under pH-uncontrolled conditions, showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	$Y(H_2/CO_2)$	$Y(H_2/S)$ (mol/C-mol))	$\%H_2$	$\%CO_2$	$\%N_2$	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic acid(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Butanediol (mmol/L)	C_balance	Degree of Reduction
0:00:00	0.00	0.000	0.000	0.0E+00	0.0E+00	0.0E+00				6.825	0.025	162.642	0.000	16.020	0.348	0.000	0.000		
0:00:54	63.43	0.000	0.000	0.0E+00	0.0E+00	0.0E+00	0.00	0.00	100.00	6.825	0.054	172.678	0.000	17.302	1.608	0.000	0.000	-0.06	
10:00:54	0.32	0.001	0.356	2.9E-12	1.8E-03	4.2E-06	0.01	6.52	93.46	6.096	0.840	106.952	0.000	22.498	150.663	21.185	0.000	0.68	0.87
12:00:54	0.43	0.006	0.423	1.1E-11	1.4E-02	1.2E-04	0.11	7.61	92.38	5.874	1.168	102.778	0.000	24.430	152.509	24.788	0.000	0.69	0.85
14:00:54	0.16	0.003	0.884	3.4E-12	2.9E-03	7.6E-05	0.04	14.58	85.38	5.655	1.308	99.991	0.000	24.463	152.010	27.111	0.000	0.79	0.83
16:00:54	0.31	0.000	0.350	1.6E-13	6.4E-04	6.5E-06	0.00	6.30	93.70	5.487	1.605	97.121	0.000	24.563	146.100	28.370	0.000	0.64	0.78
17:30:54	-0.42	0.001	0.310	7.6E-13	1.8E-03	-4.6E-05	0.01	5.59	94.40	5.394	1.302	97.876	0.000	26.778	142.060	30.258	0.000	0.66	0.79
20:00:54	0.05	0.000	0.140	1.9E-13	1.2E-03	-1.1E-04	0.00	2.60	97.40	5.251	1.362	98.032	0.000	26.395	136.020	31.561	0.000	0.61	0.78
34:00:54	0.00	0.000	0.041	0.0E+00	0.0E+00	0.0E+00	0.00	0.77	99.23	4.886	1.371	94.884	0.000	26.611	150.728	32.429	0.000	0.60	0.80

Supplement Tab.8 Values from all time points measured of *E. aerogenes* and *C. acetobutylicum* co-culture (first replicate "C1") in DOE E medium under pH-controlled conditions, showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	$Y(H_2/CO_2)$	$Y(H_2/S)$ (mol/C-mol))	% H_2	% CO_2	% N_2	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic acid(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Butanediol (mmol/L)	C_balance	Degree of Reduction
0:00:00		0.000	0.000	0.0E+00	0.0E+00	0.0E+00	0.00	0.00	0.00	6.878			0.000	16.886	0.196	0.000	0.000		
0:03:34	0.00	0.000	0.002	0.0E+00	0.0E+00	0.0E+00	0.00	0.03	99.97	6.813	0.074	131.680	0.000	15.487	0.434	0.673	0.000	0.00	0.00
9:33:34	0.05	0.000	0.001	0.0E+00	0.0E+00	0.0E+00	0.00	0.02	99.98	6.782	0.091	146.434	0.000	23.664	4.149	3.364	0.000	0.19	0.20
13:33:34	-0.05	0.000	0.005	0.0E+00	0.0E+00	0.0E+00	0.00	0.09	99.91	6.782	0.083	127.190	0.000	17.286	2.368	1.541	0.000	0.03	0.03
23:33:34	0.09	0.000	0.001	0.0E+00	0.0E+00	0.0E+00	0.00	0.02	99.98	6.782	0.120	139.857	0.000	31.474	1.781	2.909	0.100	0.22	0.23
24:33:34	-0.67	0.000	0.006	0.0E+00	0.0E+00	0.0E+00	0.00	0.11	99.89	6.781	0.093	147.755	0.000	24.296	0.978	1.888	0.000	0.17	0.17
27:33:34	0.04	0.002	0.418	4.0E-10	5.2E-03	1.0E-04	0.04	7.44	92.52	6.778	0.097	145.141	0.000	24.696	1.608	2.670	0.000	0.70	0.18
29:33:34	0.08	0.000	0.002	0.0E+00	0.0E+00	0.0E+00	0.00	0.04	99.96	6.774	0.103	145.468	0.000	24.696	2.325	2.887	0.000	0.18	0.19
32:33:34	0.13	0.000	0.007	0.0E+00	0.0E+00	0.0E+00	0.00	0.13	99.87	6.765	0.119	142.499	0.000	28.460	2.129	5.492	0.050	0.24	0.27
35:33:34	0.21	0.001	0.104	3.6E-11	6.2E-03	2.0E-05	0.01	1.95	98.04	6.745	0.147	138.508	0.000	29.409	6.170	8.791	0.166	0.44	0.31
47:33:34	0.08	0.002	0.305	5.1E-11	7.6E-03	8.4E-06	0.04	5.54	94.42	6.751	0.900	0.128	0.000	12.173	46.774	75.255	6.173	0.30	0.27
50:33:34	0.41	0.004	0.336	2.5E-11	1.1E-02	-2.2E-02	0.07	6.09	93.84	6.745	1.413	0.150	0.000	22.498	25.462	75.407	15.075	0.35	0.33
53:33:34	0.27	0.003	0.708	9.7E-12	4.8E-03	1.9E-02	0.06	12.02	87.92	6.750	1.841	0.128	0.000	24.396	48.577	86.933	10.715	0.58	0.36
70:33:34	0.12	0.000	0.000	0.0E+00		0.0E+00				6.891	4.833	0.117	0.000		36.107	87.324	18.419	0.25	0.34

Supplement Tab.9 Values from all time points measured of *E. aerogenes* and *C. acetobutylicum* co-culture (second replicate "C2") in DOE E medium under pH-controlled conditions, showing μ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH.

Inoculation time	**μ**	**HER (C-molar)**	**CER (C-molar)**	**qH_2 (C-molar)**	**$Y_{(H2/CO2)}$ (mol/C-mol)**	**$\%H_2$**	**$\%CO_2$**	**$\%N_2$**	**pH**
0:00:00		0.000	0.000	0.0E+00	0.0E+00				7.052
0:03:25	0.00	0.000	0.000	0.0E+00	0.0E+00	0.00	0.00	100.00	7.010
9:33:25	0.53	0.000	0.013	2.6E-13	5.2E-03	0.00	0.26	99.73	6.821
14:03:25	-0.27	0.001	0.065	1.2E-11	1.4E-02	0.02	1.27	98.71	6.774
24:03:25	0.28	0.005	0.475	4.0E-12	1.1E-02	0.09	8.53	91.37	6.782
24:33:25	0.36	0.002	0.341	1.6E-12	7.0E-03	0.04	6.24	93.72	6.782
27:33:25	0.10	0.001	0.200	4.1E-13	4.2E-03	0.02	3.77	96.21	6.773
29:33:25	0.07	0.003	0.616	1.5E-12	5.5E-03	0.06	10.68	89.26	6.765
32:33:25	0.04	0.001	0.381	5.3E-13	3.6E-03	0.03	6.90	93.07	6.739
35:33:25	0.01	0.002	0.336	6.2E-13	5.1E-03	0.03	6.14	93.83	6.748
47:33:25	-0.01	0.000	0.126	1.2E-13	2.2E-03	0.01	2.43	97.56	6.683
50:33:25	-0.03	0.000	0.225	1.7E-13	1.6E-03	0.01	4.27	95.72	6.748
53:33:25	-0.01	0.000	0.020	0.0E+00	0.0E+00	0.00	0.40	99.60	7.024
71:33:25		0.000	0.000	0.0E+00					6.881

Supplement Tab.10 Values from all time points measured of *E. aerogenes* and *C. acetobutylicum* co-culture (first replicate "C3") in DOE E medium under pH-uncontrolled conditions, showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	$Y(H_2/CO_2)$	$Y(H_2/S)$ (mol/C-mol))	$\%H_2$	$\%CO_2$	$\%N_2$	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic acid(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Lactose (mmol/L)	Fructose (mmol/L)	C_balance	Degree of Reduction
0:00:00	0.00	0.000	0.000	0.0E+00		0.0E+00				6.759		164.491	0.056	15.987	0.043	0.000		0.000		
0:35:53	1.49	0.000	0.001	0.0E+00	0.0E+00	0.0E+00	0.00	0.02	99.98	6.745	0.024	163.975	0.244	16.653	2.564	0.000	0.000	0.000	0.62	0.62
11:55:53	0.47	0.000	0.040	0.0E+00	0.0E+00	0.0E+00	0.00	0.79	99.21	6.153	0.051	125.924	12.389	24.263	1.695	16.432	0.000	0.000	0.50	0.50
23:25:53	0.01	0.000	0.371	2.1E-13	1.1E-03	2.9E-06	0.01	6.74	93.25	5.710	0.485	55.485	20.826	13.306	1.738	56.414	0.000	0.050	0.32	0.34
25:25:53	0.26	0.000	0.383	1.4E-13	1.2E-03	2.7E-06	0.01	6.93	93.06	5.666	1.815	40.981	21.925	11.507	2.216	62.557	0.000	0.061	0.32	0.33
27:55:53	0.08	0.000	0.302	7.4E-14	9.9E-04	2.4E-06	0.01	5.48	94.52	5.614	2.160	28.015	0.000	12.390	2.172	70.480	0.000	0.111	0.22	0.25
29:25:53	0.15	0.001	0.927	1.9E-13	1.1E-03	7.5E-06	0.02	15.01	84.98	5.581	2.307	19.816	0.000	14.072	2.064	78.077	0.000	0.128	0.36	0.26
32:25:53	0.07	0.002	0.973	3.1E-13	2.0E-03	2.4E-05	0.03	15.63	84.34	5.512	2.498	9.775	0.000	15.520	0.630	84.024	0.000	0.161	0.38	0.27
35:25:53	0.06	0.004	1.309	5.1E-13	2.9E-03	5.8E-05	0.06	19.83	80.11	5.439	2.706	1.388	0.000	17.386	0.174	90.797	0.000	0.194	0.46	0.28
47:55:53	0.01	0.000	0.200	0.0E+00	0.0E+00	0.0E+00	0.00	3.65	96.35	5.293	2.924	0.056	0.000	28.476	0.304	100.586	0.000	0.344	0.28	0.33

Supplement Tab.11 Values from all time points measured of *E. aerogenes* and *C. acetobutylicum* co-culture (second replicate "C4") in DOE E medium under pH-uncontrolled conditions, showing µ, HER, CER, qH_2 on a C-molar level, $Y(H_2/CO_2)$, $Y(H_2/S)$ on a C-molar level, gas composition (volumetric, relative; showing H_2, CO_2 and N_2), pH, OD_{600}, HPLC results, calculated C-balance and degree of reduction.

Inoculation time	µ	HER (C-molar)	CER (C-molar)	qH_2 (C-molar)	$Y(H_2/CO_2)$	$Y(H_2/S)$ (mol/C-mol))	% H_2	% CO_2	% N_2	pH	Absorbance OD_{600}	Glucose (mmol/L)	Lactic acid(mmol/L)	Acetic acid (mmol/L)	Formic acid (mmol/L)	Ethanol (mmol/L)	Lactose (mmol/L)	Fructose (mmol/L)	C_balance	Degree of Reduction
0:00:00	0.00	0.000	0.000	0.0E+00	0.0E+00	0.0E+00				6.819	0.042	143.009	0.255	15.853	0.217	0.000	0.000	0.000	0.00	
0:35:00	1.14	0.000	0.006	0.0E+00	0.0E+00	0.0E+00	0.00	0.12	99.88	6.780	0.063	159.750	0.144	15.920	0.000	0.000	0.000	0.000	0.78	0.00
11:56:01	0.43	0.001	0.143	7.6E-13	8.8E-03	1.6E-05	0.02	2.71	97.26	6.195	0.467	123.210	10.369	19.517	1.651	16.497	0.000	0.000	0.44	0.74
23:26:01	0.01	0.001	0.481	3.9E-13	1.5E-03	5.1E-06	0.01	8.55	91.44	5.837	1.746	57.872	19.005	10.441	1.456	52.550	0.000	0.061	0.42	0.40
25:26:01	0.22	0.001	0.456	2.4E-13	1.5E-03	4.3E-06	0.01	8.11	91.88	5.792	2.021	44.778	20.759	9.126	1.456	59.887	0.000	0.072	0.36	0.39
27:56:01	0.06	0.000	0.239	0.0E+00	0.0E+00	0.0E+00	0.00	4.37	95.63	5.741	2.117	34.304	22.691	9.342	2.020	66.920	0.000	0.094	0.39	0.39
29:26:01	0.20	0.000	0.355	4.3E-14	5.2E-04	1.3E-06	0.00	6.33	93.66	5.711	2.352	25.583	24.678	10.741	1.521	70.523	0.000	0.105	0.45	0.39
32:26:01	0.06	0.000	0.539	5.8E-14	5.6E-04	4.1E-06	0.01	9.32	90.68	5.638	2.522	16.247	25.111	11.540	1.521	79.357	0.000	0.139	0.53	0.40
35:26:01	0.05	0.001	0.680	2.4E-13	2.1E-03	1.9E-05	0.02	11.41	88.57	5.560	2.663	6.955	25.644	12.523	0.760	84.979	0.000	0.178	0.33	0.40
47:56:01	0.01	0.000	0.185	3.6E-14	1.3E-03	1.8E-05	0.00	3.38	96.61	5.288	2.800	0.078	22.069	24.713	0.217	98.242	0.000	0.244	0.33	0.44