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"Ecology and biotechnology of biohydrogen producing *Enterobacter aerogenes* and *Clostridium acetobutylicum* in mono-culture and co-culture"

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Abstract

Biologically produced biohydrogen (bio-H_2) is an energy carrier and considered as an alternative to conventional fossil fuels. The current biological bio-H_2 production technologies are still showing deficits in terms of bio-H_2 yields and volumetric production rates, which are the main bottlenecks in the yet unsuccessful implementation to largescale industry.

In bio-industry, mono-cultures have been the preferred production systems, however, interest has recently emerged in the usage of artificial microbial co-cultures. Highly dynamic microbial communities and consortia can improve bioconversion performances while providing enhanced resistance to environmental stressors. In this thesis, the ecological, physiological and biotechnological aspects that are required for engineering a defined microbial consortium of dark fermentative H_2 producers are shown. It is demonstrated, that an artificial consortium of *Enterobacter aerogenes* and *Clostridium acetobutylicum* enables enhanced H_2 production by efficient substrate to H_2 conversion, extended H_2 production in acidic medium and the utilization of complex carbon sources. This thesis emphasises defined co-culture design and engineering as a sophisticated and reproducible method for obtaining artificial microbial consortia for improved bio-H_2 production.

Kurzfassung

Biologisch hergestellter Wasserstoff (bio-H_2) stellt einen wichtigen erneuerbaren Energieträger der Zukunft dar und kann auf durch Fermentation hergestellt werden. Aktuell sind die dabei erzielten Umsatzraten und Ausbeuten allerdings unbefriedigend und eine Implementierung in den industriellen Maßstab aus wirtschaftlichen Gründen nicht sinnvoll. Für lange Zeit war das auf Monokulturen-basierende mikrobielle Zellfabrik-Konzept, die bevorzugte Produktionsroute in der industriellen Biotechnologie. In Hinsicht auf Produktions- und Prozessoptimierung besteht jedoch zunehmendes Interesse in der Umstellung auf Konsortien-basierte Zellfabriken. Im Vergleich zu Monokulturen, können hoch-dynamische mikrobielle Zellgemeinschaften erhöhte bio-H_2 Konversionsraten und Erträge erreichen, wobei zusätzlich verbesserte Resistenzeigenschaften gegenüber Umweltstressoren erzielt werden können.

In dieser Masterarbeit werden die ökologischen, physiologischen und biotechnologischen Aspekte, welche für die Disziplin des „defined microbial consortia engineering“ unentbehrlich sind, besprochen. Es wird gezeigt, dass, im direkten Vergleich zu Mono-Kulturen, die Co-Kultivierung von *Enterobacter aerogenes* und *Clostridium acetobutylicum* leistungsfähigere H_2 Produktion ermöglicht, die Konversion vom Substrat zu H_2 effizienter ist und zudem eine stabilere H_2 Produktion bei saurem Medium gewährleistet wird. Des Weiteren werden, hinsichtlich der biologischen Verwertung von organischen Reststoffen, gute Eigenschaften in der Nutzung von komplexen Kohlenhydraten nachgewiesen. Diese Masterarbeit empfiehlt „defined co-culture engineering“ als eine fortschrittliche und reproduzierbare Methode für profitable und in der Skalierbarkeit anpassbaren H_2 Produktion für zukünftige Anwendungen in der Bio-Industrie.

Eidesstattliche Erklärung

Ich erkläre eidesstattlich, dass ich die Arbeit selbstständig angerfertigt, keine anderen als die angegebenen Hilfsmittel benutzt und alle aus ungedruckten Quellen, gedruckter Literatur oder aus dem Internet im Wortlaut oder im wesentlichen Inhalt übernommenen Formulierungen und Konzepte gemäß den Richtlinien wissenschaftlicher Arbeiten zitiert und mit genauer Quellenangabe kenntlich gemacht habe. Diese schriftliche Arbeit wurde noch an keiner Stelle vorgelegt.

Ort und Datum Unterschrift

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Chapter 1

Introduction

Biohydrogen and conventional molecular hydrogen production

Burning of non-renewable fuels and its effects on the climate and environment endangers all life on earth (Cavicchioli et al., 2019). For over millions of years microbial biomass and organic matter from plants and animals were converted by geobiological processes to fossil fuels. Nowadays, almost 80% of the world's energy supply is provided by the combustion of fuel oil, natural gas and coal, which results in the liberation of greenhouse gases within a very short time period (Balachandar et al., 2013). As a consequence, the global carbon cycle became imbalanced, and atmospheric carbon dioxide (CO_2) levels are constantly rising (Lowe and Bernie, 2018).

The hydrogen molecule (H_2) is by far the most abundant interstellar molecule and substantially contributed in earth history (NASA, 2007). Moreover, H_2 is a promising energy carrier and a potential renewable alternative for fossil fuels (Das and Veziroglu, 2008). Since the Knallgas-reaction of H_2 with molecular oxygen (O_2) produces water (H_2O) as the only product, H_2 evidently represents a superior and clean energy carrier that could play a significant role in the reduction of greenhouse gas emissions. Despite the extensive beneficial features for humans and environment, H_2 storage and transmission problems need to be considered. However, research with regard to H_2 storage is ongoing (Boddien et al., 2011). To produce H_2, a variety of process technologies can be used. Current H_2-production systems are mainly based on thermo-catalytic reformation of H_2-rich compounds such as methane (CH_4). This commercially well-established method yet requires raw material such as natural gas, hence, a real benefit for atmospheric CO_2 reduction cannot be obtained (Urbaniec and Bakker, 2015). Electrolysis of H_2O is considered a more environmentally friendly approach in industrial H_2 production, provided that green electricity is utilized. Nevertheless, this method comprises an energy demanding and inefficient reaction (Rathore et al., 2019). In this respect, conventional H_2 production is still not carbon neutral and energy intensive, with associated greenhouse gas emissions and its severe effects on global climate change. However, the utilization of microbially produced biohydrogen might be the key to solve these ecological and economic problems associated with conventionally produced H_2.

Biological H_2 production

Biologically produced biohydrogen (bio-H_2) is H_2, which isproduced by either microalgae, bacteria or archaea and it is considered a promising biofuel in the future (Basile et al., 2019). There is an increasing activity in development and an effort to create a bio-H_2 economy, since H_2 offers several advantages (Hallenbeck et al., 2012). The production of H_2 through biological pathways is particularly attractive as it is carried out largely at ambient temperatures and pressures. A well-developed and efficient bio-H_2 system can be considered as a less energy-intensive and more sustainable and eco-friendly alternative compared to conventional H_2 production methods, such as electrolysis of H_2O and steam reformation of CH_4 (Chandrasekhar et al., 2015). Furthermore, the possibility of utilizing biological wastes as a potential source for H_2 generation through biological routes is offering new ways for the utilization of renewable and inexhaustible energy sources (Mohan and Pandey, 2013). Yet, biological processes in H_2 production are more sophisticated in design and performance, compared to conventional H_2 production in current industries (Benemann, 1998).

There are three main metabolic pathways for bio-H_2 production (**Figure 1**). (Akkerman et al., 2002; Azwar et al., 2014; Rittmann and Herwig, 2012a)

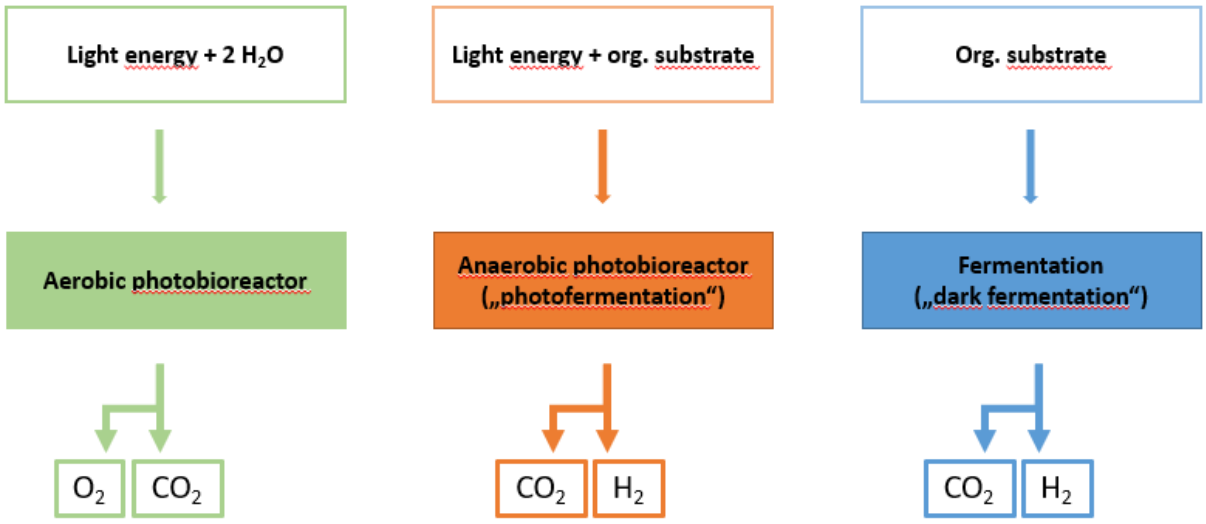

Figure 1: *Overview of the three principle biological routes for bio-H_2 production: aerobic phototrophic H_2-production requires H_2O and light energy in order to produce H_2 (left panel). Microorganisms use organic substrates or reduced sulphur bonds as electron donor and light serves as energy source (middle panel). Obligate and facultative anaerobes are using organic substances as carbon and energy source to produce H_2 (right panel).*

Water-Splitting Photosynthesis (Biophotolysis)

Currently, interest has emerged in photobiological production of H_2 by photoautotrophic microorganisms, because it represents a promising approach of generating a renewable fuel from earth's most abundant resources, light and water.

Chlorophyll a and other pigments, such as phycobilin, allow cyanobacteria or eukaryotic green microalgae to capture sunlight energy and to perform oxygenic photosynthesis in two distinct photosynthetic systems: photosystem II (PSII), which is splitting H_2O into protons (H^+), electrons (e^-) and O_2, and photosystem I (PSI), which uses light energy to generate the reducing agents nicotinamide adenine dinucleotide phosphate (NADPH) and reduced ferredoxin (Fd^{2-}). In parallel, the H^+ motive force across the thylakoid membrane drives adenosine triphosphate (ATP) production, and both ATP and NADPH, are further required for CO_2 reduction in the Calvin cycle (Nandi and Sengupta, 1998). Under specific conditions the reduced electron carrier Fd^{2-} is used by hydrogenase or nitrogenase to reduce H^+ for the formation of molecular H_2 ($2H^+ + 2Fd^- \Leftrightarrow H_2 + 2Fd$). Biophotolysis-based H_2 production can be divided into direct- and indirect biophotolysis depending on whether light is irradiated during H_2 evolution or not (Yu and Takahashi, 2007).

- Direct biophotolysis:

$$H_2O + lightenergy \rightarrow H_2 + \frac{1}{2}O_2 \quad (1)$$

At the presence of light, direct biophotolysis can be carried out by cyanobacteria and photosynthetic microalgae, including the promising H_2-producing green algae *Chlamydomonas reinhardtii* (Das and Veziroglu, 2008). Only under anaerobic conditions, O_2 sensitive hydrogenases are accepting e^- from Fd^{2-} in order to convert generated H^+ into H_2 (Nath et al., 2005). Hence, the inhibition of the H_2-evolving enzymes by O_2 creates a major barrier for sustained H_2 photoproduction by oxygenic phototrophs.

- Indirect *biophotolysis:*

$$12H_2O + 6CO_2 + lightenergy \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2)$$

and

$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2 \quad (3)$$

In indirect biophotolysis, e^- from the water-splitting process are initially stored as endogenous reserve carbohydrates (such as starch in microalgae and glycogen in cyanobacteria). Subsequently, the stored energy is released through fermentation during dark conditions and the excess "e^- pressure" can be deposited by hydrogenase on H^+, resulting into the formation of molecular H_2 (Yu and Takahashi, 2007). Hence, indirect biophotolysis is a two-staged mechanism consisting of a photosynthesis part for later carbohydrate accumulation, and a dark fermentation part for H_2 production. In addition to hydrogenase-based H_2-production, H_2 can also be formed during nitrogen (N_2) fixation catalysed by nitrogenase enzyme. In N_2-fixing cyanobacteria nitrogenase is responsible for the reduction of N_2 to ammonia (NH_3) together with the formation of H_2 as an obligatory by-product (Antal and Lindblad, 2005).

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (4)$$

Both nitrogenases and hydrogenases are extremely sensitive to O_2, and therefore N_2 fixation- and bio-H_2 production process must be separated from the oxygenic photosynthetic process. This is accomplished by time shifted assimilation of nitrogen during the night, as the cell cannot be photosynthetically active and O_2 formation is paused or O_2 and H_2 evolutions can be spatially separated by embedding hydrogenases in anaerobic heterocysts (Brentner et al., 2010).

Anoxygenic Photo-fermentation

Photo-fermentation involves the conversion of solar energy to biomass using organic compounds as carbon source. This mechanism is found among a diverse group of photosynthetic bacteria and is well-characterized in purple non-sulfur bacteria (PNSB) including *Rhodobacter* spp.. H_2 and CO_2 are formed under anaerobic and anoxic conditions using small-chain organic acids (such as like succinate, lactate, butyrate [Equation 5], malate, acetate, propionate and pyruvic acid), carbohydrates (such as glucose according to Equation 6) or reduced sulphur bonds as electron donors. The reaction is accompanied by the release of numerous by-products, such as acetic acid, butyric acid, propionic acid, acetone and butanol (Basak and Das, 2007). Similar to indirect biophotolysis, PNSB are using nitrogenases in order to transform organic substrate into H_2. PNSB have an advantage over aerobic cyanobacteria and algae because they are metabolically active solely under anaerobic conditions, which eliminates the difficulties associated with O_2 inhibition of H_2 production (Brentner et al., 2010; Das and Veziroglu, 2008).

$$CH_3COOH + 2H_2O + lightenergy \rightarrow 4H_2 + 2CO_2 \ (5)$$

$$C_6H_{12}O_6 + 6H_2O + lightenergy \rightarrow 12H_2 + 6CO_2 \ (6)$$

Theoretically, photo-fermentation is an efficient process converting organic compounds entirely into H_2. However, in practical applications there have been struggles with low light-conversion efficiencies, with a maximum theoretical solar-to-H_2 energy conversion efficiency of 10% (Eroglu and Melis, 2011). Moreover, nitrogenases also possess several deficits that affect H_2 production, such as low catalytic activity and suppression of their expression by ammonium (NH_4) ions (Chandrasekhar et al., 2015).

Dark Fermentation

Dark fermentation ubiquitously occurs under anoxic conditions and is based on the breakdown of a variety of potentially utilizable substrates such as carbohydrates, polymers or one-carbon (C1) compounds. This high variability of possible feedstocks enables the usage of biomass residues and biological waste as substrates. Hence, bio-H_2 production via dark fermentation could potentially provide a renewable H_2 stream (Hallenbeck, 2011). Dark fermentation is considered as particularly attractive in industrial applications since it does not require solar energy. Hence, bioreactor configuration and operation are less challenging (Hallenbeck et al., 2012). During fermentation of glucose, as the preferred substrate, several reactions can occur, depending on the species involved in H_2 generation.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \ (7)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 (8)$$

Equation 7 is illustrating acetate fermentation where the optimum yield of H_2 can be expected. Hence, under ideal conditions obligate anaerobes theoretically produce 4 mol H_2 per mol of glucose. This theoretical production limit, the so called “Thauer limit”, was proposed by Thauer *et al*. (1977). However, H_2 metabolism of dark fermentation is more complex in practice with several pathways which differ in their intermediary metabolites, in their formed by-products and in the types of hydrogenases involved (Das and Veziroglu, 2008).

The release of other metabolites, such as butyrate (according to Equation 8) drastically reduces the Thauer limit. Here only 2 mol H_2 mol^{-1} glucose are involved (Guo et al., 2010). On the other hand, when lactate or ethanol formation occurs (according to Equation 9 and 10), H_2 production might even be inhibited (Guo et al., 2010).

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \ (9)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2 \ (10)$$

In order to achieve high efficiencies in dark fermentative H_2 production, the formation of metabolic by-products needs to be considered. More details about strain specific physiology and its dependency on environmental conditions will be presented in later sections of this thesis.

Comparison of Bio-H_2-generating methods

Comparing the three metabolic strategies of bio-H_2 production, they all have in common that H_2 generation represents a strategy in order to maintain electrical neutrality within the cell. O_2 sensitive nitrogenases and hydrogenases are the key enzymes during these processes. Whereas dark- and photo-fermentative organisms usually do not face these O_2-caused problems of enzyme inhibition as they inherently live in anoxic environments, aerobic phototrophs had to evolve certain strategies to protect the enzymes from O_2. In respect to technical applicability, O_2 inhibition creates a major barrier for sustained H_2 photo-production. However, phototrophic microorganisms have an advantage over fermentative bio-H_2 producers in terms of yield of H_2 per substrate consumed ($Y_{(H2/S)}$) [mol mol^{-1}]. Hence, in dark fermentation the substrate is only partially used for H_2 generation and it involves the formation of various by-products. Penetrating light evenly through bioreactors, however, is a technical obstacle during oxygenic phototrophic bio-H_2 production processes. Dark fermentative bio-H_2 production is characterized by a higher H_2 evolution rate (HER) [mmol L^{-1} h^{-1}] in contrast to other bio-H_2 production processes. HER can be considered as the most crucial variable in bioprocess engineering since it depicts the fermentation volume required for production of a specific H_2 output (Ergal et al., 2018; Rittmann and Herwig, 2012). Additionally, to higher volumetric production, dark fermentative microorganisms also show up more rapid growth rates compared dark- and photo-fermentative organisms. This is accompanied by an increased qH_2, which is defined as the capacity of one gram cell dry weight of biomass to produce H_2 at a certain rate (Lee et al., 2011). Pure carbon sources are expensive raw materials for H_2 production. In respect of economic viability in industrial applications, biophotolysis exhibit substantial disadvantages as their range of utilizable substrate is limited. In contrast, both photo- and dark-fermenting organisms can use a wide range of substrates which allows conversion of cheap carbon sources originating from waste streams (Elsharnouby et al., 2013). In real life applications, photo-fermentation is considered to be economically less viable compared to dark fermentation, due its high demand of solar energy as well as the need of purchasing cost-extensive photo-bioreactors covering large surface areas (Hallenbeck, 2011). Considering the aforementioned properties, we suggest dark fermentation to be highly suitable for bio-H_2 production with anticipations for practical applications.

Despite increasing research efforts on characterization and optimization of dark fermentative H_2-systems, bio-H_2 production has not yet reached an industrial scale. For making applications economically viable, H_2 yields and production rates must considerably surpass the present achievements (Lee et al., 2011). Attempts have already been made by several researchers to find out further improvement in both yield and volumetric production rates of H_2 fermentations.

Bio-H_2 producing microorganisms

For optimizing a highly efficient bio-H_2 production system it requires the screening for promising organisms in regard to their H_2 producing properties, as well as the optimum environmental conditions. Extensive research on bio-H_2 production via dark fermentation has been conducted over the past few years, accompanied by a substantial quantity of presented data. Recently, a comprehensive review of over 300 publications including dark fermentative bio-H_2 production by Ergal *et al*. (2018) has revealed deep insights to the best performing strains under the most powerful H_2 production conditions. There, pure culture performance of microorganisms (from domain to species level) in closed batch, batch, and continuous culture was characterized in the units $Y_{(H2/S)}$ [mol mol^{-1}] (substrate conversion efficiency), HER [mmol L^{-1} h^{-1}] (volumetric productivity) and qH_2 [mmol g^{-1} h^{-1}] (biological production capacity) temperature (°C), pH, dilution rate (D) [h^{-1}], substrate type and initial substrate concentration (C-mmol L^{-1}). Herewith, we want to stress that for unambiguous result comparison throughout the scientific community, the $Y_{(H2/S)}$ and rates of bio-H_2 should be presented uniformly. Hence, results for quantitative performance attributes ($Y_{(H2/S)}$, HER and qH_2) are comparable when they are recalculated to carbon molar (C-molar) basis. Mol to C-mol conversion is accomplished by division of the molar values by the corresponding number of C-atoms which was provided as substrate.

Considering the in-depth meta-study (Ergal et al., 2018) the families of Enterobacteriaceae and Clostridiaceae are shown to be among the top performing H_2 producers. In quantitative data from continuous culture Enterobacteriaceae were found with highest mean HER of 4.76 ± 4.81 mmol L^{-1} h^{-1} (C-molar) and Clostridiaceae exhibit the second highest mean of HER (C-molar) in the ranking of total 41 Families that are identified to produce H_2 (Ergal et al., 2018). These impressive H_2 producing properties of Enterobacteriaceae and Clostridiaceae might be even enhanced by combining them into a defined microbial consortium where both contributing to the system with their individual attributes and complementing each other with the final goal to achieve higher yields and higher volumetric productivity, in contrast to mono-culture. The possibilities and advantages of co-coculture engineering will be discussed in the later sections of the thesis.

Metabolism during dark fermentation H_2 production

The metabolic strategies for H_2 generation differ between microbial strains and are strongly dependent on the environmental conditions. However, across all dark fermentative H_2-producers, glucose (as the preferred carbon source) is oxidized to pyruvate by the glycolytic pathways. During the energy yielding reactions of glycolysis, ATP is generated and oxidized nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH as follows (Alberts et al., 1998):

$$Glucose + 2NAD^+ + 2\,ADP + 2\,P_i \rightarrow 2\,Pyruvate + 2\,NADH + 2H^+ + 2\,ATP \quad (11)$$

Pyruvate is further oxidized to energy rich acetyl-CoA, which can be converted to acetate resulting in the generation of ATP. In many organisms pyruvate formate lyase (PFL) plays a central role in the conversion of pyruvate and CoA to acetyl-CoA, leading to the formation of formate as by-product (Equation 12) (Das et al., 2014), whereas other organisms rely on pyruvate oxidation to acetyl-CoA via Fd-dependent hydogenases. In order to recover Fd, hydrogenases oxidize Fd^{2-} and reduce H^+ leading to production of molecular H_2 (Equations 13 and 14) (Ferry, 2011).

$$Pyruvate + CoA \rightarrow Acetyl - CoA + Formate \quad (12)$$

$$Pyruvate + CoA + 2Fd \rightarrow Acetyl - CoA + 2Fd^{2-} + CO_2 \quad (13)$$

$$2H^+ + Fd \rightarrow H_2 + Fd^{2-} \quad (14)$$

Enterobacter aerogenes

Enterobacter aerogenes is a facultative anaerobe, non-spore-forming, gram-negative and rod-shaped gammaproteobacterium. It has size of approximately 1-3 µm in length and is capable of motility by peritrichous flagella. *E. aerogenes* has been isolated as human specimens from respiratory, urinary, blood, or gastrointestinal tract and it was also found to live in various wastes, hygienic chemicals, and soil (Zhang et al., 2011). Among all the fermentative H_2 producers, *E. aerogenes* has been one of the most widely studied model strains and has attracted much attention due to its high growth rate, easiness of culture and wide substrate range. Furthermore, *E. aerogenes* exhibited the highest H_2 production performance within the Enterobacteriaceae family with H_2 yields exceeding 2 mol H_2 mol^{-1} glucose (Cabrol et al., 2017a).

Metabolism, Thermodynamics and pH

E. aerogenes and other dark fermentative- and facultative anaerobic organisms such as *Salmonella typhimurium, Klebsiella pneumonia* and *Escherichia coli* specifically generate H_2 via pyruvate formate lyase (PFL) pathway (Garcia-Verdugo et al., 2006). The PFL pathway catalyses the aforementioned reactions, namely, to process pyruvate (which has been gained from glycolysis) into energy rich acetyl-CoA. Here, PFL is converting pyruvate and coenzyme A (HS-CoA) into formate and acetyl-CoA. Subsequently, in the H_2 generating step, formate hydrogen lyase (FHL) is further converting formate into CO_2 and H_2, as follows:

$$HCOO^- + H_2O \rightarrow \mathrm{HCO_3^-} + H_2\,,\ \Delta G^0 = +1.3\ \mathrm{kj}\ mol^{-1} \quad (15)$$

Acetyl-CoA, which is the central intermediate in PFL pathway, is further metabolized to acetate and ATP by acetate kinase and phosphotransacetylase. This final metabolic step is providing an additional crucial source of ATP for biosynthesis (Ferry, 2011).

During the H_2 generating reaction of formate lysis, bicarbonate (HCO_3^-) serves as the reaction partner rather than CO_2 in the neutral pH range from pH 6.3-10.4 (Schink et al., 2017). As shown in the equation (8), the reaction is nearly at thermodynamic equilibrium and even slightly endergonic. The reaction only becomes slightly exergonic under conditions below pH 6.3, when CO_2 is gaseous and not dissolved as bicarbonate, and beyond pH of 10.4 when the reaction partner is carbonite ion (CO_3^{2-}). Under these conditions the lysis of formate releases a Gibbs free energy of -3.4 to -7 kJ mol^{-1}. It shows that the reaction is thermodynamically feasible but only resulting in a small amount of energy that is released. A minimum amount of about 15-20 kJ mol^{-1} reaction would be necessary in order to maintain a charged cytoplasmic membrane for the energetically beneficial induction of ATP synthesis (Schink and Stams, 2013). Hence, the reaction cannot contribute to ATP generation and only under certain conditions there is no additional energy required to make the reaction feasible. In this sense, formate lysis remains a questionable reaction which supposedly is not bringing specific benefits to the cell.

An explanation is that the reaction presumably arises as a detoxification mechanism to avoid formate accumulation (Jones and Woods, 1986). Undissociated acids, such as formate and butyrate are partitioning the cell membrane and have an uncoupling effect which enables H^+ to enter the cell from the medium. Sufficient concentrations of formate might lead to a full collapse of the pH gradient across the membrane (Jones and Woods, 1986). pH is a crucial chemical parameter associated with any biochemical process and it is widely controlling the enzymatic machinery of microorganisms. A pH decline is severely affecting glycolytic enzymes and also the supporting key enzymes of PFL pathway. Enzyme activity might initially slow

down under decreasing pH conditions until the total shutdown of activity at very low pH in the range of 3.8-4.2 (Sinha et al., 2015).

A different strategic countermeasure against other partial oxidation products (such as acetate) is 2,3-butanediol fermentation (Geckil et al., 2004). This catabolic pathway is commonly found in facultative anaerobes and specifically in the genus of Enterobacter extraordinarily high rates of 2,3-butanediol production have been observed. In 2,3-butandiol fermentation two molecules of pyruvate are condensed to acetyllactate which is further decarboxylated to acetonin. From here, butanediol-dehydrogenase is catalyzing 2,3-butanediol formation as the end product, which is achieved via oxidation of NADH to NAD^+. 2,3-butandiol fermentation is conducted as an alternate to the ATP-yielding transformation of pyruvate to acetate. The overall reaction of 2,3-butanediol fermentation is not energetically favourable but it leads to a neutral product that has a minor effect on pH and is acting less inhibitory (Thapa et al., 2019).

Clostridium acetobutylicum.

Clostridium acetobutylicum is a gram-positive and rod-shaped bacterium belonging to the phylum firmicutes. It is ubiquitously found in different environments such as soil or the human gut. *C. acetobutylicum* lives under strict anaerobic conditions with the ability of spore formation in response to O_2 or other unfavourable environmental conditions, such as lack of nutrients or rising temperature (Hawkes et al., 2002).

Metabolism, Thermodynamics and pH

In contrast to PFL pathway of facultative anaerobic *E. aerogenes*, strictly anaerobic *C. acetobutylicum* uses the pyruvate ferredoxin oxidoreductase (PFOR) pathway to convert pyruvate into acetyl-CoA. These reactions involve the reduction of Fd to Fd^{2-} or NAD^+ to NADH together with the release of CO_2 and H_2. The key enzymes for H_2 generation are membrane bound Fd^{2-} hydrogenase or NADH hydrogenase, respectively (Furdui and Ragsdale, 2000).

To yield the maximum amount of ATP for an organism, the metabolized carbohydrates need to be oxidized entirely via glycolysis and PFOR pathway to acetate. Nevertheless, these reactions require a continuous availability of recovered redox equivalents (NAD^+ and Fd) for reduction. In theory, this is easily accomplished when all the reducing equivalents (NADH and Fd^{2-}) are transformed to H_2, which would also result the optimum of 4 mol of H_2 per 1 mol of glucose (Wolfe, 2005). However, under standard conditions the oxidation of Fd^{2-} or NADH to H_2 has a positive standard Gibbs free energy change ($\Delta G^{0'}$=3.1 kJ mol^{-1} and $\Delta G^{0'}$=18.1 kJ mol^{-1}, respectively) making the reactions thermodynamically unfavourable. Thus, in PFOR

pathways alternative reactions need to occur which are thermodynamically more feasible, in order to recover the redox equivalents. One recovering reaction is lactate formation by reduction of pyruvate, which does not involve H_2 formation and is also less efficient in ATP generation as energy yielding steps are skipped. Alcohol formation negatively affects H_2 formation as the molecules contain additional H-atoms which are not present in acetate (Hawkes et al., 2002). Despite the thermodynamically more favourable by-product formation of lactate and alcohols, the amount of H_2 produced has been practically shown to reach up to 2 to 4 mol mol^{-1} glucose for *C. acetobutylicum* (Verhaart et al., 2010). Hence, there must be a way for these organisms to use NADH and Fd^{2-} for H^+ reduction to form H_2, even under supposedly unfavourable conditions. One explanation is that ΔG^0 values were calculated under standard conditions with H_2 partial pressure of $1 \cdot 10^5$ Pa. However, ΔG^0 is not a fixed value but depends on the concentration of the reactants. Under physiological conditions the H_2 partial pressure is typically lower which results to a negative ΔG^0 for H_2 generation from Fd^{2-} oxidation. In this sense, the reaction can occur spontaneously (Amend and Shock, 2001).

Nevertheless, ΔG^0 for H_2 generation from NADH remains positive, even under physiological conditions with low H_2 partial pressure. Certain dark fermenting bio-H_2 producers including *C. acetobutylicum* have found a way to couple exergonic electron transfer reactions to drive NADH oxidation. The overall reaction is termed flavin-based electron bifurcation. It represents a mechanism that links the reduction of recipient molecules to the oxidation of other electron donor molecules. Hence, H_2 is simultaneously produced by electron supply from both NADH and Fd^{2-} while also reducing crotonyl-CoA (which derives from acetyl-CoA). By such means NADH recovery through H_2 generation is thermodynamically feasible and even though there is no acetate production (but mostly formation of butyrate or butanol as end-products), there is still 3.3 mol of ATP generated (Buckel and Thauer, 2013). Organisms with the capability to perform flavin-based bifurcation are suggested to be especially practical for bio-H_2 production systems because they bring high $Y_{(H2/S)}$, even under conditions that would be restrictive for H_2 generation in other species.

Furthermore, *Clostridium* spp. have evolved a specific metabolic mechanism that is distinguished in 2 phases: acidogenesis and solventogenesis. These metabolic traits have emerged during by-product assessment of *C. acetobutylicum*, where a shift in the metabolism has been observed (Kim and Zeikus, 1984). They noticed that *C. acetobutylicum* is starting from acid-production and is transitioning into solvent production (mainly acetone and butanol) as the fermentation process progresses. During acidogenesis, these bacteria are converting glucose via the PFOR pathway into organic acids, commonly butyrate and acetate. The steadily changing influence of decreasing pH conditions and increasing undissociated butyrate levels are suggested to be the main trigger for the switch that causes the initiation of solvent

production. For several strains of *C. acetobutylicum*, it has been observed to produce solvents only below about pH of 5.0. Furthermore, the optimum pH for these strains has been reported to be about pH 4.3 and solvent production was still occurring at a pH low as 3.8 (Jones and Woods, 1986). The switch to solvent production decreases intracellular concentrations of acetate and butyrate by regulating the activity of the enzymes involved in the acid and solvent producing pathways. It was shown that during solventogenesis the concentrations of terminal enzymes of the acetate and butyrate pathways were two- to six-fold lower compared to extracts from acid-producing cells (Jones and Woods, 1986). At this point, genes that encode for solvent forming enzymes (catalysing butanol-, ethanol- or acetone-formation) will be upregulated.

The initiation of enhanced solvent production is most likely performed as a countermeasure against intracellular enrichment of acetate and butyrate accompanied by decreasing pH. The switch to solvent formation also results to an alternate metabolic pathway, which enables the uptake and recovery of acetate and butyrate from the medium. Reutilization of organic acids requires metabolization of minimum 2 moles glucose for each mole acid consumed, in order to obtain a balance between electron and carbon flow (Hüsemann and Papoutsakis, 1988). However, regarding the H_2 generating efficiencies during solventogenesis, minor deficits were observed. Compared to acid-producing metabolism where there is a rapid flow of electrons from redox equivalent to produce molecular H_2, solvent-producing phase only shows approximately half of the reduced Fd^{2-} is recovered for H_2 formation. This comes along by a lower H_2 yield of below 2 moles per mole glucose compared to 3.3 mol reached during acidogenesis. Moreover, there is a reduction in in the net amount of ATP generated from approximately 3.25 mol mol^{-1} of glucose during acid production to 2 mol mol^{-1} of glucose when switched to solvent production (Rogers, 1986).

The shift to solvent production in *C. acetobutylicum* and related species is suggested to be an adaptive response to the inhibitory effects produced by acid end-products. Likewise, to the formate breakdown in the PFL pathway, it can be considered a detoxification mechanism. However, the trait of converting acids such as acetate, which is the most commonly produced end-product among dark fermentation, into solvents and H_2 might be beneficial for H_2 production systems as stable and efficient volumetric production is required under acidifying conditions.

To summarize the metabolic properties of PFOR pathway, it can be generally stated that the transformation of glucose to sole acetate is providing the highest ATP yields. Organism with improved ATP generation are also accompanied by high $Y_{(H2/S)}$. On the opposite, ATP production in the PFL pathway has not been shown to be directly linked to H_2 generation. Here, H_2 and CO_2 are merely produced through formate degradation as a detoxification

measure which does not involve ATP synthesis. In order to improve dark fermenting H_2 production one must understand the influences of the environmental conditions, together with the underlying metabolism of the respective organism. The dependency between $Y_{(H2/S)}$ and the metabolic pathways are obvious, but in regard to HER, as a crucial variable for industrial bio-H_2 production, it is very hard to predict for well performing strains.

Bio-H_2 production in biotechnological applications

Despite the promising properties of microorganisms capable of performing dark fermentative H_2 production, serious challenges need to be overcome. Following approaches are involving the broad substrate utilization potential of dark fermentative H_2 producers as well as the ecological principle of cell-cell interactions in co-culture. The goal is a synthetic ecological system with the capability of efficient H_2 generation by conversion of environmentally favorable substrates.

Dark fermentative H_2-production from waste materials

The main constraint of fermentative bio-H_2 process is the lower yield of H_2, maximally 4 mol mol^{-1} glucose, compared to other processes. However, this economic deficit can be compensated by the usage of cheap waste material. Dark fermentation can be applied to a wide range of feedstock (industrial wastewater, agricultural residues, food wastes, etc.), which are combining the objectives of cost effective and sustainable waste management with simultaneous on-site energy recovery (Cabrol et al., 2017a). Abundant fermentable waste from plant residues contains non-edible and renewable lignocellulosic substrates witch cellulose as its major component. Cellulose is enzymatically hydrolysed to (via endoglucanase and exoglucanase) to cellobiose, a β-1,4-linked glucose dimer. Hence, the cleavage of cellobiose by β-glucosidase generates two molecules of glucose (Parisutham et al., 2017). For an experimental set-up including waste conversion, cellobiose is considered a suitable carbon source, as it represents the smallest repetitive unit of cellulose. Complex cellulose polysaccharide consisting of several hundred to many thousand glucose molecules, would be a less practical as carbon source. In contrast, the simple disaccharide structure of cellobiose allows adequate quantification of molecules for conversion efficiency assessment.

Undefined microbial consortia

Microbial consortia are present in all environments on our planet, from the microbial mats (a type of biofilm) that were probably the first ecosystems in the early Archean (about 3850–3500

million years ago) to the complex microbiota of the intestinal tract of different animals (Berlanga and Guerrero, 2016). Even the simplest characterized consortia may contain from ten to thousands of species, each of them inevitably confronted to interact within close proximity and to share resources. Compared to pure cultures, microbial consortia usually possess a larger pool of genes which allows the performance of more complex tasks and an enhanced robustness to environmental fluctuations, compared to monocultures (Ben Said and Or, 2017).

Commensalistic-mutualistic associations are the key to survival during microbial coexistence. Cell to cell communication is ubiquitously employed by microbial communities and involves the exchange of dedicated signal molecules (quorum sensing signals) within or between single populations. In that way, different physiological processes and population behaviours are coordinated, including biofilm formation or virulence (Berlanga and Guerrero, 2016; Zhang and Li, 2016). Cross-feeding interactions, in which bacterial cells exchange valuable and essential metabolites for the benefit of other species, facilitates an improved substrate-product thermodynamic in microbial ecosystems (Pande et al., 2015). In respect to industrial bioprocesses, cross-feeding can reduce the accumulation of inhibitory by-products.

Wang *et al*. (2008) demonstrated that in continuous stirred-tank reactors, a natural consortium of fermentative H_2 producers *C. acetobutylicum and Ethanoligenens harbinense* has been shown a considerable resistance to changing environmental conditions. Hence, the individual strains could maintain at the same abundance, independently of the pH and other potential disturbance factors.

Despite these reported remarkable results of enhanced H_2 production by undefined co-cultures, one has to consider that these natural derived consortia are enormously complex, sometimes unstable, and hard to maintain during bioprocessing (Wang et al., 2019).

Defined microbial consortia engineering

While natural microbial communities are composed of a mix of microbes with often unknown genetic backgrounds of many wild-type species, synthetic and defined microbial consortia are determined in their composition and biological functions. Defined consortia microbes are performing particular tasks, which make the application more specific in its function with higher production efficiencies than natural consortia (Großkopf and Soyer, 2014). Defined microbial consortia hold great promises for dark fermentative H_2 production applications, however, it requires a detailed and comprehensive understanding of molecular mechanisms between species interactions to further allow the design and construction of a productive and functioning consortium (Liu et al., 2011). The maintenance of quality and stability in populations can

demand great efforts in defined consortium engineering and is still a key challenge at the industrial scale (Sharma et al., 2019).

Recent studies demonstrate the effectiveness of defined consortia to implement fermentative H_2 processes. Beckers *et al*. (2010) have investigated increased H_2 yields in co-culturing *Clostridium butyricum* and *Citrobacter freundii* on starch-containing medium. However, this study has also shown that the cell concentration of *C. freundii* was dramatically decreased as they were outcompeted and overgrown by *C. butyricum* after 48 h of fermentation. It stresses the importance of investigation and quantification of specific bacterial growth in experiments and emphasizes the problem of natural competition for co-culture applications.

Based on these observations, it is obvious that H_2 producing properties of dark fermentative strains can be enhanced by combining them into a defined microbial consortium. However, we emphasize that there is still room for further improvements. Defined microbial consortia are complex ecological systems with interactions happening at different length- and time scales. Due to different specific growth rates of different strains, population sizes in a co-culture often differ enormously resulting to one being the dominant population. Hence, in co-cultures the population ratios need to be optimized in order to obtain a stable culture so that one cell type does not eliminate the others. We propose that an overall balanced microbial culture success is accompanied by the most powerful H_2 production system. Moreover, predictability of populations facilitates reproducibility of the system which is of great importance for industrial applications. This intention requires more efforts in sophisticated population monitoring together with large-scale data collection, as well as defined inoculum design.

Dark fermentation processes operated with co-cultures might bring economically viable bio-H_2 yields, however, in order to make process as well ecologically favourable, one should consider the capability of dark fermenting organism to convert many different types of organic matter.

The idea behind co-culturing *C. acetobutylicum* and *E. aerogenes*

When combining *E. aerogenes* with its superior formate-based H_2 production in terms of HER, and faster generation times together with *C. acetobutylicum, E. aerogenes* will presumably take over productivity in the early state of the fermentation process. As the pH decreases, it can be assumed that during the release of acidic metabolic end-products, *E. aerogenes* strain E.82005 shifts the metabolism from acid production to non-acid production below a pH of 5.8, which results in a reduction of H_2 production (Tanisho et al., 1987). However, ideal conditions would then be created for proliferation of *C. acetobutylicum*. Due to its metabolic properties, *C. acetobutylicum* is switching to solvent production under acidic conditions but H_2 production is still maintained. After delayed appearance due to a longer lag phase, *C. acetobutylicum* will

have the opportunity to assist *E. aerogenes* in H_2 production while parallelly stabilize the pH conditions.

The aim is to obtain a stable and reproducible H_2 generating-system in closed batch mode. High specific growth rates of *E. aerogenes* provides early H_2 formation in a co-culture which is are especially beneficial in biotechnological applications as undesired lag-phases can be reduced during batch runs. However, fast growing properties of certain populations in a co-culture may require suppression in order to maintain population stability in a system. Unequal inoculation ratios might impede the growth of *E. aerogenes* at an extent that is necessary to facilitate proliferation of slow growing *C. acetobutylicum*.

Main goals

My master thesis research aimed the investigation and design of a bio-H_2 producing co-culture system with a defined set-up and superior characteristics compared to mono-culture systems. Multiple experiments were conducted with the focus on these three major goals:

- *Co-cultivation of* C. acetobutylicum *and* E. aerogenes *into a microbial consortium to comprise higher bio-H_2 production rates (HER) [mmol L^{-1} h^{-1}]*
- *Investigation and optimization of inoculation ratios of each microorganism in order to achieve ideal co-culture outcome*
- *Maintained H_2 productivity at acidic conditions due to solventogenic metabolic characteristics of* C. acetobutylicum
- *Design of an ecologically stable microbial consortium of* C. acetobutylicum *and* E. aerogenes *with uninterrupted H_2 production that is initiated earlier compared to mono-culture*

Chapter II

Contribution

My scientific contribution to this manuscript was performing the pure-culture and co-culture cultivation experiments in serum bottles, determining cell densities (optical density measurements, qPCR), extracting DNA and measuring headspace pressure. Additionally, I performed gas chromatography and fluorescence in situ hybridization (FISH) experiments processed and assembled the microscopic images and contributed to data analysis.

Article

Biohydrogen production beyond the Thauer limit by precision design of artificial microbial consortia

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Abstract

Dark fermentative biohydrogen (H_2) production could become a key technology for providing renewable energy. However, until now the H_2 yield is restricted to 4 moles of H_2 per mole of glucose, referred to as the “Thauer limit”. Here we show, that precision design of artificial microbial consortia increased the H_2 yield to 5.6 mol mol^{-1} glucose, 40% higher than the Thauer limit. In addition, the volumetric H_2 production rates of our defined artificial consortia are superior compared to any mono-, co- or multi-culture system reported to date. This study is a major leap forward in the engineering of artificial microbial consortia through precision design and means a breakthrough in energy science, biotechnology, and ecology. Constructing artificial consortia with this drawing-board approach could in future increase volumetric production rates and yields of other bioprocesses. Our artificial consortia engineering blueprint might pave the way for the development of a H_2 production bioindustry.

Keywords

Biotechnology, microbiology, physiology, dark fermentation, *Enterobacter aerogenes*, *Clostridium acetobutylicum*, artificial consortium

1. Introduction

Microorganisms thrive in almost all habitats on Earth, where they fulfil important ecosystem functions as complex and highly dynamic microbial communities(Fuhrman, 2009; Whitman et al., 1998). Microbial communities exist in high levels of biodiversity, where they cooperate and interact with each other in functional metabolic networks(Konopka, 2009). Compared to mono-cultures, a microbial consortium empowers complex metabolic tasks due to the multitude of possible metabolic reactions and interaction partners. The streamlined syntrophic interactions or commensal relationships among the microorganisms in microbial consortia were shown to enable an efficient utilisation of unrefined substrates(Fu et al., 2009; Hays et al., 2015), to resist to environmental stressors(Burmolle et al., 2006; Hays et al., 2015) and to display high productivity or yield(Sabra et al., 2010; Zhang and Wang, 2016). In nature, a modest undefined consortium may contain thousands of species(Curtis et al., 2002). However, for efficiently performing bioconversions in natural or artificial ecosystems, the specific metabolic reactions of individual species in the consortium are more relevant than the species richness(Cabrol et al., 2017b).

In environmental, biopharmaceuticals, or energy biotechnology, most of the bioprocesses are developed and optimised through targeted bioprocess development, utilising metabolically engineered or wild-type organisms, or even undefined microbial consortia of organisms. The emphasis lies in the optimisation of productivity and/or yield by using different types of bioreactors and organisms/undefined consortia. However, every organism, even a metabolically-engineered organism, possesses specific metabolic bottlenecks, which limit a full substrate to target product conversion. In many cases, the production of the target compound is accompanied by excretion of several metabolic by-products, which balance cellular homeostasis, reducing yield and/or productivity. Moreover, bioprocess development relies on established bioreactors and cultivation pipelines.

Synthetic or artificial microbial consortia are regarded as part of the solution to debottleneck the inherent physiological limitations of wild-type or metabolically-engineered mono-culture and undefined consortia bioprocesses, such as enabling the breakdown of complex carbon sources(Dwidar et al., 2013), efficient substrate utilisation(Kurosawa et al., 2015), reducing by-

product inhibition through operational stability(Bhatia et al., 2015), and high productivities(Bernstein et al., 2012). This can be achieved through selection, design and assembly of microorganisms with specific metabolic (e.g. cellulose utilisers) or ecological (e.g. biofilm forming microorganisms) functions. Additionally, by employing an artificial consortium of selected microorganisms, precision design of a defined microbial co- or multi-culture provides a comprehensive understanding of organismal interactions and allows examining the molecular and eco-physiological basis of community-level functions(Godheja et al., 2014; Raes and Bork, 2008). The developments in the field of artificial microbial ecosystem engineering allowed advancing in the aspects of ecology, such as soil bioremediation(Chapalamadugu and Chaudhry, 1991) and biotechnology e.g. fine chemical(Bischoff et al., 2009; Wang et al., 2013), biopolymer(Shalin et al., 2013), enzyme(Dong et al., 2012), food additive(Mapari et al., 2010), antimicrobial(Lozo et al., 2004), biofuel(Zuroff and Curtis, 2012) and biohydrogen production(Chen et al., 2015; Zeidan and van Niel, 2010; Zeidan and Van Niel, 2009). However, to achieve supreme efficiency of the bioprocess, a precision design strategy to form an artificial consortium of selected microorganisms was not yet considered.

Molecular hydrogen (H_2) is considered as an alternative source of energy. Biological production of H_2, referred as biohydrogen production, provides a sustainable and environmentally friendly method for energy generation(Ergal et al., 2018b; Müller, 2019; Rittmann and Herwig, 2012b). Dark fermentative H_2 production is promising due to high H_2 evolution rates (HERs) compared to photobiological H_2 production processes(Ergal et al., 2018b; Rittmann and Herwig, 2012b). However, the yield of H_2 per substrate consumed ($Y_{(H2/S)}$) is limited by metabolic constraints of dark fermentative H_2 producing microorganisms. According to the Thauer-limit, 4 moles H_2 can be produced per one mole of glucose consumed during dark fermentation when acetate is produced as by-product(R K Thauer et al., 1977). Depending on the microbial group, H_2 formation may occur either via the pyruvate-formate-lyase (PFL) pathway or the pyruvate ferredoxin oxidoreductase (PFOR) pathway(Ergal et al., 2018b). The PFL pathway is operative in Enterobacteriaceae and the PFOR pathway is operative in Clostridiaceae. Up to now, yields obtained by dark fermentative biohydrogen producing wild-type or metabolically-engineered mono-cultures were not successful in improving $Y_{(H2/S)}$ beyond the Thauer limit(Hallenbeck, 2009; Song et al., 2019; Zeidan and Van Niel, 2009). Therefore, to boost $Y_{(H2/S)}$, undefined microbial consortia or defined co- and multi-cultures of H_2-producing microbes were examined in complex or defined medium(Arumugam et al., 2014; Chookaew et al., 2015; Liu et al., 2013; Thompson et al., 2008). However, control of microbial community composition, media compounds and their concentration through precision design of an artificial microbial consortium were not yet the focus in any study.

In our quantitative analysis of pure culture dark fermentative H_2 production, we linked physiological and biotechnological characteristics of H_2 producing microorganism through comprehensive meta-data analysis and modelling(Ergal et al., 2018b). Our analysis revealed, that Enterobacteriaceae exhibit very high HERs and Clostridiaceae are mesophilic organisms with the highest reported $Y_{(H2/S)}$ on a C-molar level on saccharides. Therefore, we hypothesised that precision design of an artificial microbial consortium composed of Enterobacteriaceae and Clostridiaceae improves $Y_{(H2/S)}$ beyond the Thauer limit.

Here, we present results from a drawing board-like precision design of artificial microbial consortium of microorganisms with improved HER and $Y_{(H2/S)}$ beyond the Thauer limit of two H_2-producing species, the facultative anaerobic *Enterobacter aerogenes* and the obligate anaerobic *Clostridium acetobutylicum*. For the design of this defined artificial consortium, three different major community function-determining parameters were individually and syntrophically investigated: initial substrate concentration of glucose or cellobiose, designing and optimising a mutual medium, and control of the activity and concentration of initial cell densities. First, initial optimum substrate concentration was investigated for individual strains and a mutual defined medium was designed by applying Design of experiments (DoE). Then, different consortia were created using active inoculum with different initial cell densities of each microorganism. Our interdisciplinary research combines physiology, ecology and biotechnology and provides valuable insights for ecosystem functionality and enhancing H_2 production by constructing a defined artificial consortium.

2. Materials and methods

2.1. Microorganisms and medium composition

In this study, *C. acetobutylicum* DSM 792 and *E. aerogenes* DSM 30053 were used. A modified Clostridium-specific medium without yeast extract was used for growth of mono-culture *C. acetobutylicum* as previously described in detail elsewhere(Qureshi et al., 1999). The medium was prepared containing (per L): 0.5 g of KH_2PO_4, 0.5 g of K_2HPO_4 and 2.2 g of NH_4CH_3COO and glucose or cellobiose were added at a concentration of 999 carbon mmol (C-mmol). The pH was arranged with 1 mol L^{-1} NaOH to 6.8. Trace elements solution was prepared as stock 100x solution containing (per L): 0.2 g of $MgSO_4 \cdot 7\ H_2O$, 0.01 g of $MnSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of NaCl. Vitamin solution was prepared as stock 200x solution containing (per L): 0.9 g of thiamine, 0.002 g of biotin and 0.2 g of 4-aminobenzoic acid. Same trace elements solution and vitamin solution were used for all experiments. Mono-culture of *E. aerogenes* was grown in a defined Enterobacter-specific medium, as described elsewhere(DeLisa et al., 1999). Glucose and cellobiose were prepared as stock solutions.

Media, trace element solution, glucose and cellobiose solutions were flushed with sterile N_2 to make the solutions anaerobic and sterilised separately at 121ºC for 20 min. Sterile anaerobic solutions of glucose or cellobiose, trace elements solution and filter sterilised vitamin solution were added into the media before the inoculation inside the sterilised biological safety cabinet (BH-EN 2005, Faster Srl, Ferrara, Italy).

2.2. Design of experiments (DoE)

A mutual medium accommodating the nutritional requirements of both organisms, was designed by using the DoE approach. The buffer compositions of two species specific media described above were analysed and the optimum concentrations of ammonium chloride (AC) (NH_4Cl), sodium acetate (SA) (Na^+ acetate) and phosphate buffer (PB) (KH_2PO_4/K_2HPO_4) capacity were investigated. The setting of DoE for concentration effect of AC, SA and PB capacity was based on 29 randomised runs within concentration range from 3-30 mmol L^{-1} of AC, 3-150 mmol L^{-1} of KH_2PO_4 and 10-120 mmol L^{-1} of SA **(Supplementary Table 1)**. The reason for providing an acetate source in the medium was due to the possibility to add an acetate oxidising microorganism to the co-culture consortium, which was not performed in the context of this study.

2.3. Closed batch cultivations

Cultures of *E. aerogenes* and *C. acetobutylicum* were grown anaerobically at 0.3 bar in a 100 Vol.-% N_2 atmosphere in a closed batch set-up (Rittmann and Herwig, 2012b). Mono-culture and consortium closed batch experiments were conducted with the final volume of 50 mL medium in 120 mL serum bottles (Ochs Glasgerätebau, Langerwehe, Germany). Each serum bottle contained 45 mL Clostridium-specific medium, Enterobacter-specific medium or E-medium, 0.25 mL vitamin solution, 3.0 mL glucose or cellobiose stock solution, 0.5 mL trace elements solution and 1.25 mL inoculum. For consortium experiments different inoculum ratios were tested and initial cell concentrations were arranged with the ratios of (*E. aerogenes* : *C. acetobutylicum*) 1:2, 1:10, 1:100, 1:1000, 1:10000, 1:100000 at a temperature of 37 ºC. Pre-culture of *E. aerogenes* was diluted in DOE E-medium (see **Supplementary Table 1**) to inoculate the organism at cell densities of aforementioned ratios.

2.4. Cell counting, absorption measurements, DNA extraction, quantitative PCR

A volume of 1 mL of liquid sample was collected by using sterile syringes at regular intervals for monitoring biomass growth by measuring the absorbance (optical density at 600 nm (OD_{600})) using a spectrophotometer (Beckman Coulter Fullerton, CA, USA). Every

sampling operation was done inside the sterilised biological safety cabinet (BH-EN 2005, Faster Srl, Ferrara, Italy).

E. aerogenes and *C. acetobutylicum* cells were counted using a Nikon Eclipse 50i microscope (Nikon, Amsterdam, Netherlands) at each liquid/biomass sampling point. The samples for cell count were taken from each individual closed batch run using syringes (Soft-Ject, Henke Sass Wolf, Tuttlingen, Germany) and hypodermic needles (Sterican size 14, B. Braun, Melsungen, Germany). 10 µL of sample were applied onto a Neubauer improved cell counting chamber (Superior Marienfeld, Lauda-Königshofen, Germany) with a grid depth of 0.1 mm.

DNA for quantitative PCR (qPCR) was extracted from 1 mL culture samples by centrifugation at 4 °C and 13,400 revolutions per minute (rpm) for 30 min. The following steps were applied for DNA extraction; (1) cells were resuspended in pre-warmed (65 ºC) 1% sodium dodecyl sulfate (SDS) extraction buffer and (2) transferred to Lysing Matrix E tubes (MP Biomedicals, Santa Ana, CA, USA) containing an equal volume of phenol/chloroform/isoamylalcohol (25:24:1). (3) Cell lysis was performed in a FastPrep-24 (MP-Biomedicals, NY, USA) device with speed setting 4 for 30 s, and the lysate was centrifuged at 13,400 rpm for 10 min. (4) an equal volume of chloroform/isoamylalcohol (24:1) was added to the supernatant of the lysate, followed by centrifugation at 13,400 rpm for 10 min and collection of the aqueous phase. (5) Nucleic acids were precipitated with double volume of polyethylenglycol (PEG) solution (30% PEG, 1.6 mol L^{-1} NaCl) and 1 µL glycogen (20 mg mL^{-1}) as carrier, incubated for 2 h at room temperature. (6) Following centrifugation at 13,400 rpm for 1 h, nucleic acid pellets were washed with 1 mL cold 70% ethanol, dried at 30 °C using a SpeedVac centrifuge (Thermo Scientific, Dreieich, Germany), eluted in Tris-EDTA buffer and stored at -20 °C until further analysis. Nucleic acid quantification was performed with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). qPCR assays were developed for quantifying *E. aerogenes* and *C. acetobutylicum* in consortium. The primer pairs were designed by targeting species specific genes (see **Supplementary Table 2**) to prevent false positive amplification and sequences of genes were compared for identifying optimal primer using the ClustalW2 multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/). qPCR assays were performed in Eppendorf Mastercycler epgradientS realplex^2 (Eppendorf, Hamburg, Germany). The PCR mixture (20 µL) contained 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 sterile DEPC water and 1 µL of DNA template. Negative controls containing sterile DEPC water as a replacement for the DNA templates and DNA template of the non-targeted species were included separately in each run. The amplification protocol started with an initial denaturation at 95 ºC for 2 min, followed by 45 cycles of denaturation at 95 ºC for 30 s, annealing and fluorescence acquisition

at 60 ºC for 30 s, and elongation at 72 ºC for 30 s. A melting-curve analysis (from 60 ºC to 95 ºC at a transition rate of 1 ºC every 10 s) was performed to determine the specificity of the amplification. All amplification reactions were performed in triplicates. A standard curve was generated as described elsewhere(Zeidan and Van Niel, 2009). Culture samples of each organism were collected at different time intervals for cell count and genomic DNA extraction cell density of each strain were determined by cell counting under microscope during growth and subsequent gDNA extraction was applied to reflect absolute quantification. Six tenfold dilution standards were prepared and a linear regression analysis was performed between qPCR reads and cell counts and OD_{600} measurements.

2.5. Quantification of gas composition

Gas chromatography (GC) measurements were performed from serum bottles that remained without any manipulation after inoculation until the first time-point GC measurement. After every GC measurement, remaining gas was released from the serum bottles. The gas compositions were analysed by using a GC (7890A GC System, Agilent Technologies, Santa Clara, USA) with a 19808 Shin Carbon ST Micropacked Column (Restek GmbH, Bad Homburg, Germany) and provided with a gas injection and control unit (Joint Analytical System GmbH, Moers, Germany) as described before (Reischl et al., 2018a). The standard test gas employed in GC comprised the following composition: 0.01 Vol.-% CH_4; 0.08 Vol.-% CO_2 in N_2 (Messer GmbH, Wien, Austria). All chemicals were of highest grade available. H_2, CO_2, N_2, 20 Vol.-% H_2 in CO_2 and 20 Vol.-% CO_2 in N_2, were of test gas quality (Air Liquide, Schwechat, Austria).

2.6. Quantification of liquid metabolites

Quantification of sugars, volatile fatty acids (VFAs), and alcohols were performed with high performance liquid chromatography (HPLC) system (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, a G1362A RID refractive index detector, measuring at 45°C (all modules were from Agilent 1100 (Agilent Technologies, CA, USA). The measurement was performed with 0.005 mol L^{-1} H_2SO_4 as solvent, with a flow rate of 0.325 mL min^{-1} and a pressure of 48-49 bar. The injection volume was 40 µL.

2.7. Data analysis

For the quantitative analysis, the maximum specific growth rate (μ_{max} [h^{-1}]) and mean specific growth rate (μ_{mean} [h^{-1}]) were calculated as follows: $N = N^0 \cdot e^{\mu t}$ with N, cell number [cells ml^{-1}]; N^0, initial cell number [cells ml^{-1}]; t, time [h] and e, Euler's number. According to the delta cell counts in between sample points, µ was assessed. The $Y_{(H2/S)}$ [mol mol^{-1}], HER [mmol L^{-1} h^{-1}],

specific H_2 production rate (qH_2) [mmol g^{-1} h^{-1}](Ergal et al., 2018b) was calculated from each time-point gas composition of serum bottles. The elementary composition was used for the calculation of the mean molar weight, carbon balance (C-balance) and the degree of reduction (DoR) balance of the corresponding biomass(Martinez-Porqueras et al., 2013). Yields of by-products were determined after HPLC measurement. Values were normalised according to zero control. Moreover, the Shannon diversity index (H) was calculated to interpret the changes in microbial diversity, accounting for both richness (S), the number of species present, and abundance of different species. Relative abundance of two species was evaluated according to the calculated evenness (E_H) values(Shannon, 1948).

2.8. Fluorescence *in situ* hybridisation

Sample pre-treatment and fixation

For Fluorescence *in Situ* Hybridisation (FISH), samples of 2 mL were harvested for cell fixation. The samples were centrifuged in micro-centrifuge (5415-R, Eppendorf, Hamburg, Germany) for 10 min at 13,200 rpm and pellets were resuspended in 0.5 mL phosphate-buffered saline (PBS) (10 mmol L^{-1} of Na_2HPO_4/NaH_2PO, 130 mmol L^{-1} of NaCl, pH of 7.2-7.4). After repeating this procedure twice, 0.5 mL ice cold absolute ethanol was added to the 0.5 mL PBS/cell mixture. The ethanol fixed samples were thoroughly mixed and then stored at -20 ºC. Poly-L-lysine solution (0.01 % (v/v)) was used for coating the microscope slides (76·26·1mm, Marienfeld-Superior, Lauda-Königshofen, Germany) containing 10 reaction-wells separated by an epoxy-layer. After dipping the slide into the solution for 5 min, residual poly-L-lysin from the slides was removed by draining well, followed by air-drying for several minutes. Cells were immobilised on prepared slides by adding samples (1-10 µL) on each well and air-drying. Then for the cell dehydration, the slides were impregnated with ethanol concentration of 50 % (v/v), 80% (v/v) and 96% (v/v), respectively. The slides were dipped into each solution for 3 min, starting from the lowest concentration.

The EUB338 probe(Amann et al., 1990) was used to target specific 16S rRNA found in almost all organisms belonging to the domain of bacteria.(Daims et al., 1999) The GAM42a probe specifically binds to target regions of gammaproteobacterial 23S rRNA(Manz et al., 1992) (**Supplementary Table 3**). Both probes were diluted with DEPC water to a certain extent depending on the fluorescence label. Cy3 labelled EUB338 was diluted to a probe concentration of 30 ng DNA μL^{-1}, whereas FLUOS labelled GAM42a was adjusted to a final concentration of 50 ng DNA μL^{-1}. For hybridisation of the probe, 20 µL of hybridisation buffer (900 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris/HCl, 30% formamide (v/v), 0.01% SDS (v/v)) and 2 µL of diluted probe solution were added into each well. The hybridisation reaction (46ºC,

overnight) was facilitated using an air-tight hybridisation chamber (50 mL centrifuge tube) to prevent dehydration.

A stringent washing step was performed at 48ºC for 10 min in pre-warmed 50 mL washing buffer (100 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris/HCl, 5 mmol L^{-1} EDTA). Afterwards, the slides were dried up and a mounting medium (Antifade Mounting Medium, Vectashield Vector Laboratories, CA, USA) was added to each well. The slides were sealed with a cover glass and examined under phase contrast microscope (Nikon Eclipse Ni equipped with Lumen 200 Fluorescence Illumination Systems) using filter sets TRITC (557/576) (max. excitation/emission in nm) for cy3 labelled EUB338 probe and FITC (490/525) for FLUOS labelled GAM42a probes by a 100×1.45 numerical aperture microscope objective (CFI Plan Apo Lambda DM 100X Oil; Nikon Corp., Japan).

3. Results

3.1 Optimising the initial substrate concentration

The first step of assembling the artificial consortium was optimising the initial glucose concentration and to identify the essential nutritional compounds with each of the mono-cultures to prevent substrate inhibition effect on H_2 production(Ciranna et al., 2014; Ginkel et al., 2001). To be able to subsequently design the mutual medium, *E. aerogenes* and *C. acetobutylicum* were grown separately in their own microorganism-specific medium and different initial glucose concentrations ranging from 5 to 35 g L^{-1} (**Supplementary Fig. 1**). Highest OD_{600} of 1.4 and 1.5, and cumulative pressure of 3 bar and 6.8 bar from *E. aerogenes* and *C. acetobutylicum*, respectively, were measured at glucose concentration of 30 g L^{-1}. Substrate inhibition was observed at the concentrations higher than 30 g L^{-1}. Therefore, all of the further experiments were conducted at concentration of 166.5 mmol L^{-1} (30 g L^{-1} or 999 C- mmol L^{-1}) glucose or at same the carbon level equivalence of cellobiose 83.3 mmol L^{-1} (28.5 g L^{-1} or 999 C- mmol L^{-1}).

3.2 Mutual medium design and optimisation

Each microorganism was tested in the medium of the other organisms at a glucose concentration of 30 g L^{-1}. While *E. aerogenes* displayed approximately 2-fold lower cell density (max. OD_{600} of 0.6) and gas production (cumulative pressure of 1.3 bar) in Clostridium-specific medium compared to Enterobacter-specific medium, *C. acetobutylicum* did not grow in Enterobacter-specific medium (**Supplementary Fig. 2**). Hence, is was necessary to precision design a mutual medium to accommodate the nutritional needs of both microorganisms, with an emphasis examining PB capacity, AC concentration, and SA concentration. These factors were investigated in a DoE setting that compromised 8 sets of runs (triplicate) and 1 set of

additional run (pentaplicate) (A,B,C,D,F,G,H,I and E) (**Supplementary Fig. 3**). Concentrations of aforementioned compounds in DoE media are presented in **Supplementary Table 1**. The highest OD_{600} values of *C. acetobutylicum* and *E. aerogenes* were observed when E-medium was used (**Supplementary Fig. 3 (a-c))**. Even though gas production reached higher values in other medium compositions (medium; C, H and I), E-medium was superior due to an earlier onset of gas production by *E. aerogenes* (**Supplementary Fig. 3 (b-d))**. Through analyses of specific growth rate (μ) and cumulative gas production, the physiological response of each of the organisms to the multi-parameter settings was modelled. The overlay of the response surface plots visualising the models of **Supplementary Fig. 3** are shown in **Fig. 2**. Models for cumulative pressure and μ_{mean} for each of the microorganisms indicated that only AC and PB significantly contributed to the model significance. The model for cumulative pressure (R^2=0.89, p-value= < 0.0001, **Supplementary Table 4**) of *C. acetobutylicum* is based on an optimum AC concentration, which is due to a quadratic model term, and on a linear dependence of the PB capacity (**Supplementary Table 4**). Decreasing the PB capacity has a linear positive influence on cumulative pressure increase of *C. acetobutylicum.* This could be due to an accumulation of excreted organic acids and/or an increase of soluble CO_2 concentration with increasing cumulative gas pressure at low buffer capacity, as the low PB capacity cannot keep the pH stable. A low pH value of the medium is known to decrease the activity of [Fe–Fe] hydrogenases of *C. acetobutylicum*, which changes the metabolic pathway from acidogenesis and acetogenesis to solventogenesis resulting in lower gas generation.(Gadhe et al., 2013) Moreover, it has been observed that a metabolic shift occurred from lactate and acetate production to butyrate production with pH increase from 5.3 to 6.3 during *Clostridium tyrobutyricum* fermentation.(Zhu and Yang, 2004) The PB capacity is directly influencing the pH value. *C. acetobutylicum* can grow at a broad range of pH from 4.5 to 7(Zigová and Šturdík, 2000). It has been observed that *C. acetobutylicum* grown at pH 4.5 had higher intracellular concentrations of acetate, butyrate, and butanol compared to the culture grown at pH 6.5(Huang et al., 1985). The model for μ_{mean} (R^2= 0.80, p-value= < 0.0001, **Supplementary Table 4**) of *C. acetobutylicum* indicates that increasing AC concentration has a linear negative influence on μ_{mean} (**Fig. 2**, **Supplementary Fig. 4a-b**). It has been reported that *E. aerogenes* strain E.82005 shifts the metabolic pathway from acid production (e.g. acetic acid production) to non-acid production (e.g. butanediol production) below pH of 5.8, which results in a reduction of H_2 production(Tanisho et al., 1987). This response was also observed in our model for cumulative pressure (R^2=0.99, p-value= < 0.0001, **Supplementary Table 5**) of *E. aerogenes*, where an increasing PB capacity had a linear positive influence on gas generation. The model for μ_{mean} (R^2= 0.67, p-value= < 0.0001, **Supplementary Table 5**) of *E. aerogenes* is based on an optimum AC concentration, which is due to a quadratic model term, and on a linear dependence of the PB capacity. By examining the response surface plots of

μ_{mean} and cumulative gas production by each of the organisms (compare response surface plots in **Supplementary Fig. 4 (a-b**)) optimum medium for high µ and cumulative gas production was identified to be medium E. Hereafter, all experiments were conducted with E-medium containing AC, SA and PB at concentrations of 65, 76.5, 16.5 mmol L^{-1}, respectively.

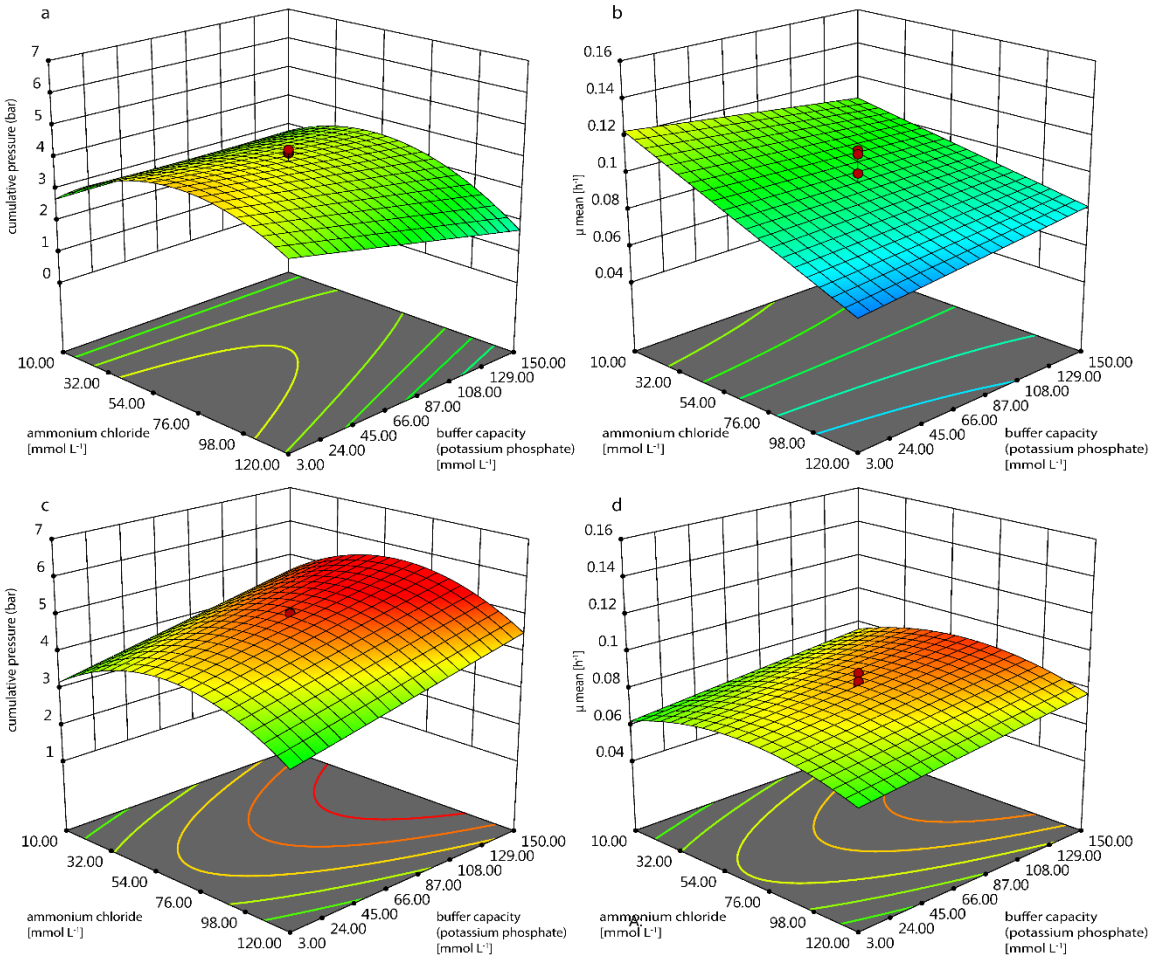

Figure 2: Response surface plots of C. acetobutylicum and E. aerogenes in different DOE media. Overlay of cumulative pressure as function of ammonium chloride and buffer capacity of C. acetobutylicum and E. aerogenes is shown in a, and µ mean as function of ammonium chloride and buffer capacity of C. acetobutylicum and E. aerogenes is shown in b. Response surface plots of C. acetobutylicum and E. aerogenes for cumulative pressure and µ mean are presented separately in ***Supplementary Fig. 4****.*

3.3 Design of the optimum artificial consortium with different initial cell densities

- Mono-culture experiments

Before the design of the optimum artificial consortium, qPCR assays were developed due to the lack of morphological differences between the two species to monitor the population dynamics by following the abundance of the individual species of *E. aerogenes* and *C. acetobutylicum* consortium. The correlation between qPCR reads and absolute number of cells

was determined by mono-culture cell counting. Initially, mono-culture cultivations of *E. aerogenes* and *C. acetobutylicum* were conducted on newly designed E-medium containing glucose or cellobiose. Growth kinetics, by-product formation, substrate uptake and HER of *E. aerogenes* on glucose and cellobiose are shown in **Fig. 3** (a-b). H_2 production by *E. aerogenes* commenced at 40 h on glucose and on cellobiose. Moreover, in **Fig. 3** (c-d) growth kinetics, by-product formation, substrate uptake and HER of *C. acetobutylicum* on glucose and on cellobiose, respectively, are presented. H_2 production by *C. acetobutylicum* started after 62 h and 28 h on glucose or cellobiose, respectively. The global substrate uptake, yields of all by-products, the mass balance analyses of the experiments are presented in **Table 1**. H_2 and CO_2 productivities and yields at each time point from mono-culture cultivations are presented in **Table 2**. Mono-cultures of *E. aerogenes* and *C. acetobutylicum* resulted in maximum $Y_{(H2/S)}$ of 0.13 mol C-mol $^{-1}$ and 0.33 mol C-mol^{-1} on glucose, and 0.04 mol C-mol^{-1} and 0.43 mol C-mol^{-1} on cellobiose, respectively. Growth of *E. aerogenes* on glucose or cellobiose was accompanied by formic acid, butanediol and ethanol production, while butyric acid, acetic acid and ethanol were produced during growth of *C. acetobutylicum* on glucose or cellobiose (**Supplementary Table 6**). Additionally, at the time point, where the highest HER was detected during growth of each of the mono-cultures on glucose, the community composition of mono-cultures was visualised with FISH. The results of FISH analysis are shown in **Fig. 4**. *E. aerogenes* was visualised by FITC and TRITC signals, hence an overlay of two probes (blue) represents *E. aerogenes*. *C. acetobutylicum* was detected by TRITC pink signals.

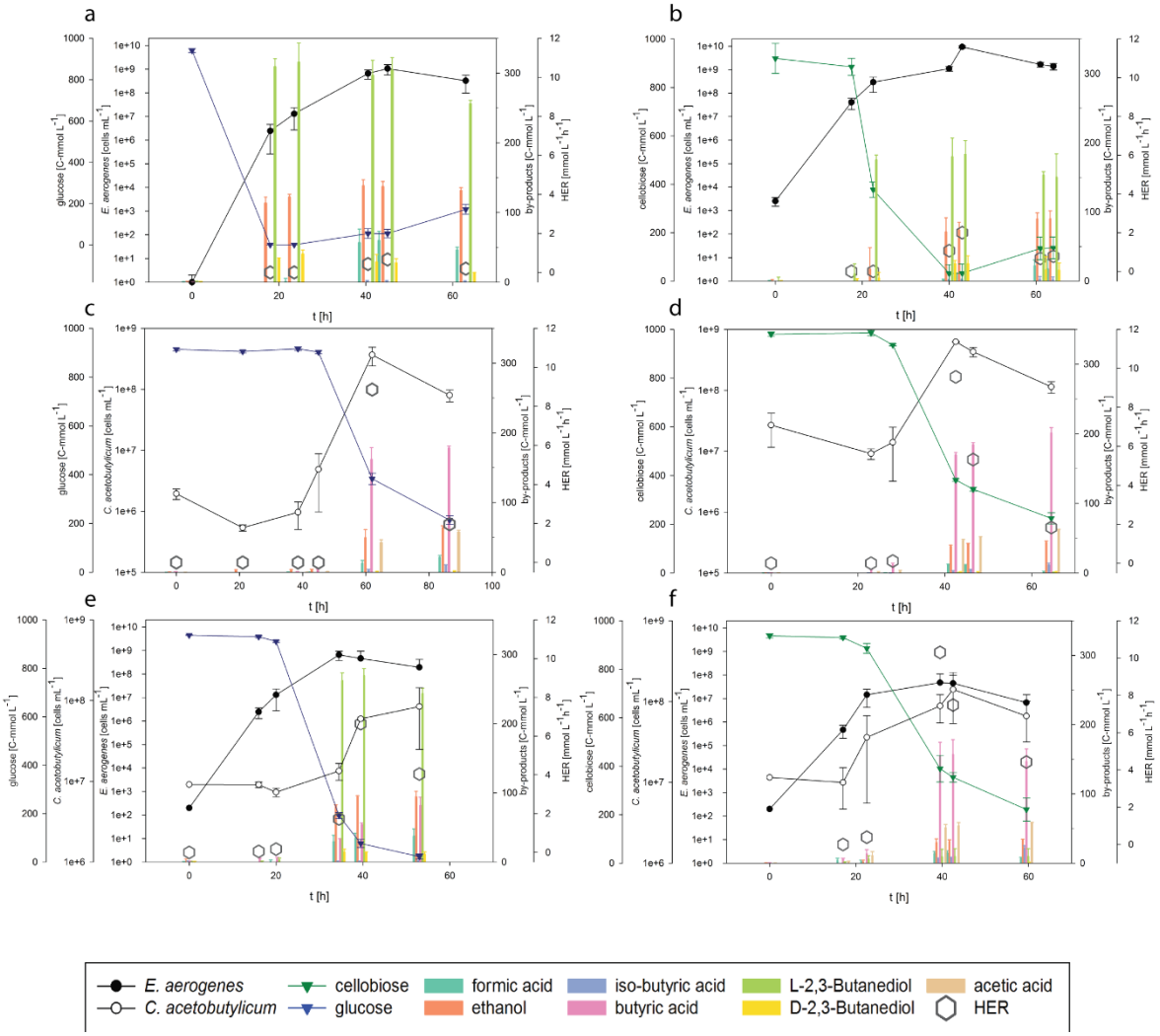

Figure 3: Growth, substrate uptake, and production kinetics of, E. aerogenes (a-b), C. acetobutylicum (c-d) and the consortium (e-f) on 999 C-mmol L^{-1} glucose and cellobiose. The results indicate that amount of produced by-product was decreased and higher HER values were reached during the in consortium cultivation compare to mono-culture on glucose and cellobiose.

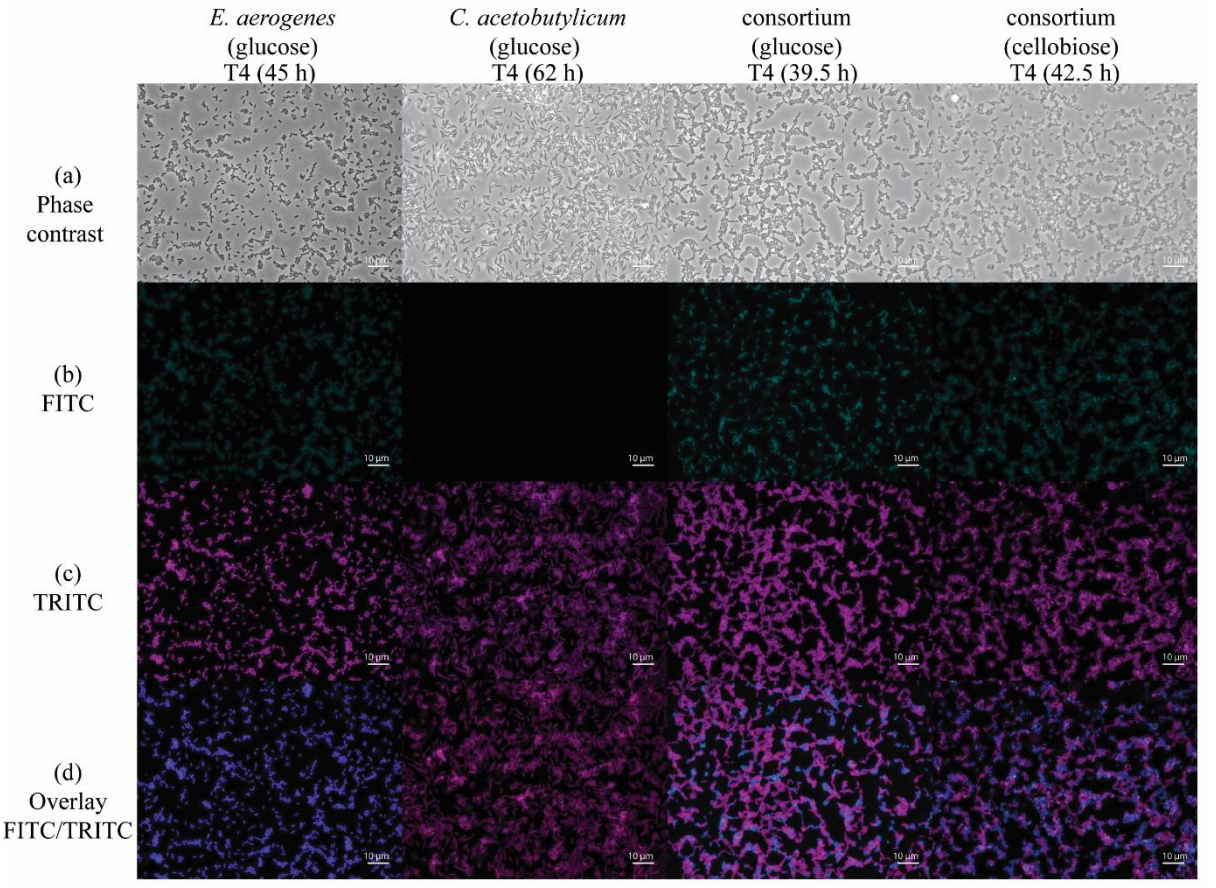

Figure 4: In situ hybridisation of E. aerogenes and C. acetobutylicum mono-cultures on glucose and the consortium on glucose and cellobiose. The phase-contrast images (a), FITC filter set images (b), TRITC filter set images (c) and images from overlay of FITC/TRITC filter sets (d) are shown at the time point where gas production was the highest (T4).

- Design of the defined artificial consortium

After optimising the substrate concentration, composing and optimising the mutual medium, and investigating growth, substrate uptake and production kinetics of both strains, the design of the defined artificial consortium was performed with regard to the eco-physiology and biotechnological characteristics of initial ratios of microorganisms. Initial cell densities were examined with OD_{600} measurements and further each time points were examined with qPCR. The first attempt was initiating the system with almost equal cell densities (1:2) of *E. aerogenes* and *C. acetobutylicum.* When the initial inoculum comprised almost equal cell densities of both strains, *E. aerogenes* rapidly overgrew *C. acetobutylicum* (data not shown). Therefore, the microorganisms were inoculated at different initial cell densities (*E. aerogenes* to *C. acetobutylicum* ratios of 1:100, 1:1,000 and 1:10,000), and to prioritise the highest productive defined artificial consortium their growth, substrate uptake, and gas production kinetics were examined (**Supplementary Fig. 5**). *C. acetobutylicum* was introduced to the system directly from a pre-culture in exponential growth phase grown in E-medium, to prevent spore formation.

The consortium comprising an inoculum ratio of 1:10,000 (*E. aerogenes* : *C. acetobutylicum*) showed the highest maximum HER of 6.64 mmol L^{-1} h^{-1} (at 39.5 h) and 10.3 mmol L^{-1} h^{-1} (at 39.5 h) on glucose and cellobiose, respectively **(Table 1, Table 2 and Fig. 3).** Furthermore, H_2 production was initiated earlier in the consortium (during the first 16 h on glucose, 22.5 h on cellobiose) compared to both mono-culture cultivations on each of the substrates (**Supplementary Table 4**). These findings clearly indicate that the engineered artificial microbial consortium with an inoculum ratio of 1:10,000 (*E. aerogenes* : *C. acetobutylicum*) reached higher HER values on both substrates **(Fig. 3)** compared to the other inoculum ratios**.** The quantities of excreted liquid metabolic by-products were also decreased **(Fig. 3)**, when the 1:10,000 mixing ratio was employed. The consortium displayed 1.24-fold lower butanediol production compared to mono-culture of *E. aerogenes*, and 1.57-fold lower butyric acid production compared to mono-culture of *C. acetobutylicum* on glucose*.* Lower amounts of ethanol and higher amounts of acetic acid and formic acid production were also detected during our consortium experiments (**Table 1**). Validity of the H_2 production and productivities of the by-products was also confirmed by calculating the C- and DoR-balances. Global by-product formation rates, substrate uptake, the mass balance analyses of all experiments and growth kinetics are shown in **Table 1**. The substrate uptake rate was higher in the consortium experiments compared to mono-culture experiments, for both substrates. HER, yields of gasses ($Y_{(H2/S)}$, $Y_{(CO2/S)}$) and qH_2 are shown in **Table 2** for each time point. The optimum consortium comprising an inoculum ratio of 1:10,000 (*E. aerogenes* : *C. acetobutylicum*) showed $Y_{(H2/S)}$ of 0.93 $mol_{(H2)}$ C-mol^{-1} on glucose, which is equal to 5.58 $mol_{(H2)}$ mol^{-1}, and 0.73 $mol_{(H2)}$ C-mol^{-1} (4.38 $mol_{(H2)}$ $mol^{-1}_{(C6\ sugar\text{-}equivalent)}$) on cellobiose (**Table 2** and **Supplementary Table 7**). This is the first study which describes an improvement of $Y_{(H2/S)}$ beyond the Thauer limit in defined medium without active gas removal techniques. These results indicate that precision design of substrate concentration, medium compounds, activity and ratio of organisms must be fine-tuned to meet the eco-physiological prerequisites of the utilised organisms to improve substrate uptake, growth, and production kinetics cleary beyond reported values.

Then we performed FISH to snapshot the population composition and visualise the interaction during the cultivation of the consortium on glucose. Both *E. aerogenes* (blue) and *C. acetobutylicum* (pink) were visualised for each sample taken from different time points (from 0 h (time point zero, after inoculation) to 53 h (time point 5)) (**Supplementary Fig. 6**). FISH confirmed that intact cells of both microorganisms were contributing to artificial microbial community and the homogenous distribution of the microorganisms obtained from qPCR reads (**Fig. 3**). At these time points, ecological indicators were also assessed. From the Shannon-Index (H) and species richness (S), the evenness (E_H) was calculated. The results (**Supplementary Table 8)** indicate that the microbial community was almost evenly distributed

during the time point of maximum $Y_{(H2/S)}$ on cellobiose (E_H = 0.79) and slightly less diverse at the maximum $Y_{(H2/S)}$ on glucose (E_H = 0.51).

Table 1: Global substrate uptake rate, by-product production rates and the mass balance analyses of the mono-cultures and consortium on glucose and cellobiose

Glucose												
Time [h]	**Glucose uptake rate [C-mmol L^{-1} h^{-1}]**	**L-2,3-Butanediol production rate [C-mmol L^{-1} h^{-1}]**	**Acetic acid production rate [C-mmol L^{-1} h^{-1}]**	**Formic acid production rate [C-mmol L^{-1} h^{-1}]**	**Isobutyric acid production rate [C-mmol L^{-1} h^{-1}]**	**Lactic acid production rate [C-mmol L^{-1} h^{-1}]**	**Ethanol production rate [C-mmol L^{-1} h^{-1}]**	**D-2,3-Butanediol production rate [C-mmol L^{-1} h^{-1}]**	**Butyric acid production rate [C-mmol L^{-1} h^{-1}]**	**Biomass (x) [C-mmol L^{-1}]**	**C-balance +**	**DoR #**
E. aerogenes												
63	12.21±0.43	4.06±0.08		0.73±0.08	0.03±0.003	0.12±0.02	2.09±0.08	0.21±0.02		3.64±0.20	0.96±0.02	1.12±0.02
C. acetobutylicum												
62	8.61±0.65		0.74±0.03	0.23±0.04	0.06±0.02		0.81±0.07		2.58±0.32	3.86±0.04	1.12±0.1	1.03±0.02
Consortium												
53	17.25±0.05	3.23±0.60	0.91±0.01	0.80±0.15	0.03±0.02		0.86±0.27	0.21±0.11	1.54±0.20	2.61±0.54	0.95±0.04	1.13±0.14
Cellobiose												
Time [h]	**Cellobiose uptake rate [C-mmol L^{-1} h^{-1}]**	**L-2,3-Butanediol production rate [C-mmol L^{-1} h^{-1}]**	**Acetic acid production rate [C-mmol L^{-1} h^{-1}]**	**Formic acid production rate [C-mmol L^{-1} h^{-1}]**	**Isobutyric acid production rate [C-mmol L^{-1} h^{-1}]**	**Glucose production rate [C-mmol L^{-1} h^{-1}]**	**Ethanol production rate [C-mmol L^{-1} h^{-1}]**	**D-2,3-Butanediol production rate [C-mmol L^{-1} h^{-1}]**	**Butyric acid production rate [C-mmol L^{-1} h^{-1}]**	**Biomass (x) [C-mmol L^{-1}]**	**C-balance +**	**DoR #**
E. aerogenes												
61	7.77±0.7	2.65±0.21		0.36±0.11	0.01±0.001	0.34±0.08	1.52±0.20	0.27±0.17		1.69±0.004	1.05±0.03	1.12±0.03
C. acetobutylicum												
46.6	13.96±0.27		1.11±0.03	0.26±0.02	3.96±0.05	0.11±0.03	0.90±0.05		0.11±0.03	4.26±0.07	1.02±0.02	1.10±0.02
Consortium												
42.5	14.18±0.23	0.33±0.10	1.26±0.10	0.34±0.07	0.18±0.06		0.74±0.12		3.69±0.40	1.84±0.30	1.00±0.08	1.02±0.1

Table 2: Productivities and $Y_{(H2/S)}$ of the mono-cultures and consortium grown on glucose and cellobiose

Glucose					
Time [h]	**$Y_{(CO2/S)}$ [mol Cmol^{-1}]**	**$Y_{(H2/S)}$ [mol Cmol^{-1}]**	**HER [mmol L^{-1} h^{-1}]**	**qH_2 [mmol h^{-1} g^{-1}]**	**CER [mmol L^{-1} h^{-1}]**
E. aerogenes					
18					
23.5					
40.5	0.03±0.003	0.02±0.002	0.43±0.14		0.61±0.13
45	0.23±0.06	0.14±0.04	0.66±0.32	11.34±0.65	1.18±0.46
63	0.10±0.03	0.05±0.02	0.19±0.05		0.35±0.08
C. acetobutylicum					
21					
38.5					
45					
62	0.17±0.03	0.30±0.06	8.86±0.60	86.41±4.51	5.03±0.7
86.5	0.46±0.17	0.33±0.12	1.96±0.84		2.75±0.50
Consortium					
16.0	0.09±0.01	0.12±0.05	0.05±0.01		0.06±0.01
20.0	0.08±0.05	0.05±0.03	0.17±0.02	68.54±5.4	0.27±0.01
34.5	0.09±0.01	0.04±0.01	1.73±0.5	9.60±3.2	4.15±0.5
39.5	0.43±0.02	0.44±0.0.1	6.64±0.25	213.98±8.8	16.19±0.4
53.0	0.67±0.28	0.93±0.29	4.04±0.22		5.84±0.21
Cellobiose					
Time [h]	**$Y_{(CO2/S)}$ [mol Cmol^{-1}]**	**$Y_{(H2/S)}$ [mol Cmol^{-1}]**	**HER [mmol L^{-1} h^{-1}]**	**qH_2 [mmol h^{-1} g^{-1}]**	**CER [mmol L^{-1} h^{-1}]**
E. aerogenes					
17.5					
22.5					
40	0.08±0.01	0.04±0.003	1.07±0.05	4.68±0.91	2.44±0.23
43	0.09±0.01	0.02±0.002	2.00±0.3	0.88±0.71	9.35±0.61
61	0.18±0.02	0.04±0.004	0.67±0.06		2.96±0.19
64	0.06±0.01	0.01±0.002	0.78±0.03		3.30±0.24
C. acetobutylicum					
23					
28	0.01±0.002	0.01±0.002	0.11±0.04	83.43±4.31	0.10±0.03
42.5	0.14±0.001	0.25±0.002	9.57±0.13	55.78±9.74	5.43±0.10
46.5	0.38±0.01	0.43±0.01	5.32±0.31		7.64±0.25
64.5	0.41±0.02	0.27±0.03	1.83±0.12		2.80±0.13
Consortium					
17					
22.5	0.01±0.01	0.06±0.03	0.39±0.01	34.77±3.5	0.06±0.01
39.5	0.12±0.02	0.36±0.05	10.31±0.22	433.95±10.1	3.32±0.31
42.5	0.72±0.11	0.73±0.18	7.48±0.16	528.81±8.11	7.30±.013
59.5	0.13±0.06	0.63±0.28	4.42±0.20		0.94±0.32

4. Discussion

Renewably produced H_2 could be implemented as one of the main energy carriers of the 21st century(Elbeshbishy et al., 2017). To gain biological H_2 production at the theoretical $Y_{(H2/S)}$, different methods (e.g. reactor configurations(Etchebehere et al., 2016), metabolic engineering(Kim et al., 2015), modelling and optimisation(Gadhamshetty et al., 2010), statistical analysis(Rittmann and Herwig, 2012b), pre-treatment strategies for spore germination, nutrient formulations, substrate composition and concentration(Rafieenia et al., 2018)) were proposed and/or already investigated. Using H_2-producing defined or undefined consortia was considered as one of the auspicious approaches(Hallenbeck, 2009), So far, numerous studies on dark fermentative H_2 production were conducted with undefined consortia (see **Supplementary Table 9**). However, an undefined consortium fetches many technical problems due to the reaction complexity, whole process kinetics, difficulties of optimisation and various process parameters (e.g. pH, temperature) as well as the ecological and functional aspects of the system(Sabra et al., 2010). Furthermore, H_2 formation is not the prime aim of microbes, but the microorganism aims on optimising the energy yield. These two aspects might be in conflict to a certain extent, but a defined consortium allows better control regarding H_2 formation whereas an undefined mix will tend to optimise energy formation. Therefore, an artificial/defined consortium, with well-studied microorganisms, is essential to further understand the relationship among microorganisms and to allow sophisticated process control, as the physiologies of the members of microbial community can be examined in depth and individually as well as mutually optimised. So far, artificial dark fermentative H_2 producing consortia were utilised in over 40 studies, which we summarised with respect to dark fermentative H_2 production and their main parameters (**Supplementary Table 9**). The highest reported $Y_{(H2/S)}$ was 4.42 $mol_{(H2)}$ $mol^{-1}_{(glucose)}$ which corresponds to 0.74 $mol_{(H2)}$ C-mol^{-1}) from a consortium of *Caldicellulosiruptor saccharolyticus* and *Caldicellulosiruptor owensensis*(Pawar et al., 2015a), followed by a thermophilic consortium composed of *C. saccharolyticus* and *Caldicellulosiruptor kristjanssonii* comprising 3.8 $mol_{(H2)}$ $mol^{-1}_{(C6\ sugar\text{-}equivalent)}$ (0.63 $mol_{(H2)}$ C-mol^{-1})(Zeidan and Van Niel, 2009). Both studies were conducted on complex medium containing yeast extract. The highest $Y_{(H2/S)}$ reported from a mesophilic consortium of *Enterobacter cloacae* and *Bacillus cereus,* was a $Y_{(H2/S)}$ of 3 $mol_{(H2)}$ $mol^{-1}_{(glucose)}$,which is the equivalent to 0.5 $mol_{(H2)}$ C-mol^{-1} (Patel et al., 2014), followed by a consortium of *E. aerogenes* and *Clostridium butylicum*, with a $Y_{(H2/S)}$ of 2.7 $mol_{(H2)}$ $mol^{-1}_{(glucose)}$ (0.45 $mol_{(H2)}$ C-mol^{-1})(Yokoi et al., 2002).

Our study is the first of its kind, which considered and integrated results from several physiological, ecological, and biotechnological levels: (1) meta-data analysis and modelling

pipeline of dark fermentative H_2 producers(Ergal et al., 2018b), (2) physiological, ecological and biotechnological aspects of mono- and co-culture design, (3) optimisation of H_2 production by subsequently investigating the effect of substrate concentration on growth and gas production, (4) employing DoE method to design a mutual defined medium (E-medium), and finally (5) engineering a defined artificial consortium by examining different initial ratios of microorganisms in defined medium. Here, we present an optimum consortium comprising two species with an inoculum ratio of 1:10,000 (*E. aerogenes* : *C. acetobutylicum*) with a $Y_{(H2/S)}$ of 5.58 $mol_{(H2)}$ $mol^{-1}_{(glucose)}$ (0.93 $mol_{(H2)}$ C-mol^{-1}) and 4.38 $mol_{(H2)}$ $mol^{-1}_{(C6\ sugar\text{-}equivalent)}$ (0.73 $mol_{(H2)}$ C-mol^{-1}) on glucose and cellobiose, respectively. This precisely engineered consortium comprised the highest ever reported $Y_{(H2/S)}$ and clearly surpassed the Thauer limit. Our findings point at a yet unidentified synergistic effect of the two strains that improves H_2 production.

The E-medium composition had a major effect on the metabolism of the microorganisms. The obtained metabolic by-products highlight the active metabolic routes of the microorganisms. On Enterobacter-specific medium we showed that by-products of *E. aerogenes* were mainly acetate and ethanol, which were also reported as main by-products of *E. aerogenes*(Martinez-Porqueras et al., 2013). In our study, the *E. aerogenes* mono-culture produced high amounts of 2,3-butanediol, which is an industrial chemical and liquid fuel and is used in food, cosmetics, and medicine industries(Celińska and Grajek, 2009; Kim et al., 2014). It has been reported that, 2,3-butanediol is produced by *E. aerogenes* under oxygen limited and anaerobic conditions(Converti and Perego, 2002; Kosaric and Lyng, 1988) and initial acetate source induces the butanediol production by catalysing the breakdown of pyruvate to butanediol(Singh and Mishra, 1995). E-medium contains acetate and that might be the reason of production of this compound here. Additionally, a higher level of CO_2 was observed during mono-culture *E. aerogenes* cultivation which is again confirming the butanediol fermentation. 2,3-butanediol production could not be detected during *C. acetobutylicum* mono-culture cultivation (**Fig. 3, Table 1**). Compared to the mono-culture experiments, it was observed that the release of metabolic end products of the two species changed, during the consortium experiments. Lower amounts of 2,3-butanediol were also detected during the consortium cultivation on glucose compared to mono-culture of *E. aerogenes* (**Fig. 3, Table 1**). This is another indication of an operative consortium where both members were metabolically functional. Moreover, during the consortium experiments production of acetic acid was higher and ethanol production was decreased which most likely provided room for H_2 production. Another aspect of the precision design of the medium on consortium was the PB capacity. At pH of 5.5, the consortium was able to produce H_2 due to the activity of *C. acetobutylicum*. In biohydrogen production, pH values less than 4.5 lead to changes in the metabolic pathways towards decreased concentrations of undissociated forms of organic acids, which cause possible inhibition of hydrogenase activity(Ghimire et al., 2015; Ruggeri et al., 2015), affecting ferredoxin's capacity

to donate electrons to reduce protons(Girbal et al., 1994; Ruggeri et al., 2015) and affect microbial growth(Ruggeri et al., 2015; Yokoi et al., 1995). Hence, low pH induces the sporulation of *C. acetobutylicum*, which can be observed in the last time point FISH images in **Supplementary Fig. 6**. The concentration of *C. acetobutylicum* (coloured pink) at the time-point 5 (T5) was drastically decreased at the FISH image (**Supplementary Fig. 6**) as well as the qPCR reads (**Fig. 3**).

Another investigated aspect in this study was the initial cell densities of each microorganism. In previous consortium studies, and most of the cases, an equal suspension volume with an unknown amount of living / active cells of each organism has been used for inoculation(Zeidan and van Niel, 2010; Zeidan and Van Niel, 2009). To our knowledge, this is the first study in which consortium was engineered by introducing active microorganisms at initial cell densities of five orders of magnitude difference into the system. The functional co-existence of two bacteria was shown, when they were introduced to the system at all aforementioned inoculum ratios. Expectedly, cell densities of microorganisms and gas production values differed at each inoculum ratio (**Supplementary Fig. 5**). This was an additional indication of the importance for precision design of the consortium including biotic and abiotic factors. Furthermore, H_2 production was initiated earlier in the consortium (during the first 16 h on glucose, 22.5 h on cellobiose) compared to both mono-culture cultivations on each of the substrates. These findings clearly indicate that the engineered consortium with an inoculum ratio of 1:10,000 (*E. aerogenes* : *C. acetobutylicum*) reached higher HER values on different substrates, and H_2 production kinetics are superior **(Fig. 3).**

This study presents an interdisciplinary approach to improve H_2 production beyond the Thauer limit from the molecular to the process level, and enlightens a systematic and engineering understanding and description of the kinetic and mechanistic aspects, which are responsible for design and definition of this efficient artificial microbial consortium. Constructing the consortium with this approach could also improve the productivity/yield of natural or undefined consortia and provide controllable, stable, predictable biotechnological processes over currently existing systems. Precision design of microbial communities might be employed for the targeted enrichment of microorganisms in undefined microbial populations, or for the restoration of microbial ecosystems in plant, animal, and human health, or in bioremediation. Design of synthetic microbial communities for the targeted conversion of complex biopolymers or surplus electricity to biofuels or intermediate storage molecules such as formic acid will benefit from the specific development of communities of well characterised pure cultures with known growth, substrate uptake, and production kinetics to communities, which are aligned by selecting appropriate concentrations of substrate, pH, reduction potential, salt concentration,

inoculum size, and co-substrate availability, or the mutual exchange of metabolic by-products between the syntrophic partners in the synthetic microbial community.

The present study is a major leap forward in the design of an artificial microbial consortium through precision engineering. Our improvement route is unprecedented and delivers an active, balanced and highly functional co-existence of two bacteria with improved H_2 production kinetics. The H_2 production characteristics of this defined artificial consortium is superior compared to any mono-, co- or multi-culture system reported to date. The system could be further improved to enhance H_2 production by introducing other microorganisms into the consortium, and the stability of the system can be boosted by H_2 milking technology(Ji et al., 2018; Lu et al., 2007), or can be combined with methanogens to stimulate syntrophic growth. Moreover, precision design of an artificial microbial consortium could even serve as a template for conversion of cellulosic biomass to gaseous and liquid biofuels. Our blueprint for a precision design consortium could hence be further extended for the development of consolidated bioprocesses for targeted conversion of lignocellulosic biomass to liquid biofuels, for the development of start-up communities in anaerobic digestion, for the conversion complex gas mixtures, food waste utilisation, or (bio)plastic recycling.

5. Conclusions

- The precision engineered consortium exhibited highly efficient H_2 production from glucose and cellobiose compared to the mono-cultures of either microorganism under optimal conditions or compared to any consortium reported in literature.

- Our drawing board-like design of a defined artificial microbial consortium of microorganisms improved HER beyond reported values. The engineered consortium breaking the Thauer limit displayed 6.6- and 2.8-fold higher maximum $Y_{(H2/S)}$ on glucose and 18.3- and 1.7-fold higher maximum $Y_{(H2/S)}$ on cellobiose compared to mono-cultures of *E. aerogenes* and *C. acetobutylicum*, respectively.

- The precision design of artificial microbial consortia, which considers results from *a priori* physiological and biotechnological knowledge from meta-data analysis will lead to a breakthrough in biotechnology by improving productivity and yield. However, this study indicates that the precision design of artificial microbial consortia might only be efficacious when nutrient demands of the individual members are individually as well as mutually aligned with the eco-physiological characteristics of the organisms.

- The eco-physiological requirements of microorganisms in undefined ecosystems have to be considered at a strain level to be able to improve the performance of the individual players in the community and to achieve high production rates and yields.

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Author contributions

İpek Ergal and Simon K.-M. R. Rittmann conceived and planned the experiments and wrote the manuscript. İpek Ergal carried out the data analysis and calculations. İpek Ergal designed and optimised all the methods and co-supervised the experiments. Simon K.-M. R. Rittmann supervised research. İpek Ergal and Oliver Gräf conducted cultivation of the mono-culture and consortium, qPCR, FISH experiments and GC measurement. Michael Steiner contributed medium design, mono-culture and FISH experiments. Sonja Vukotic contributed to pre-culture and PCR experiments. Benedikt Hasibar, Günther Bochmann, Werner Fuchs provided the HPLC method optimisation and HPLC data. All authors provided critical feedback during the progress of the projects and helped to shape the research, analysis of data and helped to draft the manuscript.

Conflicts of interest

There are no conflicts of interest to declare.

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Supplementary Information

Supplementary Tables

Supplementary Table 1: Concentrations of the compounds in DOE media.

	Ammonium Source (AC)	**Acetate Source (SA)**	**Phosphate Buffer (PB) Capacity**	
DOE Buffer	**NH_4Cl**	**$NaCH_3COO$**	**KH_2PO_4**	**K_2HPO_4**
	(mmol L^{-1})	**(mmol L^{-1})**	**(mmol L^{-1})**	**(mmol L^{-1})**
A	120	3	150	60
B	120	3	3	1.2
C	120	30	3	1.2
D	120	30	150	60
E	65	16.5	76.5	30.5
F	10	30	150	60
G	10	3	3	1.2
H	10	30	3	1.2
I	10	3	150	60

Supplementary Table 2: qPCR primers for quantification of C. acetobutylicum and E. aerogenes

Target	**Primer**	**DNA Sequence (5'-3')**	**Gene**	**Product Length (nt)**
C. acetobutylicum	C_F C_R	TGG CAC AGT CAG TCG GCT ACC GCG TGA TGC ACC TAA CCC AGC	ABC transporter (permease) (AEI33449.1)	108
E. aerogenes	E_F E_R	GCG TTG TGG GGT TGC ACG ATTGG CGC GCG AGC ACA TTT TC	Cation diffusion facilitator family transporter (AEG98846.1)	106

Supplementary Table 3: FISH probes for the in situ monitoring of E. aerogenes, C. acetobutylicum and the consortium

Target	Probe	DNA Sequence (5'-3')	Fluorophore	Reference
C. acetobutylicum *E. aerogenes*	EUB338	GCTGCCTCCCGTAGGAGT	Cy3	(Amann et al., 1990)
E. aerogenes	GAM42a	GCCTTCCCACATCGTTT	Fluos	(Manz et al., 1992)

Supplementary Table 4: Models for cumulative pressure and μ_{mean} for C. acetobutylicum

Response	**Cumulative pressure**					
These Rows Were Ignored for this Analysis: 8	ANOVA for Response Surface Reduced Quadratic Model			Analysis of variance table [Partial sum of squares - Type III]		
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	66.65	6	11.11	29.64	< 0.0001	significant
A-Ammonium chloride	0.2214	1	0.2214	0.5907	0.4507	
B-KH2PO4	3.63	1	3.63	9.68	0.0053	
C-Sodium acetate	0.3274	1	0.3274	0.8734	0.3606	
AB	4.58	1	4.58	12.23	0.0021	
BC	51.45	1	51.45	137.28	< 0.0001	
A^2	6.35	1	6.35	16.95	0.0005	
Residual	7.87	21	0.3748			
Lack of Fit	0.8221	2	0.4111	1.11	0.3506	not significant
Pure Error	7.05	19	0.371			
Cor Total	74.53	27				
Std. Dev.	0.6122		R-Squared	0.8944		
Mean	2.94		Adj R-Squared	0.8642		
C.V. %	20.82		Pred R-Squared	0.8124		

			Adeq Precision	15.3724		
Final Equation in Terms of Actual Factors:	Cumulative pressure	=				
	0.088345					
	0.060201	Ammonium chloride				
	0.026799	KH2PO4				
	0.124868	Sodium acetate				
	-0.000111	Ammonium chloride * KH2PO4				
	-0.001516	KH2PO4 * Sodium acetate				
	-0.000412	Ammonium chloride²				
Response	**μ mean**					
These Rows Were Ignored for this Analysis: 8	ANOVA for Reduced 2FI model			Analysis of variance table [Partial sum of squares - Type III]		
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	0.0111	5	0.0022	17.25	< 0.0001	significant
A-Ammonium chloride	0.009	1	0.009	70.11	< 0.0001	
B-KH2PO4	4.19E-06	1	4.19E-06	0.0325	0.8586	
C-Sodium acetate	0	1	0	0.232	0.6348	
AB	0.0011	1	0.0011	8.84	0.007	
AC	0.0007	1	0.0007	5.78	0.0251	

Residual	0.0028	22	0.0001			
Lack of Fit	0.0002	3	0.0001	0.3807	0.768	not significant
Pure Error	0.0027	19	0.0001			
Cor Total	0.014	27				
Std. Dev.	0.0114		R-Squared	0.7968		
Mean	0.0949		Adj R-Squared	0.7506		
C.V. %	11.97		Pred R-Squared	0.686		
			Adeq Precision	12.4556		
Final Equation in Terms of Actual Factors:	μ mean	=				
	0.11866					
	-0.000369	Ammonium chloride				
	-0.00012	KH2PO4				
	0.000586	Sodium acetate				
	1.75E-06	Ammonium chloride * KH2PO4				
	-7.71E-06	Ammonium chloride * Sodium acetate				

Supplementary Table 5: Models for cumulative pressure and μ_{mean} for E. aerogenes.

Response	**Cumulative pressure**					
These Rows Were Ignored for this Analysis: 29	ANOVA for Response Surface Reduced Quadratic Model			Analysis of variance table [Partial sum of squares - Type III]		
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	53.99	5	10.8	500.23	< 0.0001	significant
A-Ammonium chloride	0.0194	1	0.0194	0.899	0.3533	
B-KH2PO4	11.17	1	11.17	517.26	< 0.0001	
C-Sodium acetate	16.14	1	16.14	747.77	< 0.0001	
BC	19.96	1	19.96	924.83	< 0.0001	
A^2	5.03	1	5.03	233.17	< 0.0001	
Residual	0.4749	22	0.0216			
Lack of Fit	0.1339	3	0.0446	2.49	0.0917	not significant
Pure Error	0.3411	19	0.018			
Cor Total	54.47	27				
Std. Dev.	0.1469		R-Squared	0.9913		
Mean	4.02		Adj R-Squared	0.9893		
C.V. %	3.65		Pred R-Squared	0.9853		

			Adeq Precision	53.1379		
Final Equation in Terms of Actual Factors:	Cumulative pressure	=				
	0.511464					
	0.047083	Ammonium chloride				
	0.025082	KH2PO4				
	0.134472	Sodium acetate				
	-0.000943	KH2PO4 * Sodium acetate				
	-0.000366	Ammonium chloride²				
Response	**μ mean**					
These Rows Were Ignored for this Analysis: 8	ANOVA for Response Surface Reduced Quadratic Model			Analysis of variance table [Partial sum of squares - Type III]		
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	0.0046	5	0.0009	9.15	< 0.0001	significant
A-Ammonium chloride	9.88E-06	1	9.88E-06	0.0981	0.757	
B-KH2PO4	1.10E-03	1	1.10E-03	10.91	0.0032	
C-Sodium acetate	0.0003	1	0.0003	3.05	0.0946	
BC	0.0024	1	0.0024	23.64	< 0.0001	

AÂ²	0.0006	1	0.0006	6.16	0.0212	
Residual	0.0022	22	0.0001			
Lack of Fit	0.0002	3	0.0001	0.7781	0.5206	not significant
Pure Error	0.002	19	0.0001			
Cor Total	0.0068	27				
Std. Dev.	0.01		R-Squared	0.6752		
Mean	0.0719		Adj R-Squared	0.6013		
C.V. %	13.96		Pred R-Squared	0.4714		
			Adeq Precision	7.6716		
Final Equation in Terms of Actual Factors:	µ mean	=				
	0.039551					
	0.00054	Ammonium chloride				
	0.000264	KH2PO4				
	0.00106	Sodium acetate				
	-1.00E-05	KH2PO4 * Sodium acetate				
	-4.07E-06	Ammonium chloride²				

Supplementary Table 6: Global yields, substrate uptake rate and mass balance analyses of the pure cultures (E. aerogenes and C. acetobutylicum) and consortium grown on glucose and cellobiose during the cultivation.

***E. aerogenes* on Glucose**

Time	Glucose uptake rate [C-mmol L^{-1} h^{-1}]	Y (L-BD/s) *	Y (Ac/s) *	Y (Form/s) *	Y (IBa/s) *	Y (Citr/s) *	Y (Et/s) *	Y (D-BD/s) *	Y (Ba/s) *	Y (x/s) *	C-balance +	DoR #
18	52.24±0.52	0.33±0.01					0.12±0.01	0.04±0.001			0.50±0.01	0.79±0.01
23.5	40.01±0.39	0.34±0.03					0.13±0.004	0.04±0.004		0.01±0.001	0.54±0.03	1.01±0.04
40.5	21.87±1.93	0.34±0.01		0.06±0.01			0.16±0.003	0.03±0.006		0.01±0.0004	0.63±0.02	1.21±0.04
45	19.68±1.57	0.34±0.01		0.07±0.01			0.15±0.004	0.03±0.006		0.01±0.0004	0.70±0.01	1.41±0.06
63	12.21±0.43	0.33±0.01		0.06±0.01			0.17±0.001	0.02±0.001			0.96±0.02	1.12±0.02

***E. aerogenes* on Cellobiose**

Time	Cellobiose uptake rate [C-mmol L^{-1} h^{-1}]	Y (L-BD/s) *	Y (Ac/s) *	Y (Form/s) *	Y (IBa/s) *	Y (Citr/s) *	Y (Et/s) *	Y (D-BD/s) *	Y (Glu/s) *	Y (x/s) *	C-balance +	DoR #
17.5	1.96±6.8	0.89±0.40				0.02±0.01	0.03±0.02	0.24±0.4	0.20±0.05		0.76±0.31	0.84±0.53
22.5	14.75±4.2	0.56±0.08				0.01±0.01	0.06±0.03	0.01±0.01	0.02±0.03	0.01±0.003	0.65±0.05	0.89±0.09
40	13.37±1.29	0.35±0.02					0.14±0.04	0.05±0.03		0.02±0.002	0.72±0.03	0.79±0.03
43	12.42±1.21	0.36±0.01		0.02±0.03			0.15±0.03	0.05±0.03		0.18±0.02	1.44±0.1	1.05±0.06

61	7.77±0.76	0.34±0.01		0.05±0.03			0.20±0.04	0.03±0.02	0.05±0.09	0.04±0.004	1.05±0.03	1.12±0.03
64	7.39±0.79	0.34±0.02		0.04±0.03			0.20±0.04	0.03±0.02	0.05±0.01	0.03±0.004	1.10±0.06	1.17±0.05

C. acetobutylicum **on Glucose**

Time	**Glucose uptake rate [C-mmol L^{-1} h^{-1}]**	**Y (N-acet/s) ***	**Y (Ac/s) ***	**Y (Form/s) ***	**Y (IBa/s) ***	**Y (Citr/s) ***	**Y (Et/s) ***	**Y (D-BD/s) ***	**Y (Ba/s) ***	**Y (x/s) ***	**C-balance +**	**DoR #**
21	0.43±0.04			0.04±0.01			0.05±0.01		0.10±0.1		0.01±0.001	1.36±0.2
38.5	0.01±0.01	0.23±0.47	0.13±0.01	0.12±0.09	0.04±0.02		0.25±0.06		0.20±0.04		0.57±0.63	5.11±4.13
45	0.32±0.16	0.03±0.02	0.05±0.04	0.02±0.01	0.04±0.02		0.05±0.04		0.18±0.10	0.002	0.24±0.26	0.24±0.17
62	8.61±1.42		0.08±0.01	0.02±0.01	0.05±0.003		0.09±0.01		0.31±0.02	0.007	1.12±0.12	0.91±0.10
86.5	8.13±0.19		0.08±0.004	0.03±0.003	0.04±0.001		0.09±0.003		0.25±0.01	0.001	1.01±0.02	1.03±0.02

C. acetobutylicum **on Cellobiose**

Time	**Cellobiose uptake rate [C-mmol L^{-1} h^{-1}]**	**Y (L-BD/s) ***	**Y (Ac/s) ***	**Y (Form/s) ***	**Y (IBa/s) ***	**Y (Citr/s) ***	**Y (Et/s) ***	**Y (D-BD/s) ***	**Y (Glu/s) ***	**Y (x/s) ***	**C-balance +**	**DoR #**
23	0.01±0.05			0.06±0.11			0.14±0.1		0.59±0.9	0.02±0.03	0.39±0.81	1.05±0.13

28	1.74±0.30		0.08±0.02	0.01±0.01	0.28±0.07					0.001±0.00	0.37±0.08	0.25±0.06
42.5	14.48±0.22		0.08±0.03	0.02±0.00	0.28±0.01		0.06±0.03		0.01±0.01	0.01±0.00	0.85±0.02	1.15±0.03
46.5	14.02±0.11		0.08±0.02	0.02±0.00	0.28±0.01		0.06±0.01		0.01±0.00	0.13±0.07	1.02±0.02	1.20±0.02
64.5	11.94±0.04		0.08±0.01		0.26±0.02		0.06±0.01		0.01±0.00	0.01±0.00	0.66±0.02	1.10±0.02

Consortium on Glucose

Time	**Glucose uptake rate [C-mmol L^{-1} h^{-1}]**	**Y (L-BD/s) ***	**Y (Ac/s) ***	**Y (Form/s) ***	**Y (IBa/s) ***	**Y (Citr/s) ***	**Y (Et/s) ***	**Y (D-BD/s) ***	**Y (Ba/s) ***	**Y (x/s) ***	**C-balance +**	**DoR #**
16.0	0.39±0.44	2.73±1.35		1.96±0.33	3.63±0.63		5.18±2.33	2.64±1.86		0.31±0.29	0.48±0.32	0.49±0.18
20.0	1.27±0.81	0.03±0.19	0.12±0.04			0.08±0.07			0.25±0.15	0.01±0.00	0.50±0.01	0.16±0.04
34.5	21.58±3.85	0.35±0.06		0.04±0.01			0.11±0.04	0.02±0.01	0.05±0.07	0.01±0.00	0.74±0.00	1.08±0.08
39.5	21.77±0.64	0.31±0.10		0.04±0.01			0.11±0.05	0.02±0.01	0.06±0.08	0.04±0.02	1.27±0.04	1.25±0.17
53.0	17.25±0.05	0.27±0.07		0.04±0.01			0.10±0.05	0.01±0.01	0.09±0.07	0.05±0.02	0.93±0.02	1.13±0.14

Consortium on Cellobiose

Time	**Cellobiose uptake rate**	**Y (L-BD/s) ***	**Y (Ac/s) ***	**Y (Form/s) ***	**Y (IBa/s) ***	**Y (Citr/s) ***	**Y (Et/s) ***	**Y (D-BD/s) ***	**Y (Ba/s) ***	**Y (x/s) ***	**C-balance +**	**DoR #**

	[C-mmol L^{-1} h^{-1}]										
23	0.41±0.24	0.22±0.2	0.45±0.31	0.56±0.51		0.17±0.20		1.02±0.84		0.92±0.2	0.59±0.31
28	2.30±0.67	0.10±0.08	0.20±0.07	0.03±0.03	0.01±0.00	0.01±0.04	0.02±0.01	0.27±0.11	0.01±0.01	0.58±0.15	0.57±0.15
42.5	13.88±1.42	0.02±0.02	0.09±0.01	0.03±0.00	0.01±0.00		0.05±0.00	0.28±0.02	0.01±0.00	0.72±0.05	0.75±0.06
46.5	13.74±0.23	0.02±0.02	0.09±0.01	0.02±0.00	0.01±0.00		0.05±0.00	0.27±0.03	0.06±0.03	1.00±0.08	1.02±0.09
64.5	12.05±1.35	0.01±0.01	0.08±0.01	0.01±0.00	0.03±0.01		0.05±0.00	0.21±0.04	0.01±0.00	0.47±0.05	1.24±0.14

*Y; Yield of product (L-Butanediol (L-BD), Acetic acid (Ac), Formic acid (Form), Isobutyric acid (IBa), Citric acid (Citr) Ethanol (Et), D-Butanediol (D-BD), Butyric acid (Ba), x (biomass), CO_2, H_2, N-acetyl-D-glucosamine (N-acet)) C-mol per C-mol substrate consumed

+Carbon balance

#Degree of reduction balance

Supplementary Table 7: Reactions for each organism and consortium on glucose and cellobiose at the time point where maximum $Y_{(H2/S)}$ was produced.

Glucose

E. aerogenes

$C_6H_{12}O_6 + 0.39C_2H_4O_2 + 0.27C_2H_5OH + 0.06C_3H_6O_3 + H_2O \rightarrow 1.67CH_2O_2 + 0.81C_4H_{10}O_2 + 1.38CO_2 + 0.84H_2 + x + error$

Glucose+ 0.39acetic acid + 0.27ethanol + 0.06lactic acid + $H_2O \rightarrow$ 1.67formic acid + 0.81butanediol + $1.38CO_2 + 0.84H_2$ + biomass + error

C. acetobutylicum

$C_6H_{12}O_6 + 2H_2O \rightarrow 0.31CH_2O_2 + 0.27C_2H_4O_2 + 0.25C_2H_5OH + 0.20C_4H_8O_2 + 2.76CO_2 + 1.98H_2 + x + error$

Glucose + $2H_2O$ → 0.31formic acid + 0.27acetic acid + 0.25ethanol + 0.20butyric acid + $2.76CO_2$ + $1.98H_2$ + biomass + error

Consortium

$C_6H_{12}O_6$+ $0.95C_4H_{10}O_2$ + $0.07C_2H_5OH$ + $0.04C_6H_8O_7$ + $4H_2O$ → $0.53CH_2O_2$ + $0.40C_2H_4O_2$ + $0.99C_4H_8O_2$ + $4.04CO_2$ + $5.58H_2$ + x + error

Glucose + 0.95butanediol + 0.07ethanol + 0.04citric acid + $4H_2O$ → 0.53formic acid + 0.40acetic acid + 0.99butyric acid + $4.04CO_2$ + $5.58H_2$ + biomass + error

Cellobiose

E. aerogenes

$C_{12}H_{22}O_{11}$ +$0.26C_2H_4O_2$ + $0.06C_6H_{12}O_6$ → $0.2CH_2O_2$ + $0.37C_4H_{10}O_2$ + $1.59C_2H_5OH$ + $0.96CO_2$ + $0.48H_2$ + x + error

Cellobiose + 0.26acetic acid + 0.06glucose → 0.2formic acid + 0.37butanediol + 1.59ethanol + $0.96CO_2$ + $0.48H_2$ + biomass + error

C. acetobutylicum

$C_{12}H_{22}O_{11}$ + $0.03C_4H_{10}O_2$ + $0.19C_6H_{12}O_6$ + $2H_2O$ → $0.83C_2H_4O_2$ + $0.56C_2H_5OH$ + $1.31C_4H_8O_2$ + $4.56CO_2$ + $5.16H_2$ + x + error

Cellobiose + 0.03butanediol+ 0.19glucose + $2H_2O$ → 0.83acetic acid + 0.56ethanol + 1.31butyric acid + $4.56CO_2$ + $5.16H_2$ + biomass + error

Consortium

$C_{12}H_{22}O_{11}$ + $0.03CH_2O_2$ + $8H_2O$ → $0.37C_2H_4O_2$ + $0.04C_4H_{10}O_2$ + $0.22C_2H_5OH$ + $0.25C_4H_8O_2$ + $0.01C_6H_{12}O_6$ + $8.64CO_2$ + $8.76H_2$ + x + error

Cellobiose + 0.03formic acid + $8H_2O$ →+ 0.37acetic acid + 0.04butanediol + 0.22ethanol + 0.25butyric acid + 0.01glucose + $8.64CO_2$ + $8.76H_2$ + biomass + error

Supplementary Table 8: Shannon index (H), species richness (S) and evenness (E_H) values of consortium on glucose and cellobiose.

Consortium on Glucose

Time	H	S	E_H
0	0.00	2	0.00
16	0.75	2	0.75
20	0.94	2	0.94
34.5	0.14	2	0.14
39.5	0.51	2	0.51
53	0.89	2	0.89

Consortium on Cellobiose

Time	H	S	E_H
0	0.00	2	0.00
17	0.26	2	0.26
22.5	0.86	2	0.86
39.5	0.93	2	0.93
42.5	0.79	2	0.79
59.5	0.44	2	0.44

Supplementary Table 9: Previous consortia studies with respect to dark fermentative H_2 production and their main parameters.

Genus	Species	Strain	$Y_{(H2/S)}$ [mol/ mol]	$Y_{(H2/S)}$ [mol/ Cmol]	qH_2 [mmol/g*h]	HER [mmol/ L* h]	Temperature (°C):	pH	Dilution rate (1/h)	Carbohydrate	Medium Class	Cultivation Condition	Reference
Enterobacter	*aerogenes*	IAM12348T	0.16	0.03		0.54	35	5.5	0.17	bean curd waste	complex	continuous	(Noike et al., 2005)
	cloacae	IAM12349T											
	sakazakii	IAM12660T											
Enterobacter	*aerogenes*	IAM12348T	0.52	0.09		2.23	35	5.5	0.18	bean curd waste	complex	continuous	(Noike et al., 2005)
	cloacae	IAM12349T											
	sakazakii	IAM12660T											
Enterobacter	*aerogenes*	IAM12348T	0.62	0.10		1.56	35	5.5	0.19	bean curd waste	complex	continuous	(Noike et al., 2005)
	cloacae	IAM12349T											
	sakazakii	IAM12660T											
Clostridium	*butyricum*	IFO 13949	1.7	0.28			37	5.25		starch	complex	batch	(Yokoi et al., 2001)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949	1.5	0.25			37	5.25		starch	complex	batch	(Yokoi et al., 2001)
Enterobacter	*aerogenes*	HO-39											

Clostridium	*butyricum*	IFO 13949	2.4	0.40			37	5.25		starch	complex	batch	(Yokoi et al., 2001)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949	2.4	0.40			37	5.25		starch	complex	batch	(Yokoi et al., 2001)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949	2.3	0.38			37	5.25		starch	complex	batch	(Yokoi et al., 2001)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949	2.3	0.38			37	5.25		starch	complex	batch	(Yokoi et al., 2001)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949	2.4	0.40			37	5.25		starch	complex	batch	(Yokoi et al., 2001)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949	2.7	0.45			37	5.25		starch	complex	batch	(Yokoi et al., 2002)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 3847					30	7.9		glutmate, lactate	complex	closed batch	(Zhu, 2001)
Rhodobacter	*sphaeroides*	RV											
Escherichia	*coli*						37	5.5		glucose	defined	closed batch	(Roychowdhury et al., 1988)
Citrobacter	*freundii*	ATCC 6750											

Escherichia	*coli*						37	5.5		filter paper	defined	closed batch	(Roychowdhury et al., 1988)
Citrobacter	*freundii*	ATCC 6750											
Clostridium	*butyricum*	IFO 13949	2.1	0.35		22.3	36	5.2	0.2	starch	complex	continuous	(Yokoi et al., 1998a)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949	2.5	0.42		35.7	36	5.2	0.5	starch	complex	continuous	(Yokoi et al., 1998a)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949	2.6	0.43		58	36	5.2	1	starch	complex	continuous	(Yokoi et al., 1998a)
Enterobacter	*aerogenes*	HO-39											
Halobacterium	*halobium*	S-9				2.99	30	7			complex	batch	(Patel, 1995)
Phormidium	*valderianum*	BDU 20041											
Escherichia	*coli*	NCL-2065											
Halobacterium	*halobium*	S-9				3.18	30				complex	batch	(Patel, 1995)
Phormidium	*valderianum*	BDU 20041											
Escherichia	*coli*	NCL-2065											
Halobacterium	*halobium*	S-9				4	30	7.5			complex	batch	(Patel, 1995)
Phormidium	*valderianum*	BDU 20041											

Escherichia	*coli*	NCL-2065											
Citrobacter	*freundii*	Cf1	2.33	0.19		35	37		0.108	sucrose	defined	continuous	(Thompson et al., 2008)
Enterobacter	*cloacae*	Ecl											
Citrobacter	*freundii*	Cf1	2.33	0.19		180	37		0.217	sucrose	defined	continuous	(Thompson et al., 2008)
Enterobacter	*cloacae*	Ecl											
Clostridium	*butyricum*	M1				0.2	40			brewery yeast waste	complex	closed batch	(Jen et al., 2007)
Bacillus	*thermoamylovorans*	I											
Ruminococcus	*albus*	strain 7	3.76	0.63				6.75		glucose	complex	continuous	(Iannotti et al., 1973)
Vibrio/Wolinella	*succinogenes*												
Ruminococcus	*albus*	strain 7	3.59	0.60				6.75		glucose	complex	continuous	(Iannotti et al., 1973)
Vibrio/ Wolinella	*succinogenes*												
Ruminococcus	*albus*	strain 7	3.98	0.66				6.75		glucose	complex	continuous	(Iannotti et al., 1973)
Vibrio/Wolinella	*succinogenes*												
Ruminococcus	*albus*	strain 7	3.91	0.65				6.75		glucose	complex	continuous	(Iannotti et al., 1973)
Vibrio/ Wolinella	*succinogenes*												
Ruminococcus	*albus*	strain 7	3.83	0.64				6.75		glucose	complex	continuous	

Vibrio/ Wolinella	*succinogenes*												(Iannotti et al., 1973)
Ruminococcus	*albus*	strain 7	4.59	0.77				6.75		glucose	complex	continuous	(Iannotti et al., 1973)
Vibrio/ Wolinella	*succinogenes*												
Ruminococcus	*albus*	strain 7	3.89	0.65				6.75		glucose	complex	continuous	(Iannotti et al., 1973)
Vibrio/ Wolinella	*succinogenes*												
Ruminococcus	*albus*	strain 7	3.13	0.52				6.75		glucose	complex	continuous	(Iannotti et al., 1973)
Vibrio/ Wolinella	*succinogenes*												
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.5		12.7	3.8	70	6.7	0.04	glucose, xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.6		14.2	4.3	70	6.7	0.06	glucose, xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.5		15.5	6.2	70	6.7	0.08	glucose, xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.1		15	8.3	70	6.7	0.12		complex	continuous	

Caldicellulosiruptor	*kristjanssonii*	DSM 12137								glucose, xylose			(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	2.9		18.4	10.3	70	6.7	0.15	glucose, xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	2.8		18.3	11	70	6.7	0.2	glucose, xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	2.9		21	11.6	70	6.7	0.25	glucose, xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	2.5		21	11.6	70	6.7	0.3	glucose, xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.6		10.2	4.5	70	6.7	0.06	glucose, xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.2		24.7	8.6	70	6.7	0.15		complex	continuous	

Caldicellulosiruptor	*kristjanssonii*	DSM 12137								glucose, xylose			(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.7	0.62	14.8	4.8	70	6.7	0.06	glucose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.5	0.58	21.4	10.4	70	6.7	0.15	glucose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	2.7	0.45	22.6	3	70	6.7	0.06	xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	2.7	0.45	33	8.5	70	6.7	0.06	xylose	complex	continuous	(Zeidan and van Niel, 2010)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Clostridium	sp.	R1	2.6	0.22			30	6		cellobiose	defined	closed batch	(Ho et al., 2010)
Clostridium	*butyricum*												
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.3		38	16	70	6.5		glucose, xylose	complex	batch	(Zeidan and Van
Caldicellulosiruptor	*owensensis*	DSM 13100											

													Niel, 2009)
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.8		19	17	70	6.5		glucose, xylose	complex	batch	(Zeidan and Van Niel, 2009)
Caldicellulosiruptor	*kristjanssonii*	DSM 12137											
Clostridium	*butyricum*	IFO 13949					36	6.5		starch	complex	batch	(Yokoi et al., 1998a)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*butyricum*	IFO 13949					36	6.5		starch	complex	batch	(Yokoi et al., 1998a)
Enterobacter	*aerogenes*	HO-39											
Clostridium	*thermocellum*	JN4	1.8	0.15			60			microcrystalline cellulose	complex	closed batch	(Liu et al., 2008)
Thermoanaerobacterium	*thermosaccharolyticum*	GD17											
Clostridium	*thermocellum*	JN4					60			corn stalk powder	complex	closed batch	(Liu et al., 2008)
Thermoanaerobacterium	*thermosaccharolyticum*	GD17											
Clostridium	*thermocellum*	JN4					60			corn cob powder	complex	closed batch	(Liu et al., 2008)
Thermoanaerobacterium	*thermosaccharolyticum*	GD17											
Clostridium	*thermocellum*	JN4					60				complex	closed batch	

Thermoanaerobacterium	*thermosaccharolyticum*	GD17								corn stalk powder			(Liu et al., 2008)
Clostridium	*thermocellum*	JN4					60			corn cob powder	complex	closed batch	(Liu et al., 2008)
Thermoanaerobacterium	*thermosaccharolyticum*	GD17											
Clostridium	*thermocellum*	DSM 1237					55	7		cellulose	complex	closed batch	(Geng et al., 2010)
Clostridium	*thermopalmarium*	DSM 5974											
Clostridium	sp.	AK 15	0.39	0.07		0.78	60	6	0.16	glucose	complex	continuous	(Koskinen et al., 2008)
Thermoanaerobacterium	sp.	AK 17											
Clostridium	sp.	AK 15	0.53	0.09		1.5	60	6	0.17	glucose	complex	continuous	(Koskinen et al., 2008)
Thermoanaerobacterium	sp.	AK 17											
Clostridium	sp.	AK 15	0.55	0.09		2.18	60	6	0.18	glucose	complex	continuous	(Koskinen et al., 2008)
Thermoanaerobacterium	sp.	AK 17											
Clostridium	sp.	AK 15	0.8	0.13		4.43	60	6	0.238	glucose	complex	continuous	(Koskinen et al., 2008)
Thermoanaerobacterium	sp.	AK 17											
Clostridium	sp.	AK 15	0.8	0.13		6.1	60	6	0.323	glucose	complex	continuous	(Koskinen et al., 2008)
Thermoanaerobacterium	sp.	AK 17											
Clostridium	sp.	AK 15	0.63	0.11		5.31	60	6	0.357	glucose	complex	continuous	

Thermoanaerobacterium	sp.	AK 17											(Koskinen et al., 2008)
Clostridium	*acetobutylicum*	X9			55.4		37	4.8		microcrystalline cellulose	complex	batch	(Wang, 2008a)
Ethanoligenens	*harbinense*	B49											
Enterobacter	*cloacae*	ATCC 13047				1.84	35	7		glucose, fructose	complex	batch	(Chen et al., 2015)
Kluyveromyces	*marxianus*	15D											
Clostridium	*acetobutylicum*	ATCC 824											
Klebsiella	*pneumoniae*	IIT-BT 08	2.07	0.35		19.04	37	6.5		glucose	complex	batch	(Mishra et al., 2015)
Citrobacter	*freundii*	IIT-BT L139											
Enterobacter	*aerogenes*	NRRL B-407	0.002				37	6.5		glycerol, apple pomace hydrolysate	complex	closed batch	(Pachapur et al., 2015)
Clostridium	*butyricum*	NRRL B-41122											
Klebsiella	sp.	TR17				0.021	40	8		glycerol	defined	closed batch	(Chookaew et al., 2015)
Rhodopseudomonas	*palustris*	TN1											
Enterobacter	*aerogenes*	MTCC 8558					30	5.1		Calophyllum inophyllum oil cake	defined	batch	(Arumugam et al., 2014)
Rhodobacter	*sphaeroides*	MTCC 9765											
Chlorella	*vulgaris*	ESP6 (HM070293)	0.94				37	5.5			defined	batch	

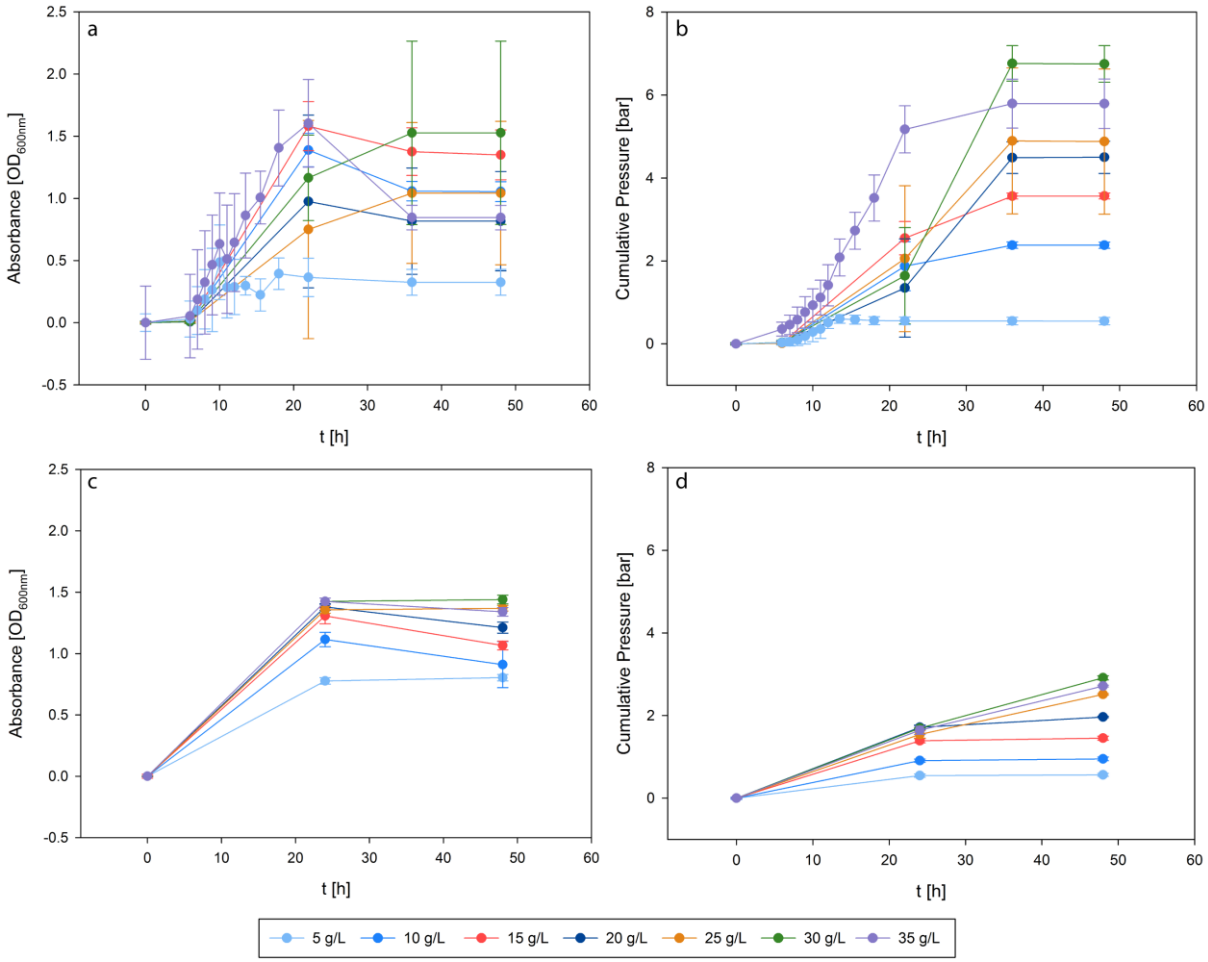
Clostridium	*butyricum*	CGS5 (AY540109)								glucose, xylose			(Liu et al., 2013)
Clostridium	*beijerinckii*	NCIMB 8052	1.88	0.38			30	6.5		xylose	defined	closed batch	(Zhang et al., 2013)
Geobacter	*metallireducens*	GS-15											
Clostridium	sp.	LE37	1.35				37	7		S. obliquus	complex	closed batch	(Ortigueira et al., 2015)
Clostridium	*butyricum*	DSM 10702											
Clostridium	sp.	LE37	1.67				37	7		S. obliquus	complex	closed batch	(Ortigueira et al., 2015)
Clostridium	*butyricum*	DSM 10702											
Clostridium	sp.	LE37	2.01				37	7		S. obliquus	complex	closed batch	(Ortigueira et al., 2015)
Clostridium	*butyricum*	DSM 10702											
Clostridium	*butyricum*	M1					40			brewery yeast waste	complex	closed batch	(Jen et al., 2007)
Bacillus	*thermoamylovorans*	I											
Clostridium	*beijerinckii*	L9					40			brewery yeast waste	complex	batch	(Chang et al., 2008)
Bacillus	*thermoamylovorans*	I											
Clostridium	*thermocellum*	ATCC 27405					55			sugarcane bagasse	complex	closed batch	(Cheng and Zhu, 2013)
Thermoanaerobacterium	*aotearoense*	SCUT27											

Clostridium	*butyricum*	CWBI1009	2.12	0.35			30	5.3		glucose	complex	batch	(Masset et al., 2012)
Clostridium	*pasteurianum*	DSM525											
Clostridium	*felsineum*	DSM749	1.71	0.29			30	5.3		glucose	complex	batch	(Masset et al., 2012)
Clostridium	*pasteurianum*	DSM525											
Clostridium	*butyricum*	CWBI1009	1.62	0.27			30	5.3		glucose	complex	batch	(Masset et al., 2012)
Clostridium	*felsineum*	DSM749											
Clostridium	*butyricum*	CWBI1009	2.32				30	5.3		starch	complex	batch	(Masset et al., 2012)
Clostridium	*pasteurianum*	DSM525											
Clostridium	*felsineum*	DSM749	2.08				30	5.3		starch	complex	batch	(Masset et al., 2012)
Clostridium	*pasteurianum*	DSM525											
Clostridium	*butyricum*	CWBI1009	1.6				30	5.3		starch	complex	batch	(Masset et al., 2012)
Clostridium	*felsineum*	DSM749											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	4.42	0.74		5.46	70	6.5	0.03	glucose	complex	continuous	(Pawar et al., 2015a)
Caldicellulosiruptor	*owensensis*	DSM 13100											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	4.13	0.69		6.7	70	6.5	0.05	glucose	complex	continuous	(Pawar et al., 2015a)
Caldicellulosiruptor	*owensensis*	DSM 13100											

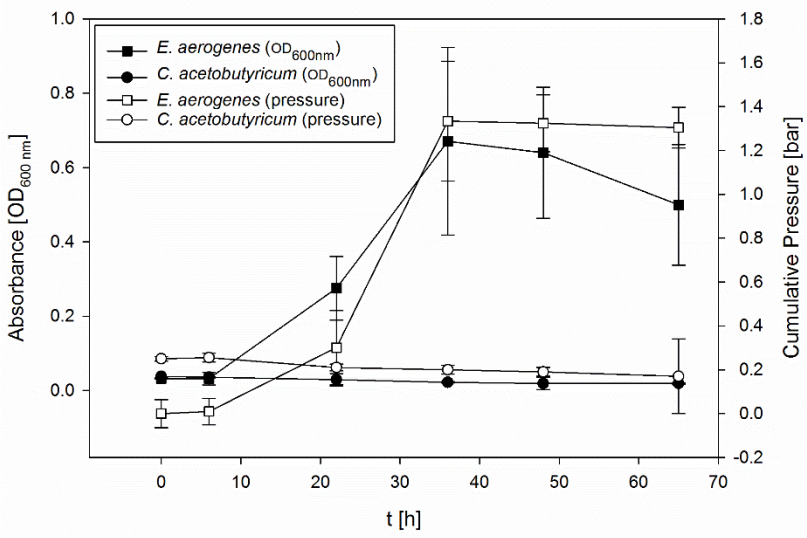
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	3.58	0.60		15.76	70	6.5	1	glucose	complex	continuous	(Pawar et al., 2015a)
Caldicellulosiruptor	*owensensis*	DSM 13100											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	1.84	0.31			70	6.5	0.8	glucose	complex	continuous	(Pawar et al., 2015a)
Caldicellulosiruptor	*owensensis*	DSM 13100											
Caldicellulosiruptor	*saccharolyticus*	DSM 8903	2.12	0.35			70	6.5	1	glucose	complex	continuous	(Pawar et al., 2015a)
Caldicellulosiruptor	*owensensis*	DSM 13100											
Clostridium	*termitidis*	ATCC 51846	2.05				37	7.2		cellulose	complex	closed batch	(Gomez-Flores et al., 2017)
Clostridium	*beijerinckii*	DSM 1820											
Clostridium	*beijerinckii*	ST1				1.04	37	6.5		sucrose	complex	closed batch	(Thi Hoang et al., 2018)
Clostridium	*bifermentans*	ST4											
Clostridium	*butyricum*	ST5											
Enterobacter	*aerogenes*	ATCC 13048				39	33	7		pineapple substrate	complex	closed batch	(Abd Jalil et al., 2018)
Clostridium	*sporogenes*	ATCC 19404											
Bacillus	*cereus*	A1 (CP015727)	1.5	0.25			37			starch			(Ma et al., 2017)
Brevumdimonas	*naejangsanensis*	B1 (CP015614)											
Escherichia	*coli*	CECT432	1.26	0.42			37	6.34		glycerol	complex	batch	

Escherichia	*coli*	CECT434											(Maru et al., 2016)
Enterobacter	*cloacae*	MCM2/1											
Thermatoga	*neapolitana*	DSM-4359	2.8	0.47		36.02	75.00	7.00		glucose	complex	closed batch	(Okonkwo et al., 2018)
Caldicellulosiruptor	*saccharolyticus*	DSM-8903											
Clostridium	*beijerinckii*	DSM 1820	2.00				37.00			glucose	complex	closed batch	(Nasr et al., 2017)
Clostridium	*saccharoperbutyl acetonicum*	DSM 14923											
Clostridium	*beijerinckii*	DSM 1820	1.00				37.00			starch	complex	closed batch	(Nasr et al., 2017)
Clostridium	*saccharoperbutyl acetonicum*	DSM 14923											
Enterobacter	*cloacae*	HPC123	3.00	0.50			37.00	7.00		glucose	complex	batch	(Patel et al., 2014)
Bacillus	*cereus*	EGU43											
Escherichia	*coli*	K-12 MG1655	1.65	0.28			37.00			glucose	complex	closed batch	(Seppälä et al., 2011)
Clostridium	*butyricum*	DSM2478											
Clostridium	*cellulolyticum*	DSM 5812	1.97				37.00	7.20		Corn stover	complex	closed batch	(Zhang and Wang, 2016)
Clostridium	*paraputrificum*	M-21											
Enterobacter	*cancerogeous*	HG6 2A	1.20	0.20			37.00			activated carbon		closed batch	(Zhang et al., 2017)
Enterobacter	*homaechei*	83											

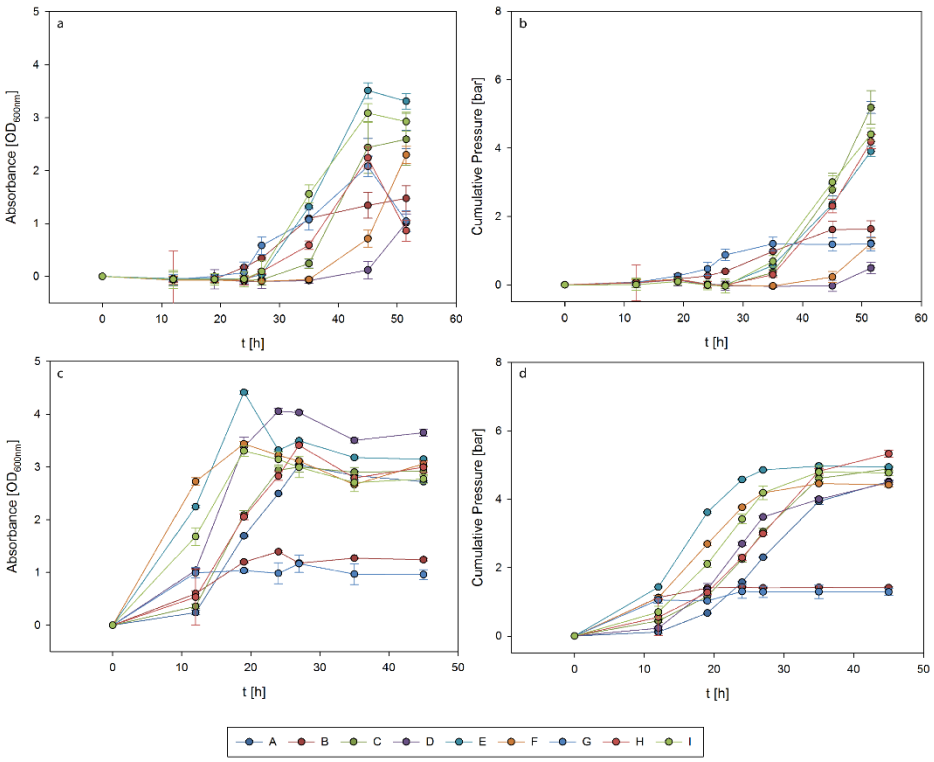
Supplementary Figures



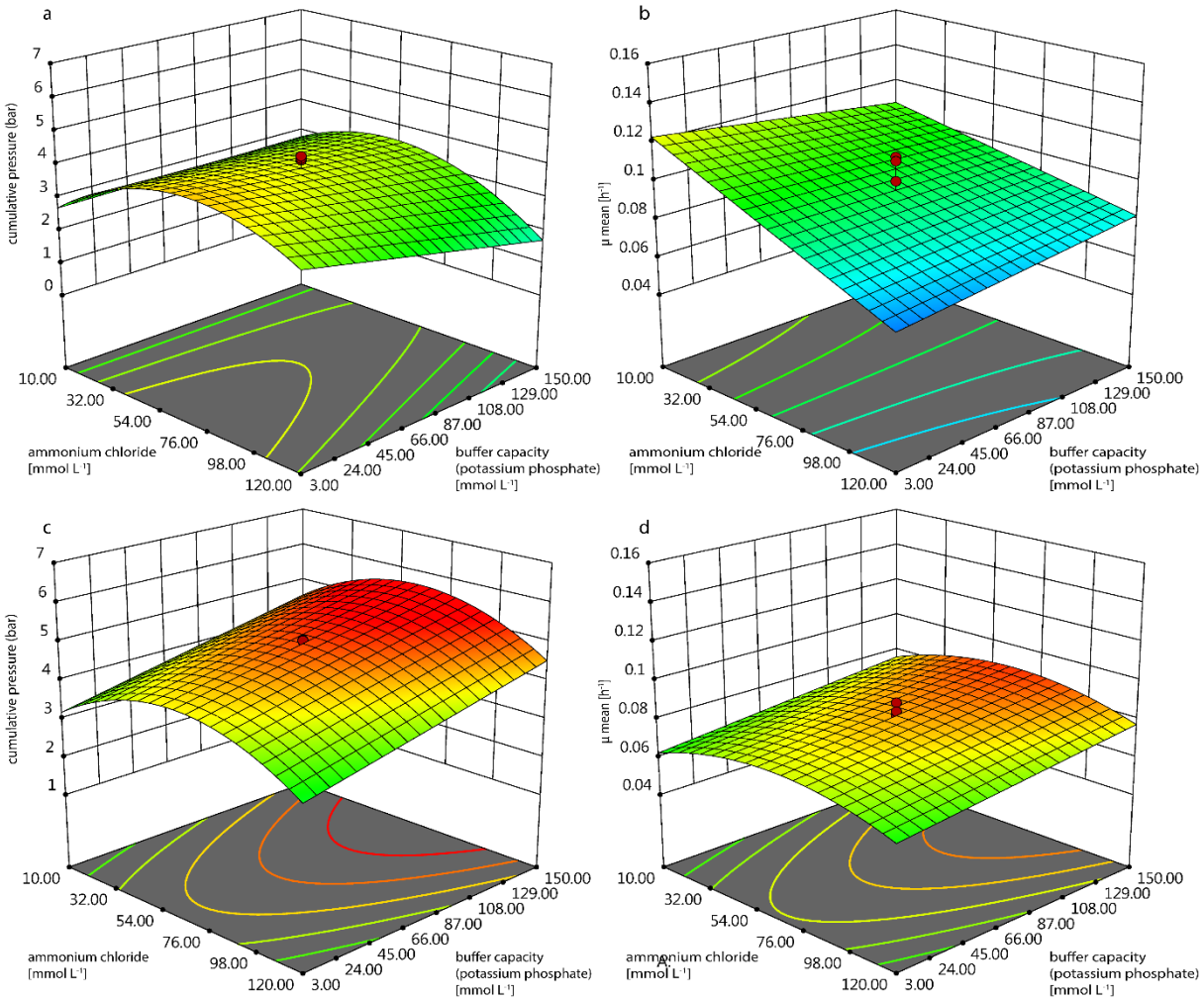
Supplementary Fig. 1: Optical density and cumulative pressure measurements of C. acetobutylicum on Clostridia-specific medium (a-b) and E. aerogenes on Enterobacter-specific medium (c-d) with different glucose concentrations ranging from 5 to 35 g L^{-1}



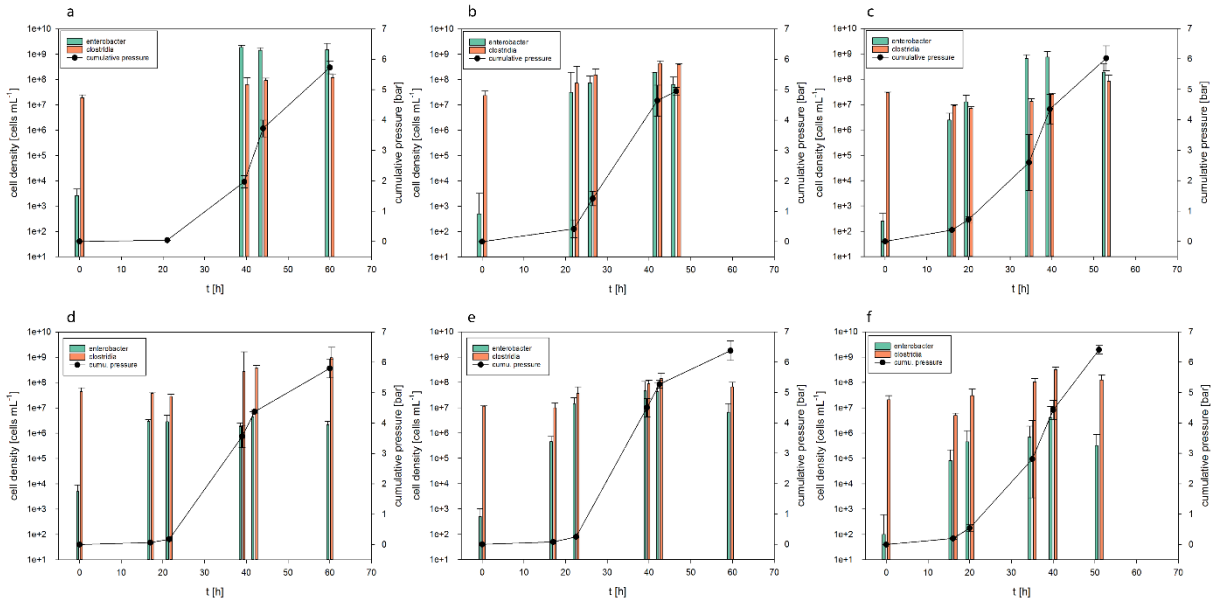
Supplementary Fig. 2: Optical density and cumulative pressure measurements of C. acetobutylicum on Enterobacter-specific medium and E. aerogenes on Clostridia-specific medium. C. acetobutylicum did not grow in Enterobacter-specific medium.



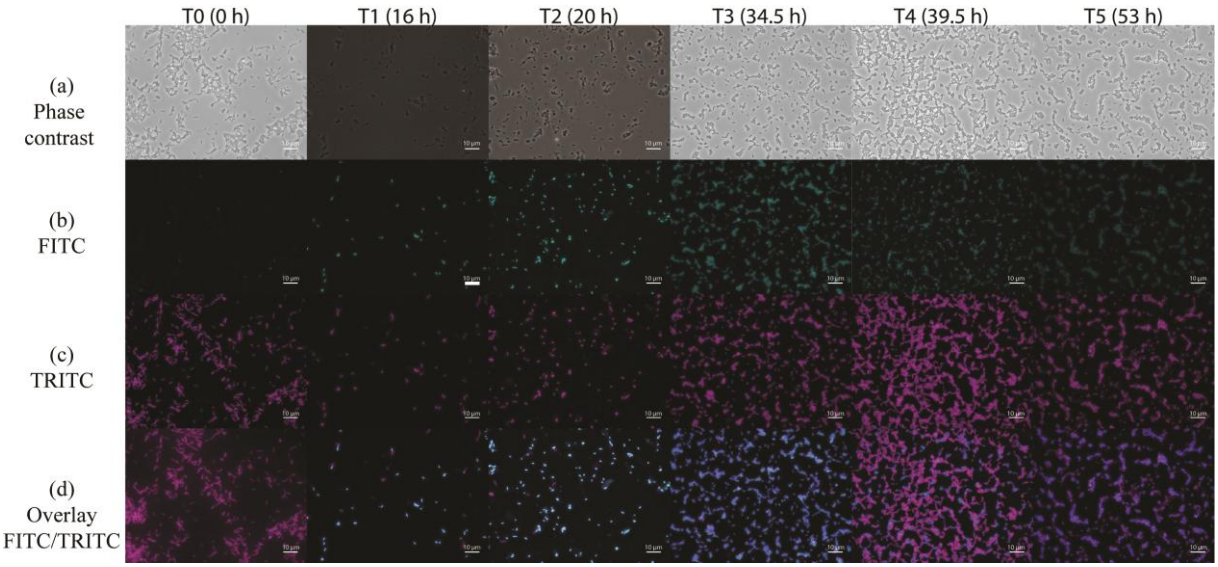
Supplementary Fig. 3: Optical density and cumulative pressure measurements of C. acetobutylicum (a-b) and E. aerogenes (c-d) on different DoE medium (A,B,C,D,F,G,H,I and E).



Supplementary Fig. 4: Response surface plots of C. acetobutylicum (a-b) and E. aerogenes (c-d) in different DoE media. Cumulative pressure and μ mean as function of ammonium chloride and buffer capacity of C. acetobutylicum are shown in a and b, and cumulative pressure and μ mean as function of ammonium chloride and buffer capacity of E. aerogenes are shown in c and d.



Supplementary Fig. 5: Growth and gas production of consortia inoculated with different initial cell densities E. aerogenes to C. acetobutylicum ratios of 1:100 (a-d), 1:1,000 (b-e) and 1:10,000 (c-f) on glucose and cellobiose, respectively.



Supplementary Fig. 6: In situ hybridisation of the consortium on glucose during cultivation. Samples (T0 to T5) taken during cultivation were visualised for detecting the growth pattern of E. aerogenes and C. acetobutylicum in consortium. The phase contrast images (a), FITC filter set images (representing E. aerogenes (green) labelled with GAM42a probe) (b), TRITC filter set images (representing both E. aerogenes and C. acetobutylicum (pink) hybridised with EUB338 probe) (c) and images from overlay of FITC/TRITC filter sets (after overlay E. aerogenes appears blue, C. acetobutylicum appears pink) (d) are represented.

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Chapter III

Discussion

Dark fermentative H_2 producing organisms are particularly suited to novel biotechnological applications and processes owing to their advantages with respect to high productivity, a wide range of utilizable substrates and simple handling characteristics for industry. Despite the promising properties of dark fermentative H_2 producing organisms, serious challenges need to be overcome in order to reach industrial scale. Several studies have already shown that poor productivity characteristics can be boosted by the usage of microbial consortia, where tasks can be divided and cells can be specialized. Wang *et al*. (2008) reported that in continuous stirred-tank reactors, a natural consortium containing *C. acetobutylicum and E. harbinense* resulted with H_2 yield of 2.8 mol H_2 per mol cellulose. This value represents a 4 times higher substrate conversion efficiency compared to yields of same organisms in mono-culture systems (Wang, 2008b). However, the species composition in an undefined consortium is enormously complex. An undefined consortium is accompanied by a multitude of unknown metabolic reactions, which is making it difficult to control the process from a biotechnological perspective. A defined and well-designed consortium, in contrast, is scalable, predictable, reproducible, as well as highly efficient in production and profitable due to its versatility in terms of feedstock (Padmaperuma et al., 2018). In another study Pawar *et al*. (2015) have reached the highest yield ever reported of 4.42 mol H_2 per mol glucose from a consortium of *Caldicellulosiruptor saccharolyticus* and *Caldicellulosiruptor owensensis.* However, the cultivation was conducted on complex medium containing yeast extract which does not allow calculations for a comprehensive H_2 production analysis due to the lack of requisite variables. In order to allow a sophisticated assessment of H_2 productivity characteristics ($Y_{(H2/S)}$, HER and qH_2 based on a C-molar mass balance), the analysis of bacterial performance must be conducted on defined media. Moreover, yeast extract is an expensive resource which would constitute an undesirable cost pressure in industry.

In this study, we aimed the construction of a synthetic ecological system with the capability of an efficient H_2 generation by conversion of glucose or lignocellulosic sources which is widely available form forestry-, agricultural-, and agro-industrial wastes. For extending the natural phenomenon of co-habitation into a defined and precisely engineered consortium for bio-manufacturing industry, the consideration of the population growth was a crucial aspect in order to maintain a functional and balanced co-culture. Common challenges in co-culture engineering are over yielding or under yielding growth effects caused by dominant species monopolizing the nutrients (Bhatia et al., 2018) In our initial experiments, *E. aerogenes*

overgrew *C. acetobutylicum* in an early cultivation state when both strains were inoculated with equal cell densities. As a response, we drastically decreased the number of inoculated cells of *E. aerogenes* which restrained its early cell growth and allowed *C. acetobutylicum* to thrive in co-existence with *E. aerogenes*. In this sense, the examination of different inoculum ratios was one of the key tasks in our study. To avoid introducing *C. acetobutylicum* spores into the system, which would lead to delayed and unpredictable growth, pre-culture of *C. acetobutylicum* at the exponential phase was used as inoculum. By this means we could ensure consistent and comparable experimental conditions. By a number of experiments we approached the ideal initial cell composition in an experimental setup and presented an optimum inoculum ratio of 1:10,000 (*E. aerogenes* : *C. acetobutylicum*) where both organisms could coexist without one outcompeting the other. Both, qPCR assays for absolute cell quantification and FISH experiments for visualization of the population dynamics could confirm an even distribution of the microorganisms throughout the co-cultivation. However, due to the dramatically decreased FISH signals and qPCR reads of *C. acetobutylicum* at the latest sample-point after 60 h incubation time, it must be assumed that acidic conditions below pH 4.6 are initiating the reversion of *C. acetobutylicum* to spore state. The signal loss in FISH experiment presumably is due to the disability of the EUB338 probe to enter the impermeable spore formed cells, whereas the insufficient lysis properties of SDS used for DNA extraction method might explain the low DNA content originating from sporulated *C. acetobutylicum,* based on the qPCR results. In our studies, we could point out the necessity in determining the optimum cell ratio in resulting a functioning co-culture and the number of inoculated cells can enormously differ depending on the strains. By comparison, Zeidan *et al*. (2010) inoculated an equal amount two *Caldicellulosiruptor* sp. to reach optimum coexistence in a *de novo* constructed H_2-producing co-culture.

Here, the 1:10,000 ratio also showed the highest maximum HER of 6.64 mmol L^{-1} h^{-1} (at 39.5 h) and 10.3 mmol L^{-1} h^{-1} (at 39.5 h) on glucose and cellobiose medium, respectively, compared to the values reached when other inoculum ratios were used. As expected, due to the activity of *C. acetobutylicum* the consortium could produce H_2 in high volumetric rates at even low pH conditions (pH 5.5) and the HER could be maintained down to lowest pH 4.5 as the shutdown of hydrogenase activity may be initiated (Sinha et al., 2015). Apart from these optimum production properties, that were provided by the optimum defined consortium, it has also been shown that H_2 production was initiated earlier in the consortium compared to both mono-culture cultivations on each of the substrates. This characteristic might be especially beneficial from an economic perspective, as non-productive phases in fermentation can be reduced, which saves resource-costs for bioreactor maintenance.

In our study, we could point out the necessity in determining the optimum cell ratio to result in a functioning co-culture by inoculating cells at an enormously differing ratio. By comparison Another interesting aspect for industry is that functional co-habitation often affects the environmental conditions which might have a beneficial influence on the system performance (Zeidan et al., 2010). Yokoi *et al*. (1998) have demonstrated a high-rate H_2 production of 58 mmol L^{-1} h^{-1} in a continuous co-culture system of *Clostridium butyricum* and *E. aerogenes*. In their studies they also investigated the beneficial effect that trace amounts of O_2 present in media may be consumed by facultative anaerobic *E. aerogenes*. This created anaerobic conditions that favoured the propagation of obligate anaerobic *C. acetobutylicum* (Balachandar et al. , 2013). In this sense, production failures or diminishing $Y_{(H2/S)}$ in industry caused by O_2-contamination can be conveniently prevented by applying an artificial consortium including facultative anaerobe *E. aerogenes* (Zeidan et al., 2010). According to species evenness calculations of *C. acetobutylicum* and *E. aerogenes* in our co-culture studies we could demonstrate that the maximum $Y_{(H2/S)}$ was achieved during a time point as the microbial community was almost evenly distributed. However, the investigated growth pattern from the co-cultivations clearly shows the quantitative dominance of *E. aerogenes* at early cultivation state, even though both strains have been initially inoculated from an exponentially active pre-culture. In contrast, *C. acetobutylicum* paused in a lag phase and could be visualized by FISH only at an advanced stage of cultivation. It can be assumed that the late and abrupt thriving of *C. acetobutylicum* is due to altered environmental conditions which was gradually created by *E. aerogenes* in lowering media pH and eliminating potential traces of O_2 (Zeidan et al., 2010).

Microbial bio-H_2 production from renewable biomass represents a sustainable opportunity for bioenergy generation. Agricultural and food industry wastes are rich in lignocellulolytic compounds which are containing cellulose, hemicellulose and lignin (Chong et al., 2009), however, the processes in converting inherently complex and heterogenic plant material into its constituent sugars have always been a problematic factor for industrial fermentation. In previous studies it has been shown that microbial communities that work efficiently and often synergistically accomplish degradation of such raw biomass (Cortes-Tolalpa et al., 2017). Ueno *et al*., 1995 took natural consortia from an anaerobic digestion sludge to degrade cellulose and generate H_2. However, due to the immense complexity of a natural consortium, the presence of H_2 consumers was ubiquitous and H_2 transfer reactions with H_2 producers were directly linked, which resulted in a release of only traces of H_2. In our defined consortium study, we could present that the usage of cellobiose as carbon source has no influence on the growth patterns and general structure of the co-culture and that there were only minor performance deficits in terms of $Y_{(H2/S)}$ and HER, compared to the values that were achieved by fermentation on glucose medium. Hence, the functional properties of co-cultured *C. acetobutylicum* with *E.*

aerogenes presumably provide ideal requirements for efficient and sustainable biofuel production from waste material.

Conclusions

Within this thesis, the initial cell composition of *C. acetobutylicum* and *E. aerogenes* for highly functional H_2-generating consortium was investigated. The ratio of 1:10.000 (*E. aerogenes* : *C. acetobutylicum*) resulted with superior H_2 production properties and was accompanied by a homogenous growth distribution of both strains. The defined consortium reached higher HER on glucose and cellobiose substrate compared to mono-culture experiments of *E. aerogenes* and *C. acetobutylicum*, respectively. Moreover, it comprised higher $Y_{(H2/S)}$ compared to mono-culture and the Thauer limit of 4 mol H_2 per mol of glucose was clearly surpassed. H_2 production was initiated at an earlier stage during fermentation in relation to mono-culture *C. acetobutylicum.* Furthermore, the system proved enhanced H_2 productivity under acidic environmental conditions in maintaining high HER above pH 4.5. The defined consortium offers environmentally sustainable routes for bio-H_2 production due to its feasibility of converting lignocellulosic biomass to H_2. In this sense we successfully constructed a defined consortium comprising superior features for enhanced bio-H_2 production. We could demonstrate the promising industrial benefits in the design of artificial microbial consortium which requires ecological, physiological and biotechnological knowledge to unleash its full performance potential.

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