universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Dark fermentative biohydrogen production in artificial co-culture"

verfasst von / submitted by

Elisa Zech, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien 2022/Vienna 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 830
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Mikrobiologie, Mikrobielle Ökologie und Immunbiologie
Betreut von / Supervisor:	Mag. Mag. Dr. Simon K.-M. R. Rittmann, Privatdoz. Bakk.

Contents

Abstract

Molecular hydrogen (H_2) is an attractive alternative energy carrier compared to fossil fuels. It can be produced biologically using different strains of microbes to replace current energy intensive methods. However, major drawbacks include low production efficiencies and limited yields that underlie the metabolic constraints of the organisms used.

A possible remedy to this issue is the application of artificial co-cultures, as symbiotic interactions increase resistance to environmental stress factors and can improve the production efficiency. In-depth meta-data analysis, statistics, and modelling enabled identifying strains to design an artificial consortium with an optimized inoculation ratio and substrate concentration to surpass the theoretical maximum yield of 4 mol H_2 mol^{-1} glucose by 1.58 mol H_2 mol^{-1} glucose.

The aim of this thesis was to up-scale this highly efficient co-fermentation using *Enterobacter aerogenes* and *Clostridium acetobutylicum*. It was demonstrated, that similarly high bio-H_2 production efficiencies can also be achieved at larger volumes. In fact, the implementation of batch cultivation resulted in H_2 yields surpassing those from closed batch fermentations, giving 6.8 and 4.45 mol H_2 mol^{-1} glucose when pH was controlled and uncontrolled, respectively. Nevertheless, the higher complexity of the system needs fine tuning and an excellent understanding of the organisms' needs, as only slight changes of the workflow can affect the performance of the system. Still, the designed microbial consortium approach for biotechnological applications does hold very promising opportunities for efficient and environmentally friendly energy production in the future.

Kurzfassung

Molekularer Wasserstoff (H_2) bietet eine vielversprechende und umweltfreundliche Alternative zur Energiegewinnung durch fossile Brennstoffe. Die Produktion durch mikrobielle Aktivität hat den Vorteil, energieeffizienter zu sein als herkömmliche Herstellungsprozesse. Ein großer Nachteil ist jedoch der geringe Ertrag an H_2 pro Substrat, der den metabolischen Limitationen der Mikroorganismen zugrunde liegt.

Eine vielversprechende Lösung für dieses Problem ist die Anwendung von Co-Kulturen, also die Kombination verschiedener Mikroorganismen. Symbiotische Interaktionen zwischen den mikrobiellen Partnern können zu einer größeren Resistenz gegenüber Umwelteinflüssen und -störungen beitragen. Noch dazu können sie sich gegenseitig metabolisch ergänzen, Stoffwechselwege in Richtung Wasserstoffproduktion lenken und die Produktionseffizienz somit erhöhen. Mithilfe eingehender Metadatenanalyse, Statistik und Modellierung gelang es Stämme auszuwählen, diese in ein künstliches mikrobielles Konsortium zu vereinen, und dadurch den theoretisch maximalen Ertrag an 4 mol H_2 mol^{-1} Glucose, um 1.58 mol H_2 mol^{-1} Glucose zu übertreffen.

Das Ziel dieser Arbeit ist der Versuch diese hocheffiziente Co-Kultur der Organismen *Enterobacter aerogenes* und *Clostridium acetobutylicum* zu erweitern und in größeren Volumen zu implementieren. Tatsächlich gelang es, einen hohen Wasserstoff Ertrag in Bioreaktoren zu erzielen, der sogar den Ertrag der künstlichen Ko-Kultur übertrifft, die in Serumflaschen gezogen wurden. Es wurden 6.16 und 4.45 mol H_2 mol^{-1} Glucose durch die Ko-Kulturen in jeweils einem pH-kontrollierten und einem pH-unkontrolliertem Reaktor produziert. Im Vergleich zur Kultivierung von Mikroben im geschlossenen Satzverfahren in Serumfläschchen gibt es jedoch viele zusätzliche Faktoren und Parameter zu beachten, wenn eine Maßstabsübertragung in den Bioreaktor erfolgt. Die höhere Komplexität der Fahrweise von Bioreaktoren benötigt Feinjustierung und eine genaue Kenntnis der Bedürfnisse der verwendeten Organismen, da selbst kleinste Änderungen die Leistung des Systems beeinträchtigen können. Dennoch wurde durch die Maßstabsübertragung der Wasserstoffproduktion in das Satzverfahren gezeigt, dass die Anwendung künstlicher mikrobieller Ko-Kulturen für biotechnologische Prozesse viele Vorteile und attraktive Möglichkeiten für eine nachhaltige zukünftige Energieproduktion besitzt.

Eidesstaatliche Erklärung

Ich erkläre eidesstattlich, dass ich die Arbeit selbständig angefertigt, 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, durch Fußnoten gekennzeichnet bzw. mit genauer Quellenangabe kenntlich gemacht habe. Diese schriftliche Arbeit wurde noch an keiner anderen Stelle vorgelegt.

Ort, Datum Unterschrift

Acknowledgements

First of all, I would like to thank my supervisor and principal investigator, Dr. Simon K.-M. R. Rittmann, Privatdoz. for giving me the opportunity to work and complete my master's thesis in his group. His encouragement, fairness, and acknowledgement towards his master's and PhD students is remarkable and made working in this group a pleasure.

Many thanks to our Head of Department, Professor Christa Schleper, for giving me the opportunity to be a part of her amazing group, her support, and motivation.

Most of all, I am very grateful to my co-supervisor, Ipek Ergal, PhD, for her patience, kindness, and limitless support, not only as a supervisor, but also as a friend. I feel very fortunate to have been able to learn from such a great scientist.

Thanks to all my group members, especially to Barbara Reischl, for answering all of the many questions I had, and of course Barbara's uplifting personality.

I would like to thank each and every one of the Archaea Physiology & Biotechnology Group, who made these last years a very enriching and unforgettable experience. This work would not have been possible without their expertise and support.

I am very grateful for my friends and family. For their encouragement and patience and for making my studies a wonderful and enjoyable experience. Thanks to my mother, sister, and brother, for their love and guidance which led me to where I am.

Dedication

This work is dedicated to my Father, who unfortunately will never have the chance to read this. But even though he left this world too early to witness the personal or professional choices I made and will make in life, he taught me to follow my heart and gave me all the strength and courage I need to make the most out of the time that has been given to me.

Chapter I

1. Introduction

1.1. Hydrogen as CO_2 neutral and renewable energy carrier

Energy demands are rising all over the industrial world. Current global energy generation mostly depends on depleting fossil fuel reserves, leading to severe air pollution problems (Marbán and Valdés-Solís 2007). According to the fifth Assessment Report of the Intergovernmental Panel on Climate Change (Collins et al. 2013; IPCC 2013) atmospheric carbon dioxide (CO_2) level will have reached a level between 794 and 1142 ppm by the end of this century (in comparison, preindustrial levels were around 280 (Wigley 1983)). This would cause a continuous rise in the global mean surface temperature, changes in atmospheric circulation, the water cycle, cryosphere, ocean, and carbon cycle (Collins et al. 2013). We are therefore in urgent need of renewable and CO_2-neutral alternatives to fossil fuel.

The use of hydrogen (H_2) is widely considered an environmentally friendly and clean alternative (Zajic et al. 1979; Peraldo Bicelli 1986; Bockris 2002). Its combustion yields 2.75 times more energy (122kJ/g) than hydrocarbons (ranging from 38 to 48 kJ/g), and this can be easily stored as electricity in fuel cells. The only side-product is water vapor instead of greenhouse gases (Hay et al. 2013; Lloyd and Davenport 1980).

Nevertheless, there are disadvantages and challenges that still need to be addressed. This includes transportation and safe handling of this explosive gas, as well as its production (Marbán and Valdés-Solís 2007). H_2 can be generated thermochemically by steam reforming, partial oxidation, autothermal reforming, gasification, and pyrolysis (Nanda et al. 2017). The majority of H_2 used is produced by fossil fuels (Kothari et al. 2008), driving severe CO_2 emissions into the atmosphere each year (Levin and Chahine 2010). Therefore, the generation of H_2 from readily available and low-cost renewable sources such as lignocellulosic material with environmentally friendly technologies is lucrative for future large-scale industrial application.

Depending on the cleanliness and associated greenhouse gas emission, different H_2 production pathways can be categorized by colours (Germscheidt et al. 2021). In this context, grey H_2 includes steam reforming processes based on fossil fuel combustion without any restriction on CO_2 emissions (*i.e.,* steam methane reforming, partial oxidation processes, and autothermal reforming). Grey H_2 production pathways can be coupled with a carbon capture and storage process, changing the colouration from grey to blue. In contrast to that, using water and renewable energies for water electrolysis, also called water splitting, is referred to as green H_2. H_2 produced using a combination of renewable and non-renewable energy sources is classified as yellow H_2. White colour is used to describe H_2 from natural resources with no commercial interest as it is only scarcely found in larger amounts. However, the definition of white H_2 is still open and includes biologically or thermochemically produced H_2 occurring naturally.

The main challenges for the commercialisation of water electrolysis cells lie in the proper selection of membranes, development of catalysts, and the source of the input energy. Lots of research has been directed towards renewable and sustainable generation of energy to fuel the water splitting process, including wind power utilization and solar energy. In spite of all these efforts, green H_2 remains a costly and challenging process that is not yet competitive enough for efficient industrial applications (Germscheidt et al. 2021). A very interesting aspect of water electrolysis is the possibility of using wastewater or seawater as feedstock. A great advantage thereof would be the on-site treatment of water and energy storage as H_2 at the same time (Nath et al. 2021; Lu and Ren 2016; Dionigi et al. 2016). Both wastewater electrolysis cells (WEC) and microbial electrolysis cells (MEC) can use wastewater as the source substrate, with the latter making use of microbial metabolic activity. Microorganisms are able to convert biodegradable substrates and produce the electrical current as well as the protons (H^+) necessary for H_2 production. WECs and MECs have great potential for future H_2 production technologies and on-site water treatment. However, a better understanding of their syntropy and competition is still needed to minimize energy and product losses (Lu and Ren 2016).

1.2. Biological H_2 production

Being less energy intensive and almost non-CO_2 emitting, the biological conversion of biomass into H_2, called biohydrogen production (bio-H_2 production), has gained significant interest over the last few decades (Vinoth Kanna and Paturu 2020; Rathore et al. 2019).

H_2 is a key compound of microbial metabolic pathways, being either an electron donor to drive energy generation or is produced as a means to dispose of excessive electrons via the reduction of protons. Thus, bio-H_2 production is taxonomically widely distributed. As a consequence, there are multiple equally suitable candidate organisms, both autotrophic and heterotrophic, to perform H_2 generation. Defining specific criteria regarding growth and production performance would narrow the selection of the most favourable H_2 generating pathway and facilitate the optimisation of the production process.

For an objective comparison of the different H_2 production strategies, a set of physiological, scalable parameters has been proposed (Rittmann and Herwig 2012). The H_2 evolution rate (HER) in mmol L^{-1} h^{-1} represents the volumetric H_2 evolution over time, making it an important variable for bioprocess engineering. For biotechnological applications, the H_2 yield ($Y_{(H2/S)}$) is another important variable, defined as how many moles of H_2 can be produced per mole of substrate. High yields are desired as it reduces the amount of substrate required to generate a specific H_2 output (in mol mol^{-1}). Hence, $Y_{(H2/S)}$ reflects the substrate conversion efficiency. Regarding the individual organisms, a third variable that has to be considered is the specific H_2 production rate (qH_2), which defines how many grams of cell dry weight (x) is needed in order to produce H_2 at a certain rate (mmol g^{-1} h^{-1}). qH_2 can be defined as HER divided by x, and so they are directly correlated. For an un-biased comparison, presentation of results on a carbon (C)-molar basis of the substrate is desirable. As different substrates are used in the literature, data analysis on a molar level would result in an under- or overestimation of substrate consumption. This is shown in Ergal et al. (2018), where the results of the highest $Y_{(H2/S)}$ on a molar level are not consistent with those obtained on a C-molar level and offer a misleading interpretation of the data.

To date, there are three main processes known to biologically produce H_2: bio-photolysis of water using algae and cyanobacteria, photodecomposition of organic compounds by photosynthetic bacteria, and dark fermentation from organic compounds using obligate or facultative anaerobic microbes (Lopes Pinto F. A et al. 2002; Nandi and Sengupta 1998; Hallenbeck and Benemann 2002; Das 2001; Schütz et al. 2004; Melis et al. 2006), as shown in figure 1.

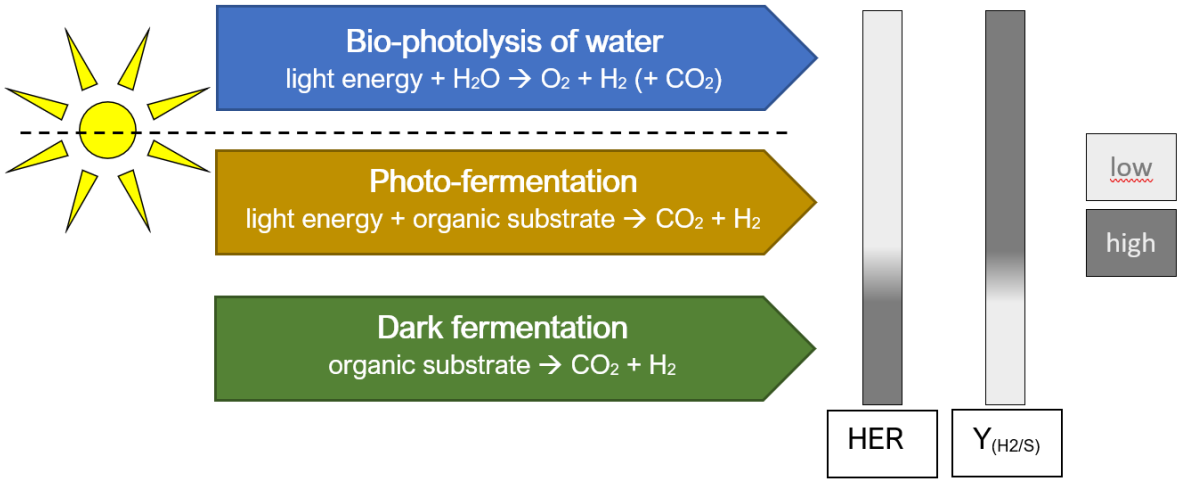

Figure 1: Shown are the three main routes for bio-H_2 production plus their respective overall reaction. The sun symbol indicates the light dependency of the two upper processes (Bio-photolysis of water and Photo-fermentation). The dashed line separates the aerobic process (Bio-photolysis of water) from the anaerobic processes (Photo-fermentation and Dark fermentation). Two main features, HER and $Y_{(H2/S)}$, are indicated.

It is worth noting that hybrid systems composed of photosynthetic and fermentative bacteria exist as well. This combination of dark fermentation by fermentative bacteria and photo-fermentation by, for example, purple non-sulphur (PNS) photosynthetic bacteria, can enhance the overall H_2 yield to a great extent (Morsy 2017; Basak and Das 2007).

1.2.1. Bio-photolysis of water

Many photosynthetic organisms, both eukaryotic and prokaryotic, are able to perform bio-H_2 production via photolysis of water.

(1) $12H_2O \rightarrow 2H_2 + 6O_2$ (Green Algae)

(2) $CO + H_2O \rightarrow H_2 + CO_2$ (Photosynthetic bacteria)

This is considered a very clean energy conversion based on two globally available and abundant resources: water and sunlight. Cyanobacteria and green microalgae possess different pigments to capture light energy and generate both reducing agents and ATP that are used for carbon dioxide fixation. During this process, the reducing equivalents generated during photosynthesis can be used for the reduction of protons via hydrogenase or nitrogenase. Biophotolytic bio-H_2 production can be further divided into direct or indirect biophotolysis (Yu and Takahashi 2007). Light harvesting pigments in photosystem I and/or photosystem II absorb the light energy to raise the energy level of electrons from water oxidation. During direct biophotolysis these electrons are passed on to redox equivalents and eventually produce H_2. Microorganisms performing indirect biophotolysis use these electrons first for the fixation of CO_2 to produce carbohydrates (such as starch or glycogen). The stored energy is released through fermentation under dark conditions and excess electrons are passed on to protons (H^+) forming molecular H_2. This two-step process allows either temporal or spatial separation of molecular oxygen (O_2) and H_2 evolution. This is favourable for bio-H_2 production as the H_2 generating enzymes, hydrogenases and nitrogenases, are highly sensitive to the oxygen evolving from the water splitting process - even low concentrations of O_2 can lead to their deactivation. Furthermore, this separation simplifies H_2 purification. It does, however, present some challenges. Although being a clean and direct energy conversion, biophotolysis of water is limited by its relatively low H_2 and energy productivities, resulting in low H_2 yields per substrate (Yu and Takahashi 2007).

1.2.2. Photo-fermentation of organic compounds

Photosynthetic bacteria are able to use light energy for the conversion of most organic acids or volatile fatty acids (VFAs) to bio-H_2 and carbon dioxide under anaerobic conditions.

(1) $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$ (Phototrophic bacteria)

This anoxygenic photo-fermentation is well-established in PNS bacteria, where this metabolism is mainly associated with the action of hydrogenase and nitrogenase enzymes (Hay et al. 2013). The usage of PNS bacteria for bio-H_2 production has multiple advantages. In addition to a high substrate to product conversion yield and the ability to use a wide light spectrum, they also lack O_2-evolving activity. Furthermore, the possibility to ferment organic substrates has potential applications in bioremediation processes (Basak and Das 2007).

Both bio-photolysis of water and photo-fermentation are limited in their application due to the necessity of providing sufficient light energy to the cultivation vessel. In addition, the solar energy conversion efficiency under full sunlight is quite low (Bolton 1996), placing intense economic restrictions on light-driven processes (Hallenbeck and Benemann 2002). Regarding bio-photolysis another potentially limiting factor for industrial applications is the presence of O_2 which impairs hydrogenase and nitrogenase activity and would therefore have to be removed, or physically or temporally separated from the H_2 production site (Rathore et al. 2019).

1.2.3. Dark fermentative bio-H2 production

During dark fermentative bio-H_2 production, carbohydrate-rich substrates are broken down anaerobically by H_2 producing organisms, including both facultative and obligate anaerobes. H_2 evolves during the process of disposing excessive electrons generated by the oxidation of organic substrates (Ghimire et al. 2015).

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

Compared to other bio-H_2 production processes, dark fermentative bio-H_2 generation has gained a lot of attention since many different types of organic matter, including even waste streams, can serve as potential substrates for H_2 generation (Hay et al. 2013). Further advantages include higher HER rates, lack of O_2 evolution, and dispensability of providing light energy to the process, which in turn facilitates application in volumetrically large vessels (Hallenbeck and Benemann 2002). Nevertheless, due to metabolic restraints, the $Y_{(H2/S)}$ is lower compared to bio-photolysis and photo-fermentation (Rittmann and Herwig 2012; Lee et al. 2011; Thauer et al. 1977; Hallenbeck 2005).

1.3. Metabolism and limitations in dark fermentative bio-H_2 production

H_2 metabolism, including H_2-sensing, electron-bifurcation, or using H_2 as an energy vector, is very diverse and widely distributed among prokaryotes (Greening et al. 2016). Microorganisms synthesizing H_2 are commonly found in environments such as soil, wastewater sludge, and other waste streams. Samples thereof can directly serve as an inoculum for fermentative H_2 production (Li and Fang 2007; Chang et al. 2008).

Dark fermentative microorganisms generate H_2 by consuming carbohydrates and producing organic acids and alcohols as by-products. Organic compounds that can be used as substrates include carbohydrates, polymers, organic waste, carbohydrate-rich substrates from industrial or domestic sources, and C1 compounds (Rittmann et al. 2015; Rittmann and Herwig 2012). In literature, most of the pure culture fermentations were performed using monosaccharides, followed by disaccharides, polysaccharides, alcohols, organic acids, formate, mixtures of individual compounds, and complex compounds. Lacking O_2 as a final electron acceptor, anaerobic fermenters reduce metabolic intermediates (e.g., pyruvate) instead, resulting in different metabolic end products.

Another possibility to dispose of the “excess” electrons is via the formation of H_2 (Das 2001). There are two noteworthy points to consider for this process: Firstly, H_2 is never produced as the single reduced compound, but is instead produced in combination

with multiple other by-products, mainly VFAs and alcohols; secondly, microbes do not benefit from H_2 evolution per se, but it is necessary to prevent an accumulation of excess electrons that might disturb the electron flow within the cell.

When fermenting glucose, substrate level phosphorylation generates adenosine triphosphate (ATP). Simultaneously, 2 moles of reduced redox equivalents (e.g., NADH) and pyruvate are formed.

(3) $C_6H_{12}O_6 + 2NAD^+ + 2ADP + 2Pi \rightarrow$

$2CH_3COCOO^- + 2NADH + 2ATP + 2H_2O + 2H^+$

To recover electron accepting redox equivalents (e.g. NAD^+), the electrons are passed on to pyruvate, eventually leading to the formation of different metabolic end products, like ethanol or lactate in facultative anaerobes, or ethanol, butyrate, butanol, and acetone in strict anaerobes (Hallenbeck and Ghosh 2012).

Alternatively, pyruvate can be converted to acetyl coenzyme A (acetyl-CoA) together with reduced ferredoxin (Fd_{2-}) and CO_2, or formate.

(4) Pyruvate + CoA+ 2Fd $\rightarrow$ acetyl-CoA + CO_2 + $2Fd^{2-}$ (PFOR)

(5) Pyruvate + CoA $\rightarrow$ acetyl-CoA + formate (PFL)

The two general metabolic pathways performing this conversion are 1) the pyruvate formate lyase (PFL) pathway; and 2) the pyruvate ferredoxin oxidoreductase (PFOR) pathway (Cabrol et al. 2017).

In both of these biological systems, the pyruvate generated by glycolysis can be used to produce acetyl-CoA in the absence of O_2. Acetyl-CoA is further reduced to a range of different fermentation products or split into ATP and acetate (Hallenbeck 2005). The formation of acetate therefore represents the route yielding the maximum amount of ATP. Missing a recovery step for the reduced redox equivalents, the excessive electrons can be passed on to H_2 ions (H^+) generating H_2 (Wolfe 2005).

1.3.1. Pyruvate ferredoxin oxidoreductase and pyruvate formate lyase pathway

Following the PFOR pathway, the Fd^{2-} is oxidised by a ferredoxin-dependent hydrogenase which leads to the formation of H_2.

(6) $Fd^{2-} + 2H^+ \rightarrow Fd + H_2$, $\Delta G°' = -1.14$ kJ $(mol\ H_2)^{-1}$ (Jay et al. 2020)

Reduced ferredoxin can also be generated by oxidizing the NADH produced during glycolysis via NADH:ferredoxin oxidoreductases (Vardar-Schara et al. 2008). The regeneration of NAD+ is an important requirement for the oxidation of glucose. During fermentation acetyl-CoA follows different metabolic pathways using up reducing equivalents. As the NADH is redirected towards the generation of specific side products, these compete with the H_2 producing pathway. The maximum H_2 yield that can be obtained via the PFOR pathway is 2 moles per one mol of glucose. If the H_2 partial pressure is low (below 0.1 kPa) two additional moles of H_2 can be produced by reoxidizing the NADH produced during glycolysis.

Following the PFL pathway, a membrane-bound formate H_2 lyase (FHL) complex splits formate into H_2 and CO_2 (Amend and Shock 2001):

(7) $HCOO^- + H_2O \rightarrow HCO_3 + H_2$, $\Delta G°' = +1.3$ kJ $(mol\ H_2)^{-1}$

Initially, the FHL complex evolved to prevent the accumulation of formate, which in higher amounts is toxic to the cell (Bagramyan et al. 2002). Accumulation of dissociated acids might impair the pH gradient across the membrane and inhibit metabolic and cellular functions (Herrero et al. 1985). This effect is enhanced under acidic conditions, which is why under lower pH some organisms tend to re-uptake formate for detoxification, which might speed up formate-based H_2 generation (Hakobyan et al. 2005; Ergal et al. 2018). The enzymatic activity of FHL is highly dependent on high formate concentrations, which is why formate degradation is often incomplete, leading to less than stoichiometric H_2 yield. Organisms capable of the PFL pathway are in theory limited to only 2 moles of H_2 per glucose since 2 moles of pyruvate are produced by one mole of glucose (see eq. 1).

Some microbes possess other hydrogenases and are able to directly re-oxidise the NADH generated during glycolysis, producing additional H_2 molecules.

(8) $NAD(P)H + H^+ \rightarrow NAD(P)^+ + H_2$,

$\Delta G^{\circ\prime} = +18.2$ kJ $(mol\ H_2)^{-1}$ (Jay et al. 2020)

In contrast to H_2 generation via Fd^{2-} and formate, the transfer of electrons from NAD(P)H to H^+ has a relatively high positive standard potential even under physiological conditions, where the H_2 partial pressure is low. Being thermodynamically unfavourable, this reaction would need energy input to take place (Lee et al. 2008a; Verhaart et al. 2010). This leads to NADH recovery through electron transfer to temporary metabolites and eventually ethanol, lactate, or butyrate. As a consequence, less acetyl-CoA would be available for the energy yielding conversion into acetate and only 2 moles of ATP would be produced (van de Werken et al. 2008).

These H_2 producing pathways can be carried out by various dark fermentative microorganisms, including strict anaerobes (e.g. Clostridia), facultative anaerobes (e.g. Enterobacter, Escherichia coli, or Citrobacter) and even aerobes (Alcaligenes, Bacillus) (Li and Fang 2007). While many organisms contain both systems, one usually domains during the fermentation process (Hallenbeck 2009). Strict anaerobic organisms perform the PFOR pathway, while the PFL pathway is active in facultative anaerobes (van de Werken et al. 2008; Verhaart et al. 2010). H_2 production via NADH-dependent hydrogenases has been described for both obligate anaerobes (Soboh et al. 2004; Losey et al. 2017) and facultative anaerobes (Nakashimada et al. 2002).

1.3.2. Electron bifurcation

Certain microbial species are able to couple the exergonic oxidation of Fd^{2-} with the endergonic electron transfer through NADH. Organisms performing this mechanism are capable of using electrons from both NADH and Fd^{2-} simultaneously for H_2 production (Buckel and Thauer 2013; Li et al. 2008; Jay et al. 2020).

(9) $2Fd^{2-} + NAD(P)H + 3H^+ = 2\ Fd + NAD(P)^+ + 2H_2$,

$\Delta G^{\circ\prime} = +8.51$ kJ $(mol\ H_2)^{-1}$

These specialised hydrogenase complexes can recover most of the NAD(P)H and increase the ATP yield. Under optimal environmental conditions, 4 moles of ATP can

be produced, with acetate being the main metabolic end product (Schut and Adams 2009). Other complexes concomitantly reduce butyryl-CoA from crotonyl-CoA. This is called flavin-based electron bifurcation and yields, in total, 3.3 moles of ATP (Buckel and Thauer 2013; Li et al. 2008).

If the NAD(P)H to Fd^{2-} ratio is not in equilibrium, the electron bifurcation can be coupled to H^+/Na^+ translocation over the cytoplasmic membrane (Buckel and Thauer 2013). Alternatively, in some organisms this gradient can be built up by membrane bound hydrogenases using Fd^{2-} formed via activity of glyceraldehyde pyruvate oxidoreductase (Schäfer and Schönheit 1991). Adenosine triphosphatases then use this gradient to produce ATP (van de Werken et al. 2008).

In all these different metabolic strategies, improved $Y_{(H2/S)}$ is generally coupled to higher yields of ATP per substrate consumed. As mentioned before, the end product of the pathway with the highest energy yield when anaerobically fermenting glucose, is acetate. The overall metabolic reaction yields 4 moles of H_2.

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

Compared to this, the production of ethanol, lactic acid, or butyrate via glucose fermentation would use up redox equivalents and yield less or no H_2.

(11) $C_6H_{12}O_6 \rightarrow 2CH_3CH(OH)COO^-$

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

(13) $C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2H_2$

Theoretically, 12 moles of H_2 could be produced with the complete oxidation of glucose.

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

However, dark fermentative bio-H_2 production is performed under anaerobic conditions. The actual maximum is therefore limited to 4 mol. This theoretical limit is referred to as the Thauer limit, proposed by Thauer *et al.* (1977). *In vivo* this theoretical limit is never reached, since H_2 generation via NADH oxidation is thermodynamically unfavourable, as discussed above.

1.3.3. Limiting parameters

It is important to keep in mind that not only the underlying physiological constraints of the organism determine the $Y_{(H2/S)}$. The amount of H_2 produced, including HER and qH_2, depends on the environmental or bioprocess conditions present in the fermentation vessel. pH, temperature, H_2 partial pressure, substrate composition and concentration, and initial cell concentrations are among the most influential parameters for microbial growth, metabolism, and production of H_2.

1.3.3.1. Limiting effect of pH

The pH is among the most important factors influencing the performance of bio-H_2 production, having a key role in the regulation of metabolic pathways (Chandrasekhar et al. 2015). Internal pH, proton motive force, and membrane potential are all directly impacted by the acidity or alkalinity of the medium. Furthermore, the ambient pH influences metabolic pathways and the durations of lag-phases, and affects the FeFe-hydrogenase activity (Tanisho et al. 1987; Dabrock et al. 1992). As H_2 generating microorganisms produce organic acids during dark fermentation, the pH decreases. Some publications report a delay in reaching inhibiting acidic conditions when the initial pH is set higher (Zhu and Yang 2004; Mitchell 1997). A lower initial pH (4.0 - 4.5) might extend the lag phase (Khanal et al. 2004), whereas a higher initial pH (8.0 - 9.0) decrease the lag phase, but with lower $Y_{(H2/S)}$ (Zhang et al. 2003).

Additionally, enzymatically catalysed reactions during metabolism are affected by the concentrations of H^+ ions as they influence the ionization state of the involved amino acids. This can therefore influence the rate at which the reaction takes place (Fabiano and Perego 2002).

Acidic or alkaline conditions directly influence the microbial community composition, particularly the H_2 producing fraction, in the bioreactor (Yasin et al. 2011). pH acts as an ecologically critical factor, determining the availability and/or toxicity of the present compounds (Li et al. 2007; Cai et al. 2010).

In terms of bio-H_2, the best pH for maximum H_2 gas production and minimum solvent production has been reported to be in the range 5.5-6.0 (Chandrasekhar et al. 2015). Indeed, pH values within or close to this range have been widely used and found to yield optimum H_2 production. For example, Tanisho et al. (1987) reported the strong influence of pH on the H_2 productivity of *Enterobacter aerogenes*, as only half of the production rate was possible at a pH of 7.0 compared to the rate at the optimum pH of 6.0-6.5. Similarly, the optimum pH for an *E. aerogenes* and *Clostridium butyricum* co-culture was around 5.5-6.5. Below a pH of 5.5, *Clostridium butyricum* hardly showed growth with H_2 production (Pachapur et al. 2017).

1.3.3.2. Limiting effect of temperature

The ambient temperature during fermentation largely influences the H_2 production rate, substrate consumption, formation of metabolites, and performance of the involved organisms. Even though thermophiles show the best results for $Y_{(H2/S)}$, mesophilic conditions are more favourable regarding HER, technical set up, and process expenses (Rittmann and Herwig 2012; Hallenbeck 2009). One disadvantage of operating within mesophilic temperature ranges lower hydrolysis rate of complex substrates compared to thermophilic conditions (Singh et al. 2015). Furthermore, at lower temperatures, the different reactions during H_2 production are more endergonic, and therefore less favourable (Conrad and Wetter 1990).

H_2 production rate, substrate consumption, and the formation of metabolites are directly influenced by the ambient temperature. It is therefore of crucial importance to investigate the optimum temperature for H_2 generation in each set up and each organism.

1.3.3.3. Limiting effect of H_2 partial pressure

As hydrogenase enzymes are highly sensitive to feedback inhibition, the H_2 partial pressure plays another key role in the efficiency of the H_2 production (Skidmore et al. 2013). An increased level of dissolved H_2 in the medium leads to thermodynamically unfavourable conditions for oxidation of redox equivalents, decreased H_2 production, and a shift from acidogenesis to solventogenesis (van de Werken et al. 2008; Mohan et al. 2013; Nath and Das 2004). To remedy this, H_2 gas from the headspace can be removed to maintain a low H_2 partial pressure, increasing the productivity (Mohan et

al. 2013). This has been further demonstrated by Angenent et al. (2004), who achieved higher H_2 yields under low H_2 partial pressure.

1.3.3.4. Limiting effect of substrate

As most research in fermentative bio-H_2 production is still ongoing, lab-scale experiments often use pure or synthetic substrates to evaluate the potential of the respective fermentation processes. Next to cellulose, cellobiose, arabinose, xylose, and glycerol, the model substrates most commonly used are glucose, sucrose and starch. Plant-based substrates, such as agricultural waste (Guo et al. 2010), being renewable, widely available, and inexpensive, do have the disadvantage of containing high amounts of cellulose, hemicellulose and lignin, which are barely degradable (Nowak et al. 2005) and would require pre-treatment or consolidated bioprocessing.

Alternatively, industrial and municipal waste streams, including distillery wastewater, food and beverage processing wastewater, municipal solid waste, domestic wastewater, sludge, etc. have been used for anaerobic fermentation (Mishra and Das 2014; Li et al. 2014; Kothari et al. 2017). These forms of organic material have a high potential for sustainable bio-H_2 production. Carbohydrates, proteins, and lipids are highly concentrated in these substrates, which may impair or slow H_2 generation since initial digestion and break-down into simpler sugars is necessary.

Another aspect of the substrate used that is likely even more important is its initial concentration. Multiple studies on dark fermentative bio-H_2 production have shown that neither substrate excess nor limitation favour maximum H_2 production (Fabiano and Perego 2002; Jo et al. 2008a; Jo et al. 2008b; van Niel et al. 2003). Under substrate limiting conditions, the organism needs to gain as much energy as possible. Nevertheless, this limitation affects the metabolic flux rate, and the fermentation process is slowed down if the sugar concentrations are low. At very high initial concentrations however, the substrate causes inhibitive effects. Under these conditions, metabolic pathways are shortened and lead to the generation of other side products without additional ATP, such as lactate or ethanol ith respect to dark fermentative H_2 production, Hawkes et al. (2007) estimated the upper limit of substrate concentration for sucrose or glucose to be around 30 g L^{-1}. Higher concentrations might lead to a decrease in the molar H_2 yield.

The selection for the most suitable substrate type and concentration is therefore highly dependent on the organism's enzymatic repertoire and the respective H_2 generation pathway.

1.3.3.5. Limiting effect of initial cell concentration

An additional factor that greatly influences the system's performance is the initial concentration of the H_2 producers. Too little microbial biomass at the beginning of the process might result in a very long lag phase before significant growth and production initiates. If the cell concentration too high, imbalance and competition may impair high H_2 productivity. This is especially true for co-culture approaches, where two or more organisms are inoculated together to establish a stable co-existence through symbiotic interactions, thus increasing the $Y_{(H2/S)}$ (Ergal et al. 2018).

Pachapur et al. (2017) implemented a co-culture in closed batch to produce H_2 from crude glycerol, a waste product of biodiesel generation. Using *E. aerogenes* and *Clostridium butyricum* for the closed batch fermentation, they investigated the effect of different inoculum ratios of these two organisms. It was clearly shown that, among other parameters, the initial concentration of the microorganisms had a significant impact on the H_2 productivity. The fast-growing *E*. *aerognenes* might cause unfavourable conditions for *C. butyricum*, which is why increasing the initial concentration of the latter enables a more stable co-existence. Of all the initial ratios tested, ranging from 1:11 to 3:1 (Enterbacter to Clostridim), the highest one in favour of *C. butyricum*, resulted in the highest H_2 production.

Similarly, Geng et al. (2010) reported a slight increase in H_2 production when adjusting the initial ratio of *C. thermopalmarium* to *C. thermocellum.*

All these parameters significantly influence the production performance. Therefore, the best strategy to improve dark fermentative bio-H_2 generation is through detailed understanding and optimisation of the environmental conditions. As these are very individual for each strain, it is crucial to know about each of the organisms' specific needs to ensure maximum H_2 production.

1.4. Dark fermentative bio-H_2 in literature

Ergal et al. (2018) performed a comprehensive literature review, collecting data from 305 scientific publications on dark fermentative bio-H_2 production from pure cultures. The authors collected information on $Y_{(H2/S)}$, HER, qH_2, temperature (°C), pH, dilution rate (D, [h^{-1}]), substrate, substrate type, medium type (defined or complex), initial substrate concentration (mmol L^{-1}), and microorganisms, with the intention of gathering the available information and identifying the best parameter settings for optimal H_2 production for each taxonomic family.

Comparing the different substrates used for H_2 production, the authors clearly concluded that, on a C-molar level, formate-based fermentation obtained the highest values for $Y_{(H2/S)}$, HER, and qH_2. This is probably due to the fact that when using formate as a substrate, high throughput is necessary for the organism to avoid toxification and to compensate for the low energy gain.

Analysing the huge variety of microbial groups performing H_2 production, the authors found that the majority of the studies on dark fermentative bio-H_2 production focused on two bacterial groups: *Clostridiaceae* and *Enterobacteriaceae*. They point out that, to them, it is not obvious why this trend is observed in literature, as many other bacterial, archaeal, and even eukaryal groups can be considered for H_2 production. This is particularly surprising since their meta-analysis revealed a member of the archaeal group *Thermococcaceae* to be the best performing organism: when grown on formate, *Thermococcus onnurineus* exhibited the highest $Y_{(H2/S)}$, HER, and qH_2 (Ergal et al. 2018; Lee et al. 2012; Lim et al. 2012). One reason for this, according to Hallenbeck (2005), is that higher $Y_{(H2/S)}$ in hyperthermophilic organisms is related to the thermodynamics of enzymatic reactions.

A similar trend regarding the performance of known microbial families for bio-H_2 production has been found by Kothari et al. (2017). Their literature survey revealed that *Thermoanaerobacterium spp*., among thermophiles, and *Enterobacteriaceae* and *Clostridiaceae* among mesophiles are the most popular bacterial H_2 producers.

Within mesophilic strains, *Enterobacteriaceae* and *Clostridiaceae* also revealed high HER values on a C-molar basis. Members of these groups have been shown to play

key roles in H_2 producing systems with high productivity, as discussed below. In addition to that, the wide range of publications available for these organisms allows specific meta-analysis of their performance, growth properties, and optimal parameter settings. Because of this, Ergal et al. (2020) chose two members of the bacterial groups *Clostridiaceae* and *Enterobacteriaceae* growing on glucose for H_2 production in co-culture: *Clostridium acetobutylicum* and *E. aerogenes*.

1.4.1. Clostridium acetobutylicum

Members of the *Clostridiaceae* are known to be among the most abundant and efficient H_2 producing bacteria (BHP) (Maintinguer et al. 2008; Chu et al. 2009; Hung et al. 2011; Masset et al. 2012; Laothanachareon et al. 2014; Chang et al. 2008). For example, in a study by Fang *et al.* (2002), they found that more than half of all the microorganisms within a mesophilic mixed culture obtained from wastewater sludge used for dark fermentative H_2 production belonged to *Clostridium*. Moreover, high H_2 yields (using saccharides; measured on a C-molar level) and HER within mesophilic prokaryotes have been reported for these obligate anaerobes (Ergal et al. 2018; Pan et al. 2008). H_2 production is performed *via* the PFOR pathway and enhanced through a flavin-based electron bifurcation complex (Li et al. 2008; Buckel and Thauer 2013).

Members of *Clostridium* are gram-positive, endospore forming bacteria, including toxin-producers as well as non-pathogens. Some terrestrial strains are of biotechnological relevance, such as lactate, butyrate, ethanol, acetate, and butanol producers (Latifi et al. 2019). The model organism performing these biosyntheses is *C. acetobutylicum.* It was discovered in the early 1920's and naturally produces acetone, butanol, and ethanol during solventogenesis (Weizmann and Rosenfeld 1937; Lütke-Eversloh and Bahl 2011). Equally diverse is the variety of carbohydrates that can be used as substrate, such as pentoses, hexoses, oligosaccharides, and polysaccharides (Servinsky et al. 2010). Cellulose cannot act as a carbon source, in spite of cellulosome genes being present and expressed (Lütke-Eversloh and Bahl 2011).

The life cycle of *C. acetobutylicum* is comprised of three different growth phases: acidogenesis, solventogenesis, and endospore formation. During the first phase cell

growth is exponential, easily fermentable carbon sources are depleted, and the produced metabolites are mainly acetic and butyric acid. Accumulation of these fermentation products cause a drop in the pH below 5 and a fundamental switch of the organism's metabolism as the culture approaches stationary phase. To prevent further acidification of the media, the organism switches from an acidogenic to a solventogenic metabolism, during which the previously produced acids are taken up again and transformed to solvents, mainly acetone and butanol (Lee et al. 2008b). During this stationary phase, also called the "clostridial stage" cells start to synthesize granulose as an intracellular storage compound. Subsequently and independently from solventogenesis, sporulation is initiated (Scotcher and Bennett 2005). The produced granulose presumably serves as an energy and carbon source during endospore formation (Reysenbach et al. 1986).

As mentioned before, H_2 production by *Clostridia* is performed by the PFOR pathway, where reduced ferredoxin is the physiological electron donor for different hydrogenases to reduce protons and generate H_2. From the three different hydrogenases known (Fe-, NiFe- or FeFe-hydrogenases) monomeric Fe-Fe-hydrogenases together with a wide range of putative hydrogenases (including both NiFe- and FeFe-type) are active in *Clostridia*. Highly specific H_2 uptake activity and catalytic efficiencies have been reported for the *C. acetobutylicum* FeFe-hydrogenase, producing H_2 from reduced ferredoxin or flavodoxin. This high potential in H_2-related activities is conserved with different electron carriers and has promising technological applications (Demuez et al. 2007). The diversity of hydrogenases in Clostridiaceae demonstrates the central role these enzymes play in metabolism and the complexity of H_2 generation in this phylogenetic group (Calusinska et al. 2010).

For *C. acetobutylicum* the optimum glucose concentration for bio-H_2 production in closed batch has been reported to be 8 g L^{-1} (Shaterzadeh and Ataei 2017). Similarly, Alshiyab et al. (2008b) found that an initial glucose concentration of 5 g L^{-1} led to maximum H_2 yields. Additional reported optimal culture conditions are a pH of 6-7 and a temperature of ~37°C (Chin et al. 2003; Shaterzadeh and Ataei 2017; Alshiyab et al. 2008b). Deviation of these factors drives a decrease in H_2 production. Lowering the pH leads to a shift in the carbon flux towards butanol production and in the cell's internal

pH, meaning little to no net H_2 production. In alkaline conditions on the other hand, the cells tend to clump, decreasing glucose uptake. This organism's H_2 production is further prone to inhibition resulting from accumulation of butyrate or acetate (Chin et al. 2003).

1.4.2. Enterobacter aerogenes

Next to obligate anaerobic fermenters, much research has been directed towards facultative anaerobic fermentative bacteria. This is largely driven by their lower sensitivity to O_2, which can be quite advantageous for their application in bio-H_2 production. Moreover, their high H_2 productivity is comparable to those of *Clostridium* sp. cultures (Patel et al. 2014).

One family worth mentioning here would be the *Enterobacteriaceae,* which produce H_2 by the PFL pathway and have been reported to be very abundant in H_2 producing mixed cultures growing on organic waste. (Marone et al. 2014). Based on a quantitative review by Ergal *et al.* (2018) members of Enterobacter are among the best performing phylogenetic groups regarding HER (Ito et al. 2005; Shin et al. 2010) and qH_2 (Seol et al. 2008; Martinez-Porqueras et al. 2013). Among these, *E. aerogenes* is among the most well-studied model organisms for dark fermentative H_2 production and was found to exhibit extraordinarily high H_2 production activity compared to other members of this microbial family (Seol et al. 2008; Zhang et al. 2011; Jayasinghearachchi et al. 2009). This non-spore forming, gram-negative, and rod-shaped gamma proteobacterium has been applied in multiple dark fermentative H_2 production studies so far. Being a facultative anaerobe, this organism's growth can be easily manipulated. The metabolism of *E. aerogenes* is well established and its H_2 production can also be enhanced through genetic modifications (Zhao et al. 2009). Given its high growth rate and H_2 production rate, *E. aerogenes* has the potential to be applied in large-scale H_2 production (Zhang et al. 2011). This organism has been found to produce H_2 using crude glycerol as a sole substrate, in the absence of additional media supplements (Sarma et al. 2013).

There are two distinct routes of bio-H_2 production operating in this organism: the formate pathway and, depending on the thermodynamic equilibrium, the enzymatic conversion of NADH and protons to H_2. The formate hydrogen lyase (FHL) pathway can yield 2 moles of H_2 per one mol of glucose by converting 2 moles of formic acid into CO_2 and H_2. The formic acid is generated via the PFL enzyme from the pyruvate produced during glycolysis, which is also converted to acetate, ethanol, CO_2, H_2, 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol. Under anaerobic conditions, 4 moles of NAD(P)H are generated from one mole of glucose that would potentially be available for H_2 generation. Nonetheless, this pathway is thermodynamically limited by the partial pressure of H_2 in the cell, which has to be less than 42 Pa in order for the process to take place. Flavin-based electron bifurcation to couple NADH oxidation to that of reduced ferredoxin is not present in *Enterobacteriaceae*. Increasing temperature and decreasing pH are two possible ways to increase this partial pressure limit. Therefore, the application of thermotolerant and acid tolerant mutants would prove beneficial for H_2 production. NADH levels predominantly influence the H_2 yields, which linearly increase with the substrates' degree of reduction. The production of by-products consumes reduction equivalents, which is why mutants deficient in acid or alcohol production showed improved H_2 production (Ito et al. 2004; Zhang et al. 2009; Zhang et al. 2011).

Fabiano and Perego (2002) reported optimum H_2 productivities for *E. aerogenes* at a pH range of 6.1-6.6 and a temperature of 40°C. Up until 40°C H_2 productivity linearly increased, but rapidly decreased after this threshold. The authors also observed that maximum productivity was accomplished with initial glucose concentrations of 20-30 g L^{-1} (Fabiano and Perego 2002). Jo et al. (2008a) reported similar results, with an optimum pH of 6.13, optimum temperature of 38°C, and an optimum glucose concentration of around 21 g L^{-1}.

1.5. Strategies to enhance the process efficiency

As mentioned above, the maximum theoretical yield for bio-H_2 production is limited to 4 moles of H_2 per mole of glucose (Thauer et al. 1977). Part of the substrate is utilized

for other cellular activities, such as biomass production or substrate degradation. This implies that natural microbial systems never reach theoretical maxima regarding H_2 productivity (Hallenbeck 2005).

To make the process more feasible for large-scale implementation, ongoing research in the field of bio-H_2 production has given rise to several different approaches: selection of suitable (pre-treated) substrates, inoculum enrichment strategies, bioaugmentation, hybrid applications, or the engineering of the organisms' metabolism and/or the ecosystem in which the producers co-exist. It is important to keep in mind, and this is true of any approach, that the optimal strategy has to be determined for each specific condition individually (Cabrol et al. 2017).

1.5.1. Pre-treatment methods

Conventional pre-treatment methods of the inoculum can ensure the presence of spore forming H_2 producers, such as *Clostridiaceae* in self-selecting microbiomes for H_2 production. These organisms have an advantage over non-spore forming bacteria, such as methanogens, as they can survive harsh conditions and germinate again under favourable conditions. For example, heat treatment of mixed cultures is a simple, inexpensive and effective method for pre-treating and selecting such spore-formers (Wang and Wan 2009; Ren et al. 2008). Other methods include selectively inhibiting methanogens present in anaerobic sludge, such as by chemical pre-treatment and aeration (Wang and Wan 2008). It must be mentioned here, however, that undesirable microbial groups, such as methanogens or lactic acid bacteria, can persist within the fermenter, even if the inoculum has been heat-shock pre-treated and the fermentation is carried out at low pH (Chu et al. 2009; Monlau et al. 2013; Oh et al. 2003).

Furthermore, in environments where the main H_2 producers are not equally adapted to environmental stress conditions or where non-spore formers play a crucial role in facilitating H_2 production, these methods could have negative consequences for the performance of the fermenter (Ohnishi et al. 2010). Adequate pre-treatment strategies are necessary to reduce the abundance of microbial competitors, but might not be

sufficient to ensure the presence of the best performing H_2 producers in the system (Kotay and Das 2009).

1.5.2. Bioaugmentation

As an alternative to pre-treatment of the inoculum, bioaugmentation has been proposed to artificially increase the proportion of H_2 producers. Through enriching complex inocula with specific H_2 producing microbes, the metabolic pathways would presumably be driven towards the desired product. The exact microbial composition of these Enrichment cultures is unspecified making this approach only suitable for undefined consortia. Pattra *et al*. (2011) reported increased H_2 productivities as a bioaugmented *Clostridium* strain coexisted with indigenous microbes. Similarly, additional inoculation with *C. butyricum* shortened the lag phase and doubled the H_2 production rate and concentration in a brewery yeast waste fermentation system (Jen et al. 2007). Even at large pilot scale, bioaugmentation strategies proved effective. Co-cultures of *Citrobacter freundii*, *E. aerogenes,* and *Rhodopseudomonas palustris* increased the $Y_{(H2/S)}$ and exceeded those of the pure cultures (Vatsala et al. 2008). Izzo *et al*. (2014) used a mixed Enterobacter and *Clostridium* inoculum for bioaugmentation to efficiently produce H_2 in an anaerobic glucose fed reactor with high yields and the ability to use a wide range of complex substrates. These findings further indicate the beneficial association of facultative and strict anaerobes.

However, bioaugmentation strategies require previous and mostly time-consuming isolation and culture steps. These might be hampered as well by the limited cultivation possibilities of the natural microbial diversity *in vitro*, leading to the loss of less abundant species. Therefore, different acclimatization strategies without the need to isolate pure strains have been suggested and successfully implemented in batch and continuous batch fermentations (Ren et al. 2010; Varrone et al. 2013). In any case, it is essential that the strains of interest, performing key functions (bio-H_2 production, cellobiose degradation, *etc.*), are of sufficient abundance in the original mixed community, in order to guarantee their presence in the reduced consortium used for inoculation (Cabrol et al. 2017).

1.5.3. Metabolic engineering

Genetic modification of bio-H_2 producing organisms has been successfully carried out for a number of different taxonomic families, including both *Enterobacteriaceae* and *Clostridiaceae*. Given the central role of the hydrogenase enzyme for the H_2 production pathways, enhancing hydrogenase activity presumably improves H_2 yields. Both Morimoto *et al*. (2005) and Mishra *et al*. (2004) reported an increased H_2 and acetic acid production when overexpressing the FeFe-hydrogenase gene for *Clostridium paraputrificum* and *Enterobacter cloacae*, respectively. Abendroth et al. (2008) reported high H_2 production in *C. acetobutylicum* when overexpressing the FeFe-hydrogenase via homologous recombination. Modifications during the PFL pathway were carried out in *Escherichia coli* to improve formate splitting. Inactivation of the formate hydrogen lyase repressor, combined with the overexpression of its activator, led to 4 fold increase in H_2 production rates compared to the control strain (Bohnenkamp et al. 2021).

Another approach is the elimination of unfavourable pathways that compete with the production of H_2. As mentioned above, the production of butyrate is consuming a big proportion of the produced NADH that could otherwise be used for the reduction of protons to H_2. The disruption of the *hbd* gene encoding the β-hydroxybutyryl-CoA dehydrogenase enzyme, involved in the butyrate formation pathway, was shown to increase H_2 and decrease ethanol production in *C. butyricum* (Cai et al. 2011). Nevertheless, as the authors reported, the changes in H_2 and ethanol production were highly dependent on the partial pressure of H_2 in the system. Other genetic modifications, such as deletion of a negative regulator for FHL and the genes encoding uptake hydrogenase, lactate dehydrogenase, and fumarate reductase, proved to enhance H_2 yields in *E. coli* (Manish et al. 2007; Kim et al. 2009). Similarly, downregulating the expression of uptake hydrogenases, as well as overexpression of FeFe-hydrogenase encoding genes have shown to boost H_2 productivities (Nakayama et al. 2008; Jo et al. 2010).

The ability to degrade lignocellulosic material concurrently to H_2 production would open up opportunities for using waste streams as feedstock. Overexpression of hemicellulases, cellulases, and lignases has been performed (Chandel et al. 2012; Thomas et al. 2014). Cloning and expression of xylose-utilization genes in *Clostridium thermocellum* allowed the co-fermentation of sugars derived from cellulose and hemicelluslose and increased the H_2 production (Xiong et al. 2018).

However, regardless of many successful applications, metabolic engineering of H_2 producers is still facing many challenges in improving H_2 yields. As Kim *et al.* (2009) discussed in their work, genetically modified cell requirements might be incompatible with the reactor conditions. Furthermore, when applying genetically engineered strains in continuous large-scale bioreactors, fed with unsterile biowaste substrates, contamination and out-competition by substrate endogenous strains, or even dispersion of the modified strains into the environment, can pose serious problems (Cabrol et al. 2017).

1.5.4. Hybrid application

As discussed earlier, photo-fermentative microorganisms are able to utilize short-chain organic acids, accumulated during dark fermentation, as a substrate for H_2 production. Therefore, combining dark and photo-fermentation might increase the H_2 production and the product conversion efficiency. Elevated H_2 yields during these hybrid applications have already been reported by several studies (Tao et al. 2007; Chen et al. 2010; Morsy 2017; Chen et al. 2008).

A major drawback of the combined process is the inhibitive effect of the substrates arising during the photo-fermentative process. One solution would be the dilution of the dark fermentation effluent (Chen et al. 2010). During the operation, the uniform distribution of light into volumetrically large vessels needed for photosynthesis is an additional obstacle that needs to be overcome. With increasing cell density, the light intensity decreases rapidly as it gets absorbed by the outer biofilm layers (Basak et al. 2014). To make hybrid applications economically more feasible, the efficient utilization of solar light is desirable, although difficult to control (Kim et al. 1997).

Morsy *et al.* (2017) described continuous production of H_2 gas by dark and photo-fermentation. Their experiment consisted of five connected fermenters, one dark- and four photo-fermenting systems. *Escherichia coli* in co-culture with *C. acetobutylicum* performed anaerobic dark fermentative H_2 production from molasses in the first reactor. *Rhodobacter capsulatus* was used for subsequent uptake of the produced VFAs and photo-fermentative H_2 generation in the other four reactors. The authors reported full consumption of the substrates with high H_2 yields (5.65 mol H_2 mol^{-1} hexose), done in a continuous manner (Morsy 2017). This exceeded yields from previous hybrid fermentation studies (Liu et al. 2010; Ding et al. 2009; Tao et al. 2007).

Hybrid applications are promising systems for application in industrial H_2 production. Future research into continuous fermentation using both dark- and photo-fermentative steps will be of interest, as batch fermentation is resource- and time-consuming and thus not suitable for active industrial production.

1.5.5. Operating mode

Rittmann and Herwig (2012) introduced a categorization system for the different cultivation techniques, defining the conduction of experiments in sealed vials as “closed batch”. This operating mode has the advantage that multiple closed batch cultivations can be performed in parallel. Additionally, a detailed analysis of physiological and biotechnological parameters is possible (Rittmann and Herwig 2012; Rittmann et al. 2015). It can be used to “screen” organisms for their H_2 producing ability, as well as for analysing their optimum growth conditions. A major drawback, due to there being no exchange of gasses and liquids, is that this system is subjected to substrate or feedback inhibition (van Niel et al. 2002; Wang et al. 2007; Park et al. 2005).

Compared to closed-batch cultivation, open bioreactor-like systems, also referred to as “batch” cultivation, cannot be easily parallelized and are more expensive. However, continuous monitoring, control of growth parameters (temperature, pH, stirring, ORP *etc.*) and analysis of liquid metabolic end products throughout the experiment are

possible. Furthermore, sparging with gasses prevents gaseous feedback inhibition (Rittmann and Herwig 2012; Rittmann et al. 2015). Preventing product inhibition in the liquid phase has been observed (Bohnenkamp et al. 2021), but is not straightforward, as minimal exchange of liquids in the fermentation vessel can achieve high biomass concentration but entails possible harmful accumulation of the metabolic end products.

Within the various bioreactor systems, stirred tank reactors (STR) have been used in the majority of studies. Many others used anaerobic fluidized bed reactors (AFBR), anaerobic sequencing batch reactors (ASBR), fixed or packed bed reactors, upflow anaerobic sludge blanket (UASB) reactors, leaching bed reactors, anaerobic baffled reactors, or membrane bioreactors (MBR). Sludge granulation and biofilm systems increase the concentration of bacteria in the reactor. As the volumetric H_2 production rate of a bioreactor increases with microbial density, the application of UASB reactors, such as those used for wastewater treatment plants, with granular biomass retention is promising. However, CSTR systems are preferable if high H_2 yields (mmol H_2/mol glucose) are desired. (Gavala et al. 2006). When working with mixed cultures, H_2-consumers, mostly methanogenic organisms, prevent the accumulation of H_2 as the final product of the metabolic flow. Working with short hydraulic retention times (HRT or increased dilution rates) in CSTR systems, can improve H_2 yields via washout of methanogens. Nevertheless, if the HRT is set too low, H_2-producing bacteria might be subjected to washout as well (Chen et al. 2001). Hung *et al*. (2007) established a fermentation process carried out using an agitated granular sludge bed reactor (AGSBR) that could achieve relatively high H_2 production rates by keeping the biomass concentrations of H_2 producers high enough to cope with very short retention times. Similarly, Zhang et al. (2008) reported the advantages of granule-based systems over biofilm-based ones in terms of biomass retention whilst having less wash-out of the biomass support carriers.

Batch cultivation can be seen as an intermediate step between initial closed batch technique and the ultimate goal of uninterrupted production of H_2. Hence, the key technique for future industrialisation is continuous culture cultivation. During continuous fermentation, inflow and outflow rates of the system are precisely controlled allowing the exact adjustment of the culture conditions. Whilst its application and set-

up are more complicated, a continuous batch fermentation system allows time-independent analysis of the physiological processes as well as H_2 production under steady state conditions.

1.5.6. Application of co-cultures

Another way to enhance the $Y_{(H2/S)}$ is the application of microbial consortia (Pachapur et al. 2015a; Kleerebezem and van Loosdrecht 2007). As opposed to monocultures, the combination of different microbial strains, either defined or undefined, can ameliorate the metabolic and genetic reservoir present in a fermentation system. Within a functional co-culture, symbiotic microbial partners ideally complement each other's metabolisms, either by consuming and processing an otherwise inaccessible substrate or by clearing away molecules that could be harmful for others. Other beneficial aspects of co-cultures is the reduction of the lag phase, as well as higher resistance and stability within a fluctuating environment (Pachapur et al. 2015b; Chang et al. 2008; Pachapur et al. 2017). There are two main approaches for the biotechnological application of co-cultures. Using natural, undefined, and mixed microbial consortia on the one hand, or generating synthetic, defined systems on the other.

1.5.6.1. Undefined consortia

Performing H_2 production in a mixed culture or microbial consortium can ensure the presence of hydrolysers capable of breaking down large organic molecules. This critical step is necessary for the use of waste streams, including lignocellulosic biowaste, as a substrate for bio-H_2 production (Patel et al. 2012). This might make otherwise necessary pre-treatment steps or the use of expensive reducing agents obsolete, improving the cost-effectiveness of the system (Bader et al. 2010; Pachapur et al. 2015b). As an example, a strain closely affiliated with *C. acetobutylicum* has been shown to improve cellulose hydrolysis and subsequent H_2 production rates in co-culture with *Ethanoigenens harbinense* (Wang et al. 2008).

Equally beneficial for H_2 production in mixed culture bioreactors are strains of *Enterobacteriaceae*. In association with *Clostridiaceae*, which play the dominant H_2 producing role, they increase the system's resistance to environmental fluctuations,

especially those in pH (Song et al. 2011), as well as the H_2 production at the beginning of the fermentation process (Tolvanen et al. 2010). Moreover, as facultative anaerobes, *Enterobacteriaceae* can act as O_2 consumers within mixed cultures and provide anaerobic conditions for obligate anaerobes such as *Clostridiaceae* (Chojnacka et al. 2011; Hung et al. 2007). It is therefore possible to perform H_2 fermentation processes without the addition of costly reducing agents, as has been shown for *E. aerogenes* and *C. butyricum* (Yokoi et al. 1998).

Undefined co-cultures prove to be more practical for environmental engineering, can economize asepsis costs and are easier to control (Wang and Wan 2009; Li and Fang 2007). However, the presence of H_2 consuming organisms, such as methanogens, might lower H_2 yields in undefined systems by interfering with the biochemical pathways for its synthesis (Li and Fang 2007). It is therefore necessary to reduce the activity of unwanted organisms by inoculum pre-treatment, different fermentation strategies such as working at a pH below 6 (Ghimire et al. 2015), or by enrichment of mixed cultures to enhance bio-H_2 production whilst inhibiting H_2 consumers.

1.5.6.2. Defined consortia

Synthetic and defined microbial consortia, as opposed to mixed cultures, are created artificially by co-culturing of selected species in a defined medium. These abstractions of natural systems allow us to study and control the different interactions and metabolisms present. Defined consortia do not need pre-treatment, are less complex, and easier to work with than undefined mixed cultures (Grosskopf and Soyer 2014). The application of synthetic microbial communities for the generation of biofuels or other biotechnologically important substrates is very promising (Brenner et al. 2008). Indeed, this has already been successfully tried. Yokoi et al. (2001) reported improvements of the fermentation process and H_2 productivity when applying co-cultures. Their defined co-culture of *Clostridium butyricum* and *E. aerogenes* achieved a $Y_{(H2/S)}$ of 2.4 mol H_2/mol glucose. Similarly, Benomar et al. (2015) observed beneficial physical interactions increasing the H_2 production rate and improving the metabolic fluxes in the cells in an artificial consortium of *C. acetobutylicum* and *Desulfovibrio vulgaris*.

Nevertheless, a detailed understanding of the organisms' metabolism and growth behaviour is necessary. Furthermore, a key challenge at the industrial scale remains in the maintenance of the system characteristics and population stability. These might include controlling species competition, as one species of the co-culture might out-compete the other, as was observed in Beckers *et al.* (2010).

1.6. "Biohydrogen production beyond the Thauer limit by precision design of artificial microbial consortia" (Ergal et al. 2020)

A very promising implementation of a synthetic consortium to surpass the theoretical H_2 production limit of 4 moles of H_2 per one mole glucose consumed during dark fermentation, the aforementioned Thauer limit, has been performed in a previous work (Ergal et al. 2020). Through specific selection of microbial partners, sophisticated design of experiments (DoE), growth medium optimization, and investigation into substrate usage and by-products, artificial microbial consortia were able to perform higher productivities and yields than theoretical limits.

As a matter of course, the main idea behind the combination of *E. aerogenes* and *C. acetobutylicum* was to enhance the overall bio-H_2 productivity by making use of the two metabolically and ecologically different strategies of the organisms. Due to its high HER values and fast generation times, *E. aerogenes* would presumably boost H_2 productivity during the early phase of fermentation. As a result of acidogenic growth, the pH decreases. Whilst this implies a shift in the metabolism of *E. aerogenes* to non-acid production entailed by a reduced H_2 production, it creates ideal growth conditions for *C. acetobutylicum*, which will then take over the productivity during this second stage of the fermentation process. As the pH drops further down, solventogenesis replaces the acidogenetic growth behaviour of *C. acetobutylicum*. However, this would not impair H_2 production. Therefore, both organisms would be assisting each other in the production of H_2, whilst the facultative anaerobe *E. aerogenes* maintains anaerobic conditions and *C. acetobutylicum* stabilizes the pH conditions.

These ideas proved to be successful, as the H_2 yield of the co-culture exceeded the theoretical limit by 1.58 moles of H_2 mol^{-1} glucose. This was obtained by implementing

an inoculation ratio of 1:10.000 in favour of *Clostridium* sp. to compensate for its slow growing behaviour compared to the co-organism.

1.7. One step further: Up-scaling of the fermentation process

The next challenge towards the industrial usage of bio-H_2 as an alternative to fossil fuels is to up-scale this successful application of microbial consortia. The experiments in Ergal et al. (2020) were performed in sealed 120 mL serum bottles with a working volume of 50 mL. To pave the way for a future H_2 economy, we need to produce similar yields in high volume systems, from bench bioreactors on the scale of litres, to eventual pilot scale systems with upwards of hundreds of litres. Most studies on dark fermentative bio-H_2 production were carried out in smaller laboratory scale batch, semi-continuous, or continuous reactors, and only a limited number of studies has been done on pilot-scale applications. Nevertheless, dark fermentation has the potential to be the basis of commercially feasible bio-H_2 production systems due to favourable production rates.

The main requirement that must be met in order to make bio-H_2 production an industrially feasible process is the continuous provision of high volumetric H_2 production rates, HER. The HER is directly dependent on the $Y_{(H2/S)}$, which can be significantly improved with the right experimental design, as clearly demonstrated by Ergal et al. (2020). As mentioned however, these results have been obtained discontinuously in volumetrically small, closed batch experiments.

In the present work, we describe the application of the same fermentative co-culture in volumetrically larger bioreactor systems. The operation in open batch mode will provide important implications of the culture performance in larger volumes and further improve the fine-tuning of the system that is crucial for future application in continuous bioreactor systems. We hope that this work will guide subsequent attempts to upscale H_2 production.

2. Hypotheses and aims

We hypothesize that *E. aerogenes* and *C. acetobutylicum* grown in a batch setup will establish a stable co-existence and symbiosis when the correct inoculation ratio is implemented. This will eventually allow them to exceed the Thauer limit of 4 mol H_2 per C-mol substrate, which was already seen in closed-batch.

The main goal for these experiments will be to observe the same improved HER (mmol H_2 L^{-1} h^{-1}) and $Y_{(H2/S)}$ in the co-culture in batch as was seen in closed batch. The chosen organisms, *E. aerogenes* and *Clostridium acetobutylicum*, have already shown a strong symbiotic behaviour in closed-batch, resulting in production rates that even exceeded the Thauer limit of 4 mol H_2 per C-mol substrate.

Shifting the fermentation method from closed batch to batch setup, these experiments can be seen as an up-scaling, with the aim of eventually being implemented in industrial bio-H_2 production.

Chapter II

1. Contribution

My scientific contribution to this manuscript was assisting in the co-culture batch fermentation, involving media preparation and bioreactor set-up as well as continuous sampling thereof (liquid and gas) and analysis of the samples. This included the assessment of the growth behaviour by optical density measurements and DNA extractions followed by quantitative PCR. Next to gas chromatography and further calculation of H_2 productivities.

2. Article

Scale-up of dark fermentative biohydrogen production by artificial microbial co-cultures

İpek Ergal [1,†,*], Elisa Zech [1,†], Nikola Hanišáková [2], Ivan Kushkevych [2], Werner Fuchs [3], Tomáš Vítěz [2], Monika Vítězová [2], Günther Bochmann [3] and Simon K.-M. R. Rittmann [1,*]

[1]Archaea Physiology & Biotechnology Group, Department of Functional and Evolutionary Ecology, University of Vienna, Djerassiplatz 1, 1030 Vienna, Austria
[2]Laboratory of Anaerobic Microorganisms, Section of Microbiology, Department of Experimental Biology, Faculty of Science, Masaryk University, Kamenice 5, 63500 Brno, Czech Republic
[3]Institute of Environmental Biotechnology, Department for Agrobiotechnology, University of Natural Resources and Life Sciences Vienna, Konrad Lorenz-Str. 20, 3430 Tulln a.d. Donau, Austria

[†] These authors contributed equally to this work.

[*] Corresponding authors: Dr. İpek Ergal and Dr. Simon K.-M. R. Rittmann, Privatdoz.

Abstract

As a renewable energy carrier dark fermentative biohydrogen (H_2) represents a promising future alternative to fossil fuels. Recently, the limited H_2 yield of 4 moles of H_2 per mole glucose, the so-called “Thauer limit”, was surpassed by a defined artificial consortium. In this article we demonstrate the upscaling of this drawing board design, from serum bottles to laboratory scale bioreactors. Our results illustrate that this designed microbial co-culture can be successfully implemented in batch mode with maximum H_2 yields of 6.18 and 4.45 mol mol^{-1} glucose. Furthermore, we report volumetric H_2 productivities of 105.6 and 80.76 mmol H_2 L^{-1} h^{-1}. These rates are higher than for any other dark fermentative H_2 production system using a synthetic microbial co-culture applied in batch mode on a defined medium. Our study is an important step

forward for the application of artificial microbial consortia in future biotechnology and energy production systems.

Keywords

Dark fermentative biohydrogen production; artificial microbial consortia; upscaling

1. Introduction

Molecular hydrogen (H_2) is an energy carrier with high combustion yields [1]. Biologically produced H_2, referred to as biohydrogen production (BHP), is considered an environmentally friendly clean alternative with near zero carbon emission and has potential to replace fossil fuels as energy carriers [2]. Comparing different BHP systems, two main parameters must be considered. The H_2 evolution rate (HER / mmol H_2 L^{-1} h^{-1}) represents the volumetric productivity over time and is independent of the respective culture used, as opposed to the substrate conversion efficiency ($Y_{(H2/S)}$ / mol H_2 mol^{-1} substrate). Taking these units into consideration, the high HER, rapid cell growth, and relatively simple implementation due to non-requirement of light energy, advocate the use of dark fermentative H_2 production (DFHP) over photobiological H_2 production processes [3,4]. However, the low $Y_{(H2/S)}$ is the major drawback of DFHP, which is restricted to a theoretical maximum of 4 moles H_2 produced per one mole of glucose consumed in microbial pure cultures and microbial enrichment cultures when acetate is produced as a by-product [5]. DFHP can be carried out by various organisms using mainly two different H_2 generating pathways. Strictly anaerobic H_2 producers perform the pyruvate ferredoxin oxidoreductase (PFOR) pathway, whereas the pyruvate formate lyase (PFL) pathway is active in facultative anaerobes [3]. As the name already implies, in the course of the PFOR pathway H_2 is generated by a ferredoxin dependent hydrogenase enzyme. Depending on the organism, reduction equivalents that may originate from glycolysis and from the conversion of pyruvate to acetyl-CoA and reduced ferredoxin [6]. Alternatively, pyruvate is converted into formate via the activity of the PFL enzyme and formate is then split into H_2 and CO_2 [6].

These two main H_2 generating metabolic routes are active in multiple microbial species. Members of *Enterobacteriaceae* and *Clostridiaceae* are both very extensively studied and successful H_2 producing microbes [3,7,8]. Among these, *Enterobacter aerogenes* and *Clostridium acetobutylicum* have shown high H_2 productivities in pure culture. Nevertheless, the maximum $Y_{(H2/S)}$ of 3.14 and 2.16 mol H_2 mol^{-1} substrate, by *C. acetobutylicum* and *E. aerogenes* respectively [9,10], are still below the theoretical limit.

Interspecies interactions within microbial communities have shown positive effects on the productivity of fermentation systems [11,12]. Hence, *E. aerogenes* and *C. acetobutylicum* were grown in a co-culture as an attempt to increase the $Y_{(H2/S)}$. This defined artificial microbial consortium surmounted the restriction of 4 moles H_2 mol^{-1} glucose in DFHP [13]. In a drawing board like approach to establish a pipeline for design and engineering of artificial microbial consortia for DFHP [14], the cultivation parameters were pre-selected by considering *a priori* physiological and biotechnological knowledge from a preceding meta-data analysis [3]. With the design of experiments (DoE) approach a mutual medium was developed, taking into consideration each of the organisms' nutritional requirements and the buffer capacity of the medium. In addition, refinement of initial substrate and cell concentrations was performed to prevent substrate inhibition and ensure a stable coexistence of the two organisms. This precision design of an artificial microbial consortium has resulted in the proliferation of both organisms and in exceeding the previously described physiological limit by reaching $Y_{(H2/S)}$ of 5.58 mol mol^{-1} [13]. As most studies on BHP, this study was also conducted in serum bottles, hence a closed batch cultivation mode without controlling cultivation parameters such as pH. Being fast and simple in application, the cultivation in closed systems can be used for screening of the microbial strains used [4]. Yet, DFHP in closed batch systems are limited in their growth and H_2 productivity due to a decrease in pH and high H_2 partial pressure, which reduces HER [15]. To overcome these drawbacks, N_2 sparging and pH control [16,17] may be applied to enhance DFHP [18]. Thus, the DFHP should be performed in bioreactors to assess their suitability for subsequent scale-up.

Even though DFHP has already been investigated for more than a century [3,4,19], there are only a limited number of results of batch and in continuous culture

experiments available for pure cultures and for defined microbial DFHP ecosystems. While the bioprocess parameters e.g., pH, substrate concentration, and temperature can be controlled, the identification of suitable scale-up procedures and parameters in batch cultivation mode, as well as data on long-term bioprocess stability of BHP in continuous culture, are urgently required. Ideally, after screening for the basic requirements during closed batch cultivation, the microbes can then be implemented in laboratory scale bioreactors to examine their physiological potential for high quantitative BHP. These insights will help for future scale-up of the process to pilot scale bioreactors and possible industrialization. It has already been shown that cultivation in batch mode can increase H_2 productivity compared to closed batch cultivation [18] and that careful strain selection and optimization of the culture conditions genuinely affect the bioreactor performance [20]. Besides, some successful pilot scale DFHP experiments have been performed already. $Y_{(H2/S)}$ of 2.12 and 2.76 mol H_2 mol^{-1} glucose were obtained by a consortium of *C. butyricum* and *C. pasteurianum* in 20 L batch bioreactors [7] and a tri-culture of *Citrobacter freundii*, *Enterobacter aerogenes* and *Rhodopseudomonas palustris* in a 100 L vessel [21], respectively. Two consortia of *Enterobacter cloacae* plus *Bacillus cereus* and *E. cloacae* plus *Klebsiella* sp., produced 3.2 and 3 mol H_2 per mol glucose, respectively, at a working volume of 4 L in 5 L bioreactors [22].

The aim of this study was to examine if a drawing board like design of an artificial microbial DFHP co-culture can be propagated towards future industrial scale fermentation processes. Therefore, the scale-up experiment was carried out in laboratory-scale bioreactors rather than closed batch serum bottles, to follow a gradual scale up strategy in bioreactors. Apart from examining the performance in volumetrically larger vessels, this enabled the manual and controlled adjustment of the most crucial fermentation variables including pH, temperature, N_2 gassing rate, and agitation speed. We hypothesized that a defined microbial consortium of *E. aerogenes* and *C. acetobutylicum* can be scaled-up regarding HER / mmol H_2 L^{-1} h^{-1} and $Y_{(H2/S)}$ / mol H_2 mol^{-1} glucose from closed batch to batch.

2. Materials and Methods

2.1. Chemicals

CO_2, N_2, and H_2 were 99.999 Vol.-%. In addition 20 Vol.-% CO_2 in N_2 was used (Air Liquide, Schwechat, Austria). All other chemicals were of highest grade available.

2.2. Experimental set-up

Cultures of *Clostridium acetobutylicum* DSM 792 and *Enterobacter aerogenes* DSM 30053 were used for pure culture and consortium experiments. Both microorganisms were cultivated strict anaerobically in a DASGIP parallel bioreactor system in 2 L bioreactors (Eppendorf AG, Hamburg, Germany) using 1.5 L working volume. A defined medium was used for all the experiments (including pre-cultures), as previously described in detail elsewhere [13], containing (per L): 3.47 g of NH_4Cl, 10.41 g of KH_2PO_4, 5.31 g of K_2HPO_4, 1.35 g of NaCl. To each bioreactor 7.5 mL of a 200x vitamin stock solution was added, containing (per L): 0.2 g of 4-amino-benzoic acid, 0.9 g thiamine, 0.002 g biotin, as well as 15 mL of a 100x mineral stock solution, containing (per L): 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $MnCl_2 \cdot 4H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of NaCl. Glucose served as carbon source for H_2 production batch experiments at a concentration of 30 g L^{-1}. Before inoculation, glucose and mineral solution were sterilized separately at 121 ºC for 20 min, vitamin solution was sterilized by filtration (0.2 µm pore size). Anaerobic conditions inside the bioreactors were obtained by flushing the vessels with N_2 prior to inoculation. Pre-cultures were grown anaerobically at 0.3 bar in a N_2 atmosphere in a closed batch set-up. Inoculation was performed using the required amount of *C. acetobutylicum* pre-culture to reach an optical density of 0.3 in the bioreactor (ranging from 150 to 200 mL) and 0.01% (v/v) of *E. aerogenes* DSM 30053 (15 mL) of an anaerobically and aseptically transferred inoculum from the pre-culture vessels to the bioreactor.

The experiments were performed once (N = 1) with controlled pH and twice (N = 2) with uncontrolled pH and both sets were performed in duplicates (n = 2). Temperature was set at 37±0.5 °C, agitation speed at 100 and 200 rpm, and N_2 inflow rate at 1 sL h^{-1}. A pH probe (Mettler Toledo GmbH, Wien, Austria) and a redox probe (Mettler Toledo

GmbH, Wien, Austria) were used to observe the pH and oxidation reduction potential (ORP), respectively.

2.3. OD measurements and cell counting

At each time point, 1 mL of liquid sample was collected from the bioreactors and the optical density (600 nm (OD600)) was measured with a spectrophotometer (Specord 200 Plus, AnalytikJena, Jena, Germany). After increased growth of the culture the samples were diluted 1:10 to ensure an exact measurement in the linear absorption range.

To determine the cell concentrations in the pre-cultures, 1 mL samples were retrieved using sterile syringes (Soft-Ject, Henke SassWolf, Tuttlingen, Germany) and hypodermic needles (Sterican size 14, B. Braun, Melsungen, Germany).

Cells were counted using a Nikon Eclipse 50i microscope (Nikon, Amsterdam, Netherlands) at each sampling point. 12 µL of each sample (non-, 1:10, 1:50 or 1:100 diluted) were applied onto a Neubauer improved cell counting chamber (Superior Marienfeld, Lauda-Königshofen, Germany) with a grid depth of 0.1 mm.

2.4. Quantification of gas composition

Gas samples were taken in a gas bag (10 L SKC Quality Sampling Bag, SKC Inc., USA) connected to the off-gas tubing at each time point. Once the gas bag was filled, the gas was collected and transferred into sealed (Butyl rubber 20 mm, Chemglass Life Science LLC, Vineland, USA) and crimped 120 mL glass serum bottles (Ochs Glasgerätebau, Langerwehe, Germany) which were flushed with the fermentation off-gas for 5 min applying hyperdermic needles (Sterican size 14, B. Braun, Melsungen, Germany) and appropriate tubing.

The compositions of the collected gas samples were analyzed using gas chromatography (GC) (7890A GC System, Agilent Technologies, Santa Clara, USA) with a 19808 Shin Carbon ST Micropacked Column (Restek GmbH, Bad Homburg, Germany). The measurements were accomplished with a gas injection and control unit (Joint Analytical System GmbH, Moers, Germany) as described before [23]. A thermal conductivity detector was used for the measurements and the gases were separated

at 170 °C using helium as the carrier gas. The reference flow setting was 10 mL min^{-1}. The makeup flow was set to 1 mL min^{-1}. The standard gasses for GC measurements were 99.999 Vol.-% H_2, 99.999 Vol.-% CO_2, 99.999 Vol.-% N_2, 20 Vol.-% CO_2 in H_2, 20 Vol.-% CO_2 in N_2, a test gas containing 4.5 Vol.-% H_2 in N_2, a test gas containing 22.4 Vol.-% H_2; 19.7 Vol.-% CO_2; 15.5 Vol.-% N_2 14.1 Vol.-% CH_4 in CO, and a test gas containing 22.4 Vol.-% H_2; 19.7 Vol.-% CO_2; 12.2 Vol.-% N_2 (Air Liquide GmbH, Schwechat, Austria). Another standard test gas for GC measurements comprised the following composition: 0.01 Vol.-% CH_4; 0.08 Vol.-% CO_2 in N_2 (Messer GmbH, Wien, Austria). Standard GC curves with an R^2 of 0.99 or higher were obtained with aforementioned standard gases.

2.5. DNA extraction and qPCR

DNA was extracted from 1 mL culture samples at each time point as follows: After centrifugation (at 4 °C and 13,400 revolutions per minute (rpm) for 30 min) and resuspension in pre-warmed (65 °C) 1% sodium dodecyl sulfate (SDS) extraction buffer, the cells were transferred to Lysing Matrix E tubes (MP Biomedicals, Santa Ana, CA, USA) containing equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and around 0.5 g Bulk B Beads, and lysed in a FastPrep-24 (MP-Biomedicals, NY, USA) device (speed setting 4 for 30 s). This was followed by centrifugation at 13,400 rpm for 10 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the supernatant and the mixture was then centrifuged again at 13,400 rpm for 10 min. Addition of 1 µL glycogen (20 mg mL^{-1}) and double volume of polyethylene glycol (PEG) solution (30% PEG, 1.6 mol L^{-1} NaCl) allowed the DNA to precipitate, which was performed overnight at 4 °C. Nucleic acid pellets were retrieved by centrifugation at 13,400 rpm for 30 min, followed by washing with 70% cold ethanol solution, drying in a SpeedVac at 30 °C (Thermo Scientific, Dreieich, Germany) and resuspension in 40 µL Tris-EDTA buffer. The extracted DNA was stored at -20°C until further analysis. Quantification of Nucleic Acid was performed with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

For the qPCR diluted DNA equivalents (1:300) were used for analysis. Additionally, negative controls with sterile DEPC water as a replacement for the DNA templates were run in parallel. 6 standards with previously determined cell concentrations at different

dilutions, ranging from 1:10 to 1: $1 \cdot 10^6$, were amplified simultaneously and used both as a positive control and to generate a standard curve as described elsewhere [13]. All amplification reactions were run in triplicates.

To prevent false positive amplification, primer design was done by targeting species specific genes. Using the ClustalW2 multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/) optimal primers were identified by sequence comparison of the genes.

For *E. aerogenes* DSM 30053 forward primer 5' - GCG TTG TGG GGT TGC ACG AT - 3' and reverse primer 5' - TGG CGC GCG AGC ACA TTT TC - 3', for *C. acetobutylicum* DSM 792 forward primer 5' - TGG CAC AGT CAG TCG GCT ACC - 3' and reverse primer 5' - GCG TGA TGC ACC TAA CCC AGC - 3' were used.

Reactions were set up using SYBR Green labelled Luna Universal qPCR Master Mix (M3003L, New England Biolabs) following the manufacturer's protocol and performed in Eppendorf Mastercycler epgradientS realplex2 (Eppendorf, Hamburg, Germany). Amplification protocol was run as described in detail elsewhere [13].

2.6. Data analysis

To determine the specific growth rate (μ [h^{-1}]) for each bioreactor, the following equation was used: $X = X^0 \cdot e^{\mu t}$ with X, cell number / cells mL^{-1}; X^0, initial cell number [cells mL^{-1}]; t, time [h] and e, Euler number. Calculation of HER / mmol H_2 L^{-1} h^{-1} was done by taking into consideration the total gas flow rate / sL h^{-1}, the respective concentration of H_2, the ideal gas law, and the inert gas flow correction factor.

3. Results and discussion

To establish the artificial consortium of *C. acetobutylicum* and *E. aerogenes* in bioreactors, the culturing conditions were at large kept identical to those described before [13].

We anticipated that the initial ratio that was optimized for the closed batch runs had to be adjusted to the batch set-up. Nevertheless, neither an increase nor a decrease of

the initial *E. aerogenes* cell concentration (ratios ranging from 1:10 to 1:10.000.000; *E. aerogenes* : *C. acetobutylicum*) resulted in stable growth or significant H_2 production (Figure S1, see in Supplementary Materials).

This initial inoculation ratio of 1:10.000 in favour of *C. acetobutylicum* can therefore be considered as the optimum inoculation ratio both for closed and batch cultivation. Therefore, medium, substrate, and cell concentrations were kept the same for the up-scaling experiments in the bioreactors.

To further optimise H_2 production we adjusted the system pH. During the first cultivations, the initial pH was set to 6. However, a rapid pH decrease was observed due to the accumulation of acidic metabolic end products for the experiments with uncontrolled pH. When pH was controlled at 6, an increase in $Y_{(H2/S)}$ and HER were observed (Figure 1).

The highest $Y_{(H2/S)}$ of 6.18 mol H_2 mol^{-1} glucose and HER of 105.6 mmol H_2 L^{-1} h^{-1} was achieved when the pH was controlled in the interval between 20-25 h after inoculation. Whereas the second-highest $Y_{(H2/S)}$ of 4.45 mol H_2 mol^{-1} glucose and a HER of 80.76 mmol H_2 L^{-1} h^{-1} was observed under non-controlled conditions between 25 to 29 h after inoculation (Figure 1). Both values clearly surpass the theoretical limit of 4 mol H_2 mol^{-1} glucose and also the highest $Y_{(H2/S)}$ and HER values that had been obtained in closed batch before.

To our knowledge, this is the first report of a synthetic co-culture cultivated in batch mode describing an improvement of $Y_{(H2/S)}$ beyond the Thauer limit. For comparison, Table 1 lists different studies on DFHP using synthetic consortia operated in batch cultivation systems. With the application of *Caldicellulosiruptor kristjanssonii* and *C. saccharolyticus* Zeidan and Van Niel [24] reported a $Y_{(H2/S)}$ that is very close to the theoretical limit (3.8 mol H_2 mol^{-1} C6 sugar equivalent). This result was achieved by using extreme thermophilic organisms which, due to thermodynamics, usually produce higher yields than mesophiles [7,25]. Furthermore, and like all other studies on co-cultures listed in Table 1, the experiments were conducted using a complex medium rather than a defined medium, making a comprehensive H_2 production analysis challenging.

Apart from using complex or defined media, earlier reports on DFHP using artificial consortia in batch reactors deviate from the current study in the working volume within the biorector. Working volumes range from only 100 mL [9] to as much as 18 L [7] (Table 1). Also varying among the different studies is the pH ranging from 5.25 [26] to 7 [9,27]. Optimum pH values for maximum H_2 production were found to be slightly acidic around 6.5 [24,28,29]. This parameter directly affects the hydrogenase activity, metabolic by-products and $Y_{(H2/S)}$ [30] which is why the ability to monitor and control the in-situ acidity/alkalinity during the fermentation procedure is a very convenient feature of the batch fermentation set-up.

Clostridium sp. have been shown to stably produce H_2 at a pH of 5.5-6 [31,32], while the peak substrate conversion rate of *E. aerogenes* is found between pH 6 and 7 [33]. With no base or acid inflow to keep the pH steady, we observed a rapid acidification of the medium due to acidogenic growth properties of the organisms. This acidification lead at first to an increased H_2 production, but very acidic conditions will eventually initiate a metabolic switch to solventogenesis or even the formation of spores in *Clostridium* sp. with decreased H_2 productivities. Both spore formation and production of solvents rather than acids can be seen as preventive actions to keep harmful effects of undissociated acids at bay [34,35]. Hence, keeping the pH stable at 6 would favour the acetate pathway and prevent the metabolic shift. Our results confirm this assumption as the maximum $Y_{(H2/S)}$ (6.18 mol H_2 mol^{-1} glucose) was observed under controlled pH conditions and a lower $Y_{(H2/S)}$ (4.45 mol H_2 mol^{-1} glucose) was achieved when the pH was allowed to decrease. Interestingly, this second-best result was obtained rather late in the experiment (Figure 1) when the pH dropped below the optimum value.

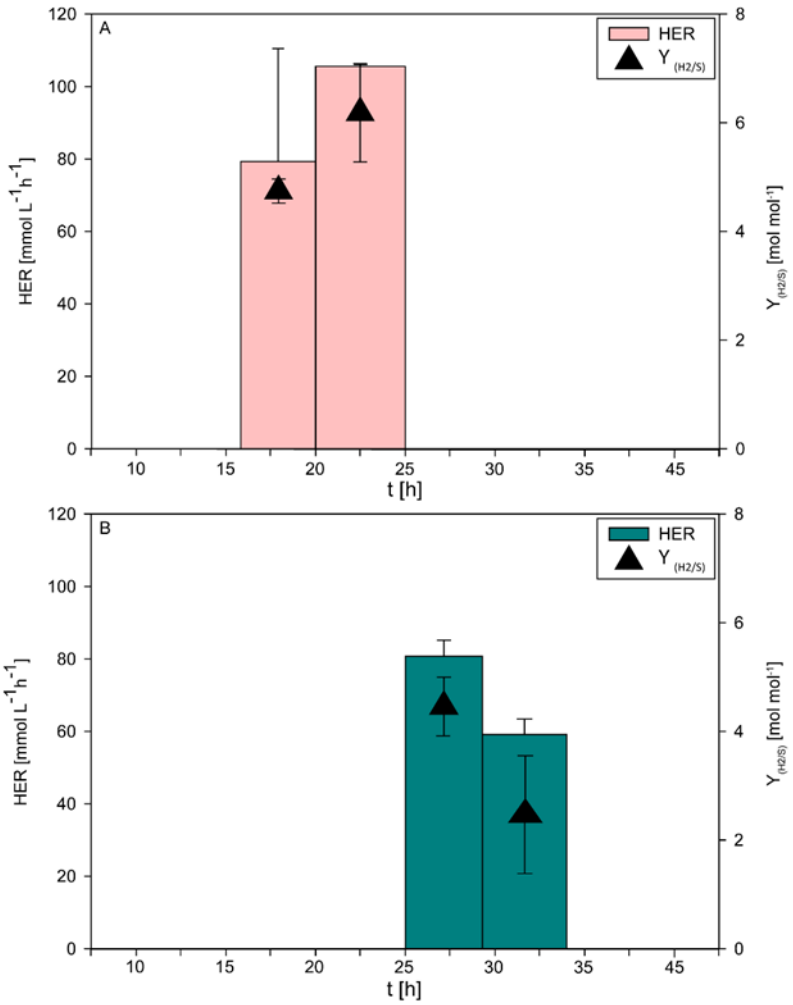

Figure 1. HER and $Y_{(H2/S)}$ results over time for the two best bioreactor runs. The displayed results were achieved when the pH was kept stable at 6, in the top chart, and when the pH was not controlled, in the lower chart. Bars indicate HER; single data plots represent the $Y_{(H2/S)}$; standard deviations are given as error bars.

In addition to the pH, other environmental parameters have a crucial influence on the system performance. Ergal *et al*. [13] found that only a remarkable discrepancy in the initial cell concentration allowed the stable co-existence of *C. acetobutylicum* and *E. aerogenes*. This was necessary as the fast-growing *E. aerogenes* threatened to quickly overgrow the *C. acetobutylicum* population. Since different strains show different growth behaviour, it is important to counteract possible imbalances by compensating with varying initial cell concentrations. Once the proliferation of subdominant species can be guaranteed, the requirements for a stable and well-functioning synthetic

consortium are provided. Yet, the initial cell ratio of 1:10.000 that enabled high H_2 yield and productivity in Ergal *et al*. [13] is quite unique. Usually, inoculation ratios do not exceed 1:1 or 1:2 [7,22,29]. Within this work we show that the designed co-culture can be successfully applied and produces $Y_{(H2/S)}$ beyond theoretical limits after the initial report in closed batch mode in serum bottles and here also performed in batch mode in bioreactors.

Synthetic microbial consortia are in fact well applicable for increased H_2 production compared to monocultures. Still, the theoretical limit of 4 mol H_2 per mol glucose during DFHP can barely be met and is almost never exceeded. In this regard, thermophilic strains are more favourable over mesophilic ones as higher temperatures favour increased specific H_2 productivities [24,39]. Mesophilic H_2 producers have the advantage of being extensively studied and the literature provides a broad and detailed understanding of their physiological and genetic properties. These insights are required to design specific microbial consortia and set up a suitable environment that meets the organisms' requirements and enables high biofuel production. Following this drawing board like approach Ergal *et al*. [13] succeeded to implement a successful synthetic microbial consortia producing $Y_{(H2/S)}$ that surpassed the Thauer limit. It is also what distinguishes their study from other reports on BHP by artificial consortia.

Table 1. Overview of studies on DFHP with pure cultures and co-cultures

Micro-organism	Feeding substrate	pH	Temperature	Medium composition (complex/ defined)	$Y_{(H2/S)}$ / mol mol^{-1}	HER [mmol L^{-1} h^{-1}]	Operating conditions	Ref.
Pure cultures								
Clostridium acetobutylicum	glucose	7	30°C	complex	3.14	*NA*	100 mL in 500 mL Scotch bottle	[9]
Clostridium acetobutylicum	cassava wastewater	7	36°C	complex	2.41	*NA*	300 mL bioreactor	[27]
Clostridium acetobutylicum	sugarcane molasses	6.5	30°C	complex	1.3	*NA*	1950 mL in 2 L MultiGen fermentor	[36]
Enterobacter aerogenes	maltose	6.5	35°C	complex	2.16	*NA*	52 mL culture in Erlenmeyer flask	[10]
Enterobacter aerogenes	corn starch	5.5	40°C	complex	1.8	5.2	1.5 L in 2 L Gallenkamp FBL-195 bioreactor	[37]
Enterobacter aerogenes	glucose	uncontrolled (initial pH 6.9)	37°C	defined	1.36	*NA*	3 L in 5 L bioreactor	[38]
Co-cultures								
Clostridium acetobutylicum and *Desulfovibrio vulgaris*	glucose	*NA*	37°C	complex	3.46	*NA*	cultivated in Hungate tubes	[24]
Clostridium butyricum and *Enterobacter aerogenes*	sweet potato starch	5.25	37°C	complex	2.4	*NA*	200 mL in 250 mL stirred reactor	[26]
Clostridium butyricum and *Clostridium pasteurianum*	starch	5.3	30°C	complex	2.32	*NA*	18 L in 20 L stainless steel tank bioreactor	[7]
Clostridium butyricum and *Clostridium pasteurianum*	glucose	5.3	30°C	complex	2.12	*NA*	18 L in 20 L stainless steel tank bioreactor	[7]
Klebsiella pneumoniae and *Citrobacter freundii*	glucose	6.5	37°C	complex	2.07	*NA*	2 L in a controlled fermenter	[29]
Enterobacter aerogenes and *Clostridium acetobutylicum*	glucose	6	37°C	defined	6.18	105.59	1.5 L in 2 L stirred tank reactor	This study
Enterobacter aerogenes and *Clostridium acetobutylicum*	glucose	uncontrolled	37°C	defined	4.45	80.76	1.5 L in 2 L stirred tank reactor	This study

4. Conclusions

Artificial microbial ecosystems can be effectively used for scale-up of DFHP from closed batch to lab scale bioreactors. In this study, we obtained the highest $Y_{(H2/S)}$ and the highest HER on glucose for any DFHP system using a synthetic microbial co-culture on a defined medium in batch mode up to date. This work provides the fundamentals for further scale-up of our DFHP bioprocess, which is required to unravel the scaling criterion aiming to establish DFHP at industrial scale. Further studies on design and engineering of artificial microbial consortia for DFHP regarding substrates such as lignocellulose, lipid waste, and food waste will drive our understanding of their functioning. Moreover, the development of more sophisticated techniques to control the physical space and environment of engineered microbial consortia might lead to a further improvement of HER and $Y_{(H2/S)}$. This study is another step forward in demonstrating the application possibilities of artificial microbial consortia in future biotechnology and energy production systems.

Author Contributions: Conceptualization, İ.E., W.F., T.M., M.V., G.B. and S.K.-M.R.R.; methodology, I.K., W.F. M.V., G.B. and S.K.-M.R.R.; validation, İ.E. and S.K.-M.R.R.; formal analysis, İ.E; investigation, İ.E, E.Z., N.H. and I.K.; resources, W.F, M.V., G.B. and S.K.-M.R.R.; data curation, İ.E. and E.Z.; writing original draft preparation, İ.E., E.Z. and S.K.-M.R.R.; writing review and editing, İ.E., E.Z., I.K., W.F., T.V., M.V. and S.K.-M.R.R.; visualization, İ.E. and E.Z.; supervision, W.F., M.V., G.B and S.K.-M.R.R.; project administration, M.V. and S.K.-M.R.R.; funding acquisition, W.F., T.V., M.V., G.B and S.K.-M.R.R.. All authors have read and agreed to the published version of the manuscript

Funding: This research was funded by the Austrian Research Promotion Agency (Forschungsförderungsgesellschaft (FFG)), project H2.AT, grant number 853618; and funded by BMBWF, project WTZ, grant number CZ 08/2020.

Data Availability Statement: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hay, J.X.W.; Wu, T.Y.; Juan, J.C.; Md. Jahim, J. Biohydrogen Production through Photo Fermentation or Dark Fermentation Using Waste as a Substrate: Overview, Economics, and Future Prospects of Hydrogen Usage. *Biofuels, Bioprod. Bioref*. **2013**, *7*, 334–352, doi:10.1002/bbb.1403.
2. Vinoth Kanna, I.; Paturu, P. A Study of Hydrogen as an Alternative Fuel. *International Journal of Ambient Energy* **2020**, *41*, 1433–1436, doi:10.1080/01430750.2018.1484803.
3. Ergal, İ.; Fuchs, W.; Hasibar, B.; Thallinger, B.; Bochmann, G.; Rittmann, S.K.-M.R. The Physiology and Biotechnology of Dark Fermentative Biohydrogen Production. *Biotechnology Advances* **2018**, *36*, 2165–2186, doi:10.1016/j.biotechadv.2018.10.005.
4. Rittmann, S.; Herwig, C. A Comprehensive and Quantitative Review of Dark Fermentative Biohydrogen Production. *Microb Cell Fact* **2012**, *11*, 115, doi:10.1186/1475-2859-11-115.
5. Thauer, R.K.; Jungermann, K.; Decker, K. Energy Conservation in Chemotrophic Anaerobic Bacteria. *Bacteriol Rev* **1977**, *41*, 100–180, doi:10.1128/br.41.1.100-180.1977.
6. Hallenbeck, P.C. Fermentative Hydrogen Production: Principles, Progress, and Prognosis. *International Journal of Hydrogen Energy* **2009**, *34*, 7379–7389, doi:10.1016/j.ijhydene.2008.12.080.
7. Masset, J.; Calusinska, M.; Hamilton, C.; Hiligsmann, S.; Joris, B.; Wilmotte, A.; Thonart, P. Fermentative Hydrogen Production from Glucose and Starch Using Pure Strains and

Artificial Co-Cultures OfClostridium Spp. *Biotechnol Biofuels* **2012**, *5*, 35, doi:10.1186/1754-6834-5-35.

8. Patel, S.K.S.; Kumar, P.; Mehariya, S.; Purohit, H.J.; Lee, J.-K.; Kalia, V.C. Enhancement in Hydrogen Production by Co-Cultures of Bacillus and Enterobacter. *International Journal of Hydrogen Energy* **2014**, *39*, 14663–14668, doi:10.1016/j.ijhydene.2014.07.084.

9. Alshiyab, H.; Kalil, M.S.; Hamid, A.A.; Yusoff, W.M.W. Effect of Salts Addition on Hydrogen Production by C. Acetobutylicum. *Pakistan J. of Biological Sciences* **2008**, *11*, 2193–2200, doi:10.3923/pjbs.2008.2193.2200.

10. Yokoi, H.; Ohkawara, T.; Hirose, J.; Hayashi, S.; Takasaki, Y. Characteristics of Hydrogen Production by Aciduric Enterobacter Aerogenes Strain HO-39. *Journal of Fermentation and Bioengineering* **1995**, *80*, 571–574, doi:10.1016/0922-338X(96)87733-6.

11. Pachapur, V.L.; Kutty, P.; Pachapur, P.; Brar, S.K.; Le Bihan, Y.; Galvez-Cloutier, R.; Buelna, G. Seed Pretreatment for Increased Hydrogen Production Using Mixed-Culture Systems with Advantages over Pure-Culture Systems. *Energies* **2019**, *12*, 530, doi:10.3390/en12030530.

12. Pachapur, V.L.; Sarma, S.J.; Brar, S.K.; Bihan, Y.L.; Buelna, G.; Verma, M. Hydrogen Production from Biodiesel Industry Waste by Using a Co-Culture of Enterobacter Aerogenes and Clostridium Butyricum. *Biofuels* **2017**, *8*, 651–662, doi:10.1080/17597269.2015.1122471.

13. Ergal, İ.; Gräf, O.; Hasibar, B.; Steiner, M.; Vukotić, S.; Bochmann, G.; Fuchs, W.; Rittmann, S.K.-M.R. Biohydrogen Production beyond the Thauer Limit by Precision Design of Artificial Microbial Consortia. *Commun Biol* **2020**, *3*, 443, doi:10.1038/s42003-020-01159-x.

14. Ergal, İ.; Bochmann, G.; Fuchs, W.; Rittmann, S.K.-M. Design and Engineering of Artificial Microbial Consortia for Biohydrogen Production. *Current Opinion in Biotechnology* **2022**, *73*, 74–80, doi:10.1016/j.copbio.2021.07.010.

15. Chong, M.-L.; Sabaratnam, V.; Shirai, Y.; Hassan, M.A. Biohydrogen Production from Biomass and Industrial Wastes by Dark Fermentation. *International Journal of Hydrogen Energy* **2009**, *34*, 3277–3287, doi:10.1016/j.ijhydene.2009.02.010.

16. Eriksen, N.T.; Nielsen, T.M.; Iversen, N. Hydrogen Production in Anaerobic and Microaerobic Thermotoga Neapolitana. *Biotechnol Lett* **2007**, *30*, 103–109, doi:10.1007/s10529-007-9520-5.

17. Nguyen, T.-A.D.; Han, S.J.; Kim, J.P.; Kim, M.S.; Sim, S.J. Hydrogen Production of the Hyperthermophilic Eubacterium, Thermotoga Neapolitana under N2 Sparging Condition. *Bioresource Technology* **2010**, *101*, S38–S41, doi:10.1016/j.biortech.2009.03.041.

18. Ngo, T.A.; Nguyen, T.H.; Bui, H.T.V. Thermophilic Fermentative Hydrogen Production from Xylose by Thermotoga Neapolitana DSM 4359. *Renewable Energy* **2012**, *37*, 174–179, doi:10.1016/j.renene.2011.06.015.

19. Rittmann, S.K.-M.R.; Lee, H.S.; Lim, J.K.; Kim, T.W.; Lee, J.-H.; Kang, S.G. One-Carbon Substrate-Based Biohydrogen Production: Microbes, Mechanism, and Productivity. *Biotechnology Advances* **2015**, *33*, 165–177, doi:10.1016/j.biotechadv.2014.11.004.

20. Hamilton, C.; Hiligsmann, S.; Beckers, L.; Masset, J.; Wilmotte, A.; Thonart, P. Optimization of Culture Conditions for Biological Hydrogen Production by Citrobacter Freundii CWBI952 in Batch, Sequenced-Batch and Semicontinuous Operating Mode. *International Journal of Hydrogen Energy* **2010**, *35*, 1089–1098, doi:10.1016/j.ijhydene.2009.10.073.

21. Vatsala, T.M.; Raj, S.M.; Manimaran, A. A Pilot-Scale Study of Biohydrogen Production from Distillery Effluent Using Defined Bacterial Co-Culture. *International Journal of Hydrogen Energy* **2008**, *33*, 5404–5415, doi:10.1016/j.ijhydene.2008.07.015.

22. Patel, S.K.S.; Singh, M.; Kumar, P.; Purohit, H.J.; Kalia, V.C. Exploitation of Defined Bacterial Cultures for Production of Hydrogen and Polyhydroxybutyrate from Pea-Shells. *Biomass and Bioenergy* **2012**, *36*, 218–225, doi:10.1016/j.biombioe.2011.10.027.

23. Reischl, B.; Ergal, İ.; Rittmann, S.K.-M.R. Biohydrogen Production Characteristics of Desulfurococcus Amylolyticus DSM 16532. *International Journal of Hydrogen Energy* **2018**, *43*, 8747–8753, doi:10.1016/j.ijhydene.2018.03.121.

24. Zeidan, A.A.; Van Niel, E.W.J. Developing a Thermophilic Hydrogen-Producing Co-Culture for Efficient Utilization of Mixed Sugars. *International Journal of Hydrogen Energy* **2009**, *34*, 4524–4528, doi:10.1016/j.ijhydene.2008.07.092.

25. Wang, A.; Ren, N.; Shi, Y.; Lee, D.-J. Bioaugmented Hydrogen Production from Microcrystalline Cellulose Using Co-Culture—Clostridium Acetobutylicum X9 and Ethanoigenens Harbinense B49. *International Journal of Hydrogen Energy* **2008**, *33*, 912–917, doi:10.1016/j.ijhydene.2007.10.017.

26. Yokoi, H.; Saitsu, A.; Uchida, H.; Hirose, J.; Hayashi, S.; Takasaki, Y. Microbial Hydrogen Production from Sweet Potato Starch Residue. *Journal of Bioscience and Bioengineering* **2001**, *91*, 58–63, doi:10.1016/S1389-1723(01)80112-2.

27. Cappelletti, B.M.; Reginatto, V.; Amante, E.R.; Antônio, R.V. Fermentative Production of Hydrogen from Cassava Processing Wastewater by Clostridium Acetobutylicum. *Renewable Energy* **2011**, *36*, 3367–3372, doi:10.1016/j.renene.2011.05.015.

28. Pawar, S.S.; Vongkumpeang, T.; Grey, C.; van Niel, E. Biofilm Formation by Designed Co-Cultures of Caldicellulosiruptor Species as a Means to Improve Hydrogen Productivity. *Biotechnol Biofuels* **2015**, *8*, 19, doi:10.1186/s13068-015-0201-7.

29. Mishra, P.; Roy, S.; Das, D. Comparative Evaluation of the Hydrogen Production by Mixed Consortium, Synthetic Co-Culture and Pure Culture Using Distillery Effluent. *Bioresource Technology* **2015**, *198*, 593–602, doi:10.1016/j.biortech.2015.09.074.

30. Ghimire, A.; Frunzo, L.; Pirozzi, F.; Trably, E.; Escudie, R.; Lens, P.N.L.; Esposito, G. A Review on Dark Fermentative Biohydrogen Production from Organic Biomass: Process Parameters and Use of by-Products. *Applied Energy* **2015**, *144*, 73–95, doi:10.1016/j.apenergy.2015.01.045.

31. Lin, C.-Y.; Wu, S.-Y.; Lin, P.-J.; Chang, J.-S.; Hung, C.-H.; Lee, K.-S.; Chang, F.-Y.; Chu, C.-Y.; Cheng, C.-H.; Lay, C.-H.; et al. Pilot-Scale Hydrogen Fermentation System Start-up Performance. *International Journal of Hydrogen Energy* **2010**, *35*, 13452–13457, doi:10.1016/j.ijhydene.2009.11.123.

32. Lee, H.-S.; Salerno, M.B.; Rittmann, B.E. Thermodynamic Evaluation on H2 Production in Glucose Fermentation. *Environ. Sci. Technol.* **2008**, *42*, 2401–2407, doi:10.1021/es702610v.

33. Fabiano, B.; Perego, P. Thermodynamic Study and Optimization of Hydrogen Production by Enterobacter Aerogenes. *International Journal of Hydrogen Energy* **2002**, *27*, 149–156, doi:10.1016/S0360-3199(01)00102-1.

34. Dürre, P. Physiology and Sporulation in Clostridium. *Microbiology Spectrum* **2014**, doi:10.1128/microbiolspec.TBS-0010-2012.

35. Van Ginkel, S.; Logan, B.E. Inhibition of Biohydrogen Production by Undissociated Acetic and Butyric Acids. *Environ. Sci. Technol.* **2005**, *39*, 9351–9356, doi:10.1021/es0510515.

36. Bagy, M.M.K.; Abd-Alla, M.H.; Morsy, F.M.; Hassan, E.A. Two Stage Biodiesel and Hydrogen Production from Molasses by Oleaginous Fungi and Clostridium Acetobutylicum ATCC 824. *International Journal of Hydrogen Energy* **2014**, *39*, 3185–3197, doi:10.1016/j.ijhydene.2013.12.106.

37. Perego, P.; Fabiano, B.; Ponzano, G.P.; Palazzi, E. Experimental Study of Hydrogen Kinetics from Agroindustrial By-Product: Optimal Conditions for Production and Fuel Cell Feeding. *Bioprocess Engineering* **1998**, 7.

38. Zhao, H.; Ma, K.; Lu, Y.; Zhang, C.; Wang, L.; Xing, X.-H. Cloning and Knockout of Formate Hydrogen Lyase and H2-Uptake Hydrogenase Genes in Enterobacter Aerogenes for Enhanced Hydrogen Production. *International Journal of Hydrogen Energy* **2009**, *34*, 186–194, doi:10.1016/j.ijhydene.2008.10.025.

39. Zeidan, A.A.; Rådström, P.; van Niel, E.W. Stable Coexistence of Two Caldicellulosiruptor Species in a de Novo Constructed Hydrogen-Producing Co-Culture. *Microbial Cell Factories* **2010**, *9*, 102, doi:10.1186/1475-2859-9-102.

Supplementary Material

Supplementary Figure S1

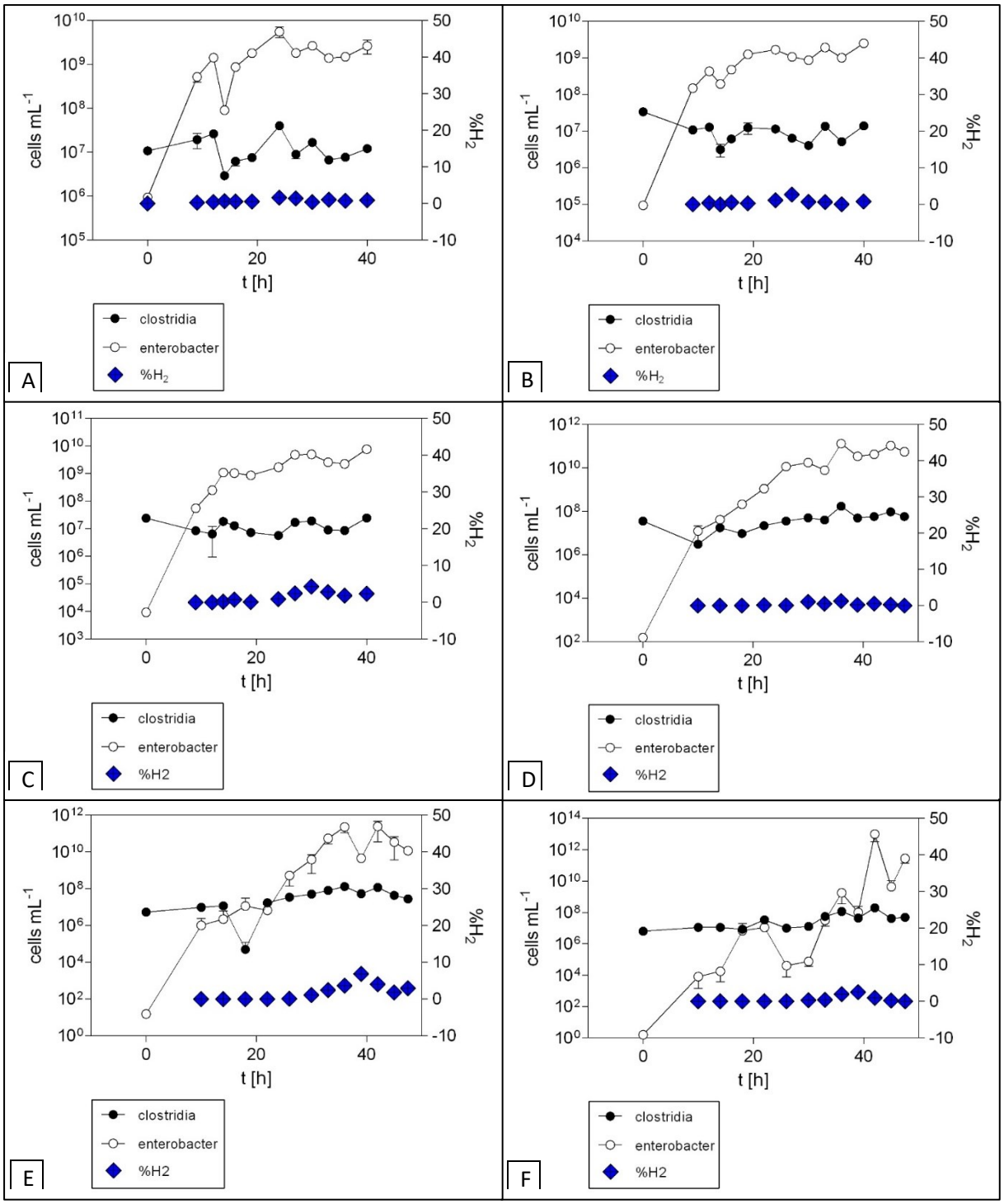

Figure S1: Different bioreactor runs with varying initial cell concentrations. A, 1:10; B, 1:100; C, 1:1.000; D, 1:100.000; E, 1:1.000.000; F, 1:10.000.000 for *C. acetobutylicum : E. aerogenes*. Depicted are the respective cell concentrations of *Clostridium acetobutylicum* and *Enterobacter aerogenes,* measured with qPCR, as well as the H_2 concentration in Vol% of the headspace, measured with GC, over the time course of the experiment.

Chapter III

1. Discussion

Biohydrogen produced through dark fermentative microorganisms is a very promising future biofuel alternative, due to high productivities and a wide substrate range. Major obstacles that prevent current processes from industrial application lie in the low substrate conversion efficiency and insufficient volumetric H_2 productivity. However, metabolic limitations inherent with the organisms' physiology can be overcome by the specific design of an artificial co-culture, as has been shown in a previous paper (Ergal et al. 2020). This defined and well-designed microbial consortium proved to be an attractive approach for the production of biofuels. Through the specific selection and combination of the most suitable producing strains, it was possible to create an artificial environment that favours growth and production of the desired end products.

Several previous studies reported higher biogas productivities when applying synthetic co-cultures compared to monocultures. For example, the synthetic consortium of *Caldicellulosiruptor kristjanssonii* and *C. saccharolyticus* achieved a $Y_{(H2/S)}$ of 3.8 mol H_2 per mol C6 sugar equivalent, surpassing those obtained with their respective pure cultures (Zeidan and van Niel 2009). Another study applied different *Caldicellulosiruptor* species in a designed consortium to improve dark fermentative H_2 production resulting in a maximum $Y_{(H2/S)}$ of 4.42 mol H_2 per mol glucose (Pawar et al. 2015). Even though this is the highest value for H_2 yield by a consortium of *C. owenensis* and *C. saccharolyticus*, the fermentations were carried out using complex media containing yeast extract. Only when cultivated in a defined medium, exact calculations of the H_2 production parameters ($Y_{(H2/S)}$, HER, and qH_2) on a C-molar level are possible. Their study differs from Ergal et al. (2020) further in the application of extreme thermophilic strains, whereas the synthetic consortium used in the latter yielded 5.6 mol H_2 per mol glucose in a defined medium and was composed of mesophilic strains. In addition, the consortium was established using different initial cell concentrations of the two strains. The inoculation ratio was tailored strongly in favour of *C. acetobutylicum* to ensure a stable growth next to fast growing *E. aerogenes.*

In our current paper, we tried to extend this drawing board design even further towards future industrial scale fermentation processes. To achieve this, the experiment was carried out in bench-scale bioreactors rather than closed batch serum bottles. This did not only allow us to test the performance of the co-culture at larger volumes, but also to control the most important environmental factors, such as pH, redox potential, temperature, gassing, and the stirring of the culture broth.

Just as seen in closed batch, the co-culture achieved H_2 yields above the theoretical limits when grown in batch bioreactor system. The Thauer limit was surpassed by 2.18 and 0.45 mol H_2 mol^{-1} glucose when pH was controlled and uncontrolled, respectively. H_2 productivity exceeded those reached with the respective pure cultures of the organisms. The maximum HER (105.6 mmol H_2 h^{-1} L^{-1}) of the artificial consortium was significantly higher than those observed by *C. acetobutylicum* and *E. aerogenes* alone. In continuous culture, the maximum HER when grown on glucose observed using monocultures of *C. acetobutylicum* is 19 mmol H_2 h^{-1} L^{-1} (Vasconcelos et al. 1994). *E. aerogenes* on the other hand obtained a volumetric H_2 productivity of 80 mmol H_2 h^{-1} L^{-1} in continuous cultivation using glycerol as feedstock (Ito et al. 2005). When grown on glucose, *E. aerogenes* produced a HER of 58 mmol H_2 h^{-1} L^{-1} (Rachman et al. 1998). Both organisms have shown to produce high yields when grown in pure culture. For example, 3.14 mol H_2 mol^{-1} glucose in batch mode has been reported for *C. acetobutylicum* (Alshiyab et al. 2008a). However, this $Y_{(H2/S)}$ was achieved in a complex medium. On a defined medium, batch cultures of *E. aerogenes* yielded a maximum of 1.16 mol H_2 mol^{-1} glucose (Zhao et al. 2009), compared to 1 mol H_2 mol^{-1} glucose on a complex medium (Yokoi et al. 1995).

Here we have shown that our artificial consortium, designed by Ergal et al. (2020), can be successfully applied in batch cultivation mode in bioreactor systems with H_2 yields beyond theoretical limits.

The fermentation in batch mode can be seen as one step closer to future industrialization and has been widely used for dark fermentative bio-H_2 production. Both mono- and co-cultures of meso- and thermophilic heterotrophs were applied on a variety of substrates in batch fermentations. Zeidan and van Niel (2009) used pure and co-cultures of thermophilic *Caldicellulosiruptor* species. *C. saccharolyticus*, *C. owenensis*, and *C. kristjanssonii*, as well as co-cultures of *C. saccharolyticus* with

enriched compost microflora in 3 L stirred-tank bioreactors with a working volume of 1 L at 70°C. With the synthetic consortium of *C. kristjanssonii* and *C. saccharolyticus* the authors reported a $Y_{(H2/S)}$ of 3.8 mol H_2 per mol C6 sugar equivalent. As mentioned above, fermentation at higher temperatures increases H_2 productivity, which is why mesophilic organisms grown in batch fermentation systems achieve lower H_2 yields. For example, a natural consortium containing *C. acetobutylicum* and *Ethanoigenens harbinense* grown at 37°C in continuous stirred tank reactors obtained a $Y_{(H2/S)}$ of 2.8 mol H_2 per mol cellulose (Wang et al. 2008). Similar yields ranging from 2.1 to 2.3 mol H_2 per mol glucose were also observed using different pure and synthetic co-cultures of *Clostridium* species (Masset et al. 2012). Again, both Wang et al. (2008) and Masset et al. (2012) reported improved H_2 productivities when using co-cultures for biogas generation, with the latter study additionally focussing on the determination of the optimum pH prior to the experiments.

The pH directly affects the hydrogenase activity, metabolic by-products and biohydrogen yields, making it one of the most crucial parameters that determine the performance of H_2 synthetic pathways (Ghimire et al. 2015). Hence, the surveillance and control of the *in situ* acidity/alkalinity of the system is big advantage of the fermentation in batch mode. Masset et al. (2012) found the highest H_2 production rate at a pH of 5.3. Other experiments on dark fermentative H_2 production using co-cultures were carried out with a slightly higher pH of 6.5 (Pawar et al. 2015; Zeidan and van Niel 2009; Mishra et al. 2015). In the majority of our runs, we observed a rapid drop in the pH of the system due to acidogenic growth of the organisms. This is also where we monitored the highest H_2 production, as was expected from the organisms' metabolism. With the switch to solventogenesis H_2 productivities decreased. To prevent this, we tried to favour the acetate pathway by keeping a permanent neutral pH of 7. Later in the experiment we also tried to hold the pH at 6, as stable H_2 production by *Clostridium* sp. have been shown at a pH of 5.5-6 (Lin et al. 2010) (Lee et al. 2008a). Furthermore, Liu et al. (2011) stated that for H_2 production by Clostridia a pH of 7 is metabolically and thermodynamically unfavourable.

This might explain the high H_2 yields obtained when controlling the pH at 6. When the pH was kept stable, the maximum yield of 6.18 mol H_2 mol^{-1} glucose was reached 15 to 20 h after inoculation. When the pH was not controlled, the second highest yield we

obtained, of 4.45 mol H_2 mol^{-1} glucose, was reached later in the experiment, 25 to 29 h after inoculation,

These results are interesting since the rapid acidification of the medium is thought to prevent H_2 production. Our results, though, were contrary to this. When the pH was left unregulated the yield peaked after the medium pH dropped below 6, which is where the maximum yield was found under stable pH conditions.

As Fabiano and Perego (2002) reported, the substrate conversion rate of *E. aerogenes* culminates within the narrow interval of pH 6 to 7. Outside of that range, the H_2 yield sharply decreases. For *C. acetobutylicum* the optimum for H_2 production is slightly lower at pH 6 (Chin et al. 2003). Without preventing the pH from dropping too much though, the acidity will eventually lead to the inactivation of hydrogenases and the formation of spores in *C. acetobutylicum*. Under acidic conditions, undissociated acids can diffuse across the cytoplasmatic membrane and lead to a collapse of the transmembrane proton gradient (Dürre 2014). To prevent this, the organism shifts its metabolism from acetogenesis to solventogenesis. van Ginkel and Logan (2005) observed a near-complete inhibition of H_2 production with added acetic and butyric acids at a pH of 5 (concentrations of undissociated acids in the reactor: 50 and 63 mmol L^{-1}, respectively). High H_2 yields obtained at low pH conditions are presumably chiefly due to the activity of the clostridial hydrogenase. We can assume that the beneficial impacts emerging from *Enterobacter* sp. sustains the high hydrogen productivity, mainly by consuming O_2 and ensuring anaerobic conditions.

The second-best performing bioreactor did not receive any acid or base additions to keep the pH stable. Such conditions would be favourable for industrial applications since it would reduce the operational costs. However, these high production results were the exception. We performed multiple bioreactor-runs, that in theory had the same environmental conditions but differed in their performance. The successful implementation of a well-functioning artificial co-culture is dependent on various complex parameters and needs a clear understanding of the organisms' metabolisms as well as fine tuning of the process.

Ideally, not only the pH, but also initial substrate concentrations, medium composition, nutritional compounds, *etc*. are analysed prior to the fermentation experiment. This

way, the ecological niche of two possibly very distinct organisms can be correlated to ensure a stable co-existence for maximum biogas production.

In the study by Ergal et al. (2020) the initial glucose concentration for a stable co-existence of *C. acetobutylicum* and *E. aerogenes* was set at 30 g L^{-1} as indicated in the literature survey (Ergal et al. 2018). Furthermore, the initial cell concentration ratio of 1:10.000 (*E. aerogenes* : *C. acetobutylicum*) was found to enable steady growth for both organisms. The increased initial concentration of *Clostridium* sp. was necessary to prevent *Enterobacter* sp. from overgrowing and monopolizing the nutrients. At this cell concentration, H_2 production was initiated earlier in the fermentation, compared to monocultures. Further, this inoculation ratio achieved the highest values for HER (6.64 mmol L^{-1} h^{-1}) and $Y_{(H2/S)}$ (5.6 mol mol^{-1}) on glucose. Balancing out each of the organisms' growth abilities and behaviour is a crucial step for constructing a stable and well-functioning synthetic consortium. This high discrepancy in the cell concentration (used by Ergal et al. (2020)) is an exception, since most other studies on synthetic co-cultures worked with an equal proportion of the strains (Mishra et al. 2015; Masset et al. 2012; Patel et al. 2012). Still, the ratio 1:10.000 proved to generate the optimum conditions for both strains in closed batch as well as in the batch system that we used in the current study.

Next to the optimization of the process parameters, our focus also lied on implementing the fermentation in volumetrically larger reactors to test the ability of the synthetic culture to provide similar H_2 yields at an increased scale.

Dark fermentative H_2 production at larger scales is undeniably necessary for the industrialization of biogas generation and has already been successfully implemented at pilot scale. Lin et al. (2010) combined batch and continuous batch cultivation mode to up-scale a H_2 producing heat treated seed sludge consortium in a vessel of 400 L with a working volume of 380 L. A 48 h batch operation was followed by one month in continuous feeding mode. This initial step enhanced the biomass growth and helped to shorten the lag phase. H_2 yield in this pilot scale set-up reached a maximum of 2.34 mol H_2 per mol sucrose. A similar yield of 2.12 mol H_2 per mol glucose was obtained by a co-culture of *C. butyricum* and *C. pasteurianum* in 20 L batch bioreactors (Masset et al. 2012). A slightly higher yield of 2.76 mol H_2 per mol glucose could be achieved by Vatsala et al. (2008) in their attempt produce biogas with a culture volume of 100

m^3 (100.000L) using defined co-cultures of *Citrobacter freundii*, *E. aerogenes* and *Rhodopseudomonas palustris*. In a much smaller batch 5 L system, Patel et al. (2012) achieved a $Y_{(H2/S)}$ of 3.2 and 3 mol H_2 per mol glucose for synthetic co-cultures of *Enterobacter cloacae* plus *Bacillus cereus* and *Enterobacter cloacae* plus *Klebsiella* sp., respectively.

All these studies clearly show how engineered microbial consortia can be applied for higher H_2 production, surpassing those of monocultures. Nevertheless, the Thauer limit of 4 mol H_2 per mol glucose during dark fermentation, is only just reached. Thermophilic strains proved to be more efficient in providing higher yields close to the theoretical limit (Zeidan and van Niel 2009; Zeidan et al. 2010). The application of mesophilic H_2 producers on the other hand profits from the fact that these organisms have been thoroughly studied and their physiology and genetics are very well known. This is a prerequisite for the successful combination of different strains to ensure stable growth and biofuel production. Using the known information in the literature then serves to aid in designing a sophisticated fermentation set-up that provides all of the organisms' necessities. It is that precision design pipeline that enabled Ergal et al. (2020) to achieve high H_2 yields in serum bottles and differentiates their study from other experiments on synthetic co-culture bio-H_2 production.

In the same way, this strategy proved to be successful in volumetrically larger batch mode operated bioreactors, as presented in this thesis. We herein show that by intensive literature research, selecting the best performing organisms, and screening of their physiological and ecological needs, up-scaling of synthetic co-cultures is in fact possible and might pave the way for future industrialization of the process.

2. Conclusions

In this thesis, I proved that the upscaling of a previously described artificial co-culture, composed of *E. aerogenes* and *C. acetobutylicum* for dark fermentative H_2 production is possible. The yields and productivities surpassed those described for any other study on DFHP using a designed consortia in batch mode before.

With the opportunity to optimize a number of environmental conditions in bioreactors, influences of different parameters were tested. Above all, adjustments of initial inoculation ratios and pH showed that with fine-tuning of the system a significant increase in the biogas production efficiency can be achieved.

Nevertheless, the design and construction of artificial microbial consortia at higher scale is complex and requires detailed knowledge of the involved organisms and intense preparation.

We can therefore conclude that, whilst future industrial applications of an artificial consortium to produce biohydrogen is very promising, it still needs further research and a better understanding of the ongoing processes. The optimization of this highly complex approach will require ecological, physiological, and biotechnological knowledge, yet may pave the way for environmentally sustainable alternatives to fossil fuel-based energy generation.

References

Abendroth G von, Stripp S, Silakov A, Croux C, Soucaille P, Girbal L, Happe T (2008) Optimized over-expression of [FeFe] hydrogenases with high specific activity in Clostridium acetobutylicum. International Journal of Hydrogen Energy 33:6076–6081

Alshiyab H, Kalil MS, Hamid AA, WM WY (2008a) Effect of salts addition on hydrogen production by C. acetobutylicum. Pakistan journal of biological sciences : PJBS 11:2193–2200

Alshiyab H, Kalil MS, Hamid AA, Yusoff WMW (2008b) Effect of some environmental parameters on hydrogen production using C. acetobutylicum. Pakistan journal of biological sciences : PJBS 11:2073–2082

Amend JP, Shock EL (2001) Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. FEMS microbiology reviews 25:175–243

Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domíguez-Espinosa R (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends in biotechnology 22:477–485

Bader J, Mast-Gerlach E, Popović MK, Bajpai R, Stahl U (2010) Relevance of microbial coculture fermentations in biotechnology. Journal of applied microbiology 109:371–387

Bagramyan K, Mnatsakanyan N, Poladian A, Vassilian A, Trchounian A (2002) The roles of hydrogenases 3 and 4, and the F 0 F 1 -ATPase, in H 2 production by Escherichia coli at alkaline and acidic pH. FEBS Letters 516:172–178

Basak N, Das D (2007) The Prospect of Purple Non-Sulfur (PNS) Photosynthetic Bacteria for Hydrogen Production: The Present State of the Art. World J Microbiol Biotechnol 23:31–42

Basak N, Jana AK, Das D, Saikia D (2014) Photofermentative molecular biohydrogen production by purple-non-sulfur (PNS) bacteria in various modes: The present progress and future perspective. International Journal of Hydrogen Energy 39:6853–6871

Beckers L, Hiligsmann S, Hamilton C, Masset J, Thonart P (2010) Fermentative hydrogen production by Clostridium butyricum CWBI1009 and Citrobacter freundii CWBI952 in pure and mixed cultures. Biotechnologie, agronomie, société et environnement 14:541–548

Benomar S, Ranava D, Cardenas ML, Trably E, Rafrafi Y, Ducret A, Hamelin J, Lojou E, Steyer JP, Giudici-Orticoni MT (2015) Nutritional stress induces exchange of cell material and energetic coupling between bacterial species. Nat Commun 6: 6283

Bockris J (2002) The origin of ideas on a Hydrogen Economy and its solution to the decay of the environment. International Journal of Hydrogen Energy 27:731–740

Bohnenkamp AC, Wijffels RH, Kengen SWM, Weusthuis RA (2021) Co-Production of Hydrogen and Ethyl Acetate in E. Coli. Biotechnol Biofuels 14: 192

Bolton JR (1996) Solar photoproduction of hydrogen. IEA technical report of the IEA Agreement of the Production and Utilization of Hydrogen. United States.

Brenner K, You L, Arnold FH (2008) Engineering microbial consortia: a new frontier in synthetic biology. Trends in biotechnology 26:483–489

Buckel W, Thauer RK (2013) Energy conservation via electron bifurcating ferredoxin reduction and proton/Na(+) translocating ferredoxin oxidation. Biochimica et biophysica acta 1827:94–113

Cabrol L, Marone A, Tapia-Venegas E, Steyer J-P, Ruiz-Filippi G, Trably E (2017) Microbial ecology of fermentative hydrogen producing bioprocesses: useful insights for driving the ecosystem function. FEMS microbiology reviews 41:158–181

Cai G, Jin B, Saint C, Monis P (2010) Metabolic flux analysis of hydrogen production network by Clostridium butyricum W5: effect of pH and glucose concentrations. International Journal of Hydrogen Energy 35:6681–6690

Cai G, Jin B, Saint C, Monis P (2011) Genetic manipulation of butyrate formation pathways in Clostridium butyricum. Journal of biotechnology 155:269–274

Calusinska M, Happe T, Joris B, Wilmotte A (2010) The surprising diversity of clostridial hydrogenases: a comparative genomic perspective. Microbiology (Reading, England) 156:1575–1588

Chandel AK, Chandrasekhar G, Silva MB, Da Silvério Silva S (2012) The realm of cellulases in biorefinery development. Critical reviews in biotechnology 32:187–202

Chandrasekhar K, Lee Y-J, Lee D-W (2015) Biohydrogen Production: Strategies to Improve Process Efficiency through Microbial Routes. International Journal of Molecular Sciences

Chang J-J, Chou C-H, Ho C-Y, Chen W-E, Lay J-J, Huang C-C (2008) Syntrophic co-culture of aerobic Bacillus and anaerobic Clostridium for bio-fuels and bio-hydrogen production. International Journal of Hydrogen Energy 33:5137–5146

Chen CC, Lin CY, Chang JS (2001) Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Applied microbiology and biotechnology 57:56–64. https://link-springer-com.uaccess.univie.ac.at/content/pdf/10.1007/s002530100747.pdf

Chen C-Y, Yang M-H, Yeh K-L, Liu C-H, Chang J-S (2008) Biohydrogen production using sequential two-stage dark and photo fermentation processes. International Journal of Hydrogen Energy 33:4755–4762

Chen C-Y, Yeh K-L, Lo Y-C, Wang H-M, Chang J-S (2010) Engineering strategies for the enhanced photo-H2 production using effluents of dark fermentation processes as substrate. International Journal of Hydrogen Energy 35:13356–13364

Chin H-L, Chen Z-S, Chou CP (2003) Fedbatch operation using Clostridium acetobutylicum suspension culture as biocatalyst for enhancing hydrogen production. Biotechnology progress 19:383–388

Chojnacka A, Błaszczyk MK, Szczęsny P, Nowak K, Sumińska M, Tomczyk-Żak K, Zielenkiewicz U, Sikora A (2011) Comparative analysis of hydrogen-producing bacterial biofilms and granular sludge formed in continuous cultures of fermentative bacteria. Bioresource technology 102:10057–10064. https://www.sciencedirect.com/science/article/pii/S0960852411011692

Chu, Chunfeng, Ebie, Yoshitaka, Inamori, Yuhei, Kong, Hainan (2009) Effect of hydraulic retention time on the hydrogen yield and population of Clostridium in hydrogen fermentation of glucose. Journal of Environmental Sciences 21:424–428

Collins M, Knutti R, Arblaster J, Dufresne J-L, Fichefet T, Friedlingstein P, Gao X, Gutowski WJ, Johns T, Krinner G (2013) Long-term climate change: projections, commitments and irreversibility. In: IPCC 2013: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Stocker, T.F., D. Qin, G.-K. Plattner,

M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp., pp 1029–1136

Conrad R, Wetter B (1990) Influence of temperature on energetics of hydrogen metabolism in homoacetogenic, methanogenic, and other anaerobic bacteria. Archives of microbiology 155:94–98

Dabrock B, Bahl H, Gottschalk G (1992) Parameters affecting solvent production by Clostridium pasteurianum. Applied and environmental microbiology 58:1233–1239

Das D (2001) Hydrogen production by biological processes: a survey of literature. International Journal of Hydrogen Energy 26:13–28

Demuez M, Cournac L, Guerrini O, Soucaille P, Girbal L (2007) Complete activity profile of Clostridium acetobutylicum FeFe-hydrogenase and kinetic parameters for endogenous redox partners. FEMS microbiology letters 275:113–121

Ding J, Liu B-F, Ren N-Q, Xing D-F, Guo W-Q, Xu J-F, Xie G-J (2009) Hydrogen production from glucose by co-culture of Clostridium butyricum and immobilized Rhodopseudomonas faecalis RLD-53. International Journal of Hydrogen Energy 34:3647–3652

Dionigi F, Reier T, Pawolek Z, Gliech M, Strasser P (2016) Design criteria, operating conditions, and nickel-iron hydroxide catalyst materials for selective seawater electrolysis. ChemSusChem 9:962–972

Dürre P (2014) Physiology and Sporulation in Clostridium. Microbiology spectrum 2:TBS-0010-2012

Ergal İ, Fuchs W, Hasibar B, Thallinger B, Bochmann G, Rittmann SK-MR (2018) The physiology and biotechnology of dark fermentative biohydrogen production. Biotechnology advances 36:2165–2186

Ergal İ, Gräf O, Hasibar B, Steiner M, Vukotić S, Bochmann G, Fuchs W, Rittmann SK-MR (2020) Biohydrogen production beyond the Thauer limit by precision design of artificial microbial consortia. Communications biology 3:443

Fabiano B, Perego P (2002) Thermodynamic study and optimization of hydrogen production by Enterobacter aerogenes. International Journal of Hydrogen Energy 27:149–156

Fang HHP, Zhang T, Liu H (2002) Microbial diversity of a mesophilic hydrogen-producing sludge. Applied microbiology and biotechnology 58:112–118

Gavala HN, Skiadas IV, Ahring BK (2006) Biological hydrogen production in suspended and attached growth anaerobic reactor systems. International Journal of Hydrogen Energy 31:1164–1175. https://www.sciencedirect.com/science/article/pii/S036031990500296X

Geng A, He Y, Qian C, Yan X, Zhou Z (2010) Effect of key factors on hydrogen production from cellulose in a co-culture of Clostridium thermocellum and Clostridium thermopalmarium. Bioresource technology 101:4029–4033

Germscheidt RL, Moreira DEB, Yoshimura RG, Gasbarro NP, Datti E, dos Santos PL, Bonacin JA (2021) Hydrogen Environmental Benefits Depend on the Way of Production: An Overview of the Main Processes Production and Challenges by 2050. Advanced Energy and Sustainability Research:2100093

Ghimire A, Frunzo L, Pirozzi F, Trably E, Escudie R, Lens PNL, Esposito G (2015) A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. Applied Energy 144:73–95

Greening C, Biswas A, Carere CR, Jackson CJ, Taylor MC, Stott MB, Cook GM, Morales SE (2016) Genomic and metagenomic surveys of hydrogenase distribution indicate H2 is a widely utilised energy source for microbial growth and survival. The ISME journal 10:761–777

Grosskopf T, Soyer OS (2014) Synthetic microbial communities. Current opinion in microbiology 18:72–77

Guo XM, Trably E, Latrille E, Carrere H, Steyer J-P (2010) Hydrogen production from agricultural waste by dark fermentation: a review. International Journal of Hydrogen Energy 35:10660–10673

Hakobyan M, Sargsyan H, Bagramyan K (2005) Proton translocation coupled to formate oxidation in anaerobically grown fermenting Escherichia coli. Biophysical chemistry 115:55–61

Hallenbeck PC (2005) Fundamentals of the fermentative production of hydrogen. Water Science and Technology 52:21–29

Hallenbeck PC (2009) Fermentative hydrogen production: Principles, progress, and prognosis. International Journal of Hydrogen Energy 34:7379–7389

Hallenbeck PC, Ghosh D (2012) Improvements in fermentative biological hydrogen production through metabolic engineering. Journal of environmental management 95:S360-S364. https://www.sciencedirect.com/science/article/pii/S0301479710002173

Hallenbeck and Benemann (2002) Biological hydrogen production; fundamentals and limiting processes. International Journal of Hydrogen Energy 27:1185–1193

Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL (2007) Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. International Journal of Hydrogen Energy 32:172–184. https://www.sciencedirect.com/science/article/pii/S0360319906003247

Hay JXW, Wu TY, Juan JC, Md. Jahim J (2013) Biohydrogen production through photo fermentation or dark fermentation using waste as a substrate: Overview, economics, and future prospects of hydrogen usage. Biofuels, Bioprod. Bioref. 7:334–352

Herrero A, Gomez R, Snedecor B, Tolman C, Roberts M (1985) Growth inhibition of Clostridium thermocellum by carboxylic acids: A mechanism based on uncoupling by weak acids. Appl Microbiol Biotechnol 22

Hung C-H, Cheng C-H, Guan D-W, Wang S-T, Hsu S-C, Liang C-M, Lin C-Y (2011) Interactions between Clostridium sp. and other facultative anaerobes in a self-formed granular sludge hydrogen-producing bioreactor. International Journal of Hydrogen Energy 36:8704–8711

Hung C-H, Lee K-S, Cheng L-H, Huang Y-H, Lin P-J, Chang J-S (2007) Quantitative analysis of a high-rate hydrogen-producing microbial community in anaerobic agitated granular sludge bed bioreactors using glucose as substrate. Applied microbiology and biotechnology 75:693–701

Ito T, Nakashimada Y, Kakizono T, Nishio N (2004) High-yield production of hydrogen by Enterobacter aerogenes mutants with decreased α-acetolactate synthase activity. Journal of bioscience and bioengineering 97:227–232

Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N (2005) Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. Journal of bioscience and bioengineering 100:260–265

Izzo G, Rosa S, Massini G, Patriarca C, Fenice M, Fiocchetti F, Marone A, Varrone C, Signorini A (2014) From hypertrophic lagoons to bioenergy production. Journal of Environmental Protection and Ecology 15:537–546

Jay ZJ, Hunt KA, Chou KJ, Schut GJ, Maness P-C, Adams MWW, Carlson RP (2020) Integrated thermodynamic analysis of electron bifurcating FeFe-hydrogenase to inform anaerobic metabolism and H2 production. Biochimica et biophysica acta. Bioenergetics 1861:148087

Jayasinghearachchi HS, Sarma PM, Singh S, Aginihotri A, Mandal AK, Lal B (2009) Fermentative hydrogen production by two novel strains of Enterobacter aerogenes HGN-2 and HT 34 isolated from sea buried crude oil pipelines. International Journal of Hydrogen Energy 34:7197–7207

Jen CJ, Chou C-H, Hsu P-C, Yu S-J, Chen W-E, Lay J-J, Huang C-C, Wen F-S (2007) Flow-FISH analysis and isolation of clostridial strains in an anaerobic semi-solid bio-hydrogen producing system by hydrogenase gene target. Appl Microbiol Biotechnol 74:1126–1134

Jo JH, Jeon CO, Lee SY, Lee DS, Park JM (2010) Molecular characterization and homologous overexpression of [FeFe]-hydrogenase in Clostridium tyrobutyricum JM1. International Journal of Hydrogen Energy 35:1065–1073

Jo JH, Lee DS, Park D, Choe W-S, Park JM (2008a) Optimization of key process variables for enhanced hydrogen production by Enterobacter aerogenes using statistical methods. Bioresource technology 99:2061–2066

Jo JH, Lee DS, Park D, Park JM (2008b) Statistical optimization of key process variables for enhanced hydrogen production by newly isolated Clostridium tyrobutyricum JM1. International Journal of Hydrogen Energy 33:5176–5183

Khanal SK, Chen W-H, Li L, Sung S (2004) Biological hydrogen production: effects of pH and intermediate products. International Journal of Hydrogen Energy 29:1123–1131

Kim BW, Chang KP, Chang HN (1997) Effect of light source on the microbiological desulfurization in a photobioreactor. Bioprocess Engineering 17:343–348

Kim S, Seol E, Oh Y-K, Wang GY, Park S (2009) Hydrogen production and metabolic flux analysis of metabolically engineered Escherichia coli strains. International Journal of Hydrogen Energy 34:7417–7427

Kleerebezem R, van Loosdrecht MCM (2007) Mixed culture biotechnology for bioenergy production. Current opinion in biotechnology 18:207–212

Kotay SM, Das D (2009) Novel dark fermentation involving bioaugmentation with constructed bacterial consortium for enhanced biohydrogen production from pretreated sewage sludge. International Journal of Hydrogen Energy 34:7489–7496

Kothari R, Buddhi D, Sawhney RL (2008) Comparison of environmental and economic aspects of various hydrogen production methods. Renewable and Sustainable Energy Reviews 12:553–563

Kothari R, Kumar V, Pathak VV, Ahmad S, Aoyi O, Tyagi VV (2017) A critical review on factors influencing fermentative hydrogen production. Front Biosci 22:1195–1220

Laothanachareon T, Kanchanasuta S, Mhuanthong W, Phalakornkule C, Pisutpaisal N, Champreda V (2014) Analysis of microbial community adaptation in mesophilic hydrogen fermentation from food waste by tagged 16S rRNA gene pyrosequencing. Journal of environmental management 144:143–151

Latifi A, Avilan L, Brugna M (2019) Clostridial whole cell and enzyme systems for hydrogen production: current state and perspectives. Appl Microbiol Biotechnol 103:567–575

Lee D-J, Show K-Y, Su A (2011) Dark fermentation on biohydrogen production: Pure culture. Bioresource technology 102:8393–8402

Lee H-S, Salerno MB, Rittmann BE (2008a) Thermodynamic evaluation on H2 production in glucose fermentation. Environmental science & technology 42:2401–2407

Lee J, Seol E, Kaur G, Oh Y-K, Park S (2012) Hydrogen production from C1 compounds by a novel marine hyperthermophilic archaeon Thermococcus onnurineus NA1. International Journal of Hydrogen Energy 37:11113–11121

Lee J, Yun H, Feist AM, Palsson BØ, Lee SY (2008b) Genome-scale reconstruction and in silico analysis of the Clostridium acetobutylicum ATCC 824 metabolic network. Applied microbiology and biotechnology 80:849–862

Levin DB, Chahine R (2010) Challenges for renewable hydrogen production from biomass. International Journal of Hydrogen Energy 35:4962–4969

Li C, Fang HHP (2007) Fermentative Hydrogen Production From Wastewater and Solid Wastes by Mixed Cultures. Critical Reviews in Environmental Science and Technology 37:1–39

Li C, Zhang T, Fang HHP (2007) Use of TaqMan gene probe for real-time monitoring of acidophilic hydrogen-producing bacteria. Biotechnology letters 29:1753–1757

Li F, Hinderberger J, Seedorf H, Zhang J, Buckel W, Thauer RK (2008) Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from Clostridium kluyveri. Journal of bacteriology 190:843–850

Li X-H, Liang D-W, Bai Y-X, Fan Y-T, Hou H-W (2014) Enhanced H2 production from corn stalk by integrating dark fermentation and single chamber microbial electrolysis cells with double anode arrangement. International Journal of Hydrogen Energy 39:8977–8982

Lim JK, Bae SS, Kim TW, Lee J-H, Lee HS, Kang SG (2012) Thermodynamics of formate-oxidizing metabolism and implications for H2 production. Applied and environmental microbiology 78:7393–7397

Lin C-Y, Wu S-Y, Lin P-J, Chang J-S, Hung C-H, Lee K-S, Chang F-Y, Chu C-Y, Cheng C-H, Lay C-H (2010) Pilot-scale hydrogen fermentation system start-up performance. International Journal of Hydrogen Energy 35:13452–13457

Liu B-F, Ren N-Q, Xie G-J, Ding J, Guo W-Q, Xing D-F (2010) Enhanced bio-hydrogen production by the combination of dark-and photo-fermentation in batch culture. Bioresource technology 101:5325–5329

Liu I-C, Whang L-M, Ren W-J, Lin P-Y (2011) The effect of pH on the production of biohydrogen by clostridia: thermodynamic and metabolic considerations. International Journal of Hydrogen Energy 36:439–449

Lloyd WG, Davenport DA (1980) Applying thermodynamics to fossil fuels: Heats of combustion from elemental compositions. J. Chem. Educ. 57:56

Lopes Pinto F. A, Troshina O., Lindblad P. (2002) A brief look at three decades of research on cyanobacterial hydrogen evolution. International Journal of Hydrogen Energy 27:1209–1215. https://www.sciencedirect.com/science/article/pii/S0360319902000897

Losey NA, Mus F, Peters JW, Le HM, McInerney MJ (2017) Syntrophomonas wolfei Uses an NADH-Dependent, Ferredoxin-Independent FeFe-Hydrogenase To Reoxidize NADH. Applied and environmental microbiology 83

Lu L, Ren ZJ (2016) Microbial electrolysis cells for waste biorefinery: A state of the art review. Bioresource technology 215:254–264

Lütke-Eversloh T, Bahl H (2011) Metabolic engineering of Clostridium acetobutylicum: recent advances to improve butanol production. Current opinion in biotechnology 22:634–647

Maintinguer SI, Fernandes BS, Duarte ICS, Saavedra NK, Adorno MAT, Varesche MB (2008) Fermentative hydrogen production by microbial consortium. International Journal of Hydrogen Energy 33:4309–4317. https://www.sciencedirect.com/science/article/pii/S0360319908007088

Manish S, Venkatesh KV, Banerjee R (2007) Metabolic flux analysis of biological hydrogen production by Escherichia coli. International Journal of Hydrogen Energy 32:3820–3830

Marbán G, Valdés-Solís T (2007) Towards the hydrogen economy? International Journal of Hydrogen Energy 32:1625–1637

Marone A, Izzo G, Mentuccia L, Massini G, Paganin P, Rosa S, Varrone C, Signorini A (2014) Vegetable waste as substrate and source of suitable microflora for bio-hydrogen production. Renewable Energy 68:6–13

Martinez-Porqueras E, Rittmann S, Herwig C (2013) Analysis of H2 to CO2 yield and physiological key parameters of Enterobacter aerogenes and Caldicellulosiruptor saccharolyticus. International Journal of Hydrogen Energy 38:10245–10251

Masset J, Calusinska M, Hamilton C, Hiligsmann S, Joris B, Wilmotte A, Thonart P (2012) Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures ofClostridium spp. Biotechnology for biofuels 5:35

Melis, A., Melnicki (2006) Integrated biological hydrogen production. International Journal of Hydrogen Energy 31:1563–1573. https://www.sciencedirect.com/science/article/pii/S0360319906002308

Mishra J, Khurana S, Kumar N, Ghosh AK, Das D (2004) Molecular cloning, characterization, and overexpression of a novel [Fe]-hydrogenase isolated from a

high rate of hydrogen producing Enterobacter cloacae IIT-BT 08. Biochemical and biophysical research communications 324:679–685

Mishra P, Das D (2014) Biohydrogen production from Enterobacter cloacae IIT-BT 08 using distillery effluent. International Journal of Hydrogen Energy 39:7496–7507

Mishra P, Roy S, Das D (2015) Comparative evaluation of the hydrogen production by mixed consortium, synthetic co-culture and pure culture using distillery effluent. Bioresource technology 198:593–602

Mitchell WJ (1997) Physiology of carbohydrate to solvent conversion by clostridia. Advances in microbial physiology 39:31–130

Mohan SV, Chandrasekhar K, Chiranjeevi P, Babu PS (2013) Biohydrogen production from wastewater. In: Biohydrogen. Elsevier, pp 223–257

Monlau F, Aemig Q, Trably E, Hamelin J, Steyer J-P, Carrere H (2013) Specific inhibition of biohydrogen-producing Clostridium sp. after dilute-acid pretreatment of sunflower stalks. International Journal of Hydrogen Energy 38:12273–12282

Morimoto K, Kimura T, Sakka K, Ohmiya K (2005) Overexpression of a hydrogenase gene in Clostridium paraputrificum to enhance hydrogen gas production. FEMS microbiology letters 246:229–234

Morsy FM (2017) Synergistic dark and photo-fermentation continuous system for hydrogen production from molasses by Clostridium acetobutylicum ATCC 824 and Rhodobacter capsulatus DSM 1710. Journal of photochemistry and photobiology. B, Biology 169:1–6

Nakashimada Y, Rachman MA, Kakizono T, Nishio N (2002) Hydrogen production of Enterobacter aerogenes altered by extracellular and intracellular redox states. International Journal of Hydrogen Energy 27:1399–1405. https://www.sciencedirect.com/science/article/pii/S0360319902001283

Nakayama S-i, Kosaka T, Hirakawa H, Matsuura K, Yoshino S, Furukawa K (2008) Metabolic engineering for solvent productivity by downregulation of the hydrogenase gene cluster hupCBA in Clostridium saccharoperbutylacetonicum strain N1-4. Appl Microbiol Biotechnol 78:483–493

Nanda S, Li K, Abatzoglou N, Dalai AK, Kozinski JA (2017) 11 - Advancements and confinements in hydrogen production technologies. In: Dalena F, Basile A, Rossi C (ed) Bioenergy Systems for the Future. Woodhead Publishing, pp 373–418

Nandi R, Sengupta S (1998) Microbial production of hydrogen: an overview. Critical reviews in microbiology 24:61–84

Nath D, Chakraborty I, Ghangrekar MM (2021) Integrating microbial electrochemical technologies for methane-to-bioelectricity and water-splitting to impart self-sustainability to wastewater treatment plants. Bioresource Technology Reports 13:100644

Nath K, Das D (2004) Improvement of fermentative hydrogen production: various approaches. Applied microbiology and biotechnology 65:520–529

Nowak J, Florek M, Kwiatek W, Lekki J, Chevallier P, Zięba E, Mestres N, Dutkiewicz EM, Kuczumow A (2005) Composite structure of wood cells in petrified wood. Materials Science and Engineering: C 25:119–130

Oh S-E, van Ginkel S, Logan BE (2003) The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. Environmental science & technology 37:5186–5190

Ohnishi A, Bando Y, Fujimoto N, Suzuki M (2010) Development of a simple bio-hydrogen production system through dark fermentation by using unique microflora. International Journal of Hydrogen Energy 35:8544–8553

Pachapur VL, Sarma SJ, Brar SK, Le Bihan Y, Buelna G, Verma M (2015a) Biohydrogen production by co-fermentation of crude glycerol and apple pomace hydrolysate using co-culture of Enterobacter aerogenes and Clostridium butyricum. Bioresource technology 193:297–306

Pachapur VL, Sarma SJ, Brar SK, Le Bihan Y, Buelna G, Verma M (2015b) Biological hydrogen production using co-culture versus mono-culture system. Environmental Technology Reviews 4:55–70

Pachapur VL, Sarma SJ, Brar SK, Le Bihan Y, Buelna G, Verma M (2017) Hydrogen production from biodiesel industry waste by using a co-culture of Enterobacter aerogenes and Clostridium butyricum. Biofuels 8:651–662

Pan C-M, Fan Y-T, Zhao P, Hou H-W (2008) Fermentative hydrogen production by the newly isolated Clostridium beijerinckii Fanp3. International Journal of Hydrogen Energy 33:5383–5391. https://www.sciencedirect.com/science/article/pii/S036031990800551X

Park W, Hyun SH, Oh S-E, Logan BE, Kim IS (2005) Removal of headspace CO2 increases biological hydrogen production. Environmental science & technology 39:4416–4420

Patel SKS, Kumar P, Mehariya S, Purohit HJ, Lee J-K, Kalia VC (2014) Enhancement in hydrogen production by co-cultures of Bacillus and Enterobacter. International Journal of Hydrogen Energy 39:14663–14668

Patel SKS, Singh M, Kumar P, Purohit HJ, Kalia VC (2012) Exploitation of defined bacterial cultures for production of hydrogen and polyhydroxybutyrate from pea-shells. biomass and bioenergy 36:218–225

Pattra S, Lay C-H, Lin C-Y, Sompong O, Reungsang A (2011) Performance and population analysis of hydrogen production from sugarcane juice by non-sterile continuous stirred tank reactor augmented with Clostridium butyricum. International Journal of Hydrogen Energy 36:8697–8703

Pawar SS, Vongkumpeang T, Grey C, van Niel EW (2015) Biofilm formation by designed co-cultures of Caldicellulosiruptor species as a means to improve hydrogen productivity. Biotechnology for biofuels 8:19

Peraldo Bicelli L (1986) Hydrogen: A clean energy source. International Journal of Hydrogen Energy 11:555–562. https://www.sciencedirect.com/science/article/pii/0360319986901217

Rachman MA, Nakashimada Y, Kakizono T, Nishio N (1998) Hydrogen production with high yield and high evolution rate by self-flocculated cells of Enterobacter aerogenes in a packed-bed reactor. Applied microbiology and biotechnology 49:450–454

Rathore D, Singh A, Dahiya D, Singh Nigam P (2019) Sustainability of biohydrogen as fuel: Present scenario and future perspective. AIMS Energy 7:1–19

Ren N-Q, Guo W-Q, Wang X-J, Xiang W-S, Liu B-F, Wang X-Z, Ding J, Chen Z-B (2008) Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. International Journal of Hydrogen Energy 33:4318–4324

Ren N-Q, Wang D-Y, Yang C-P, Wang LU, Xu J-L, Li Y-F (2010) Selection and isolation of hydrogen-producing fermentative bacteria with high yield and rate and its bioaugmentation process. International Journal of Hydrogen Energy 35:2877–2882

Reysenbach AL, Ravenscroft N, Long S, Jones DT, Woods (1986) Characterization, biosynthesis, and regulation of granulose in Clostridium acetobutylicum. Applied and environmental microbiology 52:185–190

Rittmann S, Herwig C (2012) A comprehensive and quantitative review of dark fermentative biohydrogen production. Microbial cell factories 11:115

Rittmann SK-MR, Lee HS, Lim JK, Kim TW, Lee J-H, Kang SG (2015) One-carbon substrate-based biohydrogen production: microbes, mechanism, and productivity. Biotechnology advances 33:165–177

Sarma SJ, Brar SK, Le Bihan Y, Buelna G, Soccol CR (2013) Hydrogen production from meat processing and restaurant waste derived crude glycerol by anaerobic fermentation and utilization of the spent broth. Journal of Chemical Technology & Biotechnology 88:2264–2271

Schäfer T, Schönheit P (1991) Pyruvate metabolism of the hyperthermophilic archaebacterium Pyrococcus furiosus. Archives of microbiology 155:366–377

Schut GJ, Adams MWW (2009) The iron-hydrogenase of Thermotoga maritima utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. Journal of bacteriology 191:4451–4457

Schütz K, Happe T, Troshina O, Lindblad P, Leitão E, Oliveira P, Tamagnini P (2004) Cyanobacterial H(2) production -- a comparative analysis. Planta 218:350–359

Scotcher MC, Bennett GN (2005) SpoIIE regulates sporulation but does not directly affect solventogenesis in Clostridium acetobutylicum ATCC 824. Journal of bacteriology 187:1930–1936

Seol E, Kim S, Raj SM, Park S (2008) Comparison of hydrogen-production capability of four different Enterobacteriaceae strains under growing and non-growing conditions. International Journal of Hydrogen Energy 33:5169–5175

Servinsky MD, Kiel JT, Dupuy NF, Sund CJ (2010) Transcriptional analysis of differential carbohydrate utilization by Clostridium acetobutylicum. Microbiology 156:3478–3491

Shaterzadeh MJ, Ataei SA (2017) The effects of temperature, initial pH, and glucose concentration on biohydrogen production from Clostridium acetobutylicum. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects 39:1118–1123

Shin J-H, Yoon JH, Lee SH, Park TH (2010) Hydrogen production from formic acid in pH-stat fed-batch operation for direct supply to fuel cell. Bioresource technology 101 Suppl 1:S53-8

Singh A, Sevda S, Abu Reesh IM, Vanbroekhoven K, Rathore D, Pant D (2015) Biohydrogen production from lignocellulosic biomass: technology and sustainability. Energies 8:13062–13080

Skidmore BE, Baker RA, Banjade DR, Bray JM, Tree DR, Lewis RS (2013) Syngas fermentation to biofuels: Effects of hydrogen partial pressure on hydrogenase efficiency. biomass and bioenergy 55:156–162

Soboh B, Linder D, Hedderich R (2004) A multisubunit membrane-bound NiFe hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium Thermoanaerobacter tengcongensis. Microbiology (Reading, England) 150:2451–2463

Song J, An D, Ren N, Zhang Y, Chen Y (2011) Effects of pH and ORP on microbial ecology and kinetics for hydrogen production in continuously dark fermentation. Bioresource technology 102:10875–10880

Tanisho S, Suzuki Y, Wakao N (1987) Fermentative hydrogen evolution by Enterobacter aerogenes strain E. 82005. International Journal of Hydrogen Energy 12:623–627

Tao Y, Chen Y, Wu Y, He Y, Zhou Z (2007) High hydrogen yield from a two-step process of dark- and photo-fermentation of sucrose. International Journal of Hydrogen Energy 32:200–206. https://www.sciencedirect.com/science/article/pii/S0360319906002412

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriological Reviews 41:100–180

Thomas L, Joseph A, Gottumukkala LD (2014) Xylanase and cellulase systems of Clostridium sp.: an insight on molecular approaches for strain improvement. Bioresource technology 158:343–350

Tolvanen KES, Santala VP, Karp MT (2010) [FeFe]-hydrogenase gene quantification and melting curve analysis from hydrogen-fermenting bioreactor samples. International Journal of Hydrogen Energy 35:3433–3439

van de Werken HJG, Verhaart MRA, van Fossen AL, Willquist K, Lewis DL, Nichols JD, Goorissen HP, Mongodin EF, Nelson KE, van Niel, EWJ, Stams, AJM, de Ward, Vos WM de, van der Oost J, Kelly RM, Kengen SWM (2008) Hydrogenomics of the extremely thermophilic bacterium Caldicellulosiruptor saccharolyticus. Applied and Environonmental Microbiology 75:6720–6729

van Ginkel S, Logan BE (2005) Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environmental science & technology 39:9351–9356

van Niel EWJ, Budde MAW, Haas GG de, van der Wal FJ, Claassen PAM, Stams AJM (2002) Distinctive properties of high hydrogen producing extreme thermophiles, Caldicellulosiruptor saccharolyticus and Thermotoga elfii. International Journal of Hydrogen Energy 27:1391–1398. https://www.sciencedirect.com/science/article/pii/S0360319902001155

van Niel EWJ, Claassen P am, Stams AJM (2003) Substrate and product inhibition of hydrogen production by the extreme thermophile, Caldicellulosiruptor saccharolyticus. Biotechnology and bioengineering 81:255–262

Vardar-Schara G, Maeda T, Wood TK (2008) Metabolically engineered bacteria for producing hydrogen via fermentation. Microbial Biotechnology 1:107–125

Varrone C, Rosa S, Fiocchetti F, Giussani B, Izzo G, Massini G, Marone A, Signorini A, Wang A (2013) Enrichment of activated sludge for enhanced hydrogen production from crude glycerol. International Journal of Hydrogen Energy 38:1319–1331

Vasconcelos I, Girbal L, Soucaille P (1994) Regulation of carbon and electron flow in Clostridium acetobutylicum grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. Journal of bacteriology 176:1443–1450

Vatsala TM, Raj SM, Manimaran A (2008) A pilot-scale study of biohydrogen production from distillery effluent using defined bacterial co-culture. International Journal of Hydrogen Energy 33:5404–5415

Verhaart MRA, Bielen AAM, van der Oost J, Stams AJM, Kengen SWM (2010) Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: mechanisms for reductant disposal. Environmental technology 31:993–1003

Vinoth Kanna I, Paturu P (2020) A study of hydrogen as an alternative fuel. International Journal of Ambient Energy 41:1433–1436

Wang A, Ren N, Shi Y, Lee D-J (2008) Bioaugmented hydrogen production from microcrystalline cellulose using co-culture—Clostridium acetobutylicum X9 and Ethanoigenens harbinense B49. International Journal of Hydrogen Energy 33:912–917

Wang J, Wan W (2008) Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. International Journal of Hydrogen Energy 33:2934–2941

Wang J, Wan W (2009) Factors influencing fermentative hydrogen production: A review. International Journal of Hydrogen Energy 34:799–811. https://www.sciencedirect.com/science/article/pii/S0360319908015073

Wang XJ, Ren NQ, Xiang WS, Guo WQ (2007) Influence of gaseous end-products inhibition and nutrient limitations on the growth and hydrogen production by hydrogen-producing fermentative bacterial B49. International Journal of Hydrogen Energy 32:748–754. https://www.sciencedirect.com/science/article/pii/S0360319906002953

Weizmann C, Rosenfeld B (1937) The activation of the butanol-acetone fermentation of carbohydrates by Clostridium acetobutylicum (Weizmann). Biochemical Journal 31:619

Wigley TML (1983) The pre-industrial carbon dioxide level. Climatic Change 5:315–320

Wolfe AJ (2005) The acetate switch. Microbiology and molecular biology reviews 69:12–50

Xiong W, Reyes LH, Michener WE, Maness P-C, Chou KJ (2018) Engineering cellulolytic bacterium Clostridium thermocellum to co-ferment cellulose-and hemicellulose-derived sugars simultaneously. Biotechnology and bioengineering 115:1755–1763

Yasin NHM, Man HC, Yusoff MZM, Hassan MA (2011) Microbial characterization of hydrogen-producing bacteria in fermented food waste at different pH values. International Journal of Hydrogen Energy 36:9571–9580

Yokoi H, Ohkawara T, Hirose J, Hayashi S, Takasaki Y (1995) Characteristics of hydrogen production by aciduric Enterobacter aerogenes strain HO-39. Journal of Fermentation and Bioengineering 80:571–574

Yokoi H, Saitsu A, Uchida H, Hirose JUN, Hayashi S, Takasaki Y (2001) Microbial hydrogen production from sweet potato starch residue. Journal of bioscience and bioengineering 91:58–63

Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y (1998) H2 production from starch by a mixed culture of Clostridium butyricum and Enterobacter aerogenes. Biotechnology letters 20:143–147

Yu J, Takahashi P (2007) Biophotolysis-based hydrogen production by cyanobacteria and green microalgae. Communicating current research and educational topics and trends in applied microbiology 1:79–89

Zajic JE, Margaritis A., Brosseau JD (1979) Microbial hydrogen production from replenishable resources. International Journal of Hydrogen Energy 4:385–402

Zeidan AA, van Niel EWJ (2009) Developing a thermophilic hydrogen-producing co-culture for efficient utilization of mixed sugars. International Journal of Hydrogen Energy 34:4524–4528

Zhang C, Lv F-X, Xing X-H (2011) Bioengineering of the Enterobacter aerogenes strain for biohydrogen production. Bioresource technology 102:8344–8349

Zhang C, Ma K, Xing X-H (2009) Regulation of hydrogen production by Enterobacter aerogenes by external NADH and NAD+. International Journal of Hydrogen Energy 34:1226–1232

Zhang T, Liu H, Fang HHP (2003) Biohydrogen production from starch in wastewater under thermophilic condition. Journal of environmental management 69:149–156

Zhang Z-P, Show K-Y, Tay J-H, Liang DT, Lee D-J (2008) Biohydrogen production with anaerobic fluidized bed reactors—A comparison of biofilm-based and granule-based systems. International Journal of Hydrogen Energy 33:1559–1564. https://www.sciencedirect.com/science/article/pii/S036031990700571X

Zhao H, Ma K, Lu Y, Zhang C, Wang L, Xing X-H (2009) Cloning and knockout of formate hydrogen lyase and H2-uptake hydrogenase genes in Enterobacter aerogenes for enhanced hydrogen production. International Journal of Hydrogen Energy 34:186–194. https://www.sciencedirect.com/science/article/pii/S0360319908013852

Zhu Y, Yang S-T (2004) Effect of pH on metabolic pathway shift in fermentation of xylose by Clostridium tyrobutyricum. Journal of biotechnology 110:143–157