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Extremophile mikrobielle Zellfabriken zur hocheffizienten Produktion von Biowasserstoff

H2.AT

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1 Inhaltsverzeichnis

2 Einleitung

Ziel des Projekts war es, ein definiertes mikrobielles Konsortium in Form eines Biofilms zu etablieren, das höhere organische Verbindungen vollständig zu Biowasserstoff umsetzen kann. Dabei wurde organisches Substrat durch Mikroorganismen hydrolysiert und zu H_2 und Acetat abgebaut, und in weiterer Folge das gebildete Acetat ebenfalls biologisch zu H_2 oxidiert. Das Projekt gliederte sich in sechs Arbeitspakete (APs).

Die Ergebnisse des AP1 (Strukturierte Datenbank, Vorselektion von Stämmen aus Stammsammlungen) wurden erfüllt. Eine Datenbank, die 1732 Datenpunkte zur Biowasserstoffproduktion enthält, die aus 305 Publikationen extrahiert wurden, wurde erstellt. Diese Datenbank umfasst alle bis zum Mai 2016 publizierten Daten zur chemoorganoheterotrophen Biowasserstoffproduktion für die drei maßgeblichen Variablen: volumetrische H_2 Produktionsrate, spezifische H_2 Produktionsrate und den Ausbeutekoeffizienten der Biowasserstoffproduktion, produktionsassoziierte Parameter, phylogenetische und weiterführende Informationen, sowie eine Vielzahl von weiteren quantitativen und qualitativen Daten. Als das beste Substrat für die fermentative Biowasserstoffproduktion wurde Formiat identifiziert. Es konnte jedoch aus gezeigt werden, dass Zellulose und Lignozellulose interessante Substrate für die fermentative Biowasserstoffproduktion sein können. Teile der Datenbank wurden in der Fachzeitschrift *Biotechnology Advances* publiziert (Ergal *et al*. 2018, Biotechnology Advances 36(8):2165-2186).

Mittels statistischer Datenbankanalyse wurde der extremophile Stamm *Desulfurococcus amylolyticus* für die Analyse der Biowasserstoffproduktion mit Zellulose ausgewählt. Mit *D. amylolyticus* wurden Experimente im geschlossenen Zulaufsatzverfahren in Serumfläschchen und im Satzerfahren in Laborbioreaktoren durchgeführt. Hierzu konnten zwei Publikationen veröffentlicht werden (Reischl *et al*. 2018, Folia Microbiologica 63(6):713-723; Reischl *et al*. 2018, International Journal of Hydrogen Energy 43(18):8747-8753) und eine weitere Veröffentlichung von Ergal *et al*. ist derzeit „in revision“. Die in der wissenschaftlichen Literatur zu *D. amylolyticus* kolportierten H_2 Produktionscharakteristika konnten im AP2 leider nicht bestätigt werden, jedoch wurden in diesem Organismus hochinteressante physiologische Entdeckungen gemacht (Ergal *et al*., *in revision*).

Weitere heterotrophe Mikroorganismen, die für das „mikrobielle consortium engineering“ (MCE) aus der in AP1 erstellen Datenbank ausgewählt wurden, waren *Enterobacter aerogenes* und *Clostridium acetobutylicum*. Diese beiden Mikroorganismen wurden in Serumfläschchen als auch in Laborbioreaktoren kultiviert und die Charakterisierung der Wachstums- und Produktionskinetik mit folgenden analytische Methoden durchgeführt: Fluoreszenz *in situ* Hybridisierung (FISH), Mikroskopie und konfokale Mikroskopie, quantitative PCR, GC, HPLC und OD Messungen. Die Ergebnisse dieser Messungen wurden analytisch ausgewertet, um die Massenbilanzen und den quantitativen Einfluss der einzelnen MCE Organismen quantifizieren zu können. Das MCE war mit *E. aerogenes* und *C. acetobutylicum* erfolgreich und derzeit wird die Datenanalyse durchgeführt sowie das Verfassen einer Publikation vorbereitet.

Eine zweite Datenbank zu Acetat-oxidierenden Mikroorganismen wurde auch bereits im 1. Zwischenberichtzeitraum erstellt. Daraus wurden die Acetat-oxidierenden Organismen *Geobacter sufurreducens* und *Shewanella oneidensis* ausgewählt und erfolgreich in Serumfläschchen kultiviert.

Die „assisted H_2 production“ Technologie wurde im AP3 experimentell untersucht. Hier wurde der Fokus auf „H_2 milking“ und Elektrobiotechnologie gelegt. Zu Beginn des AP3 war die H_2 Entfernung leider nicht erfolgreich, jedoch konnten durch geschickte Adaptierung des experimentellen Aufbaus, und durch die Nutzung von undefinierten Mischkulturen, und später mit den in AP2 erstellten MCEs, die Experimente erfolgreich durchgeführt werden. Die Biofilmbildung wurde durch bildgebende Verfahren aus einer Kombination von FISH und konfokaler Lasermikroskopie erfolgreich durchgeführt.
Die Systemintegration von MCE-Biofilmen mit Elektrobiotechnologie bzw. „H_2 milking“ konnte erfolgreich in AP4 durchgeführt werden. Die H_2 Produktion, die Zusammensetzung der mikrobiellen Gemeinschaft wurde anhand von GC, HPLC, FISH und bildgebende Verfahren dokumentiert. Die Integration von *G. suflurreducens* oder *S. oneidensis* zu dem MCE, bestehend aus *C. acetobutylicum* und *E. aerogenes* konnte jedoch nicht erfolgreich durchgeführt werden. Hierbei besteht noch maßgeblicher und grundlegender Forschungsbedarf, um das MCE Verfahren hinsichtlich Biowasserstoffproduktion mit parallel ablaufender Acetatbildung und H_2 Produktion mittels Acetat-oxidation deutlich effizienter und zielgerichteter durchführen zu können.
Das AP5 (Management und Projektkoordination) und das AP6 (Know-How Schutz und Dissemination) wurden erfolgreich abgeschlossen. In AP5 wurden Teile der bis jetzt erzielten Forschungsergebnisse auf nationalen Arbeitsseminaren und internationalen Tagungen in Form von Postern und Vorträgen veröffentlich bzw. vorgestellt. Ein Workshop, Gas in Biotechnology, mit internationalen Teilnehmern von den Konsortialpartnern an der Universität für Bodenkultur im Jahre 2019 in Wien organisiert. Zudem wurden bereits drei Publikationen in int. Fachzeitschriften veröffentlicht und zwei bis vier weitere Veröffentlichungen in int. Fachzeitschriften sind noch vorgesehen. Alle APs wurden somit erfolgreich abgeschlossen und deren Meilensteine erfüllt.
Derzeit bereiten die Konsortialpartner zusammen mit int. akad. Projektpartnern einen FETopen Horizon 2020 Projektantrag vor, welcher sich mit der Weiterführung des MCE/“assisted H_2 production“-Verfahrens beschäftigen soll.

3 Inhaltliche Darstellung

3.1 Literature and Technology Assessment

Dark fermentative H_2 production (DFHP) characteristics of pure culture of microorganisms from more than a century of were analysed. Analysing pure culture DFHP has the advantage that the physiology and the biotechnological potential of a specific organism can be exploited with the aim to optimize and establish a straightforward H_2 production bioprocess. Essential to this effort is the analysis of reported values across phylogenetically distinct groups of microorganisms. Therefore, an extensive and in-depth meta-data analysis of DFHP from pure cultures was performed with the goals of providing: a comprehensive overview to their physiology, analysing closed batch, batch, and continuous culture DFHP from an energy production perspective, and to integrate physiology and biotechnology through comprehensive meta-data analyses, statistics, and modelling. A DFHP database is now available for further analyses (Ergal *et al*. 2018, Biotechnology Advances 36(8):2165-2186).

Our meta-data analyses revealed that a comparison of H_2 productivity and H_2 yield could unambiguously be performed on a carbon molar level. Clear dependencies between H_2 yield and the metabolic pathways of specific phylogenetic DFHP groups were identified. With respect to specific H_2 productivity and H_2 yield the superior phylogenetic group for DFHP was Thermococcaceae. Moreover, a distinct correlation between high H_2 yield and high H_2 productivity was identified. The best substrate for H_2 production was found to be formate. A statistical analysis and subsequent modelling provided the input parameter sets that could be used to improve of H_2 production of the families Clostridiaceae and Enterobacteriaceae. Therefore, a *Clostridium* sp. and an *Enterobacter* sp. were chosen for co-culture engineering. *Desulfurococcus amylolyticus* was chosen for extremophilic DFHP, due to its promising reported H_2 production characteristics.

Concerning microbial electrolysis for H_2 production about 300 papers were analysed. 40 papers concerned acetate utilization for H_2 production, only 5 papers utilized pure cultures. With respect to H_2 production with membrane contractors only one scientific work was yet published. Regarding H_2 production at nanostructured material no publication could yet be identified. Also with regard to these data assisted H_2 production technologies a database was constructed, which is compatible to the DFHP database. The literature research for acetate oxidizing bacteria led to the identification of two electroactive microorganisms, which were chosen for pure culture experiments. *G. sulfurreducens* and *S. oneidensis* both are able to use the anode inside a microbial electrolysis cell (MEC) as electron acceptor for the consumption of acetate. Both organisms have already been used in other studies on MECs and showed good results. First tests with both organisms confirm what is described in literature. Experiments with both organisms were conducted, also concerning the visualization of the formed biofilms. *G. sulfurreducens* showed the best results regarding H_2 production. Parts of the literature and technology assessment were published in the journal *Biotechnology Advances* (Ergal *et al*. 2018).

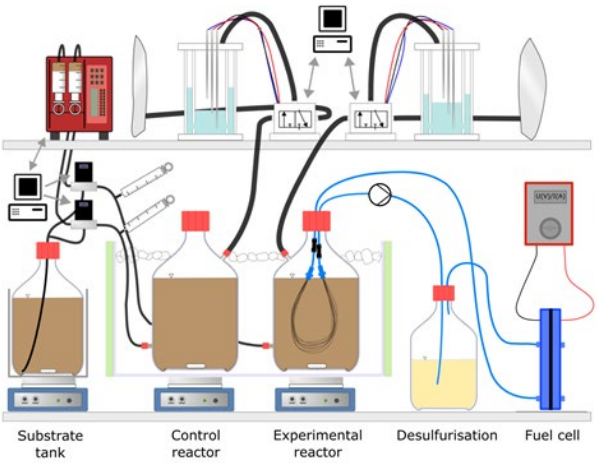
3.2 Cultivation and optimization of H_2-producing extremophiles

Desulfurococcus amylolyticus DSM 16532 was selected as a promising potential extremophilic H_2 cell factory. *D. amylolyticus* is an anaerobic and hyperthermophilic crenarchaeon. The organism is known to grow on a variety of different carbon sources, including monosaccharides and polysaccharides. *D. amylolyticus* is one of the few archaea that are known to be able to grow on cellulose. A metabolic reconstruction of *D. amylolyticus* metabolism was performed. Based on the published genome sequence, the metabolic reconstruction was completed by compiling complementary information available from KEGG, BRENDA, UniProt, NCBI and PFAM databases, and from literature. The metabolic reconstruction highlighted gaps in yet known carbon dioxide-fixation pathways. No complete carbon dioxide fixation pathway, such as the dicarboxylate-4-hydroxybutyrate cycle or the reductive citrate cycle could be identified. A closed batch experimental verification of glucose utilization was performed in chemically defined medium. All results and the outcome of the study, which were mentioned in this paragraph, were published in Reischl *et al*. 2018, Folia Microbiologica 63(6):713-723.
Based on the outcome of our study (Ergal *et al*. 2018, Biotechnology Advances 36(8):2165-2186) and due to the importance of cellulose as a second generation biofuel production substrate this compound was selected for H_2 production studies using *D. amylolyticus*. The biohydrogen production potential of *D. amylolyticus* was analysed in batch mode in lab bioreactors on cellulose and fructose. By analysing the cell specific H_2 production rates revealed that *D. amylolyticus* possesses an astonishing physiological potential to produce H_2, at rates equal or higher to other, well-known biohydrogen producing strains, however, regarding the volumetric H_2 production rate the limiting factor might be related to low cell concentrations. Given the cell-specific H_2 production rate and the metabolic physiological capacity of *D. amylolyticus*, the organism might still be envisioned to be optimized as a second generation H_2 cell factory. All results and the outcome of the study, which were mentioned in this paragraph, were published in Reischl *et al*. 2018, International Journal of Hydrogen Energy 43(18):8747-8753. More findings on the physiology of *D. amylolyticus* are currently under revision (Ergal et al.*in revision*).

3.3 Bioreactor and process technology

3.3.1 H_2 milking

Initial tests were conducted with two different sizes of membrane modules. The applied modules consisted of 7 and 17 hollow-fiber membranes. The hydrophobic membranes made of polypropylene (PP) have a nominal pore size of 0.2 µm (Accurel PP Q3 / 2, Membrana GmbH). The fibers were bonded to couplers using a two-component epoxy resin adhesive having an outer diameter of 1.8 mm and an approximate length of 160 mm at the respective ends, resulting in a membrane area of about 9 cm^2 per hollow fiber membrane. This leads to a total membrane area of about 63 cm^2 and 153 cm^2 for the modules consisting of 7 or 17 membranes. For the main experiment, the larger membrane module (see **Figure 1**) was used.



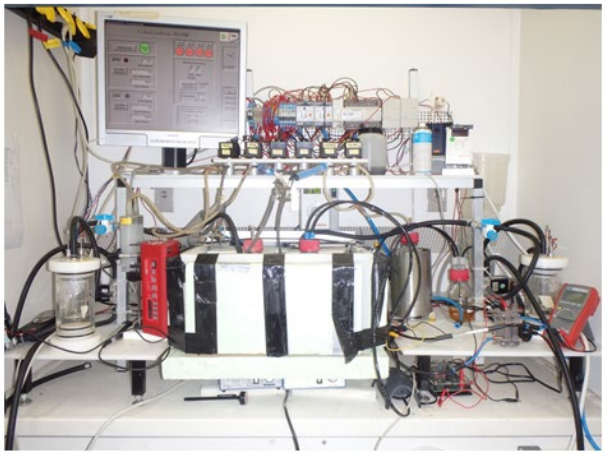


Figure 1: H_2 milking reactors. In the picture the first setup is shown, which included an experimental reactor and a control reactor.

Membrane modules

The experiments with small membrane modules (**Figure 2**) with a membrane surface area of 153 cm^2 and a reactor volume of 0.8 L did not show H_2 production. Due to a very small membrane surface to reactor volume ratio, no significant increase of H_2 production was found. This led to the acquisition of new membrane modules (Type Zena) (**Figure 3**). They consist of 1385 membranes with an external diameter of 310 µm and a length of 0.3 m and thus have a total membrane area of 0.4 m^2. The membrane module has a capacity of 25 mL and is used directly as a test reactor in these experiments. The resulting membrane surface to reactor volume ratio is much higher than in the previous modules.

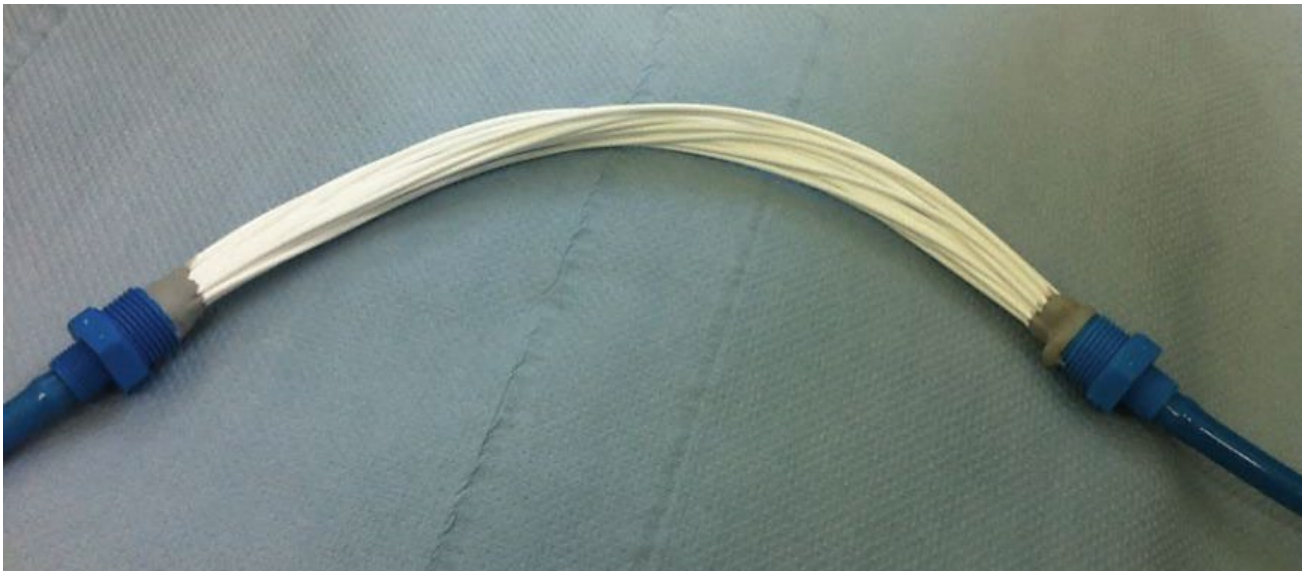


Figure 2: Small membrane module with 153 cm^2 membrane surface area.

Figure 3: Membrane Reactor - Type Zena

Experimental setup

In first experiments with the membrane modules type Zena the gas loop led to a fuel cell, which consumed the passing H_2 to produce electrical current. This worked with glucose as carbon source, because it is an easily available substrate for H_2 producing bacteria. With acetate as carbon source it did not work that well. To increase the H_2 removal rate the system was modified. The fuel cell was removed and a continuous N_2-purging (18 mL/h) was implemented. This setup minimizes the possibility of oxygen in the system and leads to a continuous removal of the produced gases H_2 and CO_2. A hydrogen sensor (qualitative, not quantitative) was implemented after the reactor, but was not feasible determining H_2 production. After some tests it was removed again. For quantitative measurements GC samples were taken twice a week, as well as liquid samples for HPLC analysis. First experiments were conducted at mesophilic conditions at 37°C. The main experiment is conducted at thermophilic conditions (50°C). The inoculum for this experiment was derived from a thermophilic anaerobic digester in Strem/Austria. The

medium consists of a phosphate buffer, minerals, vitamins, trace elements, 2-bromo-ethane-sulfunate as inhibitor of methanogenesis and acetate as the sole carbon source.

3.3.2 Electrochemically assisted H_2 production

Biological H_2 production is usually associated with metabolic end-products from mixed acid fermentation (Ergal *et al*. 2018, Biotechnology Advances 36(8):2165-2186). In the optimal case, the organic substrate is anaerobically degraded to acetate. The conversion of acetate to H_2 and CO_2 is thermodynamically not beneficial, and therefore does not occur without external energy input. Electrochemically assisted systems represent one possibility for supplying additional energy. In a microbial electrolysis cell (MEC), voltage is applied in the reactor via two electrodes. Electroactive, acetate-degrading microorganisms accumulate at the anode, H_2 is generated at the cathode. Such a set-up was constructed and it is schematically shown in **Figure 4**.

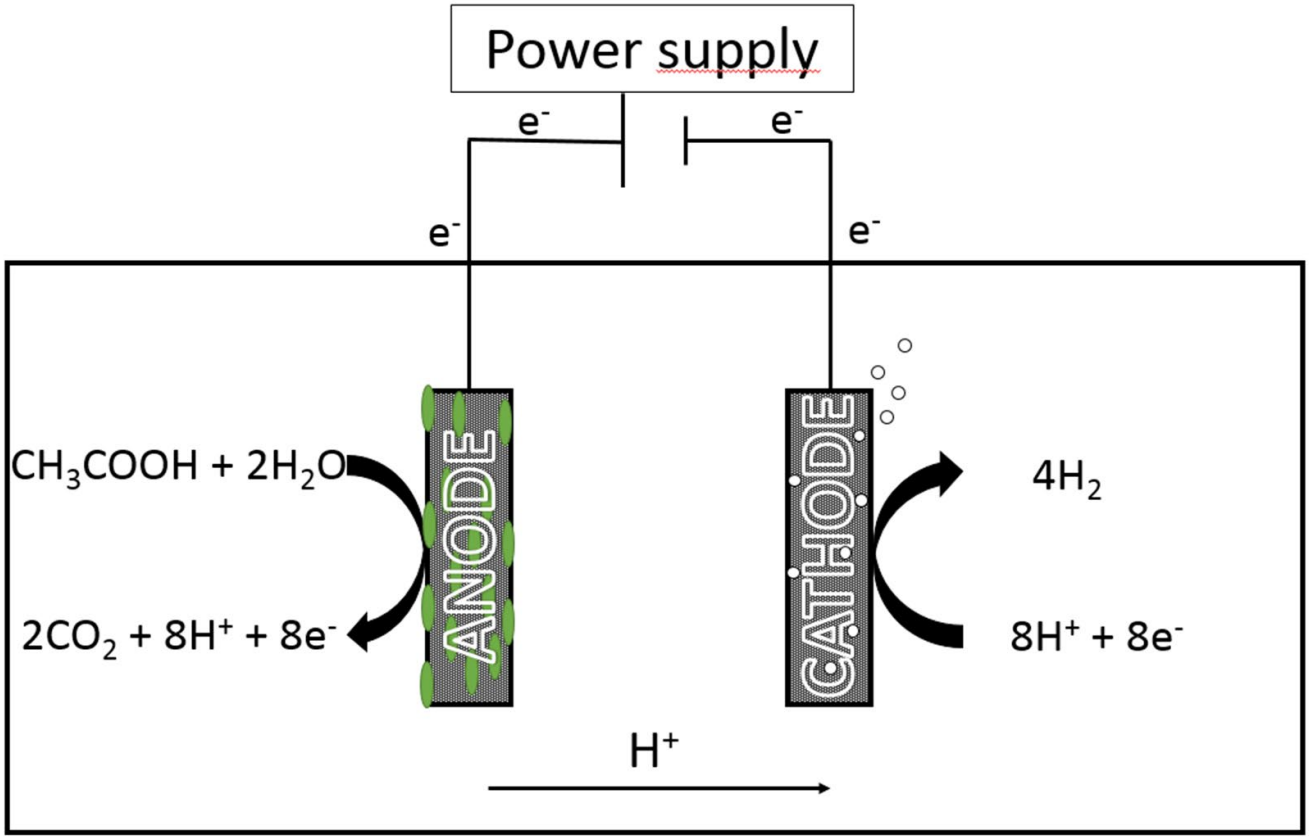


Figure 4: Principle of a microbial electrolysis cell (MEC). Electroactive microorganisms use the anode as electron acceptor for the consumption of acetate. H_2 is generated at the cathode.

Experimental setup

For the first experiments an experimental setup using the water replacement method for gas storage and quantification was used (**Figure 5**). This system was not gas tight regarding H_2, thus some adaption needed to be made. First, the gas collection system was changed to a setup with direct connection from the MEC to the gas bag (**Figure 6**). The initial system was not gastight, also other technical problems occurred. Many experiments were conducted to optimize the system. The major adaptions are shown in **Figure 6**.

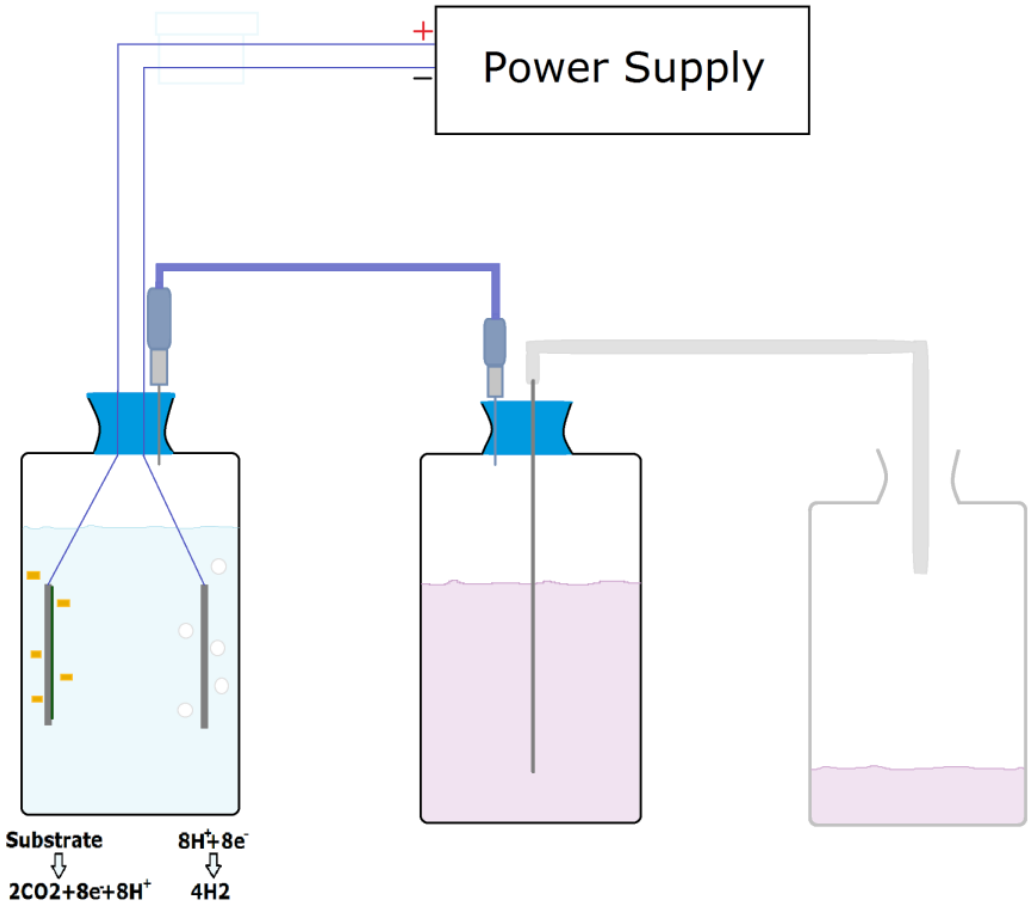


Figure 5: Experimental setup for the MECs using the water replacement method.

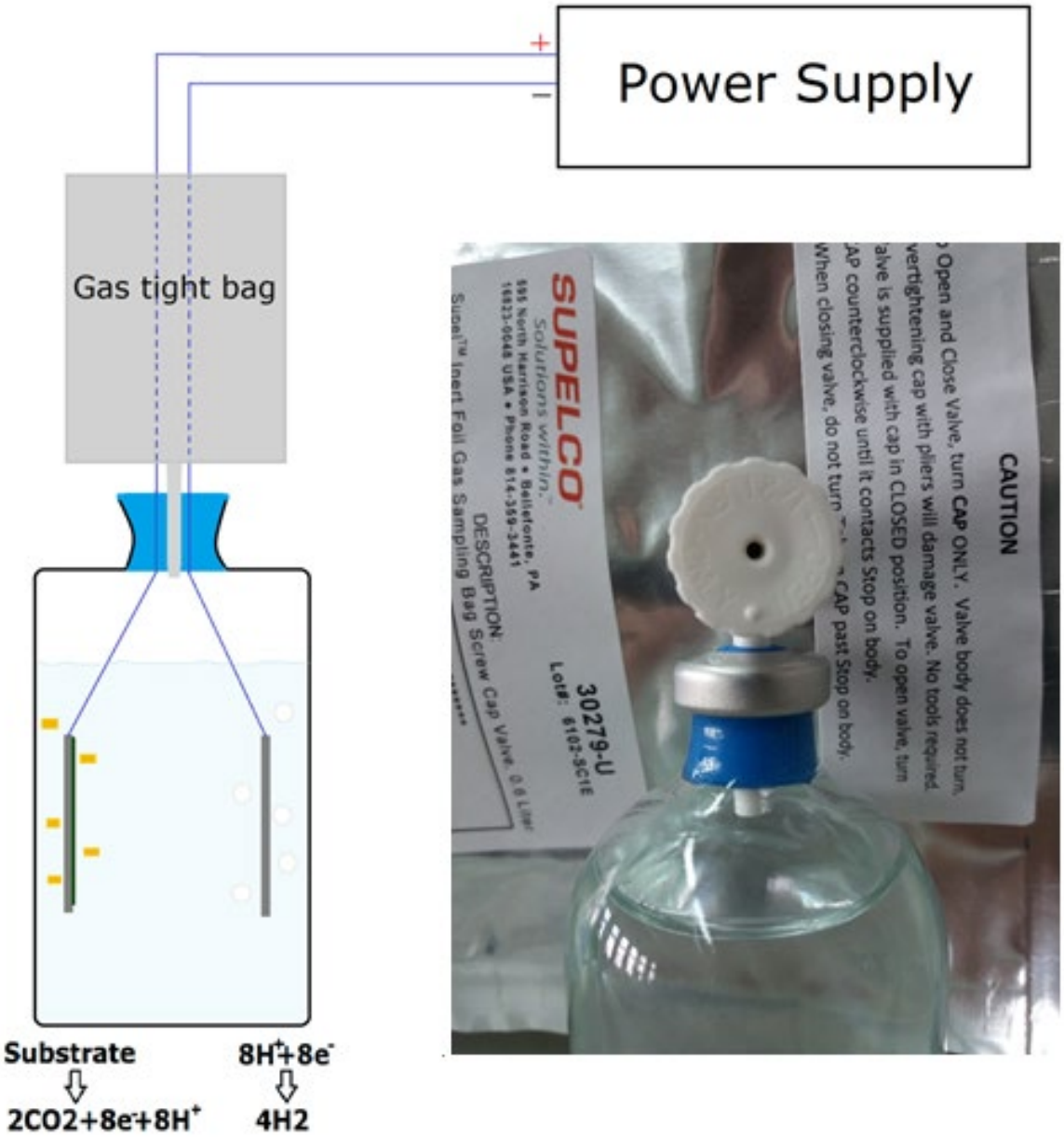


Figure 6: Experimental set-up with direct connection from the MEC to the gas bag for gas storage.

Mixed culture experiments

Several experiments were made using mixed cultures during the system optimization process. The results show that H_2 is being produced inside the MECs, but goes missing again within a few hours. Two possible reasons for this phenomenon were discussed: Leaks in the system and consumption of H_2 by homoacetogens. The implemented system adaptions deal with these two problems. With the optimized system this phenomenon could not be detected anymore. The results of the experiments with the optimized system show, that H_2 is continuously produced out of acetate. Over time the microbiological community adapted to the given conditions and the desired microorganisms colonized the anode. The initial inoculum was taken from a biogas plant. A characterization of the microbial consortium was conducted. Currently data analysis is ongoing.

Competing acetate consumption and production

As mentioned before, homoacetogens can use the produced H_2 and CO_2 to produce acetate. As the aim of this system is to produce H_2 out of acetate this reaction is unwanted. To understand and confirm this phenomenon taking place in our case, further experiments were conducted.The MECs were operated for three months before the presented experiments were started. The experiments were conducted in duplicate. One set of MECs was daily purged with N_2, the other one with a mixture of H_2/CO_2 (80:20 (v/v)). The acetate concentration as well as the gas composition and the electrical current inside the MECs was measured. The microbial consortium of the enrichment culture as well as of the anode and suspended cultures of the MECs purged with N_2 and H_2/CO_2 were determined by Amplicon sequencing using Illumina MiSeq. The change of the microbial community due to different gassing conditions is shown by the sequencing data. Data analysis is currently being performed.

Pure culture experiments

Two electroactive microorganisms were chosen based on an extensive literature research. *G. sulfurreducens* and *S. oneidensis,* both are able to use the anode as electron acceptor for the consumption of acetate, were utilized. Both organisms have already been used in studies published before, which also applied MECs and showed H_2 production. MEC experiments were operated with both organisms, using acetate as carbon source and a voltage of 0.8 V. *G. sulfurreducens* showed better growth rates and was chosen for further experiments.

3.3.3 FISH analysis/microscopic analysis of electrochemically assisted H_2 production biofilms

The microscopic analysis of the biofilms forming on carbon cloth (CC) using confocal laser scanning microscopy (CLSM) were used to elucidate the three-dimensional structure of the H_2 producing biofilm. Biofilms grown on CC electrodes in MECs were analysed concerning their 3D structure by staining the bacteria with the nucleic acid stain DAPI and the matrix with Concanavalin A –FITC (ConA). To visualize the 3D structure a z-stack (27 stacks of 1.5 µm thickness) of three different channels (ConA = green, DAPI = blue, CC rods = pink, all three colours = overlay of 3 channels) was performed and the microscope software used to overlay the channels and to create a three dimensional image. Data analysis is currently ongoing.

3.4 System integration and proof of principle

3.4.1 MEC co-culture experiments

The microorganisms were selected for co-culture experiments. Each of the microorganisms is metabolically specialized. These microorganisms should complement each other to enable highly efficient H_2 production. The species and the putative metabolic tasks are listed below:

Enterobacter aerogenes – consumption of cellobiose or glucose for H_2 and acetate production

Clostridium acetobutylicum – consumption of cellobiose or glucose and possibly volatile fatty acids for H_2 production

Geobacter sulfurreducens – consumption of glucose, volatile fatty acids (acetate) via microbial electrolysis

These organisms were tested individually and in co-culture on glucose or on cellobiose as sole carbon source. The applied voltage of 0.8 V shall shift the thermodynamic limits of acetate consumption. The anode shall serve as an electron acceptor for the exoelectrogenic bacteria inside the system. The MECs are prepared as described above. Data analysis is ongoing and the material and methods as well as results will be submitted for publication. The experimental set-up is shown in **Figure 8**.

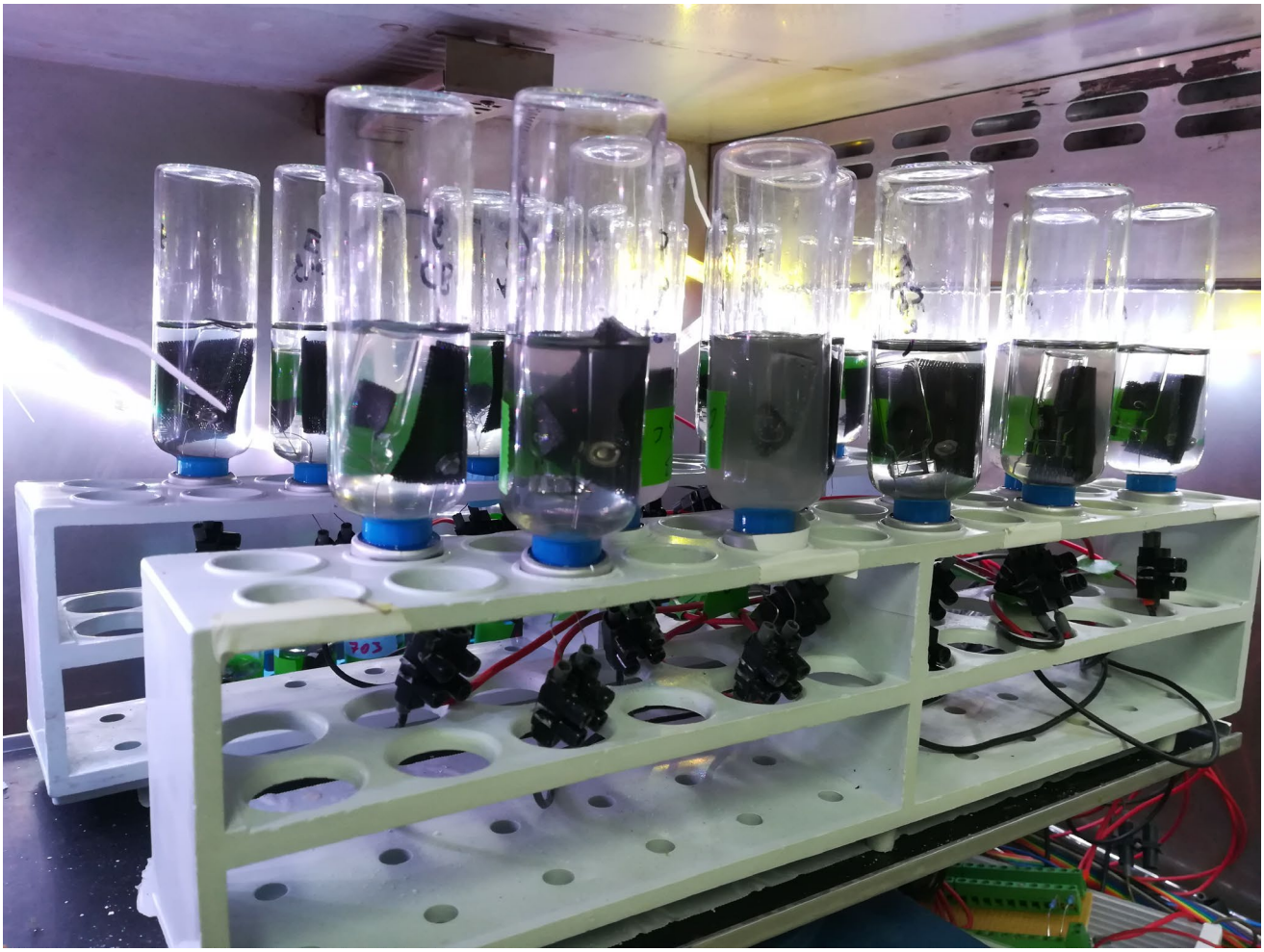


Figure 7: Experimental Setup MEC CoCulture experiments

4 Ergebnisse und Schlussfolgerungen

4.1 Literature and technology assessment

One paper was published in the journal *Biotechnology Advances* (Ergal *et al*. 2018, Biotechnology Advances 36(8):2165-2186). This paper summarises dark fermentative biohydrogen production from 117 years and more than 200 studies.

4.2 Cultivation and optimization of H_2-producing extremophiles

All experiments with respect to extremophilic H_2 production using *D. amylolyticus* DSM 16532 are finished. Two studies were published and one study is in revision stage:
Reischl *et al*. 2018, Folia Microbiologica 63(6):713-723
Reischl *et al*. 2018, International Journal of Hydrogen Energy 43(18):8747-8753)
Another manuscript was re-submitted: Ergal *et al*., *in revision*

4.3 Bioreactor and process technology

4.3.1 H_2 milking

The results of the continuously N_2-sparged reactors fed with acetate show an increase in acetate concentration over time (**Figure 9**). Also production of H_2 could be seen, but not in the expected stoichiometry (**Figure 10**).

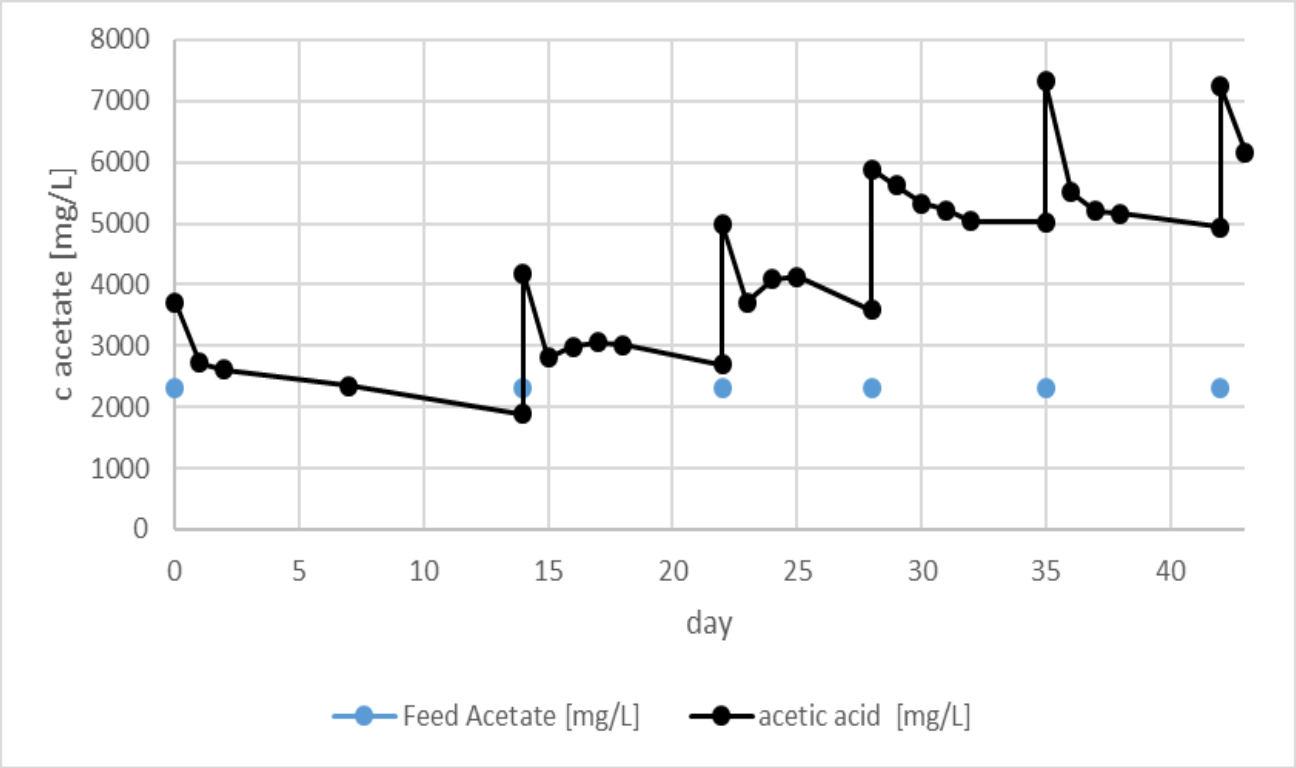


Figure 8: Acetate concentration inside the membrane reactor (ZENA) fed with acetate. Blue dots mark feeding events

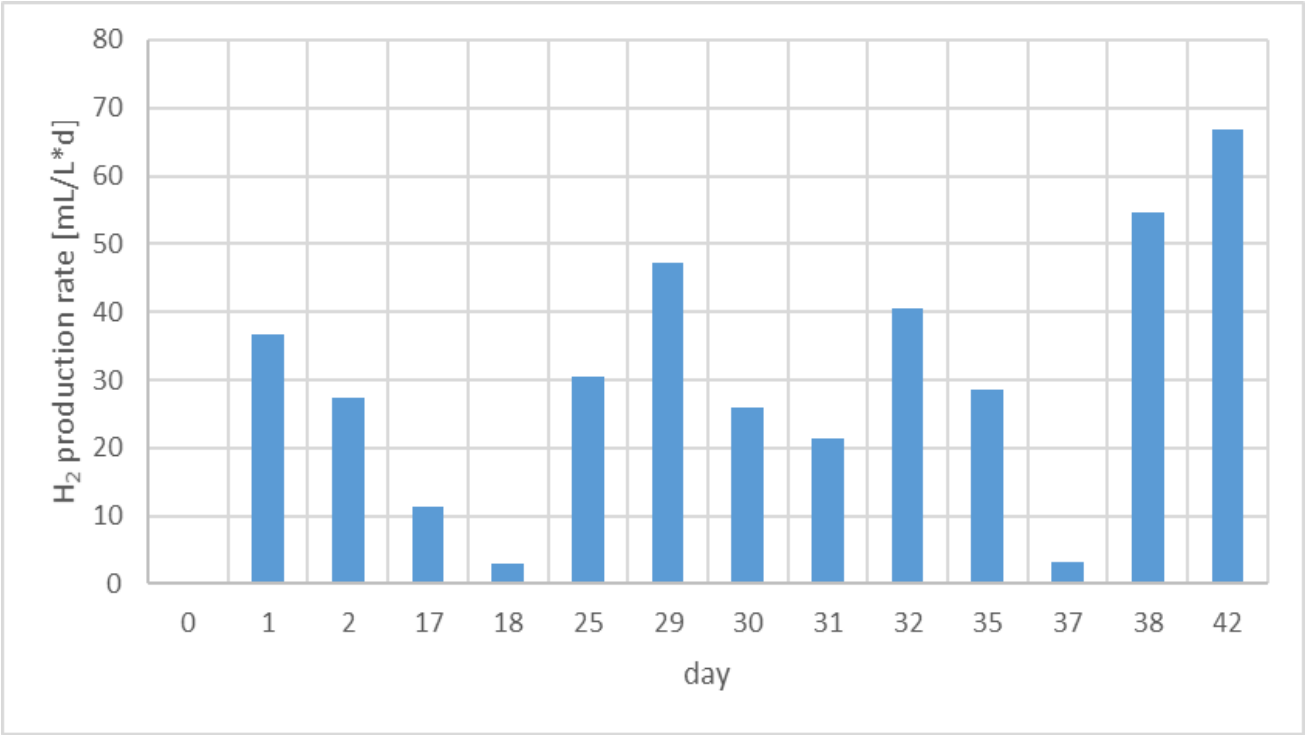


Figure 9: H_2 production rates inside the membrane reactor (ZENA) fed with acetate.

Another reactor was simultaniously conducted with the same parameters but with another carbon source (**Figure 11**). Here glucose instead of acetate was used. **Figure 11** shows a continious glucose

consumption, whereas the acetate level stays the same. Also, no other byproducts could be detected in HPLC. The slow consumption rates are due the low pH of 4.5 during this time of the experiment. With the anaerobic degradation of glucose the expected endproducts besides H_2 and CO_2 are volatile fatty acids and alcohols. But their concentration did not increase while glucose is beeing consumed. One possible explanation for this phenomenon, is, that the fatty acids are further consumed due to the lowered H_2 partial pressure. Unfortunately gas analysis could not confirm this theory.

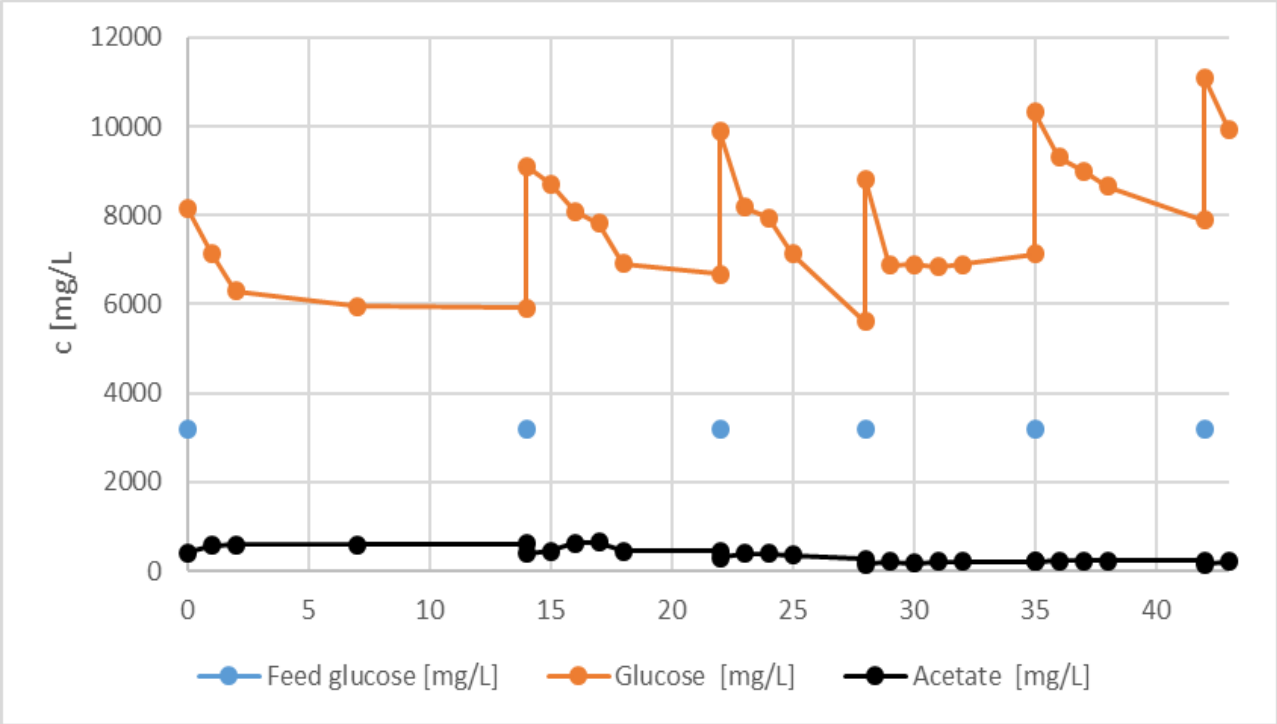


Figure 10: Glucose and acetate concentration inside the membrane reactor (ZENA) fed with glucose. Blue dots mark feeding events.

4.3.2 Electrochemically assisted H_2 production

Over 400 experiments were conducted, resulting in an intensive optimization of both mixed culture and pure culture system. Four major adaptions were made to meet the requirements for a gas tight MEC system with the possibility for continuous monitoring of the electrical current and daily sampling for GC and HPLC analysis:

1) Daily gas exchange

The daily exchange of the gas in the headspace is done for three reasons. First, it makes external gas storage unnecessary. The gas production of one day only causes a low overpressure. Thus the bottle is closed without any connection port. Second, with the daily removal of the produced H_2 the chemical equilibrium is shifted towards further H_2 production. Third, possible acetogenesis is avoided by removal of the produced H_2 and CO_2.

2) Turning the system upside down (**Figure 7A**)

To avoid any gas diffusion through the butyl rubber stopper the MEC bottles are turned upside down. Thus the headspace is enclosed by the glass vial and the butyl rubber stopper is only in contact with the liquid phase. Leakage of the rubber stopper may occur because of repeated piercing with cannulas for daily sampling.

3) Cathode inside a glass vial (**Figure 7B**)

The cathode (1x1 cm) is located inside a glass vial (2 mL) turned upside down. The vial is filled with medium. When gas production occurs on the cathode, the produced gas replaces the medium in the small bottle. Thus the productivity of the MEC can be checked visibly without disturbing the system. Especially at the beginning of an experiment (lag phase) it is beneficial to not disturb the system by taking gas or liquid samples. With the occurrence of gas inside the small vial the lag phase is over and gas production has started.

4) Continuous monitoring of the electrical current

To enable a continuous monitoring of the electrical current the voltage of each reactor is recorded separately. This makes it easy to determine the productivity of the MECs over time and also gives a warning, when a shortcut appears. For the technical setup of this system see **Figure 7C**.

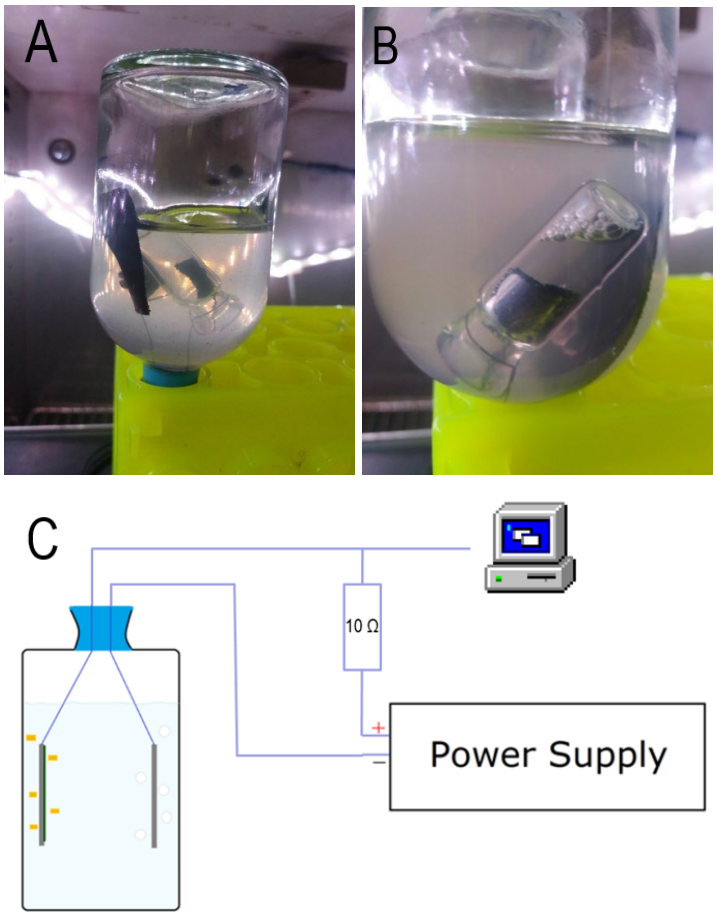


Figure 11: Adapted experimental MEC setup: (A) MEC reactor turned upside down. (B) Produced gas (H_2) collected inside glass vial. (C) Setup for continuous monitoring of the electrical current.

4.3.3 FISH analysis/microscopic analysis of electrochemically assisted H_2 production biofilms

Biofilms (inoculum: biogas sludge) grown on CC electrodes in microbial electrolysis cells were analyzed concerning their 3D structure by staining the bacteria with the nucleic acid stain DAPI and the matrix with Concanavalin A –FITC (ConA). In order to visualize the 3D structure a z-stack (27 stacks of 1.5 µm thickness) of three different channels (ConA = green, DAPI = blue, CC rods = pink, all three colours = overlay of 3 channels) was performed and the microscope software used to overlay the channels and to create a three dimensional image. The results can be seen in **Figure 11**.

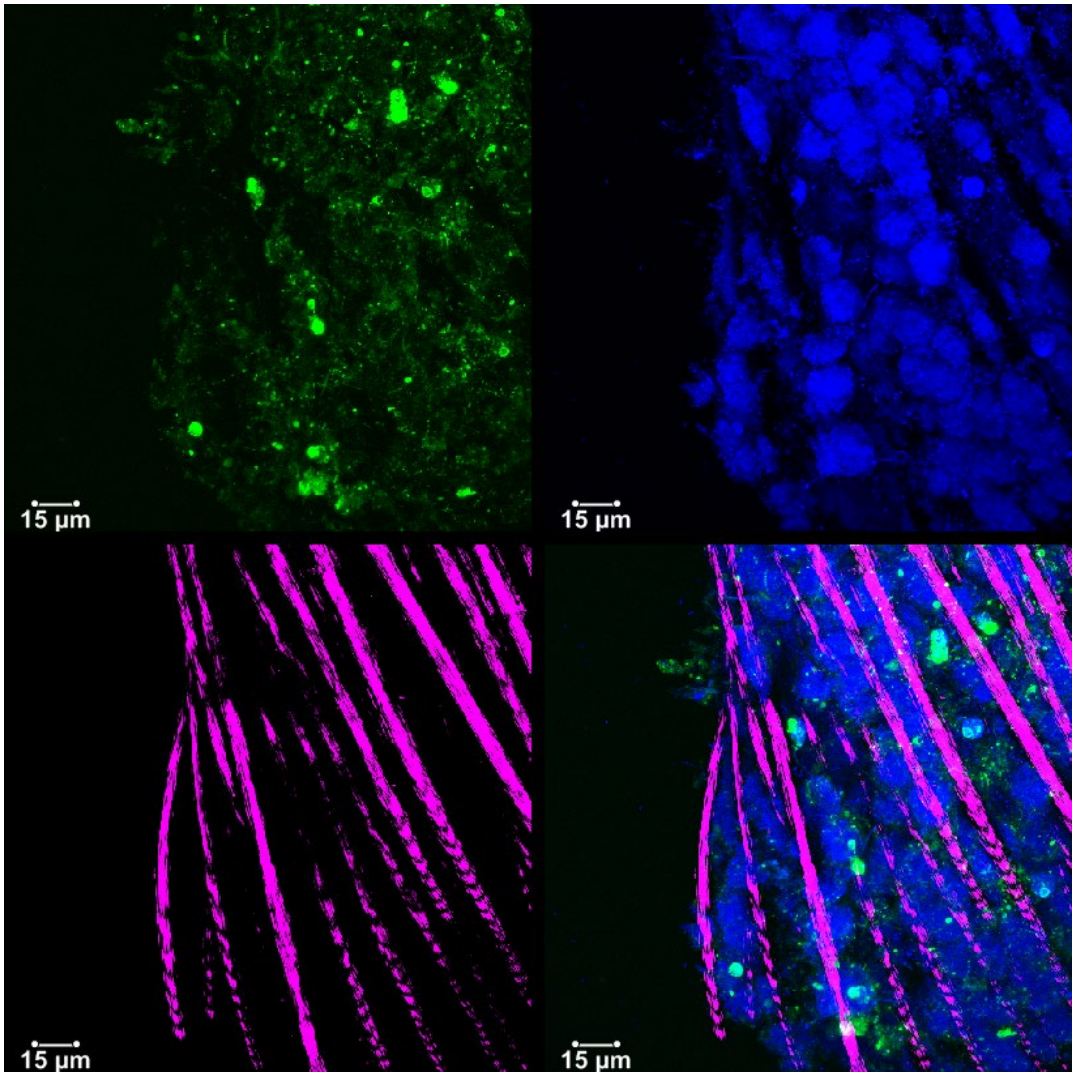


Figure 12: Z-stack of biofilm grown on carbon cloth electrodes in microbial electrolysis cells. Image shows biofilm grown on a single carbon cloth rod. (green channel = ConA, blue channel = DAPI, pink channel = CC rods)

The images shown in **Figure 11** paint a really clear picture of the 3D structure of the mixed species biofilm. CC rods consist of intertwined carbon cloth threads and the bacteria seem to appreciate this space as breeding ground. The DAPI staining shows clouds of bacteria attached to the rods partially covered by matrix components. As ConA only binds to the α-D-glucosyl and α-D-mannosyl residues of

the matrix one can assume, that the amount of matrix present within and around the biofilm is greater than what is shown by the CLSM image. These images clearly show that CC is a suitable material for the cultivation of biofilms which also does not hinder the microscopic visualization of the biofilm. In order to gain more insight, the biofilm was visualized in a higher magnification. The results are shown in **Figure 12**.

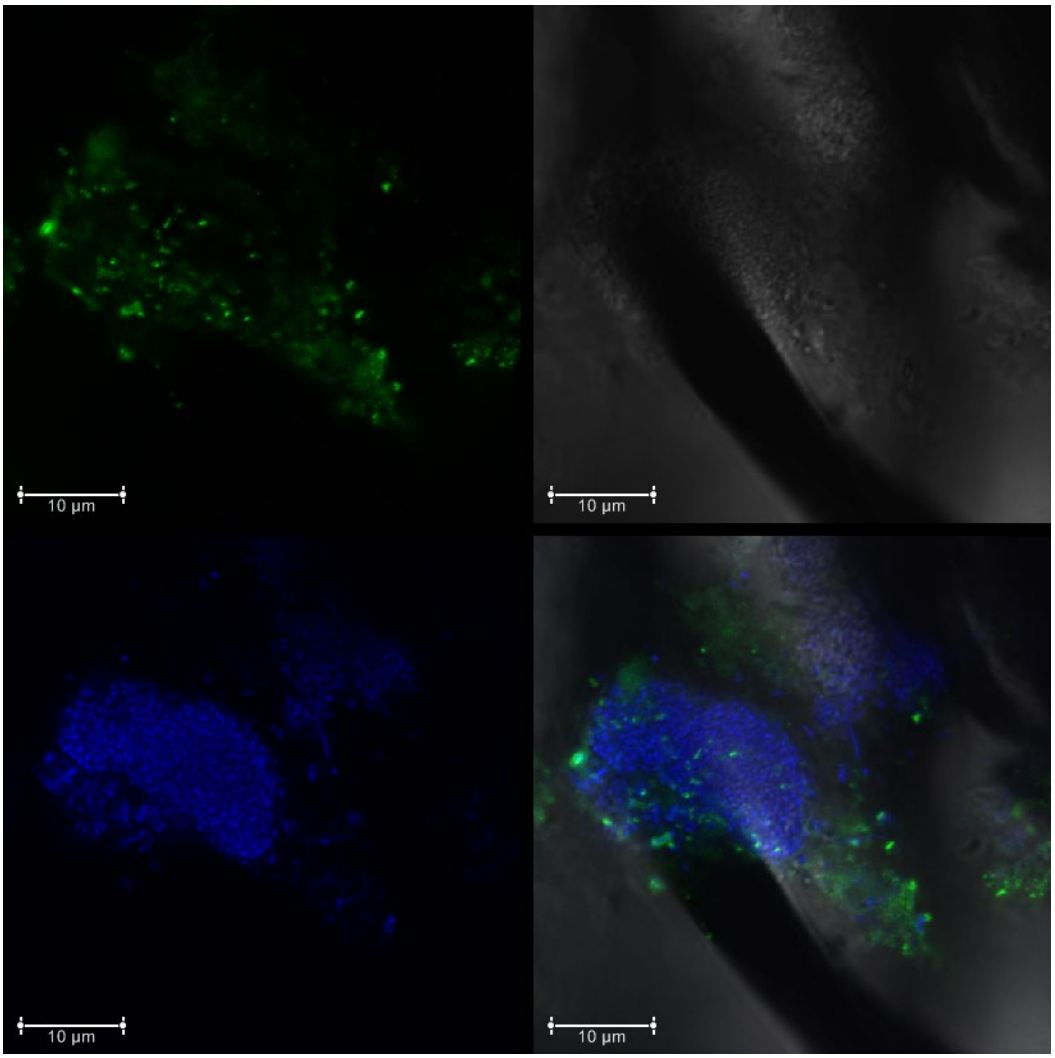


Figure 13: Higher magnification CLSM image of bacteria clouds shown in **Figure 11**. (ConA = green channel, transmission image = grey channel, DAPI = blue channel, overlay of 3 channels = grey, blue, green image)

For **Figure 12** the acquisition setup was changed and the reflexion image of the carbon cloth rods replaced by a transmission image. The higher magnification shows the blue clouds in **Figure 12** consist of single bacterial cells and that, morphologically speaking, it is a mixture of cocci- and rod-shaped bacteria. It seems like they are attached to the rod via a layer of matrix and also surrounded by matrix components. The bacteria fill out the space in between the threads, growing tightly and evenly. After the successful visualization of the general structure of the biofilm, the next step was the identification of the types of microorganisms that make up the biofilm. FISH is the method of choice if the type and localization of bacteria within a community is of interest. Fluorescently stained oligonucleotides, also

known as probes, are used to identify phyla, classes and orders of bacteria and archaea via specific regions on the 16s rDNA. In a first experiment only the general bacteria probe (equimolar mixture of Cy 2 labeled EUB I+II+III) was used. The results are shown in **Figure 13**. Figure 13 shows that the FISH staining was successful, as the area around the rods is stained yellow. The protocol has to be further improved in order to increase the fluorescent intensity and the resolution of the image as either the carbon cloth or the chemicals used throughout the FISH protocol seem to interfere with the imaging process.

The fluorescence in situ hybridization (FISH) protocol was further optimized and expanded in order to gain more insight on the composition of the biofilms formed on the carbon cloth. Figure 14 shows a high magnification image of a mixed species biofilm on a carbon cloth rod. The yellow fluorescence indicates the successful hybridization with the equimolar mixture of the general bacterial probe EUB I+II+III labelled with Cy 2. The high resolution of the image enables us to distinguish between different morphologies of bacteria (rods and cocci) and shows that mainly rods are present.

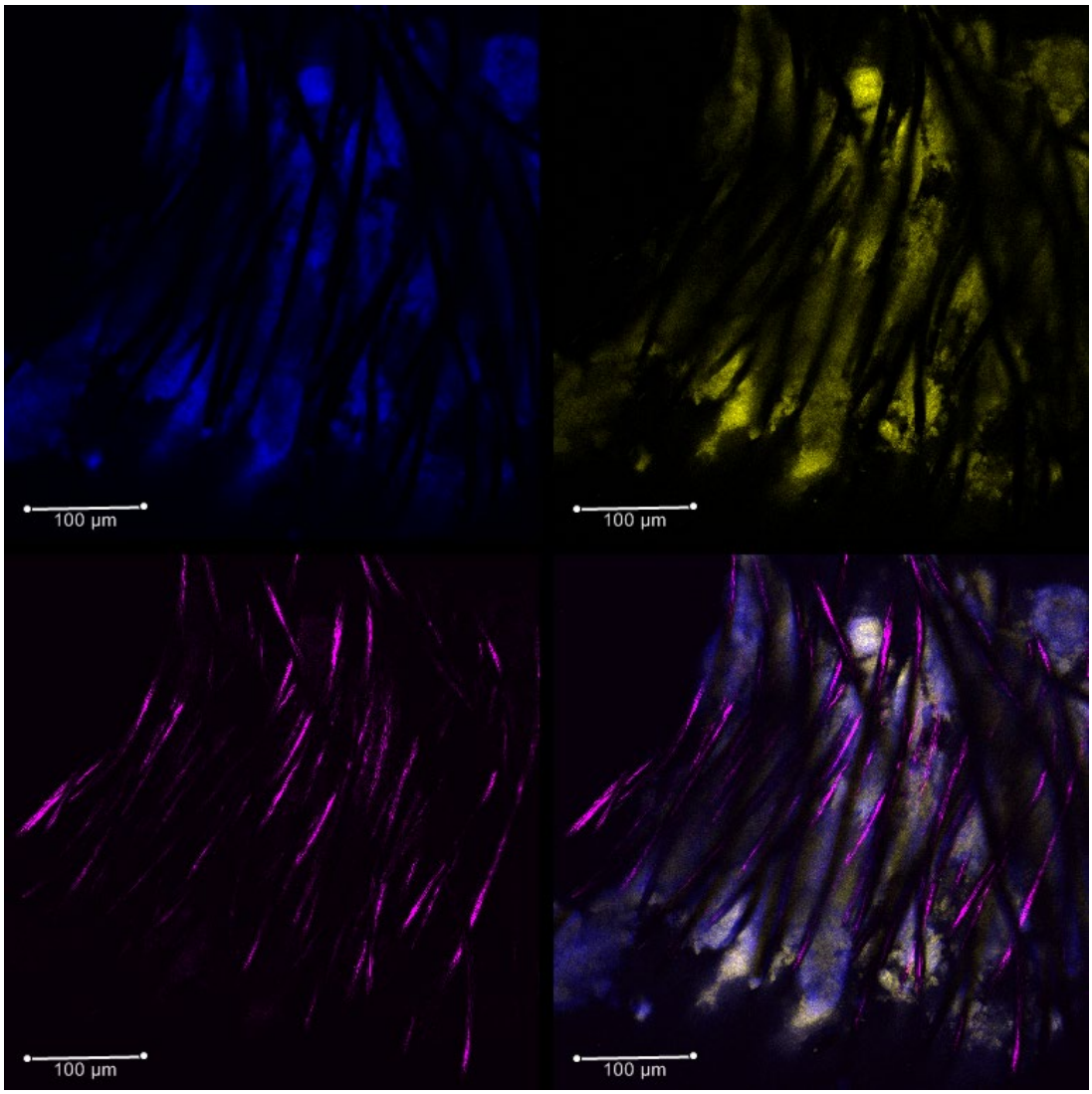


Figure 14: CLSM image after FISH experiment using DAPI for the bacteria (blue image), EUB I+II+III to identify bacteria (yellow image) and a reflexion image to visualize the CC rods (pink image). Blue, yellow and pink image shows the overlay of the three channels.

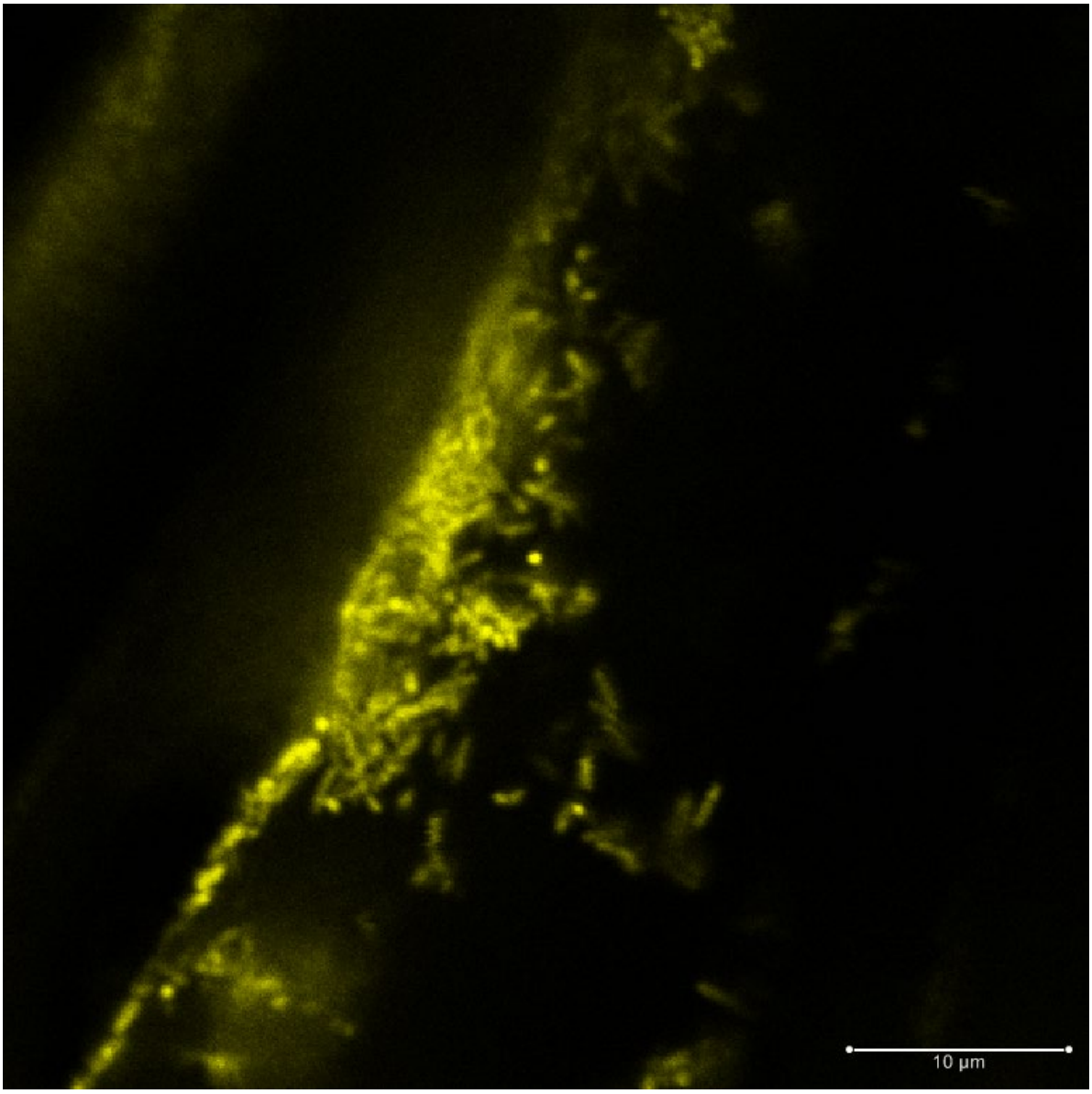


Figure 15: High resolution CLSM image of a mixed species biofilm formed on a carbon cloth rod. Bacteria are made visible through hybridization with an equimolar mix of the general bacterial probe EUB I + II + III labelled with Cy2

4.4 System integration und Proof of Principle

The experiments are finished (see also section 3.4). Currently data analysis is on-going for drafting three to four manuscripts.

4.5 Dissemination

A scientific workshop entitled Gas in Biotechnology was organized. The workshop on Gas in Biotechnology was held at the University of Natural Resources and Life Sciences (BOKU) in Vienna. Austria on the 28th and 29th of January 2019. 51 scientists from all over Europe joined this workshop (Figure 15).

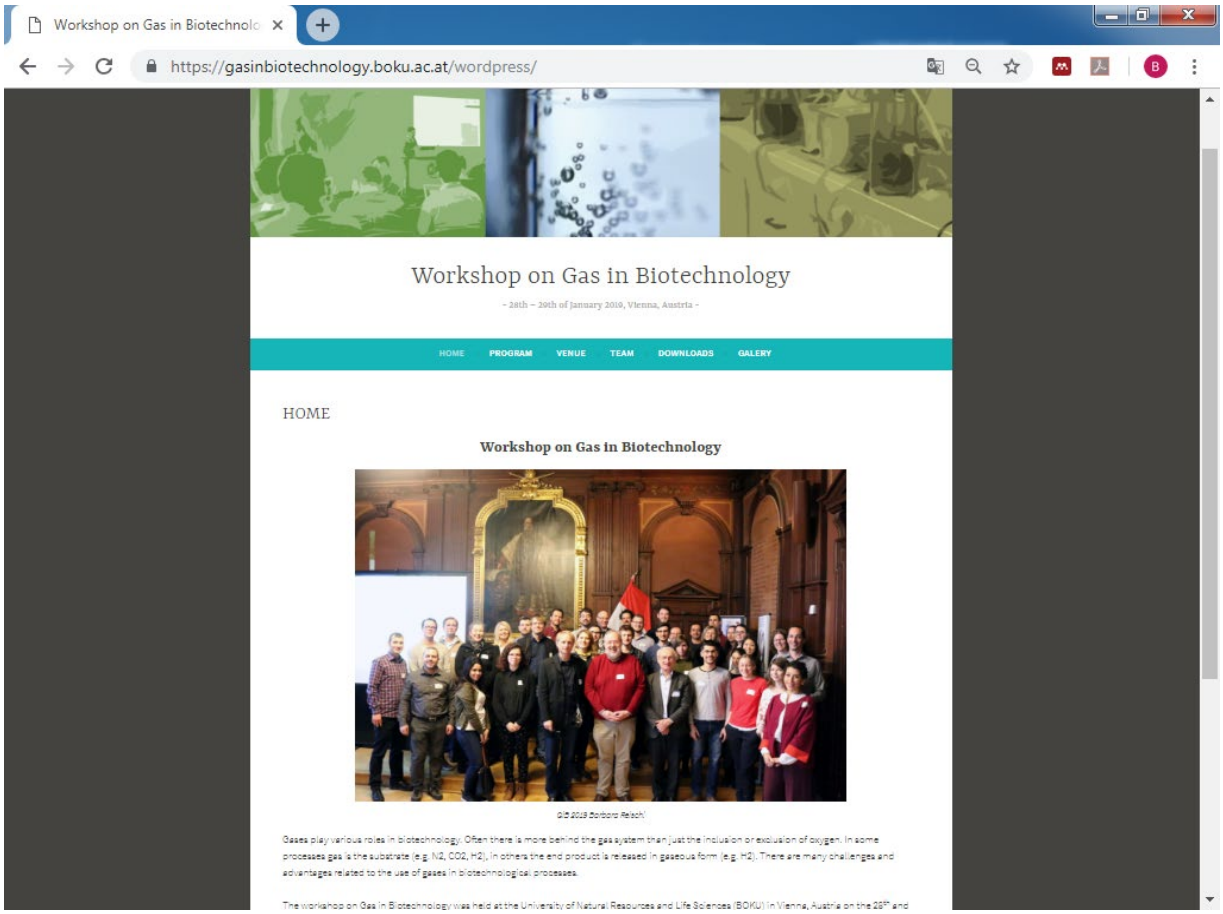


Figure 15: Webpage of the workshop "Gas in Biotechnology"

The topic focused on DFHP and gas fermentation, but also contributions from other relevant fields were presented. The key notes were given by Prof.dr.ir. Willy Verstraete (University Ghent. Belgium: Power to Biomass) and Prof. Dr. Volker Müller (Goethe-University Frankfurt. Germany: Production of molecular hydrogen with acetogenic bacteria). Talks were also given by Dr. Simon Rittmann, Prof. Dr. Werner Fuchs, İpek Ergal, MSc, and Benedikt Hasibar, MSc. Besides scientific talks and posters, also a workshop session was held, focusing on the problems and challenges experienced in anaerobic microbiology and biotechnology. Also a book of abstract was published (ISBN: 978-3-900932-60-2). It can be downloaded from the workshop webpage. The webpage for the workshop can be found here: https://gasinbiotechnology.boku.ac.at .

5 Ausblick und Empfehlungen

The results of the literature assessment show that formate is the most useful substrate for DFHP. However, compared to mono- or disaccharides also lignocellulose is a promising substrate for DFHP due to the high abundance and the DFHP potential on a C-molar level. Based on our analysis, we found that *Thermococcaceae* is the most promising family for DFHP. However, also members of the *Clostridiaceae* show high H_2 production potential. Moreover, many Eukarya, Bacteria, and Archaea have still not been investigated with respect to their DFHP potential. This will be an endeavor of high relevance but also of high effort.

In this project, the extremophilic organism *D. amylolyticus* was investigated regarding its DFHP characteristics. This organism can currently not be used for further optimization of DFHP, because of slow growth rates and low H_2 productivity.

The results of the project H2.AT indicate that the MCE/assisted H_2 production might render a possible technology combination for future biohyrogen production. However, many results of this study suggest that the technology breakthrough still requires more efforts with regard to applied basic research and development activities in the area of biological engineering. These research and development activities should be the joint endeavour of academic partners from the project H2.AT and from international academic partners. Therefore, the academic consortium members and international academic partners are currently drafting a project proposal to submit an extended, but at the same time more specialized, follow-up project proposal to a Horizon 2020 FETopen call.

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