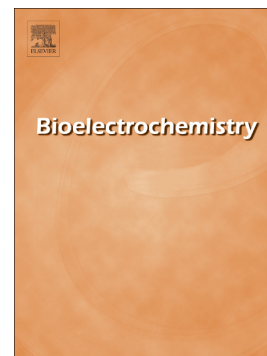# Accepted Manuscript

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Bioelectrochemistry

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Predicting and experimental evaluating bio-electrochemical synthesis - a case study with *Clostridium kluyveri*

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Abstract

Microbial electrosynthesis is a highly promising application of microbial electrochemical technologies for the sustainable production of organic compounds. At the same time a multitude of questions need to be answered and challenges to be met. Central for its further development is using appropriate electroactive microorganisms and their efficient extracellular electron transfer (EET) as well as wiring of the metabolism to EET.

Among others, Clostridia are believed to represent electroactive microbes being highly promising for microbial electrosynthesis. We investigated the potential steps and challenges for the bio-electrochemical fermentation (electro-fermentation) of mid-chain organic acids using *Clostridium kluyveri.* Starting from a metabolic model the potential limitations of the metabolism as well as beneficial scenarios for electrochemical stimulation were identified and experimentally investigated.

C. kluyveri was shown to not be able to exchange electrons with an electrode directly. Therefore, exogenous mediators (2-hydroxy-1,4-naphthoquinone, potassium ferrocyanide, neutral red, methyl

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viologen, methylene blue, and the macrocyclic cobalt hexaamine [Co(*trans*-diammac)]$^{3+}$) were tested for their toxicity and electro-fermentations performed in 1 L bioreactors covering 38 biotic and 8 abiotic runs were performed. When using *C. kluyveri* and mediators, maximum absolute current densities higher than the abiotic controls were detected for all runs. At the same time, no significant impact on the cell metabolism (product formation, carbon recovery, growth rate) was found. From this observation, we deduce general potential limitations of electro-fermentations with *C. kluyveri* and discuss strategies to successfully overcome them.

Highlights

- Metabolic modeling identified two potential beneficial scenarios for bio-electrochemical production in *C. kluyeri*
- Six exogenous mediators were tested for their toxicity on *C. kluyveri*
- Characterization of electroactivity in *C. kluyveri* in 1 L electro- fermentations
- Maximum absolute current densities higher than abiotic controls for all mediators

1. Introduction

Microbial electrosynthesis (MES) is a bio-electrochemical technology that comprises all microbially catalysed electrochemical reactions aiming at the generation of value added products. This includes the conversion of CO_2 to organic compounds based on reducing equivalents provided by a cathode but also the balancing of fermentation processes towards a desired product through an electrode-derived electron balance (electro-fermentation) [1, 2]. Accordingly, the metabolism of these so-called electroactive microorganisms is wired to a current flow at an electrode. The wiring of the electric current flow at the electrode and the metabolism can be achieved by several so-called extracellular electron transfer mechanism, see e.g. [3, 4]. In this way, sustainably produced electrical power can be utilized to improve microbial production, making MES an important future technology for a sustainable bioeconomy [5, 6].

Successful microbial electrosynthesis has been demonstrated with pure cultures (e.g. [7-9]) and natural mixed culture microbiomes (e.g. [10, 11]) but its broad application, especially regarding industrially relevant product concentrations, remains to be demonstrated. Here, a multitude of open questions and challenges has to be solved, starting from appropriate electroactive microorganisms possessing a metabolism that can efficiently and reproducibly be linked to an electrode. In the best case, this bioelectrocatalyst is also accessible to targeted genetic modifications to introduce the desired production pathways [12]. Likewise, appropriate biotechnological production platforms including upscaling options have to be available [13].

Clostridia are a physiologically diverse class of mainly gram-positive bacteria in the phylum Firmicutes [14]. They are very interesting for microbial electrosynthesis due to their metabolic potentials covering e.g. production of organic compounds from CO_2 or synthesis gas (CO/H_2) via the Wood–Ljungdahl pathway in *Clostridium ljungdahlii* and other acetogens [15, 16] as well as the production of alcohols [17, 18] and medium chain length organic acids [19]. They have been demonstrated to be accessible for genetic modifications [20-22] enabling metabolic engineering and were suggested to interact with electrodes directly [23, 24] or with the help redox mediators [9, 25] although the exact mechanisms behind the electrode interaction is not yet understood.

The current study is devoted to *Clostridium kluyveri* as potential platform organism for microbial electrosynthesis. It has a well characterized physiology and a sequenced genome [26]. Its main mode of energy generation is the reverse *β*-oxidation, a cyclic process resulting in production of medium

chain length organic acids, i.e. *n*-butyrate[1] and *n*-caproate from ethanol and acetate [19, 27]. Therefore, its natural product spectrum would offer an attractive alternative over the low-value product acetate, which is currently the major product in MES processes. As shown in Schematic 1 this process involves an oxidative (ethanol oxidation) and a reductive part (chain elongation) which result in ATP generation via substrate level phosphorylation and electron-transport phosphorylation, respectively. Further details of this process and the potential for its electrode derived redox balancing are discussed below within a theoretical framework of a metabolic model of *C. kluyveri*.

Based on the example of *C. kluyveri* the potential steps and challenges when predicting and experimentally evaluating microbial electrosynthesis are investigated covering the theoretical metabolic potential, choice of suitable and non-toxic mediators as well as electro-fermentation conditions.

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[1] we refer to the salt as all experiments were performed at neutral pH

2. Materials & Methods

2.1. General conditions

All chemicals were of at least analytical grade and were supplied from Carl Roth GmbH (Karlsruhe, Germany) and Merck KGaA (Darmstadt, Germany). De-ionized water (Millipore, Darmstadt, Germany) was used to prepare the microbial growth media. All potentials in this article refer to the Ag/AgCl reference electrode (sat. KCl, +0.197 V vs. SHE).

2.2. Microbial growth medium and cultivation conditions

C. kluyveri (DSMZ strain 555) was cultivated in serum bottles (total volume 100 ml, 50 ml medium), anaerobic tubes (total volume 16 ml, 8 ml medium) or in bio-electrochemical reactors (details below) using DSM 52 (defined medium by Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, DSMZ) as microbial growth medium (10 g K-acetate, 20 mL ethanol, 0.31 g K_2HPO_4, 0.23 g KH_2PO_4, 0.25 g NH_4Cl, 0.20 g $MgSO_4 \times 7$ H_2O, 1.0 g yeast extract, 2.5 g $NaHCO_3$, 0.25 g L-cysteine-HCl $\times$ H_2O, 0.25 g $Na_2S \times 9$ H_2O, 1 mL trace element solution SL-10, 1 mL selenite-tungstate solution, 1 mL seven vitamin solution, dissolved in 1 L water, final pH 6.8 – 7.0). For pre-experiments 1 mL of 0.1 % (w/v) Na-resazurin solution was included to ensure anoxic working procedures but then omitted for all experiments discussed in this article. The medium was purged with a gas mixture of 80 % N_2 and 20 % CO_2 (v/v) and all experiments were performed at 30°C.

2.3. Bio-electrochemical cultivation conditions

The bio-electrochemical cultivation was carried out in conventional 1 L glass bioreactors (Multifors, Infors HT, Bottmingen, Switzerland) which were extended by a bio-electrochemical upgrade kit [13, 28]. Working and counter electrode compartments were separated by a cation exchange membrane (fumasep FKE, FuMA-Tech GmbH, Bietigheim-Bissingen, Germany). Thereby, the working electrode compartment had a working volume of 850 mL and was filled with 800 mL medium. The counter electrode compartment had a working volume of 150 mL and was filled with 150 ml medium without acetate and ethanol.

Working and counter electrode were pairs of graphite rods (CP-Handels GmbH, Wachtberg, Germany) with a geometric surface area of 32.3 and 35.3 cm² inside the medium, respectively. A Ag/AgCl reference electrode (0.195 V vs. SHE, SE 11, Sensortechnik Meinsberg, Germany) was used and immersed in 70% ethanol for 2-3 h prior use for sterilization.

Before each experiment the bioreactors including the complete bio-electrochemical setup (except reference electrode) and the microbial growth medium (without acetate, ethanol, vitamins, $NaHCO_3$, L-cysteine-HCl $\times$ H_2O, $Na_2S \times 9$ H_2O) were autoclaved. Afterwards, the medium was cooled down

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while the headspace was purged with a gas mixture of 80 % N_2 and 20 % CO_2 (v/v). After adding the missing medium components and the mediator (where applicable), the bioreactors were inoculated with 50 ml of a 4 day old *C. kluyveri* preculture in early stationary phase.

All experiments were carried out under potentiostatic control using a multi-channel Potentiostat/Galvanostat (VSP, BioLogic Science Instruments, Claix, France). Control measurements were performed using the identical setup but without any potential applied (open circuit voltage, OCV). Chronoamperometry (details Table 1) was continuously applied for the complete experiment being only interrupted every 23 h for three cycles of cyclic voltammetry (CV) at a scan rate of 1 mV s^{-1}.

2.4. Mediators

Different redox mediators were assessed: 2-hydroxy-1,4-naphthoquinone (HNQ, Alfa Aesar), potassium ferrocyanide (FECY, Sigma Aldrich), neutral red (NR, Carl Roth GmbH), methyl viologen (MV, Sigma Aldrich), methylene blue (MB, FERAK Berlin GmbH) and the macrocyclic cobalt hexaamine [Co(*trans*-diammac)]$^{3+}$ (CoHex, synthesized as detailed in [29]). Stock solutions (25 mM) were freshly prepared prior to the each experiment in 66.6 mM phosphate buffer pH 7 (3.54 g L^{-1} KH_2PO_4, 5.77 g L^{-1} Na_2HPO_4). All stock solutions were filter sterilized (0.2 µm cellulose-acetate filter, LLG Labware). The working solutions contained 0.1 mM, 0.5 mM or 2 mM of the respective mediator in the microbial growth medium.

The mediators were characterized in the complete microbial growth medium using cyclic voltammetry and a mediator concentration of 0.1 mM (HNQ, MB, CoHex) or 0.5 mM (FECY, NR). Additionally, HNQ and FECY were also tested with the microbial growth medium without the reducing agents cysteine and Na_2S and at pH 6.0. The potential range was between -0.6 and 0.4 V (details Supplementary Table 1). The formal potential of the mediators was determined in the complete microbial growth medium at pH 7.0 as described in [30] using a scan rate of 1 mV s^{-1}.

2.5. Analytics

2.5.1. Microbial growth and pH

The microbial growth was determined using the optical density at 600 nm (OD_{600}). The anaerobic tubes were directly measured in a photometer (DSM Cuvette^{TM}, LAXCO, USA). Samples from serum bottles and bioreactor samples were measured with a spectrophotometer (Ultrospec 3100 pro, GE Healthcare, Amersham, UK) using microbial growth medium as a reference. Samples with high optical density were diluted with microbial growth medium to be in the OD_{600} range of 0.1 to 0.5. The pH in the bioreactors was continuously monitored with an integrated electrode (EasyFerm Plus PHI K8 180,

Hamilton, Germany). For all other samples, the pH was determined with a pH-meter (H138 miniLab™ Elite, HACH-Lange, Germany) that was calibrated on a daily basis.

2.5.2. Substrate conversion and product synthesis

The concentrations of ethanol, acetate, *n*-butyrate and *n*-caproate in the liquid samples were determined using HPLC (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a refractive index detector RID-10A and a HiPlex H column 300 x 7.7 mm (Agilent Technolgies, Inc. CA, USA) with a pre-column SecurityGuard Cartidge Carbo-H 4 x 3.0mm (Phenomenex, USA). All samples were centrifuged at 13,000 g for 10 minutes, filtered with a 0.2 µm nylon filter and stored at 4°C until analysis (usually within 4 days). The liquid phase was 0.01 N sulfuric acid. The samples were run for 75 min isocratically at a flow rate of 0.5 mL min^{-1} and an oven temperature of 50°C. Peak calibration and identification was carried out with external standards (three point calibration from 0.1 g L^{-1} to 1 g L^{-1}, $R^2 = 0,999$)

2.6. Calculations

The growth rate μ was determined in the exponential growth phase considering OD_{600} values up to 1.4 (Microsoft Excel 2010, Microsoft Corporation, USA). The carbon recovery from the substrates acetate and ethanol into the products *n*-butyrate and *n*-caproate was determined as proportion of carbon from the substrates bound in the products (Eq. 1).

$$\text{Carbon recovery [\%]} = \left| \frac{\Delta C\text{-mol}_{n\text{-butyrate} + n\text{-caproate}}}{\Delta C\text{-mol}_{\text{ethanol} + \text{acetate}}} \cdot 100 \right| \qquad \text{(Eq. 1)}$$

with ΔC-mol being the mol of carbon bound either in the substrates or in the products. If not stated otherwise, the carbon recovery was averaged over all samples from exponential and stationary phase.

The product yield was determined in mol of carbon bound in the products. Based on this value the biomass product yield coefficient (Y_C) was calculated (Eq. 2), considering the optical density as measure for biomass production.

$$\text{Yield coefficient} \left[\text{mol } OD_{600}{}^{-1} \right] = \frac{\Delta C\text{-mol}_{n\text{-butyrate} + n\text{-caproate}}}{OD_{600}} \qquad \text{(Eq. 2)}$$

The total charge, *q*, transferred per batch was calculated from the chronoamperograms by integration. The coulombic efficiency, *CE*, was calculated as the percentage of measured charge compared to the theoretical maximum value. Thereby, also the turnover number TN_{Med} of the

mediator, representing an average value how often each mediator molecule is oxidized and reduced, was calculated (Eq. 3):

$$TN_{Med} = \frac{q}{q_{MedHNQ}} = \frac{q}{n_{Med} \cdot z \cdot F} \qquad \text{(Eq. 3)}$$

with q_{Med} being the theoretical charge from the mediator if all molecules would interact with the electrode once, n_{Med} the amount (mol) of the Mediator, z the number of transferred electrons per oxidation/reduction of the mediator and F the Faraday constant (96 485 C mol^{-1}).

The theoretical contribution of the reducing agents to current flow was considered with 1 electron per molecule of L-Cysteine (total $q_{th\ L-Cysteine}$ = 103 C) and 2 electrons per molecule Na$_2$S (total $q_{th\ Na2S}$ = 154 C) (details Supplementary Information S1).

Statistical analysis was performed using student's t-test (Microsoft Excel 2010, Microsoft Corporation, USA) and a significance level of p = 0.05. All values are provided as mean±standard deviation.

2.7. Metabolic modeling

The stoichiometric analysis of a metabolic network of *C. kluyveri* was performed based on the elementary mode analysis framework introduced by Schuster *et al*. [31]. The java implemented free software *efmtool* (version 4.7.1) [32] was used within MATLAB, MathWorks (version R2015b), to compute for each network the elementary flux modes (*efm*s) which represent all possible and unique steady-state flux distributions the network could have based on reaction stoichiometry. The basic *C. kluyveri* core model used for the *in silico* analysis is based on the metabolic study by Seedorf at al. [26] and available genomic information on databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes). It includes: Embden-Meyerhof-Parnas-pathway/glycolysis, pentose phosphate pathway, (incomplete) tricarboxylic acid cycle, anaerobic fermentation, fatty acid elongation, reactions for energy and redox pools, import and export reactions and interaction with a soluble electron carrier (see Schematic 1 and Additional file 1). Several possible reactions between electrodes and metabolic redox carriers were proposed to investigate the effect of different possible mechanisms of extracellular electron transfer. The exact biomass composition for *C. kluyveri* is not available in literature. Therefore, analogous to previous work on *C. autoethanogenum* [25] and a published metabolic model for *C. acetobutylicum* [33], the function for biomass production was based on the assumption that the macromolecular composition is similar to that of *B. subtilis* [34]. All reactions used to condense the macromolecular composition back to essential metabolites that are

part of the core network can be found in Additional file 1. The final function is given as R41 in Schematic 1 and the essential biomass precursors are also highlighted with blue outline.

The main network includes 68 metabolites, 85 Reactions (32 reversible) and up to 7,800 *efms* per scenario were calculated. The full network can be found in the supplementary information (Additional file 1).

Maximal theoretical yields for biomass or a certain product are obtained by calculating the carbon balance of all carbon containing substrates entering the network and the carbon containing products leaving the network (Eq. 4):

$$Yield_{product} \ [\%] = \frac{flux_{product} \cdot carbon_{product}}{flux_{substrate} \cdot carbon_{substrate}} \cdot 100\% \qquad\qquad \text{(Eq. 4)}$$

3. Results & Discussion

3.1. Theoretical framework

Chain elongation to medium chain length organic acids is a desirable target conversion for microbial electrosynthesis and the proof of concept has been demonstrated in mixed cultures [11, 35].

To investigate the theoretical benefits and limitations of electrical stimulation on chain elongation we used elementary mode analysis as we did in an earlier study to identify beneficial substrate-product combinations for MES [36]. Here, a theoretical network of the model strain for chain elongation, *Clostridium kluyveri,* was constructed that represents the cellular core carbon metabolism. The model is based on its genomic information and can be found in the Supplementary Information as Additional file 1. A schematic pathway overview is given in Schematic 1. The theoretical analysis regards the cells in *steady state* condition and calculates all possible flux distributions based on reaction stoichiometry. By changing reactions in the network, e.g. adding reactions for interaction with an electrode, the theoretical effect of these changes on the cellular metabolism is assessed. This enables to capture changes in active reactions, pathway branches and effects on growth and production. Here, the computational model is used to estimate the effect of cathodic electron feed and an anode as an electron sink, respectively. Due to the unknown mechanisms of extracellular electron transfer (EET) in Clostridia, and *C. kluyveri* specifically, several different reactions were added to the network alternatively to investigate their effect on the metabolic balances. This was done as demonstrated before [36], for investigating the effect of different EET mechanisms on electrically stimulated production in *E. coli*. The proposed EET pathways display electron transport to and from the NADH pool, while being coupled and uncoupled from energy metabolism.

The products of interest in this study, the medium chain length organic acids *n*-butyrate and *n*-caproate, are formed by the cyclic process of reverse β-oxidation (see Schematic 1). The preferred carbon sources are ethanol and acetate, while a certain amount of CO_2 co-utilization is observed that is essential to sustain growth in *C. kluyveri* [19, 37]. In the metabolic pathway for chain elongation, ethanol is oxidized in two steps to acetyl Coenzyme-A (Ac-CoA), which generates the reducing equivalents that are used for chain elongation. In the second pathway branch, acetate is activated by acetate kinase (Ack) to acetyl phosphate (ACP) and then converted to Ac-CoA (see reactions R72, R73 and R74, R75 in Schematic 1). Chain elongation progresses as Ac-CoA addition under NAD(P)H consumption. To meet the overall energy requirements of the pathway, a part of the alcohol uptake is used for additional acetate production, which results in a theoretical optimum ratio between ethanol and acetate of 3:2 (overall reaction: 6 ethanol + 4 acetate $\rightarrow$ 5 *n*-butyrate). Further

elongation to *n*-caproate proceeds analogously and is included in the metabolic network (see Additional file 1) but is not shown in Schematic 1 to maintain clarity.
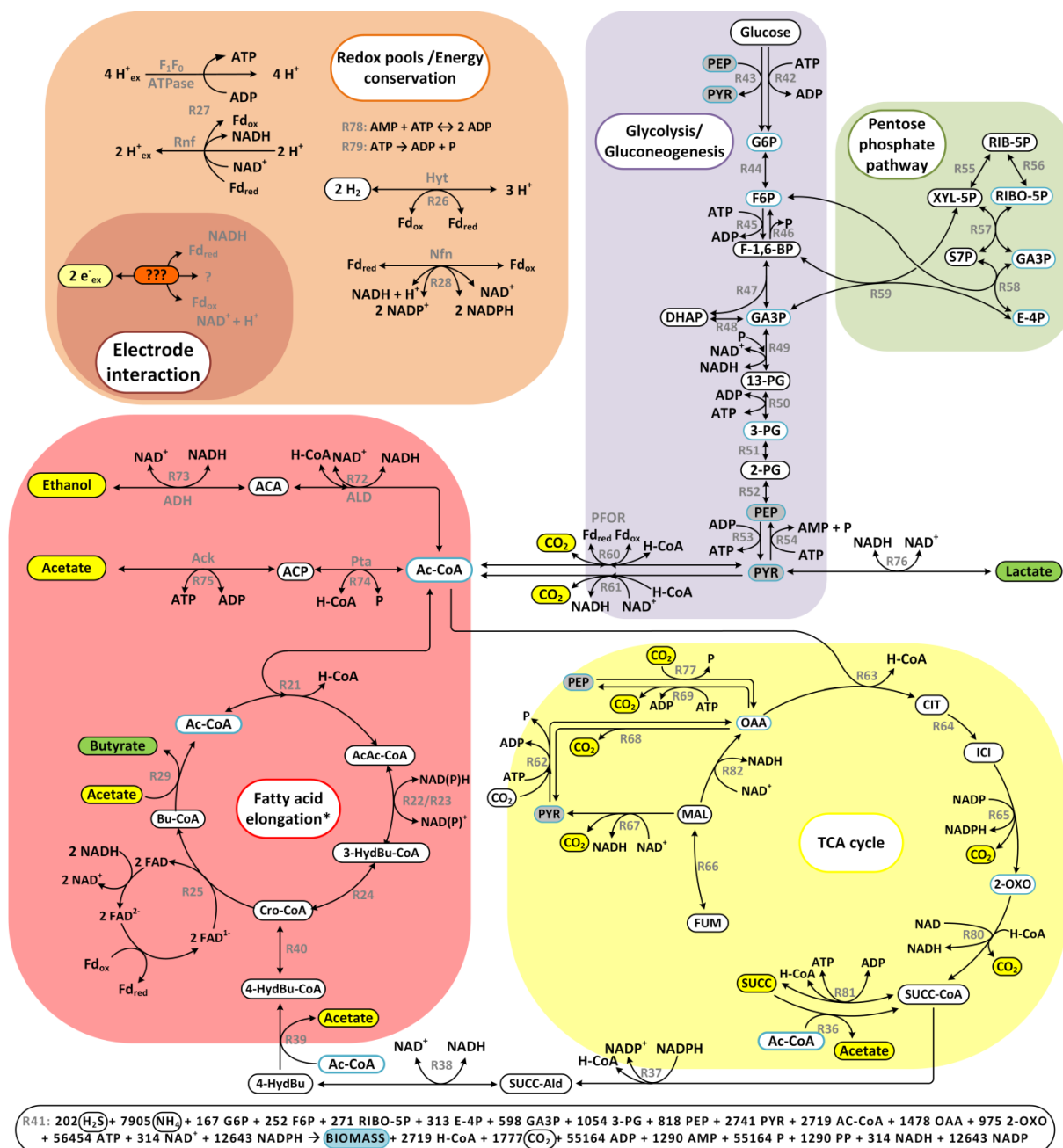
Schematic 1: Metabolic network of *C. kluyveri* including major carbon pathways. Substrates are highlighted in yellow, products in green. Reaction numbers relate to the complete metabolic network given in Additional file 1. Biomass precursors are highlighted with blue border and biomass equation is given as R41. *Chain elongation is presented for the first cycle of *n*-butyrate production only.

The metabolic model was validated to be representable for the general metabolic behavior of *C. kluyveri*: The optimum ratio of ethanol and acetate feed results in a theoretical maximum yield of

100% for *n*-butyrate (based on reaction directionality and stoichiometry) and CO_2 was found to be essential for biomass production.

The incorporation of reactions for EET into the network identified two possible scenarios that could replace full pathway branches: i) a cathode serving as alternative to the ethanol pathway branch and ii) an anode serving as electron sink promoting a growth benefit and enabling chain elongation from ethanol only.

In the first scenario, it was assumed that a cathode serves as electron donor for the cellular NADH pool, which is redundant to the NADH usually accumulating from ethanol oxidation (R72,73). However, as mentioned above the natural pathway is only balanced in its energy requirements if a part of the ethanol feed is used for energy production via substrate-level phosphorylation. Therefore, a cathode can "replace" ethanol co-feed only if the electron uptake is coupled to ATP production, which is essentially needed for acetate activation. The connection of energy and redox pools has been identified as crucial in the past, especially with respect to cathodic electron uptake [36, 38]. There are proteins that catalyze ATP synthesis from reducing equivalents indirectly such as the Rnf-complex (see R27 in Schematic 1). Therefore, it might be possible that electron feed from a cathode results in simultaneous NADH and ATP production, e.g. by an EET mechanism that involves the Rnf complex or similar protein-complexes that link redox pools to ATP synthesis [25]. Even though the chain elongation from acetate and cathodic electrons has been demonstrated for microbiomes [11, 35], the underlying EET mechanisms remain pure speculation to date.

The second scenario resulting from the theoretical analysis is the chain elongation from ethanol only. Here, an anode that functions as an electron sink is predicted to sustain *n*-butyrate and *n*-caproate production without acetate addition. This prediction is based on the assumption that an anode is accepting electrons from NADH. If ethanol is the only carbon source, the needed acetate for chain elongation (R29 Schematic 1) can be synthesized from ethanol as well at the cost of NADH accumulation. The metabolic model shows that theoretically *n*-butyrate production is possible from ethanol as sole substrate with the following overall reaction: 2 ethanol + ADP + P + NAD + oxidized ferredoxin → *n*-butyrate + ATP + NADH + reduced ferredoxin. The NADH production is balanced by the anode, while reduced ferredoxin can be oxidized by the Rnf complex, producing more NADH and indirectly ATP *via* the membrane gradient (see R27 in Schematic 1). As a result, a growth benefit is predicted for this scenario.

To test if any of the predicted trends could be observed in *in vivo* fermentations, we started pure culture fermentations of *C. kluyveri* in bio-electrochemical systems. These included the investigation of potential electrode interactions without and with addition of exogenous mediators as well as a selected scenario of the theoretical analysis.

3.2. Characterization of electroactivity in *C. kluyveri*

3.2.1. Direct electron transfer of *C. kluyveri*

C. kluyveri is able to convert ethanol and acetate to *n*-butyrate and *n*-caproate under anaerobic conditions based on reverse *β*-oxidation. This is the only mode of energy generation for the organism [19, 26]. Figure 1 shows the typical growth and substrate conversion under standard conditions. The conversion of ethanol and acetate to *n*-butyrate followed by *n*-caproate is going along with the increase in optical density and a decrease of pH from 7.2 to 6.2 until a stationary phase is reached after about 40 h. The carbon recovery is 90.3±9.3%.

Our preliminary experiments verified that *C. kluyveri* is not able to perform direct electron transfer under the conditions applied. The potential interaction with the electrode was tested in the bioreactor at -0.13 V (anodic conditions, n=5) and -0.80 V (cathodic conditions, n=2) (see Table 1). The bioreactors showed a maximum current density of 0.01 mA cm^{-2} (anodic conditions) and -0,037 mA cm^{-2} (cathodic conditions) (Supplementary Information S2b and S2c) with an average current density of 0.003±0.002 mA cm^{-2} (anodic conditions) and -0.019±0.008 (cathodic conditions). This is comparable to the current densities achieved for abiotic incubation of medium under the same conditions. The applied potential did not significantly impact the microbial growth (Table 1) compared to OCV control conditions.
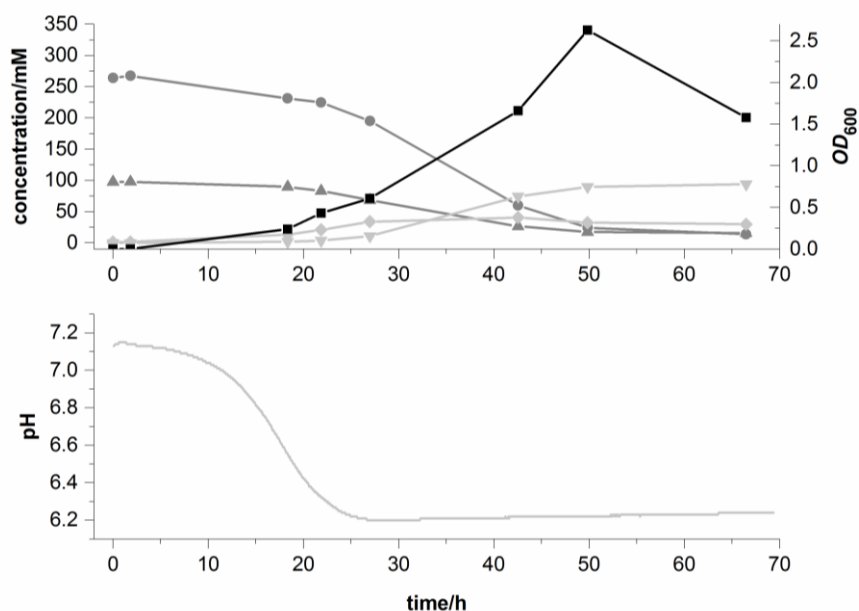

Figure 1: Representative growth curve of *C. kluyveri* on acetate and ethanol. The cultivation was performed in 1 L bioreactors and at open circuit potential. Acetate (▲) and ethanol (●) are converted to *n*-butyrate (◆) and *n*-caproate (▼) based on reverse *β*-oxidation, the only mode of

energy generation for the organism. Growth was determined based on optical density (OD_{600}, ■). Further replicates are given in Supplementary Information S2.

3.2.2. Mediated electron transfer of *C. kluyveri*

As we have shown that *C. kluyveri* is not able to perform direct electron transfer, mediated electron transfer was studied as a potential mode of electrode interaction.

The suitable mediator has to fulfill several requirements: It has to be non-toxic to the microorganisms and possess the suitable formal potential to accept/donate electrons from/to the microbial metabolism. Moreover, the mediator has to be able to physically reach the potential electron transfer site of the microbial cell. The chosen mediators for the current study differ in their formal potentials enabling electron transfer at different energy levels as well as in their molecular structures leading to different membrane permeabilities. The here studied compounds are 2-hydroxy-1,4-naphthoquinone (HNQ), methylene blue (MB), neutral red (NR), methyl viologen (MV), cobalt hexaamine [Co(*trans*-diammac)]$^{3+}$ (CoHex) and potassium ferrocyanide (FECY). All of these molecules have already either been applied for bio-electrochemical studies with other microorganisms (HNQ [28], MB [39]) as well as for Clostridia specifically (NR, MV[9], CoHex [25]) or served in biosensors and as electrochemical model compounds in general (FECY[40]).

The toxicity of the mediators was tested based on the impact on the microbial growth using different mediator concentrations (0.1 mM, 0.5 mM and 2 mM). The complete results with all growth curves are given in Supplementary Figure S1. The major results are summarized in Table 2. For all mediators a concentration of 2 mM severely affected the microbial growth and hence 2mM or even higher mediator concentration is not suitable for application. MV and NR severely inhibited microbial growth even at the lowest tested concentration of 0.1mM. Therefore, we regarded both compounds as not suitable as mediator for electro-fermentations with *C. kluyveri*. HNQ, FECY, MB and CoHex did not or only to a minor degree affect the growth (optical density, C recovery) at concentrations of 0.5 mM and 0.1 mM (Table 2, Figure S1). The mediator concentration of 0.1 mM was then chosen for all later bio-electrochemical cultivations.

Table 1: Results of bio-electrochemical cultivations in 1 L bioreactors. All mediators were applied in a concentration of 0.1 mM. The growth parameters at open circuit voltage (OCV) without any mediator were considered as control for all calculations. A statistically significant difference to the control was labeled with an asterisk.

Abbreviations: HNQ: 2-hydroxy-1,4-naphthoquinone, FECY: potassium ferrocyanide, MB: methylene blue, CoHex: cobalt hexaamine [Co(*trans*-diammac)]$^{3+}$

Condition	Mediator	Applied potential	Number of replicate reactors	Growth rate	Growth rate	C recovery	C recovery	Charge[3]	Coulombic efficiency	C4 formation	C6 formation
		[V vs. Ag/AgCl]		[h^{-1}]	[% control]	[%]	[% control]	[C]	[%]	[mM]	[mM]
OCV (=control)	-	-	5	0.104±0.009	100	90.3±9.3	100	-	-	28.6±4.5	81.7±12.0
anodic	-	-0.13	5	0.092±0.021	89	92.0±5.1	102	0.0002±0.0001	3,8E-10	25.9±2.7	93.6±5.4
cathodic	-	-0.80	2	0.086±0.009	83	81.3±11.3	90	-295±99	5,6E-04	24.1±0.1	100.7±8.2
anodic	HNQ	-0.13	5	0.098±0.014	94	94.3±8.1	104	347±38	6,5E-04	29.7±1.2	88.9±2.7
cathodic	HNQ	-0.53	3	0.071±0.006	68*	92.1±2.4[2] (57.4±8.8)	102	-0.002±0.001	3,6E-09	25.2±3.8	85.7±5.9
OCV	HNQ	-	2	0.073±0.005	70	88.9±8.3	98	-	-	28.7±5.5	71.3±13.1
anodic without acetate	HNQ	-0.13	3	0.001±0.000[1]	1*	52.7±1.4	58*	394±52 (256±5)[4]	4,5E-03 (2,9E-03)[4]	-2.5±0.1[1]	0.1±0.2[1]
anodic	FECY	0.40	2	0.109±0.013	105	86.8±5.5	96	246±168	5,9E-04	33.8±0.6	63.3±3.8
cathodic	FECY	-0.01	2	0.117±0.005	113	89.9±7.2	99	41±20	8,7E-05	31.5±1.5	71.1±6.1
anodic	MB	-0.05	2	n.a.[5]	n.a.[5]	89.3±8.0	99	214±15	3,8E-04	23.4±1.3	96.3±6.6
cathodic	MB	-0.50	2	n.a.[5]	n.a.[5]	93.4±1.3	103	-27±14	4,7E-05	23.7±0.5	100.9±2.5
cathodic	CoHex	-0.80	3	0.096±0.010	92	91.9±20.1	102	-280±157	4,9E-04	26.5±4.5	93.5±10.2
cathodic without ethanol	CoHex	-0.80	2	0.004±0.001	3*	88.5±3.2	98	-641±80 (-178±5)[4]	7,2E-02 (2,0E-02)[4]	6.1±0.3[6]	0.3±0.3[6]

[1] minimal growth and product formation can be attributed to small amounts of acetate transferred with the inoculum

[2] carbon recovery of late exponential and stationary phase, in brackets average of all sampling points

[3] measured value (the contribution of the abiotic current flow was not yet subtracted)

[4] considering only the first 60 hours of the batch in accordance with the other run times

[5] not applicable - optical density could not be determined in methylene blue experiments

[6] minimal growth and product formation can be attributed to small amounts of ethanol transferred with the inoculum

Table 2: Toxicity test. Suitable mediators were tested for their effect on the microbial growth in anaerobic tubes. Given below is their formal potential at pH 7.0 under cultivation conditions, the applied concentration in the individual growth tests and found growth rate (given as % of growth rate of the control without mediator) and maximum optical density (OD_{600}^{max}, given as % of the maximum OD of the control without mediator). All experiments were performed in three independent biological replicates and the results are given as mean±standard deviation. A statistically significant difference to the control was labeled with an asterisk. All growth curves are shown in Supplementary Figure S1.

Mediator	Abbreviation	Formal potential[1] [V]	Concentration [mM]	OD_{600}^{max} [% control]	C recovery [% control]
2-hydroxy-1,4-naphthoquinone	HNQ	-0.283	0.1	88±8	97±4
			0.5	79±2*	96±0*
			2.0	6±0*	n.d.
neutral red	NR	0.140	0.1	n.d.	—[2]
			0.5	n.d.	—[2]
			2.0	n.d.	—[2]
potassium ferrocyanide	FECY	0.254	0.1	110±4*	104±7
			0.5	112±1*	101±2
			2.0	17±2*	n.d.
methylene blue	MB	-0,223	0.1	n.d.	94±5
			0.5	n.d.	96±1
			2.0	n.d.	63±22
cobalt hexaamine [Co(*trans*-diammac)]$^{3+}$	CoHex	-0,768	0.1	102±3	95±1
			0.5	105±1	98±0.5
			2.0	96±3	90±3
methyl viologen	MV	n.d.	0.1	19±3	n.d.
			0.5	21±5	n.d.

[1] formal potential is given vs. Ag/AgCl reference electrode.
[2] no substrate consumption
n.d. not determined

The chemical stability of the mediators was tested, as the addition of the mediators to the microbial growth medium could affect their electrochemical characteristics. Therefore, the mediators were analyzed by CV in the microbial growth medium with and without reducing agents as well as under different pH conditions simulating the course of the experiment. HNQ and FECY in the medium without reducing agents showed the expected CV curves with oxidation and reduction resulting in formal potentials of -0.283 V (HNQ) and 0.254 V (FECY). The addition of the reducing agents resulted

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in a catalytic curve for both mediators with e.g. for HNQ maximum values of 0.04 mA cm^{-2} at a potential of 0.3 V compared to 0.004 mA cm^{-2} without the reducing agents. This indicates that there is already an electrode interaction under abiotic conditions which could also result in a current flow during chronoamperometry. Therefore, it is mandatory to include abiotic runs for chronoamperometry to estimate the abiotic contribution of the reducing agents. In our experiments, the abiotic runs resulted in a total charge flow of 29±7 C (HNQ), 148±19 C (FECY), 108±16 C (MB) and -234±7 C (CoHex). This value should be subtracted from the biotic experiments.

The change of the pH from 7.0 to 6.0 resulted in a shift of the redox peaks for HNQ but not for FECY, as can be expected from the NERNST-equation. This observation is in accordance with the observed shift of the formal potential in the CVs of HNQ during the biotic experiments.

3.3. Mediated electro-fermentations for production of medium chain fatty acids

Bio-electrochemical cultivation of *C. kluyveri* in 1 L bioreactors was performed under anodic and cathodic conditions using HNQ, FECY, MB and CoHex as redox mediator. The details and results of all performed experiments are summarized in Table 1 and the complete growth curves and concomitant process parameters are given as Supplementary Information S2.

Abiotic and OCV control

The abiotic bioreactors showed a maximum current density of 0.005 mA cm^{-2} (HNQ), 0.255 mA cm^{-2} (FECY), 0.036 mA cm^{-2} (MB) and -0.034 mA cm^{-2} (CoHex) (Supplementary Information S2n to S2q). Applying OCV, the results of the toxicity test were confirmed. Using HNQ, the growth rate decreased while the carbon recovery was similar to the control (Table 1).

Cathodic cultivation with HNQ

The inhibitory effect of HNQ on the microbial growth was also observed under cathodic conditions (constant potential of –0.53 V applied). The growth rate was significantly reduced by 32% compared to the control. Nevertheless, the efficiency of the substrate conversion was not affected with a carbon recovery of 92.1±2.4%. Noteworthy, carbon recovery values above 45% were only found at the end of the exponential phase. That means that the carbon recovery was delayed in comparison to most other experimental conditions under which high carbon recovery values were found during the complete exponential growth phase. The total product concentrations of *n*-butyrate and *n*-caproate at the end of the batch did not significantly differ from the control.

The substrate conversion to *n*-butyrate and *n*-caproate is the only way of *C. kluyveri* to generate ATP. Therefore, the finding of a lower growth rate but unaffected *n*-butyrate and *n*-caproate production

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indicates that the cells required more energy for their growth under cathodic conditions with HNQ. From a production perspective, the biomass product yield coefficient was higher under cathodic conditions with HNQ (Y_C = 320.66) compared to the control (Y_C = 290.88) indicating that the production per individual cell increased. But this effect seems to be only a result of the inhibition caused by HNQ and is not linked to the cathodic bio-electrochemical cultivation conditions (Supplementary Table S2).

A maximum cathodic current density of -0.03 mA cm^{-2} was measured after 23 h and decreased afterwards independently from the microbial growth behavior (Supplementary Information S2e). The charge transfer of the complete batch was -0.002±0.001 C which would represent less than 3.6·10^{-9} % of the electrons included in the converted products. In conclusion, the experiments confirmed that the cathodic cultivation does neither support the microbial growth nor the product conversion.

Anodic cultivation with HNQ

The inhibitory effect of HNQ on the microbial growth was alleviated under anodic conditions. The growth rate with HNQ (μ = 0.098±0.014 h^{-1}) as well as carbon recovery (94.3±8.1%) did not significantly differ from the control. A maximum anodic current density of 0.077 mA cm^{-2} was found after 21 h (Supplementary Information S2d). The total charge transfer per batch was 347±43 C, representing 6.5·10^{-4}% of the electrons included in the converted products. This represents about 4 turnovers of each HNQ molecule, therefore indicating an interaction between microbial cells and mediator. While no impact on the growth or product conversion was found, the anodically oxidized mediator seemed to be less toxic for *C. kluyveri* which could be explained by a change of the charge of the HNQ molecule.

Under the standard cultivation conditions acetate is utilized as electron acceptor in the reductive part of the *β*-oxidation. The metabolic model indicated a potential benefit under anodic conditions, where the electrode could serve as alternative electron acceptor in case acetate is not available. Therefore, this specific cultivation condition with only ethanol as substrate and only a very small amount of acetate from the inoculation was investigated under anodic conditions with HNQ. The growth under these conditions was negligible (1% of control) and was only found as long as acetate (small leftovers from the inoculum culture) was available. The carbon recovery was only 52.7±1.4%. In conclusion, the microbial metabolism could not be coupled to the electrode in the performed experiments. Further investigations are necessary on how a successful coupling of the microbial metabolism to the electrode can be realized (see discussion below). Interestingly, the current density was still higher compared to the abiotic control with an overall charge transfer of 236±24 C over 60 hours of incubation indicating that the current flow was neither linked to the number of microbial cells nor to their state of growth. Due to the small amount of substrate turnover and comparably

high current flow, the calculated coulombic efficiency ($4.5 \cdot 10^{-3}$%) is higher than in all other experiments. Nevertheless, one cannot take this number to point to physiologically relevant interactions, as it was not found to be connected to the expected product formation. It seems to be rather unspecific - but linked to the presence of microbial cells.

Cultivation with FECY

The bio-electrochemical cultivation with FECY did not significantly impact the growth rate or the carbon recovery. An anodic current flow was found under both applied conditions. Applying an anodic potential a maximum current density of 0.34 mA cm^{-2} was found directly after starting (Supplementary Information S2h) and a total charge per batch of 230±71 C was transferred, representing $5.9 \cdot 10^{-4}$% of the electrons included in the converted products. Applying a cathodic potential (being 200 mV below the formal potential) the maximum current density was 0.03 mA cm^{-2} (Supplementary Information S2i) and a total charge transfer per batch of 41±20 C was found, representing a columbic efficiency of $8.7 \cdot 10^{-5}$%.

Cultivation with MB

The bio-electrochemical cultivation with MB did not significantly impact the growth rate or the carbon recovery. An anodic or cathodic current flow was found under the respective conditions. Applying an anodic potential a maximum current density of 0.124mA cm^{-2} was found directly after starting (Supplementary Information S2j) and a total charge per batch of 214±71 C was transferred under anodic conditions, representing $3.8 \cdot 10^{-4}$% of the electrons included in the converted products. Applying a cathodic potential the maximum current density was -0.001 mA cm^{-2} (Supplementary Information S2k) and a total charge transfer per batch of -27±14 C was found.

Cultivation with CoHex

The bio-electrochemical cultivation with CoHex did not significantly impact the growth rate or the carbon recovery under the tested cathodic conditions. A potential impact of hydrogen production at the applied potential of -0.8 V vs Ag/AgCl was also not found (independent of presence of CoHex). A maximum current density of -0,045 mA cm^{-2} was measured (Supplementary Information S2l) and a total charge per batch of -280±157 C transferred, representing $4.9 \cdot 10^{-4}$% of the electrons included in the converted products.

Under the non-bio-electrochemical cultivation conditions ethanol is utilized as electron donor in the microbial metabolism. The metabolic model indicated a potential benefit under cathodic conditions, as the electrode may serve as alternative electron donor. Therefore, specifically cultivation using only

acetate as substrate and only a very small amount of ethanol from the inoculation was investigated under cathodic conditions with CoHex.

The growth under these conditions was negligible (3% of control) and was only found at the beginning of the experiment when a small amount of ethanol from the inoculum culture was available. The carbon recovery was 88.5±3.2% and hence not significantly different from the control conditions or the cathodic conditions with ethanol, acetate and CoHex.

Conclusions for bio-electrochemical cultivation

From the experimental results it is clear that *C. kluyveri* is not able to directly interact with electrodes. Consequently, different exogenous mediators were investigated for their suitability to mediate the electron transfer and wire the microbial metabolism to a current flow. FECY, HNQ, MB and CoHex were identified as non-toxic (FECY, MB, CoHex) or only minor toxic (HNQ) at concentrations up to 0.5 mM. While no specific interaction between the microbial cells and the electrodes was found, a current flow higher than the abiotic control was detected when the microbial cells and the mediators were present. This was not related to the cell metabolism represented by product formation, carbon recovery or growth rate.

4. Perspective

Pure culture studies are highly promising for the electrification of white biotechnology [2], especially, if these are well known production hosts like *C. kluyveri*. Metabolic modelling has predicted a scenario with a potential technological benefit using bio-electrochemical cultivation for *C. kluyveri*. Specifically, it was predicted that *C. kluyveri* can perform chain elongation and growth, when only ethanol serves as substrate and the anode as selective NADH (i.e. electron) sink. However, in the current study using the wildtype strain of *C. kluyveri* in combination with several redox mediators this could not be experimentally proven. In the following paragraph, we propose potential reasons for our observations and discuss possibilities to assess these in future.

In contrast to *C. pasteurianum* [23], *C. ljungdahlii* and *C. aceticum* [24], the studied *C. klyuveri* was not found to directly interact with electrodes. For a more efficient electro-fermentation, endogenous electroactivity has to be established, e.g. by the integration of electron transfer mechanism components like transmembrane redox proteins of well-studied electroactive species [12]. Further, the electroactivity has to be coupled to the metabolism. Proof-of-concept has been shown for *Escherichia coli* [39, 41] and *Pseudomonas putida* [42], covering the integration of direct as well as mediated electron transfer mechanisms. This is a very complex task, especially for Clostridia. Here *C. acetobutylicum* and *C. ljungdahlii* have been shown to be accessible for genetic modifications [20-

22]. Nevertheless, the integration of electron transfer mechanisms from gram-negative into gram-positive bacteria still has to be demonstrated.

Alternative to establishing direct electron transfer, exogenous mediators can be applied to successfully shuttle the electron between the microbial cells and the electrode. This path was also followed in this study. The choice of a suitable mediator is not simple – an exemplary workflow for its assessment has been shown here. As discussed, it has to be non-toxic for the cell and at the same time enable adequate wiring of the metabolism and the current flow. Especially challenging is that the specific modes of interaction at the subcellular level are hardly studied for most mediators. Therefore, the choice can often only be based on experimental assessment, especially as also species specific differences have to be expected. Most of the so far studied electroactive microorganisms were gram-negative [43] and potential electron transfer mechanisms of gram-positive bacteria are only studied poorly [44-46]. Two studies have shown potential exogenous mediator based interaction of Clostridia with electrodes [9, 25]. Although, the application of MV for *C. tyrobutyricum* was demonstrated [9], it was not successful for *C. kluyveri* in this study and did even result in complete growth inhibition. Kracke *et al*. [25] have tested several macrocyclic cobalt hexaamines as potential mediators and found only the one with the lowest formal potential to influence the redox balance in *C. autoethanogenum*, probably by triggering a regulatory switch rather than participating in the fermentative electron transfer. This mediator was tested here for *C. kluyveri* and did not change the metabolism. Similar to direct extracellular electron transfer, the challenge for mediated extracellular electron transfer is also to subsequently couple EET to metabolic conversions.

An alternative to the optimization of pure culture fermentation is the utilization of microbiomes. Mixed culture microbial communities bear the potential of combining the individual capacities of different species to an emerging function of the community that cannot be provided by any of the single members [47-49]. This was also the case for microbiomes performing chain elongation in bio-electrochemical systems [10, 11, 50] although the specific mode of microbe-electrode interaction and the relevance of *C. kluyveri* was not investigated. Relevant interactions in the microbiome include the natural production of a mediator by one species and its utilization by another [51, 52] but also an electrode interaction and production of a compound by the first species which is then further utilized or even upgraded by a second species [10, 53, 54]. Therefore, one may speculate that defined co-cultures of *C. kluyveri* with other microorganisms can be a way to realize electricity driven microbial chain-elongation.

All of the above discussed options demonstrate that we can be optimistic that it will possible to use microbial electrosynthesis as a sustainable technology for the production of organic compounds in future. But further studies on the exact mechanisms behind the microbial electrode interaction are

required to improve the extracellular electron transfer and its specific wiring to the microbial metabolism. Further on, microbial resource mining will certainly help to leverage the potential of the high diversity of electroactive microbes [43] and result in the identification and characterization of so far undiscovered electroactive species.

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6. References

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