Supplementary Material to

In situ electrochemical H_2 production for efficient and stable power-to-gas electromethanogenesis

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Contents

Supplementary discussion of experimental conditions

The three reactors to study long-term electromethanogenesis, E-Chem1, E-Chem2 and H_2-Control were operated twice for the period of about a month (see Methods section in main manuscript). Here, we want to provide additional discussion regarding the ratio of electron to carbon source (H_2/CO_2) in the feed gas of H_2-Control, which was changed from 80/20 vol% in Exp1 to 75/25 vol% in Exp2.

Methanogenesis rates, as all microbial processes, are slowing down when the substrates become limiting. To optimise turnover rates, it is beneficial to limit only one substrate, either H_2 or CO_2. Therefore, one trade-off to consider for power-to-gas applications is, whether hydrogen or CO_2 is the more acceptable impurity in the product gas stream.

In Exp1, we supplied the standard gas mix for growing methanogens containing H_2/CO_2 in a ratio of 80/20 vol% to the H_2-Control and matched the H_2 supply rate to the *in situ* hydrogen production rates in the E-Chem reactors. However, this likely caused CO_2 limitation in this reactor. Methanogens are routinely grown with a gaseous H_2/CO_2-mix of 80/20 vol% according to the stoichiometry of methanogenesis: $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$. This, however, neglects biomass formation, which requires only two H_2 to reduce one CO_2 cell mass at an average oxidation state of around zero compared to -4 for CH_4. Further, it has been shown previously that the affinity of methanogens for CO_2 are is with a partial pressure of about 20 mbar about two orders of magnitudes lower than for H_2[1-3] with threshold partial pressures of less than 0.1 mbar. Therefore, at the gas feed ratio of H_2 and CO_2 for methanogenesis of 80% H_2 and 20% CO_2, CO_2-supply becomes rate limiting before H_2 does.

This resulted in reduced volumetric methane production rates in the H_2-Control of 1.15 ±0.01 L L^{-1} day^{-1} in Exp1 compared to 1.29 ±0.06 L L^{-1} day^{-1}in Exp2 (cf. Table S3), higher amount of residual, unused hydrogen (average concentrations of H_2in the reactor off-gas: 4.1% in Exp1 compared to 2.8% in Exp2), and changes in microbial physiology (see main manuscript).

To avoid carbon limitation and thus favour a maximized use of electrons during electromethanogenesis, E-Chem1 and E-Chem2 were operated under constant current at a ratio of 75/25 vol% electric-H_2/CO_2. Indeed, during both long-term experiments the overall electron recovery of the integrated system was extremely high with an average CE of 97 ± 7 % during the stable period of Exp1 and 98 ± 2% in Exp2 (cf. Table S3).

Supplementary material and methods of the proteomic analysis

Protein samples were precipitated with 4X volume of acetone and incubated at -80°C overnight. Air-dried protein pellets were reconstituted in 200ul of 100mM triethylammonium bicarbonate buffer, sonicated, and vortexed to solubilize proteins. The samples were then reduced with 10mM DTT at 55^{o}C for 30 minutes followed by alkylation with 30mM acrylamide for 30 minutes at room temperature. 0.5µg of Trypsin/LysC protease (Promega) was added to each sample for digestion at 37^{o}C overnight.

Peptide quantification was performed with the Pierce Quantitative Fluorometric Peptide Assay kit (Thermo Fisher Scientific). 10µg aliquots of the digested peptide samples were each labeled with a different channel (126-130C) from a TMT 10-plex Isobaric Label Reagent set (Thermo Fisher Scientific), with the last 131 channel used as a pool channel for all samples. After 1-hour incubation at room temperature, the reactions were quenched with 5% hydroxylamine, combined into one series, and de-salted with C18 Monospin Reverse Phase Columns (GL Sciences). The peptide mixture was dried by speed vac before dissolution into 50µL of reconstitution buffer (2% acetonitrile with 0.1% formic acid). 2 µl was used for subsequent LC-MS/MS analysis.

Mass spectrometry experiment was performed using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, San Jose, CA) attached to an Acquity M-Class UPLC system (Waters Corporation, Milford, MA). A pulled-and-packed fused silica C18 reverse phase column containing Dr. Maisch 1.8 micron C18 beads and a length of ~25 cm was used over a 180 minute gradient. A flow rate of 450 nL/min was used with the mobile phase A consisting of aqueous 0.2% formic acid and mobile phase B consisting of 0.2% formic acid in acetonitrile. Peptides were directly injected onto the analytical column with a gradient of 2-45% mobile phase B followed by a high-B wash. The mass spectrometer was operated in a data-dependent mode using MS3 HCD and CID fragmentation for spectral generation.

Raw data were imported into MaxQuant Version 1.6.14.0 and analysis was performed using the default parameters. Isobaric labeling TMT10plex was selected. The reference proteome file for Methanococcus maripaludis (strain S2/LL) was obtained from Uniprot (UP000000590_267377). The output file “protein Groups” of the combined MaxQuant analysis of both experiments the columns “reporter intensity corrected” were manually renamed to the corresponding. Proteins annotated with “Only identified by site“, “Reverse “, and “Potential contaminant” were omitted from further analysis. Intensities were then normalized to the total intensity of identified proteins (by dividing every intensity value by the sum of all intensities in that sample and then multiplied by the average sum of intensities of all samples) to correct for differences in total protein used for analysis. Normalized intensities were imported into Perseus

Version 1.6.10.50. Rows containing missing values were removed. Statistical analysis was performed on log(2) transformed data comparing the combined E-Chem 1&2 reactor data to the H_2-Control using the "Volcano Plot" function of Perseus based on a t-test with 1000 randomizations, a false discovery rate of 0.05, and an S0 of 0.5. The entire analysis has been deposited to the ProteomeXchange Consortium via the PRIDE[4] ref partner repository with the dataset identifier PXD019312 and 10.6019/PXD019312.

Proteomic comarison of the combined samples of E-Chem 1 & 2 to H_2-Control can be found as additional file 1 (.xls).

Figure S1: Methane and hydrogen production rates and coulombic efficiencies during long-term electromethanogenesis Exp1

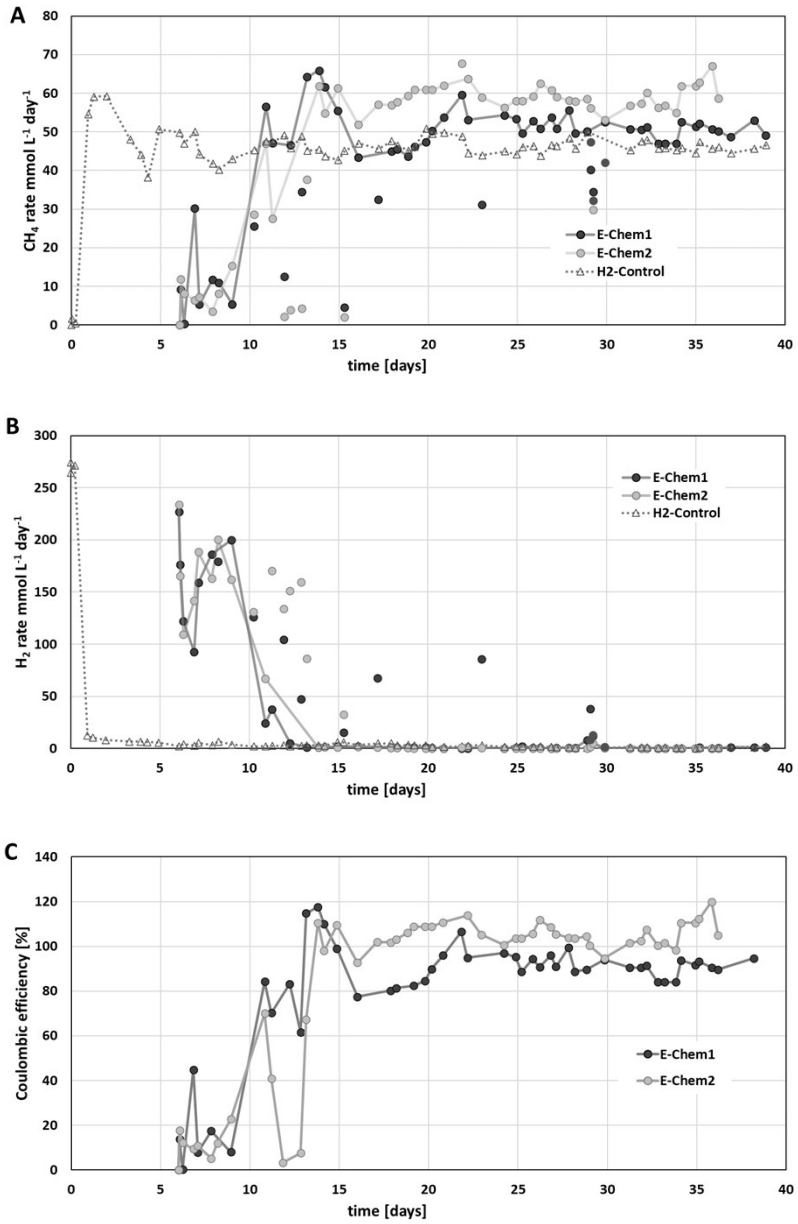

Fig. S1: Methane production rates **(A)** and hydrogen flow rates **(B)** in the reactor off-gas as well as corresponding coulombic efficiencies **(C)** during long-term experiment Exp1 with *M. maripaludis*. E-Chem1 and E-Chem-2 are bio-electrochemical reactors using NiMo-cathodes and H_2-Conrol presents a reactor featuring external H_2-feed. Data points representing measurements taken while reactor performance was impaired (e.g. insufficient mixing) are shown but not connected with lines to aid visualization.

Figure S2: Nickel and Molybdenum concentration in the catholyte

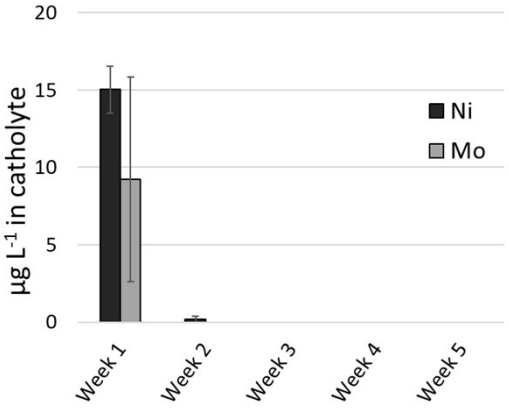

Fig. S2: ICP-OES measurements to monitor potential dissolution of the electrochemical NiMo-catalyst during Exp1. Liquid samples taken from the catholyte chambers of E-Chem 1 and E-Chem2 three times weekly. Displayed values are mean of all measurments from both reactors with the error displaying the corresponding standard deviation.

Figure S3: Cathode potential during long-term electromethanogenesis

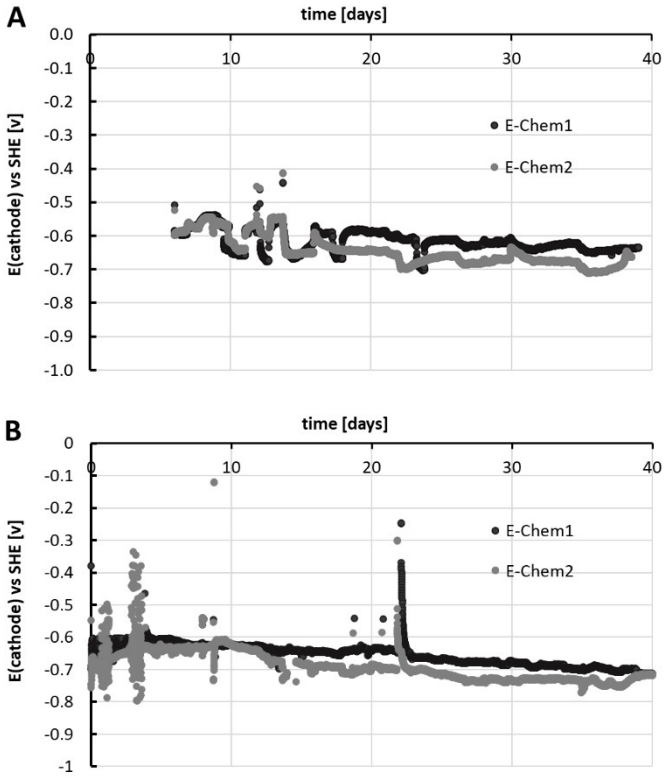

Fig. S3: Cathode potential of the NiMo cathodes of the two bio-electrochemical systems E-Chem1 and E-Chem2 during Exp1 **(A)** and Exp2 **(B)**. The system was operated under constant current of -50 mA (-1 mA cm^{-2}).

Figure S4: Images of cathode modification

Fig. S4: Digital photograph of a carbon rod cathode, unmodified (left) and NiMo-coated via electroplating (right).

Table S2: Summary of performance parameters during Exp1 and Exp2

Table S2: Summary of the system performance during long-term electromethanogenesis Exp1 and Exp2. E-Chem1 and E-Chem-2 are dublicate bio-electrochemical reactors integrating NiMo-cathodes and *M. maripaludis*; H_2-Conrol presents a reactor featuring external H_2-feed. Given values are mean ± SD averaged over all measurements during the stable experiment period. Measurements during times of technical difficulties, such as impaired mixing, were excluded.

parameter	**Exp1**	**Exp2**
Total experiment duration	5 weeks / 35 days	6 weeks / 42 days
Duration of stable performance	3 weeks	5 weeks
Volumetric methane production rate [L/Lday]	H_2-Control: 1.15 ± 0.01 E-Chem1&2: 1.38 ± 0.09	H_2-Control: 1.29 ± 0.06 E-Chem1&2: 1.38 ± 0.07
Specific methane production rate [mL/g_{CDW}min]	H_2-Control: 0.78 ± 0.11 E-Chem1&2: 1.53 ± 0.26	H_2-Control: 0.88 ± 0.07 E-Chem1&2: 1.05 ± 0.11
Coulombic efficiency [%]	98 ± 6	98 ± 2

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