Supporting information

Selective enrichment establishes a stable performing community for microbial electrosynthesis of acetate from CO_2

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S-1 MATERIALS AND METHODS

All microbiological and bioelectrochemical experiments were conducted using anaerobic culturing techniques.[1] The medium replenishments were done under constant N_2:CO_2 gas flow in the cathode chamber. The tubing and connections were assembled in such a way that allowed easy replenishments and no oxygen intrusion due to a constant overpressure in the cathodic chamber.

Bioelectrochemical reactor set-up. The bioelectrochemical experiments were conducted using custom-made glass reactors (250 mL) with five necks (Figure S1) and a three-electrode arrangement consisting of a working electrode, an Ag/AgCl reference electrode and a counter electrode. The working electrode or cathode was a carbon felt (projected surface area, 4 cm^2; Alfa Aesar, Germany) and the counter electrode or anode was a dimensionally stable titanium-coated TaO_2/IrO_2 (35/65 %) mesh or rod (5x2x0.1 cm or 15x1 cm; m-270, Magneto Special Anodes, The Netherlands). Titanium wire (Ø1 mm, Advent Research Materials, UK) was used to establish external connections between the working electrode and the potentiostat. Before use, the carbon felt was pre-treated with 1 M HCl and then with 1M NaOH (each step for 24 h), and finally washed with deionized water to remove any possible organic and metal contamination. The Brunauer–Emmett–Teller surface area of the treated carbon felt was 2.03 m^2 g^{-1}. The counter electrode was separated from the working electrode compartment by a cation exchange membrane (0.8 cm^2; Ultrex CMI-7000, Membranes International Inc., USA) fixed with a punctured rubber stopper and an aluminium crimp seal in a glass serum culture tube (Balch tube[2]). The other end of the tube was cut-off in order to accommodate the counter electrode. This tube was assembled in the central neck of the reactor. The membrane was pretreated by soaking it in 4% NaCl for at least 24 h. The working volume of the catholyte was maintained at 125 mL. Separate sampling ports were used for liquid and gas samples.

Chemical analyses and calculations. During the enrichment phase of the autotrophic community, VFAs were extracted by using ether extraction protocol according to Holdeman *et al.*[3] Extracted samples were analysed on a Gas Chromatogrpah (GC-2014, Shimadzu) with a flame ionization detector using an Alltech EC-1000 Econo-cap column (25m x 0.53 mm). N_2 was used as the carrier gas. Headspace CH_4 and CO_2 were analysed during the enrichment phase on a GC (GC 14-B, Shimadzu) with a TCD using a Hayesep Q 80-100 column (2.74m x 2 mm) connected to an integrator (C-R8A, Shimadzu).

During MES experiments, liquid samples were taken three times a week for monitoring VFAs, pH and bicarbonate. Optical density of these samples was measured with a UV-VIS spectrophotometer (Isis 9000, Dr Lange, Germany) at 610 nm. The filtered samples (0.22 µM) were analysed for VFAs (formate to butyrate) using a Dionex DX 500 ion chromatography system (Dionex-IC, Wommelgem, Belgium) equipped with an IonPac ICE-AS1 column and an ED50 conductivity detector. The mobile phase was 0.4 mM HCl with a flow rate of 0.8 mL min^{-1}. Representative liquid samples from MES reactors were also processed for detection of other organic acids (valerate & caproate) and alcohols. For organic acids, the samples were processed by ether extraction protocol and then analysed with GC as explained before. For alcohols, filtered samples were analysed using a high performance liquid chromatography (Prostar Varian, Walnut Creek, USA) equipped with Rezex ROA-Organic Acid-H+ column. The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL min^{-1}. The detection limit for ethanol was 200 mg L^{-1}.

The flow rate of N_2:CO_2 was monitored by water displacement measurements during each experiment. Effluent gases from reactors were analysed on a Compact GC (Global Analyser Solutions, Breda, The Netherlands), equipped with a Molsieve 5A pre-column and Porabond column (CH_4, O_2, H_2 and N_2) and a Rt-Q-bond pre-column and column (CO_2, N_2O and H_2S). Concentrations of gases were determined by means of a thermal conductivity detector. The carrier gases were N_2 for H_2 channel and He for all other gases. The detection limit for these gases was 100 ppmv (1 % = 10000 ppmv). For estimating bicarbonate concentrations as CO_2, 1 mL of spent catholyte was added to 1 mL 1M H_2SO_4 in a vacutainer. The contents of vacutainers were mixed vigorously for a couple of minutes and then the headspace CO_2 was determined by Compact-GC. These CO_2 values were correlated with a standard curve of CO_2/bicarbonate determined with the same method in order to calculate the bicarbonate concentration. Suspended biomass was estimated using chemical oxygen demand (COD) analysis as the difference between total and soluble (i.e., 0.22 µm filtered) COD using standard kits according to the manufacturer's instructions (Nanocolor® COD, Macherey-Nagel, Germany). pH was measured using a handheld probe (SP10T, Consort, Belgium).

Electron recoveries were calculated by dividing electrons recovered in products by total electrons supplied as current. "Total electrons supplied" was calculated by dividing theoretical coulombs supplied (calculated using current and time relationship) by Faraday's constant. For the calculations, samples from periods where acetate concentrations reached at the maximum or a stable plateau were considered. The electron recovery calculations for H_2 are based on its residual concentration in the headspace of the reactors. The production rates

of acetate were calculated by dividing its concentration by projected cathode surface area and the time to reach this concentration.

Confocal microscopy. At the end of the MES experiments the cathode samples from two reactors (R3 and R4) were processed for biofilm growth analysis by confocal microscopy. The cathode samples were treated with 20 µl of live/dead staining solution (a mixture of PI (200 µM) and SYBR green (SG; 200X diluted from stock, Invitrogen) for 30 min at 37°C. Subsequently, these stained biocathode samples were visualized and z-stacks were captured using a confocal laser scanning microscope (CLSM, Nikon C1, The Netherlands). The CLSM images were processed using open source software "Fiji".

Analyses of the enriched acetogenic culture and electrosynthetic microbial communities. Genomic DNA was extracted from 2 mL samples using a fastprep method according to the protocol described by Vilchez-Vargas *et al.*[4] The concentration of extracted DNA was measured using a nanodrop ND1000 spectrophotometer (Thermo scientific, USA) and Quant-iT dsDNA BR Assay kits with a Qubit fluorometer (Life Technologies, VIC Australia). The V6 to V8 region of the 16S rRNA gene was amplified using the 926F (5'-AAACTYAAAKGAATTGRCGG-3') & 1392R (5'-ACGGGCGGTGWGTRC-3') primers.[5] Library preparation was performed according to the Illumina workflow (#15044223 Rev.B), with the substitution of Q5 Hot Stat High-fidelity 2x Mastermix (NEB) in standard PCR conditions.[5] Indexing of the purified DNA with unique 8bp barcodes was performed using the Illumina Nextera XT 96 sample Index kit (Illumina, CA, USA). Indexed amplicons were sequenced at the Australian Centre for Ecogenomics on the MiSeq Sequencing System (Illumina, CA, USA) using the MiSeq Reagent Kit v3 (600 cycle, #MS-102-3003) for paired-end sequencing according to the manufacturer's protocol. The forward and reverse reads were independently examined to confirm the taxonomic composition of the communities, however, only the results from the forward reads were used in the subsequent analysis. Adaptor sequences were removed and reads were hard trimmed to 250bp using Trimmomatic,[6] discarding reads below 190 bp. Sequence cluster representatives were selected using the QIIME v.1.8.0 pick_open_reference_otus.py script[7] with the Greengenes database[8] to assign taxonomy. Sequences were clustered at 97 % identity and operational taxonomic units (OTUs) matching less than 0.01 % of total reads were discarded. An OTU table was generated and a heatmap showing relative abundances was created in RStudio v2.15.0 with the RColorBrewer package.[9] The sequences used to create the heatmap were deposited under accession number SRR1577794 in the National Center for Biotechnology Information (NCBI) short read archive.

Table S-1. Composition of modified homoacetogenic medium adapted from Leclerc *et al.*[10]

K_2HPO_4	0.2 g L^{-1}
NH_4Cl	0.25 g L^{-1}
KCl	0.5 g L^{-1}
$CaCl_2.2H_2O$	0.15 g L^{-1}
$MgCl_2.6H_2O$	0.6 g L^{-1}
NaCl	1.2 g L^{-1}
$NaHCO_3$	30 mL from 84 g L^{-1} stock
Trace metal solution*	1 mL
Vitamin solution**	2.5 mL
Tungstate-selenium solution***	0.1 mL

***Composition of trace metal solution** (g L^{-1})[11]

Nitrilotriacetic acid (dissolve with KOH; pH 6.5)	1.5
$Mg_2Cl_2.6H_2O$	3.0
$MnCl_2.2H_2O$	0.5
NaCl	1
$FeCl_2$	0.1
$CoCl_2$	0.1
$CaCl_2.2H_2O$	0.1
$ZnCl_2$	0.1
$CuCl_2$	0.01
$AlCl_3.6H_2O$	0.01
H_3BO_3	0.01
$Na_2MoO_4.2H_2O$	0.01

****Composition of vitamin solution** (mg L^{-1})[12]

Sodium ascorbate	10
Biotin	4
Folic acid	4
Pyridoxine hydrochoride	20
Thiamine hydrocloride	10
Riboflavin	10
Nicotinic acid	10
DL-calcium pantothenate	10
Vitamin B12	0.2
p-aminobenzoic acid	10
Lipoic(thioctic) acid	10
Myo-inositol	10
Choline chloride	10
Niacinamide	10
Pyridoxal hydrochloride	10

***<u>Composition of tungstate-selenium solution</u>:

0.1mM Na_2WO_4 + 0.1mM Na_2SeO_3 in 20mM NaOH

Table S-2. Experimental details and objectives for six microbial electrosynthesis (MES) reactors (R1 to R6) used in this study.

Reactors		**R1**	**R2**	**R3**	**R4**	**R5**	**R6**
Experimental details	Gas flow rate[#] (L d^{-1})	5	6.5	6	7	1.25±0.25	
	Reported experimental duration (days)	63	72	24	24	36	38
	No. of batch cycles	3	3	1	1	1	1
	Catholyte pH (start-up)	7.57	7.50	7.69	7.69	7.78	7.79
	Experimental aims	Production profiles, Production rates, Microbial community analysis		Testing reproducibility, Electrochemical & microscopic analyses of biocathodes		Production capacity, Investigate factors limiting product concentrations and rates	
Cathode potential*		-1.2±0.2	-1.2±0.2	-1.35±0.1	-1.3±0.1	-1.26±0.1	-1.3±0.08

[#]R1 to R4 were operated at high gas flow rates (>5 L d^{-1}). It is important to stress that different gas flow rates didn't affect the acetate concentrations in these reactors. However, as discussed in results section in the main paper it affected the electron recoveries in acetate and H_2. For reactors R5 and R6, very low gas flow rates (>1.5 L d^{-1}) were used for checking its influence on electron recovery.

* V (vs. Ag/AgCl, 3 M KCl) during MES experiments. The slight differences in measured potential values can be attributed to real-time pH of the medium, more specifically close to the electrode surfaces. Furthermore, the data for R1 and R2 is based on three batch cycles, whereas for other reactors it is based on a single batch cycle. The long-term operation of these reactors likely resulted in a decrease in the overpotential at biocathodes as also indicated by the CV scans.

Note: The pH of the anolyte remained in the range of 1.75±0.75 during these experiments.

Table S-3. VFAs produced by the representative enriched mixed microbial culture (E3, grown as static culture at 30 °C) in serum bottles with CO_2:H_2 growth conditions. The analysis was conducted at different time intervals during incubation. No methane production was observed with the enriched culture. Data is presented for the representative culture.

VFAs (mg/L)	**Day 3**	**Day 6**	**Day 16**	**Day 31**
Formate	65	89	594	587
Acetate	602	1488	1923	1850
Propionate	7	106	135	160
Butyrate	46	343	730	743
Methane	-	-	-	-

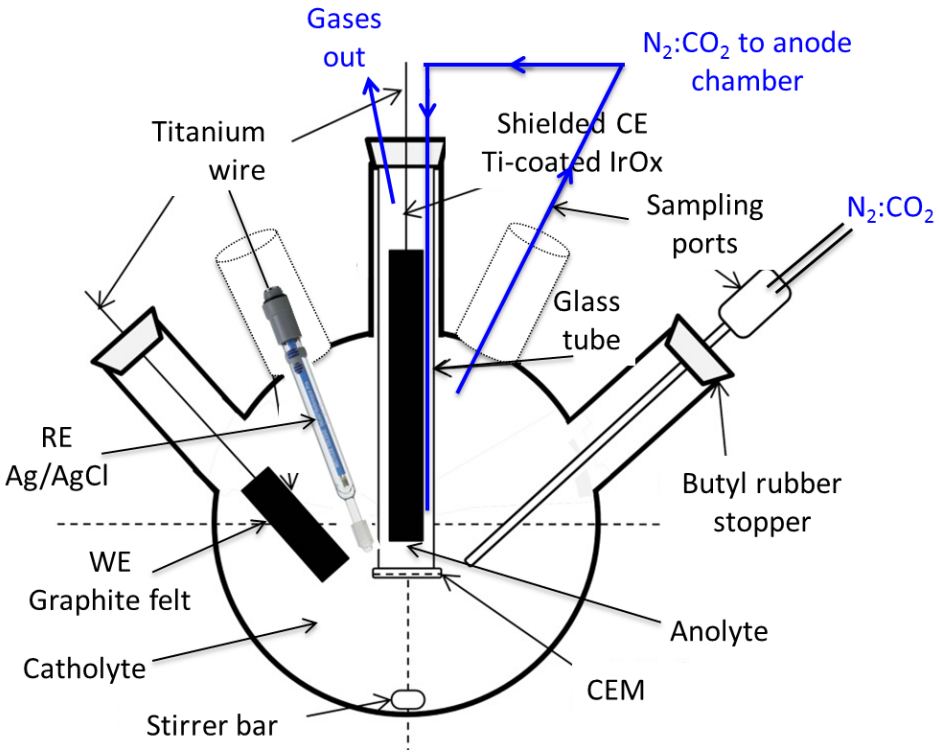

Figure S-1. Schematic overview of the bioelectrochemical system used in this work (design based on[13, 14]). (RE/WE/CE: reference/working/counter electrode; CEM: cation exchange membrane).

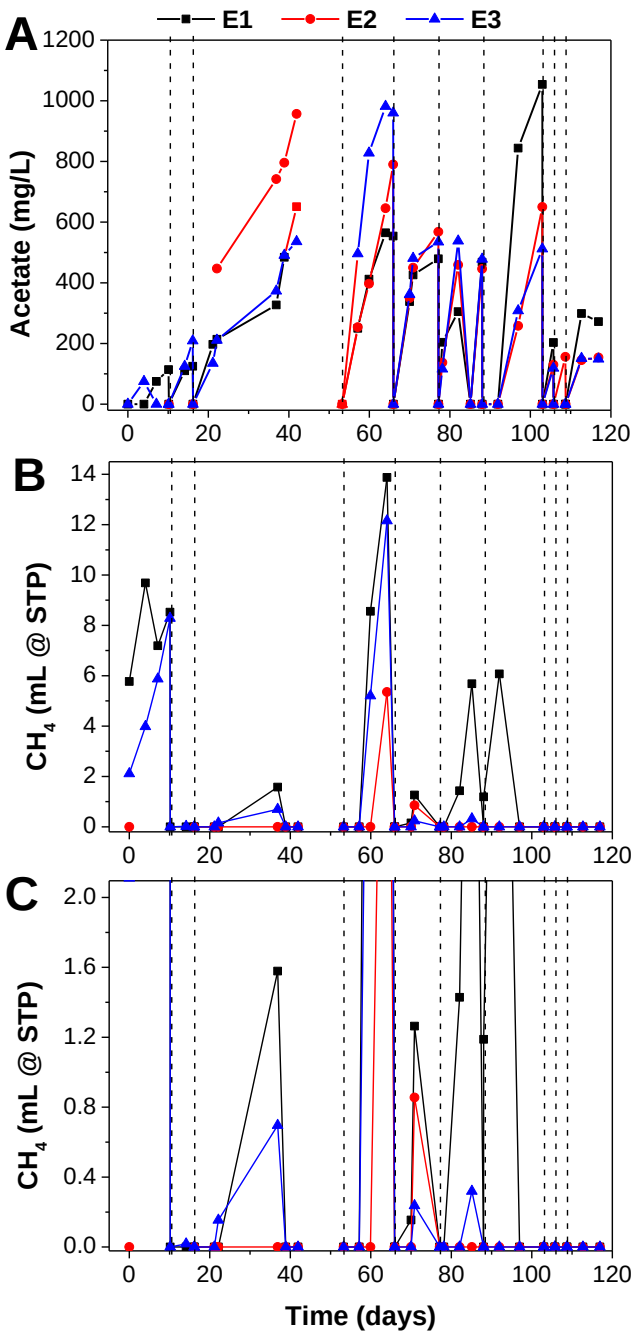

Figure S-2. I) Product profiles for three replicate cultures (denoted as E1, E2 and E3) during the initial enrichment phase. A) Acetate concentration, B) Methane concentration in the headspace, C) close-up view of B. Vertical dotted lines indicate a culture transfer step to fresh medium. The earlier transfers (during enrichment phase until day 110) were based on the experimental observations of acetate production. The quick culture transfers (every 2-3 days) were done at the end of the enrichment phase (i.e starting at day 110) when it became clear that methanogenic activity was still present in the culture flasks.

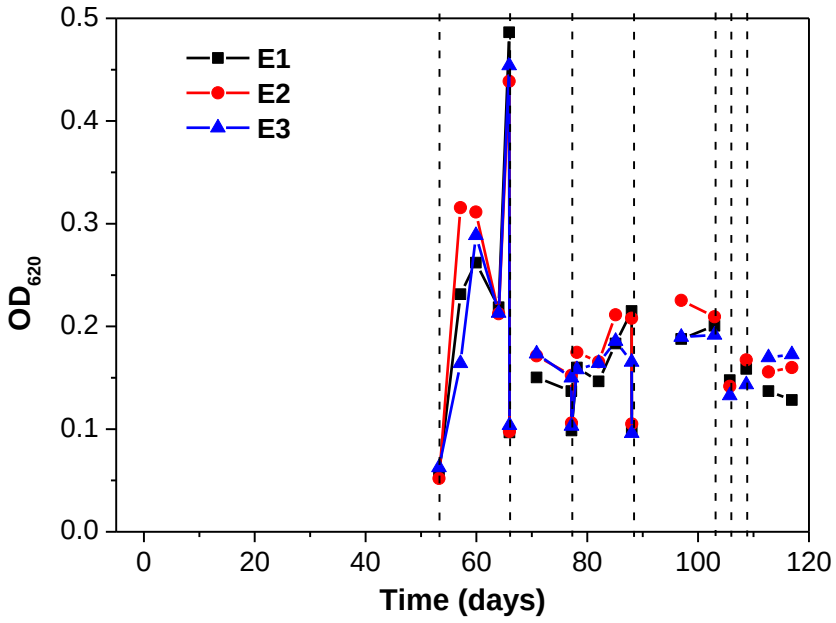

Figure S-2. II) Optical density (OD_{620}) data for three replicate cultures (E1 to E3) during the initial enrichment phase (data shown from transfer cycle 3). OD of 200 µl sample from serum flasks was determined in a microtiter plate at 620 nm using a Tecan Sunrise absorbance Reader with XFluor4 v.4.51 software (Tecan, Austria). Actively growing cultures were distinguished by observing an increase in absorbance.

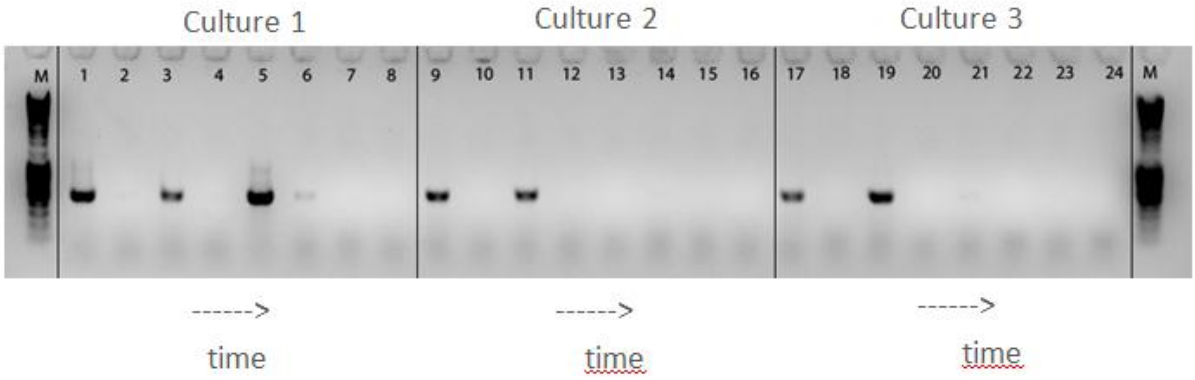

Figure S-3. PCR detection of archaea during the quick-transfer phase of the enrichments in the serum bottles (S2). Odd numbers are samples taken before transfer, even numbers after transfer. Archaeal PCR was conducted using primerset GC-ARCH 915/ Uni-B-rev according to Yu *et al.*[15]

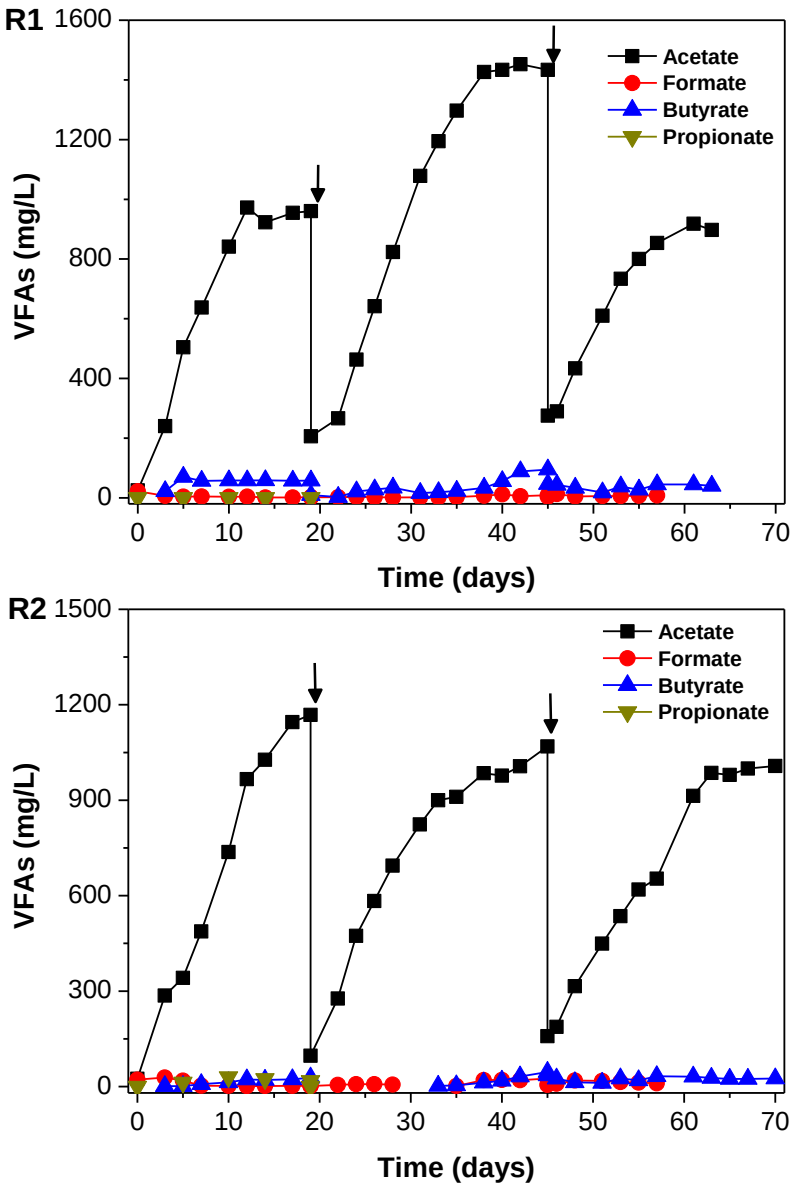

Figure S-4. Production of volatile fatty acids (VFAs) in reactors R1 and R2 operated at similar experimental conditions (except N_2:CO_2 flow rates). Arrows indicate medium replenishments.

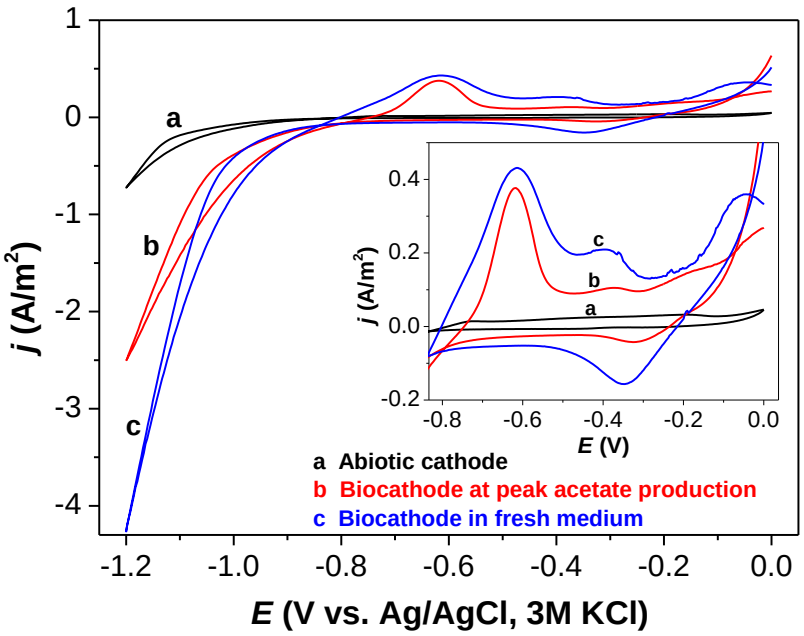

Figure S-5. Cyclic voltammograms obtained with reactor R4: before the inoculation; abiotic cathode (black trace; a), biocathode at the peak acetate production (red trace; b) and biocathode immediate after medium change/in a fresh medium (blue trace; c). Scan rate 1 mV/s. Inset shows the zoom in of small section of the main figure.

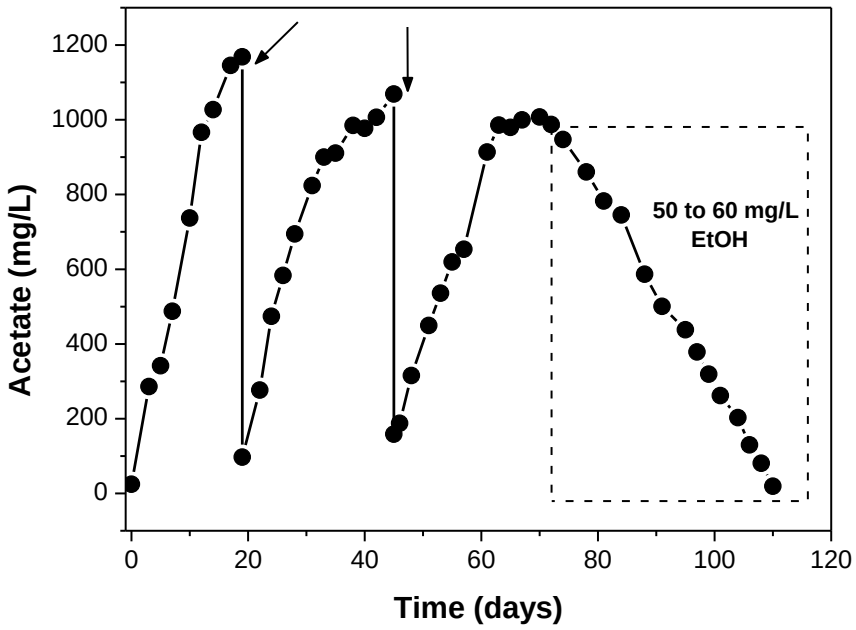

Figure S-6. Microbial electrosynthesis with reactor R2 with a prolonged batch cycle, showing the transition of production from acetate to ethanol during the third batch cycle (box with dotted lines). Arrows indicate medium replenishments.

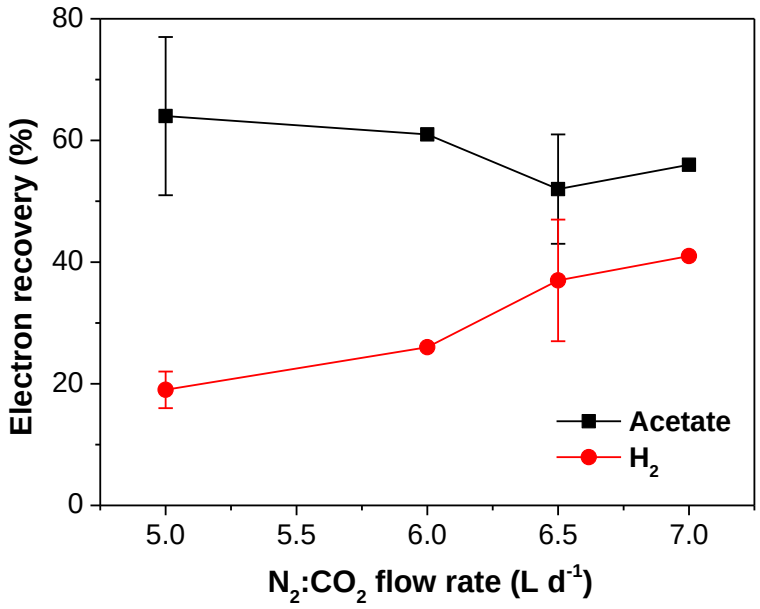

Figure S-7. Influence of gas flow rates on the electron recoveries in acetate and H_2 as determined over R1-R4. The data for R1 & R2 (flow rates: 5.0 and 6.5 L d^{-1}) are averages and standard deviations based on three fed-batch cycles shown in Figure 1.

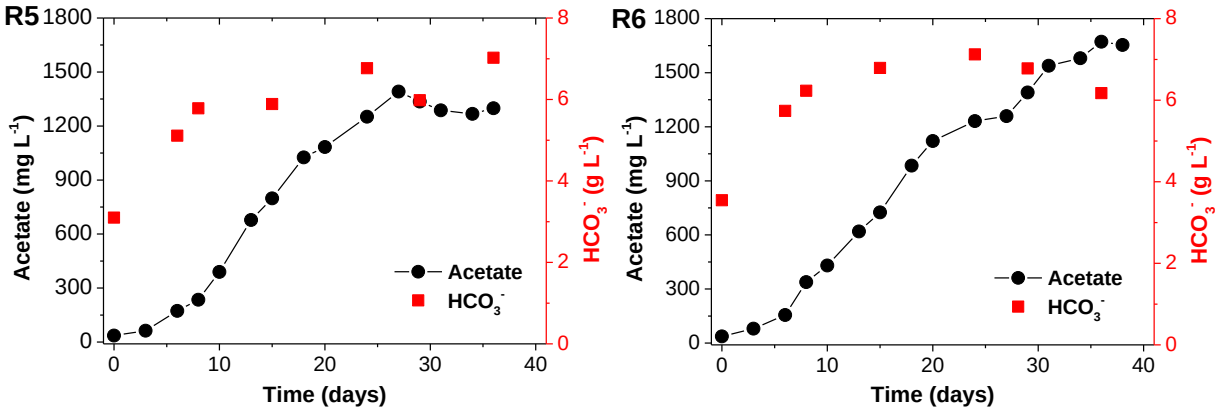

Figure S-8. Acetate production and bicarbonate concentration profiles for reactors R5 and R6. The pH in both reactors was maintained in the range of 8.0±0.4 by dosing 1N NaOH and/or 1 N HCl as needed.

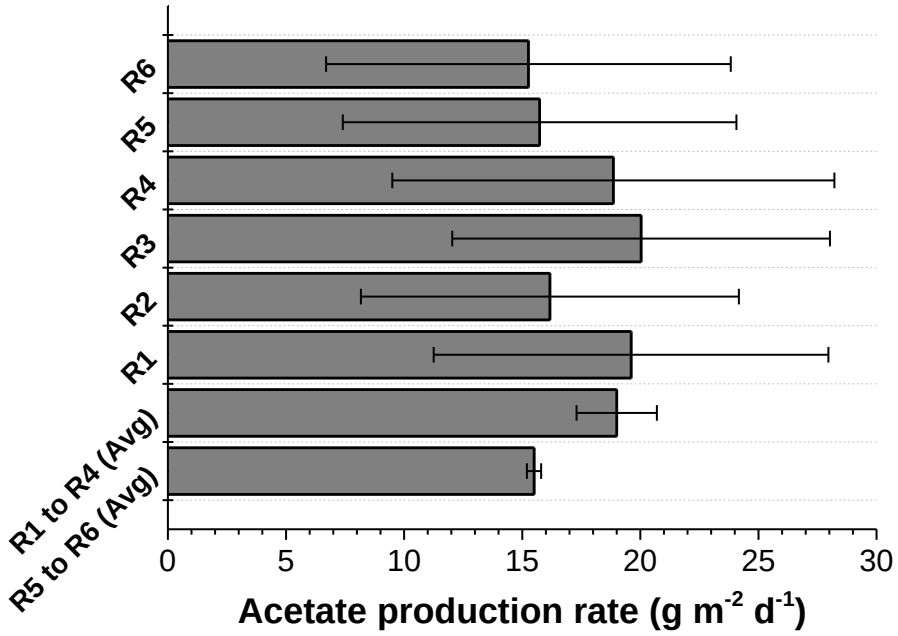

Figure S-9. Acetate production rates achieved with reactors R1 to R6. The rates are calculated relative to the projected cathode surface area and based on the difference in concentration and time between each sampling event. The data for R1 and R2 is based on the production rates of three batch cycles calculated separately whereas the data for other reactors (R3 to R6) is based on a single batch production cycle. Experiments with R1 to R4 were conducted at high N_2:CO_2 flow rates (>5 L d^{-1}) and with R5 to R6 at low gas flow rates (<1.5 L d^{-1}). Multiply values with 0.0004 m^2/0.125 L to obtain production rates in terms of g L^{-1} d^{-1}. The rates are calculated until maximum acetate concentration is reached.

Calculation S-1: Estimation of biomass in the reactor

Based on confocal analysis of the biofilm (thickness; ~20 µm, coverage 75%), relative abundance of methanogens in the biofilm (~45%), a total outer electrode surface area of 10 cm^2 (rectangle of 2.5*1.6*0.25 cm) and the example provided by Arends and Verstraete[16] an approximation of the total methanogenic biomass in the reactor can be made. This approximation is about 1.35 x 10^8 methanogenic cells in the biofilm.

Over the total reactor operation about 20 mgCOD L^{-1} could be quantified as suspended COD in the reactor effluent which can be interpreted as biomass. Using the numbers and uncertainties provided by Gaudy *et al.*[17] and verified by Heddle and Tavener,[18] the percentage of methanogens of the total biomass max. 13%. However, this calculation is based on the assumption that all unidentified COD is active biomass therefore, the number might still be lower.

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