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Selective Enrichment Establishes a Stable Performing Community for Microbial Electrosynthesis of Acetate from CO_2

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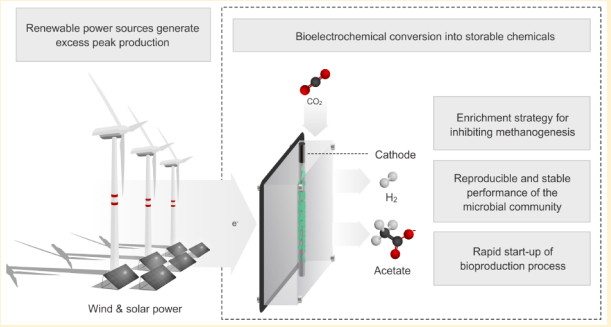
Supporting Information

ABSTRACT: The advent of renewable energy conversion systems exacerbates the existing issue of intermittent excess power. Microbial electrosynthesis can use this power to capture CO_2 and produce multicarbon compounds as a form of energy storage. As catalysts, microbial populations can be used, provided side reactions such as methanogenesis are avoided. Here a simple but effective approach is presented based on enrichment of a robust microbial community via several culture transfers with H_2:CO_2 conditions. This culture produced acetate at a concentration of 1.29 ± 0.15 g L^{-1} (maximum up to 1.5 g L^{-1}; 25 mM) from CO_2 at a fixed current of −5 Am^{-2} in fed-batch bioelectrochemical reactors at high N_2:CO_2 flow rates. Continuous supply of reducing equivalents enabled acetate production at a rate of 19 ± 2 $gm^{-2}d^{-1}$ (projected cathode area) in several independent experiments. This is a considerably high rate compared with other unmodified carbon-based cathodes. 58 ± 5% of the electrons was recovered in acetate, whereas 30 ± 10% of the electrons was recovered in H_2 as a secondary product. The bioproduction was most likely H_2 based; however, electrochemical, confocal microscopy, and community analyses of the cathodes suggested the possible involvement of the cathodic biofilm. Together, the enrichment approach and galvanostatic operation enabled instant start-up of the electrosynthesis process and reproducible acetate production profiles.



INTRODUCTION

The electricity-driven production of high-value chemicals and fuels from CO_2[1−3] and/or organic substrates[4,5] using microorganisms is a developing technology concept for bioproduction and storage of excess energy as chemicals.[6] It is a far less land intensive approach compared to a crop-based bioproduction scheme. This concept is referred to as microbial electrosynthesis (MES) and typically relies on the capability of microorganisms to grow on the cathode surfaces or in the bulk of bioelectrochemical systems (BESs) by consuming reducing equivalents released from the electrode as their energy source.[7,8] Due to inadequate knowledge on microbial inoculum sources today, obtaining novel microorganisms for cathodic bioproduction either through screening of pure strains or through enrichment-selection strategies from mixed inoculum sources is of crucial importance for the development of MES.[9] The need for good inoculum sources as well as thoroughly engineered reactor systems becomes clear as production rates of current systems are low (Table 1).

For acetate bioproduction processes starting from CO_2, homoacetogens are the obvious choice of microorganisms because of their ability to fix CO_2 via the efficient Wood-Ljungdahl pathway.[6,10] Only a few pure strains have been reported to use electricity for converting CO_2 into valuable chemicals such as acetate.[1,11] Mixed microbial inoculum sources have also successfully been applied for MES of mainly acetate.[12−15] The use of mixed microbial inocula offers some advantages over pure cultures such as the possibility to operate reactors with limited or no sterile conditions, no risk of strain degradation, resistance to operational perturbations and adaptive capacity of the biocatalyst to its diverse composition. However, when using mixed inoculum sources the production of acetate via MES has to compete with the production of methane in the original inoculum.[13,16,17] The practice of using methanogenic inhibitors such as 2-bromoethanesulfonate can result in improvement of acetate production rates.[13,16,17] Short-term suppression of methanogens by such inhibitors and the need for continued addition of such inhibitors to the reactors limits their use for long-term and large-scale applications.[6] Moreover, delays in start-up of bioproduction (up to 40 days) have been reported with the use of a mixed inoculum sources

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Table 1. Comparison of Reported Data on Microbial Electrosynthesis of Acetate[a]

				electrochemical variables of cathode		acetate production				
	microbial inoculum/reactor operation		cathode material	$J_{applied/produced}$ (A/m^2)[b]	$E_{cathode}$ (V vs SHE)	volumetric production rate (mM/day (g/L/day))	surface based rate ($g/m^2_{cathode}/day$)[b]	max. product titer (g/L)	coulombic efficiency in acetate (%)	reference
pure cultures	*Sporomusa ovata*	continuous	graphite stick	−0.208	−0.4	0.17 (0.045)	1.3	0.063[c]	85	1
			Ni-coated graphite stick	−0.63		1.13 (0.068)	3.38	0.094[c]	82 ± 14	45
	Sporomusa sphaeriodes		graphite stick	−0.017		0.01	0.06	0.003[c]	84	11
	Morella thermoacetica			−0.009		0.01	0.10	0.005[c]	84	11
	Acetobacterium woodii	fed-batch or batch	stainless steel felt	−1.5	−0.690	(0.046)	12.8	0.127	81	18
mixed cultures	brewery wastewater sludge		graphite granules	nd	−0.590	4.0 (0.240)	nd	1.71	67 (incl. H_2)	13
	adapted brewery WW sludge					17.25 (1.0)	nd	10.5	69	17
					−0.800	52 (3.1)	nd	8.7	nd	28
			graphite rod	∼−1.2	−0.600	3.6 (0.22)	10.8	0.35	77 ± 9.8 (incl. H_2)	
	domestic WWTP sludge		carbon felt	−2.96	−0.903	2.35 (0.14)	10	4.7	90	16
	domestic WWTP sludge[d]		carbon felt	∼−19	−0.953	6.58 (0.39)	19	0.095	15	12
	enriched culture (sediment from a bog)[e]		carbon fiber rod	−0.03 ± 0.006	−0.4	0.05 (0.0037)	0.063 ± 0.008	0.02 ± 0.0025	35.2 ± 4.4	15
	pond sediments and anaerobic WWTP sludge[f]		carbon plate	−1.76 ± 0.01	−0.85	0.38 ± 0.09 (0.02 ± 0.005)	5.9 ± 1.4	4.2∼	49.5	14
			nanoweb RVC	−37 ± 3		0.42 ± 0.06 (0.03 ± 0.004)	195 ± 30	1.65∼	78.5	
	enriched culture (labscale anode and algae UASB sludge)		carbon felt	−5.0	−1.26 ± 0.08	1.0 ± 0.09 (0.06 ± 0.006)	19 ± 1.7	1.29 ± 0.15	58 ± 5 30 ± 10 in H_2	This study[g]
					−1.28 ± 0.03	0.83 ± 0.02 (0.05 ± 0.001)	15.5 ± 0.3	1.5 ± 0.2	44 ± 5 5.5 ± 0.7 in H_2	This study[h]

[a]Note: It is practical to consider projected surface area of the cathode for calculating rates since this is the main driver determining the reactor geometry and size. Therefore, the rates are compared considering the projected cathode surface area (in addition to volume-based rates) irrespective of the used electrode material or reactor design. [b]calculated per projected cathode surface. [c]estimated based on linear production rate and reported flow rate; n.d.: not defined. [d]based on 24 h production test. [e]estimated based on the data reported in the paper for autotrophic bioproduction (stoichiometric calculations based on consumed electrons). [f]based on the last phase of the batch experiments (38 days/140 days); RVC: Reticulated vitreous carbon; ∼data provided by Jourdin et al., 2014 [g]High N_2:CO_2 flow rate (>5 L d^{-1}); [h]Low N_2:CO_2 flow rate (<1.5 L d^{-1})

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directly from their natural habitat.[12−14] In most of these cases, microbial enrichments were directly achieved in BES cathodes. Recently, a successful pre-enrichment strategy comprising the growth of inoculum first in serum cultures and then in cathode chamber of BESs has been reported for enhancing the start-up times of autotrophic biocathodes.[15] However, glucose was used as carbon source during these enrichment phases, which consequently affected the overall bioproduction into a wide variety of products.[15] It is noteworthy to stress here that this particular study reported a generalized method for pre-enrichment using alternative electron donors to develop anaerobic biocathodes for electrosynthesis in BESs.

The goal of this study was to investigate if (i) it would be possible to enrich a mixed microbial community from an anaerobic environment on H_2:CO_2 for only acetate production and (ii) how this enriched microbial community would behave in a galvanostastically operated cathode. The enrichment approach comprised of the addition of 2-bromoethanesulfonate only once in the initial phase followed by several culture transfers across serum flasks in quick succession to eliminate methanogenic activity. A resulting enriched community used in BES cathodes lead to an instant start-up of the bioproduction process. Different from previous studies that applied potentiostatic control for a CO_2 to acetate process, here, the BESs were operated in galvanostatic mode to ensure a constant flux of electrons toward the microorganisms.[5,18]

MATERIALS AND METHODS

Enrichment Experiments. As a source of microbial inoculum, several mixtures of anodic effluent of a microbial fuel cell[19] and the effluent of a lab-scale upflow anaerobic sludge blanket reactor (UASB) digesting microalgae[20] were used. The selection of these two inoculum sources was based on the theoretical possibility of the presence of the needed functional traits within the microbial community, that is, interaction with an electrode (anode biomass) and the formation of products from CO_2 as a carbon source (UASB biomass). The enrichment experiments were conducted in serum bottles (120 mL) filled with 40 mL growth medium based on Leclerc et al.[21] and with a H_2:CO_2 (70:30) headspace at 50 kPa overpressure (data for three enrichment cultures E1-E3; Supporting Information (SI) Figure S2). The composition of the modified growth medium is provided in SI Table S1. All incubations were done at 28 °C on a rotary shaker maintained at 100 rpm. 2-bromoethanesulfonate (0.5 mM) was added only once in the medium during the start-up of enrichment phase to inhibit methanogenesis.[22] The initial transfers (during the enrichment phase) were based on the experimental observations of acetate production (SI Figure S2). Subsequent quick culture transfers (1000× dilution every 2 days for three consecutive cycles) were then performed at the end of the enrichment phase (i.e starting at day 110, SI Figure S2). In order to keep track of acetogenesis and methanogenesis, the production of VFAs and headspace gas composition and pressure were monitored by analyzing samples every 2 days.

Microbial Electrosynthesis Experiments. All MES experiments were carried out under galvanostatic control (Potentiostat/Galvanostat Model VMP3, Biologic Science Instruments, France) using custom-made glass reactors (250 mL) with five necks (SI Figure S1; based on refs 23 and 24). A modified homoacetogenic medium (125 mL) lacking yeast extract, tryptone, resazurin and Na_2S with pH 7.6 ± 0.2 was used as production medium (referred to as catholyte) in the cathode chamber (SI Table S1). CO_2 was initially supplied in the form of sodium bicarbonate (30 mM) as the sole carbon source in the medium. The anolyte was 0.5 M Na_2SO_4 with pH 2.5 (adjusted with 1 M H_2SO_4). In order to avoid limitations from anodic reactions, dimensionally stable anodes and abiotic conditions were used.[18] To ensure similar start-up conditions, the enriched acetogenic culture (E3) was revived from a −80 °C stock culture (40% glycerol) under H_2/CO_2 atmosphere in serum flasks to check activity. Cathode chambers were inoculated to a final density of ∼10^7 intact cells per mL. The cell count was determined by viability staining method using flow cytometry (Accuri C6 flow cytometer; BD Biosciences).[25] The procedure was adjusted to 4 μM propidium iodide (PI) and incubation for 13 min at 37 °C. The cathode chamber was continuously purged with N_2:CO_2 (90:10) using gas flow meters (OMA-1, Dwyer, UK) in order to maintain anaerobic conditions in reactors. This also served as a constant source of CO_2 and buffering agent in the medium. The effluent gas from the cathode chamber was sent through the anode chamber to strip O_2 produced at the anode. Different N_2:CO_2 flow rates (high; >5 L d^{-1} and low; <1.5 L d^{-1}) were used in order to investigate its effect on the electron recovery in acetate and residual H_2 (SI Table S2). The gas flow rates were monitored and determined by using the water displacement method. The applied gas flow rates did not result in a change in catholyte volume due to evaporation.

Electrosynthesis was facilitated by providing a constant cathodic electron supply by means of setting a fixed current of −5 A m^{-2} using chronopotentiometry, that is, galvanostatic conditions. (With the reactors used in this study, −5 A m^{-2} translates, assuming 100% conversion to acetate, to ∼33 g $m^{-2}_{cathode}$ d^{-1}. The aim behind using −5 A m^{-2} was not to be limited on supply of reducing equivalents which was clearly the case as maximum production rates in our experiments were in the range of 19 g m^{-2} d^{-1} (see Results and Discussion)). The use of galvanostatic operation leads to the production of H_2 at the cathode. This is of high importance to ensure quick consumption of large currents in the framework of excess electrical energy storage. Despite the low solubility of H_2 at ambient conditions (0.002 g L^{-1} water,[26] although in situ production of H_2 may lead to supersaturation), and its retention or distribution in reactors,[27] this approach offers some interesting possibilities. First, it can enable the participation of planktonic cells in electrosynthesis processes. Second, the excess H_2 can be coupled to conventional biorefineries for supporting further conversions. Consequently, it can contribute in bringing down the cost of H_2 production in conventional chemical refineries. Finally, as H_2 is a gaseous product, it separates naturally and therefore does not interfere with the downstream processing of soluble products.

These experiments were conducted in a fed-batch or batch mode at 28 ± 2 °C. In batch mode, all removed liquid during sampling was replaced with an equal amount of anaerobic sterile medium. In fed-batch experiments, medium exchanges after each batch cycle were accomplished by replacing 90% of the spent medium with an equal volume of fresh medium. The remaining 10% of the spent medium acted as an inoculum source for the subsequent cycle. Furthermore, both anolyte and membrane were replaced during medium replenishments. Cyclic voltammograms (CVs) were recorded at a scan rate of 1 mV s^{-1} at different time points in order to get mechanistic insights into the electrochemical reactions occurring at the cathodes. All CVs were recorded under constant N_2:CO_2

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purging conditions. The CV scans were started from "0" V vs Ag/AgCl and were recorded for two cycles in order to obtain reproducible and stable voltammograms. The second cycle is reported in the paper. The most important operational parameters and main objectives of each experiment with these reactors (R1 to R6) are presented in SI Table S2.

Unless stated otherwise, all potentials provided in this manuscript refer to the Ag/AgCl reference electrode (3 M KCl, (0.240 V vs standard hydrogen electrode (SHE)); RE-1B, Biologic Science Instruments, France). The observations and results of MES experiments presented in this work are based on six independent reactors (R1 to R4, operated at high gas flow rates of >5 L d^{-1} and R5 to R6, operated at low gas flow rate of <1.5 L d^{-1}). For details on bioelectrochemical reactor setup, chemical analyses and microscopy please refer to SI Section S1.

Analyses of the Enriched Acetogenic Culture and Electrosynthetic Microbial Communities. The enriched acetogenic culture grown in serum bottles with CO_2:H_2 as well as samples (both catholyte and biocathode) from the MES reactors (R1 to R2) taken at different time points as elaborated further below were processed for microbial community analysis. The samples were taken at day 19 of the batch cycle 1 from both reactors, and at day 63 and day 70 of the batch cycle 3 from R1 and R2, respectively (Figure 1A, B). In addition, a cathode sample was taken at the end of the experiment of R2 (day 110; SI Figure S6). For further details on molecular biology protocols please refer SI Section S1.

RESULTS AND DISCUSSION

Enrichment of the Mixed Microbial Culture Lacking Methanogenic Activity. The enrichment of an acetogenic culture was assessed by volatile fatty acids (VFAs) and methane production in actively growing cultures provided with CO_2 and H_2. Methane production was only detected after accumulation of acetate (data for three replicate enrichment cultures E1-E3; SI Figure S2), leading to the hypothesis that acetoclastic methanogenesis was the dominant process in the start-up inoculum used. Therefore, multiple transfers in quick succession (after 2 days incubation, 1000x dilution) were made in fresh medium, which eventually resulted in a washout of methanogenic activity (SI Figures S2, S3).

A representative enriched acetogenic culture (E3), which was revived from −80 °C storage and used for subsequent tests, produced independent of the enrichment phase acetate and other VFAs, but no detectable methane at static conditions (SI Table S3). Methane was not detected even after incubation of these cultures for more than a month, thus clearly indicating the inhibition of methanogenic activity. These observations were initially confirmed by PCR analysis (SI Figure S3 for methods and results) and further corroborated by analyzing enriched communities through 16S rRNA gene amplicon sequencing (Figure 5). In the presented case the enrichment phase lasted 110 days. However, we would like to stress here that such a long duration is not needed for the initial enrichment phase. The quick culture transfer steps (SI Figure S2) can be done at the moment acetate production occurs in the serum flasks thus shortening the enrichment phase considerably.

Microbial Electrosynthesis of Acetate From CO_2. Abiotic (uninoculated, connected) and biotic (inoculated but disconnected) control experiments were conducted for >2 weeks under constant N_2:CO_2 flow but did not result in production or accumulation of acetate or other VFAs (data not shown). In order to check the efficacy of the enriched acetogenic culture for bioproduction of acetate from CO_2 and electrical current, initially, two BESs, R1 and R2, were operated for three fed-batch cycles. Acetate production started immediately in both reactors (Figure 1). This can be attributed to the immediate activity of acetogenic microorganisms in CO_2 reduction and also to the fact that a constant current was supplied to the cathode. In both reactors, an increase in acetate concentration over time and eventual stabilization in the range of 1−1.5 g L^{-1} (16−25 mM) in all fed-batch cycles was observed. The maximum concentrations of acetate produced in R1 and R2 were 1.5 g L^{-1} and 1.2 g L^{-1} respectively. Other VFAs such as formate, propionate and butyrate were also

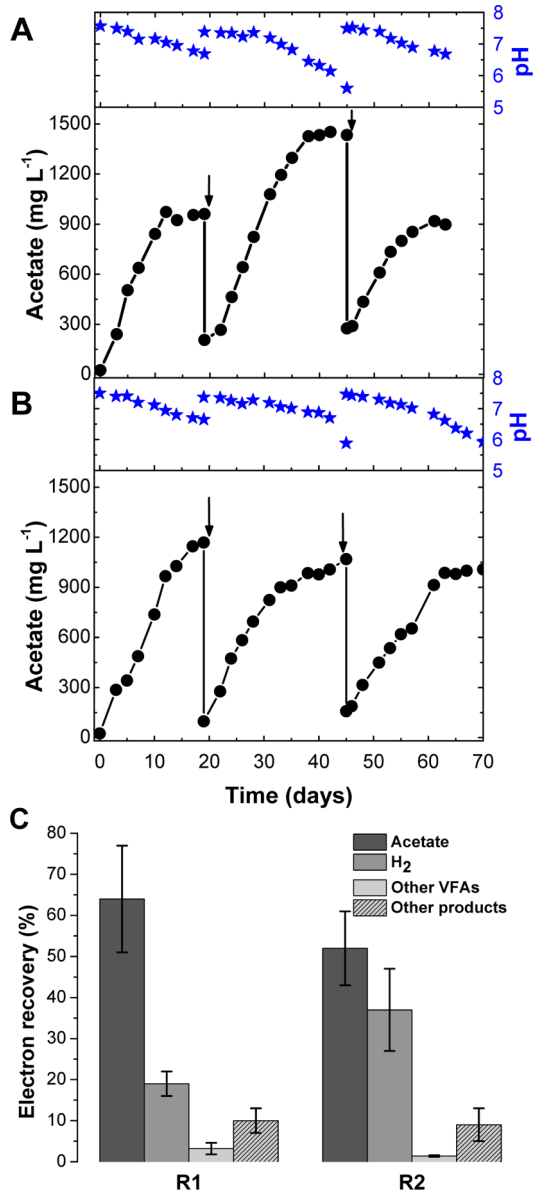

Figure 1. Acetate production and pH profiles of reactors (A) R1 and (B) R2 operated with similar experimental conditions (except N_2:CO_2 flow rates). Arrows indicate medium replenishments. (C) Electron recovery (Coulombic efficiency) in different products achieved with reactors R1 and R2. Electron recovery is based on the final measured products, that is, soluble components and H_2 in the effluent gas. The data are averages and standard deviations based on three fed-batch cycles shown in A and B.

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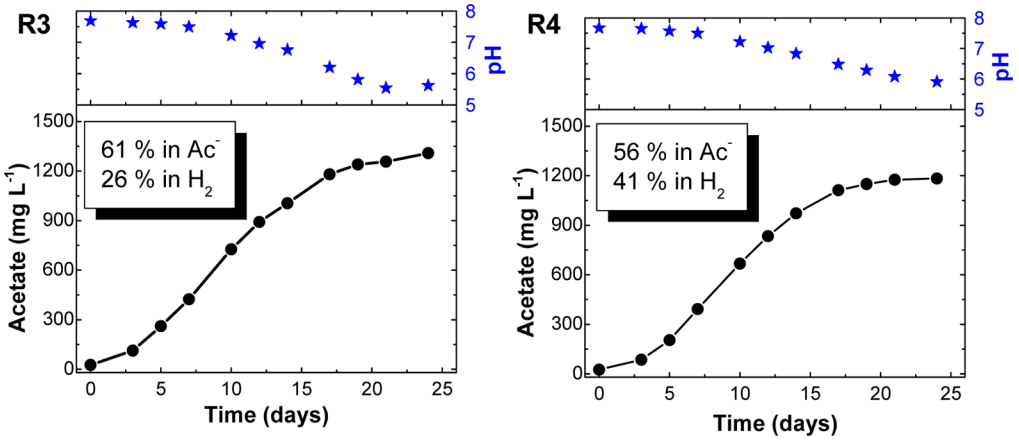

Figure 2. Acetate production and pH profiles of reactors R3 and R4. The values in boxes represent the electron recovery in acetate (Ac^-) and H_2 for respective reactors. Electron recovery is based on the final measured products, that is, soluble components and H_2 in the effluent gas.

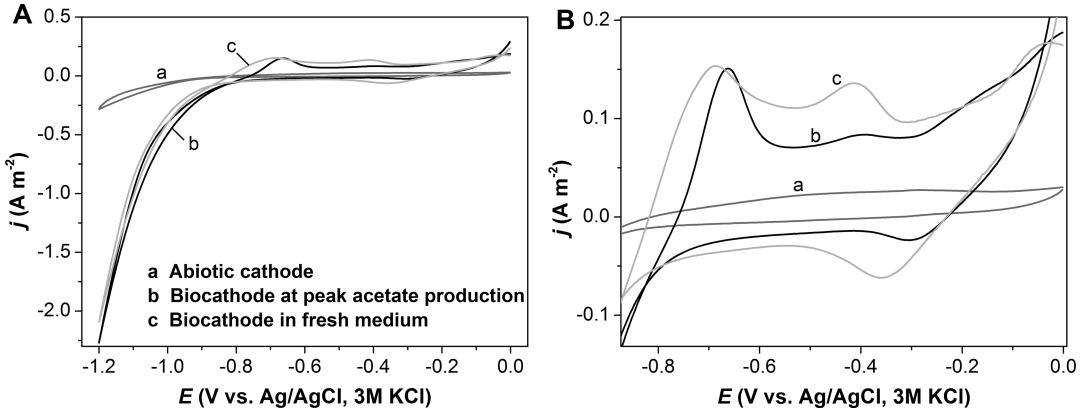

Figure 3. (A) Cyclic voltammograms recorded on the biocathode of reactor R3: before inoculation; abiotic cathode (black trace; a), biocathode at the peak acetate concentration (red trace; b) and biocathode immediately after medium change or in a fresh medium (blue trace; c). Scan rate: 1 mV s^{-1}. (B) Zoom in of small section of the Figure A.

produced, but in low concentrations (<0.06 g L^{-1}) (SI Figure S4). No ethanol was detected at this stage of the MES process. These results indicate the ability of the enriched acetogenic culture for the MES of acetate as a major product. The production of acetate in both reactors was accompanied by a gradual decrease in the pH of the catholyte (from 7.5 to <6.0), attributed to the accumulation of VFAs in these reactors. The pH drop was not associated with the diffusion of protons from the anode chamber as confirmed with abiotic control tests conducted with electrically disconnected reactors at similar conditions (data not shown). No methane was detected in these reactors for two fed-batch cycles. However, during the prolonged duration of experiments after attaining maximum production in the third batch cycle, methane production was observed (though below quantification limit). It is important to note that methane was not detected at the end of the second batch cycle even after closing the gas flow for 2 days. Appearance of limited methane will likely influence the overall production efficiency on the long run. When operating the MES reactors as a batch production process with reactor or electrode cleaning in between, the influence of methanogenesis can be further limited. Inoculation of a new or cleaned reactor with this particular enrichment delivers reproducible results (see SI Figure S9).

In case of R1, 64 ± 13% and 19 ± 3% of the electrons were recovered in acetate and H_2, respectively (Figure 1C). Only 3.2 ± 1.4% electrons were recovered in other VFAs. In addition, 10 ± 3% electrons appeared in other products determined as soluble COD. With R2, 52 ± 9%, 37 ± 10%, and 1.4 ± 0.2% electrons were recovered in acetate, H_2 and other VFAs, respectively (Figure 1C). In this case also a similar amount of electrons (9 ± 4%) appeared in other soluble products. Biomass formation most likely accounted for the rest of the electrons. The electron recovery calculations for H_2 are based on its residual concentration in the headspace of the reactors.

Microscopic and Electrochemical Analyses of the Biocathodes. *Reproducibility of the Enriched Acetogenic Culture for MES Process.* Two additional BESs (R3 and R4) were operated under similar conditions to assess the reproducibility of the enriched acetogenic culture for MES of acetate. Both reactors exhibited reproducible behavior compared to R1 and R2 in terms of start-up time, acetate production and pH drop (Figure 2). Up to 1.3 g L^{-1} (21.5 mM) and 1.2 g L^{-1} (20 mM) of acetate production was achieved with R3 and R4 respectively. The gradual increase in acetate concentration was accompanied by the increase in OD_{610} in these reactors (from 0.03 to 0.27 in R3 and from 0.03 to 0.31 in R4). Other VFAs, mainly butyrate up to 0.06 g L^{-1}, were again present in low amounts. Electron recoveries in acetate with R3 and R4 were up to 61% and 56% respectively. Similar to R1 and R2, a large portion of the electrons was recovered in H_2 (26% in R3 and 41% in R4). A substantial

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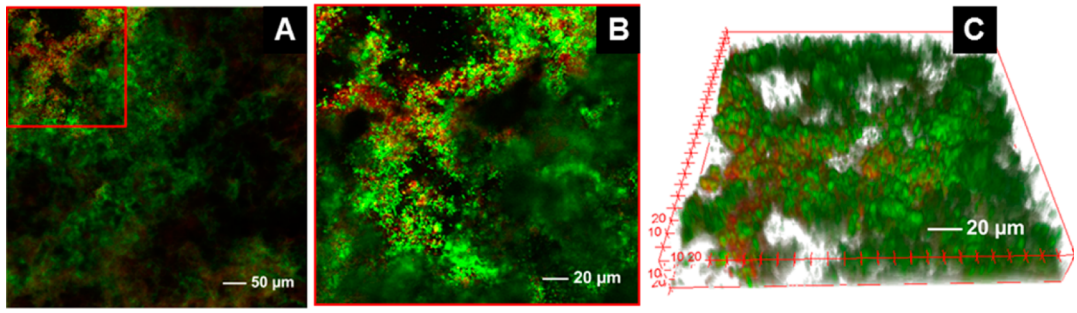

Figure 4. (A) Representative confocal microscopy images of the biocathode from R3 processed at the end of the experimental run (Figure 3). (B) zoom in of the red frame of image A, (C) 3D view of 16 stacks of the selected section from image A. Green fluorescence indicates intact microbes and red fluorescence indicates microbes with damaged membranes.

amount of the electrons was also recovered in other products as soluble COD (13% in R3 and 12% in R4). In agreement with earlier observations, no methane was detected in the off-gas of these reactors. When acetate production reached its maximum, these reactors were subjected to further analyses (microscopy and CV) as will be discussed in the following sections.

Analyses of Electrochemical Processes Involved in Bioproduction. The abiotic control CV recorded in growth medium (without inoculum) confirmed that no redox active components were present in the catholyte (Figure 3; trace a). The onset potential of H_2 evolution at the abiotic carbon felt cathode was around −0.9 V. No redox peaks were observed in the CVs recorded immediately after inoculation of these reactors with the enriched culture (data not shown). A clear shift in the onset potential of H_2 evolution potential toward a higher potential of approximately −0.8 V was observed at the time when maximum acetate concentration was present in the catholyte (Figure 3; trace b). The pH of the catholyte was close to 5.6 at the time the CV was recorded (R3 in Figure 2). The electrochemical potential of the biological redox active components is pH dependent.[24] Therefore, in order to clarify the role of pH in the shift in H_2 evolution potential and also the role of soluble mediators in the electron exchange process, another CV was recorded in a fresh catholyte (pH 7.6) with the same biocathode (Figure 3; trace c). As evident from this CV, the potential of H_2 evolution remained at around −0.8 V thereby suggesting that the shift was not associated with the pH decrease nor with soluble mediators. It indicates that the pH did not affect the thermodynamic performance considerably for the minor difference between CV traces b and c. These results are well in agreement with the recent report by LaBelle et al.[28] that highlights the possible role of an enriched electrosynthetic community in lowering the H_2 evolution overpotential by 0.250 V on biocathodes.

The CV data also reveals that the current draw at −1.2 V in the case of a biocathode (−2 to −2.5 A m^{-2}) is 8−10 times higher than the abiotic cathode (−0.25 A m^{-2}). The increased current production in the biotic scans strongly supports the involvement of microbial catalysis in current draw from the electrode. This can be due to an enhanced removal of H_2 from the cathode surface at a faster rate by the microorganisms. In addition, Figure 3B reveals that for the electrosynthetic biocathode in fresh medium, one redox reaction is found between −0.05 and −0.35 V, one irreversible oxidation reaction at −0.4 V and one oxidation reaction at −0.7 V. It seems unlikely that these redox-active moieties played any important role in the electron uptake process as the actual potential recorded during the MES experiments was −1.35 ± 0.1 V (SI Table S2). However, substantiating this hypothesis would require further investigation. The sustained appearance of a redox active moiety upon replacing the reactor medium by fresh medium (Figure 3B; trace c) indicates that no soluble mediator was involved in this particular electron exchange process.[29] Similar electrochemical behavior was also observed in the biocathode of reactor R4 as evident from the CVs recorded at comparable conditions and time points (SI Figure S5).

Microscopic Analyses of the Biocathodes. Biofilm growth was clearly present on the cathode surface of R3 (Figure 4A). Individual cells can be distinguished in the cathodic biofilm (Figure 4B). The 3D image of the biocathode shows ∼75% coverage of the electrode by microbial biomass (Figure 4C). Biofilm growth on the cathode and lowered overpotential of cathode indicate microorganisms played an important role in the consumption of reducing equivalents from the cathode.

Composition of the Enriched Acetogenic Culture and Electrosynthetic Microbial Communities. The enriched acetogenic culture grown on H_2:CO_2 was dominated by populations belonging to the order *Clostridiales* at a total relative abundance of up to 77% (Figure 5). Members of the order *Clostridiales* are metabolically versatile and known (homo)acetogens.[30,31] Methanogens were present at a low relative abundance (<0.1%) in enriched cultures, consistent with the lack of methane production in serum cultures. These results demonstrate the efficacy of performing multiple culture transfers in quick succession for promoting acetogenic activity over methanogenic activity.[32] The transfer of a similar enriched culture to medium with fructose resulted in 2.5% and 14% relative abundance of *Methanosaeta* and *Methanobacterium*, respectively, indicating that not all methanogens were completely removed from the enrichment; rather, they were successfully repressed. The presence of *Methanobacterium*, known for its hydrogenotrophic metabolism,[32] indicates that there might still be competition for the substrates of acetogenic metabolism in the serum bottles.

The microbial communities in the effluents of the first and third batch cycles of reactors (R1 and R2; see Materials and Methods for sampling days) were dominated by populations belonging to the bacterial order *Rhodocyclales* (up to 40% relative abundance). Up to 20% of the total population belonged to *Anaerovorax* and *Sphaerochateaceae* in the samples of the first batch cycle. The most abundant population after cycle 3 in both R1 and R2 belonged to the genus *Azovibrio* which is capable of N_2-fixation.[33] Remarkably, the genus *Arcobacter* was present in the effluent of R2 up to 12% relative abundance. This genus has been implicated to harbor species

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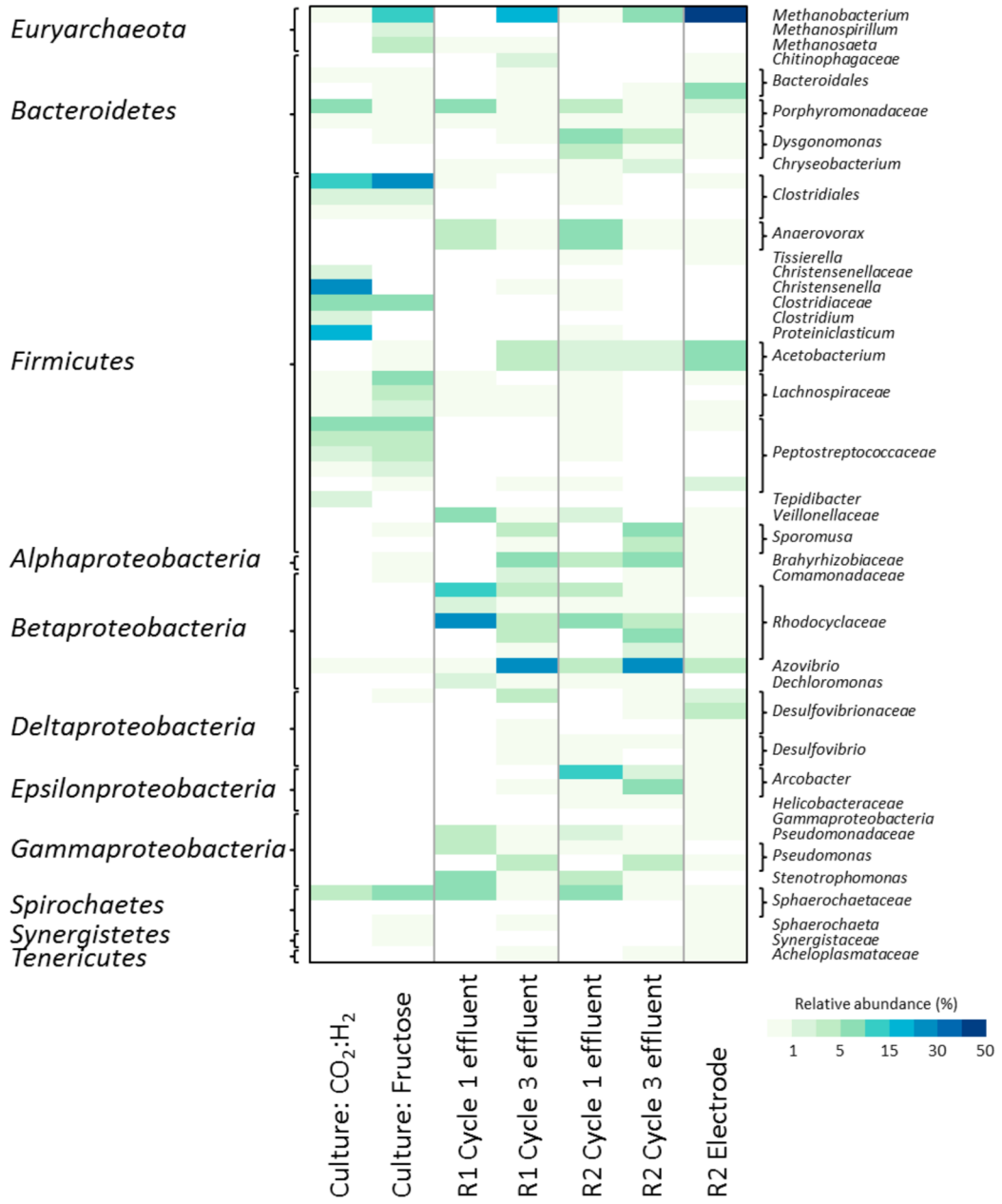

Figure 5. Microbial community composition of the enrichment cultures grown on CO_2:H_2 and fructose, R1 (effluents cycle 1 and 3), R2 (effluents cycle 1 and 3) and R2 (cathode electrode). The heatmap shows the relative abundance of operational taxonomic units (OTUs) at >1% relative abundance in at least one of the samples. Taxonomy at the phylum level is indicated in the left column whereas the right column shows taxonomy at the lowest classification level.

capable of electron transfer to an electrode.[47] It is likely that *Arcobacter*, in this case, does not perform a unique, essential function as it was only present in the effluent of one reactor and not in the biofilm. Known homoacetogens[34] such as *Sporomusa* and *Acetobacterium* were present at relatively low abundance (<10%), suggesting unknown or biofilm based acetogens may have been involved in the MES process. Marshall et al.[13,17] and LaBelle et al.[28] have also observed a low relative abundance of *Acetobacterium* in the supernatant compared to a relative enrichment of *Acetobacterium* in the biofilm on the electrodes. A decrease in species richness from the supernatant toward the biofilm was also observed in this work (i.e., 31% less OTUs were recovered from the biofilm compared to the effluent of reactor R2; Figure 5).

Methanogen abundance in the effluents of R1 and R2 was low at the end of the first batch cycle (<0.02%), and increased to 17% after cycle 3. The relative abundance of *Methanobacterium* was higher in the cathode biofilm and reached up to 47% toward the end of the experiment. However, the total biomass of methanogens was relatively low compared to the total bacterial biomass (Max. <13% cell dry weight; SI calculation S1).

As the cathode samples were analyzed at the end of the experiments (>3.5 months; SI Figure S6), the prolonged duration of the experiments may have resulted in the revival of methanogens and other microbial groups that were present in low abundance in the initial enriched culture. Interestingly, *Methanobacterium* has been enriched on anaerobic mixed culture biocathodes before,[35] also in conjunction with

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Acetobacterium.[13,28] In this work an increased relative abundance of *Acetobacterium* from 2.7% in the suspension to 16.7% in the biofilm was observed. Although *Methanobacterium* is able to produce activated acetate (Acetyl CoA) from CO_2,[36] large amounts of extracellular acetate production via this pathway is rather unlikely because this seems merely an assimilatory process rather than an acetate excreting process such as the Wood-Ljungdahl pathway. A more plausible explanation for the high abundance of *Methanobacterium* on cathodes (here without quantifiable CH_4 production) is the enhanced production of H_2 as proposed for a new *Methanobacterium* isolate IM1.[37] In the resulting model from this theory, current is converted into molecular H_2 by means of the hydrogenases on the cell wall of *Methanobacterium* or by other enzymes released in the biofilm by the methanogenic biomass[44,48] which is subsequently scavenged by acetogens before it can be transformed into CH_4. The IM1 isolate has recently been shown to produce H_2 instead of methane when confronted with an excess supply of reducing equivalents on a cathode.[38] The hypothesis put forward here is plausible since (i) there is a constant, relatively high, supply of reducing equivalents due to galvanostatic conditions, (ii) the substrate affinities (K_s) for H_2 point to a possible competitive advantage of acetogens over hydrogenotrophic methanogens, for example, K_s of *Acetobacterium bakii* (4 μM)[39] is similar or an order of magnitude lower compared to hydrogenotrophic methanogens (4−120 μM),[40−43] and (iii) also nonviable cell(component)s of methanogenic microorganisms can be used to facilitate H_2 and even formate production on cathodes.[38,44,48] Since the reactors were operated with live biomass in this study, the individual influence of cellular components and intact cells on the cathodic biocatalysis remains to be investigated.[48] To the best of our knowledge, substrate affinities or half saturation constants are not widely available to make a definite statement on this issue. The action of the hydrogenases and other enzymes on the electrode or the cell wall of the methanogen can, in this case, be considered as merely (bio)catalytic instead of being linked to the metabolism of the methanogen.[44,48] This hypothesis can explain the low production of methane (below detection limit) in the effluent due to competitive scavenging of any produced H_2. The fact that *Methanobacterium* was enriched on the cathode indicates that it is still able to use part of the provided energy to sustain some growth. The proposed (bio)catalysis pathway opens a new avenue for linking nonelectroactive acetogens onto a cathode, with possible implications for research on microbial induced corrosion processes, but needs further thorough experimental verification.

High Gas Flow Rates Favors Electron Recovery in H_2 over Acetate. During the aforementioned four reactor runs, different N_2:CO_2 flow regimes were applied. These gas flow rates influenced the electron recovery in the main products, that is, acetate and H_2 (SI Figure S7). At the low gas flow rate of 5 L d^{-1}, more electrons (64 ± 13%) were recovered in acetate and comparatively less electrons were recovered in H_2 (19 ± 3%). At increased gas flow rates (6.0 and 6.5 L d^{-1}), the percentage recovery of electrons in H_2 increased up to 37%. It increased further to more than 40% at 7 L d^{-1} flow rate. Flushing H_2 at faster rates from reactors clearly affected the electron recovery in acetate as it restricted the retention of H_2 in the cathode. Very low gas flow rate (1.25 ± 0.25 L d^{-1} for R5 and R6, see below) affected electron recovery in H_2 (<10%) but no effect was observed on the electron recovery in acetate (44 ± 5%). These observations highlight the importance of fine-tuning the gas flow rates depending upon the product(s) of interest. However, gas flow rates had no substantial effect on the concentration of acetate all these reactors (R1 to R6; 1.37 ± 0.19 g L^{-1}).

pH, CO_2, Current and Growth Factors Did Not Limit Acetate Concentrations. With the enriched acetogenic culture, the acetate production in all these reactors was stabilized at maximum 1.5 g L^{-1}. A decrease in the pH (<6.0) of the catholyte (Figure 1 and 2) was initially suspected to be the reason for the limited acetate production. Therefore, two additional BESs were started (R5 and R6) to understand the influence of pH on the maximum acetate concentration. In reactors R5 and R6, the catholyte pH was kept in the range of 7.6 to 8.4, which resulted in acetate concentrations (1.4 to 1.7 g L^{-1}; SI Figure S8) in a similar range as observed in R1−R4. These observations suggest that the observed low pH was not the major factor limiting the acetate concentrations in these reactors. However, lower pH values (~5.0) than observed in this work have been recently reported to limit acetate production.[28] Furthermore, the bicarbonate concentration in these reactors remained in the range of 2.5 to 6.5 g L^{-1} (SI Figure S8), thereby ruling out possible carbon limitations. Preliminary observations with additional supply of current and growth factors (vitamins and trace metals) also indicated their nonlimiting role in acetate production (data not shown). Continuing the operation of batch cycle for longer period did not improve acetate concentrations (SI Figure S6). Instead, it lead to the transition of production from acetate to ethanol during the third batch cycle of R2. Further analyses of the products and mechanisms in this case were beyond the scope of this study.

Therefore, acetate concentrations achieved at the end of each batch cycle in this study are most likely due to the reactor design, the electrode to volume ratio, and retention or availability of H_2 that acts as an energy source for acetogenic microorganisms. On the basis of our observations, strategies such as (i) increasing the cathode surface area relative to the production medium, (ii) exploiting efficient H_2 producing cathode materials, (iii) testing different reactor designs, and (iv) more inocula from different sources need to be investigated to establish the key factor limiting the concentration of the desired product.

Acetate Production Rates. On average, 19 ± 2 g m^{-2} d^{-1} (maximum up to 28 g m^{-2} d^{-1}) acetate was produced with carbon felt cathodes in reactors R1−R4 (SI Figure S9). As presented in Table 1, this is the highest, reproducible acetate production rate achieved so far with unmodified carbon-based electrodes in MES process, irrespective of the microbial inoculum source. This can be attributed to the porous and fibrous nature of the unmodified carbon felt cathode that allows efficient mass transfer, possess high real surface area, provides habitat for the growth of bacteria besides its conductive and biocompatible properties. Rates are compared based on projected cathode surface area as this will likely be the main parameter for scale up; that is, a parallel plate reactor with as high as possible surface to volume ratio. Although Jiang et al.[12] achieved similar surface based rates (19 g m^{-2} d^{-1}), they did not show the robustness of their enrichment, moreover the start-up time to achieve these rates was in the order of 40 days. In addition, similar to the work of Marshall and co-workers[13,17] methane was still present in the headspace or had to be suppressed by using 2-bromoethansulfonate. A 10-fold higher acetate production rate (195 ± 30 g m^{-2} d^{-1}) was achieved

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with multiwalled-carbon-nanotubes modified reticulated vitreous carbon (nanoweb-RVC) cathodes.[14] In this case, the high rate was attributed to the nanostructure and the high surface area to volume ratio of the modified RVC cathodes compared to their unmodified counterparts. Such nanostructured electrodes[14,45] and the use of an enriched culture as presented here, could result in improved start-up times consequently leading to high production rates and stable, reliable process operation over longer periods. In case of the use of electrodes with nondefined surface area, the performance of MES processes can be compared considering the volumetric production rates as presented in Table 1. Volumetric production rates of acetate via H_2:CO_2 conversions by a genetically modified *A. woodii*[49] have reached to 28 g L^{-1} d^{-1} whereas via chemical synthesis starting from methanol can reach values, up to 7200−67 680 g L^{-1} d^{-1} (Lab scale representation of commercial process; 190 °C; 22−28 barg).[50] These values indicate the possibilities for development of MES and the need for positioning of MES in an appropriate economical niche.

On the Use of Enrichment Approach and Mixed Culture Inoculum Sources for MES Processes. Even though mixed cultures offer some operational advantages, similar to mixed culture fermentations their use in MES processes is associated with the production of a mixture of products. Such issues can be addressed by using an initial enrichment strategy (as presented here), selective removal of the target product(s)[46] and/or long-term culture adaptation toward the production of specific products.[17] Our strategy of pre-enrichment of the acetogenic microbial community under selective pressure (H_2:CO_2 conditions) prior to reactor operation shows promising results as far as start-up phase and the production of acetate from CO_2 and electrical current is concerned. The presence of methanogens in the catholyte solution and in the biofilm on the cathode was evident after prolonged reactor operation although this did not notably affect product outcomes. Though the methanogenic activity was irrelevant for more than two months of reactor operation, these results also suggest that the enrichment strategy presented here to eliminate methanogens on the long-term is not successful. These findings point to a need for better strategies to prevent methanogenesis or methanogens on the longer term. Nevertheless, based on acetate production rates and profiles, a batch production process where production is stopped upon a maximum concentration of product, the reactor is cleaned and a new batch is started, as is done in industrial fermentation processes, can be envisioned. Using this approach with an active start-up inoculum and limited methanogenic activity will ensure a dedicated process without interference of side reactions.

Bioproduction in a cathode can be facilitated by operating reactors either in a potentiostatic or a galvanostaic mode. In a potentiostatic mode, the potential at the working electrode is set at more positive value (usually >−600 mV vs Ag/AgCl) depending upon the electrode materials to avoid abiotic H_2 production and to facilitate direct electron uptake process. Lower potentials can also be set using the potentiostatic mode. The energy input in using more positive potentials is usually lower. In a galvanostatic mode of reactor operation, as presented in this study, the current is fixed to enable a constant flow of electrons to the microorganisms. However, this can lead to more negative cathode potentials leading to a higher energy input. The ultimate measure to determine the optimal strategy is the amount of energy input per gram of product produced.

In summary, in addition to providing a promising pre-enrichment strategy for a robust microbial community for autotrophic acetate production, these results expand the existing knowledge on electricity-driven bioproduction processes. The enriched acetogenic culture exhibited consistency in the production of acetate via MES in several independent experiments. The combined use of (i) a galvanostatic approach for continuous supply of reducing equivalents and (ii) an enriched culture enabled rapid start-up of electrosynthetic biocathodes and high acetate production rates (up to 28 g m^{-2} d^{-1}). The galvanostatic approach also leads to the production of H_2, the excess of which can be used for other catalytic or production processes. These results also suggest that a useful bioproduction system could be developed irrespective of the presence or the absence of direct electron uptake mechanism. Overall, these two approaches pave the way for a fast conversion of excess electrical energy into storable energy carriers.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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