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Bioelectrochemical Chain Elongation of Short-Chain Fatty Acids Creates Steering Opportunities for Selective Formation of *n*-Butyrate, *n*-Valerate or *n*-Caproate

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Valorization of organic residual streams that produce short-chain fatty acids (SCFA) require an energetic electron donor to form more valuable elongated products. By microbial electrosynthesis such electrons donor is supplied by an electrode. Here we show that bioelectrochemical chain elongation (BCE) of SCFA was steered to high selective product formation efficiencies depending on the supplied fatty acid. *n*-Butyrate, *n*-valerate, *n*-caproate were in different experimental conditions formed at respectively 94.1, 95.4 and 83.4% carbon-based selectivity. The reactor microbiomes adapted to the new feeding conditions within a few days. Remarkably, propionate elongation appeared to be preferred over acetate elongation. Propionate elongation resulted in highly selective formation of the odd-chain fatty acid *n*-valerate; this seems contradictory to ethanol chain elongation studies in which acetate is concurrently formed leading to straight fatty acids as by products.

Introduction

The European Union plans a transition to a more circular economy wherein the value of products, materials and resources is maintained in the economy, and the generation of waste is minimized.[1] Evidently, usage of organic residual streams as feedstock to produce recyclable products contributes to this venture. Concentrated, complex and variable organic residual streams are in practice typically handled by undefined mixed cultures which produce biogas via anaerobic digestion (AD).[2] Biogas is often combusted to produce electricity although the methane and present carbon dioxide can be used as resource too. Instead of producing biogas one can also use digestible feedstocks for other bioprocesses and broaden the product spectrum according to the so called carboxylate platform.[3,4] As such, various anaerobic bioprocesses, which are sometimes combined with (bio)(electro) chemical processes, are under development to produce chemicals like carboxylic acids, alcohols, alkanes or products like biodegradable plastics.[5–7] For example, mixed microbial cultures first hydrolysed and acidified the organic fraction of municipal solid waste into a mixture of short-chain fatty acids (SCFAs) like acetate and butyrate. These SCFAs are valuable chemicals when separated, however, they were used with supply of ethanol in a biological chain elongation process to produce more valuable caproate at 72% product selectivity.[8]

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Caproate is a medium chain fatty acid (MCFA) and is useful in feed additive as well as building block in other emerging applications.[6] Various anaerobic microbial pathways are known which elongate carbon chains to longer multi-carbon chemicals.[9] Chain elongation does for example occur via the reversed *beta*-oxidation pathway which requires the availability of an energetic electron donor like ethanol. A life-cycle-assessment (LCA) on a developed pilot-scale chain elongation caproate producing factory revealed that corn-based exogenous ethanol supply was contributing at least 20% to each of the live-cycle impact categories (including global warming potential, acidification potential & eutrophication potential).[10] The latter was mainly due to the production and transportation of the corn grain. This insight supports the proposition that alternative electron donors, which are not the result of a primary agricultural process (e.g. electrons and/or hydrogen which is supplied from a cathode electrode), may become more environmental friendly than usage of corn ethanol.[11] Obviously, these alternative electron donors will also have an impact as electrodes and other materials are used. Further LCA should be done to evaluate which electron donor(s) are attractive for the environment.

The latter process which uses cathode supplied electrons is called bioelectrochemical chain elongation (BCE).[11] This process elongates for example the SCFA acetate into the longer multi-carbon butyrate. BCE can also start with elongating of CO_2 to acetate and 2-oxobutyrate or even to *n*-caproate.[12,13] BCE is considered to be a standalone process but it also can become complementary to biogas production once it uses the digestate or the CO_2 of the process.[14]

Bioelectrochemical chain elongation starting from supplied SCFA is only shown with open mixed cultures. Besides formation of elongated fatty acids also alcohols and a broad spectrum of other valuable products were reported in either major or minor amounts (note that we report the form of

isomer once available). Steinbusch *et al.* showed acetate conversion into ethanol, propionate and *n*-butyrate with supplemented methyl viologen as redox shuttle.[15] The same group reported acetate and K_2CO_3 use for temporary production of ethanol, butyrate, caproate and caprylate.[16] That work was followed up with reactors continuously chain elongating acetate to *n*-butyrate including formation of traces of propionate, lactate, isobutyrate, b & *n*-valerate and *n*-caproate.[11] Sharma *et al.* showed the formation of a mix of ethanol, butanol, methanol, propionic acid, caproate, acetone and propanol from the supplied acetate and butyrate.[17] In a follow-up study the same substrate was supplied leading to formation of succinate, ethanol, hydrogen, glycerol and propionate while within a sealed reactor acetone, propionate, isopropanol, propanol, isobutyrate, isovalerate, valerate and heptanoate were produced once the headspace was flushed with dinitrogen.[18] Real anaerobic digestion effluent which included acetate and butyrate revealed formation of a range of products including methanol, ethanol, propanol and butanol, but also formate, lactate, propionate and glycerol.[19] Other studies which supplied acetate and butyrate reported sole formation of alcohols including methanol, ethanol and propanol while no chain elongation product like caproate were shown.[20,21] Jiang *et al.* supplied besides the electrons from the electrode also ethanol and CO_2 which resulted into enhanced caproate formation up to a product selectivity of 91% compared to 32% without ethanol supply.[22] More-over acetate and ethanol were fermented with relative small amounts of current via electro-fermentation to alcohols (propanol, butanol, hexanol) and fatty acids propionate, butyrate, valerate and caproate. Due to the supply of current caproate selectivity improved from 28 to 36%.[23]

Its intriguing to note that BCE from SCFA is resulting into a broad spectrum of industrial relevant bio-based chemicals. By comparing some of the previous mentioned BCE from SCFA studies it's evident that the supply of acetate and butyrate does not always lead to the formation of the same products.[17,18,20] With the various substrates and products formed several metabolic networks, biochemical conversions and electron transfer means have been proposed.[16,17]

From a bioengineering point of view it is key to understand and control the actual electron and carbon flow to be able to steer to the desired product(s) as well as to optimise the processes on performance. Open culture processes like BCE and other fermentative chain elongation processes can be controlled by putting certain selection pressure on the bioprocess to stimulate the desired process and reduce the competing process.[24] Coma *et al.* used fermentative chain elongation and studied product diversity in batch reactors that resulted from several combinations of electron donors (methanol, ethanol, propanol, butanol) and electron acceptors (acetate, propionate, butyrate, etc.).[25] It was concluded that multiple substrates can be used for chain elongation and that the chain elongation process is carried out by highly similar organisms. Evidently, the supplied substrate provided the selection pressure to produce the specific products. This results supports the hypothesis that BCE from SCFA may as well be able to switch between electron acceptor i.e. SCFA use.

In our previous study we show a BCE process of acetate into *n*-butyrate.[26] The objective of present study was to further investigate the role and selection pressure of different supplied SCFA combinations on the product formation. Hereby the substrate spectrum was extended by using propionate as new substrate. In this study continuous triplicate BCE reactors were sequentially supplied with I) acetate, II) acetate and propionate, III) acetate and *n*-butyrate, and IV) a mixture of acetate, propionate and *n*-butyrate. The effects on SCFA utilisation and continuous product formation resulted into surprisingly high carbon-based formation selectivity on either *n*-butyrate, *n*-valerate or *n*-caproate. By studying the consumed and produced fatty acids the reaction stoichiometry of the various processes could be proposed. From these processes the thermodynamics showed that the hypothetical direct use of electrons could be energetically more favourable for the micro-organisms than usage of in-situ produced hydrogen. By placing present BCE study in perspective to fermentative chain elongation studies it was revealed that odd chain fatty acid formation is more selective, though concentrations are still rather low.

Results and Discussion

Figure 1 shows the concentration (in mMC; millimole carbon of a specific fatty acid per Liter) and production rate profiles over time of one of the triplicate reactors. The data for the other two reactors can be found in Figure S1. Unless stated otherwise, the replicate reactors exhibited similar behavior.

Supply of acetate and propionate promotes formation of *n*–valerate over *n*–butyrate

In phase I when only acetate was supplied as substrate for BCE, *n*-butyrate (nC4) was the only identified chain elongation product (Figure 1B and C). The nC4 concentration was 39

mMC (0.9 g L^{-1}), and was produced at 39.7 mMC d^{-1} with a carbon selectivity of 94% (Table 1; selectivity data for other two reactors can be found in Supplementary info). This nC4

Table 1. Formation selectivity of bioelectrochemical chain elongation to products during the four experimental phases. Values presented are averages and standard deviations of the last three values per phase, except for phase IV where the last two data points were averaged (indicated with *).

	Selectivity carbon [%]			Selectivity electron [%]		
Phase	nC4	nC5	nC6	nC4	nC5	nC6
I	94.1 ± 0.3	0.7 ± 0.1	4.6 ± 0.9	93.8 ± 1.7	0.8 ± 0.1	4.9 ± 1.0
II	23.7 ± 0.9	73.8 ± 0.8	2.5 ± 0.5	23.0 ± 0.9	74.4 ± 0.8	2.6 ± 0.6
III	14.5 ± 2.7	5.8 ± 0.5	83.4 ± 7.4	13.8 ± 2.6	5.7 ± 0.5	84.1 ± 7.2
IV*	-	95.4 ± 1.9	4.6 ± 0.1	-	95.3 ± 1.9	4.7 ± 0.1

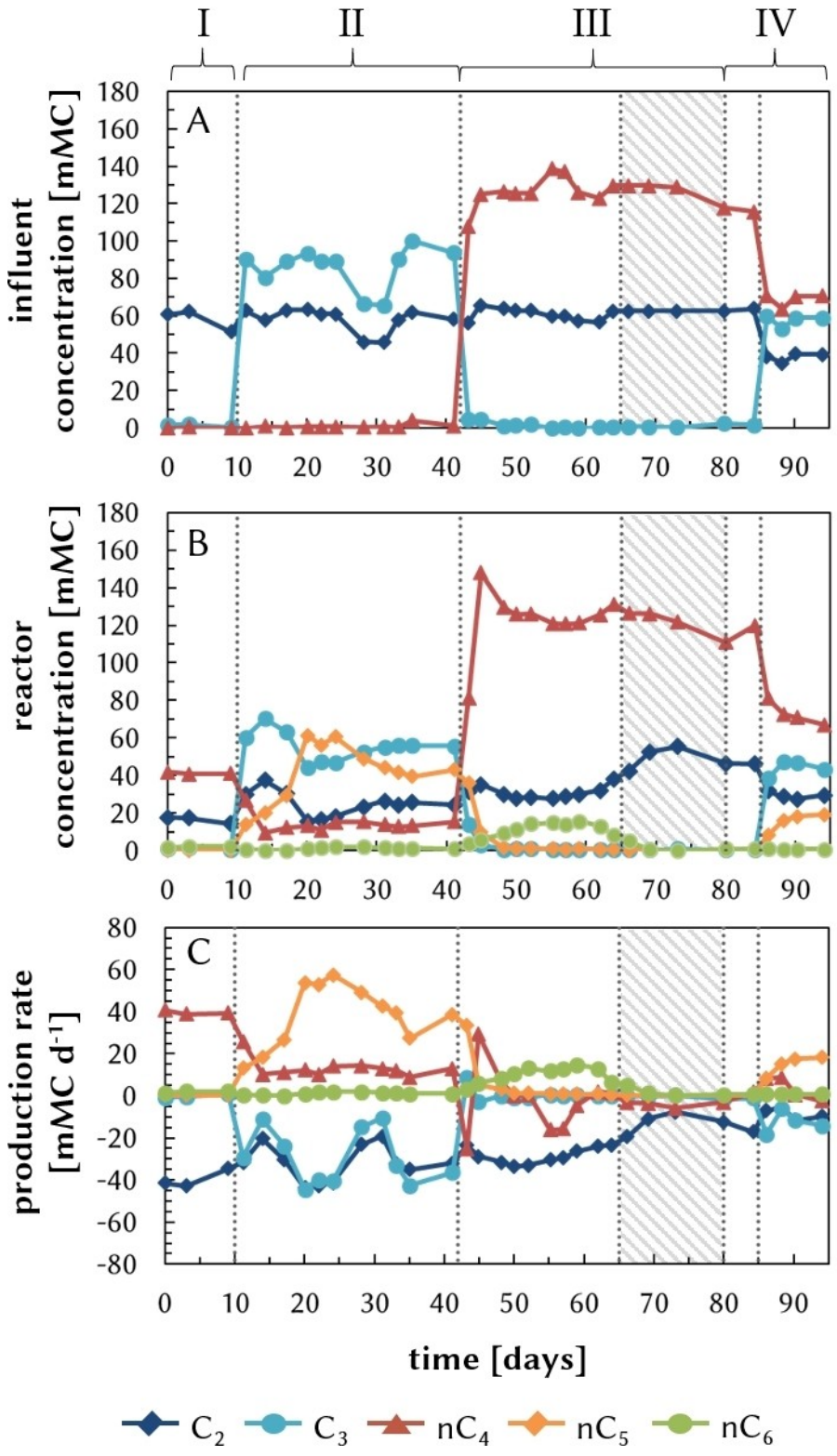

Figure 1. Concentration of organic acids in the influent (A) and in the reactor (B) over time and the corresponding production rate (C). The reactor was operated at 18.1 A m^{-2}. Roman numbers indicate different feeding strategies as indicated in the text: I) 30 mM acetate, II) 30 mM acetate + 30 mM propionate, III) 30 mM acetate + 30 mM *n*-butyrate, IV) 20 mM acetate + 20 mM propionate + 20 mM *n*-butyrate. Shaded area in phase III indicates the amperostatic control was impaired.

concentration was slightly improved (0.59 g L^{-1}) compared to the earlier operation of the same reactor.[11] From here selectivity is carbon based unless stated otherwise. Within 2 days after adding propionate (start phase II – day 10), it was consumed and valerate (nC5) production was initiated. The nC5 concentration reached a maximum concentration of 60.6 mMC (1.2 g L^{-1}) at day 24, at a production rate of 57.5 mMC d^{-1} with a remarkable high 73.8% formation selectivity (Table 2). Whilst, propionate was consumed at 40.5 mMC d^{-1}. Apparently, the biomass was capable to change its metabolism from acetate elongation to propionate elongation in short time. These observations are in line with the experiments of Grootscholten et al., who also showed nC5 production directly (i.e. without lag phase) after propionate addition.[27]

Besides nC5, also nC4 was produced but at lower rates compared to phase I (14.1 mMC d^{-1} vs. 39.7 mMC d^{-1}). Although the exact mechanism of acetate conversion in BCE systems is not yet unravelled, it can be hypothesised based on the consumed substrates and formed compounds, that acetate was not only consumed for propionate elongation, but was partly elongated to nC4 as well (Supplementary info Table S4 reaction 2 and 1 respectively). This co-production of nC4 was observed before in open chain elongation reactors when fed with propionate and ethanol.[25,27,28] Additionally, propanol formation was also observed in these previous studies, presumably as a result of propionate reduction.[28,30] Here, we did not detect any alcohol formation, which was in agreement with our previous BCE study.[11] In phase II also traces of *n*-caproate were measured, of which the maximal concentration was 2.1 mMC (0.040 g L^{-1}) corresponding to a 2.5% selectivity.

Interestingly, in this phase the consumption patterns of acetate and propionate were highly similar (Figure 1C). Similar consumption in mMC translates to 1.5 times more consumption of propionate than acetate based on molar concentrations. The molar stoichiometric reactions (Table S5, reaction 2) correspond to a carbon-molar stoichiometry for nC5 production of 0.4 molC acetate to 0.6 molC propionate resulting in 1 molC of nC5. Stoichiometric analysis of the consumption and production rates in this phase is shown in Table 2. The conversion of both acetate to nC4 and the production of nC5 from acetate and propionate accounts on day 24 for 90.4% and 85.3% for the acetate and propionate consumption respectively. This stoichiometric analysis fits well with the formation of nC5 and nC4 and the observed consumption of acetate and propionate. The addition of propionate to the medium did not significantly affect the cathode potential (Figure S3 & Table S3). Even though in this phase more electrons were supplied via the influent by addition of 60 mMC propionate, the electron recovery increased in phase II from 38% to 65% (Figure 2). This electron recovery included as well the electrons supplied by the substrates in the influent.

Table 2. Production rates at day 24, when nC5 production was the highest [all in mMC d^{-1}]. Last three columns represent the percentage of consumed C2 or C3 which was converted to the products nC4 or nC5. Reactor was applied with 18.1 A m-2 and at that moment fed with 30 mM acetate and 30 mM propionate in the influent.

		Production rate [mMC d^{-1}]				Substrate conversion [%]		
Day	C2	C3	nC4	nC5	nC6	C2 to nC4	C2 to nC5	C3 to nC5
24	-41.1	-40.5	14.1	57.5	2.0	34.4	56.0	85.3

Supply of *n*–butyrate and acetate steers to more selective *n*–caproate formation

In phase III nC4 (~120 mMC) was co-supplied with acetate to the reactors starting on day 43 (Figure 1A). Upon the change of medium, propionate and nC5 were washed out of the reactor. As a result of less acetate consumption, the acetate concentration in the reactor increased in the first 5 days of this third phase. The nC4 concentration was for the first days higher than the influent concentration, signifying that conversion of acetate to nC4 occurred at a higher rate than nC4 was consumed. After ~10 days a stable *n*-caproate (nC6) production was observed. The maximum nC6 concentration was 15.8 mMC (0.3 g L^{-1}), at a production rate of 13.3 mMC d^{-1} and formation selectivity of 83.4%. During nC6 production, the nC4 consumption rate was fluctuating around 0 mMC d^{-1}. Because nC4 was supplied via the influent, this value means that possibly nC4 was consumed and produced at a similar rate, resulting in a net consumption rate of 0 mMC d^{-1}. Compared to phase I when solely acetate was supplied, the co-supply of nC4 steered to more selective

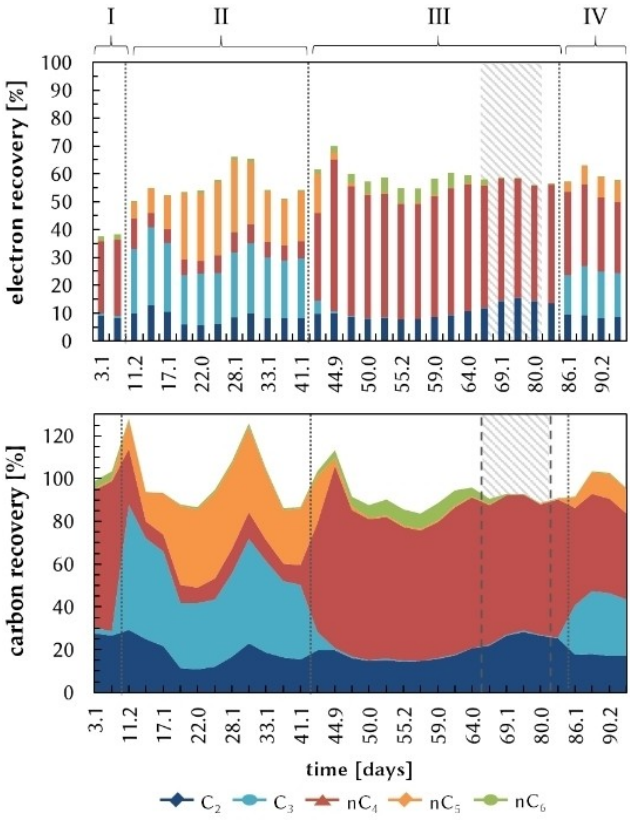

Figure 2. Electron recovery and carbon recovery over time.

formation of nC6 (from 4.6% to 83.4%). Comparing the nC4 production in phase I to the nC6 production in phase III, 39.7 mMC d^{-1} compared to 13.3 mMC d^{-1}, the elongation rate decreased with increasing chain length.

Although the precise chain elongation carbon fluxes remain hypothetical, possibly simultaneously nC4 was produced from acetate elongation as well as nC4 was elongated towards nC6 (Table S5 reaction 1 and 4 respectively). Alternatively, it could be hypothesised that acetate was directly elongated to *n*-caproate (Table S5 reaction 3). In both the possible reactions (reactions 3 and 4), the origin of 1 molC of nC6 can be 1 molC of acetate or 0.33 molC acetate and 0.67 molC nC4. The elongation of acetate to nC4 has also a 1 molC acetate to 1 molC of nC4, so based on stoichiometric analysis the exact elongation route could not be determined in this study.

n-Caproate production was sustained until a period where amperostatic control was twice temporary impaired (shaded area in Figure 1 and Figure 2). As a consequence of two short periods of no applied current (day 65.5 till 66; day 70 till day 72), protons from the acid anolyte (pH 1.5 to 2.0) could diffuse through the membrane to the catholyte. These technical issues led to a ceasing acetate consumption and as a consequence at day 70 the acetate concentration in the reactor was similar to the influent concentration. Together with the ceased acetate consumption, the associated nC4 and nC6 production were ceased as well. At day 72 the amperostatic control was re-established, and after a week (from day 80) the elongation process had started again. At day 84 the substrate in the influent was changed to a mixture of acetate, propionate and nC4 to investigate the effect of a mixture on the product spectrum.

Electron equivalent supply by electrode steers towards selective odd-chain fatty acid production

In phase IV the three electron acceptors were supplied in a molar ratio of 1 to 1 to 1. Upon re-addition of propionate in the reactors after 41 days feeding on nC4 and acetate, directly propionate was consumed and nC5 production commenced (Figure 1, phase IV). The nC5 concentration at the end of the experiment reached 19.3 mMC (0.4 g L^{-1}). The acetate concentration in the reactor was decreased again to concentrations lower than the influent, signifying its consumption. *n*-Caproate production was not re-established before the end of the experimental period.

This last phase in which a mixture of three SCFAs was fed to BCE reactors resulted in a highly selective nC5 production. *n*-Valerate was produced at a rate of 18.3 mMC d^{-1} at a selectivity of 95.4%. Propionate was consumed at 14 mMC d^{-1}. Both the propionate consumption and the nC5 production in phase IV were approximately three times lower than in phase II. Stoichiometric analysis showed that 74.7% and 77.8% of respectively the acetate and propionate consumption could be explained by nC5 production. In open culture fermentative chain elongation reactors where propionate and ethanol were fed, the selectivity towards *n*-valerate and *n*-heptanoate production together was maximally 56%.[28]

When using ethanol as electron donor, the selectivity towards odd-chain fatty acids (nC5 and nC7) has an observed limitation since intrinsic ethanol oxidation to acetate and subsequent butyrate and caproate production does occur (26.9 % selectivity towards nC4 and nC6).[28] Similar loss of selectivity towards odd-chain fatty acids was observed by Grootscholten et al., where nC5 selectivity was maximally 39% (electron based).[27] In the reverse *beta*-oxidation pathway, 6 moles of ethanol can be oxidised to 5 moles of acetyl-CoA and 1 mole of acetate.[6] Therefore, when applying ethanol as electron donor, even-chain fatty acid production cannot be prevented and decreases the selectivity towards odd-chain products. Ethanol oxidation in the reverse beta-oxidation generates reducing power in the form of NADH to elongate a carboxylate with two carbon atoms. The observations here in this study indicate that an electrode can supply the chain elongation process with reducing equivalents without the production of acetate. Therefore, the usage of an electrode for the chain elongation process might be beneficial when high selectivities towards odd-chain products is required.

Additionally Roghair et al. posed the hypothesis that acetate and propionate compete for the same enzyme system in chain elongating microorganisms, and that this enzyme system could have a higher affinity for acetate.[28] Presuming similar dominant microbial metabolic pathways in our microbiome, the results obtained in this study indicate a contradiction of Roghair et al. Here propionate elongation appeared to be preferred over acetate elongation in phase II and in phase IV. The addition of propionate to the medium from phase I to phase II, showed that the microbiome was capable of utilising both substrates, which is in accordance to literature.[25,27,30] Assuming the same microbes to elongate acetate as well as propionate, the enzyme affinity hypothesis does not explain the propionate preference observed here. A recent open microbiome chain elongation study showed that not the reverse beta-oxidation pathway played a role in the production of MCFAs, but another pathway: the fatty acid biosynthesis (FAB) pathway.[31] In that study ethanol and acetate were supplied in a fermentative batch CE reaction, producing a mixture of propionate, nC4, nC5 and nC6. Based on metagenomic and metatranscriptomic analysis the authors demonstrated that the FAB was more active in the chain elongation to *n*-caproate than the reverse beta-oxidation pathway. Even though the present study was not designed to elucidate the exact chain elongation pathways, together with the study of Han et al. it emphasises that the CE pathways involved are yet insufficiently characterised.[31] Asides, the concentrations (e.g. nC6 at max 0.3 g L^{-1}) on fatty acids production in present study, are rather low compared to fermentative chain elongation studies which did e.g. achieve caproate concentrations up to 25.7 g/L. Further bioengineering using more concentrated SCFA streams and a longer HRT may lead to higher concentrations as similar shown in BCE from CO_2 and fermentative chain elongation studies.[32,33] Hereby one could also investigate on how the electron recovery could be affected since still 40% of the electrons were not recovered in the formed fatty acids. A higher SCFA vs. current loading rate

may lead to more use of supplied electrons presuming the present electron supply is not limiting the BCE process. In addition, in case more concentrated SCFA streams are supplied also the product selectivity may be affected since the (more) exact kinetics of the various proposed BCE processes are unknown.

Thermodynamics favouring electrons as electron donor for chain elongation

Step by step progress is being made to elucidate how exactly electrodes are used as electron donor for microbial metabolism in biocathode processes, e.g. via (bio)electrochemically induced H_2 or other redox active molecules.[34,35] Earlier electrochemical analysis with impedance spectroscopy and cyclic voltammetry supported that membrane-bound redox enzymes of sulfate reducing micro-organisms may facilitate direct electron transfer in BCE processes from SCFA.[17] To examine whether H_2 or electrons could thermodynamically serve as electron donor for the chain elongation reactions, thermodynamic calculations were performed (Table S5). The Gibbs free energy of the presumed elongation reaction in every experimental phase was calculated with either H_2 or electrons as electron donor. For this the average reactor conditions and cathode potentials for each experimental phase were used (Table S3).

All the included chain elongation reactions (1a to 4b,Table S5) showed to be thermodynamically feasible, which seems to contradict the kinetic study of Gonzalez-Cabaleiro.[36] The study of Gonzalez-Cabaleiro and co-workers modelled the described metabolic pathway for acetate elongation to *n*-butyrate with H_2 as electron donor. Their metabolic model included a threshold energy yield needed to transport protons across the membrane and subsequently produce ATP. H_2 could not achieve enough energy to support the needed proton gradient. Therefore they concluded that H_2 itself could not serve as electron donor. However, in electrode driven systems, such as BCE, it is not yet understood how energy is conserved by the microorganisms, nor if this happens via the same mechanisms as in fermentative conditions with a soluble electron donor. The thermodynamic calculation here thus demonstrates only the feasibility of a certain bioprocess, of which the exact steps need to be elucidated yet.

Interestingly, electron driven reactions (all reactions b) yield a higher energy gain compared to H_2 driven ones (all reactions a). Which is in a sense logical, as in these reactions no ΔG_f^0 for protons and H_2 were incorporated. Therefore, there is a clear energetic benefit of using an electrode as (direct) electron donor for microbial metabolism instead of H_2.

Acetate elongation towards *n*-butyrate in phase I (reaction 1, Table S5) yield similar energy gains compared to propionate elongation in phase II (reaction 2, Table S5) regardless of the electron donor employed. The Gibbs free energies for these reactions (1 and 2) were similar as well in phase IV when the three SCFAs were simultaneously supplied. The observed preference for propionate elongation over acetate elongation during phase IV, can thus not be explained by thermodynamics, but is likely related to enzyme specificity and/or bioenergetics of the microbial pathway(s). Noteworthy hereby is that there was some variation, but no significant difference, on average cathode potentials during all tested phases (Figure S3 & Table S3). As it is well known that cathode potential does affect performance of biocathodes for e.g. oxygen reduction or hydrogen formation.[37,38] In present study the cathode potential was rather stable and therefore likely not responsible for the observed changes on formation selectivities. *n*-Caproate formation can occur via solely acetate elongation (reaction 3, Table S5) or directly via *n*-butyrate (reaction 4, Table S5). The energy gain for the hypothesised reaction of solely acetate towards caproate yields the most energy. Even though this theoretical higher energy gain, in phase IV mainly *n*-valerate was produced. This might indicate that direct caproate formation from acetate (reaction 3) did not occur. Alternatively, the energy yield of reaction 4 during phase IV is slightly higher than reaction 1 and reaction 2. In contrast to the observed *n*-valerate production preference in the last experimental phase, caproate formation via reaction 4 is thermodynamically more favourable.

Conclusions

Bioelectrochemical chain elongation of short-chain fatty acids creates steering opportunities for selective formation of *n*-butyrate, *n*-valerate or *n*-caproate. A rapid switch between substrate use is feasible. The observed preference for propionate elongation over both *n*-butyrate formation or caproate formation is in contrast to fermentative chain elongation studies. Thermodynamics support that direct electron transfer of various chain elongating processes is overall energetically feasible. Further research in which mixtures of SCFAs derived from real fermentation broths are used could validate whether these curious preferences occur as well and whether electrodes could be employed to steer towards the desired elongated chemicals.

Supporting Information Summary

In supporting information one can find the experimental section as well as figures and tables with experimental data of the duplicate/triplicate bioreactors, and outcome of thermodynamic calculations.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: bioelectrochemical chain elongation · electron acceptor · microbial electrosynthesis · electrochemistry · biocatalysis

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