Branched Medium Chain Fatty Acids: Iso-Caproate Formation from Iso-Butyrate Broadens the Product Spectrum for Microbial Chain Elongation

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ABSTRACT: Chain elongation fermentation can be used to convert organic residues into biobased chemicals. This research aimed to develop a bioprocess for branched medium chain fatty acids (MCFAs) production. A long-term continuous reactor experiment showed that iso-caproate (4-methyl pentanoate, i-C6) can be produced via ethanol based chain elongation. The enriched microbiome formed iso-caproate from iso-butryate at a rate of 44 ± 6 mmol C L⁻¹ day⁻¹ during the last phase. This amounted to 20% of all formed compounds based on carbon atoms. The main fermentation product was n-caproate (55% of all carbon), as a result of acetate and subsequent n-butyrate elongation. The microbiome preferred straight-chain elongation over branched-chain elongation. Lowering the acetate concentration in the influent led to an increase of excessive ethanol oxidation (EEO) into electron equivalents (e.g., H₂) and acetate. The formed acetate in turn stimulated straight chain elongation, but the resulting lower net acetate supply rate towards straight chain elongation led to an increased selectivity towards and productivity of i-C6. The electrons produced via oxidation routes and chain elongation were apparently utilized by hydrogenotrophic methanogens, homoacetogens, and carboxylate-to-alcohol reducing bacteria. Further improvements could be achieved if the acetate-producing EEO was minimized and limitations of ethanol and CO₂ were prevented.

INTRODUCTION

Sustainable alternative technologies need to be developed to transform the linear fossil-based economy into a circular economy.¹ Biomass residues, organic waste streams, and gaseous CO₂ streams offer microbial convertible feedstocks for procuring new biochemicals. The carboxylate platform is regarded as a potent tool that can supply alternatives to some of the still widely used fossil-derived products.²,³ One emerging bioprocess is the elongation of short chain fatty acids (SCFA, carbon length C₁−C₇) into medium chain fatty acids (MCFAs, carbon length C₆−C₁₂) via microbial chain elongation.⁴ It provides the means to catalyze the conversion of complex organic residues into a broad range of useful chemicals.⁵⁻⁷ MCFAs as potential new platform chemicals can be used as feedstocks for the fuel industry, chemicals for antimicrobial agents, additives in animal feed,⁸ flavor additives, lubricants, and plasticizers.⁹ The global market size for MCFAs shows a growing trend, and it was predicted to grow from 5.32 billion USD in 2014 to ~8 billion USD by the end of 2023.¹⁰ Of all MCFAs, C₆ has by far the lowest availability in traditional resources (C₆ constitutes around 2% of the total fatty acids in palm and kernel oil.¹¹) and thus its scarcity provides a window of opportunity for alternative production by microbial chain elongation. A recent research effort has led to the implementation of chain elongation technology on a large scale in The Netherlands. ChainCraft B.V. is building the first large-scale demonstration plant in Amsterdam that will produce MCFAs from organic waste streams via a multistep fermentation process at a few metric tons per year.¹²

During chain elongation microbes that employ reverse beta-oxidation use an electron donor, such as ethanol, to elongate SCFAs to MCFAs.⁹,¹³ The elongation is always done with two carbon atoms via an acetyl-CoA condensation step. Besides for the production of n-caproate (n-C₆), the microbes can also be used to produce n-valerate (n-C₅), n-heptanoate (n-C₇), and n-caprylate (n-C₈).¹⁴,¹⁵ To date, reports have mainly mentioned straight-chained (n-forms) MCFAs production via open culture chain elongation with the focus on n-C₆ as dominant product, whereas branched chained (iso-forms) MCFAs have been reported to be produced in low amounts only.¹⁶ Moreover, in the processes studied it was not clear if i-C₆ had been produced via either chain elongation or protein degradation. So far the only known iso-C₆ bioprocess has been created while using a pure culture of genetically modified Escherichia coli, which converted glucose to i-C₆ at a high titer of 13 g per liter.¹⁷

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Higher oxidative stability. For example, branched MCFAs can be obtained from organic residues via a mixed culture methanol chain elongation bioprocess that simultaneously stimulates isomerization. Such a precursor can be added in the substrate mix for Kolbe electrolysis to increase iso-butyrate titers. Alternatively, as recently shown in a ethanol chain elongation reactor that produced n-C6 caproate production of iso-butyrate, methanol, acetate, and vitamin B12 Cobalamin. During each of these six phases the medium composition was changed, as shown in Table 1. The reactor system was characterized by analysis of the steady state mass balances for the total amount of carbon and electrons in the liquid and gas phase.

**Reactor Setup and Operation.** The experiment was performed in a lab-scale continuous up-flow anaerobic reactor (schematic depiction shown in SI Figure S1) with a height of 35 cm and an internal column diameter of 6.5 cm (working liquid volume of 1 L with a headspace of 0.15 L). The medium inflow was set at 25 mL hour\(^{-1}\). This flow led to a hydraulic retention time (HRT) of approximately 40 ± 2 h, which, at the start of the experiment when no biofilms were observed, was low enough to provide a selective pressure against dominant growth of suspended methanogens. The liquid phase was partially mixed by internal recirculation (150 mL minute\(^{-1}\)) with a pump (Watson-Marlow 323DZ, UK). Biofilms were allowed to grow in the reactor, which then decoupled the HRT from the SRT. In the first five phases biomass growth was mainly suspended, while a thin biofilm grew on the wall of the reactor. In phase VI black granular biofilms developed and started to accumulate in the recirculation tubes and at the bottom of the reactor. The temperature was kept constant at 35 °C, using a water jacket and water bath (Fisher Scientific Polystat 37). The pH was controlled (pH 6.5 ± 0.1) to maintain a suitable pH for chain elongation by automatic titration using 1 M KOH as base. CO\(_2\) was continuously added (at a rate of 240 mL\(\text{min}^{-1}\)) with the aim of maintaining a sufficient (>1 kPa) CO\(_2\) partial pressure needed for growth of ethanol chain elongating bacteria.

**Inoculum.** The reactor was inoculated with a mixture of two undefined anaerobic cultures that originated (1) from an ethanol based chain elongation reactor and (2) from a protein-rich waste leads to formation of branched fatty acids, such as iso- butyrate, iso-valerate, and iso-caproate. Valine, leucine, and iso-leucine are substrates for branched carboxylic acids formation in this fashion, and represent a small but significant fraction of protein mass in organic residues. Moreover, (de)isomerization—of n-isoo-butyrate in particular—is stimulated in some acidifications that are associated with methanogenesis, which leads to temporarily increased iso-butyrate titers. Alternatively, as recently shown i-C\(_4\) feedstock can be obtained from organic residues via a mixed culture methanol chain elongation bioprocess that simultaneously stimulates isomerization. Such a precursor could potentially be used to produce i-C\(_6\) from any type of fermentable organic residue.

**Materials and Methods.** This study aimed to develop a chain elongation bioreactor system capable of producing branched MCFAs. The experiment was divided into six phases to investigate the effect on iso-caproate production of iso-butyrate, methanol, acetate, and vitamin B12 Cobalamin. During each of these six phases the medium composition was changed, as shown in Table 1. The reactor system was characterized by analysis of the steady state mass balances for the total amount of carbon and electrons in the liquid and gas phase.
reactor performing methanol based chain elongation to produce iso-butyrate.

**Medium.** The synthetic growth medium contained the following macro nutrients (g L\(^{-1}\)): NH\(_4\)H\(_2\)PO\(_4\) 3.60; MgCl\(_2\)-6H\(_2\)O 0.33; MgSO\(_4\)-7H\(_2\)O 0.20; CaCl\(_2\)-2H\(_2\)O 0.20; KCl 0.20. In addition, the micro nutrients (Pfenning trace metals and B-vitamins) of the designed basal medium described in Phillips et al. (1993)\(^{39}\) were used. The B-vitamin composition of this medium differed in phases I, II, IV, V, and VI. The vitamin B\(_12\) cobalamin concentration in these phases was 30 times higher than in phase III (0.375 mg L\(^{-1}\) compared to 0.0125 mg L\(^{-1}\)). The carbon sources were acetate, methanol, ethanol, n-butylate, and i-butylate. The composition of the carbon sources during each of the six phases changed as shown in Table 1. The reason for the shifts in vitamin B\(_12\) concentration and the addition/removal of methanol are given in the Supporting Information (SI) section.

**Sampling and Measurement.** The reactor was sampled approximately three times per week. Liquid samples were stored at \(-20^\circ\text{C}\) and analyzed once every 2 weeks using gas chromatography according to the procedure described by Jourdin (2018).\(^{40}\) This method allowed the quantification of volatile fatty acids from n-C\(_5\) to n-C\(_6\), i-C\(_5\), b-C\(_5\) (both 2- and 3-methylbutanoic acid have the same retention time) and i-C\(_6\) (4-methyl-pentanoic acid). The i-C\(_6\) isof orm, 4-methyl-pentanoic acid, has a different retention time than 2-methyl-pentanoic acid, as is shown in SI Figure S2. In addition to the volatile fatty acids, the alcohols methanol up to and including hexanol, as well as iso-butanol and iso-hexanol could be quantified. Prior to injection, samples were acidified in a final concentration of 1.5 wt % formic acid.

The headspace was analyzed every time a liquid sample was taken. The concentrations of O\(_2\), N\(_2\), CH\(_4\), H\(_2\), and CO\(_2\) were determined via gas chromatography using 2 GC systems with a standardized method.\(^{44,45}\) The headspace was connected to a gas meter (µFlow Bioprocess Control, Sweden) to measure gas production. In this internal recirculation loop the turbidity was continuously measured to indicate the suspended biomass concentration.

**Steady State Characterization.** During all six phases the reactor was assumed to be in a steady state when it met the following criteria: (i) the reactor had been operating at the same conditions for at least 5 times the HRT value and (ii) the main metabolite concentrations and corresponding conversion rates were relatively constant. The second criteria was defined as when the confidence interval was less than 20% of the steady state average values. Confidence intervals were determined with an α of 0.01 and are indicated with ±.

**Calculation for Excessive Ethanol Oxidation.** There are various competing excessive ethanol oxidation (EEO) routes that lower the efficiency of ethanol usage in chain elongation processes (see SI Table S1). Moreover ethanol oxidation yields acetate that directly causes a favorable selective pressure toward straight chain elongation, which competes with branched chain elongation. The percentage of ethanol that was not used for chain elongation could be quantified via stoichiometric analysis of chain elongation activity (using the measured formation of n-C\(_5\), n-C\(_6\)OH, n/i-C\(_5\), n/i-C\(_6\)OH, and n-C\(_6\)) and the measured ethanol consumption. The stoichiometry of chain elongation was generalized as reverse beta-oxidation combined with ethanol oxidation coupled in a 5 (n = S) to 1 (p = 1), or 4(n = 4) to 1(p = 1) ratio (reaction 1 for acetate and 2 for longer carboxylates):\(^{37}\)

\[
\begin{align*}
(n_{\text{C2}} + p)\text{CH}_3\text{CH}_2\text{OH} + (n_{\text{C2}} - p)\text{CH}_3\text{COO}^- & \rightarrow n_{\text{C2}}\text{C}_5\text{H}_9\text{COO}^- + 2\text{H}_2 + (n_{\text{C2}} - p)\text{H}_2\text{O} + \text{pH}^+ \\
(n_{\text{C2}} + p)\text{CH}_3\text{CH}_2\text{OH} + n_{\text{C6}}\text{H}(\text{C2}_{\text{C2}})\text{COO}^- & \rightarrow n_{\text{C6}}\text{C}_{\text{C2}}\text{H}(\text{C2}_{\text{C2}}+2)\text{COO}^- + 2\text{H}_2 + (n_{\text{C2}} - p)\text{H}_2\text{O} + \text{pH}^+ + \text{pCH}_3\text{COO}^- 
\end{align*}
\]

where \(\sum_{\text{chain elongation activity}}\) is the amount of ethanol necessary to perform all observed chain elongation and as such

\[
\text{EEO(%) = } \left(1 - \frac{\sum_{\text{chain elongation activity}}}{\text{observed ethanol consumption}}\right) \times 100
\]

Furthermore, for the calculation the following assumptions were made:

- Consumed methanol was used for methanol based chain elongation of acetate to butyrate (reaction 9 in SI Table S1).
- EEO was calculated using two different stoichiometries for chain elongation (i) where \(n = 5\) and \(p = 1\) and (ii) where \(n = 4\) and \(p = 1\). For simplicity it was assumed that elongation of externally added carboxylates, besides acetate, occurs with similar stoichiometry. The letter \(n\) stands for the amount of ethanol used for the summed chain elongation activity and \(p\) stand for the amount of ethanol used for substrate level phosphorylation and acetate+H\(_2\) formation. Both ratios were used because thermodynamic calculations of the chain elongation reaction (SI Figure S5) showed that the reaction Gibbs free energy was not always sufficient for generation of 2.5 ATP.\(^{38}\)
- If \(\beta\)-oxidation was occurring, then this will cause a net increase of EEO(%). Chain elongation (reaction 1) followed by five times \(\beta\)-oxidation (reaction 5 in SI Table S1) yields the same stoichiometric outcome as six times direct ethanol oxidation (reaction 2 in SI Table S1). Thereby potential \(\beta\)-oxidation activity is incorporated within this calculation.

**Microbial Community Analysis.** Biomass samples during the steady states in phase I, II, IV, and VI (for VI both suspended biomass and granules) were used for 16s rDNA analysis to determine the microbial compositions. The biomass samples were taken, in duplicate, by spinning down ~20 mL (for suspended growth) and ~1 mL (wet granular biomass) in 2 mL Eppendorf centrifuge tubes for 5 min at 9300 RCF, snap freezing the pellets using liquid nitrogen and storing the frozen pellets at ~80 °C until DNA extraction. The samples taken in duplicate were then analyzed separately.

DNA was extracted from the pellets applying a Powersoil DNA isolation kit, according to the instruction manual. The isolated DNA was used as template for amplifying the V3–V4 region of 16S rDNA via Illumia sequencing using the primer sets described by Takahashi et al.\(^{42}\) This allowed simultaneous amplification of the both bacterial and archaean 16s rRNA gene regions. The 16S rDNA analysis and subsequent taxonomic analysis was performed using QIIME software version 1.9.\(^{43,45}\) (For more details see Supporting Information.) This bioinformatics process was performed on the December 31, 2017.
From the acquired data a heat map was made using Microsoft Excel. Open source software Rstudio v3.5.0 was used to sort the data and create quantitative OTU tables that belonged to the chosen taxonomic group. This allowed counting the most abundant OTU’s that were classified within a single genus. The rDNA sequences of selected abundant OTUs were then used for Megablast to search within the NCBI nucleotide database on September 27, 2018.

It is important to emphasize that the performed 16s rDNA analysis limits the microbial community analysis to taxonomic and phylogenetic determination. Functional roles within the microbiome cannot be attributed to the observed taxa using this data. Instead the microbial community analysis was used to link the stoichiometric analysis of the experiment to existing literature. Moreover, the relative abundances estimated by this NGS method are an indication but should not be used for quantitative analyses.

RESULTS AND DISCUSSION

Elongation of Iso-Butyrate with Ethanol to Iso- Caproate. A reactor system with an ethanol based chain elongation microbiome was developed that formed isocaproate (i-C6) continuously for 250 days (Figure 1). The highest volumetric production rate was $44 \pm 6 \text{ mmol C L}^{-1} \text{ day}^{-1}$ (0.86 ± 0.1 g L$^{-1}$ day$^{-1}$) during the last phase of operation with a broth concentration of 73 ± 7 mM C or 1.4 ± 0.1 g L$^{-1}$. This amounted to 20% of all the formed compounds based on carbon atoms. The complete concentration profiles of

Figure 1. Graph a, b and c show the reactor broth concentrations (mM C) of the soluble compounds throughout the six operation phases. Carboxylic acids are indicated according to their carbon number and straight (n-) or branched (i-) form. Alcohols are indicated by name or the OH suffix. Graph d shows the headspace gas partial pressures throughout the phases, excluding nitrogen gas and oxygen (which was kept under 1%).
all measured carbon compounds, as well as the headspace gas pressures, are shown in Figure 1. From these profiles it can be seen that the presence of i-C₄ steers toward i-C₆ formation.

The measured C₆ isoform, 4-methyl pentanoate, matches the predicted compound for i-C₄ elongation. According to the described mechanism for ethanol based chain elongation,⁹ the carboxylates are always elongated with two carbon chain units via acetyl-CoA condensation. In the case of ethanol based chain elongation this suggests that the first step is a thiolase driven catalysis in which the alkyl-transfer reaction allows for i-C₄ to covalently bind one of the cysteines in the catalytic site. Next, a Claisen-type condensation reaction adds the subsequent acetyl-CoA.⁴⁶,⁴⁷ For i-C₄ elongation, reduction of the acquired 3-keto-4-methyl-pentanoyl-CoA should then occur via 3-hydroxy-4-methyl-pentanoyl-CoA, 4-methyl-2-pentenoyl-CoA to 4-methyl-pentanoate, as shown in SI Figures S3 and S4.

During start up, in phase I when both n-C₄ and i-C₄ were fed into the system, only small amounts of i-C₆ were formed (6 ± 2 mmol C L⁻¹ day⁻¹). Mainly straight (n-)C₆ was formed. The i-C₆ formation stopped completely in the following phase (II), when i-C₄ was removed from the influent and replaced by the same amount of n-C₄. In the phase thereafter (III) the n-C₄ was replaced by i-C₄ and branched (i-)C₆ was formed again. In SI Table S2 the molar ratios of i-C₄-derived production (e.g., the formation of i-C₆OH, i-C₆₂, i-C₆₄OH), and i-C₄ consumption are given for each steady state during the phases. In phases III, IV, V, and VI these ratio were between 98% and 112%. The fact that i-C₆ formation was equivalent to i-C₄ consumption further supports the proposed mechanism for ethanol based i-C₄ elongation and suggests that in these phases no desomerization took place.

**Iso-Caproate Formation without Elevated B₁₂ or Methanol.** As earlier work on i-C₄ formation indicated the necessity for methanol and high vitamin B₁₂ concentrations,³¹ the reactor system was started with similar amounts in the influent. The vitamin B₁₂ concentration was lowered (phase III) and methanol was removed from the influent (phase V). Figure 1b shows that lowering the B₁₂ concentration and removing methanol from the influent did not affect i-C₆ formation.

**Straight Chained MCFA n-C₆ Formation Was Preferred Over i-C₆ Formation.** In Figure 2 the volumetric conversion rates show that throughout the six phases n-C₆ was the most abundantly formed compound. When n-C₄ and i-C₄ were present in equal ratios (phase I), there was 26 times as much n-C₆ formation than i-C₆ formation (0.038 i-C₆ per n-C₆). Even when no n-C₄ was fed (from phase III onward) the major part of the formed MCFAs was n-C₆. Formation of this n-C₆ could be attributed to regular chain elongation of C₂ via n-C₄ to n-C₆. There is a preference for C₂ and straight-C₄ over branched-C₄ during ethanol based chain elongation. Straight chain elongation should thus be prevented in order develop a more selective branched MCFA bioprocess.

In the final phase (VI) the effect of a lowered acetate concentration in the influent (reduction from 4.5 g L⁻¹ to 0.42 g L⁻¹) was studied with the aim to prevent n-C₆ formation via C₂ and to increase i-C₆ formation. However, it was observed that direct (excessive) ethanol oxidation (EEO) was stimulated which resulted into the formation of acetate and subsequent elongation to n-C₄ and n-C₆. Yet, less n-C₆ was formed than at the high acetate load during preceding phases. Still, the ratio of i-C₆ per n-C₆ in the final phase turned out the be the highest ever achieved in this system (0.4 i-C₆ per 1 n-C₆).

**Excessive Ethanol Oxidation and Acetate Limitations.** The excessive ethanol oxidation in Figure 3 shows a decreasing trend during the first five phases. This coincides with a low ethanol broth concentration (0 to 0.5 g L⁻¹) and a C₂ broth concentration of 1.0 to 2.5 g L⁻¹. Moreover, when the EEO was near zero during phases IV and V, the headspace hydrogen partial pressure (Pₗ₂) was regularly above 1 kPa (Figure 1d), during peaks around day 196, 210, 240 in phase IV and constantly above 1 kPa during phase V. A Pₗ₂ higher than...
Increased CO₂ supply (i.e., greater availability) does enhance chain elongation (EEO) and can compensate for insufficient bicarbonate for chain elongation microbes (SI Figure S5) would lower the amount of internally produced acetate by stoichiometry. In this final phase branched chain elongation had - relative to straight chain elongation—a higher probability to occur than in the previous phase because more ethanol was present in conjunction with the high i-C₄ concentration, while there was less acetate supply, both by influent and by production of the microbiome itself.

**Concurrent Methanogenesis, Acetogenesis, and Carboxylate-to Alcohol Reduction.** In the scenario of CO₂ limitation, excess reducing equivalent (e.g., hydrogen released during ethanol oxidation, β-oxidation and chain elongation) is present without sufficient bicarbonate for hydrogenotrophic methano- and/or acetogenesis. Therefore, an alternative electron acceptor must be reduced. The remaining electron equivalents were utilized by carboxylate reducers that performed biohydrogenation of fatty acids to alcohols, or ethanol was consumed via direct carboxyl-hydroxyl exchange (reaction 14 in SI Table S1). By these processes, iso-butanol could be formed up to a concentration of 0.5 g L⁻¹ and n-hexanol up to 0.3 g L⁻¹; also trace amounts of n-butanol and iso-hexanol were observed. This is the first prospective observation of branched carboxylic acid and n-caproate reduction at these levels during mixed culture chain elongation, without other electron donors being supplied besides ethanol (e.g., carbon monoxide or via biocathodes). Production rates of higher alcohols were similar to those of a chain elongation coculture growing on hydrogen, carbon monoxide, and carbon dioxide. Besides the carboxylate-to-alcohol reduction mechanism as energy yielding pathway, chain elongating bacteria could also be responsible for the observed n-butanol and n-hexanol formation throughout the operation period, where it acts as an additional electron sink.

Thermodynamic calculations of the carboxylate-to-alcohol reduction suggest that a high reactant to product ratio for hydroxyl-carboxyl exchange is necessary to maintain a ΔG lower than −20 kJ reaction⁻¹ (SI Table S1) when the hydrogen pressure is insufficient and pH too high to drive the reduction. During earlier phases (I and II) where EEO was also around 20–40%, less carboxylate-to-alcohol reduction occurred. This could be explained by the too low reactant-to-product ratio in these phases and abundance of CO₂ as external electron acceptor (SI Figure S6). Moreover, during phase VI small black granules were observed. The emergence of these granules, together with the microbiota data, suggests a syntropic culture that created its own niche to facilitate otherwise thermodynamically unfavorable reactions (see SI Table S1). Close proximity of various microbes within a...
biofilm facilitates syntrophic interactions.\textsuperscript{55} It is known that effective electron transfer (via hydrogen or direct interspecies electron transfer (DIET)) between ethanol (or $\beta$-) oxidizers and syntrophic partner organisms, such as methanogens, is feasible.\textsuperscript{56–58} Therefore, we can hypothesize that here the ethanol oxidation, or possibly beta-oxidation, was linked via reducing equivalent transfer (hydrogen, formate, or other), or DIET to ethanol oxidation, or possibly beta-oxidation, was linked via reducing equivalents, such as hydrogen gas that can be utilized for phases I, II, IV, and VI. In SI Table S4, the heatmap shows the relative abundances of orders within the microbiome extended toward genus level. OTU counts and BLAST results are shown in SI Tables S5 and S6). The heat map shows that in all analyzed samples Clostridiales is the most abundant order, in which the most abundant OTU belonged to 
\textit{Clostridium kluyveri} (100% coverage and 98% similarity, SI Tables S5 and S6). C. \textit{kluyveri} is a well described bacterium that performs the chain elongation. It is likely that in this case a strain of \textit{C. kluyveri} is present that can perform the branched chain elongation. Hypothetically the elongation is done using the same enzymes that are used for straight-chain elongation, albeit that these enzymes have different affinities for the metabolites involved in the branched-chain elongation. Alternatively a different microbe could be responsible for iC\textsubscript{5} formation. By isolating and characterizing the responsible bacteria, a definitive explanation could be given.

As mentioned earlier, the microbiome contains several other functional groups apart from chain elongation. SI Figure S7 shows a putative overview of possible metabolic activities of the presented microbiome. Some conversions were attributed to microorganisms that were identified within the community. Among these are bioprocesses such as chain elongation, albeit that these enzymes have different affinities for the metabolites involved in the branched-chain elongation. Alternatively a different microbe could be responsible for iC\textsubscript{5} formation. By isolating and characterizing the responsible bacteria, a definitive explanation could be given.

Microbial Community analysis. In Table 2 a heat map shows the relative abundances of orders within the microbiome for phases I, II, IV, and VI. In SI Table S4, the heatmap is extended toward genus level. OTU counts and BLAST results are shown in SI Tables S5–S28. The black granules (0.5–1 mm diameter) that were formed during phase VI were also analyzed and are referred to as "VI granule".

The heat map shows that in all analyzed samples Clostridiales is the most abundant order, in which the most abundant OTU belonged to \textit{Clostridium kluyveri} (100% coverage and 98% similarity, SI Tables S5 and S6). C. \textit{kluyveri} is a well described bacterium that performs the chain elongation. It is likely that in this case a strain of \textit{C. kluyveri} is present that can perform the branched chain elongation. Hypothetically the elongation is done using the same enzymes that are used for straight-chain elongation, albeit that these enzymes have different affinities for the metabolites involved in the branched-chain elongation. Alternatively a different microbe could be responsible for iC\textsubscript{5} formation. By isolating and characterizing the responsible bacteria, a definitive explanation could be given.

As mentioned earlier, the microbiome contains several other functional groups apart from chain elongation. SI Figure S7 shows a putative overview of possible metabolic activities of the presented microbiome. Some conversions were attributed to microorganisms that were identified within the community. Among these are bioprocesses such as beta-oxidation and direct ethanol oxidation.\textsuperscript{59} These bioconversions form reducing equivalents, such as hydrogen gas that can be utilized via hydrogenotrophic methanogenesis, acetogenesis, and/or carboxylate-to-alcohol reduction.

It should be noted that the reactor was not a sole ideally mixed system because some biofilms (in the form of wall growth and granules) were present. Niche formation, which is underlined by the granule formation in phase VI, increases the possibility for these syntrophic processes to occur. Biofilm growth decouples solids retention time from hydraulic retention time and allows microbes that grow slower than the reactor dilution rate to be maintained in the reactor. The $\beta$-oxidizers \textit{Syntrophomonadaceae} in phase I had some similarity (100% coverage, 96% similarity, SI Table S8) with \textit{Syntrophomonas zehnderi} OL-4. These microbes are thermodynamically only able to oxidize fatty acids at a partial H\textsubscript{2} pressure below 1 Pa (SI Table S1),\textsuperscript{66,60} although higher hydrogen partial pressures were measured. Due to local differences and fluctuations in the micro environment around the bacteria, thermodynamic calculations done with macroscopic data (e.g., headspace partial pressures) should therefore be interpreted with a wider range of uncertainty. In phase VI the granules contained a syntrophic culture of $\beta$-oxidizing microbes within the \textit{Anaerolineaceae} UCG-001 order and methane forming \textit{Methanooccus palmolei} (99% coverage, 99% similarity, SI Table S28, Methanomicrobiales order).\textsuperscript{61,62}

The \textit{Methanooccus} genus has been reported to perform hydrogenotrophic methanogenesis in other mixed culture chain elongation microbiomes.\textsuperscript{63,69} In contrast to \textit{Methano- coccus palmoi}, that had a high presence within the granular biofilm, the \textit{Thermo- plasmatales} had a relatively low abundance in the granular biofilm compared to their abundance within the suspended biomass. The OTU classification of the \textit{Thermoplasmatales} showed very low similarity to \textit{Methanomassiliicoccus luminyensis} B10 (99% coverage, 88% similarity, \textit{Candidatus methanogranum} genus). On account of its low abundance in the granular biofilm compared to the abundance in suspension this unknown C. \textit{methanogranum} is hypothesized to not be a syntrophic reduction partner. Instead, assuming it is a methane producing organism, it could be involved in either alcohol-otrophic or acetoclastic methanogenesis (reaction 4 and 13 respectively in SI Table S1), both which are thermodynamically feasible independent of hydrogen partial pressure.

The observed \textit{Desulfovibrio} most likely work in conjunction with the \textit{Anaerolineaceae} ICG-001 and \textit{Methanooccus palmolei}. \textit{Desulfovibrio} are known to be able to perform $\beta$-oxidation in the absence of an extracellular electron acceptor.\textsuperscript{64,65} The most abundant \textit{Desulfovibrio} OTU was identified to resemble \textit{Desulfovibrio legallii} H1 (95% coverage and 99% similarity, SI Table S28).
similarity, SI Table S20), which is also known to be able to perform direct ethanol oxidation.65 These type of sulfate reducing bacteria are versatile in bioelectrochemical processes and can donate or take up electrons from electrodes while converting various organics or CO₂. Possibly the Desulfovibrio species were (partly) responsible for the usage of electrons or reducing CO₂ to acetate and/or forming branched alcohols.66,67 In addition to these fermentation processes, the Desulfovibrio could also have been responsible for the black coloration of the granules by causing black precipitation of FeS after reducing the little amounts of present sulfate and iron(III).68,69 Some types of FeS compounds have been shown to enhance direct interspecies electron transfer.58

Future Outlook. Here we report on iso-caproate bioformation via chain elongation. We also observed that higher alcohols such as iso-butanol, hexanol, and iso-hexanol were formed. Branched alcohol formation has not been reported in earlier chain elongation research work. Higher branched alcohols could be interesting fermentation products because of their advantages over bioethanol such as higher energy density, lower hygroscopicity, lower vapor pressure, and compatibility with existing transportation infrastructures.70 The developed mixed culture microbiome represents a complex mix of microbial processes with several possible competing and syntrophic interactions. The reactor system was not operated with conditions for maximal production rate and product titers. By increasing ethanol loads and preventing CO₂ to become limiting for chain elongation higher MCFA production rates and concentrations can likely be achieved. In addition, a more stringent selective pressure could be achieved by lowering the hydraulic retention time4,34 and by increasing the mixing to prevent niche-formation.

In order to improve the selectivity of i-C₆ production via i-C₄ elongation, the microbiome must be enriched to the point where C₇/n-C₄/n-C₆ elongation is no longer the dominant chain elongation process. Excessive ethanol oxidation and beta-oxidation should be prevented to the point where the resulting acetate production in turn does not allow for dominant C₇/n-C₆ elongation. A 100% selective production process of i-C₆ is impossible in principle, due to the fact that some C₇ is always present. There will always be acetate production during the reverse beta-oxidation process, due to substrate level phosphorylation coupled to ethanol oxidation.9

The feedstock to produce i-C₆ could be derived from an i-C₄ bioproduction process that uses methanol and organic waste (e.g., outdated food waste).31 The presence of methanol remaining from such a first stage will likely not hamper the i-C₄ formation, as in this study methanol did not have an effect on i-C₄ formation. Hypothetically, earlier formed iso-valerate too could be elongated to i-C₇ within the developed microbiome.

Further studies will be required to exploit the formation of the branched MCFA's and alcohols. The i-C₆ production rate of 0.86 g L⁻¹ day⁻¹ is still 65 times lower than optimized n-C₆ (55.8 g n-caproate L⁻¹ day⁻¹) chain elongation systems32 and thus needs to be increased. Moreover, the i-C₆ broth concentration (1.4 g L⁻¹) in this study was 10 times lower than the maximum solubility of the protonated acid-form. Concentrations in this higher range are necessary for effective extraction.72 Once these criteria are met, efficient downstream processing methods can be developed that will allow for branched chain elongation to become viable in industry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b07256.

Additional information regarding Materials and Methods, a summary table with bioprocesses and thermodynamic calculations, additional depictions for Results and Discussion, 13 figures, and 29 tables (PDF)

Accession Codes

All data generated or analyzed during this study are included in this published article (and its Supporting Information files). Microbiota data (raw 16s rDNA amplicon sequences) is submitted to the EBI database (https://www.ebi.ac.uk/ena) under accession number PRJEB32171.

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K.D.L. planned and performed the experiments, analyzed the results, and wrote the manuscript. D.S. & C.B. organized the design of the study, supported the data analysis, and revised the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SCFAs short chain fatty acids
MCFAs medium chain fatty acids
HRT hydraulic retention time
EEO excessive ethanol oxidation
OTU operational taxonomic unit
MeOH methanol
EtOH ethanol
n-C₆ straight monocarboxylic acids with x carbon atoms
i-C₆ branched monocarboxylic acids with x carbon atoms
n-C₆ OH straight primary monoalcohols with x carbon atoms
i-C₆ OH branched primary monoalcohols with x carbon atoms

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