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Research Article

Methanol-Based Chain Elongation with Acetate to n-Butyrate and Isobutyrate at Varying Selectivities Dependent on pH

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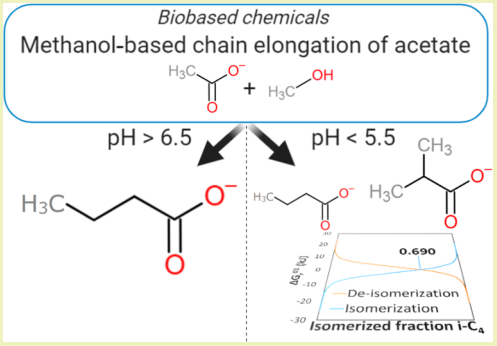
ABSTRACT: Biomass fermentation technologies offer alternative methods to produce platform chemicals that currently originate from fossil sources. This research showed that an enriched microbiome was capable to produce isobutyrate (i-C_4) from acetate via methanol-based chain elongation. A long-term continuous reactor experiment showed that the selectivity for i-C_4 and/or n-butyrate (n-C_4) could be reversibly adjusted by changing the reactor pH. A reactor pH of 6.75 led to formation of (carbon per total carbon of products) 0.78 n-C_4 and 0.024 i-C_4, whereas a reactor pH of 5.2 led to a selectivity of 0.24 n-C_4 and 0.65 i-C_4. This shift in product spectrum was also represented by a shift in microbial composition. The results suggest that a *Eubacterium* genus is responsible for the formation of n-C_4, whereas a *Clostridium luticellarii* strain is responsible for the formation of a mixture of i-C_4 and n-C_4. The formation of n-C_4 and i-C_4 at a low pH was observed to be coupled according to the thermodynamics of isomerization. At a reactor pH of 5.5 and 5.2, the product ratio of i-C_4:n-C_4 approached 0.69 i-C_4:0.31 n-C_4, which is the theoretical ratio that would be achieved when determined by the equilibrium of isomerization. Various batch experiments at pH 5.5 and 5.2 confirmed that addition of either n-C_4 or i-C_4 at the start of the batch would directly lead to the formation of the other butyrate component. Moreover, batch experiments performed at pH 6.5 produced mainly n-C_4 and led to the development of a completely different microbiome. The imposed pH is a strong selection pressure that can facilitate changes in product selectivities for n-C_4 and i-C_4 during methanol-based chain elongation of acetate.



KEYWORDS: *Chain elongation, Selection pressure, Open-culture fermentation, Biobased chemicals, Methanol, n-Butyrate, Isobutyrate*

INTRODUCTION

The necessity to transition toward a circular economy has driven researchers to explore new biomass fermentation processes for alternative production of currently oil-derived and palm-derived chemicals.[1−4] Microbial chain elongation fermentations have potential to become alternative and sustainable technologies for acquiring platform chemicals, such as short chain carboxylates (SCCs) and medium chain carboxylates (MCCs).[5−7] Biomass residues and CO_2 are used as favorable substrates for these bioprocesses. The carbon streams can be more efficiently recycled which could help lessen environmental harmful expansion of agricultural areas. Microbiomes that catalyze chain elongation processes have been enriched using different selection pressures, for which the electron donor is an important factor.[4,8] Methanol is an exceptionally interesting electron donor, because it can be electrochemically synthesized from CO_2 or thermochemically produced from lignocellulosic biomass.[9−12]

Recently it has been shown that isobutyrate (i-C_4) can be formed during methanol-based microbial chain elongation.[13,14] It was observed that a high concentration of n-butyrate (n-C_4) is necessary to stimulate isomerization toward i-C_4 within the methanol-based chain elongation microbiome. It was also shown that addition of methanol to n-C_4-rich fermented supermarket waste residue induced the formation of i-C_4. The precise metabolic route for the biochemical i-C_4 formation has not been fully elucidated, but it was suggested that it takes place via methanol chain elongation and subsequent isomerization of n-C_4 to i-C_4. Alternatively i-C_4 can be formed via microbial electrosynthesis; however, for now, its formation was only observed as a side product.[15,16] Also biological production of i-C_4 from sugars has been shown using metabolically engineered strains.[17,18] Currently, i-C_4 is produced chemically from fossil-based feedstocks and other compounds such as alkali metal bases or metal alcoholates.[19] Isobutyrate can be used in the production processes of transparent thermoplastics, emulsifiers, vitamins, antibiotics, and organic solvents.[17,20,21] It

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has a market size of around 2.7×10^6 ton/year.[18] The newly discovered methanol chain elongation bioprocess within a mixed culture is a prospective alternative route toward i-C_4 production.[13] By using only acetate and methanol as sole substrates and by using a mixed culture, a versatile organic residue-based fermentation process can be developed.[22] Methanol-based chain elongation experimentally complied to the following stoichiometry (eq 1)[13,23,24]

$$2\,\text{methanol} + \text{acetate} \rightarrow \text{n-butyrate} + 2\,H_2O$$
$$\Delta G_r^{01} = -106.8\,\text{kJ} \quad (1)$$

The objectives of this study were to further elucidate the selection pressure involved in enriching the i-C_4 forming microbiome and to develop this microbiome without supplying exogenous n-C_4. A long-term continuous reactor system was operated in order to study varying pH levels (from 6.75 to 5.2). The necessity for high n-C_4 concentrations in earlier studies was hypothesized as a toxic effect on the microbiome that induced isomerization.[13] Hypothetically, a similar selection pressure could be achieved by high amounts of in situ butyrate formation from acetate and methanol. Additionally, lowering the pH and thus increasing the amount of undissociated acids in the broth could further amplify the toxicity of the formed butyrates toward microorganisms.[25,26] We show with this experimental work that at pH 5.2 the microbiome produced i-C_4 with a 65% carbon selectivity from acetate and methanol as sole substrates. A selection pressure tool is presented that can be used to form i-C_4 with a mixed culture from simple molecules such as acetate and methanol.

In addition to the continuous reactor experiment, a series of batch experiments was done to gain insight into the microbial activity and microbiome enrichment under varying starting pH levels, different n-/i-C_4 starting concentrations, and different substrate (acetate and methanol) concentrations. These batch series give insight into how the i-C_4 production is coupled to catabolic conversion of the chain elongation substrates and how the isomer formation specificity is affected.

MATERIALS AND METHODS

This research aimed to investigate what selection pressure is needed for a methanol chain elongation microbiome to produce isobutyrate (i-C_4) and/or n-butyrate (n-C_4) from acetate (C_2) and methanol (MeOH). A continuous reactor was operated throughout seven phases to investigate the effect of different pH levels (pH 5.2, 5.5, 6.0, and 6.75). Moreover, CO_2 addition was removed (phase I → II). A slight increase and consecutive decrease of acetate in the influent were executed (II → III → IV), and the vitamin B12 supply was dropped by a factor of 30 (III → IV). A description of the operating conditions during the seven phases is 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 phases.

In addition to the continuous reactor, batch experiments were performed to investigate (i) the immediate effect of two different pH levels (5.2 and 6.5) on product formation and microbial composition, (ii) whether initial n-C_4 or i-C_4 addition affects the product selectivity, (iii) how the absence or presence of carbon sources affect the (de)isomerization of n-C_4 and i-C_4, and (iv) how different substrate concentrations affect the methanol chain elongation process. An overview of the batch experiments is given in Table 2.

Medium. The synthetic growth medium contained the following macronutrients (g L^{-1}): $NH_4H_2PO_4$, 3.60; $MgCl_2{\cdot}6H_2O$, 0.33; $MgSO_4{\cdot}7H_2O$, 0.20; $CaCl_2{\cdot}2H_2O$, 0.20; KCl, 0.20, and yeast extract, 1.0. Micronutrients were used as described in Phillips et al. (designed basal medium, Pfennig trace metals, and B-vitamins).[27] The B-vitamin

Table 1. Overview Includes Short Description of Each Phase, Phase Duration, Steady State Period per Phase, Temperature, CO_2 inflow, pH Set Points and Set HRTs

	Phase I	Phase II	Phase III	Phase IV	Phase V	Phase VI	Phase VII
Description	Changed pH set point 5.5, CO_2 flow set to 120 mLn day^{-1}	no CO_2 input	Changed pH set point from 5.5 to 6, increased acetate from 9.0 to 10.8 g L^{-1}	Decreased acetate from 10.8 to 9.0 g L^{-1}, decreased vitB12 from 362.5 to 12.5 μg L^{-1}	Changed pH set point from 6.0 to 6.75	Changed pH set point from 6.75 to 5.50	Changed pH set point from 5.50 to 5.20
Phase period	day 1 to 48	day 48 to 111	day 111 to 142	day 142 to 181	day 181 to 327	day 327 to 399	day 399 to 489
Steady state period	day 40 to 48	day 94 to 111	day 123 to 135	day 166 to 181	day 311 to 327	day 345 to 364	day 472 to 489
Temperature (°C)	35	35	35	35	35	35	35
CO_2 inflow	120 mLn day^{-1}	–	–	–	–	–	–
pH set point	5.5	5.5	6.0	6.0	6.75	5.5	5.2
Set HRT (h)	40	40	40	40	40	40	40

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Table 2. Overview of Performed Batches

Inoculum	Methanol, mM	Acetate (mM)	pH	i-C_4 (mM)	n-C_4 (mM)	Batch code
Phase VII (suspended cells)	50	25	5.2		50	I
	50	25	6.5		50	II
	50	25	5.2	50		III
	50	25	6.5	50		IV
Phase VII (suspended cells)	–	–	5.2		50	V
	–	–	6.5		50	VI
	–	–	5.2	50		VII
	–	–	6.5	50		VIII
Repeated batch (suspended cells)	300	150	5.5	0	–	A
	300	150	5.5	5	–	B
	300	150	5.5	20	–	C
	300	150	5.5	50	–	D
none	300	150	5.5	0	–	E
Repeated batch (suspended cells)	0	0	5.5	0	–	F
Repeated batch (suspended cells)	50	25	5.5	50	–	G
	100	50	5.5	50	–	H
	200	200	5.5	50	–	J
	300	150	5.5	50	–	K
	50	25	5.2	50	–	L (III repeat)
	0	0	5.5	50	–	M
Eubacterium limosum	300	150	5.5	–	–	EL batch

composition of this medium differed in phases I, II, and III. The vitamin B_{12} cobalamin concentration in these phases was 30 times higher than in the following phases (0.375 mg L^{-1} compared to 0.0125 mg L^{-1}). The higher vitamin concentration was initially used in concordance to the earlier done research on methanol-based formation i-C_4.[13] The vitamin concentration was lowered in order to rule out the necessity of these high amounts. The carbon sources during the whole study were acetate and methanol. For the continuous reactor experiment in phases I, II, IV, V, VI, and VII, the concentrations were 150 mM (8.9 g L^{-1}) acetate and 300 mM (9.6 g L^{-1}) methanol, and during phase III, the acetate concentration was temporarily increased to 180 mM (10.8 g L^{-1}). The starting concentrations for the batch experiments ranged from 50 and 25 mM to, respectively, 300 and 200 mM for methanol and acetate (see Table 2 for the exact starting concentrations for each batch).

Inoculum. The reactor was inoculated with an undefined anaerobic culture that originated from a reactor that performed methanol chain elongation from n-C_4 to n-caproate and also formed i-C_4.[13] A complete 1 L broth was used at the start of this experiment. The batches of the first series (I–VIII, Table 2) were inoculated with suspended biomass from the continuous reactor during the end of phase VII. The batches of the second series were inoculated with biomass that originated from batch III and was subsequently kept active with a repeated batch. A more detailed description of the inoculation is described in the Supporting Information, Materials and Methods.

Reactor Setup and Operation. The reactor experiment was performed for 489 days in a lab-scale continuous up flow anaerobic reactor setup (1 L working volume) as described in an earlier chain elongation research.[28] Table 1 summarizes the operating parameters (HRT, pH, CO_2). The temperature was kept constant at 35 °C. The medium inflow was set at 25 mL h^{-1}, which led to an HRT of around 40–46 h. During the first four phases, the HRT slowly increased due to diminished flow rate caused by clogging of the influent tubes. This problem was solved from phase V and onward. The aim of the chosen HRT value was to provide sufficient retention for methanol-based chain elongation organisms, while simultaneously suppressing suspended methanogenic growth.[9,29] Biofilms were allowed to grow in the reactor; periodically (approximately once per month), sludge-like biomass that accumulated in the pH control unit was removed. The reactor pH was regulated by automatic titration using 1 M KOH as the base. In the first phase, (120 mLn day^{-1}) CO_2 was supplied, because earlier experiments showed successful methanol-based chain elongation under CO_2 supplementation.[9,23] However, CO_2 can act as electron acceptor for acetate-producing methylotrophs which compete for methanol as the substrate and reduce selectivity toward butyrates.[24] Additionally, sufficient CO_2 was produced in the reactor itself, via possibly yeast extract conversion and/or methanogenesis. Therefore, its supply was stopped from phase II and onward. The reactor was partially mixed via internal recirculation at 150 mL min^{-1}. A schematic overview of the reactor setup is shown in Figure S1.

Steady State Characterization. During the seven phases, a steady state was assumed when the following criteria were met: (i) For at least five times, the HRT no setup change had occurred. (ii) Main metabolite concentrations and corresponding conversion rates were relatively constant. Relatively constant is 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 a $\pm$.

Batch Experiments. At the end of the seventh phase, a series of eight batch experiments was carried out in duplo to investigate how the formation of n-C_4 and i-C_4 is coupled to methanol-based acetate elongation under various conditions (Table 2).

First, batches were performed at pH 6.5 (batches II, IV, VI, and VIII) and at pH 5.2 (batches I, III, V, and VII). Second, the effect of initially present chain elongation products on formation selectivity was investigated: either 50 mM n-C_4 (batches I, II, V, and VI) or 50 mM i-C_4 (batches III, IV, VII, and VIII) was added. Third (V to III), a control series was performed without methanol and acetate in the medium, because it was unclear if i-C_4 was formed via a secondary isomerization coupled to degradation[30–34] or via a catabolically (and seemingly irreversible) driven formation. After this first series, the microbiome from batch III was used for a second series of 12 batch experiments in duplicate (batches A to M, Table 2) to determine how different substrate concentrations affect the methanol-based chain elongation. In addition to these batches, a pure culture *Eubacterium limosum* 2A (DSM 2593) was used as inoculum to perform a batch at pH 5.5 and methanol and acetate as substrates. The aim was to check if this culture was able to also form i-C_4 under these conditions, as it is known that *E. limosum* can form n-C_4.[35] The protocols for the batch experiments are described in the Supporting Information, Materials and Methods, and Tables S1–S6.

Sampling and Measurement. Continuous reactor samples were taken approximately two or three times per week. Batch samples were taken three times per week in the beginning and less frequently as the batch progressed. The pH of the samples was checked using an external pH meter, which allowed periodic recalibration of the internal pH controller in the continuous system. The liquid samples were analyzed for primary alcohols (methanol to hexanol), isobutanol, and volatile fatty acids (acetic acid to n-caprylic acid) composition via gas chromatography by an earlier described method.[36] The headspace gas 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 two gas chromatography systems as described earlier.[7,9] The headspace of the continuous reactor was connected to a gas meter (μFlow Bioprocess Control, Sweden) to measure gas production. The headspace pressure in the batches was measured (pressure meter GMH 3151).

Microbial Community Analysis. Biomass samples during the steady states in phases II, IV, V, VI, and VII, as well as biomass samples taken at the end of batches (I to VIII) were used to analyze the microbial compositions. Biomass was acquired by spinning down approximately 25 mL of suspended broth (either from the continuous reactor or at the end of a batch run) and subsequently snap freezing

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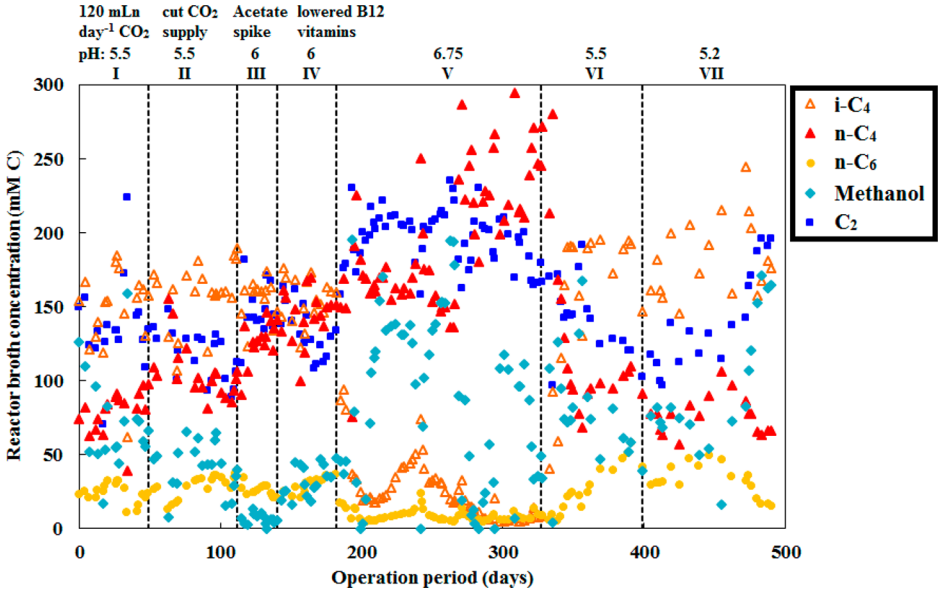

Figure 1. Graph shows the reactor broth concentrations of the main metabolites. A short summary of each phase is depicted above the graph. For phase VII, the pH set point value 5.2 was stably maintained only for the last 50 days (10 measurement points) due to pH probe calibration issues.

the pellet using liquid nitrogen and storing it at −80 °C. In the case of the biofilm sample in phase VI, 1 mL of biomass sludge was used for the DNA extraction. All biomass samples were taken in duplo and analyzed separately. DNA was extracted using a Powersoil DNA isolation kit. The isolated DNA was used as a template for amplifying the V3−V4 region of the 16S rRNA gene using the illumina library generation method described by Takahashi et al.[37] Exactly the same method for the 16s rRNA gene analysis was used as described earlier.[28,38−40] Sequences of frequently found OTUs were used for a NCBI BLAST query. Canoco 5 was used to make two redundancy triplots (Figures S21 and S22) to support discussed correlations. Sequencing data are submitted to the ENA database and can be found with the accession number PRJEB36205.

RESULTS

Reactor pH Influences Formation Selectivities of n-C_4 and i-C_4 during Methanol-Based Chain Elongation of Acetate. The continuous reactor system enriched microbiomes that performed methanol-based chain elongation of acetate (C_2) to isobutyrate (i-C_4) and n-butyrate (n-C_4) as main products in varying ratios dependent on reactor pH. Side products were n-caproate (n-C_6) and methane (CH_4). Figure 1 shows the reactor broth concentrations of the main metabolites, and Table 3 summarizes the performance of the reactor. The results from phases IV to VII are particularly important because the only varied reactor parameter in the phases following phase IV was the broth pH. During the shift from phase IV to V, it took around 100 days for the i-C_4 formation rate to drop and stabilize at around 4 mmol C L^{-1} day^{-1}. Consequently, n-C_4 formation increased with 86% to 142 ± 13 mmol C L^{-1} day^{-1} or 3.1 ± 0.3 g L^{-1} day^{-1}. With a concentration of 237 ± 22 mM C or 5.2 ± 0.5 g L^{-1}, n-C_4 was the dominant product in phase V (78% selectivity based on carbon atoms). The reactor pH set point was lowered to pH 5.5 in phase VI in order to investigate the reversibility of using pH as a selection pressure to enrich an i-C_4 forming microbiome. It took 18 days (about 11 times the HRT) for the i-C_4 formation rate to return back to the levels achieved in phases I and II. In the last phase (VII), the pH set point was set to 5.2. This led to the highest achieved steady state average volumetric production rate for i-C_4, with 111 ± 15 mmol C L^{-1} day^{-1} or 2.4 ± 0.3 g L^{-1} day^{-1}, an average broth concentration of 191 ± 28 mM C or 4.2 ± 0.6 g L^{-1}, and 65% selectivity (based on carbon atoms).

Methanogenic Activity Was Strongly Sensitive to Change between pH 5.6 and 5.2. From day 374 in phase VI to day 455 in phase VII, there were calibration issues of the automatic titration setup that led to increased hydroxide dosage and subsequently to a broth pH that was higher than the set point (Figure S4). As a result, prior to the steady state in phase VII (at pH 5.23 ± 0.07), the pH rose slowly from pH 5.24 up to 5.64 on day 455. An increase and decrease in methane formation rate were observed concomitant with this pH rise and fall (Figure S4). During the steady state of phase VII, the average methane formation rate had dropped by 90% compared to the steady state value in phase VI (at pH 5.61 ± 0.06). The i-C_4 formation rate and concentration did not change significantly compared to phase VI, whereas the n-C_4 formation rate had dropped by 20%.

Cutting CO_2 Supply (II) and Reducing Vitamin B12 Feed (IV) Did Not Adversely Affect Chain Elongation Performance. In the second phase, the CO_2 addition was ceased, which led to a 26% decrease in CH_4 formation and a 47% increase in n-C_6 formation. The steady state average CO_2 partial pressure did drop from 23.7 ± 1.4 (phase I) to 17.2 ± 1.3 kPa (phase II). Gas headspace partial pressures are shown in Figure S5. Conversion of the main metabolites (substrates MeOH, C_2 and products n-C_4 and i-C_4) was not significantly affected by cutting the CO_2 dosage. Lowering the vitamin B12 concentration from 0.375 to 0.0125 mg L^{-1} in phase IV also did not significantly affect the overall performance of the reactor (Table 3). Confidence intervals of the conversion rates overlap for phases II and VI, where the only difference is the influent B12 concentration.

Batch Experiments Show That at pH 5.2 i-C_4 Formation Is Caused by the Presence of n-C_4 and Vice Versa. The results of the first batch series are summarized in Figure 2. The concentration profiles and gas headspace partial pressures for each separate batch are given in

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Table 3. Overview Includes Measured Steady State Averaged Values of pH, HRT, Volumetric Conversion Rates, Organic Loading Rate (OLR), i-C_4 to n-C_4 Ratios, Main Product Selectivites, Percentages of Unconverted Substrate, and Carbon/Electron Balances[a]

	Phase I	Phase II	Phase III	Phase IV	Phase V	Phase VI	Phase VII
Description	Changed pH set point 5.5, CO_2 flow set to 120 mLn day^{-1}	no CO_2 input	Changed pH set point from 5.5 to 6, increased acetate from 9.0 to 10.8 g L^{-1}	Decreased acetate from 10.8 to 9.0 g L^{-1}, decreased vitB12 from 362.5 to 12.5 μg L^{-1}	Changed pH set point from 6.0 to 6.75	Changed pH set point from 6.75 to 5.50	Changed pH set point from 5.50 to 5.20
Steady state period	day 40 to 48	day 94 to 111	day 123 to 135	day 166 to 181	day 217 to 233	day 345 to 364	day 472 to 489
pH	5.79 ± 0.03	5.65 ± 0.04	6.10 ± 0.03	6.02 ± 0.5	6.76 ± 0.8	5.61 ± 0.06	5.23 ± 0.07
HRT	42.8 ± 2.2	45.6 ± 5.2	45.5 ± 1.0	46.3 ± 1.9	40.9 ± 0.2	40.2 ± 0.8	41.5 ± 0.9
			Volumetric C molar conversion rates and OLR (mmol C L^{-1} day^{-1})				
i-C_4	87 ± 12	90 ± 11	86 ± 3	80 ± 4	4 ± 2	106 ± 12	111 ± 14.5
n-C_4	49 ± 4	53 ± 6	69 ± 3	76 ± 2	142 ± 13	54 ± 7	42 ± 4
n-C_6	10 ± 3	19 ± 2	14 ± 1	17 ± 1	5.0 ± 1	14 ± 2	14 ± 4.4
CH_4	31 ± 1	23 ± 3	31 ± 5	21 ± 1	22 ± 8	20 ± 5	2 ± 1.8
CO_2	11 ± 1	9.1 ± 1	11 ± 1	6.2 ± 0.5	3.1 ± 0.9	8 ± 1.3	1 ± 0.4
MeOH	−129 ± 6	−137 ± 14	−154 ± 3	−134 ± 3	−134 ± 17	−120 ± 18	−93 ± 17
C_2	−91 ± 10	−103 ± 16	−110 ± 7	−90 ± 4	−67 ± 8	−84 ± 11	−68 ± 10
Yeast extract	−14 ± 0.9	−13.8 ± 1.3	−14 ± 0.3	−13.3 ± 0.2	−15.3 ± 0.4	−15.6 ± 0.3	−15.0 ± 0.3
OLR	347 ± 21	335 ± 33	361 ± 8	319 ± 4	367 ± 8	373 ± 6	359 ± 6
			i-C4 to n-C4 ratios, selectivities, and percentages of unconverted substrates				
i-C_4 to n-C_4 ratios (molar)	0.64:0.36	0.63:0.37	0.55:0.45	0.51:0.49	0.03:0.97	0.66:0.34	0.73:0.27
i-C_4/total products (carbon)	46%	46%	40%	39%	2%	51%	65%
n-C_4/total products (carbon)	26%	27%	32%	37%	78%	26%	24%
Unconverted C_2	45%	38%	42%	41%	61%	53%	60%
Unconverted MeOH	22%	14%	3%	12%	24%	33%	46%
			Balances				
C-balance	86% ± 7%	82% ± 4%	81% ± 3%	87% ± 5%	89% ± 3%	95% ± 2%	98% ± 2%
e-balance	86% ± 7%	82% ± 4%	82% ± 3%	88% ± 6%	89% ± 4%	95% ± 2%	99% ± 2%

[a]Yeast extract (YE) was assumed to be completely consumed (using 0.4148 g C g^{-1}).[41] Propionate and b/n-valerate are not shown (<3 mmol C L^{-1} day^{-1}).

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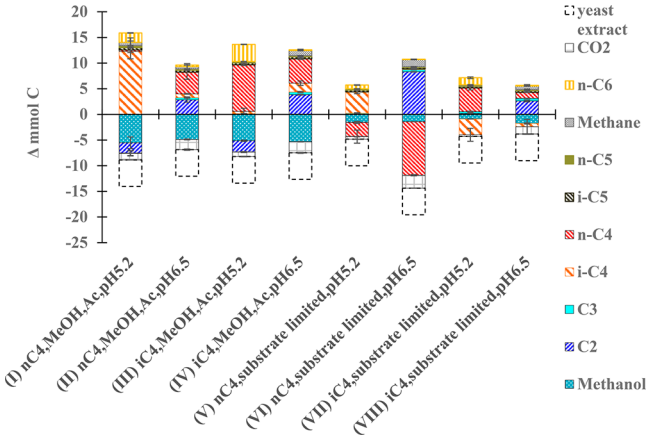

Figure 2. Formation and consumption of carbon-containing components during the batch experiments. Error bars show the difference between the duplicate experiments. The yeast extract that was added to the medium was assumed to be fully consumed.[41] The total formation and consumption after 77 days are shown.

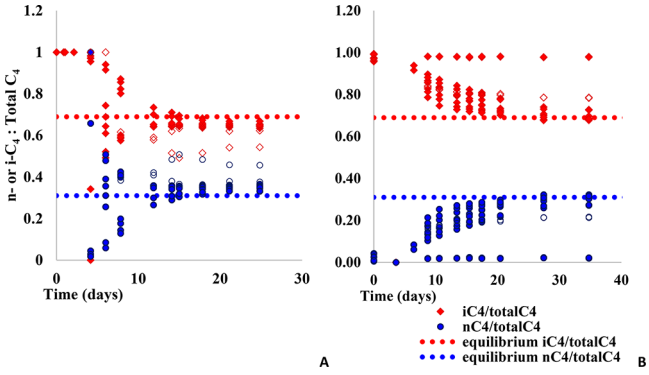

Figure 3. (A) (batches A–E, negative control F excluded) and (B) (batches G–L) show i-C_4 and n-C_4 to total C_4 ratios as measured during all batches throughout the operation period. Dotted lines represent the calculated equilibrium ratio using an isomerization ΔG_r of −1.98 kJ.[42,43] The concentrations of n-C_4 and i-C_4 are interdependent and approach a ratio that is determined by equilibrium of isomerization as the batches progress. In panel (A), the unfilled symbols represent the batch that was started without inoculum (but apparently was contaminated). In panel (B), the unfilled symbols represent the batch that started with 300 mMC methanol, acetate, and 50 mM i-C_4. The two series are highlighted in this way because they show a deviating behavior compared to the other batches (not seeming to approach the calculated equilibrium ratios).

Figures S8 to S11 in the Supporting Information. The results show that at pH 5.2, i-C_4 formation is triggered by the presence of n-C_4 (I, V) and that n-C_4 formation is triggered by the presence of i-C_4 (III, VII). Additionally, a pH of 5.2 induces n-C_6 formation, whereas at pH 6.5, hardly any n-C_6 is formed. At pH 6.5, there is mainly acetate and n-C_4 formation from methanol and CO_2 (II and IV).

In batches V to VIII, substrate was limited to the amount that came from the inoculum. Here, methanol was quickly consumed in three batches (V, VI, and VIII) and more slowly in the batch at pH 5.2 with added i-C_4 (VII). The batch with added n-C_4 at pH 6.5 (VI) showed significant n-C_4 consumption (45% of the supplied n-C_4 was consumed), and acetate was formed together with methane. In contrast, the batch with added i-C_4 at pH 6.5 (VIII) showed very little i-C_4 degradation (~98% remained) within the time frame of the experiment (77 days). The substrate-limited batches show that (de)isomerization occurred at pH 5.2 (V, VII) as well as at pH 6.5 (VIII). However, the concentration profiles (Figure S9) of these batches show that (de)isomerization activity occurred only during chain elongation activity when methanol was present but not after methanol was depleted. This is emphasized by the calculated conversions that occurred after methanol was depleted (shown in Figure S7).

Determining That i-C_4 and n-C_4 Ratios Meet Thermodynamic Equilibrium of Isomerization. A second batch series was performed (A–M, Table 2) at pH 5.5 to further investigate the impact of methanol, acetate, and i-C_4. Batches A–F started with different initial amounts of i-C_4 (0–50 mM) and batches G–M started with different initial amounts of acetate and methanol (50–300 mMC) at t = 0. The results of each individual batch are shown in Figures S12–S19 (concentration profiles, as well as headspace partial pressure profiles). All batches showed i-C_4 and n-C_4 formation from acetate and methanol with a similar pattern as during the first series: presence of initial i-C_4 (A–F) caused n-C_4 to be formed first, followed by simultaneous formation of both butyrate species. The results of batches A–F and G–M are summarized in Figure 3A and B, respectively, which show the ratios of i-C_4 and n-C_4 over total C_4 as measured during all batches throughout the operation period. In addition, a horizontal line is added to the figure that represents the ratio at isomerization equilibrium conditions, which is calculated as shown on pages S10 and S11 in the Supporting Information. Eventually the concentrations of the butyrate species seem to approach this equilibrium in most cases. The progress of i-C_4 and n-C_4 to total C_4 ratios in the continuous reactor system are shown in Figure S6 and follow the same relation when operated at low pH (5.2–5.5).

Microbial Community Analysis: Key Players in Reactor Are Related to *Clostridium luticellari* and *Eubacterium limosum*. The biomass from phases II, IV, V, VI, and VII of the continuous reactor, as well as biomass from batches I–VIII were harvested to be used for a 16s rRNA gene microbial community analysis. A heat map of the microbial community analysis data is shown in Table 4 (Table S7 in the Supporting Information shows the complete data). The results show that operating the reactor at increasing pH levels causes a gradual shift in *Clostridiales* composition from *Clostridiaceae 1* (at pH 5.2) to *Eubacteriaceae* (at pH 6.75). The shift was reversed when reactor pH was decreased again. The batch experiments (I–VIII) that were inoculated with biomass from phase VII (pH 5.2) show a selection toward different microbiomes at the end of the batch.

In the reactor at pH 6.75, the most dominant genus is *Eubacterium*. The most abundant OTU (40% relative abundance, Table S8) showed a slight similarity (94.8% identity, 100% query cover) with *Eubacterium limosum*. Operating the reactor at pH 5.2 caused an apparent complete washout of the *Eubacterium* order and strictly selected for *Clostridium sensu stricto 12* dominance. The most abundant *Clostridium sensu stricto 12* OTU (Table S9) showed a very high similarity (99.7% identity, 100% query cover) with *Clostridium luticellarii*.

The batch series showed a microbial selection that was similar to the selection in the continuous reactor to some extent. *Clostridium luticellarii* presence is maintained at pH 5.2, albeit at a lower relative abundance than within the continuous

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Table 4. Heat Map Showing Relative Abundances of All Orders and for *Clostridiales* also All Observed Genera

Order	Family	Genus	Reactor Phases					Batches I-VIII							
			II	IV	V	VI	VII	I	II	III	IV	V	VI	VII	VIII
								Carbon source added				No carbon source added			
			pH 5.5	pH 6.0	pH 6.75	pH 5.5	pH 5.2	pH 5.2	pH 6.5	pH 5.2	pH 6.5	pH 5.2	pH 6.5	pH 5.2	pH 6.5
								n-C4	n-C4	i-C4	i-C4	n-C4	n-C4	i-C4	i-C4
Methanomicrobiales (Archaea)	*Methanomicrobiaceae*	*Methanoculleus*	1%	5%	3%	3%	0%	0%	1%	0%	0%	0%	7%	0%	4%
Thermoplasmatales (Archaea)	*Thermoplasmatales Incertae Se*	*Candidatus Methanogranu*	14%	8%	4%	14%	6%	0%	11%	0%	4%	0%	4%	0%	2%
Clostridiales	*Clostridiaceae 1*	*Clostridium sensu stricto 1*	37%	19%	0%	27%	61%	12%	0%	20%	0%	21%	0%	51%	0%
		Clostridium sensu stricto 1	0%	0%	3%	0%	0%	0%	0%	0%	1%	0%	1%	0%	3%
		Oxobacter	0%	0%	0%	0%	0%	27%	0%	17%	1%	0%	2%	2%	0%
	Clostridiales Incertae S	*Proteiniborus*	0%	0%	0%	0%	0%	0%	1%	0%	5%	0%	0%	0%	0%
	Eubacteriaceae	*Eubacterium*	3%	23%	40%	6%	0%	0%	8%	0%	9%	0%	1%	2%	2%
	Family XI	*Sporanaerobacter*	7%	4%	1%	12%	1%	0%	16%	0%	6%	0%	7%	1%	13%
		uncultured	3%	4%	0%	4%	3%	9%	8%	7%	12%	29%	16%	19%	11%
	Family XIII	*uncultured*	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	Gracilibacteraceae	*Lutispora*	0%	0%	0%	0%	0%	0%	3%	0%	2%	0%	2%	0%	3%
	Lachnospiraceae	*Lachnoclostridium*	0%	1%	3%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		Tyzzerella	0%	0%	3%	0%	0%	0%	1%	0%	5%	0%	0%	0%	3%
	Peptococcaceae	*Desulfitobacterium*	1%	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	10%
		uncultured	0%	0%	1%	0%	0%	0%	1%	0%	2%	0%	3%	0%	1%
	Ruminococcaceae	*Caproiciproducens*	3%	1%	0%	2%	4%	8%	3%	6%	4%	16%	2%	7%	3%
		Hydrogenoanaerobacteriu	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		Oscillibacter	6%	8%	3%	5%	2%	3%	14%	4%	16%	1%	1%	1%	2%
		Ruminiclostridium 1	2%	1%	0%	3%	0%	0%	0%	0%	0%	0%	0%	0%	0%
		uncultured	1%	0%	0%	1%	1%	6%	0%	6%	0%	0%	0%	0%	0%
		other	1%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	Syntrophomonadaceae	*Syntrophomonas*	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	19%	0%	1%
		uncultured	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%	4%
Micrococcales	*Microbacteriaceae*	*Pseudoclavibacter*	0%	0%	0%	0%	1%	8%	0%	10%	0%	3%	0%	1%	0%
Coriobacteriales	*Coriobacteriaceae*	*multiple*	1%	0%	0%	1%	2%	2%	0%	1%	0%	1%	0%	0%	1%
Bacteroidales	*multiple*	*multiple*	9%	14%	18%	11%	9%	7%	8%	5%	2%	8%	5%	4%	7%
Sphingobacteriales	*Lentimicrobiaceae*	*Lentimicrobium*	0%	0%	3%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Bacillales	*Sporolactobacillaceae*	*Sporolactobacillus*	1%	0%	0%	0%	0%	0%	0%	6%	0%	1%	0%	0%	0%
Lactobacillales	*Lactobacillaceae*	*multiple*	0%	0%	1%	0%	0%	1%	0%	1%	0%	1%	0%	0%	1%
Erysipelotrichales	*Erysipelotrichaceae*	*multiple*	1%	1%	1%	1%	4%	2%	0%	2%	0%	1%	1%	1%	0%
Selenomonadales	*multiple*	*multiple*	1%	1%	0%	1%	1%	5%	0%	3%	0%	6%	0%	2%	0%
Burkholderiales	*Alcaligenaceae*	*multiple*	2%	2%	0%	1%	1%	2%	1%	4%	0%	5%	1%	2%	0%
Desulfovibrionales	*Desulfovibrionaceae*	*multiple*	1%	1%	1%	1%	0%	0%	12%	0%	11%	1%	8%	3%	8%
Enterobacteriales	*Enterobacteriaceae*	*multiple*	0%	0%	3%	0%	0%	0%	0%	0%	0%	0%	4%	0%	0%
Spirochaetales	*Spirochaetaceae*	*multiple*	0%	0%	0%	0%	0%	3%	0%	3%	0%	3%	0%	1%	0%
Synergistales	*Synergistaceae*	*multiple*	0%	0%	0%	0%	0%	0%	5%	0%	6%	0%	6%	0%	10%
Mollicutes RF9	*uncultured*	*uncultured*	0%	0%	0%	0%	0%	0%	0%	0%	5%	0%	0%	0%	0%
NB1-n	*uncultured*	*uncultured*	0%	1%	3%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Sum of shown genera			96%	95%	94%	96%	98%	97%	95%	97%	94%	98%	95%	97%	92%
Other			4%	5%	6%	4%	2%	3%	5%	3%	6%	2%	5%	3%	8%
Total OTUs			106774	111347	114771	98517	172237	131170	135252	106025	98354	95327	96885	106780	119243

0 - 10% | 10 -100%

reactor. At pH 6.5, the relative abundance of the *Eubacterium* order increased from 0% (phase VII used as inoculum) to 8% and 9% in batches II and IV, respectively. In addition to these microbes, a large amount of other *Clostridiales* emerged in the batches among which the most abundant were *Oxobacter* (at pH 5.2), *Family XI*, and *Oscillibacter* (at pH 6.5).

■ DISCUSSION

Methanol-Based Chain Elongation with Acetate to n-Butyrate and Isobutyrate at Varying (high) Selectivities Dependent on pH. This study shows that the product spectrum of methanol-based chain elongation can be adjusted using pH as a selection pressure tool. A pH of around 6.75 in the continuous reactor facilitated the dominance of an unknown *Eubacterium* that resembled *Eubacterium limosum*, which is a known methanol-based chain elongation microbe.[35] The results suggest that the microbiome dominated by *Eubacterium* does not form isobutyrate (i-C_4) under these conditions. A pure culture experiment with *E. limosum* done at pH 5.5 also showed no i-C_4 formation, only n-butyrate (n-C_4) formation (Figure S20).

Lowering the pH in the reactor toward 5.5 changed the microbiome toward a state where a *Clostridium luticellarii*-related species was dominant. At this pH, there was still a small amount of *Eubacteria* present (6%) and more so in the sampled biofilm that had grown within the pH control volume (Figure

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S1 and Table S7). Further lowering the pH to 5.2 led to a steady state in phase VII without the *Eubacterium* genus (0% relative abundance) and with the OTU related to *C. luticellarii* at a 55.5% relative abundance. A pure culture of *C. luticellarii* has recently been shown to be able to form i-C_4 and n-C_4 under supplementation of acetate and methanol.[44]

Suppressing Methanogenesis at pH 5.2 Increased Chain Elongation Selectivity. Shifting the reactor pH from 5.5 to 5.2 significantly lowered methanogenic activity as well as the relative abundances of *Archaea*. The microbiome analysis showed two potential candidates for methanogenic activity: (i) hydrogenotrophic methanogenesis by the *Methanoculleus* genus (98.8% identity, 99% query cover, *Methanoculleus palmolei* DSM 4273),[45] and (ii) methylotrophic methanogenesis by an unknown *Candidatus methanogranum* (closest similarity to *Methanomassiliicoccus luminyensis*, 87.76% identity, 99% query cover).[46,47] The decrease in methanogenic activity was simultaneous with a decrease in methanol consumption, suggesting that the observed methanogenesis at pH 5.5 was of a methylotrophic nature.[48] This shift from pH 5.5 to 5.2 effectively lowered methanogenic activity, while maintaining chain elongation activity. Methylotrophic methanogenensis can occur down to pH 4.2.[49] However, its inhibition is known to be amplified by high concentrations of undissociated acids[26,50] and by sufficient mixing that prevents niche and biofilm formation.[51] As a result, total chain elongation selectivity increased from 84% to 96% (percentage of the summed formed i-C_4, n-C_4, and n-C_6 per total amount of formed products, based on carbon atoms). Overall, the selection pressure in the reactor was sufficient to select for methanol-based chain elongators and to prevent methanogenesis from becoming the dominant bioprocess.

i-C_4 and n-C_4 Ratios Meet Thermodynamic Equilibrium of Isomerization. In the continuous reactor experiment, the formation of i-C_4 and n-C_4 at pH 5.5, as well as at pH 5.2, seemed to occur toward concentrations that approached the thermodynamic equilibrium of isomerization (Figure S6A and S6B). The batch series A−M also showed i-C_4 and n-C_4 formation progressing toward a similar ratio, as well as a previous study on methanol-based chain elongation.[23] Moreover, an experiment that was done with cell extracts and was designed to determine butyryl-CoA:isobutyryl-CoA mutase activity showed that in this setup isobutyryl-CoA to butyryl-CoA ratios approach a similar value.[34] The genome of *C. luticellarii* (DSM 29923) has recently been sequenced and harbors genes encoding for enzymes within the isobutyryl-CoA mutase (ICM)-like subfamily (NCBI Reference Sequence: WP_106007777.1 and WP_106008210.1).[52] In contrast, the sequences of two known *Eubacterium limosum* species (taxid: 1736 and 903814) do not contain any similarities to genes of the ICM-like subfamily (NCBI blastp queries with various fused isobutyryl-CoA mutases, icmF, show no similarity). On the basis of the stoichiometric and microbial analysis of this research and the work on pure *C. luticellarii* (DSM 29923),[44] it is suggested that this species forms both i-C_4 and n-C_4 during methanol-based chain elongation of acetate coupled to a bidirectional isomerization reaction dictated by thermodynamic conditions. For this fermentation, i-C_4 and n-C_4 are effectively the main end products (apart from the formation of small n-C_6 amounts), which allows their ratios to approach thermodynamic equilibrium. In contrast, during other isobutyrate-forming fermentations, e.g., methanogenesis systems[30,31] and bioelectrochemical systems,[15,16] the metabolic fluxes are also geared toward the formation of alternative products from butyrate as the intermediate, which could prevent equilibrium of isomerization from being established.

Isobutyrate Formation Was Observed to Be Coupled to Catabolic Activity of Methanol Chain Elongation at Low pH. Isomerization of n-C_4 and i-C_4 can occur in both directions and is regularly observed within methanogenic cultures.[34,53] Within methanogenic cultures, (de)isomerization of butyrate species is coupled to beta-oxidation and consecutive hydrogenotrophic and acetotrophic methanogenesis.[30,33] In this research, however, the microbiome performed methanol-based chain elongation, and methanogenesis was mainly methylotrophic. It took the microbiome 3 weeks in a batch (VI, at pH 6.5, Figure S9) to develop observable beta-oxidation activity, which shows this activity was negligible during continuous reactor operation. The increase in relative abundance of *Syntrophomonas* and *Methanoculleus*[45,54] (Table 4, batch VI) supports this observation. Moreover, deisomerization of i-C_4 occurred slowly, if not at all (batches VII and VIII).

In the first batch series (I−VIII), which was done at low substrate concentrations, equilibrium of isomerization could not be achieved before the substrate was depleted; in the absence of methanol, catabolic activity (chain elongation) was halted and so was (de)isomerization activity. It shows that the i-C_4 formation is driven by the active formation of the butyrate species via methanol-based chain elongation. The enzyme butyryl-CoA:isobutyryl-CoA mutase that performs reversible isomerization requires activation of the substrates via a Coenzyme A bond.[55] It is known that transition from exponential phase (during substrate abundance) to stationary phase (when energy sources become limited) can lead to drastic changes of the metabolic fluxes,[56] accompanied by large changes in intracellular CoA-derivative concentrations.[57,58] The metabolic changes during substrate limitation, combined with other regulatory mechanisms,[59,60] could potentially have reduced the flux toward (de)isomerization and explain the lack of passive (de)isomerization. However, to experimentally verify this hypothesis, pure culture experiments with *Clostridium luticellarii* to determine intracellular concentrations and enzyme activity assays to determine K_m and k_{cat} values would need to be performed.

Isomerization of n-C_4 to i-C_4 May Provide a Competitive Advantage to Chain Elongation Microbes at Low pH and a High Undissociated Acid Concentration. A physiological reason for i-C_4 formation can be found in exploring what selection advantage is gained under the supplied conditions. Isobutyrate formation was stimulated in an environment with a high concentration of fatty acids and a pH that approaches the pK_a values of these acids (see Figure S4 for the undissociated acids concentration in the reactor). It is known that branched fatty acids have a lower toxic effect on microorganisms than unbranched forms.[61] Partly, this can be explained because i-C_4 is predicted to have a lower diffusivity through cellular membranes compared to n-C_4,[62] thereby lessening the strain on maintenance due to futile cycling.[26] Thus, hypothetically, switching toward an i-C_4 forming catabolism could give a competitive advantage to the chain elongation microbe that is growing at low pH and at high undissociated acid concentrations. This would explain the dominance of i-C_4 formation at the lower applied pH, and its absence at a higher pH where an anabolic investment of

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making an additional enzyme by organisms to perform the isomerization is not energetically rewarded.

Future Outlook. During the last phase (VII, at pH 5.2) of the reactor, the highest i-C_4 volumetric productivity was 111 ± 15 mmol C L^{-1} day^{-1} or 2.4 ± 0.3 g L^{-1} day^{-1} with an average broth concentration of 191 ± 28 mM C or 4.2 ± 0.6 g L^{-1} and a selectivity toward i-C_4 of 0.65 carbon per carbon of total products. The achieved volumetric productivity was 20% higher (continuous reactor), and the achieved concentration was 80% higher (batch) than in an earlier study on i-C_4 formation via methanol-based chain elongation.[13] The batch experiments showed i-C_4 production up to a concentration of around 400 mM C, 8.8 g L^{-1} (and n-C_4 up to a concentration of around 150 mM C, 3.3 g L^{-1}), where chain elongation became constrained despite acetate and methanol still being available. It shows the limitations of methanol-based chain elongation in the batch set up but also that a higher broth concentration within the continuous system can likely be achieved. The necessary increased productivity may be reached by an increase in the amount of active biomass via biomass retention with, for example, granulation techniques.[63,64] Further exploring the metabolic pathway(s) and investigating the potential physiological reasons for i-C_4 formation may also give more insight in the fundamental principles that drive the selection of the developed reactor microbiome. Finally, more researches on effective product recovery methods and specific case-tailored solutions are necessary to determine how valorization of organic residues by chain elongation can become economically feasible.[65]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c00907.

Additional information regarding Materials and Methods and additional figures and graphs for the Results and Discussion, 22 figures, and 13 tables (PDF)

Accession Codes

All data generated or analyzed during this study are included in this published article (and the Supporting Information). Microbiota data (raw 16s rRNA gene amplicon sequences) are submitted to the EBI database (https://www.ebi.ac.uk/ena) under accession number PRJEB36205.

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Author Contributions

K.D.d.L. planned and performed the continuous reactor experiment, planned and helped executed the batch experiments, analyzed the results, and wrote the manuscript. S.M.d.S. contributed to the experimental design of the first batch series, its execution, data interpretation, drafted the results sections, and revised the manuscript. S.v.O. contributed to the experimental design of the second batch series, its execution, data interpretation, drafted the results sections, and revised the manuscript. M.J.M. contributed with many preliminary batch experiments that led to the design of this research, among which the pure EL culture batch. He helped with the data interpretation and revised the manuscript. C.J.N.B. participated as a coapplicant of the ALWGR.2015.8 project and planning of the study, contributed to the experimental design and data interpretation, and revised the manuscript. D.P.B.T.B.S. is principal investigator and designer of the ALWGR.2015.8 project, secured project funding, planned the study, contributed to the experimental design and data interpretation, and revised the manuscript. All authors have read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SCCs = short chain carboxylates
MCCs = medium chain carboxylates
C_2 = acetate
C_3 = propionate
n-C_4 = n-butyrate
i-C_4 = isobutyrate
n-C_6 = caproate
YE = yeast extract
HRT = hydraulic retention time
OTU = operational taxonomic unit
MeOH = methanol

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