INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19F–131/2019/22–6–1613–1622
DOI: 10.17957/IJAB/15.1242
http://www.fspublishers.org

Full Length Article

Valorization of Mixed Volatile Fatty Acids by Chain Elongation: Performances, Kinetics and Microbial Community

Shuai Bao[1], Guangming Zhang[2], Panyue Zhang[1,3*], Qingyan Wang[1], Yazhou Zhou[1], Xue Tao[1], Siqi Wang[1], Mohammad Nabi[1], Yajing Cai[1] and Wei Fang[1]

[1]*College of Environmental Science and Engineering, Beijing Forestry University, Beijing 100083, China*
[2]*School of Environment and Resource, Renmin University of China, Beijing 100872, China*
[3]*School of Environmental and Chemical Engineering, Chongqing Three Gorges University, Chongqing 404632, China*
[*]For correspondence: baoshuai16@163.com

Abstract

Chain elongation of short-chain carboxylates into medium-chain carboxylates is an emerging bioenergy technology. Most work focus on single acid chain elongation, however, primary fermentation products from abundant biomass are mixed volatile fatty acids. Chain elongation was carried out with mixture of acetate, propionate, *n*-butyrate as electron acceptor and with ethanol as electron donor. The mixed acids showed the best chain elongation performances with a targeted *n*-caproate production of 56.69 mmol L^{-1}, much higher than those with single acid. With the mixed acids as electron acceptor, the chain elongation was promoted and the substrate oxidation as competition process was significantly relieved. The chain elongation bacteria *Clostridium sensu stricto* 12 was highly enriched with abundance up to about 55%, while some acidogens and hydrotrophic methanogens for competition processes were restrained. These results can provide a novel technical route combined biomass fermentation and chain elongation to valorize fermentation intermediates, and to improve the economic and sustainability of biomass utilization. © 2019 Friends Science Publishers

Keywords: Chain elongation; Competition processes; Microbial community; Mixed acids

Introduction

Biomass wastes, such as sewage sludge, kitchen wastes, organic municipal solid wastes, agricultural and garden wastes, contain abundant organic matters with a large amount of energy (Wang *et al.*, 2018a; Ye *et al.*, 2018). Recycling this part of energy into beneficial biofuel is critical to sustainable development (Tilman *et al.*, 2009). Traditional utilization technology of biomass wastes is anaerobic digestion and product is biogas with methane as main energetic component, which contains a lower energy density and is of low economic value (Zaks *et al.*, 2011). Thus, intermediates of biomass fermentation, volatile fatty acids (VFAs), has caught more and more attentions in recent years (Chen *et al.*, 2017; Huang *et al.*, 2018), which is of higher economic value than methane. Chain elongation can further valorize the VFAs to medium-chain carboxylates (MCCs), such as *n*-caproate, *n*-heptanoate, and *n*-caprylate. The MCCs can be used as antimicrobials and corrosion inhibitors, or as precursor of many important biochemicals, including biodiesel and bioplastic (Levy *et al.*, 1981; Witholt and Kessler, 1999) with higher added value or higher energy density than methane and VFAs (Spirito *et al.*, 2014). The economic value of MCCs is much higher than that of VFAs, for example, the price of *n*-caproate is 3,200 Dollars/t, much higher than that of acetate (800 Dollars/t), propionate (1,160 Dollars/t) and *n*-butyrate (1,530 Dollars/t) in industry market of China. Furthermore, the MCCs are more easily separated and extracted from fermentation broth than the VFAs, which is beneficial to subsequent utilization and industrial application (Spirito *et al.*, 2014).

As an emerging bioenergy technology, carbon chain elongation has become more and more attractive (Leng *et al.*, 2017; Wu *et al.*, 2018). The chain elongation of short-chain carboxylates (SCCs) to MCCs commonly occurs via reverse β oxidation pathway with ethanol as source of carbon, energy and reducing equivalents (Angenent *et al.*, 2016). The researches on chain elongation have shown that acetate and ethanol can be biologically converted to *n*-butyrate, *n*-caproate and hydrogen, and propionate and ethanol can be converted to acetate, *n*-butyrate, *n*-valerate, *n*-caproate, *n*-heptanoate and hydrogen (Agler *et al.*, 2014; Kucek *et al.*, 2016). Normally pure ethanol and single SCC are using as the substrates for chain elongation in open culture. Steinbusch *et al.* (2011) showed that *n*-caproate and *n*-caprylate were continuously produced for 115 d in open culture from pure ethanol and acetate. Grootscholten *et al.* (2013c) further improved the production efficiency of single acid chain elongation and displayed a *n*-caproate production

To cite this paper: Bao, S., G. Zhang, P. Zhang, Q. Wang, Y. Zhou, X. Tao, S. Wang, M. Nabi, Y. Cai and W. Fang, 2019. Valorization of mixed volatile fatty acids by chain elongation: performances, kinetics and microbial community. *Intl. J. Agric. Biol.*, 22: 1613–1622

up to 55 g L^{-1} d^{-1} with ethanol and acetate and a *n*-heptanoate production of 4.5 g L^{-1} d^{-1} with ethanol and propionate. The chain elongation of different single SCCs is feasible with satisfactory efficiency.

The natural fermentation broth of biomass, however, is normally a mixture of VFAs. Li *et al.* (2016) got a mixture of VFAs by hydrolytic acidification of excess sludge pretreated by high pressure homogenization, Wang *et al.* (2019) produced mixed VFA from grass clipping with ultrasound-calcium hydroxide pretreatment, and Fang *et al.* (2018) showed that the product from solid-state fermentation of solid digestate pretreated by white rot fungi was also a VFA mixture. All these fermentation products from biomass were mainly composed with acetate, propionate, *n*-butyrate and so on. The utilization of single acid means that the fermentation broth should be separated and purified for the production of pure single acid (Li *et al.*, 2016). Therefore, if the mixed acids can be directly used for chain elongation, the chain elongation process will become more economic and practical. But there is still a blank in the researches on chain elongation with mixed VFAs as substrate. The usage of different single acids provides a diversity of MCC products (Coma *et al.*, 2016) and Wu *et al.* (2018) verified that the mixed electron acceptors of acetate, *n*-butyrate, and *n*-caproate in liquor-making wastewater promoted the *n*-heptylate generation. These researches indicate that the mixture of acetate, propionate and *n*-butyrate as electron acceptor might be beneficial to chain elongation. And these VFAs may synergistically function to enhance MCC production due to characteristics of individual acid. It's meaningful and necessary to investigate the performances of chain elongation with these mixed acids as electron acceptor.

In this paper, a mixture of acetate, propionate and *n*-butyrate, three major VFAs in biomass fermentation broth, were used as electron acceptor, ethanol as electron donor, *n*-caproate, the most attractive product was selected as targeted product because of its high production and the high proportion of even-carbon substrate in fermentation broth (Cavalcante *et al.*, 2017). Open culture was used for its robustness. The aims of this paper were to investigate the performances, kinetics and advantages of chain elongation with mixed acids as electron acceptor. The microbial community analysis was conducted to clarify the improvement of chain elongation and inhibition of competition progress with mixed acids elongation. This research provides a promising approach for chain elongation with fermentation intermediates of biomass to produce high-valued MCCs, improving economic and sustainability of biomass utilization.

Materials and Methods

Open Culture Inocula

Initial inoculation sludge was the granular sludge from an internal circulation reactor for anaerobic fermentation in Beijing. The inoculation sludge was then acclimatized for chain elongation at 35°C in several 2 L anaerobic bottles for almost two months. Acetate and ethanol were added as the electron acceptor and donor in an acetate/ethanol molar ratio of 1:3 with an initial addition of 50 mmol L^{-1} acetate and 150 mmol L^{-1} ethanol for each batch. Then the expanded culture of inoculum was further done under the same condition for one month. After enlargement cultivation, *n*-butyrate was the main products for chain elongation. After that the inocula were collected and centrifuged (15 min, 11600 g). The supernatant was discarded to avoid interference to the substrate, and the solid was resuspended with a fixed volume of deionized water as the open culture inocula in this study.

Medium

The medium for batch chain elongation in each bottle contained 100 mL mineral stock and different VFAs and ethanol as substrate. The mineral stock contained minerals and nutrients with the following composition per liter: 3600 mg $NH_4H_2PO_4$, 600 mg $MgCl_2$, 150 mg KCl, 200 mg $CaCl_2 \cdot 2H_2O$, 4000 mg K_2CO_3 and 1 mL trace element solution (Steinbusch *et al.*, 2011). Our preliminary experiments showed that the product from anaerobic fermentation of sewage sludge was a mixture of acetate, propionate and *n*-butyrate with a proportion about 5:2:3. Therefore, in this study, we performed the carbon chain elongation batch experiments with a mixture of acetate, propionate and *n*-butyrate with a proportion of 5:2:3, to simulate the chain elongation of actual fermentation broth of sewage sludge. The sum of initial acid concentration was set as 60 mmol L^{-1}. Thus the mixed acids were a combination of 30 mmol L^{-1} acetate, 12 mmol L^{-1} propionate and 18 mmol L^{-1} *n*-butyrate.

Besides, 1 g L^{-1} yeast extract was added to improve the growth of MCC-producing bacteria (Grootscholten *et al.*, 2013b). 10 g L^{-1} 2-Bromoethanesulfonate (2-BrES) was added as the inhibitor of acetoclastic methanogenesis (Zinder *et al.*, 1984).

Batch Experiments

Batch experiments were conducted in 300 mL glass bottles with ~100 mL working volume and ~200 mL headspace. Four experimental groups were implemented using single acetate, single propionate, single *n*-butyrate and mixed acids as electron acceptor and ethanol as electron donor, respectively. The molar ratio of acid to ethanol was 1:3 according to our preliminary experiments. This ratio was set near the optimal 3:7 for pure *C. kluyveri* culture (Yin *et al.*, 2017), but higher with the consideration of ethanol oxidation. Control was carried out without any addition of electron donor and acceptor. All experiments were performed in duplicate. The initial inocula/substrate ratio was 1:4 based on volatile solids (VS). Thus each bottle contained 100 ml medium and inoculum sludge of 9.25 to

14.97 mL respectively. After inoculation, the system pH was adjusted to 6.5–7.0 with 2 mol L^{-1} NaOH or HCl, and the bottles were flushed with nitrogen gas for 3 min to remove oxygen and closed with rubber stoppers and aluminum caps before fermentation. All bottles were incubated at 35ºC in a rotating shaker (140 r min^{-1}) for 12 d. Every one (occasionally half of one) or two days, 2 mL liquid was taken out using syringe with needles for the analysis of pH change, substrate degradation, MCC formation and microbial community development.

Kinetic Analysis

Modified Gompertz model Eq. (1) was applied to assess the chain elongation performances with single and mixed acids. The specific MCC yield was fitted into the models via software of MATLAB R2016a (Yang and Wang, 2017a, b; Wang *et al.*, 2018a; Wang *et al.*, 2019).

$$H = P \exp\left\{-\exp\left[\frac{(\lambda - t)R_m e}{P} + 1\right]\right\} \qquad (1)$$

Where, H is specific MCC yield at chain elongation time (mg/L); P is specific MCC production potential (mg/L); t is chain elongation time (d); λ is lag time (d) and R_m is maximum specific MCC production rate (mg L^{-1} d^{-1}).

Chemical Analysis

The pH of liquid sample was measured by an on line pH meter M200 with a sensor of InPro3250i/SG/120 (Mettler Toledo, Switzerland). The liquid sample was centrifuged (15 min, 11000 g) and the supernatant was diluted with ultrapure water to 10% (v/v) for fatty acid analysis. Fatty acids (from acetate to *n*-caprylate) and ethanol were analyzed by gas chromatography (GC-2018, Schimadzu (China) Co., China) with a WondaCap-WAX column (30 m×0.25 mm×0.25 μm, Schimadzu, China) and a flame ionization detector (FID). 1 mL filtered aqueous samples (0.45 μm+0.22 μm) was placed in a vial together with 100 μL freshly prepared 3% phosphoric acid. Prepared sample of 0.5 μL was injected by an auto injector (AOC-20i, Schimadzu, China) at 250ºC with a split ratio of 35. The oven temperature was held at 90ºC for 1 min, increased by 10ºC min^{-1} from 90 to 240ºC and then was kept at 240ºC for 10 min. The carrier gas was nitrogen with a column flow rate of 1.6 mL min^{-1}. FID temperature was set at 250ºC and the flow rate of hydrogen and synthetic air was set as 40 and 400 mL min^{-1}, respectively.

High Throughput Sequencing of Microbial Community

To analyze composition of microbial community during chain elongation fermentation, the centrifuged solid of sludge samples were all stored at -80°C before DNA extraction. The sludge samples were collected at the end of rapid reaction phase in different chain elongation systems. Microbial DNA extraction was performed using the E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, U.S.). The V4-V5 region (Liu *et al.*, 2017) of the bacteria 16S ribosomal RNA gene were amplified by PCR using primers 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). And the primer 524F10extF_Arch958RmodR was used to obtain archaeal information. PCR reactions were performed in triplicate with 20 μL mixture containing 5×FastPfu Buffer of 4 μL, 2.5 mmol L^{-1} dNTPs of 2 μL, each primer (5 μmol L^{-1}) of 0.8 μL, FastPfu Polymerase of 0.4 μL and template DNA of 10 ng. After purification and quantification of PCR products, amplicon sequencing was conducted using an Illumina MiSeq PE250 platform (Shanghai Majorbio Bio-pharm Technology Co. Ltd., China) (Wang *et al.*, 2018b).

Fluorescent Quantitative Analysis

The extracted DNA was also carried out with fluorescent quantitative analysis by real time PCR, to obtain an absolute quantity of bacteria and archaea. The primers were same as that in high throughput sequencing. PCR reactions were performed in triplicate with 20 μL mixture containing 2×ChamQ SYBR Color qPCR Master Mix of 16.5 μL, each primer (5 μmol L^{-1}) of 0.8 μL and template DNA of 2 μL. After amplification, CT values were transformed to copies through standard curve and then converted to copies/g of each sample. The proportion of bacteria and archaea in microbial community was calculated by copies g^{-1}.

Results

Performances of Carbon Chain Elongation with Single Acid as Electron Acceptor

Concentration variations of substrates and products along 12-day chain elongation period are shown in Fig. 1. Odd-carbon carboxylates and *n*-caprylate were not all presented because of their low concentration fluctuating around zero. As shown in Fig. 1, the reaction period can be divided into lag phase, rapid reaction phase and stable phase. However, the duration of these three phases was different for the three single acids. Both the lag phase and rapid reaction phase with acetate as electron acceptor was the shortest with about 3 and 2 d, respectively and prolonged to about 4 and 2 d with *n*-butyrate as electron acceptor, while was the longest with propionate as electron acceptor with about 5 and 3 d. In the lag phase, there was hardly MCC generation, but a quantity of substrates were converted because of the chain elongation bacteria adaption, bacterial proliferation, as well as substrate oxidation. In the rapid reaction phase, the concentration of VFAs and ethanol greatly reduced and a large amount of MCCs were rapidly generated. During the stable phase, the composition of system tended to be stable, and the concentration of each compound remained basically unchanged because of substrate deficiency.

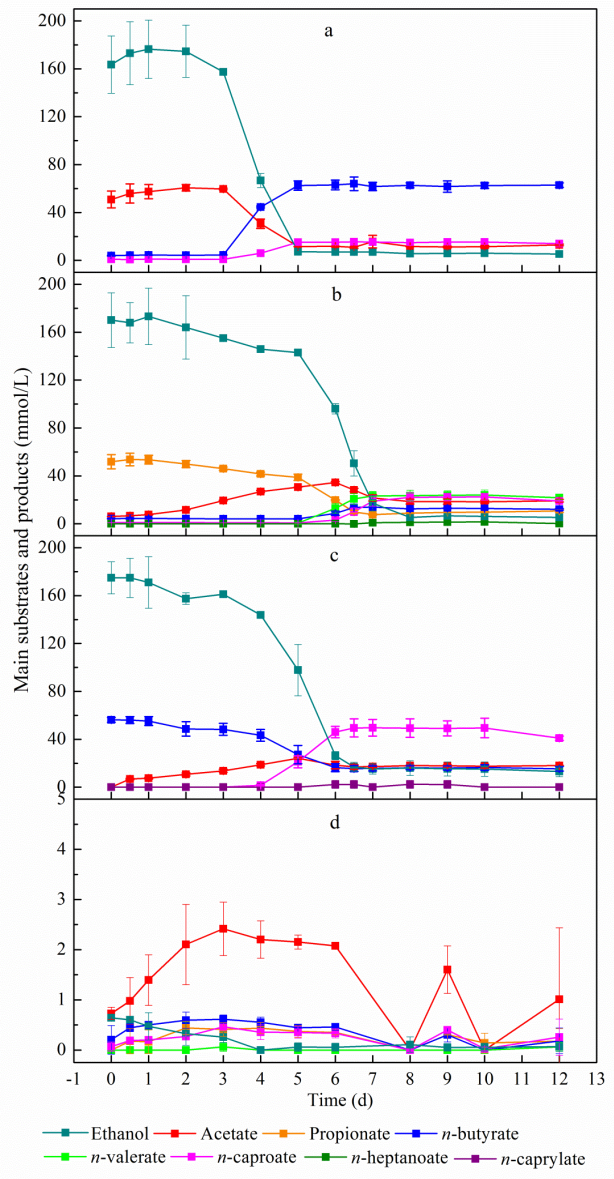

Fig. 1: Concentration variation of main substrates and products along chain elongation of single acid with an acid/ethanol ratio of 1:3 (**a**. acetate, **b**. propionate, **c**. n-butyrate, **d**. control)

As shown in Fig. 1d, the concentration of different components varied only between 0 to 2.5 mmol L^{-1} in the control, thus, the degradation of inocula can be neglected. The main products with acetate as electron acceptor were *n*-butyrate and *n*-caproate. The concentration of *n*-butyrate was clearly higher than that of other components (Fig. 1a). The highest concentration of *n*-butyrate and *n*-caproate was 63.98 and 15.57 mmol L^{-1} on day 6.5, respectively. In the rapid reaction phase, *n*-butyrate was first generated from day 3 to day 4 rapidly, and then *n*-caproate was sequentially generated from day 4 to day 6 (Fig. 1a). This phenomenon accorded with the chain elongation rule of adding two carbons at one time and with the regulation of acetate elongation to *n*-butyrate and the latter elongation to *n*-caproate. But actually, the bottleneck of conversion from *n*-butyrate to *n*-caproate was not broken with acetate as electron acceptor.

The main products with propionate as electron acceptor were not only *n*-valerate, but also *n*-butyrate and *n*-caproate (Fig. 1b). The composition of carboxylate complex reached almost stable on day 8 with a *n*-valerate concentration of 23.97 mmol L^{-1}, a *n*-butyrate concentration of 12.51 mmol L^{-1}, and a *n*-caproate concentration of 22.15 mmol L^{-1}. The acetate was continuously generated from the start of inoculation to day 6. Besides ethanol, the propionate was slowly consumed, especially without any chain elongation until day 5. Therefore, besides ethanol oxidation, anaerobic oxidation of propionate could be another important pathway for acetate production, which then enable the chain elongation of even-carbon carboxylate. On the other hand, *n*-valerate was first generated from day 5 rapidly, followed by the *n*-butyrate production from day 6 to day 6.5 and *n*-caproate production from day 6 to day 7 (Fig. 1b). Finally, there were also trace of *n*-heptanoate production by further *n*-valerate chain elongation.

The main products with *n*-butyrate as electron acceptor was *n*-caproate, and the highest *n*-caproate concentration was 49.63 mmol L^{-1}, obviously higher than that with acetate and propionate as electron acceptor (Fig. 1c). In the lag phase, the acetate was continuously generated, while the concentration of ethanol and *n*-butyrate decreased slowly almost without any chain elongation (Fig. 1c). This phenomenon is similar with that with propionate as electron acceptor, where both ethanol and propionate or *n*-butyrate oxidation occurred. In the rapid reaction phase, from day 4 on, the concentration of *n*-butyrate and ethanol began to decrease rapidly until day 6, resulting in a rapid generation of *n*-caproate by *n*-butyrate chain elongation. While the acetate concentration kept increasing to 24.11 mmol L^{-1} until day 5 and then began to decrease until day 6.5, which could be named as acetoclastic caprogenesis. It's obvious that the acetate elongation to *n*-butyrate might be driven by the *n*-butyrate reduction due to the *n*-butyrate elongation to *n*-caproate.

Performances of Carbon Chain Elongation with Mixed Acids as Electron Acceptor

Fig. 2 shows the concentration variations of substrates and products with mixed acids as electron acceptor, which showed similar trend as that with single acid. However, the *n*-caproate production was higher, and the bottleneck of conversion to *n*-caproate was broken through and the highest *n*-caproate concentration reached 56.69 mmol L^{-1}. The lag phase was further shortened to about 2 d, even shorter than that with acetate as electron acceptor; the rapid reaction phase lasted from day 2 to day 4. As shown in Fig. 2a, there was a slow consumption of substrates in the lag phase due to slight substrate oxidation.

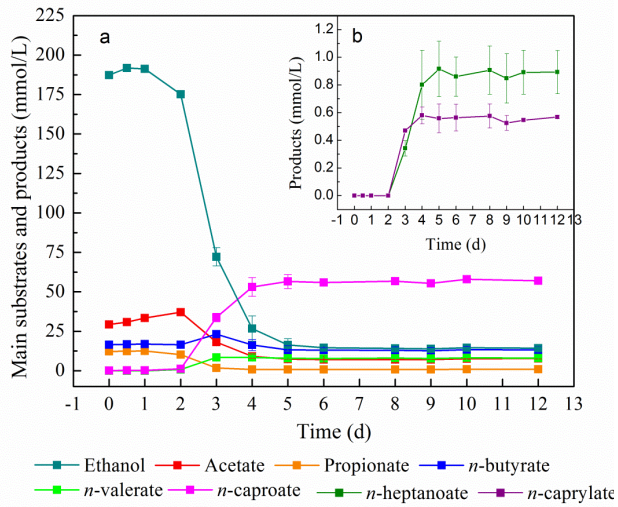

Fig. 2: Concentration variation of main substrates and products along chain elongation of mixed acids with an acid/ethanol ratio of 1:3 (**a**. production of *n*-butyrate, *n*-valerate and *n*-caproate, **b**. production of *n*-heptanoate and *n*-caprylate)

Among them, the ethanol consumption was the highest, followed by propionate and butyrate oxidation. It seems like that the propionate partially replaced *n*-butyrate to be oxidized in the mixed acids chain elongation system. The chain elongation of acetate, propionate and *n*-butyrate simultaneously started to produce *n*-butyrate, *n*-valerate and *n*-caproate, respectively. As shown in Fig. 2b, there was also a small amount of *n*-heptanoate (0.91 mmol L^{-1}) and *n*-caprylate (0.58 mmol L^{-1}) production.

Comparison of Chain Elongation with Single Acid and Mixed Acids as Electron Acceptor

We choose the highest efficiency in stable phase to compare the chain elongation performances with different electron acceptor, as shown in Table 1. It was found that the main products with acetate as electron acceptor were *n*-butyrate (63.98 mmol L^{-1}) and *n*-caproate (15.57 mmol L^{-1}) on day 6.5, with propionate as electron acceptor were *n*-butyrate (12.74 mmol L^{-1}), *n*-valerate (23.97 mmol L^{-1}) and *n*-caproate (22.48 mmol L^{-1}) on day 10, with *n*-butyrate as electron acceptor was *n*-caproate (49.36 mmol L^{-1}) on day 8, and with mixed acids as electron acceptor was *n*-caproate (56.69 mmol L^{-1}) on day 8. It is obvious that the targeted *n*-caproate production with mixed acids as electron acceptor was the highest with a relative lower ethanol consumption (165.82 mmol L^{-1}). The chain elongation with mixed acids as electron acceptor realized the breakthrough of conversion efficiency to *n*-caproate.

To further evaluate the targeted *n*-caproate production, the *n*-caproate production efficiency was

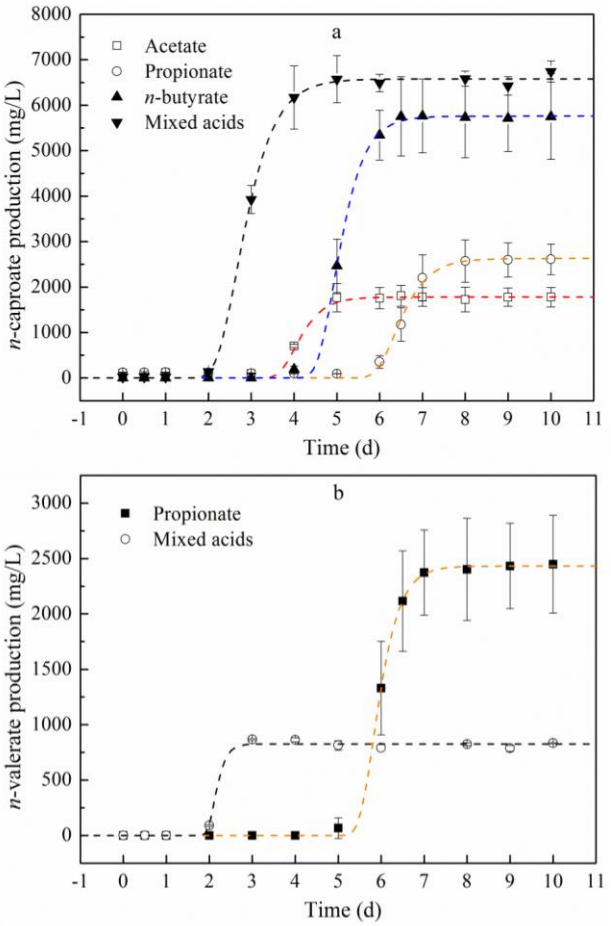

Fig. 3: Specified MCC production fitted by modified Gompertz model with single and mixed acids as electron acceptor (**a**. *n*-caproate, **b**. *n*-valerate)

defined as the ratio of carbon equivalent quantity (Ceq) of *n*-caproate produced to that of initial carbon source added, as shown in Eq. (2):

$$n\text{-caproate production efficiency} = \frac{Ceq_{n-caproate}}{(Ceq_{ethanol}+Ceq_{SCCs})_{added}} \quad (2)$$

Where $Ceq_{n\text{-caproate}}$ is the Ceq of *n*-caproate produced, $(Ceq_{ethanol}+Ceq_{SCCs})_{added}$ is the sum of initial Ceq of ethanol and SCCs added.

The *n*-caproate production efficiency with *n*-butyrate and mixed acids as electron acceptor was obviously higher with 51.88 and 63.32%, respectively, while that with acetate and propionate was only 21.16 and 25.77%, respectively, because a large portion of acetate was only elongated to *n*-butyrate and the propionate was converted to *n*-valerate by chain elongation and acetate by substrate oxidation. In a conclusion, from both concentration and production efficiency of targeted *n*-caproate, the mixed acids were the optimal option of electron acceptor.

Table 1: Concentration of main substrates and products at the highest chain elongation efficiency

Electron acceptor	Ethanol (mmol L^{-1})	Acetate (mmol L^{-1})	Propionate (mmol L^{-1})	*n*-butyrate (mmol L^{-1})	*n*-valerate (mmol L^{-1})	*n*-caproate (mmol L^{-1})
Acetate (Day 6.5)	6.99 ± 0.52	10.87 ± 0.77	-	63.98 ± 5.73	1.29 ± 0.01	15.57 ± 1.99
Propionate (Day 10)	6.15 ± 1.58	18.37 ± 1.13	9.81 ± 0.43	12.74 ± 0.29	23.97 ± 4.32	22.48 ± 2.90
n-Butyrate (Day 8)	15.94 ± 6.25	18.03 ± 0.30	-	16.24 ± 1.67	0.80 ± 1.14	49.36 ± 7.68
Mixed acids (Day 8)	14.18 ± 1.64	7.15 ± 0.02	0.80 ± 0.12	13.00 ± 2.17	8.07 ± 0.12	56.69 ± 1.41

Table 2: Kinetic analysis for specific MCC production with single acid and mixed acids as electron acceptor

Model	Production	Parameters	Acetate	Propionate	*n*-butyrate	Mixed acids
Modified Gompertz model	*n*-caproate	P (mg L^{-1})	1780	2628	5761	6577
		R_m (mg L^{-1} d^{-1})	2068	2229	5401	4965
		λ (d)	3.65	5.922	4.544	2.188
		R^2	0.9927	0.9920	0.9995	0.9994
		Adjusted R^2	0.9921	0.9904	0.9994	0.9992
	n-valerate	P (mg L^{-1})	-	2432	-	826
		R_m (mg L^{-1} d^{-1})		2665		1990
		λ (d)		5.495		1.96
		R^2		0.9997		0.9962
		Adjusted R^2		0.9996		0.9958

Table 3: Archaeal abundance of two important species in different chain elongation systems

Species abundance	Acetate	Propionate	*n*-butyrate	Mixed acids	Control
Methanobacterium beijingense	45.17%	45.09%	44.03%	48.73%	46.76%
Methanosaeta harundinacea 6Ac	6.32%	8.79%	6.67%	3.53%	3.85%

Table 4: Copies and proportions of bacteria and archaea by fluorescent quantitative analysis

Group	Copies (L g^{-1})		Proportion (%)	
	515_907	524F10extF_Arch958RmodR	515_907	524F10extF_Arch958RmodR
Acetate	3.72E+08	3.08E+08	54.71	45.29
Propionate	1.96E+09	1.23E+09	61.44	38.56
n-butyrate	1.07E+09	8.30E+08	56.32	43.68
Mixed acids	3.74E+08	1.37E+08	73.19	26.81
Control	1.67E+07	1.59E+07	51.23	48.77

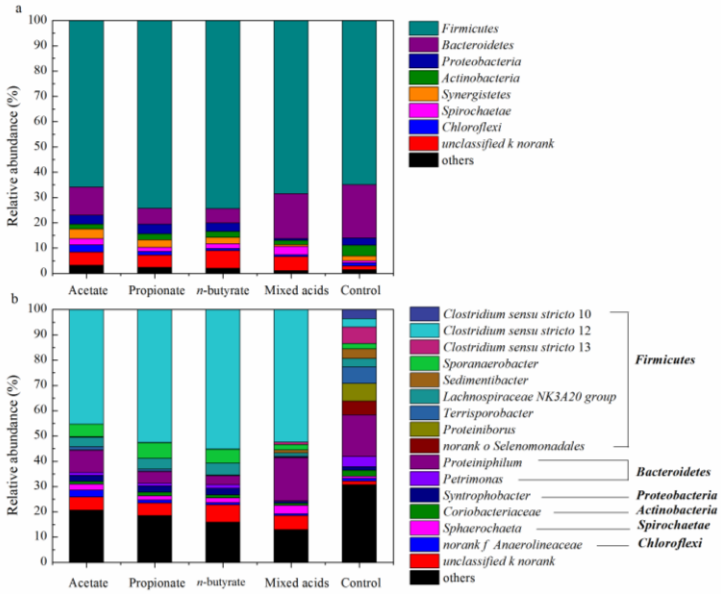

Fig. 4: Bacterial abundance in different chain elongation systems (**a**. on phylum level, **b**. on genus level)

Discussion

Some researches on chain elongation with real fermentation broths have been carried out (Grootscholten *et al.*, 2013a; Grootscholten *et al.*, 2014), however, the MCC production efficiency is difficult to be regulated and controlled because of the substrate fluctuation and the complexity of fermentation effluent. And the mechanisms of chain

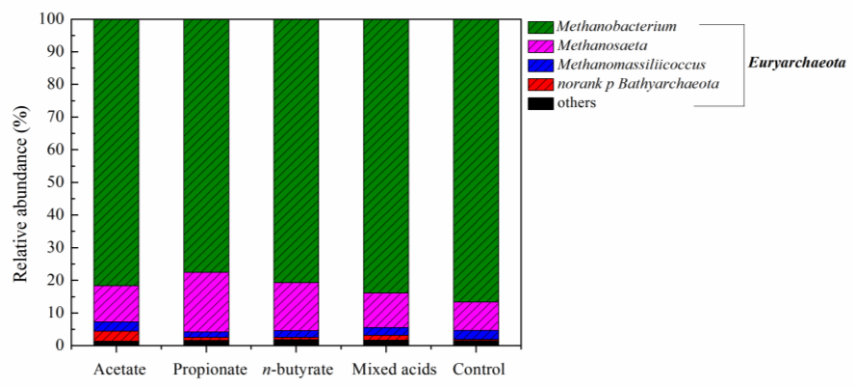

Fig. 5: Archaeal abundance on genus level in different chain elongation systems

elongation with the mixed substrates are still unclear. In this study, simulating the actual biomass fermentation broth with pure mixed VFAs, the reaction kinetic analysis and microbial community analysis were well utilized to clarify the corresponding mechanisms. What's more, the substrate oxidation process would lead to an extra substrate consumption, decreasing the chain elongation efficiency (Grootscholten *et al*., 2014; Angenent *et al*., 2016). The *n*-caproate production efficiency of mixed acids was even higher than that in a fed-batch experiment with hydrogen addition (62%) by Steinbusch *et al*. (2011), which might be derived from weaker competition process with lower substrate oxidation during shorter lag phase. Therefore, apart from the hydrogen addition, the promotion of *n*-caproate production efficiency in this study might be derived from the synergistic effect of mixed acids and weaker competition process.

Modified Gompertz model was applied intensively to fit hydrogen production and VFA production from biomass fermentation (Yang and Wang, 2017a, b; Wang *et al.*, 2018a; Wang *et al.*, 2019). We found that the modified Gompertz model was also suitable for fitting chain elongation for MCC production with different electron acceptors and R^2 was higher than 0.992. Especially the R2 for chain elongation with mixed acids was up to 0.999 (Fig. 3, Table 2). The fitted curves are shown in Fig. 3 and the the relevant kinetic parameters are displayed in Table 2. The specific MCC production potential (P) and the maximum specific MCC production rate (R_m) are crucial in evaluating the chain elongation performances. The obvious high *n*-caproate production potential (P) and maximum *n*-caproate production rate (R_m) achieved by the chain elongation with *n*-butyrate and mixed acids as electron acceptor, followed by that with propionate and acetate. The simulated results agreed with the performances of *n*-caproate yield in the results. The highest R_m of 5401 mg L^{-1} d^{-1} with *n*-butyrate as electron acceptor was very near to that with mixed acids (4965 mg L^{-1} d^{-1}), which contains only 30% (molar ratio) *n*-butyrate. One of the reasonable explanations for the high R_m is that a high *n*-butyrate concentration in substrate was favorable to saving acetate chain elongation to reach the minimum *n*-butyrate concentration required by *n*-caproate production (Cavalcante *et al.*, 2017) and the saved ethanol could promote the *n*-caprogenic process.

The *n*-valerate production potential (P) and maximum *n*-valerate production rate (R_m) were only observed in the system with propionate and mixed acids as electron acceptor. Both with mixed acids as electron acceptor were lower than those with propionate, which agreed with the performances of *n*-valerate yield in the results. The lower P with mixed acids is intelligible due to the lower propionate concentration in mixed acids (20% molar ratio), while the lower R_m was probably attributed to the suppression by even-carbon chain elongation. It is recognized that the existence of propionate goes against *n*-caproate production, because the odd-carbon acids (propionate and *n*-valerate) result in the decrease of total even-carbon acid proportion and compete for ethanol substrate due to even-carbon chain elongation. But in this study, a small portion of propionate addition did not severely inhibit the even-carbon chain elongation. Instead, the propionate chain elongation was inhibited by the chain elongation of acetate and *n*-butyrate. Furthermore, the propionate addition might be beneficial to the overall caprogenesis, because the existence of propionate was in favor of reducing the *n*-butyrate oxidation and the propionate oxidation slowly released the acetate, supplementing the even-carbon resource.

For the production of *n*-caproate and *n*-valerate, the lag time (λ) with acetate and mixed acids as electron acceptor was obviously shorter than that with propionate and *n*-butyrate. The shorter lag time represented the better acclimatization to environment and the better growth of chain elongation bacteria. It was found that the shorter chain carboxylate, acetate, was preferred to be utilized by chain elongation bacteria through reverse β oxidation pathway (Angenent *et al.*, 2016), which could stimulate and improve bacteria activity. And this preference was probably driven from the higher substrate uptake rate of acetate elongation to *n*-butyrate (Wu *et al.*, 2018). Therefore, the shorter λ of mixed acids was probably attributed to the addition of acetate, which improved the total chain elongation

efficiency from the aspect of bacteria growth and metabolism.

The modeling results indicated that the mixed acids significantly enhanced the *n*-caproate production rate and shortened the lag time and the better *n*-caproate production performance with mixed acids was actually a synergistic effect of acetate, propionate and *n*-butyrate. What's more, the mixed acids with a little lower *n*-caproate production rate than *n*-butyrate however presented a higher *n*-caproate yield and production efficiency. This phenomenon must be attributed to the much higher substrate oxidation degree of *n*-butyrate group, which then decreased the total chain elongation efficiency. Therefore, the analysis of competition process is of crucial importance to clarify the mechanism of the better *n*-caproate production promoted by mixed acids.

The microbial communities with different electron acceptors were compared using Illumina Hiseq16S DNA genes technique. The microbial communities converged after chain elongation open culture. The number of operational taxonomic units (OTUs) was 367, 348, 319 and 303 with acetate, propionate, *n*-butyrate, and mixed acids as electron acceptor, respectively, with 186 same OTUs. The usage of mixed acids as electron acceptor decreased the microbial diversity, but did not significantly change the microbial structure. This might be attributed to the better acclimatization of microorganisms with mixed acids as electron acceptor. Bacterial abundance in different chain elongation systems are shown in Fig. 4 as well as the archaeal abundance in Fig. 5.

For the bacterial community of chain elongation systems with different electron acceptors, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Synergistetes* and *Spirochaetae* were the dominant phyla, accounting for more than 90% of the total bacteria microbial sequences. And *Firmicutes* (65.89–74.50%) and *Bacteroidetes* (5.57–17.67%) were the top two phyla. At the level of phylum, the community composition with different electron acceptors was similar, but more different from the control, indicating that the stimulation of ethanol had a great influence on the transformation of microbial community.

On genus level, the dominant bacteria included *Clostridium sensu stricto* 12 (Firmicutes), *Proteiniphilum* (Bacteroidetes), *Sporanaerobacter* (Firmicutes), *Lachnospiraceae NK3A20 group* (*Firmicutes*), *Syntrophobacter* (Proteobacteria), and *Sphaerochaeta* (Spirochaetae). Among them, *Clostridium sensu stricto* 12 and *Proteiniphilum* were the two main genus in chain elongation system. *Clostridium sensu stricto* 12 was obviously enriched with different electron acceptors, compared to the control. *Clostridium sensu stricto* 12 accounted for the most part of bacteria with an abundance with 45.36, 52.58, 55.26 and 52.41%, when acetate, propionate, *n*-butyrate and mixed acids were used as electron acceptor, respectively. As stated by Angenent *et al.* (2016), most of the bacteria performing chain elongation belonged to class *Clostridia* in *Firmicutes*.

Clostridium sensu stricto 12 was reported to have the ability of chain elongation to *n*-butyrate and *n*-caproate (Wang *et al.*, 2018c; Yang *et al.*, 2018). In this study, the ability of this genus in odd-carbon chain elongation was also proved. The abundance of *Clostridium sensu stricto* 12 with acetate as electron acceptor was relative lower, which might be relevant to the lower efficiency of chain elongation from *n*-butyrate to *n*-caproate. Besides, as the genus with close relatives of *Clostrudium sensu stricto* 12, *Clostrudium sensu stricto* 10 and *Clostrudium sensu stricto* 13 may also have the ability of chain elongation. Among them, *Clostrudium sensu stricto* 10 was only found in the system with mixed acids as electron acceptor with an abundance of 0.07% and *Clostrudium sensu stricto* 13 with mixed acids showed the highest abundance of 0.99%, surpassing the abundance of 0.05% with other single acid as electron acceptor.

Proteiniphilum showed an obvious outstanding abundance of 17.13% with mixed acids, compared with single acid as electron acceptor, however, was close to the abundance of 16.49% of the control. *Proteiniphilum* use proteins and amino acids as a fermentative substrate to produce carbon dioxide, hydrogen, and acetate (Cardinali-Rezende *et al.*, 2016). The abundance of *Proteiniphilum* with different electron acceptors was almost inversely correlated with the concentration of acetate in chain elongation systems, with an acetate concentration of 11.52, 18.58. 17.01 and 7.38 mmol L^{-1} with acetate, propionate and *n*-butyrate as electron acceptor, respectively. This indicates that the acetate deficiency might drive acetate production by *Proteiniphilum* with a relatively high abundance, when the acetate and mixed acids were used as electron acceptor.

There were also some genus, such as *Sporanaerobacter*, *Petrimonas* and *Syntrophobacter*, the abundance of which was relative lower with mixed acids than that with single acid as electron acceptor. The *Syntrophobacter* as a syntrophism bacteria can form a syntrophism system only with hydrotrophic methanogens to conduct the oxidation of ethanol, propionate or *n*-butyrate to acetate and hydrogen (Liu *et al.*, 2017). The syntrophism mechanism of *Syntrophobacter* is interspecies H_2/formate transfer (IH/FT) only with the methanogens using both hydrogen and formate rather than only hydrogen (Dong and Stams, 1995). The lowest *Syntrophobacter* abundance of 0.56% with mixed acids as electron acceptor provided an explanation for its lowest extra carbon consumption in the view of microbial metabolism. While the abundance of *Syntrophobacter* reached about 2.50% with single acid as electron acceptor. What's more, family *Synergistaceae* in phylum *Synergistetes* was detected, which also contains some syntrophism bacteria utilizing acetate or other SCCs to produce hydrogen and carbon dioxide (Ito *et al.*, 2011), the abundance of which was also the lowest with mixed acids as electron acceptor.

At genus level, *Methanobacterium* and *Methanosaeta* dominated in archaeal community, accounting for more than

92% of the total archaea sequences with all electron acceptors. *Methanobacterium*, as a typical hydrotrophic methanogens, accounted for a proportion of about 80% archaeal abundance. While the acetoclastic methanogens, *Methanosaeta*, was inhibited by 2-BrES, accounting for only 11.5, 18.27, 14.65 and 10.59% with acetate, propionate, *n*-butyrate and mixed acids as electron acceptor, respectively. Furthermore, two important species, *Methanobacterium beijingense* (Methanobacterium) and *Methanosaeta harundinacea* 6Ac (Methanosaeta), were detected. The abundance of these two important species in different chain elongation systems are shown in Table 3. The species Methanobacterium beijingense accounted for about 45% with different electron acceptors, which can use H_2/CO_2 and formate for growth and methane production (Ma *et al.*, 2005). Therefore, the IH/FT syntrophism system with hydrogen-producing bacteria, such as *Syntrophobacter* (Dong and Stams, 1995), could exist in this study. The IH/FT syntrophism system made the substrate oxidation process thermodynamically feasible by consuming H_2 to maintain a relative low hydrogen partial pressure ($<10^{-1}$ atm) (Grootscholten *et al.*, 2014). Therefore, although the acetoclastic methanogens were inhibited, the carbon source (ethanol, propionate and *n*-butyrate) could still be consumed by IH/FT process.

Besides, the relative abundance of *Methanosaeta harundinacea 6Ac* was 6.32, 8.79, 6.67 and 3.53% with acetate, propionate, *n*-butyrate and mixed acids as electron acceptor, respectively. Although *Methanosaeta harundinacea* is an acetoclastic methanogen, it contains a gene to utilize carbon dioxide (Rotaru *et al.*, 2014). Thus a certain abundance of this species under 2-BrES inhibition might be relevant with the process of direct interspecies electron transfer in chain elongation system.

It's obvious that the abundance of *Methanobacterium* in archaeal community was similar with different electron acceptors, but actually the competition of methanogenesis should be relatively weak with mixed acids. Therefore, the absolute amount of bacteria and archaea was detected by fluorescent quantitative analysis. Copies number of objective genes 515_907 (for bacteria) and 524F10extF_Arch958RmodR (for archaea) was measured and relative proportion was calculated, as shown in Table 4. The archaeal abundance of 26.81% with mixed acids were significantly lower than that with single acid as electron acceptor. This was an important proof of more serious inhibition to competition processes with mixed acids as electron acceptor, because the overall syntrophic system was inhibited. It is certain that the syntrophism system is an entirety, and suppression of hydrogenotrophic methanogenesis will lead to the overall suppression through the accumulated hydrogen partial pressure. The high activity of chain elongation bacteria for higher chain elongation efficiency may inhibit the competition process of interspecies H_2/formate transfer (IH/FT) syntrophic system. It's worth noting that our results are different from the statement of Agler *et al.* (2012) that hydrogenotrophic methanogenesis and chain elongation can precede simultaneously in mixed cultures. This might be due to the existing of other uninhibited substrate-oxidizing organisms than MCC producers in the batch open culture system, and this uninhibition was closely related with a high enough CO_2 content in gas phase due to the addition of 4000 mg/L K_2CO_3, which is accorded with the statement of Grootscholten *et al.* (2014). Additionally, the high proportion of hydrotrophic methanogens with acetate as electron acceptor gave an explanation for the accumulation of *n*-butyrate, as shown in Fig. 1a, that it might be attributed to the low partial pressure of hydrogen in chain elongation systems (Steinbusch *et al.*, 2011).

Conclusion

The mixed acids as electron acceptor promoted chain elongation by open culture, the lag time shortened to 2.188 d and the total reaction time to about 5 d and the highest concentration of targeted *n*-caproate reached 56.69 mmol L^{-1} with a *n*-caproate production efficiency of 63.32%. The kinetic analysis showed the synergistic effect of mixed acids. The microbial community analysis corresponding proved the microorganisms for the chain elongation were promoted and those for competitive substrate consumption were suppressed. It is proved that the mixed acids from primary fermentation of biomass are more suitable to be as the electron acceptor of chain elongation by open culture than single acid.

Acknowledgements

This research is financially supported by the Fundamental Research Funds for the Central Universities (2017CGP017) and the National Natural Science Foundation of China (51578068).

References

Agler, M.T., C.M. Spirito, J.G. Usack, J.J. Werner and L.T. Angenent, 2014. Development of a highly specific and productive process for n-caproic acid production: applying lessons from methanogenic microbiomes. *Water Sci. Technol.*, 69: 62–68

Agler, M.T., C.M. Spirito, J.G. Usack, J.J. Werner and L.T. Angenent, 2012. Chain elongation with reactor microbiomes: upgrading dilute ethanol to medium-chain carboxylates. *Energ. Environ. Sci.*, 5: 8189–8192

Angenent, L.T., H. Richter, W. Buckel, C.M. Spirito, K.J. Steinbusch, C.M. Plugge, D.P. Strik, T.I. Grootscholten, C.J. Buisman and H.V. Hamelers, 2016. Chain elongation with reactor microbiomes: open-culture biotechnology to produce biochemicals. *Environ. Sci. Technol.*, 50: 2796–2810

Cardinali-Rezende, J., P. Rojas-Ojeda, A.M. Nascimento and J.L. Sanz, 2016. Proteolytic bacterial dominance in a full-scale municipal solid waste anaerobic reactor assessed by 454 pyrosequencing technology. *Chemosphere*, 146: 519–525

Cavalcante, W.A.D., R.C. Leitão, T.A. Gehring, L.T. Angenent and S.T. Santaella, 2017. Anaerobic fermentation for n-caproic acid production: A review. *Proc. Biochem.*, 54: 106–119

Chen, Y., X. Jiang, K. Xiao, N. Shen, R.J. Zeng and Y. Zhou, 2017. Enhanced volatile fatty acids (VFAs) production in a thermophilic fermenter with stepwise pH increase–Investigation on dissolved organic matter transformation and microbial community shift. *Water Res.*, 112: 261–268

Coma, M., R. Vilchez-Vargas, H. Roume, R. Jauregui, D.H. Pieper and K. Rabaey, 2016. Product diversity linked to substrate usage in chain elongation by mixed-culture fermentation. *Environ. Sci. Technol.*, 50: 6467–6476

Dong, X. and A.J. Stams, 1995. Evidence for H_2 and formate formation during syntrophic butyrate and propionate degradation. *Anaerobe*, 1: 35–39

Fang, W., P. Zhang, X. Zhang, X. Zhu, J.B.V. Lier and H. Spanjers, 2018. White rot fungi pretreatment to advance volatile fatty acid production from solid-state fermentation of solid digestate: Efficiency and mechanisms. *Energy*, 162: 534–541

Grootscholten, T., D. Strik, K. Steinbusch, C. Buisman and H. Hamelers, 2014. Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol. *Appl. Energ.*, 116: 223–229

Grootscholten, T., F.K.D. Borgo, H. Hamelers and C. Buisman, 2013a. Promoting chain elongation in mixed culture acidification reactors by addition of ethanol. *Biomass Bioenerg.*, 48: 10–16

Grootscholten, T., K. Steinbusch, H. Hamelers and C. Buisman, 2013b. Chain elongation of acetate and ethanol in an upflow anaerobic filter for high rate MCFA production. *Bioresour. Technol.*, 135: 440–445

Grootscholten, T., K. Steinbusch, H. Hamelers and C. Buisman, 2013c. Improving medium chain fatty acid productivity using chain elongation by reducing the hydraulic retention time in an upflow anaerobic filter. *Bioresour. Technol.*, 136: 735–738

Huang, L., Z. Chen, D. Xiong, Q. Wen and Y. Ji, 2018. Oriented acidification of wasted activated sludge (WAS) focused on odd-carbon volatile fatty acid (VFA): Regulation strategy and microbial community dynamics. *Water Res.*, 142: 256–266

Ito, T., K. Yoshiguchi, H.D. Ariesyady and S. Okabe, 2011. Identification of a novel acetate-utilizing bacterium belonging to synergistes group 4 in anaerobic digester sludge. *ISME J.*, 5: 1844–1856

Kucek, L.A., C.M. Spirito and L.T. Angenent, 2016. High n-caprylate productivities and specificities from dilute ethanol and acetate: chain elongation with microbiomes to upgrade products from syngas fermentation. *Energ. Environ. Sci.*, 9: 3482–3494

Leng, L., P. Yang, Y. Mao, Z. Wu, T. Zhang and P.H. Lee, 2017. Thermodynamic and physiological study of caproate and 1, 3-propanediol co-production through glycerol fermentation and fatty acids chain elongation. *Water Res.*, 114: 200–209

Levy, P., J. Sanderson, R. Kispert and D. Wise, 1981. Biorefining of biomass to liquid fuels and organic chemicals. *Enzyme Microb. Technol.*, 3: 207–215

Li, G., P. Zhang, Y. Wang, Y. Sheng, X. Lv and J. Yin, 2016. Enhancing biological denitrification with adding sludge liquor of hydrolytic acidification pretreated by high-pressure homogenization. *Intl. Biodeter. Biodegr.*, 113: 222–227

Liu, Y., P. He, L. Shao, H. Zhang and F. Lü, 2017. Significant enhancement by biochar of caproate production via chain elongation. *Water Res.*, 119: 150–159

Ma, K., X. Liu and X. Dong, 2005. *Methanobacterium beijingense sp. nov.*, a novel methanogen isolated from anaerobic digesters. *Intl. J. Syst. Evol. Microbiol.*, 55: 325–329

Rotaru, A.E., P.M. Shrestha, F. Liu, M. Shrestha, D. Shrestha, M. Embree, K. Zengler, C. Wardman, K.P. Nevin and D.R. Lovley, 2014. A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to Methanosaeta for the reduction of carbon dioxide to methane. *Energ. Environ. Sci.*, 7: 408–415

Spirito, C.M., H. Richter, K. Rabaey, A.J. Stams and L.T. Angenent, 2014. Chain elongation in anaerobic reactor microbiomes to recover resources from waste. *Curr. Opin. Biotechnol.*, 27: 115–122

Steinbusch, K.J., H.V. Hamelers, C.M. Plugge and C.J. Buisman, 2011. Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass. *Energ. Environ. Sci.*, 4: 216–224

Tilman, D., R. Socolow, J.A. Foley, J. Hill, E. Larson, L. Lynd, S. Pacala, J. Reilly, T. Searchinger and C. Somerville, 2009. Beneficial biofuels—the food, energy and environment trilemma. *Science*, 325: 270–271

Wang, S., F. Li, D. Wu, P. Zhang, H. Wang, X. Tao, J. Ye and M. Nabi, 2018a. Enzyme pretreatment enhancing biogas yield from corn stover: feasibility, optimization, and mechanism analysis. *J. Agric. Food Chem.*, 66: 10026–10032

Wang, S., G. Zhang, P. Zhang, X. Ma, F. Li, H. Zhang, X. Tao, J. Ye and M. Nabi, 2018b. Rumen fluid fermentation for enhancement of hydrolysis and acidification of grass clipping. *J. Environ. Manage.*, 220: 142–148

Wang, Y.Q., F. Zhang, W. Zhang, K. Dai, H.J. Wang, X. Li and R.J. Zeng, 2018c. Hydrogen and carbon dioxide mixed culture fermentation in a hollow-fiber membrane biofilm reactor at 25°C. *Bioresour. Technol.*, 249: 659–665

Wang, S., X. Tao, G. Zhang, P. Zhang, H. Wang, J. Ye, F. Li, Q. Zhang and M. Nabi, 2019. Benefit of solid-liquid separation on volatile fatty acid production from grass clipping with ultrasound-calcium hydroxide pretreatment. *Bioresour. Technol.*, 274: 97–104

Witholt, B. and B. Kessler, 1999. Perspectives of medium chain length poly (hydroxyalkanoates), a versatile set of bacterial bioplastics. *Curr. Opin. Biotechnol.*, 10: 279–285

Wu, Q., W. Guo, X. Bao, X. Meng, R. Yin, J. Du, H. Zheng, X. Feng, H. Luo and N. Ren, 2018. Upgrading liquor-making wastewater into medium chain fatty acid: Insights into co-electron donors, key microflora, and energy harvest. *Water Res.*, 145: 650–659

Yang, G. and J. Wang, 2017a. Co-fermentation of sewage sludge with ryegrass for enhancing hydrogen production: Performance evaluation and kinetic analysis. *Bioresour. Technol.*, 243: 1027–1036

Yang, G. and J. Wang, 2017b. Enhanced hydrogen production from sewage sludge by co-fermentation with forestry wastes. *Energ. Fuel.*, 31: 9633–9641

Yang, P., L. Leng, G.Y.A. Tan, C. Dong, S.Y. Leu, W.H. Chen and P.H. Lee, 2018. Upgrading lignocellulosic ethanol for caproate production via chain elongation fermentation. *Intl. Biodeter. Biodegr.*, 135: 103–109

Ye, J., A. Hu, X. Cheng, W. Lin, X. Liu, S. Zhou and Z. He, 2018. Response of enhanced sludge methanogenesis by red mud to temperature: Spectroscopic and electrochemical elucidation of endogenous redox mediators. *Water Res.*, 143: 240–249

Yin, Y., Y. Zhang, D.B. Karakashev, J. Wang and I. Angelidaki, 2017. Biological caproate production by *Clostridium kluyveri* from ethanol and acetate as carbon sources. *Bioresour. Technol.*, 241: 638–644

Zaks, D.P., N. Winchester, C.J. Kucharik, C.C. Barford, S. Paltsev and J.M. Reilly, 2011. Contribution of anaerobic digesters to emissions mitigation and electricity generation under US climate policy. *Environ. Sci. Technol.*, 45: 6735–6742

Zinder, S.H., T. Anguish and S.C. Cardwell, 1984. Selective inhibition by 2-bromoethanesulfonate of methanogenesis from acetate in a thermophilic anaerobic digestor. *Appl. Environ. Microb.*, 47: 1343–1345

[Received 08 Jul 2019; Accepted 13 Aug 2019; Published (online) 22 Dec 2019]