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Graphical Abstract

**Unveiling the mechanisms of medium-chain fatty acid production from waste
activated sludge alkaline fermentation liquor through physiological,
thermodynamic and metagenomic investigations**

Shu-Lin Wu^{1,a}, Jing Sun^{1,2,a,*}, Xueming Chen³, Wei Wei¹, Lan Song^{4,5}, Xiaohu Dai^{1,2},
Bing-Jie Ni^{1,2,*}

¹State Key Laboratory of Pollution Control and Resources Reuse, College of
Environmental Science and Engineering, Tongji University, Shanghai 200092, PR
China

²Shanghai Institute of Pollution Control and Ecological Security, Shanghai 200092, P.R.
China

³Process and Systems Engineering Center (PROSYS), Department of Chemical and
Biochemical Engineering, Technical University of Denmark, 2800 Kgs. Lyngby,
Denmark

⁴School of Environmental Science and Engineering, Southern University of Science
and Technology, Shenzhen 518055, PR China

⁵Shenzhen Institute of Sustainable Development, Shenzhen 518055, PR China

***Corresponding Authors:**

Bing-Jie Ni, Phone: +86 21 65986849; e-mail: bjni@tongji.edu.cn

Jing Sun, Phone: +86 21 65986849; e-mail: jingsun@tongji.edu.cn

a. These authors contributed equally to this work.

ABSTRACT

Effective sludge treatment with bioenergy production is attracting increasing interests as large quantities of waste activated sludge (WAS) are produced during the wastewater treatment. In this study, a new biotechnical process for converting the WAS alkaline fermentation liquor (WASAFL) into valuable, easy-separated medium chain fatty acids (MCFAs) through chain elongation (CE) was investigated, which may provide a new insight into sludge treatment. In the process, ethanol was served as the electron donor (EDs) and WASAFL were main electron acceptors (EAs). The MCFAs productions were investigated under three different ED to EA ratios (i.e., 1:2, 1:1 and 2:1). The result showed that MCFAs production was increased from 2.88 ± 0.01 to 5.28 ± 0.18 g COD/L with the increase of ED to EA ratio. However, the highest MCFA selectivity was achieved at 72.9 % when the ED to EA ratio was 1:1. The decrease in the selectivity at high ED:EA ratio is mainly due to the production of higher alcohol (i.e., n-butanol and n-hexanol). The thermodynamic analysis confirmed all CE processes for MCFAs production from WASAFL were exothermic reactions, with the spontaneity and energy release of the reactions increased with the ethanol level. The microbial community analysis showed that the relative abundances of *Clostridium*, *Oscillibacter*, *Leptolinea* and *Exilispira* were positively correlated with the MCFAs production. The metagenomic analysis suggested that both the reverse β -oxidization pathway and fatty acid biosynthesis pathway contributed to the CE process in the studied system. The functional enzymes were mainly associated within *Clostridium*, with *Clostridium Kluyveri*, *Clostridium botulinum* and *Clostridium magnum* being likely the key species responsible for the CE process.

Keywords: Waste activated sludge; alkaline fermentation liquor; medium chain fatty acids; reverse β -oxidization; fatty acid biosynthesis; metagenomics

1. Introduction

Large quantities of waste activated sludge (WAS) were produced due to the wide application of the activated sludge process for the biological treatment of wastewater. It has been estimated that, in China only, WAS production would increase from around 40 million tons to more than 60 million tons from 2015 to 2020 posing serious threats to the natural environment (Li et al. 2018, Sun et al. 2019). Thus, effective sludge treatment, especially the implementation of renewable technologies, is of great importance for sludge disposal. Although WAS has been regarded as a low-grade biomass, it contains substantial amount of organic substances and can produce energy approximately equivalent to 150 kWh/PE/year (PE: population equivalent) (Lazarova et al. 2012). Therefore, the development of processes to produce valuable bioenergy and bioresource from WAS has attracted increasing interests (Luo et al. 2016, Wang et al. 2018b).

Methane production through anaerobic digestion is a conventional method for recovering energy from WAS (Li et al. 2019). However, methane is a product of low value that may likely subside anaerobic digestion into an economically unattractive technology (Spirito et al. 2014). In addition, the potential leakage of methane during its collection and transportation could result in greenhouse effect, given that the global warming potential of methane is 30 times higher than carbon dioxide (Howarth 2014). To overcome such limitations, anaerobic WAS fermentation under alkaline conditions for the production of short chain fatty acids (SCFAs, mainly C2-C5 carboxylic acids) has been developed recently (Yuan et al., 2006, Xu et al., 2018).

During alkaline fermentation (normally pH=10), methane production is fully inhibited so that WAS could be converted to SCFAs efficiently (Yuan et al., 2006). However, SCFAs have very high solubilities in water, and thus the separation and purification of SCFAs from the fermentation broth might be complicated and energy intensive in application (Singhania et al. 2013).

In this sense, a recently developed carboxylate platform to produce medium chain fatty acids (MCFAs) via chain elongation (CE) may provide an alternative solution for bioenergy or resource recovery from WAS (Cavalcante et al. 2017, Ge et al. 2015, Lonkar et al. 2016). MCFAs, such as n-caproate (C6), n-heptanoate (C7), and n-caprylate (C8), have higher caloric value than SCFAs due to their higher carbon/oxygen ratios. They are high monetary value products and can be adopted as superior precursors for further processing to biofuels or directly as industrial commodity (Agler et al. 2012, Ge et al. 2015). Moreover, MCFAs have superior separation characteristics due to their hydrophobic carbon chains (Steinbusch et al. 2011). The separation of MCFAs from fermentation broth could be relatively easily achieved through physical extraction with much less energy consumption compared to SCFAs (Angenent et al. 2016).

In the CE process, MCFAs could be produced by anaerobic microorganisms using SCFAs as electron acceptors (EAs), mainly via the reverse β -oxidation pathway. The reverse β -oxidation pathway utilizes acetyl-CoA produced by the electron donors (EDs) as the cyclic molecule interacting with the EAs, extending its carbon chain length with two carbons every time (Spirito et al. 2014). A number of organic compounds like ethanol, methanol, lactate and glycerol have been successfully used

as EDs for CE in the past (Ge et al., 2015; Chen et al., 2016; Zhu et al., 2015; Leng et al., 2017). Among these compounds, ethanol is considered to be one of the most suitable EDs, as it could usually result in a relatively high MCFA synthesis rate (Wu et al., 2018). Also, ethanol is regarded as the most favorable substrate for n-caproate synthesis by *Clostridium kluyveri*, which is a key microorganism involved in the CE process (Zhu et al., 2015).

As aforementioned, the alkaline fermentation of WAS could produce significant quantities of SCFAs. It has been reported that the SCFA yield could reach ~250 mg COD per gram of volatile suspended solids, with acetate, propionate, butyrate and valerate being the main products (Yuan et al., 2006). Thus, the high SCFA concentration in the WAS alkaline fermentation liquor (WASAFL) suggested it could work as an emerging source of EAs for the production of MCFAs. However, so far, the feasibility of upgrading WASAFL into MCFA has not been demonstrated. Previous studies on MCFA production through the CE process were mainly use a single SCFA as the EA (Grootscholten et al., 2013a; Kucek et al., 2016b). More recently, a few researchers also investigated the MCFA synthesis using feedstocks containing mixed SCFAs, such as acid whey and food waste (Duber et al., 2018, Nzeteu et al., 2018). The SCFAs compositions and concentrations in these systems are quite different from each other, which resulted in different MCFAs production performances. This indicated that the composition of SCFAs in feedstocks can significantly affect the MCFA production efficiency and selectivity, as well as the metabolic pathways involved. As the compositions of SCFAs in WASAFL can be distinct from the systems which have been reported, the process for MCFA production from WASAFL is worthy to be investigated individually.

Therefore, in this study, we explored the feasibility of upgrading WASAFL into MCFA using ethanol as electron donor. The MCFAs production was investigated at increasing carbon-molar ratios of ED to EA, i.e., 1:2, 1:1 and 2:1. Thermodynamic analysis was conducted for assessing the potential spontaneity and competition in the CE processes. In addition, the high-throughput sequencing and metagenomic analyses were also performed to elucidate the taxonomic composition and functional genes in the culture, with the goal of identifying the key metabolic pathways in the CE process for upgrading WASAFL into MCFAs. The results of this study are expected to provide a new insight into the bioenergy and resource recovery from WAS.

2. Material and methods

2.1. Inoculum and WAS alkaline fermentation liquor

The fresh inoculum for CE experiments was harvested from a laboratory-scale semi-continuous anaerobic digester treating the WAS. The WAS used was collected from a wastewater treatment plant treating domestic wastewater located in Shanghai, China. The reactor had an effective volume of 10 L and a sludge retention time (SRT) of 20 days. Its main characteristics of the inoculum were as follows: pH 7.1 ± 0.1 , TS 16.8 ± 0.3 g/L, VS 9.2 ± 0.4 g/L, TCOD 12.5 ± 0.5 g/L, and SCOD 1.21 ± 0.015 g/L. The inoculum was withdrawn immediately before starting the CE experiments. As the function of WASAFL is to provide SCFAs as EA, the WASAFL used for CE experiments was synthetically prepared according to the SCFA concentration observed in real anaerobic WAS alkaline fermentation reactor (Yuan et al., 2006). The primary SCFA components include: acetate 1.16 g/L, propionate 0.30 g/L, butyrate 0.29 g/L and valerate 0.23 g/L. Growth medium was also applied during the incubation to supply trace elements for maintenance and growth of the

microorganisms. Detailed information regarding the medium is summarized in Supporting Information (SI).

2.2. CE experiments for converting WAS alkaline fermentation liquor into MCFAs

To investigate a wide range of carbon molar ratio of ED to EA on the CE process and to avoid the toxicity of ethanol and produced MCFAs on microorganisms, CE experiments were carried out with ethanol as the ED under three different carbon molar ratios of ED to EA, i.e. 1:2 (R1), 1:1 (R2), and 2:1 (R3). The experiments were conducted in a series of 120 ml serum bottles. Each bottle was filled with 4.8 g of inoculum and 37.5 mL of WASAFL with growth medium. Before inoculation, a stock solution of ethanol (50%) was added to the serum bottle to achieve the desired concentration (i.e., 18.82, 37.64 and 75.29 mmol/L for ED to EA ratios of 1:2, 1:1, and 2:1, respectively), while 10.5 g/L 2-bromoethanosulfonic acid (BESA) was also added as a methanogenesis inhibitor. Then pH was adjusted to 5.5 ± 0.2 with 4 M HCl solutions. The preliminary results showed that pH of the system would not significantly change during the CE process. Finally, each serum bottle was flushed with nitrogen gas for 5 min, and then was sealed with a rubber stopper retained by an aluminum crimp-cap.

The CE experiments were conducted in a shaker-incubator at 170 rpm under mesophilic conditions ($37 \pm 1^\circ\text{C}$). The bottles were well mixed by inversion prior to each sampling event. The initial concentrations of SCFAs and ethanol in each bottle were measured to confirm the successful startup of the experiment according to the designed ED to EA ratio. Afterwards, a 1-mL gas sample was taken every 2 to 4 days

to analyze the gas composition (H_2 and CH_4) and a 2-mL liquid sample was collected simultaneously for ethanol, SCFAs and MCFAs analysis, using the methods as described in Section 2.6. The CE experiments lasted over 30 days, with complete depletion of ethanol and MCFAs concentrations leveling off without further accumulation. All experiments were carried out in triplicates.

2.3. Thermodynamic analysis of the CE processes of WAS alkaline fermentation liquor

A thermodynamic analysis was conducted for assessing the spontaneity of the potential catabolic reaction of the CE process using WASAFL as the substrate. The thermodynamic calculations of a certain biochemical reaction were determined according to the method describe by Leng et al. (2017). Briefly, the transformed Gibbs free energy was calculated according to Eq (1) - Eq (2):

$$\Delta_r G_T = \Delta_r G_T^0 + RT \ln Q \quad (1)$$

$$Q = \frac{(\alpha_A)^a (\alpha_B)^b \dots (\alpha_C)^c}{(\alpha_X)^x (\alpha_Y)^y \dots (\alpha_Z)^z} \quad (2)$$

where $\Delta_r G_T^0$ is the standard transformed Gibbs free energy of a reaction at a certain temperature; R is the thermodynamic constant (8.314 J mol/K); T is the temperature of the system, which is 310 K in this study; and Q is the factor related to the activities of products A, B, C, etc. and reactants X, Y, Z, etc (Kleerebezem and Van Loosdrecht 2010).

The standard transformed Gibbs free energy of a reaction at a certain temperature (T, 310K in this study) was calculated based on the standard Gibbs free energy of formation and standard enthalpy of formation of each product and reactant. Firstly, the Gibbs free energy of formation a certain compound was adjusted according to

203 temperature (T) by Eq (3).

$$204 \quad \Delta_f G_{i,T}^0 = \Delta_f G_{i,T_s}^0 \times \frac{T}{T_s} + \Delta_f H_{i,T_s}^0 \times \frac{T_s - T}{T_s} \quad (3)$$

205 Where: $\Delta_f G_{i,T}^0$ is the Gibbs free energy of formation a certain compound after
 206 adjusting temperature; T_s is standard temperature (298.15K); $\Delta_f G_{i,T_s}^0$ is the
 207 standard Gibbs free energy of formation of a compound at the standard
 208 temperature. $\Delta_f H_{i,T_s}^0$ is standard enthalpy of formation of a certain compound. The
 209 $\Delta_f G_{i,T_s}^0$ and $\Delta_f H_{i,T_s}^0$ of all the products and reactants in this study was listed in
 210 Table S2.

211

212 Then effect of pH and ionic strength on the Gibbs free energy of formation was also
 213 considered and calculated based on the Eq (4).

$$214 \quad \Delta_f G_{i,T}'^0 = \Delta_f G_{i,T}^0 - N_{H,i} RT \ln 10^{-pH} - RT \alpha (Z_i^2 - N_{H,i}) I^{\frac{1}{2}} / (1 + B I^{\frac{1}{2}}) \quad (4)$$

215 Where : $\Delta_f G_{i,T}'^0$ is the Gibbs free energy of formation a certain compound after
 216 adjusting pH and ionic strength. $RT\alpha = 9.20483 \times 10^{-3} T - 1.28467 \times 10^{-5} T^2 + 4.95199$
 217 $\times 10^{-8} T^3$, $B = 1.6 \text{ kg}^{\frac{1}{2}} \text{ mol}^{\frac{1}{2}}$, $N_{H,i}$ is the number of hydrogen atoms in a substance, and
 218 Z_i is the charge number.

219

220 Finally, the standard transformed Gibbs free energy of a reaction was calculated based
 221 on the Gibbs free energy of formation of each reactant and product, according to Eq 5

$$222 \quad \Delta_r G_T'^0 = \sum V_i \times \Delta_f G_{i,T}'^0 \quad (5)$$

223 Where $\Delta_r G_T'^0$ is the standard transformed Gibbs free energy of a reaction at a certain
 224 temperature; V_i is the stoichiometry of each reactant and product.

225

2.4. High-throughput sequencing for microbial community analysis

High-throughput sequencing analysis was carried out to determine the microbial community structures of CE sludges for all three ED to EA ratios, as well as the original inoculum, for comparison. After the CE reaction, the bacterial genomic DNA of the four sludge samples were extracted using the FastDNATM Spin Kit for Soil (MP bio, U.S.) according to manufacturer's protocols. The V3-V4 hypervariable regions of the bacteria 16s rRNA gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGT WTCTAAT-3') by thermocycler PCR system (Gene Amp 9700, ABI, USA). Then, all the samples were sequenced on Illumina Miseq platform (Illumina, San Diego, USA). The sequences were quality filtered, trimmed to 250 base pairs and dereplicated using the QIIME pipeline. Chimeric sequences were identified and removed using UCHIME. Operational taxonomic units (OTUs) were clustered through Silva database (SSU123) with 97% similarity cutoff using UPARSE. The further analysis of OTUs were normalized firstly. The raw reads were deposited into National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database with Accession Number: SRP181066.

2.5. Metagenomic analysis for identifying the CE pathways

For metagenomic analysis, the sludge sample was taken from the CE reactor with the ED: EA ratio of 1:1 (referred as R2), in which the produced MCFAs accounted for the highest proportion among all products (i.e., highest MCFA selectivity among the three ED: EA conditions). A sludge sample from original inoculum (referred as R0) was also taken for analysis as a comparison. After sampling, the sludge samples were preserved in liquid nitrogen as soon as possible and then stored at a -80°C freezer for

the following analysis. The methods for DNA extraction, sequencing and bioinformatic analysis were described in detail in SI. The final clean reads numbers were 43,997,308 for R0 and 43,471,326 for R2. Sequence data associated with these samples have been deposited in the NCBI Short Read Archive database (Accession Number: SRP181562).

2.6. Chemical Analysis

The concentrations of H₂ and CH₄ were determined using a gas chromatography (Lunan model SP6890, CN) equipped with a thermal conductivity detector. The temperatures of the injector, detector, and column were kept at 100, 100, and 120 °C, respectively (Sun et al. 2015). Argon was used as the carrier gas. SCFAs, MCFAs and alcohols (ethanol, n-butanol and n-hexanol) were analyzed by using a gas chromatography (SHIMADAU GC 2010 Plus, Japan) with a flame ionization detector, equipped with a 30 m × 0.32 mm × 0.1 µm Stabilwax-DA polyethylene glycol capillary column. The inlet and detector temperatures were set at 250 °C and 300 °C, respectively. The column operating temperature profiles were 70 °C for 3 min, then 10 °C/min to 180 °C, then held for 6.5 min. The liquid samples were acidified with 3% (v/v) formic acid before analysis. The TS, VS, TCOD, SCOD of the inoculum were determined according to the standard methods (Wang et al. 2018a).

3. Results and discussion

3.1. MCFAs production from WAS alkaline fermentation liquor

The profiles of carboxylic acids (SCFAs and MCFAs) and alcohols (ethanol, n-butanol and n-hexanol) under different ED to EA ratios (i.e., 1:2, 1:1 and 2:1) in the CE experiments are shown in Figure 1. At the end of 33-day experimental period, the substrates in all reactors were no longer consumed with a stationary level of MCFAs

production being achieved. The changes in the total COD of carboxylic acids and alcohols from the starting to the end of the experiment were varied in about 90% - 110%, indicating that COD is almost balanced in the studied system (Table S1). Under all tested ED to EA ratios, three different types of MCFAs (i.e., n-caproate, n-heptanoate, n-caprylate) were produced. Obviously, a high ratio of ED to EA promoted the MCFAs production from WASAFL. The total cumulative MCFAs yield significantly increased from 2.88 ± 0.01 g COD/L to 5.19 ± 0.42 g COD/L with respect to the ED: EA ratio of 1:2 to 1:1 and then further increased slightly to 5.28 ± 0.18 g COD/L at the ED: EA ratio of 2:1. This indicated that the ratio of ED to EA was strongly related to MCFAs production, which was consistent with previous reports. Yin et al. (2017) reported that within the appropriate concentration range, the composition and yield of both SCFAs and MCFAs (mainly caproate) were positively related to ethanol/acetate ratio. Similarly, it is also observed by Liu et al. (2016) that the higher ratio of alcohol to acid provided sufficient ED capacity and yield high concentration of n-caproate.

As shown in Figure 1 (a, c, e), the ethanol concentration decreased gradually and depleted in 4-10 days depending on its initial concentration. The decrease of ethanol concentration was in accordance with the production of MCFAs (Figure 1b, 1d, 1f), confirming that ethanol was the primary ED of CE process in the studied system. The butyrate and valerate concentrations decreased in the first 4 days, probably involved in the CE process for the generation of n-caproate and n-heptanoate, respectively. However, afterward, the butyrate concentration increased gradually, especially in the case of ED: EA of 1:1 and 2:1. This increase could be caused by the production of butyrate due to the CE of acetate. The valerate concentration was maintained at the

same level after 4 day.

As for the propionate, the concentration decreased gradually in the first 4-7 days to zero, expect for the case of ED: EA of 1:2. The exhaustion of propionate is due to that the propionate could only serve as the substrate in the CE of carboxylic acids. Theoretically, the propionate might be generated through elongating the carbon chain of formate with two carbon atoms. However, such substance is lack in the WASAFL. In the case of ED: EA of 1:2, the propionate concentration was kept at around 0.15 g COD/L after 4 days, which probably due to that the CE process is ceased as ethanol had been used up.

Interestingly, the acetate concentration was maintained at the same level, or even slightly increased during the entire experiment period. This indicated a considerable amount of acetate was produced in the system, despite it was partially involved in CE process to produce butyrate, n-caproate and n-caprylate. The production of acetate in the studied system could mainly through two different pathways. First, the CE process was initiated with ethanol oxidation. In such process, 1 mol of acetate would be produced with the oxidation of 6 mol of ethanol (Spirito et al. 2014). In addition, H₂ is an important byproduct during the CE process (See Figure 2d and Table S3, SI), it could be served as ED for the formation of acetate through homoacetogenesis (Saady 2013(Angenent et al. 2016, Spirito et al. 2014) . In this study, significant amount of bicarbonate was existed in the growth media, which could serve as EA for homoacetogenesis. This is coincide with the situation in the real WASAFL, as biocarbonate could be produced with considerable amount in both acidogenesis and acetogenesis processes (Batstone et al. 2002).

327

328 It should also be noted that two higher alcohols, i.e., n-butanol and n-hexanol were
329 produced when the highest ED:EA ratio is applied (2:1). The n-butanol concentration
330 reached 0.49 ± 0.02 g COD/L and the hexanol was 0.30 ± 0.02 g COD/L at the end of
331 the experimental period, respectively. The n-butanol and n-hexanol could be
332 generated through the reduction of butyrate and n-caproate, respectively (Gómez and
333 Cantero 2007). Besides, they could also be produced through the CoA-dependent
334 pathway. Namely n-butanol and n-hexanol could be generated from the corresponding
335 longer chain acyl-CoA, i.e. butyryl-CoA and hexanoyl-CoA, via butyraldehyde and
336 hexaldehyde, with the process catalyzed by aldehyde/alcohol dehydrogenases (Gómez
337 and Cantero 2007, Machado et al., 2012). However, it could be speculated that the
338 CoA-dependent pathway might be the major pathway responsible for n-butanol and
339 n-hexanol production in the investigated system. Butyryl-CoA and hexanoyl-CoA is
340 key intermediate products in the CE process to produce n-caproate and n-caprylate
341 (Angenent et al., 2016). The high accumulated concentration of n-caproate and
342 n-caprylate (Figure 1f) suggested that butyryl-CoA and hexanoyl-CoA were
343 substantially produced during the CE processes, which provided essential substrates
344 for n-butanol and n-hexanol production. On the other hand, the carboxylate acid
345 reduction might not be the predominant pathway, as no n-octanol was observed even
346 the concentration of n-caprylate was higher than that of n-caproate. Also, n-butanol
347 was produced although the butyrate concentration was lower than n-caprylate
348 concentration (Figure 1e and 1f).

349

350 **3.2. MCFAs selectivity in the CE process of WAS alkaline fermentation liquor**

351 The proportions of end products in the three sets of CE experiments were calculated

and summarized in Figure 2. Although the total MCFAs production was the highest at the ratio of ED: EA of 2:1(Figure 1), the highest total proportion of MCFAs was observed at the ED: EA ratio of 1:1, accounting for 72.9 % of the total organic carbon contents. In contrast, the proportion of MCFAs was 60.2 % and 65.9 % at the ED: EA ratios of 1:2 and 2:1, respectively. The MCFAs selectivity in our system is higher than that reported by Lonkar et al. (2016), who found that MCFA production was up to 55% when used office paper and chicken manure as substrates. On the other hand, the selectivity was slightly lower than other studies which produced MCFAs from liquor-making wastewater (80%) and municipal solid waste (79%) (Wu et al., 2018, Grootscholten et al., 2014). However, it should be noted that the ethanol in our system is used up by the end of the experiment, while some SCFAs remained. Therefore, the selectivity might further increase if more ethanol was provided to the system. It should be noted that although based on the COD balance, it seems only propionate and ethanol contributed to the production of MCFAs, actually other carboxylic acids (C2-C5) in the WASAFL were also involved in MCFAs production. As these compounds could be simultaneously produced in the studied system (as discussed in Section 3.1), their final concentrations turn out to be similar to the initial concentrations. The decrease of MCFAs proportion at the highest ED:EA ratio (2:1) could be attributed to the production of higher alcohols, i.e. n-butanol and n-hexanol, which accounted for the 6.1 % and 3.7 % of the total organic carbon, respectively.

The production of higher alcohols might be related to available H_2 in the system. H_2 is an important substrate of alcohols generation, either through fatty acid reduction pathway or medium-chain-CoA dependent pathway as previous discussed in 3.1 (Dürre 2016). Therefore, the H_2 content in the headspace under three different ED:EA

ratios were monitored, with the results shown in Figure 2. Increases in H₂ proportion were observed in all reactors during the initial 4-10 days, complied with the decrease of the ethanol concentration in each system (Figure 1a 1c 1e). The accumulated H₂ in the headspace increased with the increase of ED:EA ratio, reaching the highest level at 0.65 ± 0.02 %, 6.36 ± 0.11 % and 11.85 ± 0.11 % for ED: EA ratio of 1:2, 1:1 and 2:1, respectively. H₂ was known to inhibit the acetogenic reaction at a high partial pressure (Batstone et al. 2002), but so far there was no evidence that H₂ would inhibit MCFA production. However, the results of this study showed that a higher H₂ production favors the generation of higher alcohols and the production of MCFA could be reduced. This indicated the accumulation of H₂ should be avoided in the CE process of WASAFL to ensure high MCFA selectivity. It could also be observed that H₂ percentage decreased gradually after reaching the summit, when the ethanol in the system was depleted. The decrease of H₂ confirmed it could be served as ED for CE or homoacetogenesis in the system, as no methane was produced (Saady et al., 2013, Steinbusch et al., 2011). However, since the decrease in the total mass of H₂ was only around 0.1- 0.2 mmol, the corresponding slight increase in carboxylic acids (~0.02-0.04 g COD/L in total) may not be able to precisely reflected in Figure 1 due to the detection limitation as well as the scale of the figure.

3.3. Thermodynamic analysis of the CE process of WAS alkaline fermentation liquor

The spontaneity of the potential reactions during the CE process using WASAFL as the substrate was evaluated by thermodynamic analysis. Table S3 (SI) listed the standard transformed Gibbs free energy ($\Delta G_{37^\circ\text{C}}^0$) of possible CE reactions and acetogenic reactions (anaerobic SCFA degradation) under the actual temperature (37°C)

and pH (5.5) conditions in the system. The results showed that all the CE reactions (Re.1 – Re.9) are exothermic reactions, while the most of the acetogenic reactions are endothermic reactions (Re.10 – Re.13), except for homoacetogenesis (Re.14). These indicated that the ambient conditions of studied system in general favor the CE of WASAFL rather than their break-down.

It has been recognized that the ratio of the transformed Gibbs free energy ($\Delta'_r G_T$) to the numbers of electron transfer in each reaction (Ne) could indirectly presented the competitiveness of specific reaction pathways (Wu et al., 2018). Therefore, the $\Delta'_r G_T$ /Ne ratios of different CE processes under different ethanol concentrations at the initial stage were calculated to evaluate the competition between different CE reactions as well as the effect of ethanol concentration on the competition with the results shown in Figure 3. As under the initial condition, the concentrations of MCFAs almost negligible in the system, their concentration were at a very low concentration of 0.01M for the ease of calculation. Also, the ethanol concentrations of 0-100 mM were evaluated in the thermodynamic calculation in order to cover a wide range of ED/EA ratios. The results showed that $\Delta'_r G_T$ /Ne ratios of most CE reactions were closed to each other, indicating that the competitions between these reactions are generally unapparent. The $\Delta'_r G_T$ /Ne ratio of the process for valerate production by propionate (Re.4) was slightly higher than other reactions, indicating that it was relatively thermodynamically unfavorable in the studied system. This is supported by the evidence that the consumption rate of propionate (equals to the production rate of valerate) was slower than the consumption rate of valerate, as indicated by the decrease of valerate concentration during the initial 4-7 days (Figure 1). The thermodynamically unfavorable production of the valerate from propionate (Re.4) is

probably the result of that valerate presented with a significant amount (2.24 mM), comparable to propionate concentration (4 mM) in the WASAFL, as relative high concentrations of products would commonly negatively affect the spontaneity of the biochemical reactions (Kleerebezem and Van Loosdrecht 2010).

It is also suggested by Figure 3 that the $\Delta'_r G_T/\text{Ne}$ ratios would gradually decrease with the increase of ethanol concentration, indicating a higher ethanol concentration would favor the CE processes. This is in accordance with the increase in the MCFAs production rates when ethanol level became higher (Figure 1). However, the competition between different CE reactions will not be affected by the increase of the ethanol concentration, as the relative differences in $\Delta'_r G_T/\text{Ne}$ between different reactions remained constant. When the ethanol concentration was lower than 5 mM, the $\Delta'_r G_T/\text{Ne}$ ratios would be increased drastically with the decrease of ethanol level, suggesting the concentration of ethanol would preferably higher than 5 mM to ensure the fast and successful initiation of the CE process using WASAFL. In addition, the $\Delta'_r G_T/\text{Ne}$ ratio of homoacetogenesis in the studied system was also assessed (Figure 3). It was kept at -18.4 kJ/mol e^- and would not be affected by the ethanol concentration. The low $\Delta'_r G_T/\text{Ne}$ of the homoacetogenesis process further confirmed its possible occurrence and important role in acetate production in the studied system.

3.4. Microbial communities for MCFAs production in the CE processes of WAS alkaline fermentation liquor

To further understand the mechanisms of CE process using WASAFL, the microbial community compositions with different ethanol levels, i.e. R1 (ED: EA = 1:2), R2 (ED: EA = 1:1) and R3 (ED: EA = 2:1), and the original inoculum (R0) were

analyzed and compared. The rarefaction curves of the OTU numbers in all samples at 0.03 distance was shown in Figure S1. The curves for each sample reached near saturation, indicating that the sequence data were representative and reliable. Table S4 listed the parameters related to microbial community diversity. The microbial richness and diversity of the CE sludge samples was higher than those of the initial inoculum, reflected by higher Chao and Shannon index. In addition, the principal component analysis (PCA) based on the sequencing data suggested that microbial populations in the sludge have been significantly changed after the CE process and there were also differences in microbial populations in the CE sludge at three different ED to EA ratios (Figure S2).

Specifically, the differences in the compositions of the microbiomes at the class level among the inoculum and three CE sludge samples were presented in Figure 4a. The addition of ethanol changed the microbial community structures mainly by enriching the bacteria within the class of *Clostridia*, that the relative abundance increased from 5.00 % in the inoculum to 8.17~19.43% in the CE sludge samples with different content of ethanol. The *Clostridia* class has been reported with high abundance in CE systems fed with ethanol and viable carboxylic acids (Coma et al. 2016).

The microbial compositions at genus level in different sludge sample were exhibited in Figure 4b. The relative abundance of *Clostridium sensu stricto*, a major member of *Clostridia*, was increased with the addition of ED, with average abundances being 0.17 %, 0.73 %, 0.85 % and 2.85% for R0, R1, R2 and R3, respectively. Blasting the OTUs belonged to *Clostridium sensu stricto* on National Central for Biotechnology Information (NCBI) database, some of them were belonged to *Clostridium Kluyveri*

(Identity >99%), which is by far the best-studied species known to produce MCFAs (Angenent et al., 2016; Spirito et al., 2014). The relative abundance of *C. Kluyveri* was positively related to the ethanol addition in the studied system, which increased from 0 in R0 (without ethanol addition) to 0.44% in R3 (ethanol 74 mM). This indicated that *C. Kluyveri* also played an important role in CE process using WASAFL. Other species which also have been reported capable of MCFA production through CE, such as *Clostridium* sp. BS-1, *Eubacterium pyruvativorans* and *Eubacterium limosum* were absent in the studied system (Angenent et al., 2016). However, the relative low abundance of *C. Kluyveri* suggested that other unreported species might also be responsible for CE process in the studied system

The relative abundance of other genera such as *Oscillibacter*, *Leptolinea* and *Exilispira* were also increased with the ethanol addition and MCFA production. These microorganisms are known as anaerobic fermenters. The increase of the abundance of *Oscillibacter* with MCFAs production has also been reported previously (Wu et al., 2018). Although there is no evidence of directly carrying out CE process by these fermenters, the positive correlation between their abundance and MCFA production indicated that they might be beneficial to the CE process when using ethanol as electron donor.

In addition, the key microorganisms involved in the homoacetogenesis in the studied system were mainly affiliated to the genera of *Acetobacterium*, *Acetoanaerobium*, *Eubacterium* and *Clostridium* (*C. ljungdahlii*, *C. butyricum*, *C. drakei* and so on). The relative abundance of the homoacetogens increased from 0.036% in R0 to 0.396% in R3. This result further proved that the homoacetogenesis was enhanced in the system,

contributing to the acetate generation during the CE process using WASAFL (Figure 1). It should be noted that the reactor operation time is relatively short in this study and a longer experimental period might bring more information on microbial community related to CE process, which is worthy to be investigated in future.

3.5. MCFAs production pathways and corresponding microbes in the CE processes of WAS alkaline fermentation liquor

All the functional genes identified by the metagenomics analysis were annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/pathway.html>) to carry out enzyme assays for further investigation of the CE pathways. Potential functional enzymes involved in CE processes were specifically studied and are listed in Table S5. Accordingly, two cyclic pathways, i.e. the reverse β -oxidization (RBO) pathway and fatty acid biosynthesis (FAB) pathway were constructed (Figure 5).

The RBO pathway is a model pathway for MCFA production in mixed culture, which centers on acetyl-CoA (Han et al., 2018). In the process, the acetyl-CoA serves as a carbon donor, adding two C atoms to the initial acyl-CoA to achieve CE (Han et al., 2018). During the CE of WASAFL, the acetyl-CoA could be primarily generated from ethanol oxidation as well as H_2 and CO_2 , as all these substances are abundant in the studied system. The relative abundances of most enzymes involved in RBO pathways were increased in the CE reactor (R2), suggesting this pathway is responsible for MCFA production from WASAFL.

The FAB pathway is an alternative route for MCFA production, which was less

investigated in the past (Han et al., 2018; Chan et al., 2010). However, all of the required functional enzymes involved in the FAB pathways were identified in the metagenomic profiles and the relative abundances of the most enzymes were enhanced in the CE reactor (R2), suggesting that FAB pathways are also likely to contribute to the CE of WASAFL. In the FAB pathway, the malonyl-ACP was served as the 2-C donor and acyl-carrier protein (ACP) was the intermediates of the CE process. The FAB pathway was considered less efficient than RBO pathway as acetyl-CoA should be firstly converted to malonyl-CoA and then transferred to malonyl-ACP before entering the FAB pathway cycle (Han et al., 2018). However, the relative abundance of enzymes involved in FAB pathway was in general higher than those involved in RBO pathway (Table S5). This result indicated that FAB pathway was likely more active than the RBO pathway during the CE of WASAFL.

The taxonomic origins of the functions involved in CE processes were also investigated and presented in Figure 6. In both RBO pathway and FAB pathway, most of the functional enzymes were associated with the genus of *Clostridium*, suggesting this microbe is mainly responsible for the CE process in the studied systems. However, many other microorganisms also contain parts of the functions (Figure 6). Thus, the possibility that the CE process was fulfilled by the interaction between different microorganisms could not be ruled out and is worthy for future study. The presence of functional enzymes in the predominant species in *Clostridium* was further investigated to gain more information about the microbes responsible for CE process using WASAFL as substrate. As shown in Table 1, as expected, the most of enzymes required for acetyl-CoA production and in RBO pathway and FAB pathway were assigned to *C. Kluyveri*, further confirming its crucial role in MCFA production in the

studied system. Another two species were also observed, i.e. *C. botulinum* and *C. magnum*, that associated with all the functional enzymes required for FAB pathway as well as for the acetyl-CoA production through CO₂ and H₂ (cdhA and cdhD). Therefore, it is very likely that these two microorganisms also took responsibility for the CE process in the studied system. The capability of *C. botulinum* for CE through FAB pathways has also been observed by Han et al (2018). However, to our knowledge, the function of *C. magnum* in CE was revealed for the first time in this study. Taxonomic assignments showed that *C. magnum* accounted for a significant abundance at 14% of the total *Clostridium* in the CE system (R2). Thus, it is worthwhile to conduct in-depth investigation on *C. magnum*, through multi-omic analysis for instance, to fully reveal its role in the CE process in future work.

3.6. Implication of this work

This study demonstrated the feasibility of using WASAFL as substrate and ethanol as ED for the production of MCFAs through CE process. The results showed that the increase of ethanol addition would enhance the MCFAs production, but high ED:EA ratio might result in the decline of the MCFA selectivity, due to the production of higher alcohol. Moreover, the production of alcohol was positively correlated to the H₂ fraction in the gas phase. Therefore, in practice, to enhance the MCFA production and selectivity, a higher dosage of ethanol is desirable. However, the ethanol should be added in several steps with a relatively low amount for each step to avoid the accumulation of H₂ and the production of alcohol. It also should be noted that a low ethanol concentration can decrease the CE spontaneity, especially when the concentration was lower than 5 mM (See Section 3.3). Therefore, it is desirable that the minimum ethanol dosing rate is higher than 5 mM to ensure the successful

initiation of the CE process.

The produced MCFAs during the CE of WASAFL mainly include n-caproate, n-heptanoate, n-caprylate. As indicated by thermodynamic study, the accumulation of products in the CE system could decrease the Gibbs free energy releasing and negatively affect the MCFA production efficiency. The inhibitory effect of MCFA on the CE process has also been found by other researchers. For example, n-caproate was observed to inhibit the CE process when the concentration is higher than ~7.5 mM (Coma et al., 2016). Also, n-caprylic acid would pose to toxic effect on fermentative bacteria when the concentration was over 1 mg/ml (Skrivanova et al., 2006). In this light, the produced MCFA should be regularly extracted from the CE system during the practical application. By applying liquid-liquid extraction system, different MCFAs can be separated from the CE system (Agler et al. 2012). The separated MCFAs could further be processed into fragrances (Kenealy et al., 1995), food additives (Xu et al., 2015), renewable diesel (Levy et al., 1984), and antimicrobial agent (P. Desbois, 2012) etc. To increase the ease and extraction efficiency of the separation process, future research could be dedicated to decrease the diversity of MCFAs produced from WASAFL. In addition, it should be noted that synthetic WASAFL was used for the CE experiment in this study. In the real WASAFL, it may contain some other organics, such as proteins and carbohydrates. After thorough alkaline fermentation, the remaining organics were mostly hardly biodegradable and unexpected to contribute to the MCFAs production. The potential minor effect of these organics on WASAFL production may need to be evaluated in future work.

In addition, as revealed by reactor performance data, thermodynamic calculation and microbial community analysis, the homoacetogenesis is another important biochemical process in the studied CE system. During the homoacetogenesis process, a significant amount of CO₂ would be consumed for acetate generation. Thus, the

process could contribute to the fixation of the CO₂ in WASAFL and consequently reduced the greenhouse gas emission from the system, which could be considered as an additional benefit of the proposed technology.

4. Conclusions

The feasibility and mechanisms of upgrading waste-activated sludge alkaline fermentation liquor (WASAFL) into medium-chain fatty acids (MCFAs) using ethanol as electron donors through chain elongation process was comprehensively evaluated in this work. The following conclusions can be drawn from this study:

- The MCFAs production from WASAFL was increased with the ethanol level in the system, but a high carbon molar ratio of ethanol to WASAFL could result in the decline of the MCFA selectivity, due to the production of higher alcohol.
- All CE processes for MCFAs production from WASAFL were exothermic reactions, with the spontaneity and energy release of the reactions increased with the ethanol level.
- The relative abundances of *Clostridium*, *Oscillibacter*, *Leptolinea* and *Exilispira* increased with the MCFAs production, suggesting the potential benefit of these microbes on the CE of WASAFL.
- Both the reverse β -oxidization (RBO) pathway and fatty acid biosynthesis (FAB) pathway contributed to the CE of WASAFL. The functional genes were mainly associated with *Clostridium*, including *C. Kluyveri*, *C. botulinum* and *C. magnum*.

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References

- Agler, M.T., Spirito, C.M., Usack, J.G., Werner, J.J. and Angenent, L.T. (2012) Chain elongation with reactor microbiomes: upgrading dilute ethanol to medium-chain carboxylates. *Energy & Environmental Science* 5(8), 8189-8192.
- Angenent, L.T., Richter, H., Buckel, W., Spirito, C.M., Steinbusch, K.J., Plugge, C.M., Strik, D.P., Grootscholten, T.I., Buisman, C.J. and Hamelers, H.V. (2016) Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology To Produce Biochemicals. *Environ. Sci. Technol* 50 (6), 2796-22810
- Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S.V., Pavlostathis, S.G., Rozzi, A., Sanders, W.T.M., Siegrist, H. and Vavilin, V.A. (2002) The IWA Anaerobic Digestion Model No. 1 (ADM1). *Water Science And Technology* 45(10), 75-83.
- Cavalcante, W.d.A., Leitão, R.C., Gehring, T.A., Angenent, L.T. and Santaella, S.T. (2017) Anaerobic fermentation for n-caproic acid production: A review. *Process Biochemistry* 54, 106-119.
- Chan, D.I., and Vogel, H.J. (2010) Current understanding of fatty acid biosynthesis and the acyl carrier protein. *Biochem J* 430, 1-19.
- Chen, W.S., Ye, Y., Steinbusch, K.J.J., Strik, D.P.B.T.B. and Buisman, C.J.N. (2016) Methanol as an alternative electron donor in chain elongation for butyrate and caproate formation. *Biomass and Bioenergy* 93, 201-208.
- Coma, M., Vilchez-Vargas, R., Roume, H., Jauregui, R., Pieper, D.H. and Rabaey, K. (2016) Product Diversity Linked to Substrate Usage in Chain Elongation by Mixed-Culture Fermentation. *Environ. Sci. Technol* 50(12), 6467-6476.
- Duber, A., Jaroszynski, L., Zagrodnik, R., Chwialkowska, J., Juzwa, W., Ciesielski, S. and Oleskowicz-Popiel, P. (2018) Exploiting the real wastewater potential for resource recovery – n-caproate production from acid whey. *Green Chemistry* 20(16), 3790-3803.
- Dürre, P. (2016) Butanol formation from gaseous substrates. *Fems Microbiology Letters* 363(6), fnw040.
- Ge, S., Usack, J.G., Spirito, C.M. and Angenent, L.T. (2015) Long-Term n-Caproic Acid Production from Yeast-Fermentation Beer in an Anaerobic Bioreactor with Continuous Product Extraction. *Environ. Sci. Technol* 49(13), 8012-8021.
- Gómez, J.M. and Cantero, D. (2007) Microbial Processing of Metal Sulfides. Donati, E.R. and Sand, W. (eds), pp. 287-309, Springer Netherlands, Dordrecht.
- Grootscholten, T.I., Strik, D. P. B. T. B., Steinbusch, K.J. and Hamelers, H.V. (2014) Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol. *Applied Energy* 116, 223-229.
- Han, W., He, P., Shao, L. and Lu, F. (2018) Metabolic Interactions of a Chain Elongation Microbiome. *Appl Environ Microbiol* 84(22).
- Howarth, R.W. (2014) A bridge to nowhere: methane emissions and the greenhouse gas footprint of natural gas. *Energy Science & Engineering* 2(2), 47–60.
- Kenealy, W.R., Cao, Y., Wimer, P.J., 1995. Production of caproic acid by cocultures of ruminal cellulolytic bacteria and *Clostridium Kluyveri* grown on cellulose and ethanol. *Appl. Microbial. Biotechnol.* 44(3-4), 507-513.

- 678 Kleerebezem, R. and Van Loosdrecht, M.C.M. (2010) A Generalized Method for
 679 Thermodynamic State Analysis of Environmental Systems. *Critical Reviews in*
 680 *Environmental Science and Technology* 40(1), 1-54.
- 681 Kucek, L.A., Spirito, C.M. and Angenent, L.T. (2016b) High n-caprylate
 682 productivities and specificities from dilute ethanol and acetate: chain elongation
 683 with microbiomes to upgrade products from syngas fermentation. *Energy &*
 684 *Environmental Science* 9(11), 3482-3494.
- 685 Lazarova, V., Choo, K.-H. and Cornel, P. (2012) *Water-energy interactions in water*
 686 *reuse*, IWA publishing.
- 687 Leng, L., Yang, P., Mao, Y., Wu, Z., Zhang, T. and Lee, P.-H. (2017)
 688 Thermodynamic and physiological study of caproate and 1,3-propanediol
 689 co-production through glycerol fermentation and fatty acids chain elongation.
 690 *Water Research* 114, 200-209.
- 691 Levy, P., Sanderson, J., Cheng, L., 1984. Kolbe electrolysis of mixtures of aliphatic
 692 organic acids. *J. Electrochem. Soc.* 131 (4), 773-777
- 693 Li, X., Chen, L., Mei, Q., Dong, B., Dai, X., Ding, G. and Zeng, E.Y. (2018)
 694 Microplastics in sewage sludge from the wastewater treatment plants in China.
 695 *Water Research* 142, 75-85.
- 696 Li, Y., Chen, Y. and Wu, J. (2019) Enhancement of methane production in anaerobic
 697 digestion process: A review. *Applied Energy* 240, 120-137.
- 698 Liu, Y., Lu, F., Shao, L. and He, P. (2016) Alcohol-to-acid ratio and substrate
 699 concentration affect product structure in chain elongation reactions initiated by
 700 unacclimatized inoculum. *Bioresour Technol* 218, 1140-1150.
- 701 Lonkar, S., Fu, Z. and Holtzapple, M. (2016) Optimum alcohol concentration for
 702 chain elongation in mixed-culture fermentation of cellulosic substrate.
 703 *Biotechnology And Bioengineering* 113(12), 2597-2604.
- 704 Luo, J., Chen, Y. and Feng, L. (2016) Polycyclic Aromatic Hydrocarbon Affects
 705 Acetic Acid Production during Anaerobic Fermentation of Waste Activated
 706 Sludge by Altering Activity and Viability of Acetogen. *Environ Sci Technol*
 707 50(13), 6921-6929.
- 708 Machado, H.B., Dekishima, Y., Luo, H., Lan, E.I. and Liao, J.C. (2012) A selection
 709 platform for carbon chain elongation using the CoA-dependent pathway to
 710 produce linear higher alcohols. *Metabolic Engineering* 14(5), 504-511.
- 711 Nzeteu, C. O., Trego, A. C., Abram, F., O'Flaherty, V. (2018) Reproducible,
 712 high-yielding, biological caproate production from food waste using a
 713 single-phase anaerobic reactor system. *Biotechnol Biofuels* 11,108.
- 714 P. Desbois, A., 2012. Potential applications of antimicrobial fatty acids in medicine,
 715 agriculture and other industries. *Recent Pat. Anti-Infect. Drug Discov.* 7
 716 (2),111-122
- 717 Saady, N.M.C. (2013) Homoacetogenesis during hydrogen production by mixed
 718 cultures dark fermentation: Unresolved challenge. *International Journal Of*
 719 *Hydrogen Energy* 38(30), 13172-13191.
- 720 Singhanian, R.R., Patel, A.K., Christophe, G., Fontanille, P. and Larroche, C. (2013)
 721 Biological upgrading of volatile fatty acids, key intermediates for the valorization

- of biowaste through dark anaerobic fermentation. *Bioresour Technol* 145, 166-174.
- Skrivanova, E., Marounek, M., Benda, V., Brezina, P. (2006) Susceptibility of *Escherichia coli*, *Salmonella* sp. and *Clostridium perfringens* to organic acids and monolaurin. *Veterinarni Medicina*, 51, 81-88.
- Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J. and Angenent, L.T. (2014) Chain elongation in anaerobic reactor microbiomes to recover resources from waste. *Current Opinion In Biotechnology* 27, 115-122.
- Steinbusch, K.J.J., Hamelers, H.V.M., Plugge, C.M. and Buisman, C.J.N. (2011) Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass. *Energy Environ. Sci.* 4(1), 216-224.
- Sun, J., Dai, X., Wang, Q., van Loosdrecht, M.C.M. and Ni, B.-J. (2019) Microplastics in wastewater treatment plants: Detection, occurrence and removal. *Water Research* 152, 21-37.
- Sun, J., Hu, S., Sharma, K.R., Bustamante, H. and Yuan, Z. (2015) Impact of reduced water consumption on sulfide and methane production in rising main sewers. *Journal Of Environmental Management* 154, 307-315.
- Wang, Q., Sun, J., Song, K., Zhou, X., Wei, W., Wang, D., Xie, G.-J., Gong, Y. and Zhou, B. (2018a) Combined zero valent iron and hydrogen peroxide conditioning significantly enhances the dewaterability of anaerobic digestate. *Journal of Environmental Sciences* 67, 378-386.
- Wang, Y., Zhao, J., Wang, D., Liu, Y., Wang, Q., Ni, B.J., Chen, F., Yang, Q., Li, X., Zeng, G. and Yuan, Z. (2018b) Free nitrous acid promotes hydrogen production from dark fermentation of waste activated sludge. *Water Res* 145, 113-124.
- Wu, Q., Guo, W., Bao, X., Meng, X., Yin, R., Du, J., Zheng, H., Feng, X., Luo, H. and Ren, N. (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.
- Xu, J., Guzman, J.J., Andersen, S.J., Rabaey, K., Angenent, L.T., 2015. In-line and selective phase separation of medium-chain carboxylic acids using membrane electrolysis. *Chem. Commun.* 51 (31), 6847-6850.
- Xu, Q., Liu, X., Fu, Y., Li, Y., Wang, D., Wang, Q., Liu, Y., An, H., Zhao, J., Wu, Y., Li, X., Yang, Q., Zeng, G., 2018. Feasibility of enhancing short-chain fatty acids production from waste activated sludge after free ammonia pretreatment: role and significance of rhamnolipid. *Bioresour. Technol.* 267, 141–181.
- Yin, Y., Zhang, Y., Karakashev, D.B., Wang, J. and Angelidaki, I. (2017) Biological caproate production by *Clostridium kluyveri* from ethanol and acetate as carbon sources. *Bioresour Technol* 241, 638-644.
- Yuan, H., Chen, Y., Zhang, H., Jiang, S., Zhou, Q. and Gu, G. (2006) Improved bioproduction of short-chain fatty acids (SCFAs) from excess sludge under alkaline conditions. *Environ. Sci. Technol.* 40(6), 2025-2029.
- Zhu, X., Tao, Y., Liang, C., Li, X., Wei, N., Zhang, W., Zhou, Y., Yang, Y. and Bo, T. (2015) The synthesis of n-caproate from lactate: a new efficient process for medium-chain carboxylates production. *Sci Rep* 5, 14360.

Table and Figure Legends

Table 1. Presence key enzymes involved in CE process in the predominant species in *Clostridium*.

Figure 1. Concentration of SCFAs (acetate, propionate, butyrate and valerate), MCFAs (n-caproate, n-heptanoate and n-caprylate) and alcohols (ethanol, n-butanol and n-hexanol) in three sets of CE experiments using ethanol as ED and WAS alkaline fermentation liquor as EA: (a-b) the ED to EA ratio was 1:2; (c-d) the ED to EA ratio was 1:1; and (e-f) the ED to EA ratio was 2:1.

Figure 2. The proportions of end products in the three sets of CE experiments using ethanol as ED and WAS alkaline fermentation liquor as EA: (a) the ED to EA ratio was 1:2; (b) the ED to EA ratio was 1:1; (c) the ED to EA ratio was 2:1; and (d) the percentage of H₂ in the headspace of serum bottle at three ED to EA ratios.

Figure 3. The changes in ratios of the transformed Gibbs free energy $\Delta'_r G_T$ to the numbers of electron transfer in each reaction (Re) of different CE processes with ethanol concentration in the system. The acetate (C2), propionate (C3), butyrate (C4), valerate (C5) concentration used for the calculation was the same as the initial concentration in WASAFL, i.e. 19.33 mM for acetate, 4 mM for propionate, 3.33 mM for butyrate, 2.24 mM for valerate. The concentration of n-caproate (C6), n-heptanoate (C7) and n-caprylate (C8) was assumed much lower at 0.01 Mm. The vertical dashed lines indicate the initial ethanol concentration in R1, R2 and R3.

Figure 4. Relative abundances of the microbial community at class level (a) and genus level (b) for the initial inoculum (R0) and the three CE sludge samples (R1, R2

and R3).

Figure 5. Potential metabolic chain elongation pathways with ethanol as ED and WASAFL as EA in this work: green solid circle represents acyl-carrier protein, and orange solid circle represents coenzyme A. The abundance of the enzymes in two samples are shown in the color bars and calculated based on RPKM (hit reads per kilo-base pair per million reads). The definition of enzyme number is listed in Table S5, SI.

Figure 6. Taxonomic origins of the key enzymes detected in reverse β -oxidization pathway (a) and fatty acid biosynthesis pathway (b) with ethanol to WASAFL carbon molar ratio of 1:1. The definition of enzymes were listed in Table S5. The abundance was calculated based on RPKM (hit reads per kilo-base pair per million read).

Table 1 Presence key enzymes involved in CE process in the predominant species in *Clostridium*

Name	KO entry	<i>Clostridium kluyveri</i>	<i>Clostridium magnum</i>	<i>Clostridium botulinum</i>	<i>Clostridium ljungdahlii</i>	<i>Clostridium perfringens</i>	<i>Clostridium colicanis</i>	<i>Clostridium coskatii</i>	<i>Clostridium disporicum</i>	<i>Clostridium homopropionicum</i>	<i>Clostridium lundense</i>
Acetyl-CoA production											
adh	K00001	+									
cdhA	K00192	+	+	+	+		+	+			+
cdhD	K00194	+	+	+	+		+	+			+
RBO											
ACCA2	K07508	+									
HADH	K00022										
ECHS1	K07511	+									
MECR,	K07512	+	+								
FAB											
ACACA	K11262	+	+	+	+	+	+		+	+	
accC	K01961	+	+	+	+	+				+	
fabD	K00645	+	+	+		+		+	+		
fabF	K09458	+	+	+	+	+	+		+	+	+

fabH	K00648	+	+	+	+	+		+	+	+	+
fabG	k00059	+	+	+	+	+		+	+	+	+
fabZ	k02372	+	+	+		+	+				+
fabV	k00209	+	+	+			+	+	+	+	

Shading (+) indicates the enzyme is included in the microbe.

Figure 1. Concentration of SCFAs (acetate, propionate, butyrate and valerate), MCFAs (n-caproate, n-heptanoate and n-caprylate) and alcohols (ethanol, n-butanol and n-hexanol) in three sets of CE experiments using ethanol as ED and WAS alkaline fermentation liquor as EA: **(a-b)** the ED to EA ratio was 1:2; **(c-d)** the ED to EA ratio was 1:1; and **(e-f)** the ED to EA ratio was 2:1.

Figure 2. The proportions of end products in the three sets of CE experiments using ethanol as ED and WAS alkaline fermentation liquor as EA: **(a)** the ED to EA ratio was 1:2; **(b)** the ED to EA ratio was 1:1; **(c)** the ED to EA ratio was 2:1; and **(d)** the percentage of H₂ in the headspace of serum bottle at three ED to EA ratios.

Figure 3. The changes in ratios of the transformed Gibbs free energy $\Delta_r'G_T$ to the numbers of electron transfer in each reaction (Re) of different CE processes with ethanol concentration in the system. The acetate (C2), propionate (C3), butyrate (C4), valerate (C5) concentration used for the calculation was the same as the initial concentration in WASAFL, i.e. 19.33 mM for acetate, 4 mM for propionate, 3.33 mM for butyrate, 2.24 mM for valerate. The concentration of n-caproate (C6), n-heptanoate (C7) and n-caprylate (C8) was assumed much lower at 0.01 Mm. The vertical dashed lines indicate the initial ethanol concentration in R1, R2 and R3.

Figure 4. Relative abundances of the microbial community at class level **(a)** and genus level **(b)** for the initial inoculum (R0) and the three CE sludge samples (R1, R2 and R3).

Figure 5. Potential metabolic chain elongation pathways with ethanol as ED and WASAFL as EA in this work: green solid circle represents acyl-carrier protein, and orange solid circle represents coenzyme A. The abundance of the enzymes in two samples are shown in the color bars and calculated based on RPKM (hit reads per kilo-base pair per million reads). The definition of enzyme number is listed in Table S4, SI.

Figure 6. Taxonomic origins of the key enzymes detected in reverse β -oxidation pathway **(a)** and fatty acid biosynthesis pathway **(b)** with ethanol to WASAFL carbon molar ratio of 1:1. The definition of enzymes were listed in Table S4. The abundance was calculated based on RPKM (hit reads per kilo-base pair per million reads)

Highlights

- MCFAs production from WAS alkaline fermentation liquid was achieved
- MCFA production is positively related to the level of ethanol as electron donor
- A high level of ethanol could reduce the selectivity of MCFA production
- Key functional microbes involved *C. Kluyveri*, *C. botulinum* and *C. magnum*
- Production pathways included reverse β -oxidation and fatty acid biosynthesis

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: