

# UNVEILING THE TECHNOLOGY AND MECHANISMS OF MEDIUM-CHAIN FATTY ACIDS PRODUCTION FROM WASTE ACTIVATED SLUDGE FERMENTATION LIQUOR

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## ABSTRACT

Chain elongation (CE) with open-culture microbiomes has been demonstrated to be an effective biotechnological platform to produce valuable medium chain fatty acids (MCFAs). Herein we reported a new biotechnological process for producing MCFAs by using waste-activated sludge alkaline fermentation liquor as feedstock and electron acceptors (EAs) with ethanol as electron donor (ED). Under three different carbon-molar ratios of ED to EAs (i.e., 1:2, 1:1 and 2:1), three different types of MCFAs (i.e., n-caproate, n-heptanoate and n-caprylate) were produced. Thermodynamic analysis showed the higher amount of ethanol was more favorable for MCFAs production. Microbial community analysis revealed that the ethanol participation caused microbes shift in the favorable direction for CE process. Metagenome analysis showed both reverse  $\beta$ -oxidization and fatty acid biosynthesis pathways simultaneously enhanced and occurred in the CE process.

## BACKGROUND AND RELEVANCE

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.

Recently, a newly developed carboxylate platform to produce medium chain fatty acids (MCFAs) via chain elongation (CE) may provide a promising 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 short chain fatty acids (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). 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.

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.

## MATERIAL AND METHODS

Waste activated sludge alkaline fermentation liquid (WASFL) was cultivated under alkaline condition (pH=10) with waste activated sludge from secondary sedimentation tank of municipal waste water treatment plant. After reaching the steady state, different levels of ethanol were added into fermenters as electron donor (ED). 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). Thermodynamic analysis was applied to explore the spontaneity of the CE process with WASFL as EA and ethanol as ED. Illumina Miseq sequencing was applied to explore the shift of microbial communities and functional microbes in CE process. Metagenomic analysis was applied for figuring out the pathway participated in CE process.

## RESULT AND DISCUSSION

### MCFAs Production from WASFL via CE Process

The MCFAs (i.e., n-caproate, n-heptanoate, n-caprylate) could be obtained with different carbon molar ratio of ED (ethanol) to EA (WASFL). As shown in Figure 1, the concentration of MCFAs significantly increased with ratios of ED to EA increasing from 1:2 to 2:1. The total cumulative MCFAs yield increased from  $2.88 \pm 0.01$  g COD/L to  $5.19 \pm 0.42$  g COD/L with respect to the ED: EA ratio of 1:2 to 1:1 and then further increased slightly to  $5.28 \pm 0.18$  g COD/L at the ED: EA ratio of 2:1. However, the proportion of total MCFAs (C6, C7 and C8) was highest at ED: EA ratio of 1:1, accounting for 72.9 % of the total carbon content, followed by those at ED: EA ratios of 2:1 and 1:2, each accounting for 60.2% and 65.9%, respectively.

It should also be noted that two higher alcohols, i.e., n-butanol and n-hexanol were produced when the highest ED:EA ratio is applied (2:1). The n-butanol concentration reached  $0.49 \pm 0.02$  g COD/L and the hexanol was  $0.30 \pm 0.02$  g COD/L at the end of the experimental period, respectively. The n-butanol and n-hexanol could be generated through the reduction of butyrate and n-caproate, respectively (Gomez and Cantero, 2007). Besides, they could also be produced through the CoA-dependent pathway.

### Thermodynamic calculations

The thermodynamic calculation results were shown in Figure 2. All calculated Gibbs free energy values for n-caproate, n-heptanoate and n-caprylate were negative and numerically higher than the minimum amount of energy (-20kJ per reaction) required for the survival of living cells, confirming that all of the experimental carbon molar ratio of ethanol to WASFL combinations were thermodynamically feasible in the CE processes.

### Effect of ethanol on functional microbial communities in CE process

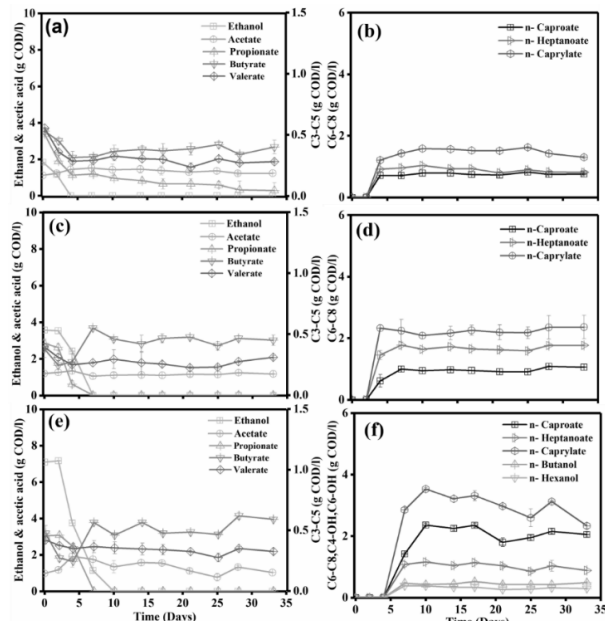
The microbial compositions at genus level in different sludge sample were exhibited in Fig. 3. The relative abundance of *Clostridium sensu stricto*, a major member of *Clostridia*, was increased with the addition of ED, with average abundances being 0.17%, 0.73%, 0.85% and 2.85% for R0, R1, R2 and R3, 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 ED.

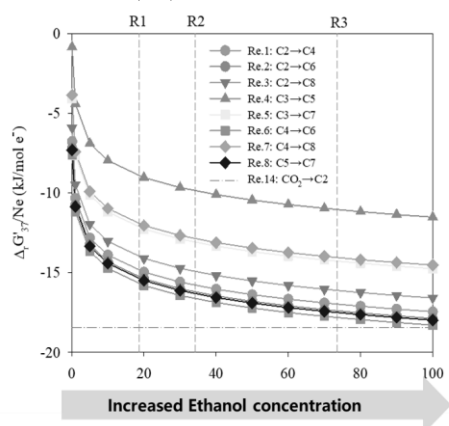
### Pathway in CE process revealed by metagenomic analysis

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. Accordingly, two cyclic pathways, i.e., the reverse  $\beta$ -oxidization (RBO) pathway and fatty acid biosynthesis (FAB) pathway were constructed (Figure 4). Most enzymes involved in the above two pathways were clearly more abundant in CE sludge sample than that in original inoculum, suggested that RBO and FAB pathway were enhanced and played important role in CE process.

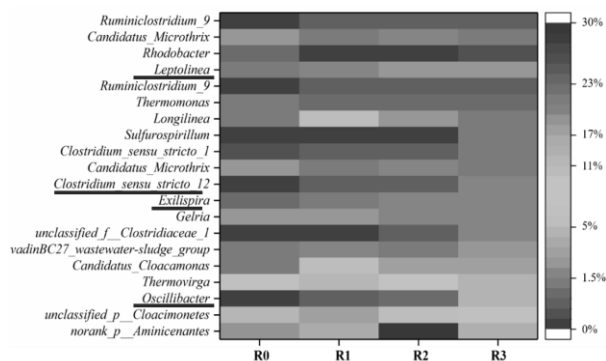
## FIGURES/ PHOTOGRAPHS



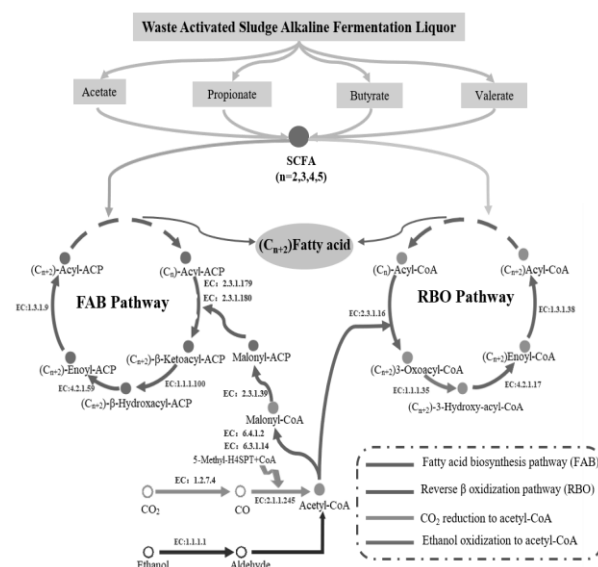
**Figure 1.** Concentrations evolution of ethanol and SCFAs (acetate, propionate, butyrate and valerate) as well as MCFAs (n-caproate, n-heptanoate and n-caprylate) in three sets of CE experiments using ethanol as ED and WAS alkaline fermentation liquid as EA: (a-b) the ED to EA ratio was 1:2; (c-d)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



**Figure 2** Thermodynamics calculation in three sets of ED to EA ratios.



**Figure 3.** Composition of relative species at genus level for the initial inoculum (R0) and the three CE sludge samples (R1, R2 and R3)



**Figure 4.** Potential metabolic chain elongation pathways with ethanol as ED and WASFL as EA in this work.

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