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Significant enhancement by casamino acids of caproate production via chain elongation

Peixian Yang [a,b], Ling Leng [b], Huichuan Zhuang [b], Po-Heng Lee [b,c,*]

[a] School of Environment and Spatial Informatics, China University of Mining and Technology, Xuzhou 221116, PR China
[b] Department of Civil and Environmental Engineering, the Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong Special Administrative Rregion of China
[c] Dept. of Civil and Environmental Eng., Imperial College London, Imperial College Road, London SW7 2BU, UK

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ABSTRACT

In this study, the effects of casamino acids, as the main component in yeast extract, on the bioproduction of caproate were evaluated. A dosage of 5 g/L casamino acids addition significantly promoted caproate production and shortened the lag phase for caproate production by 7.6-folds compared with the control. Both the net carbon and electron conversion efficiencies were enhanced. The addition of casamino acids shaped a dissimilar microbial community from that of the control and that of the inoculum. The caproate-producing *Clostridium* and *Caproiciproducens,* the proteolytic *Proteiniphilum* and *Brassicibacter,* and the saccharide-utilizing *Hydrogenoanaerobacteriu* were enriched, and their abundance were distinguished between with and without the addition of casamino acids as fermentation progressed. Further metagenomics analysis recovered genome bins of *Clostridium kluyveri*, *Clostridium cochlearium*, *Caprobacter fermentans* and *Proteiniphilum acetatigenes*, representing the majority of the functional bacteria with casamino acids addition. The complete reverse β-oxidation and amino acids (serine/glycine) metabolic pathway for caproate production and butyrate formation, respectively, were fully recovered. Furthermore, all the genes involved were highly expressed and metabolically active in meta-transcriptomics analysis. The finding proved that casamino acids was able to enhance caproate formation by promoting ethanol utilization and butyrate formation. In general, yeast extract could not only support microbial growth but also contribute to the formation of medium chain fatty acids, therefore promoting the efficiency of chain elongation process.

1. Introduction

Chain elongation is a fermentation process with undefined mixed cultures that upgrades diluted ethanol and volatile fatty acids (VFAs) into medium chain fatty acids (MCFAs, usually C_6-C_{12}) [1–3], which have wide applications, such as precursors for biodiesel production [4], bioplastic production [5] and corrosion inhibitors [6]. The properties of low solubility and high energy density make MCFAs superior bio-products to VFAs due to the longer hydrophobic carbon chain and lower oxygen/carbon ratio [1]. Different types of ethanol- and VFAs-containing waste streams, were successfully utilized to produce MCFAs via chain elongation process, such as yeast fermentation beer [2,7], lignocellulosic materials [8–10], municipal solid waste [11], acidified food waste [12], cellulosic substrate (shredded office copier paper) [13] and liquor-making wastewater [14]. Upgrading diluted ethanol into MCFAs via chain elongation could circumvent energy-intensive distillation process and sterilization step, making this bioprocess energy-efficient and cost-effective. Moreover, chain elongation offers an alternative solution for treating waste streams that are challenging for anaerobic digestion, such as the food waste and digestate from food waste containing considerable amounts of organic matter, especially protein [15].

To further improve the chain elongation process, approaches like upgrading reactor configuration [16,17], employing two-stage fermentation [18] and reducing the hydraulic retention time for high productivity of MCFA were successfully applied [19]. In particular, to support the microbial growth and improve productivity of MCFAs, yeast extract was commonly added as a supplement in the chain elongation process [3,16,19–21]. As the main composition in yeast extract, casamino acids were found to be utilized as a nitrogen source for biohydrogen

* Corresponding author at: Dept. of Civil and Environmental Eng., Imperial College London, Imperial College Road, London SW7 2BU, UK.
E-mail addresses: peixianyang@126.com (P. Yang), andy-ling.leng@polyu.edu.hk (L. Leng), zhuanghchit@gmail.com (H. Zhuang), po-heng.lee@imperial.ac.uk (P.-H. Lee).

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Table 1
Synthetic medium for mixed-culture batch fermentation.

Composition	Quantity
KH_2PO_4	0.20 g
NH_4Cl	0.50 g
NaCl	1.00 g
$MgCl_2{\cdot}6H_2O$	0.40 g
KCl	0.50 g
$CaCl_2{\cdot}2H_2O$	0.15 g
$NaHCO_3$	2.52 g
$FeCl_2{\cdot}4H_2O$	1.50 mg
H_3BO_3	0.30 mg
$CoCl_2{\cdot}4H_2O$	0.20 mg
$ZnCl_2$	0.05 mg
$MnCl_2{\cdot}4H_2O$	0.03 mg
$Na_2MoO_4{\cdot}2H_2O$	0.03 mg
$NiCl_2{\cdot}6H_2O$	0.02 mg
$CuCl_2{\cdot}2H_2O$	0.01 mg
L-cysteine	0.5 g
2-bromoethanosulfonic acid (BESA)	10 g
Resazurin	(0.1% w/v)
DI water	add to 1 L

production by *Clostridium butyricum* with the supplement of carbon source and essential elements [22]. Also, casamino acids were claimed to be the main substrate for the sulfate reducing bacteria (SRB) in the anoxic, and could stimulate the growth of SRB [23]. Presumably, the primary components of yeast extract, casamino acids and micro-nutrients, improved the MCFA production rate because it stimulates the growth of MCFAs-producing bacteria and facilitated product formation [19]. Moreover, it was claimed that the addition of yeast extract might contribute to butyrate formation, although it is unlikely used for caproate production [20]. Likewise, in a chain elongation process using yeast-fermentation beer as feedstock, the feed rich in casamino acids were speculated to be converted to acetate or butyrate, therefore facilitating the MCFA production [10]. Specifically, the caproate producer, *Clostridium kluyveri*, is able to produce caproate from ethanol with acetate via the reverse β-oxidation pathway [24,25]. In the mixed culture chain elongation processes, *C. kluyveri* was identified to be the predominant, as well as the main functional chain elongating microorganism [1,2]. To stimulate the growth and production rates of *C. kluyveri*, yeast extract was a common supplement used providing a necessary source of casamino acids and multiple microelements [26,27]. However, casamino acids, as the main composition of yeast extract, its metabolic association with the chain elongating process was not explored before.

In this study, to examine the effect of yeast extract in the chain elongation process, we focus our investigation on the effect of casamino acids – the main component of yeast extract to eliminate the contribution of other micro-nutrients, such as vitamins and biotin. Given that understanding the dynamic and metabolism of the mixed microbial population is essential for the enhancement of MCFAs formation, 16 S rRNA gene sequencing, metagenomics and meta-transcriptomics were conducted to holistically identify the microbial community structure, their interspecies interaction and their active metabolic pathways. A deeper mechanistic understanding of the chain elongation process with complex reactor microbiomes would benefit from shaping stable and functional community structures. Therefore, the knowledge gleamed offer new insights for the design and operation of an efficient and predictable engineered system.

2. Materials and methods

2.1. Experiment

The semi-continuous fermentation was conducted in 1-L fermenters anaerobically. The nutrient medium contained minerals, trace metals, a reducing agent, a methanogenesis inhibitor, and a redox indicator. The detailed information of medium was described in Table 1. In addition, absolute ethanol and Na-acetate were supplied as substrates, with an initial concentration of 286.79 mM and 45.63, respectively. Both the reactors were filled with 0.5 L of fermentation broth, and pH was adjusted and maintained to 7.0 ± 0.2 using phosphate buffer saline (PBS) solution. Specifically, additional 5 g/L of casamino acids were supplied into the reactor "AA". The reactor without extra supplement was the control fermentation. Both the reactors were maintained under an anaerobic condition. 10% (v/v) of well-mixed and pre-cultured anaerobic digesting (AD) sludge collected from Shek Wu Hui Sewage Treatment Works (STW, Sheung Shui, Hong Kong) was inoculated to each reactor. The headspaces of the reactors were flushed with N_2 gas with a final pressure of 1 atm. The reactors were incubated under 37 °C at an agitation speed of 100 rpm. The liquid samples were periodically withdrawn for chemical analyses. The produced gas was exhausted to maintain a headspace pressure of 1 atm. The semi-continuous fermentation was operated for 7 days per cycle. During each cycle, 100 mL of suspension was extracted and the same volume of substrate medium was replenished. Sampling was conducted on Day 0 (influent sampling), Day 3, and Day 7 (effluent sampling) of each cycle.

2.2. Analytical procedures

Liquid samples were centrifuged at 6000 rpm for 5 min, and the supernatant was filtered with 0.20-μm PTFE filter for components analysis. The species of alcohols, short and medium chain fatty acids in the liquid filtrate were determined with high performance liquid chromatography (HPLC, SHIMADZU Prominence, MD, USA) equipped with a refractive index detector (RID) and an HPX-87 H column (Bio-Rad Aminex HPX-87 H column, CA, USA). The operation condition was: 5 mM H_2SO_4 as mobile phase, 0.6 mL/min for pump flow rate, 50 °C for detector temperature, and 65 °C for oven temperature.

2.3. Carbon recovery and electron recovery calculation

To calculate the carbon recovery efficiency and take into account the carbon contribution of casamino acids to the products formation, the molecular formula of yeast extract, ($CH_{1.7}O_{0.5}N_{0.24}$; MW=25.1) [28], was applied to estimate the available carbon from the initial casamino acids supplement. The dosage of casamino acids was 5 g/L, therefore 172.4 mM of carbon was provided assuming a complete conversion. The carbon recovery efficiency η_c was calculated according to Eq. (1).

$$\eta_{c,i} = \frac{C_i}{C_{et} + C_{ac} + C_{aa}} \times 100\% \quad (1)$$

where $\eta_{c,i}$ is the carbon recover efficiency of product *i*,%; C_i is the mole of carbons in the product *i*; C_{et}, C_{ac} and C_{aa} is the initial mole carbons added in substrate ethanol, acetate and casamino acids, respectively. The concentrations of influent substrates were the same in both the reactors except for the casamino acids (0 for the control group).

For the calculation of products in the form of electron equivalent, the amount of electrons per molecule (mol/mol) can be derived from electron donating half reaction: 8 for acetate, 12 for ethanol, 20 for butyrate, 32 for caproate, 24 for butanol and 4.7 for yeast extract [19]. The electron recovery efficiency η_q was calculated according to Eq. (2).

$$\eta_{q,i} = \frac{q_i}{q_{et} + q_{ac} + q_{aa}} \times 100\% \quad (2)$$

where $\eta_{q,i}$ is the electron recover efficiency of product *i*,%; q_i is the mole of electron in the product *i*; q_{et}, q_{ac} and q_{aa} is the initial mole electron added in substrate ethanol, acetate and casamino acids, respectively.

2.4. DNA extraction and high-throughput sequencing

On Day 0, Day 10, Day 14, Day 21, Day 30, Day 45 and Day 51,

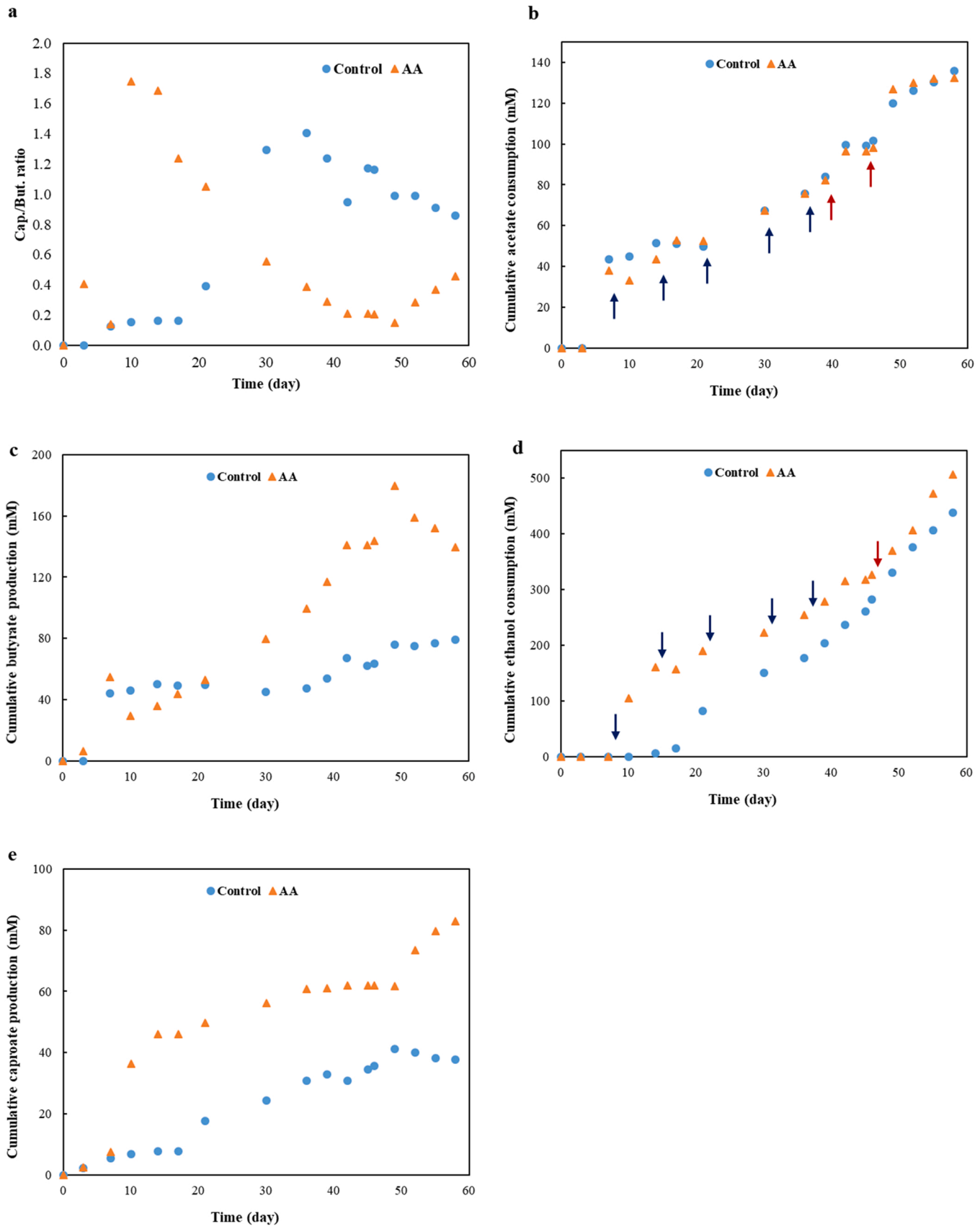

Fig. 1. Variation of carbon substrates and carboxylate products in the two reactors across the whole operation: (a) caproate/butyrate ratio, (b) acetate, (c) butyrate, (d) ethanol and (e) caproate. Blue arrow represents replenishment of fementation medium (same as the initial medium); red arrow represents replenishment of acetate stock solution in (a) and replnishment of ethanol in (b), repectively. Neither concentration of propionate nor carplyate were above detectable level.

biomass-containing broth samples were collected from each reactor and centrifuged at 10,000 rpm under 4 °C for 5 min, then the sediment were preserved at − 20 °C before DNA extraction. Genomic DNA extraction was conducted on the samples centrifugated at 16000*g* for 10 min using a kit (PowerSoil™ DNA Isolation Sample Kit, MoBio Laboratories, Inc., Carlsbad, CA). 16 S rRNA genes library construction and high throughput sequencing on Illumina MiSeq platform was performed at BGI (Shenzhen, China). A primer pair 341 F-806R [29], amplifying the V3-V4 region of the 16 S rRNA gene [30], was used for PCR amplification. On Day 14, a sample was extracted from the reactor AA for shotgun metagenomic sequencing on an Hiseq 4000 platform at BGI (Shenzhen, China). On Day 51, a sample was extracted from the reactor AA for metatranscriptomic sequencing when the fermentation reached a stable phase. It was well-mixed and submerged with RNAlater solution immediately after sample collection, and it was delivered for mRNA extraction, metatranscriptomic library construction and metatranscriptomic sequencing on Illumina Hiseq 4000 platform at BGI (Shenzhen, China).

2.5. Bioinformatics analysis

A modified version of the standard operating procedure [29] was used to process 16 S rRNA gene sequences in Mothur v.1.38.1 [31]. Raw sequences were assembled and screened, then the qualified one were aligned and filtered against the SILVA reference database (release 132) to ensure that the derived sequences were from the V3-V4 region. Next, chimeric sequences detected and removed using UCHIME algorithm [32]. Taxonomic classification was conducted using the Bayesian Classifier [33] with the SILVA database. Clustering sequences into operational taxonomic units (OTUs) with 97% identity threshold was performed to categorize bacteria groups. Sample diversity was evaluated based on operational taxonomic units (OTUs). The high Good's coverage (>0.95) of 16 S rRNA sequencing analysis suggested that sequencing depth was adequate to reflect the whole microbial community. Principal co-ordinates analysis (PCoA) was performed to evaluate the similarity among the microbial communities in Mothur v.1.38.1 [31]. The metagenomic and metatranscriptomics analysis method referred to the previous study except that assembled contigs in this study were separated into taxonomic bins using multiple binning tools – MaxBin version 22.2.7, MetaBAT version 22.15, and VamB 3.0.2 [34]. Gene expression level was evaluated based on generated Reads Per Kilobase Million (*RPKM*) and calculated as $\log_2$ RPKM values.

3. Results and discussion

3.1. Casamino acids significantly improved the production of caproate

Fig. 1a shows the caproate to butyrate ratio in the two reactors with (AA) and without (Control) the addition of casamino acids. The ratio directly reflected the degree of elongation, since butyrate is the precursor of the caproate fermentation [35]. In the initial stage of fermentation (within 20 days), the yielded caproate/butyrate ratio of the AA group was far higher than that of the control fermentation, indicating a higher level of chain elongation was accomplished in the reactor AA. It also suggested the trend of carbon distribution toward caproate with the addition of casamino acids. Additionally, the ratio reached the peak value on day 10 for the AA, indicating casamino acids accelerate the carbon conversion into the end-product in chain elongation process. As for the control fermentation, the caproate/butyrate ratio showed a great raise on day 30, corresponded to its lag phase for caproate production. Since low concentration of ethanol and accumulation of end-product inhibit the process of chain elongation [13], therefore, the caproate/butyrate ratio continued to decline after reaching its peak value. Until day 46, the replenishment of ethanol stimulated the caproate production, leading to an increase of caproate/butyrate ratio in the reactor AA correspondingly.

Fig. 1b shows the cumulative consumption of acetate in the two reactors across the whole fermentation, including acetate consumed in the initial supplement and each replenishment. Generally, concentration of acetate fluctuated in a similar trend in both the reactors. The drastic rise of acetate consumption on day 7 indicated a great utilization between day 3 and 7 for both groups. Accordingly, 44 mM and 55 mM of butyrate was detected on day 7 in the reactor of control and AA, respectively, as shown in Fig. 1c, implying an effective conversion of acetate to butyrate. From day 14–45, the consumption of acetate in both reactors gradually increased, and the concentration of acetate was at a relatively low level (below 6 mM) even with replenishment per cycle, indicating a constant utilization of acetate. Moreover, the continuous production of butyrate and caproate during this fermentation period suggested that the chain elongation process still proceeded even though acetate was at a low level, as long as the thermodynamics condition was favorable [36]. On day 45, sufficient acetate and ethanol was replenished, accordingly, an obvious rise of butyrate concentration was observed 4 days after this replenishment (day 49) in both groups (32% and 36% of increase compare with day 45), as shown in Fig. 1c. The increase of butyrate indicated an effective chain elongation, suggesting that sufficient acetate supplement was essential to support butyrate formation via chain elongation.

The variation of cumulative ethanol consumption also showed a similar trend in both reactors, as displayed in Fig. 1d. Comparatively, ethanol was consumed at an increased kinetic rate in the reactor AA than in the control one, especially between day 10 to day 20. This was consistent to the high caproate formation during this time period in the reactor AA (6-folds of the caproate formation in the control). This suggested that ethanol utilization was remarkably promoted by the addition of casamino acids. In the middle stage of fermentation (day 20–46), the ethanol consumption rate in the reactor AA slowed down, and the concentration of ethanol was at a relatively low level (127–174 mM). It was reported that very low concentration of ethanol reduces the rate of chain elongation [13]. Consequently, the insufficiency of ethanol supplement resulted in a decline of ethanol utilization rate and a decrease of the caproate formation (Fig. 1e). Moreover, the formation of caproate was always coupled with ethanol utilization, indicating that the caproate-producing step was highly ethanol-dependent.

The significant formation of butyrate on day 7 (Fig. 1c) indicated that the butyrate producers were well enriched and metabolically active within the initial stage of fermentation. The formation of butyrate in the reactor control was 25% lower than that in the reactor AA. On day 10, the reactor AA showed a sharp decrease of butyrate formation and a significant increase of caproate formation, suggesting that butyrate was efficiently converted to caproate, coupled with a great consumption of ethanol. Comparatively, from day 7–20 in the reactor control, the butyrate formation reached its plateau, corresponding to the plateau of caproate formation during the same time as shown in Fig. 1e. This suggested an inactive chain elongation process in the initial stage of fermentation in the control fermentation. After day 30, butyrate was gradually accumulated in the reactor AA. Thereafter, the limited ethanol and accumulation of end-product caproate were not thermodynamically favorable for further conversion of butyrate to caproate. Generally, the formation of caproate reached its peak (35 mM; 5.9-folds of that in the control fermentation) on day 10 in the AA. In contrast, the concentration of caproate achieved its peak value until day 30 in the control. This suggested that the lag phase for caproate production (time span between significant butyrate and caproate production) was reduced by 20 days, 7.6-folds kinetics increase for the AA (3 days) compared with the control (23 days). While the lag phase for butyrate production was 7 days for both the reactors, implying that the addition of casamino acids greatly shortened the lag phase for caproate production and accelerate the chain elongation process toward caproate production primarily.

3.2. Casamino acids improve carbon recovery efficiency and electron recovery efficiency

The carbon recovery efficiency of the reactor AA was remained higher than that in the reactor control mostly, as shown in Fig. 2a. In both the reactors, the initial concentration of acetate and ethanol was

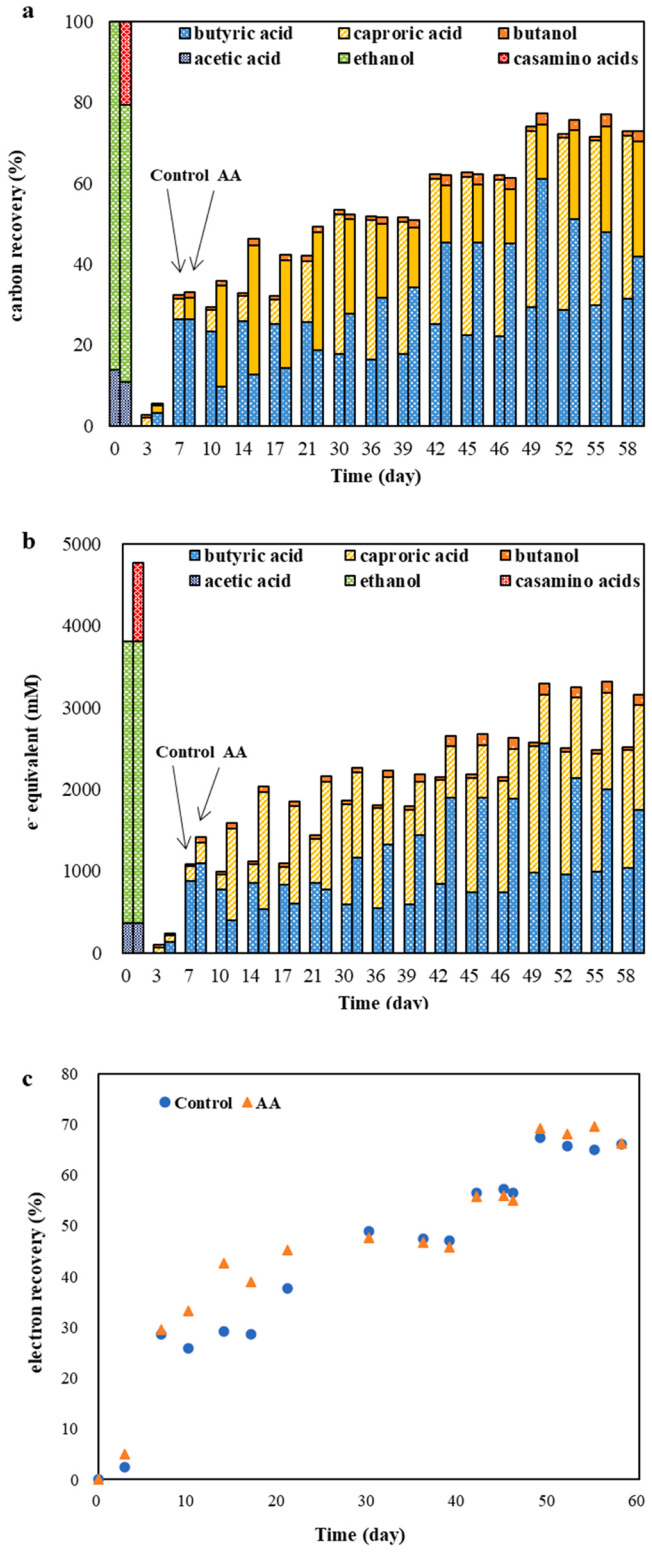

Fig. 2. Carbon and electron recovery efficiency across the whole operation in the two reactors: (a) carbon recovery efficiency; (b) variation of electron concentration based on e- equivalent; (c) electron recovery efficiency. The left and right bars in (a) and (b) represent the control group and AA group, respectively. The initial carbon as substrates was 666 mM for the reactor control and 838.4 mM for the reactor AA, and their respective carbon contribution of substrates and products was normalized and displayed as "%" in (a); calculation of e^- equivalent was based on the number of electrons contained in the reduced compounds (mol e-/mol: 8 for acetate, 12 for ethanol, 32 for caproate, 20 for butyrate, 24 for butanol and 4.7 for yeast extract). The actual concentration of products or substrates (on day 0) as e- equivalent was depicted in (b). The relative percentage of recovered electron to the initial influx of electron was depicted in (c) when casamino acids were assumed to be completely metabolized.

46 mM and 287 mM, respectively. The initial carbon available was 666 mM for the reactor control and 838.4 mM for the reactor AA (refer to method in Section 2.3). Casamino acids added were assumed to be completely metabolized, and the initial carbon contribution of substrates was displayed as "day 0″ in Fig. 2a. The recovered carbon was in the form of butyrate, caproate or butanol, and the carbon recovery percentage represents the efficiency of substrates utilization for products formation. During the middle stage of fermentation (day 30 to day 46), their efficiencies was comparable. During day 10 to day 21, the carbon recovery efficiency of group AA was 6.7–13.2% higher than the control fermentation, especially the carbon recovery contributed by caproate formation (14.2–25.8% higher than the control). Based on the observations, casamino acids efficiently promoted the chain elongation process toward the formation of caproate. While after 30-days fermentation, the limited ethanol in the reactor AA caused the decrease of caproate formation as well as the carbon recovery efficiency consequently. The observations implied that casamino acids influenced the carbon distribution in the products toward caproate formation. Generally, casamino acids not only accelerated chain elongation process, but also upgraded the overall carbon utilization efficiency by 13.2%, even casamino acids were assumed to be completely metabolized.

On the other hand, the electron recovery efficiency indicates the percentage of electrons from substrates recovered in reduced products, i.e., the number of electrons that pass from ethanol, acetate and casamino acids to caproate, butyrate and butanol. Fig. 2b shows the variation of electron concentration in the formed products in the two reactors during the whole fermentation period. The influx of electron into the two reactors was depicted on day 0, including acetate, ethanol and casamino acids. The yielded concentration of electron as products in the reactor AA was obviously higher than in the control fermentation, as shown in Fig. 2b. This was expectable considering the initial electron flow into the group AA was also higher than the control fermentation due to the electron contribution of casamino acids. While the proportion of electrons in form of caproate was much higher in the AA than in the control in the initial stage of fermentation (within 20 days), suggestion an accelerated chain elongation for caproate recovery in the AA. Fig. 2c. shows the overall percentage of recovered electron to the initial input of electron. Still, the AA group showed higher overall electron recovery efficiency than the control group even when the contribution of casamino acids was took into consideration, especially between Day 7 to Day 21. Presumably, the addition of casamino acids not only stimulated microbial growth, but also involved in the chain elongation process, attributing to substrates utilization and product formation, therefore promoted the overall electron and carbon recovery efficiency. Also, the addition of amino acids promoted electron flow for caproate recovery, especially in the initial stage of fermentation when ethanol was sufficient.

(caption on next column)

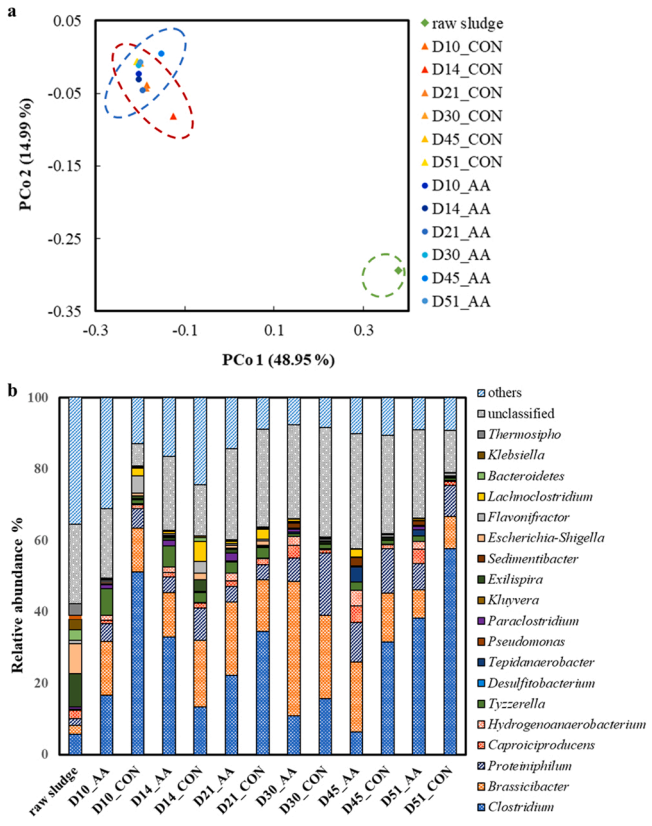

Fig. 3. Diversity and microbial community structure: (a) Principal coordinate analysis (PCoA) of community similarity comparing the raw sludge and samples from the reactor AA & control. PCo 1 and PCo 2 represents Principal coordinate 1 and Principal coordinate 2, respectively (63.94% in total); (b) Taxonomic classification of the bacterial communities of the raw sludge and the two reactors on different days at genus level. Genus occurred at abundance more than 1% in at least one sample was annotated whereas rests were grouped as "others". Each sample was named after the form of "sampling day_the reactor".

3.3. Diversity and microbial community structure of chain elongation microbiome

The Principal Co-ordinates Analysis (PCoA) result in Fig. 3a showed that dots representing the reactor control and AA were clustered closely, whereas the green dot representing raw sludge was located distantly from the other two reactors. This indicates that the microbial community structures of the two reactors were readily re-constructed and shared a similarity even within 10-days fermentation, and they were divergent from that of the raw sludge. Moreover, after 51-days fermentation, the dots representing the two reactors were clustered even closer than before, suggesting that their microbial community structures were evolved to be similar after certain time of cultivation. Additionally, alpha diversity analysis showed that microbial communities of the two reactors were much less diverse and less evenly-distributed than the raw sludge, implying an effective enrichment of microbial consortia (Table S1).

From the taxonomic distribution of microbial community at genus level, as shown in Fig. 3b, microbial communities of the reactor AA and control were remarkably predominated by *Clostridium*, *Brassicibacter* and *Proteiniphilum*, and the genera *Caproiciproducens* and *Hydrogenoanaerobacterium* were also well-enriched. The highly abundant genera in raw sludge, *Exilispira* and *Escherichia-Shigella,* later diminished as the fermentation progressed, suggesting they were outcompeted by the MCFAs-producing related microorganisms. Particularly, a gradual enrichment and absolute predominance of *Clostridium* was observed in both the reactors, reaching an abundance of 58% (in the control) and 38% (in the AA) after 51 days of cultivation. *Clostridium spp.* are strongly implicated in the synthesis of caproate and butyrate [25,37]. Such predominance of *Clostridium* was also observed in several previous chain elongation system [2,10,36]. Although *Clostridium* was highly enriched in the control, its caproate production still lagged than the AA reactor. We hypothesize that caproate formation requires tight metabolic cooperation among various microorganism and the enrichment of a single caproate producer (i.e. *Clostridium kluyveri*) not consequentially accompanied with great caproate production. Besides, the caproate-producing genus, *Caproiciproducens*, was also well-enriched, especially in the reactor AA after 30-days cultivation. *Caproiciproducens* is able to ferment various carbohydrates such as glucose, galactitol and several mono- and disaccharides to produce ethanol, acetate, butyrate and caproate [38,39]. In an anaerobic fermentation system using Chinese liquor distillers' grain as feedstock for caproate formation, *Caproiciproducens* was found to be the abundant group responsible for converting lactic acid into caproate [40]. The enrichment of *Caproiciproducens* indicated that they might utilize substrates other than ethanol for caproate formation under the fermentation condition in this study. Its higher abundance in the AA than in the control indirectly proved the speculation that diverse carbon sources might positively correlate with the abundance of *Caproiciproducens,* considering that the reactor AA was provided with extra organic substances. In addition, *Brassicibacter* was also well-enriched in both the reactors, and it could ferment amino acids and form acetate and ethanol [41]. The amino acids-rich environment in the reactor AA was more favorable for the enrichment of *Brassicibacter*, since it was provided with more sufficient amino acids than the control one, in which probably microbial debris were the main amino acids sources. Moreover, another predominant genus in both reactors, *Proteiniphilum*, is also proteolytic and capable of using various amino acids, such as glycine and L-arginine, or yeast extract, for converting to acetic acids as the main product [42]. The genus *Hydrogenoanaerobacterium* only showed dominance in the reactor AA with an abundance of 1.3% on day 10, and gradually increase as the fermentation progressed. While its abundance in the raw sludge was not even detected and was very low (< 0.3%) in the reactor control. *Hydrogenoanaerobacterium* uses various saccharides to produce ethanol, acetate, hydrogen and carbon dioxide mainly [43]. It was found to be the preeminent genera in a polysaccharidic-rich fermentation system utilizing mixed fruit wastes for acidogenesis and carboxylic chain elongation [44]. Its predominance in the AA reactor suggested that amino acids-rich condition also favored its enrichment. Generally, the predominant microbial groups in the AA and the control were similar with somewhat difference in abundance. The better performance of caproate production in the AA reactor indicated that miscellaneous substances might facilitate caproate formation indirectly.

3.4. Casamino acids facilitated caproate production pathway

A sample was taken on Day 15 for metagenomics analysis to further explore the effect of amino acids towards its interspecies metabolic interactions. The recovered high-quality genome bins that phylogenetically identified to be closely related to *Clostridium kluyveri*, *Clostridium cochlearium*, *Caprobacter fermentans* and *Proteiniphilum acetatigenes*, respectively (Fig. S3, Table S6) were analyzed further for metabolic pathways reconstruction. These draft genomes together represented the majority of the microbial community in the AA. As an exceptional anaerobe, *C. kluyveri* was reported to be capable to accomplish ethanol-acetate chain elongation using the reverse β-oxidation pathway [25], which can be characterized by three coupled reactions. Ethanol is first oxidized to acetate, then acetate together with CoA is converted to acetyl-CoA and elongated to butyrate in a cyclic pathway. Lastly, by using a similar cyclic pattern of coupling butyryl-CoA with acetyl-CoA, caproate is elongated from butyrate and ethanol [1,25]. In this fermentation study, the concentration shifted in a similar way as first ethanol was consumed, and then butyrate was produced followed by

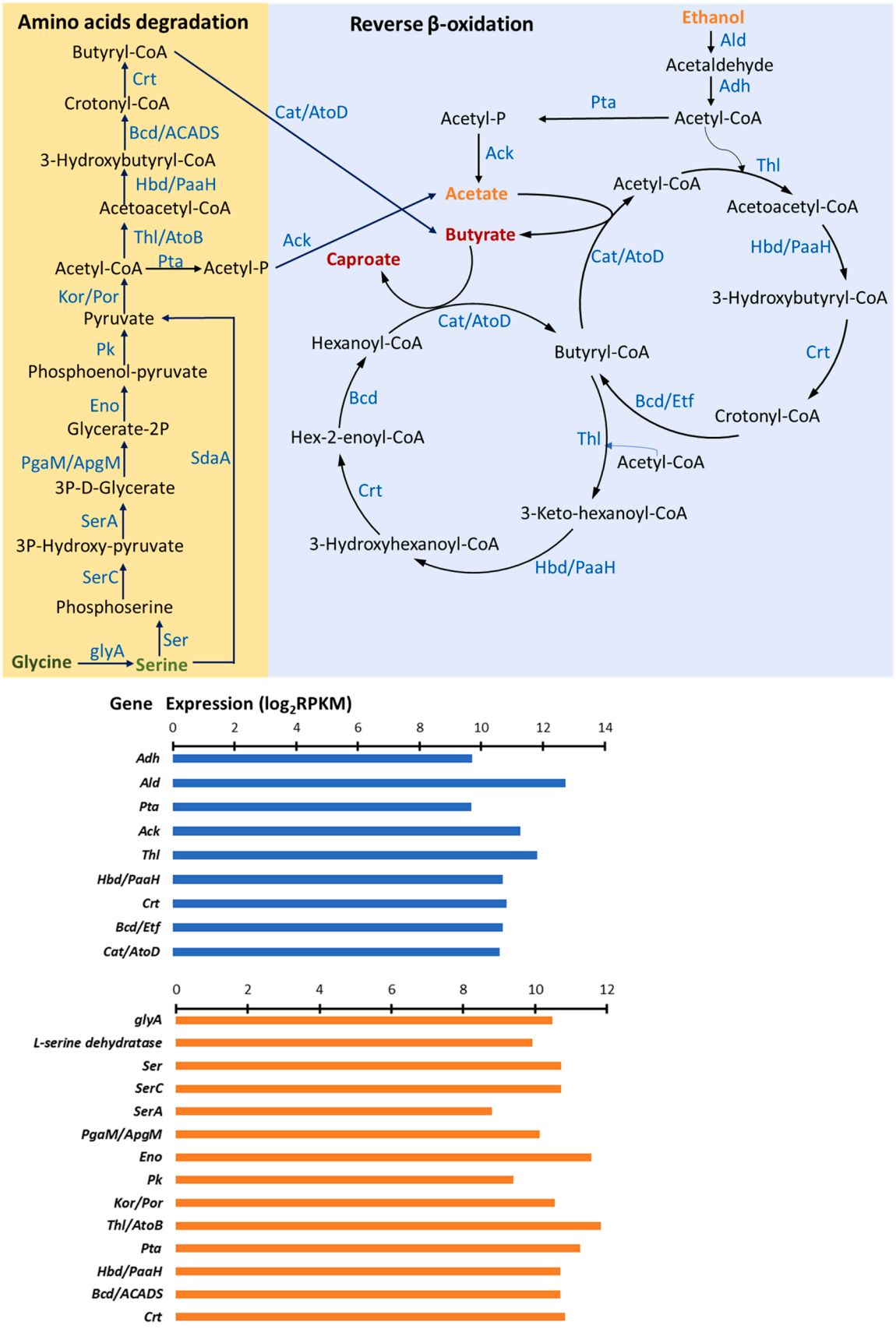

Fig. 4. Metabolic pathways of ethanol-acetate fermentation for butyrate and caproate production (in blue) and degradation of glycine and serine for butyrate production (in yellow): the sample was taken in the reactor AA on Day 15.

caproate production (Fig. 1). The complete ethanol-acetate fermentation pathway for caproate formation was fully recovered from the *C. kluyveri* (CE_AA_MaxBin.002) genome bin (Fig. 4; Table S7).

Another high-quality genome bin (CE_AA_metabat.60) phylogenetically identified to be closely related to the genomes of *C. cochlearium* was recovered. *Clostridium cochlearium* was a butyrate producer reported to be involved in the conversion of amino acids, such as glutamate, glutamine, and histidine, into butyrate, but not the synthesis of caproate [37,45]. It was found to be dominant in a single-phase anaerobic reactor system for caproate production from food waste [46]. To examine the metabolism of casamino acids and its association with the butyrate production, L-glutamate was selected as the typical amino acid to re-construct the metabolic pathway in *C. cochlearium*. The complete L-glutamate fermentation pathway for butyrate production was fully recovered from the *C. cochlearium* genome bin (Table S7). L-glutamate was degraded to acetyl-CoA, which was converted to acetate or elongated to butyryl-CoA for further MCFAs formation. *Caprobacter fermentans* (*Caproiciproducens spp.*), recovered as maxbin.022, is chain-elongating and caproate-producing bacterium that ferment several sugars into acetate, butyrate and caproate as major metabolic end products. Moreover, ethanol, CO_2 and H_2 are its metabolic products as well [38,39,47]. D-glucose was selected as a typical sugar to re-construct its metabolism in *C. fermentans* to examine its correlation with caproate production. The complete D-glucose fermentation pathway for butyrate production was fully recovered from the *C. fermentans* genome bin (Table S7). D-glucose was converted to acetyl-CoA via glycolysis and pyruvate metabolism, and was converted to acetate or elongated to butyryl-CoA for further butyrate formation. Both the formed acetate and butyrate were involved in the reverse β-oxidation pathway, then further chain elongation occurred by coupling acetate/butyrate with acetyl-CoA in a cyclic loop.

The glycine and serine fermentation pathways for acetate and butyrate formation were fully recovered from a genome bin of *Proteiniphilum acetatigenes* (CE_AA_MaxBin.018), which metabolizes yeast extract, peptone, pyruvate, glycine and L-arginine into acetic acid as the main product [42]. To examine the metabolism of casamino acids and its association with the MCFAs production, glycine and serine were selected as typical amino acids to re-construct the metabolic pathway in the system, as depicted in Fig. 4, glycine and serine are converted to pyruvate firstly, then acetyl-CoA is produced as an important intermediate in the further pyruvate metabolism. Subsequently, acetyl-CoA is converted to acetate or elongated to butyryl-CoA, which is further converted to butyrate. Both the formed acetate and butyrate were involved in the reverse β-oxidation pathway, then further chain elongation occurred by coupling acetate/butyrate with acetyl-CoA in a cyclic loop. Metatranscriptomic analyses were performed to further verify the expression of key functional genes in the reconstructed metabolic pathways. The *C. kluyveri* and *P. acetatigenes* genome bins with high gene expression level were selected to reconstruct the microbial interactions for caproate production. In the *C. kluyveri* genome bin, the genes involved in ethanol-acetate fermentation pathway for caproate formation were actively-transcribed with high $\log_2$ RPKM values of 9.7–12.7 (Fig. 4), suggesting that the pathway was metabolic-active and contributed to caproate production. In addition, gene expression patterns confirmed that the glycine and serine fermentation pathway for butyrate production was metabolically active in the *P. acetatigenes* genome bin with the high $\log_2$ RPKM values of 8.8–11.8 (Fig. 4). The results verified that casamino acids could directly contribute to butyrate production but not to caproate production. The converted butyrate is further involved in caproate formation, therefore promoting the chain elongation process. Details in genome bins recovery and gene contents of the annotated metabolic pathways are shown in supplementary information.

4. Conclusions

As the primary component in yeast extract, the effect of casamino acids introduction into a chain elongation process was investigated. By intergrating 5 g/L of casamino acids The lag phase for caproate production was significantly shortened, and the overall products recovery efficiency was imporved. Microbial community structure distinctively differented from the raw sludge was enriched, highly predominated by *Clostridium*, *Proteiniphilum* and *Brassicibacter*. It was confimed that casamino acids are directly metabolized to acetate and butyrate for further chain elongation to caproate. The optimal dosage of yeast extract worth for further investigation to maximize the chain elongation efficieny and lower down the cost of fermentation. The finding implies that the microbial community capable of caproate synthesis could be diverse and may therefore help in maintaining a stable and robust process.

CRediT authorship contribution statement

Peixian Yang: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Ling Leng**: Formal analysis, Writing – review & editing. **Huichuan Zhuang**: Formal analysis. **Po-Heng Lee**: Writing – review & editing.

Declaration of Competing Interest

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.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2023.108879.

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