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Biological caproate production by *Clostridium kluyveri* from ethanol and acetate as carbon sources

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Abstract

Caproate is a valuable industrial product and chemical precursor. In this study, batch tests were conducted to investigate the fermentative caproate production through chain elongation from acetate and ethanol. The effect of acetate/ethanol ratio and initial ethanol concentration on caproate production was examined. When substrate concentration was controlled at 100 mM total carbon, hydrogen was used as an additional electron donor. The highest caproate concentration of 3.11 g/L was obtained at an ethanol/acetate ratio of 7:3. No additional electron donor was needed upon an ethanol/acetate ratio ≥7:3. Caproate production increased with the increase of carbon source until ethanol concentration over 700 mM, which inhibited the fermentation process. The highest caproate concentration of 8.42 g/L was achieved from high ethanol strength wastewater with an ethanol/acetate ratio of 10:1 (550 mM total carbon). Results obtained in this study can pave the way towards efficient chain elongation from ethanol-rich wastewater.

Keywords: Chain elongation; n-caproate, *Clostridium kluyveri;* Fermentation; Ethanol/acetate ratio

1. Introduction

For a sustainable development, production of biochemicals and biofuels from biomass by fermentation has become a priority. Examples of such products are ethanol, biohydrogen and biomethane. Besides the final products, intermediate compounds are also produced. Such compounds are volatile fatty acids (VFA) with 2 to 5 carbon atoms which are formed during fermentation processes and often accumulate in the liquid phase (Lee et al., 2014; Yang et al., 2015; Yin and Wang, 2016). Discharge of these metabolites into environment will not only lead to pollution but also waste energy, since they could be used as precursor for biofuels. However, the high solubility of short-chain fatty acids makes them hard to be recovered and therefore the downstream processing for retrieving these intermediates has been the main reason hampering their further maturation as market products. Furthermore, they are inappropriate to be used as fuel directly owing to the high oxygen-to-carbon ratio and low energy density (Steinbusch, 2011). On the other hand , medium-chain fatty acids (carboxylic acids with 6 to 8 carbon atoms), which own the advantage of both low solubility and high energy content, are regarded as potential intermediates for further conversion to valuable chemicals or fuels (Levy et al., 1981).

Caproic acid is a six-carbon acid with the general formula $C_5H_{11}COOH$. It is a fatty acid naturally present in various animal fats and oils. Caproic acid is slightly soluble in water (10.19 g/L), thus, converting short-chain fatty acids to caproic acid can lead to efficient downstream recovery of liquid metabolites from fermentation process (Agler et al., 2014). It is not only a valuable industrial product but also a chemical precursor. It

can be used as a "green" antibiotic in agriculture at lower pH levels (pH 2.0-5.0) (Butkus et al., 2010; Agler et al., 2011; P. Desbois, 2012). Caproic acid is also a precursor for the production of flavors (Kenealy et al., 1995), liquid fuels (Harvey and Meylemans, 2014) and corresponding alcohols (Perez et al., 2013; Isom et al., 2015). Moreover it is an important flavor compound in distilled alcoholic beverage (Hu et al., 2015).

Caproate has been found as a byproduct of fermentative hydrogen or methane production from organic wastes (Steinbusch et al., 2009; Ding et al., 2010), which may be produced from the elongation of VFA present in the system. Then, several studies have followed up this assumption and explored chain elongation process by using different substrates (e.g., syngas) or process configurations (Steinbusch et al., 2011; Grootscholten et al., 2013; Kucek et al., 2016; Gildemyn et al., 2017). It has been observed that a group of anaerobic bacteria that possess fatty acid synthase complex are able to elongate ethanol and short chain fatty acids through a reaction known as the reversed β-oxidation. Several bacteria such as *Clostridium* species have been reported to be able to produce caproic acid from short chain fatty acids (Thauer et al., 1968; Seedorf et al., 2008; Weimer and Stevenson, 2012); Among all the species able to produce caproate, spore forming *Clostridium kluyveri* has been ubiquitously identified in anaerobic fermentation systems, and proved to be efficient in converting ethanol and acetate to butyrate and caproate (Ding et al., 2010). Thus, *C. kluyver* was used in this study to explore the caproate production from different concentrations of ethanol and acetate. Equation 1-3.4 shows several metabolic pathways that have been

suggested for caproate formation in anaerobic fermentation (Ding et al. 2010; Mu and Yu, 2006).

$2C_2H_5OH + CH_3COO^- \rightarrow C_5H_{11}COO^- + 2H_2O$ ΔG^0= -79.0 kJ/mol (1)

$3CH_3COO^- + 4H_2 + 2H^+ \rightarrow C_5H_{11}COO^- + 4H_2O$ ΔG^0= -86.2 kJ/mol (2)

$C_2H_5OH + CH_3COO^- \rightarrow C_3H_7COO^- + H_2O$ ΔG^0= -38.7 kJ/mol (3)

$2C_3H_7COO^- \rightarrow C_5H_{11}COO^- + CH_3COO^-$ ΔG^0= 0.1 kJ/mol (3.1)

$C_3H_7COO^- + CH_3COO^- + 2H_2 + H^+ \rightarrow C_5H_{11}COO^- + 2H_2O$ ΔG^0= -48.0 kJ/mol (3.2)

$C_3H_7COO^- + 2C_2H_5OH \rightarrow C_5H_{11}COO^- + +CH_3COO^- + H^+ + 2H_2$ ΔG^0= -48.4 kJ/mol (3.3)

$C_3H_7COO^- + 2CO_2 + 6H_2 \rightarrow C_5H_{11}COO^- + 4H_2O$ ΔG^0= -143.3 kJ/mol (3.4)

ΔG^0 (kJ/mol) is the value of ΔG at pH 7.0 under standard conditions (i.e., all solutes are at the concentration of 1 mol/L, and gases have partial pressure of 1 atm). Bornstein and Barker (1948) found that the metabolic pathways of *C. kluyver* are dependent upon concentration of acetate and ethanol, and the excess of ethanol can lead to more caproate yield than butyrate. Similar conclusion was also obtained by Kenealy and Waselefsky (1985). Weimer et al. (2012) examined the effect of different acetate and ethanol concentrations on products formation, and highest caproate production was obtained at ethanol 700 mM and acetate 120 mM. Besides, Kenealy and Waselefsky (1985) observed the linear response of both products and microbial growth with ethanol and acetate concentration up to 200 and 50 mM, and Weimer et al. (2012) found that a further increase of ethanol or acetate concentration can lead to a significant decrease on *C. kluyver* growth rate. These studies show the significant influence of acetate/ethanol ratios on products, and the contradictory effect of substrate

concentration on caproate production and microbial growth. Thus, to achieve a high caproate production, it is necessary to break through the substrate inhibition and supply a suitable acetate/ethanol ratio.

In the past few years, most studies have reported production of caproate only from low ethanol concentration (<300 mmol/L) because ethanol is inhibitory to microorganisms (Kenealy et al., 1995; Jeon et al., 2010; Weimer and Stevenson, 2012; Vasudevan et al., 2014; Weimer et al., 2015). As a consequence, with low substrate concentrations, only low caproate production rates can be achieved resulting to low caproate titers, making a production process uneconomic. Furthermore, in ethanol fermentation broths the ethanol concentration ranges from 400 to 1800 mmol/L, which is much higher than the initial ethanol level reported as substrate for caproate production (Kenealy et al., 1995; Jeon et al., 2010; Weimer and Stevenson, 2012; Vasudevan et al., 2014; Weimer et al., 2015). Thus, in order to use the ethanol fermentation broth as substrate, a fermentation process for caproate production at higher ethanol concentrations needs to be developed.

In this study, the characteristics of caproate production from the diluted fermentation effluent with relatively high level of ethanol (up to 1000 mmol/L) by *Clostridium kluyveri* were investigated. In addition, the effect of acetate and ethanol concentration on caproate production was explored. The results provide important supplementary information to the biosynthesis of caproate from industrial fermentation effluent.

2. Material and methods

2.1 Preparation of inocula

Clostridium kluyveri DSM 555 was purchased from the DSMZ (The Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), and was cultivated in DSM-52 medium. The medium was boiled for 1 min and cooled to room temperature under 80% N_2 and 20% CO_2 gas atmosphere, after which 0.25 g/L L-Cysteine-HCl•H_2O was added as reducing agent. The pH of the medium was adjusted to 7.5 using 1 M NaOH and 1 M HCl. Then, the reduced medium was dispensed in 300 mL glass bottles with 100 mL working volume. All bottles were sealed with rubber stoppers and aluminum caps to avoid gas leakage. The sealed bottles were flushed with 80% N_2 and 20% CO_2 for 30 min to provide the anaerobic environment. Bottles were autoclaved and the medium was further supplemented with a vitamin solution (DSM-503), 1 g/L yeast extract and 20 mL/L ethanol through a 0.2 um membrane filter. Then, *Clostridium kluyveri* DSM 555 was inoculated into the bottles, which were placed in a reciprocal shaker (37 °C, 150 rpm) and incubated for 3-4 days until the bacteria entered the stationary phase. Subsequently, the bacteria was transferred into fresh medium for another cultivation. Before being used in batch experiments, pure culture of *Clostridium kluyveri* was transferred for over 3 times to fully activate the bacteria and achieve a stable state.

2.2 Experimental setup

Batch experiments were conducted in 600 mL glass bottles with 100 mL working volume, rubber stoppers and aluminum caps were used to avoid gas leakage from the bottles. Acetate and ethanol was used as sole carbon sources, the composition of carbon source and additional electron donor in different batch tests is shown in Table 1. For the

batch 1 with 100 mmol/L carbon source as substrate, acetate/ethanol ratio of 1:1 was employed to examine the feasibility of hexonal production process, and then, acetate/ethanol ratio of 8:2, 7:3, 5:5, 3:7, 2:8 were explored. Acetate/ethanol ratio of 1:1 was selected as starting point, as it is within the ratio ranges reported in the previous study (Weimer and Stevenson, 2012).

10 mL of pre-cultured *C. kluyveri* was inoculated into the designed medium when the culture was at exponential growth phase (OD 600=0.8±0.02). Initial pH of all batches was set to 7.5 and the medium was flushed with 80% N_2 and 20% CO_2 for 30 min to drive away the oxygen present in the liquid and headspace. For the tests with 50 mM acetate and 50 mM ethanol as substrate, hydrogen was added in the batches as additional electron donor. Except the last test group with sole ethanol as carbon source, 480 mL hydrogen was injected in the rest 7 test groups. All the bottles were incubated in a reciprocal shaker at a speed of 150 rpm at constant temperature of 37 °C. All the batch tests were conducted in duplicate. During the batch fermentation process, the pH of the media was not controlled. Every one or two days, 1 mL gas and 2 mL of liquid were taken out using syringe with needles for the analysis of hydrogen concentration, microbial growth, pH change, substrate degradation and VFA formation.

Table 1 Experimental set up of the different batch tests.

2.3 Analytical methods

Cell dry weight was measured according to Standard Methods (APHA, 1995). The pH

159 was measured by PHM99 LAB pH meter connected to the Gel pH electrode
160 (pHC3105-8, Radiometer analytical). Hydrogen was analyzed by GC-TCD (Mikrolab,
161 Aarhus A/S, Denmark) fitted with a 4.5 m × 3 mms-m stainless column packed with
162 Molsieve SA (10/80). The temperatures of the injector, detector and oven were 190, 110,
163 and 190 ^{o}C, respectively. N_2 was used as carrier gas. Concentration of alcohols and VFA
164 was analyzed by a gas-chromatograph (HP5890 series II) equipped with a FFAP fused
165 silica capillary column (30m 0.53 mmi.d. film thickness 1.5 mm) and a flame
166 ionization detector. The carrier gas was N_2.

167 ## 3. Results and discussion

168 ### 3.1 Growth of *Clostridium kluyveri*

169 *C. kluyveri* (DSM 555) was cultivated in DSM-52 medium. Initially it showed a lag
170 phase of approx. 30 hours, and then entered the exponential growth phase which lasted
171 for approx. 40 hours before it entered the stationary phase (Fig.1). Maximum cell dry
172 weight of 0.62 g/L was obtained after 74 h of cultivation. Microbial growth rate of
173 13.16 mg/L/h was obtained. Similar, growth process was observed by Stadtman and
174 Barker (1949). Besides, shorter lag time of 16 h was obtained by Thauer et al. (1968),
175 with maximum cell dry weight of less than 0.025 g/L at 48 h. The short lag time and low
176 cell dry weight obtained by Thauer et al. (1968) may due to lower initial ethanol
177 concentration of 11.5 g/L, resulting in lower inhibition of growth, while supplying
178 lower amount of carbon source yielding in lower cell-biomass. Thus, the high cell
179 concentration and regular growth period indicated strain *C. kluyveri* was fully activated
180 and functioned well in present lab condition.

Fig. 1 Growth curve of *Clostridium kluyveri* cultivated in DSM-52 medium

3.2 Effect of substrate composition on caproate production

In batch fermentation with 50 mmol/L (2.3 g/L) acetate and 50 mmol/L (3 g/L) ethanol as initial substrate concentrations, caproate was produced by *C. kluyveri* at 37 ^{o}C and initial pH 7.5. Fig. 2 depicts the time course of microbial growth, pH change, substrate utilization and formation of VFA during the fermentation process in 30 days. As shown in Fig. 2A, there was a long lag phase of 6 days, before the bacteria entered the exponential growth phase. Microbial growth rate of 0.80 mg/L/h and maximum cell dry weight of 0.28 g/L was obtained at 20 d, and remained stable for the remaining 10 days. The microbial growth rate and cell dry weight obtained in batch fermentation were 94 % and 55 % lower in comparison with the bacteria cultivated in DSM-52 medium. The slower growth rates observed may be due to the lower initial substrate concentration used in batch fermentation than bacteria cultivation process, indicating that the substrate concentration was below the saturation concentration to achieve maximum growth rates according to Monod growth model. Lonkar et al. (2016) also observed that the increase of ethanol concentration from 0 g/L to 20 g/L resulted in increase of growth rates, and subsequently a decrease of the fermentation duration, defined as the termination of both substrate consumption and VFA generation, from around 22 d to 18 d. However, despite that stagnation of growth it was observed that not all substrates were consumed, probably due to product

inhibition or due to pH decrease. Accompanied with the microbial growth, pH decreased gradually from 7.5 to 5.7 due to the accumulation of VFA.

Studies have proved that the fermentative caproate production process was very sensitive to pH. Kenealy et al. (1995) found that substrate consumption was inhibited when pH decreased at around 5.5, and caproate production can be significantly increased through controlling pH at around 6.8. Agler et al. (2012) and Vasudevan et al. (2014) figured out that caproate could be toxic to microbes when pH was under 5.5. Coma et al. (2016) examined the detrimental effect of acidic environment ($pH \leq 4.5$-5) to *C. kluyveri*. Thus, controlling operational pH at a near-neutral range can be necessary for ahieving a higher substrate degradation and caproate production rate.

As shown in Fig. 2B, substrate consumption and VFA production was terminated in 20 h. Concentration of caproate, burytate and valerate produced were 2.82 g/L, 1.54 g/L and 0.31 g/L, respectively. The formation of VFA was consistent with the cell growth. The first 10 h only low VFA production as observed, and therefore was assumed that microbial growth was in lag phase. When the exponential growth phase was initiated, significant increase in VFA production was obtained. Formation of VFA ceased with termination of cell growth. Same time course was obtained by Thauer et al. (1968). During the fermentation process, both acetate and ethanol concentrations decreased gradually from the beginning of fermentation process and removal efficiency of 72.5 % and 85.2 % were obtained at the end of batch run. However, in other studies, substrate was consumed simultaneously with the formation of VFA (Thauer et al., 1968; Kenealy et al., 1995; Weimer and Stevenson, 2012; Jeon et al.,

2013; Lonkar et al., 2016). Possible reason of this difference was probably the different fermentation conditions. In the other studies, mixed cultures and *Clostridium* species other than *C. kluyveri* were used as inocula, led to different metabolic pathways and more diverse VFA composition. Furthermore, besides acetate and ethanol, other substrates like cellulose, galactitol and succinate were also used as substrates for caproate production, which can affect both microbial growth and VFA formation process. Formation of butyrate was 2 hours earlier than caproate, indicating that during the process, acetate and ethanol were firstly converted to butyrate as an intermediate (Equation 3), and then caproate was formed from butyrate elongation with acetate (Equation 3.1-3.4). It can be seen from Equation 1-3.4 that, sums of ΔG^o of Equation 3 and 3.1-3.4 are all more exergonic at standard conditions (negative (from -86.6 to -182.0) than Equation 1 (-79.0) and 2 (-86.2)), indicating that, caproate is more preferred to be formed from butyrate than initially from acetate and ethanol.

Fig. 2 Volatile fatty acids production from acetate (50 mmol/L) and ethanol (50 mmol/L)

To explore the effect of initial acetate/ethanol ratio on caproate production, batch tests with only acetate and ethanol at different acetate/ethanol ratios (8:2, 7:3, 5:5, 3:7, 2:8), as carbon sources were conducted (Table 1, batch 1). Hydrogen was added as extra electron donor. VFA and alcohols concentration at different acetate/ethanol ratios is shown in Fig. 3.

It can be seen that caproate production was lower than 1 g/L when acetate/ethanol was higher than 7:3. However, it increased dramatically to 3.02 g/L when acetate/ethanol decreased to 5:5, and achieved the highest concentration of 3.11 g/L when acetate/ethanol was decreased to 3:7. Many studies have also observed the negative correlation between caproate production and acetate/ethanol ratio. Liu et al. (2016) found that caproate production can be enhanced through decreasing the acetate/ethanol ratio from 2:1 to 1:3. Weimer and Stevenson (2012) observed linear decrease of caproate formation with the reduction of acetate/ethanol ratio from 5 to 0. However, in this study, both caproate and butyrate production decreased with the further increase of the ethanol share, while the concentration of valerate remained at a high level, indicating that acetate was necessary for caproate production, reduction of acetate in substrate can also inhibit the fermentative caproate production process. Similarly Diender et al. (2016) found that the production of medium-chain fatty acids can be significantly stimulated by the presence of acetate. However, the specific function of acetate is not clear yet.

As to the other VFA, butyrate showed a similar trend with caproate. Propionate production was favored at high acetate concentration, while valerate production was promoted at high ethanol concentration. Wallace et al. (2004) found that both propionate and butyrate can be turned into valerate and caproate with ethanol as electron donor. Thus, it is reasonable that the remaining propionate and butyrate concentration decreased with the increase of added ethanol. For the tests with ethanol as sole carbon source, valerate production was dramatically affected by the addition of

hydrogen.

Fig. 3 Volatile fatty acids production at different acetate/ethanol ratios

Fig. 4 shows the change of hydrogen content in each bottle. Only a little change of hydrogen content was observed in all test groups. For the test groups with acetate/ethanol ratio over 5:5, hydrogen content showed a little increase in 10 d and then decreased in 30 d. Seedorf et al. (2008) and Ding et al. (2010) have showed that both hydrogen and caproate can be produced from acetate and ethanol by *Clostridium kluyveri*. Hydrogen has been proved to be able to act as electron donor for both hexanol and ethanol production from acetate (Spirito et al., 2014; Ding et al. 2010; Mu and Yu, 2006), however, the results showed that little hydrogen was consumed during the fermentation process, which may because of the thermodynamic bottleneck for the hydrogen to be used as electron donor (Gonzálezcabaleiro et al. 2013).

For the tests with acetate/ethanol ratio less than 1:1, the hydrogen content increased along the fermentation process. Although the ΔG^{o} of hydrogen as electron donor is much more negative than ethanol as electron donor (Equation 1-3.4), the results showed that ethanol is more easily consumed as electron donor than hydrogen. This may due to the high mass transfer resistance between hydrogen and reaction phase. The results indicate that at high ethanol levels, no additional electron donors are needed for the caproate production process. Same conclusion was also made by Liu et al. (2016) that the electron donor was sufficient for the chain elongation reaction

when acid/alcohol ratio was less than 1:2.

Fig. 4 Change of hydrogen content along the fermentation process in different batches

3.3 Caproate production from high ethanol concentration wastewater

To explore the possibility for caproate production from high ethanol concentration wastewaters, 500 mmol/L (23 g/L) ethanol along with 50 mmol/L (3 g/L) acetate were used as carbon source. It can be seen from Fig. 5A that bacteria grew very fast in the medium containing high ethanol concentration. Cell dry weight achieved 0.75 g/L in 3 d cultivation. Subsequently the fast growing cells formed flocks in the solution, making determination of the cell concentration through a small amount of sample unreliable due to the inhomogeneity issues. However, we could also observe that the microbes entered exponential growth directly without experiencing a lag phase, and the fermentation process terminated in 5 d. Microbial growth rate of 12.67 mg/L/h was obtained. Comparing with the batch tests using 23 g/L ethanol and 3 g/L acetate as substrate, both microbial growth rate and maximum cell dry weight were significantly enhanced. Similar to microbial growth, pH dropped from pH 7.5 to pH 6.2 in 3 d, and then remained constant at around pH 6.1.

As shown in Fig. 5B, acetate and ethanol decreased with the microbial growth. After three days, acetate concentration decreased to an undetectable level and ethanol was at around 15 g/L. Butyrate increased in first 36 h, showing that acetate and ethanol were

first converted to butyrate (Equation 3). Subsequently, butyrate decreased to under 5 g/L due to elongation of butyrate to caproate (Equation 3.1, 3.3) (Mu and Yu, 2006; Ding et al., 2010). Caproate concentration increased significantly in first three days and highest concentration of 8.42 g/L was obtained. It can be seen that the conversion efficiency (Consumed amount /Original amount) of ethanol was around 48 %. Lonkar et al. (2016) reported that ethanol conversion efficiency decreased from over 80 % to less than 25 % when initial ethanol concentration increased from 5 to 40 g/L. The termination of fermentation process may be due to the acetate depletion, since it has been proposed that the chain elongation reaction needs to be stimulated by acetate. Another possible reason could be product inhibition. Different maximum achieved caproate concentrations through microbial fermentation, have been reported in the literature and were in the range from 0.8 g/L to 8.6 g/L (Steinbusch et al., 2011; Weimer and Stevenson, 2012; Jeon et al., 2013; Vasudevan et al., 2014; Weimer et al., 2015; Ganigué et al., 2016; Liu et al., 2016; Lonkar et al., 2016). To further enhance the caproate production, in situ recovery of the formed caproate during the fermentation process may enhance the final caproate yield (Jeon et al., 2013).In summary, 23 g/L ethanol showed no inhibition on *C. kluyveri*, and the high substrate concentration stimulated both microbial growth and fermentative caproate production.

Fig. 5 Volatile fatty acids production from acetate (50 mmol/L) and ethanol (500 mmol/L)

Caproate production from different ethanol concentrations (Table 1, batch 2) was studied. Since little hydrogen was used in batch tests with low ethanol concentration, no hydrogen was added as electron donor in this test. VFA production in different test groups is shown in Fig. 6.

Similar with the results obtained in 3.2, low concentrations of caproate was produced from sole acetate. Then, with the increase of ethanol concentration from 9.2 g/L to 23 g/L, acetate/ethanol ratio decreased from infinity to 1:10, caproate production increased from 7.66 to 8.42 g/L. However, Gildemyn et al. (2017) obtained more efficient caproate production with acetate/ethanol ratio 1:3 instead of 1:10 in continuous fermentation, indicating the optimal substrate composition is affected by the operation mode. Then, with the further increase of ethanol concentration to 36.8 g/L, caproate concentration decreased to 4.93 g/L. On the other hand, valerate showed a significant increase from 3.66 mg/L to 148.49 mg/L with the increase of ethanol concentration. The results indicate that metabolic pathway was changed from caproate formation to valerate production in *C. kluyveri* when ethanol was over 23 g/L. Butyrate production decreased from 2.56 to 0.23 g/L with the decrease of acetate/ethanol ratio, which was because less butyrate was formed with the decrease of acetate while more butyrate was turned to caproate with the increase of ethanol. When ethanol concentration was further increased to 46 g/L, little VFA production was observed, indicating that *C. kluyveri* was inhibited. Lonkar et al. (2016) also observed no chain elongation when ethanol concentration was over 40 g/L. Different from the results obtained in 3.2, no propionate was detected in all the test groups,

which may because of the absence of hydrogen.

Fig. 6 Volatile fatty acids production at different acetate/ethanol ratios

4. Conclusions

Caproate production through chain elongation by *Clostridium kluyveri* was conducted, and high ethanol concentrations (up to 46 g/L) was for the first time explored in this study. The results showed great effect of acetate/ethanol ratios ranged from 1:40 to 4:1 on biosynthesis of caproate. Caproate production can be enhanced through the increase of ethanol concentration. Follow-up studies can focus on optimizing the fermentation process considering the interactions between different parameters, like pH, acetate/ethanol ratio and temperature; exploring more strains that are capable of high efficient chain elongation as well as high tolerance to ethanol and final products. Further development of innovative bioprocess that could further convert the caproate to the corresponding alcohol would promote the wide application of the technology.

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Legends

Fig. 1 Growth curve of Clostridium kluyveri cultivated in DSM-52 medium

Fig. 2 Volatile fatty acids production from acetate (50 mmol/L) and ethanol (50 mmol/L)

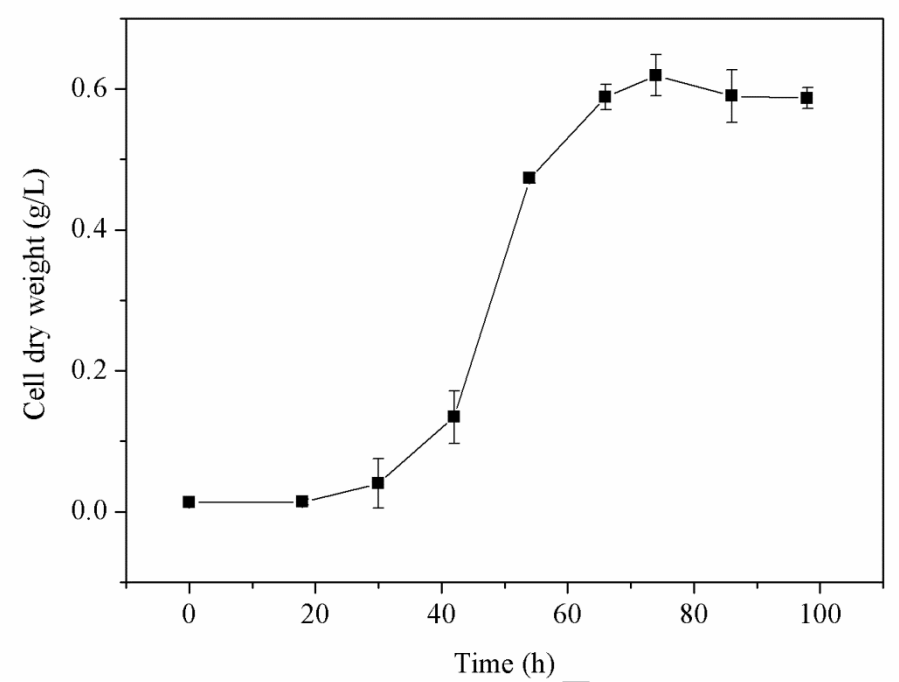
Fig. 3 Volatile fatty acids production at different acetate/ethanol ratios

Fig. 4 Change of hydrogen content along the fermentation process in different batches

Fig. 5 Volatile fatty acids production from acetate (50 mmol/L) and ethanol (500 mmol/L)

Fig. 6 Volatile fatty acids production at different acetate/ethanol ratios

Table 1 Experimental set up of the different batch tests.



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524 Fig. 1

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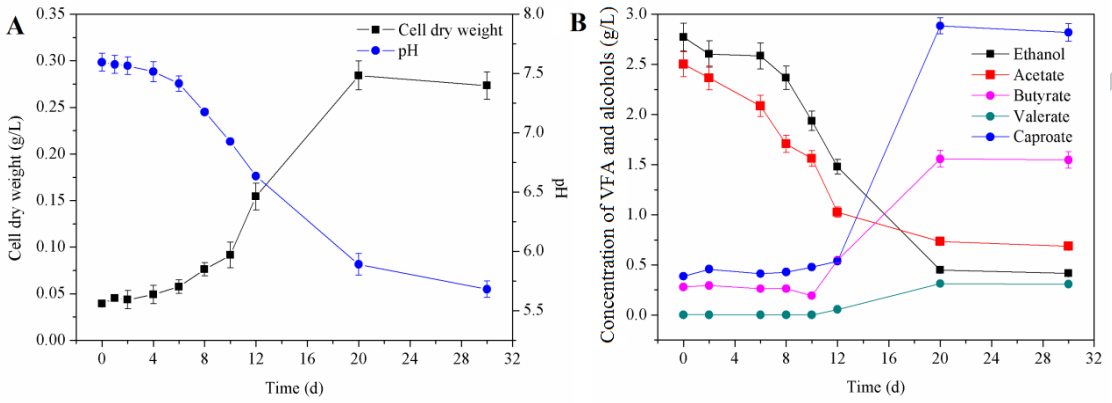
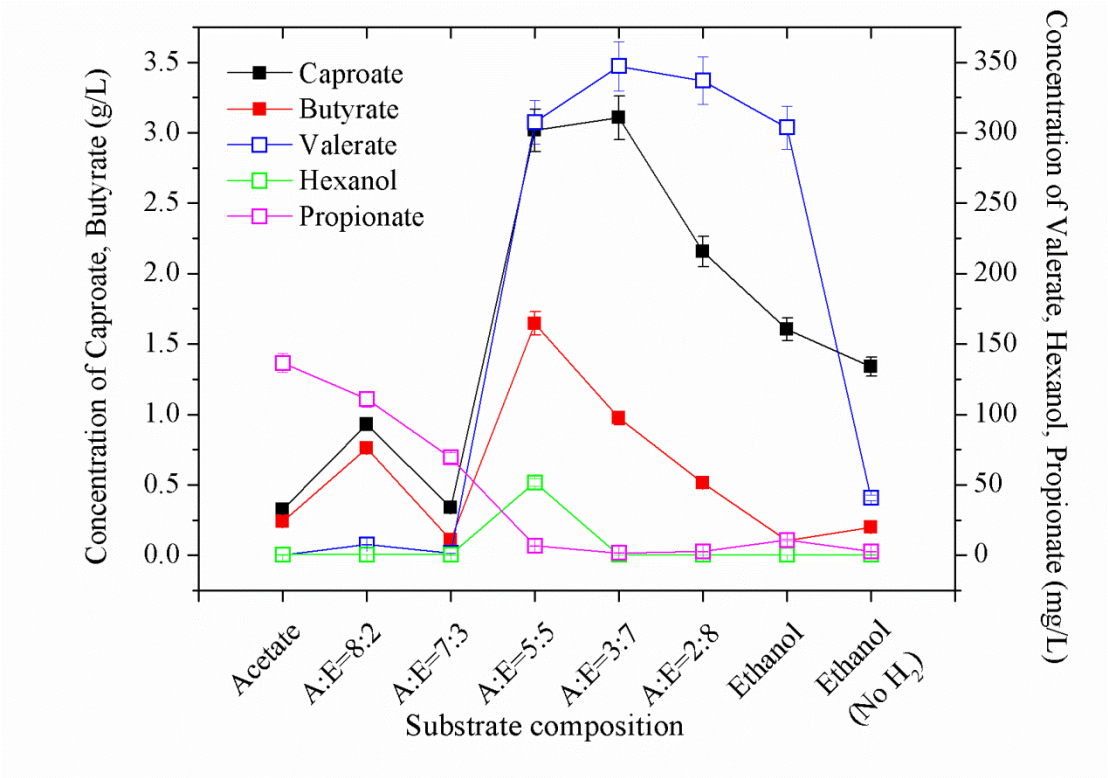


Fig. 2



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536 Fig. 3
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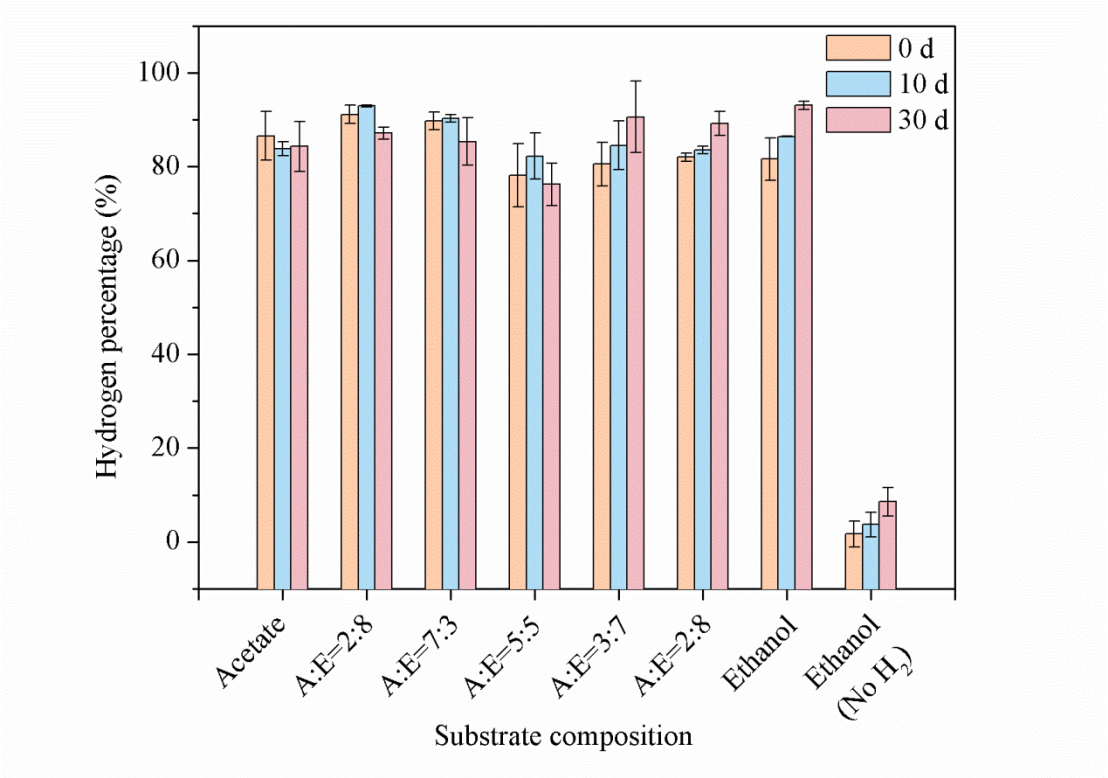

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541 Fig. 4

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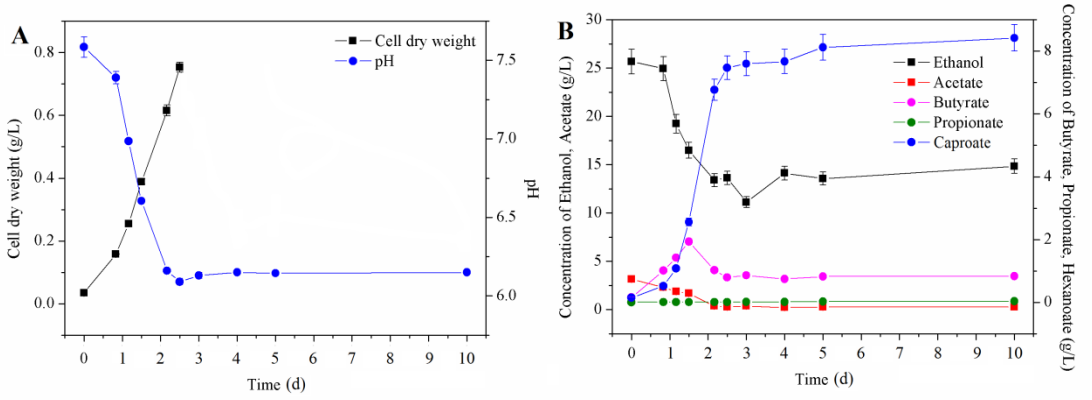

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548 Fig. 5

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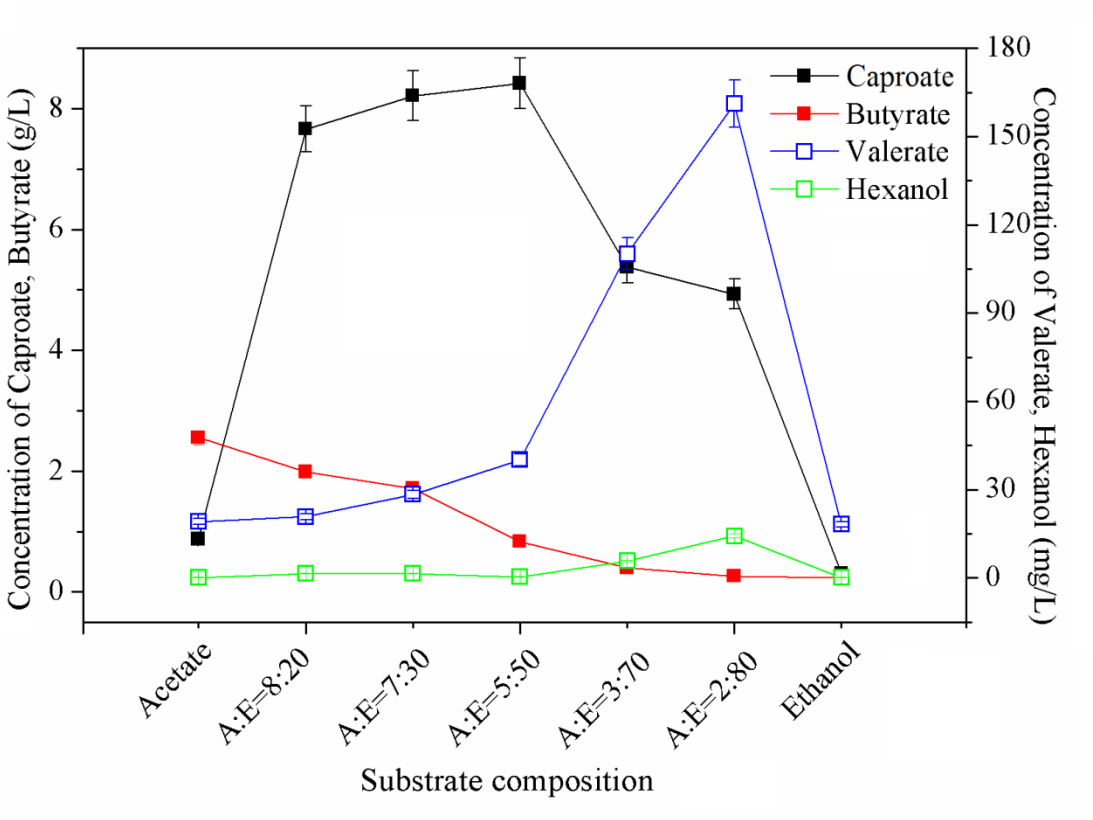

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553 Fig. 6

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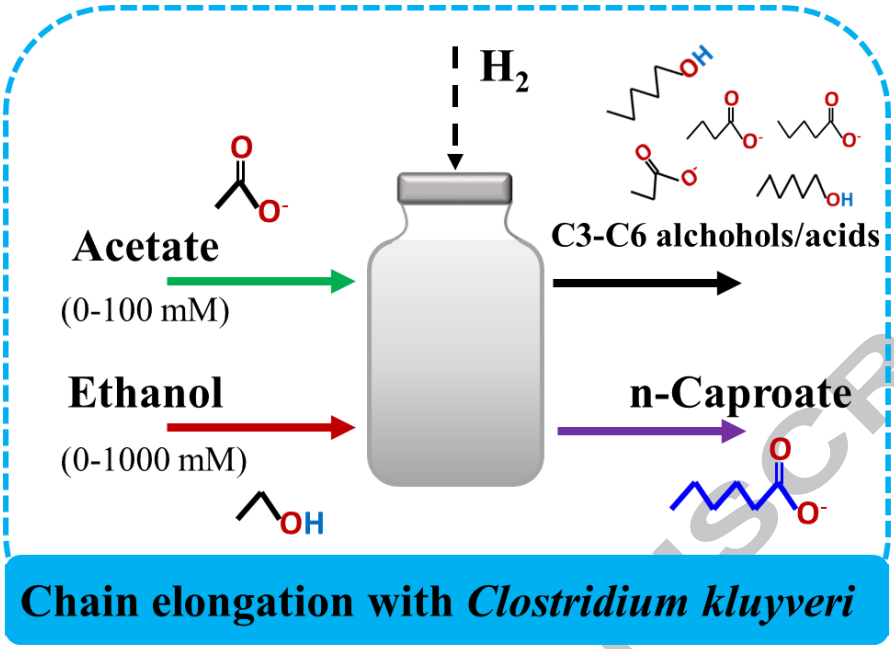
Table 1 Composition of carbon source and addition of hydrogen in different batch tests

Batch	Carbon source (mmol/L)		Additional electron donor
	Acetate	Ethanol	H_2 (mL)
Batch 1			
Acetate	100	0	480
A:E=8:2	80	20	480
A:E=7:3	70	30	480
A:E=5:5	50	50	480
A:E=3:7	30	70	480
A:E=2:8	20	80	480
Ethanol	0	100	480
Ethanol(No H_2)	0	100	0
Batch 2			
Acetate	100	0	0
A:E=8:20	80	200	0
A:E=7:30	70	300	0
A:E=5:50	50	500	0
A:E=3:70	30	700	0
A:E=2:80	20	800	0
Ethanol	0	1000	0

Highlights

- The composition and yield of VFA was influenced by acetate/ethanol ratio.
- Ethanol was used in priority as electron donor than hydrogen.
- High carbon source concentration enhanced caproate production.
- Ethanol concentration over 700 mM inhibited biosynthesis process.
- Highest caproate concentration was achieved at acetate/ethanol ratio 1:10



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