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

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11 **Abstract**

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

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

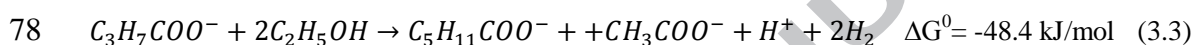
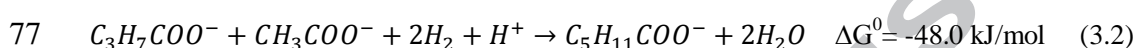
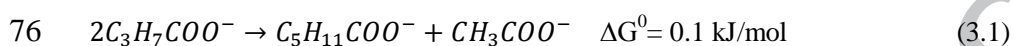
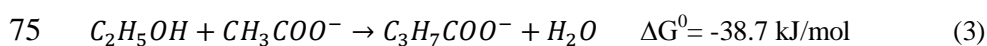
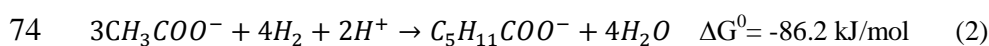
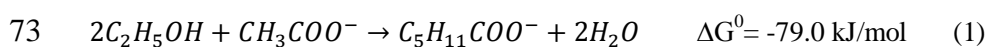
27 **1. Introduction**

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

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

49 can be used as a “green” antibiotic in agriculture at lower pH levels (pH 2.0-5.0)
50 (Butkus et al., 2010; Agler et al., 2011; P. Desbois, 2012). Caproic acid is also a
51 precursor for the production of flavors (Kenealy et al., 1995), liquid fuels (Harvey and
52 Meylemans, 2014) and corresponding alcohols (Perez et al., 2013; Isom et al., 2015).
53 Moreover it is an important flavor compound in distilled alcoholic beverage (Hu et al.,
54 2015).
55 Caproate has been found as a byproduct of fermentative hydrogen or methane
56 production from organic wastes (Steinbusch et al., 2009; Ding et al., 2010), which may
57 be produced from the elongation of VFA present in the system. Then, several studies
58 have followed up this assumption and explored chain elongation process by using
59 different substrates (e.g., syngas) or process configurations (Steinbusch et al., 2011;
60 Grootscholten et al., 2013; Kucek et al., 2016; Gildemyn et al., 2017). It has been
61 observed that a group of anaerobic bacteria that possess fatty acid synthase complex are
62 able to elongate ethanol and short chain fatty acids through a reaction known as the
63 reversed β -oxidation. Several bacteria such as *Clostridium* species have been reported
64 to be able to produce caproic acid from short chain fatty acids (Thauer et al., 1968;
65 Seedorf et al., 2008; Weimer and Stevenson, 2012); Among all the species able to
66 produce caproate, spore forming *Clostridium kluyveri* has been ubiquitously identified
67 in anaerobic fermentation systems, and proved to be efficient in converting ethanol
68 and acetate to butyrate and caproate (Ding et al., 2010). Thus, *C. kluyveri* was used in
69 this study to explore the caproate production from different concentrations of ethanol
70 and acetate. Equation 1-3.4 shows several metabolic pathways that have been

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



80 ΔG^0 (kJ/mol) is the value of ΔG at pH 7.0 under standard conditions (i.e., all solutes
81 are at the concentration of 1 mol/L, and gases have partial pressure of 1 atm).

82 Bornstein and Barker (1948) found that the metabolic pathways of *C. kluyver* are
83 dependent upon concentration of acetate and ethanol, and the excess of ethanol can
84 lead to more caproate yield than butyrate. Similar conclusion was also obtained by
85 Kenealy and Waselefsky (1985). Weimer et al. (2012) examined the effect of different
86 acetate and ethanol concentrations on products formation, and highest caproate
87 production was obtained at ethanol 700 mM and acetate 120 mM. Besides, Kenealy
88 and Waselefsky (1985) observed the linear response of both products and microbial
89 growth with ethanol and acetate concentration up to 200 and 50 mM, and Weimer et al.
90 (2012) found that a further increase of ethanol or acetate concentration can lead to a
91 significant decrease on *C. kluyver* growth rate. These studies show the significant
92 influence of acetate/ethanol ratios on products, and the contradictory effect of substrate

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

96 In the past few years, most studies have reported production of caproate only from low
97 ethanol concentration (<300 mmol/L) because ethanol is inhibitory to microorganisms
98 (Kenealy et al., 1995; Jeon et al., 2010; Weimer and Stevenson, 2012; Vasudevan et
99 al., 2014; Weimer et al., 2015). As a consequence, with low substrate concentrations,
100 only low caproate production rates can be achieved resulting to low caproate titers,
101 making a production process uneconomic. Furthermore, in ethanol fermentation broths
102 the ethanol concentration ranges from 400 to 1800 mmol/L, which is much higher than
103 the initial ethanol level reported as substrate for caproate production (Kenealy et al.,
104 1995; Jeon et al., 2010; Weimer and Stevenson, 2012; Vasudevan et al., 2014;
105 Weimer et al., 2015). Thus, in order to use the ethanol fermentation broth as substrate, a
106 fermentation process for caproate production at higher ethanol concentrations needs to
107 be developed.

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

113 **2. Material and methods**

114 **2.1 Preparation of inocula**

115 *Clostridium kluyveri* DSM 555 was purchased from the DSMZ (The Leibniz Institute
116 DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig,
117 Germany), and was cultivated in DSM-52 medium. The medium was boiled for 1 min
118 and cooled to room temperature under 80% N₂ and 20% CO₂ gas atmosphere, after
119 which 0.25 g/L L-Cysteine-HCl•H₂O was added as reducing agent. The pH of the
120 medium was adjusted to 7.5 using 1 M NaOH and 1 M HCl. Then, the reduced medium
121 was dispensed in 300 mL glass bottles with 100 mL working volume. All bottles were
122 sealed with rubber stoppers and aluminum caps to avoid gas leakage. The sealed bottles
123 were flushed with 80% N₂ and 20% CO₂ for 30 min to provide the anaerobic
124 environment. Bottles were autoclaved and the medium was further supplemented with a
125 vitamin solution (DSM-503), 1 g/L yeast extract and 20 mL/L ethanol through a 0.2 µm
126 membrane filter. Then, *Clostridium kluyveri* DSM 555 was inoculated into the bottles,
127 which were placed in a reciprocal shaker (37 °C, 150 rpm) and incubated for 3-4 days
128 until the bacteria entered the stationary phase. Subsequently, the bacteria was
129 transferred into fresh medium for another cultivation. Before being used in batch
130 experiments, pure culture of *Clostridium kluyveri* was transferred for over 3 times to
131 fully activate the bacteria and achieve a stable state.

132 **2.2 Experimental setup**

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

137 batch 1 with 100 mmol/L carbon source as substrate, acetate/ethanol ratio of 1:1 was
138 employed to examine the feasibility of hexonal production process, and then,
139 acetate/ethanol ratio of 8:2, 7:3, 5:5, 3:7, 2:8 were explored. Acetate/ethanol ratio of
140 1:1 was selected as starting point, as it is within the ratio ranges reported in the
141 previous study (Weimer and Stevenson, 2012).
142 10 mL of pre-cultured *C. kluyveri* was inoculated into the designed medium when the
143 culture was at exponential growth phase ($OD_{600}=0.8\pm 0.02$). Initial pH of all batches
144 was set to 7.5 and the medium was flushed with 80% N_2 and 20% CO_2 for 30 min to
145 drive away the oxygen present in the liquid and headspace. For the tests with 50 mM
146 acetate and 50 mM ethanol as substrate, hydrogen was added in the batches as
147 additional electron donor. Except the last test group with sole ethanol as carbon
148 source, 480 mL hydrogen was injected in the rest 7 test groups. All the bottles were
149 incubated in a reciprocal shaker at a speed of 150 rpm at constant temperature of 37 °C.
150 All the batch tests were conducted in duplicate. During the batch fermentation process,
151 the pH of the media was not controlled. Every one or two days, 1 mL gas and 2 mL of
152 liquid were taken out using syringe with needles for the analysis of hydrogen
153 concentration, microbial growth, pH change, substrate degradation and VFA formation.

154

155 **Table 1 Experimental set up of the different batch tests.**

156

157 **2.3 Analytical methods**

158 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 °C, respectively. N₂ 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 mm.i.d. film thickness 1.5 mm) and a flame
166 ionization detector. The carrier gas was N₂.

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.

181

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

183

184 **3.2 Effect of substrate composition on caproate production**

185 In batch fermentation with 50 mmol/L (2.3 g/L) acetate and 50 mmol/L (3 g/L)
186 ethanol as initial substrate concentrations, caproate was produced by *C. kluyveri* at 37
187 °C and initial pH 7.5. Fig. 2 depicts the time course of microbial growth, pH change,
188 substrate utilization and formation of VFA during the fermentation process in 30 days.
189 As shown in Fig. 2A, there was a long lag phase of 6 days, before the bacteria entered
190 the exponential growth phase. Microbial growth rate of 0.80 mg/L/h and maximum
191 cell dry weight of 0.28 g/L was obtained at 20 d, and remained stable for the
192 remaining 10 days. The microbial growth rate and cell dry weight obtained in batch
193 fermentation were 94 % and 55 % lower in comparison with the bacteria cultivated in
194 DSM-52 medium. The slower growth rates observed may be due to the lower initial
195 substrate concentration used in batch fermentation than bacteria cultivation process,
196 indicating that the substrate concentration was below the saturation concentration to
197 achieve maximum growth rates according to Monod growth model. Lonkar et al.
198 (2016) also observed that the increase of ethanol concentration from 0 g/L to 20 g/L
199 resulted in increase of growth rates, and subsequently a decrease of the fermentation
200 duration, defined as the termination of both substrate consumption and VFA
201 generation, from around 22 d to 18 d. However, despite that stagnation of growth it
202 was observed that not all substrates were consumed, probably due to product

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

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

213 As shown in Fig. 2B, substrate consumption and VFA production was terminated in
214 20 h. Concentration of caproate, butyrate and valerate produced were 2.82 g/L, 1.54
215 g/L and 0.31 g/L, respectively. The formation of VFA was consistent with the cell
216 growth. The first 10 h only low VFA production as observed, and therefore was
217 assumed that microbial growth was in lag phase. When the exponential growth phase
218 was initiated, significant increase in VFA production was obtained. Formation of VFA
219 ceased with termination of cell growth. Same time course was obtained by Thauer et
220 al. (1968). During the fermentation process, both acetate and ethanol concentrations
221 decreased gradually from the beginning of fermentation process and removal
222 efficiency of 72.5 % and 85.2 % were obtained at the end of batch run. However, in
223 other studies, substrate was consumed simultaneously with the formation of VFA
224 (Thauer et al., 1968; Kenealy et al., 1995; Weimer and Stevenson, 2012; Jeon et al.,

225 2013; Lonkar et al., 2016). Possible reason of this difference was probably the
226 different fermentation conditions. In the other studies, mixed cultures and *Clostridium*
227 species other than *C. kluyveri* were used as inocula, led to different metabolic
228 pathways and more diverse VFA composition. Furthermore, besides acetate and
229 ethanol, other substrates like cellulose, galactitol and succinate were also used as
230 substrates for caproate production, which can affect both microbial growth and VFA
231 formation process. Formation of butyrate was 2 hours earlier than caproate, indicating
232 that during the process, acetate and ethanol were firstly converted to butyrate as an
233 intermediate (Equation 3), and then caproate was formed from butyrate elongation
234 with acetate (Equation 3.1-3.4). It can be seen from Equation 1-3.4 that, sums of Δ
235 G° of Equation 3 and 3.1-3.4 are all more exergonic at standard conditions (negative
236 (from -86.6 to -182.0) than Equation 1 (-79.0) and 2 (-86.2)), indicating that, caproate
237 is more preferred to be formed from butyrate than initially from acetate and ethanol.

238

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

241

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

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

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

269 hydrogen.

270

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

272

273 Fig. 4 shows the change of hydrogen content in each bottle. Only a little change of
274 hydrogen content was observed in all test groups. For the test groups with
275 acetate/ethanol ratio over 5:5, hydrogen content showed a little increase in 10 d and
276 then decreased in 30 d. Seedorf et al. (2008) and Ding et al. (2010) have showed that
277 both hydrogen and caproate can be produced from acetate and ethanol by *Clostridium*
278 *kluveri*. Hydrogen has been proved to be able to act as electron donor for both
279 hexanol and ethanol production from acetate (Spirito et al., 2014; Ding et al. 2010; Mu
280 and Yu, 2006), however, the results showed that little hydrogen was consumed during
281 the fermentation process, which may because of the thermodynamic bottleneck for the
282 hydrogen to be used as electron donor (Gonzálezcabaleiro et al. 2013).
283 For the tests with acetate/ethanol ratio less than 1:1, the hydrogen content increased
284 along the fermentation process. Although the ΔG° of hydrogen as electron donor is
285 much more negative than ethanol as electron donor (Equation 1-3.4), the results
286 showed that ethanol is more easily consumed as electron donor than hydrogen. This
287 may due to the high mass transfer resistance between hydrogen and reaction phase.
288 The results indicate that at high ethanol levels, no additional electron donors are
289 needed for the caproate production process. Same conclusion was also made by Liu et
290 al. (2016) that the electron donor was sufficient for the chain elongation reaction

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

292

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

294 **batches**

295

296 **3.3 Caproate production from high ethanol concentration wastewater**

297 To explore the possibility for caproate production from high ethanol concentration

298 wastewaters, 500 mmol/L (23 g/L) ethanol along with 50 mmol/L (3 g/L) acetate were

299 used as carbon source. It can be seen from Fig. 5A that bacteria grew very fast in the

300 medium containing high ethanol concentration. Cell dry weight achieved 0.75 g/L in 3

301 d cultivation. Subsequently the fast growing cells formed flocks in the solution,

302 making determination of the cell concentration through a small amount of sample

303 unreliable due to the inhomogeneity issues. However, we could also observe that the

304 microbes entered exponential growth directly without experiencing a lag phase, and

305 the fermentation process terminated in 5 d. Microbial growth rate of 12.67 mg/L/h

306 was obtained. Comparing with the batch tests using 23 g/L ethanol and 3 g/L acetate

307 as substrate, both microbial growth rate and maximum cell dry weight were

308 significantly enhanced. Similar to microbial growth, pH dropped from pH 7.5 to pH

309 6.2 in 3 d, and then remained constant at around pH 6.1.

310 As shown in Fig. 5B, acetate and ethanol decreased with the microbial growth. After

311 three days, acetate concentration decreased to an undetectable level and ethanol was at

312 around 15 g/L. Butyrate increased in first 36 h, showing that acetate and ethanol were

313 first converted to butyrate (Equation 3). Subsequently, butyrate decreased to under 5
314 g/L due to elongation of butyrate to caproate (Equation 3.1, 3.3) (Mu and Yu, 2006;
315 Ding et al., 2010). Caproate concentration increased significantly in first three days
316 and highest concentration of 8.42 g/L was obtained. It can be seen that the conversion
317 efficiency (Consumed amount /Original amount) of ethanol was around 48 %. Lonkar
318 et al. (2016) reported that ethanol conversion efficiency decreased from over 80 % to
319 less than 25 % when initial ethanol concentration increased from 5 to 40 g/L. The
320 termination of fermentation process may be due to the acetate depletion, since it has
321 been proposed that the chain elongation reaction needs to be stimulated by acetate.
322 Another possible reason could be product inhibition. Different maximum achieved
323 caproate concentrations through microbial fermentation, have been reported in the
324 literature and were in the range from 0.8 g/L to 8.6 g/L (Steinbusch et al., 2011;
325 Weimer and Stevenson, 2012; Jeon et al., 2013; Vasudevan et al., 2014; Weimer et al.,
326 2015; Ganigué et al., 2016; Liu et al., 2016; Lonkar et al., 2016). To further enhance
327 the caproate production, in situ recovery of the formed caproate during the
328 fermentation process may enhance the final caproate yield (Jeon et al., 2013). In
329 summary, 23 g/L ethanol showed no inhibition on *C. kluyveri*, and the high substrate
330 concentration stimulated both microbial growth and fermentative caproate production.

331

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

334

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

339 Similar with the results obtained in 3.2, low concentrations of caproate was produced
340 from sole acetate. Then, with the increase of ethanol concentration from 9.2 g/L to 23
341 g/L, acetate/ethanol ratio decreased from infinity to 1:10, caproate production
342 increased from 7.66 to 8.42 g/L. However, Gildemyn et al. (2017) obtained more
343 efficient caproate production with acetate/ethanol ratio 1:3 instead of 1:10 in
344 continuous fermentation, indicating the optimal substrate composition is affected by
345 the operation mode. Then, with the further increase of ethanol concentration to 36.8
346 g/L, caproate concentration decreased to 4.93 g/L. On the other hand, valerate showed
347 a significant increase from 3.66 mg/L to 148.49 mg/L with the increase of ethanol
348 concentration. The results indicate that metabolic pathway was changed from caproate
349 formation to valerate production in *C. kluyveri* when ethanol was over 23 g/L.

350 Butyrate production decreased from 2.56 to 0.23 g/L with the decrease of
351 acetate/ethanol ratio, which was because less butyrate was formed with the decrease
352 of acetate while more butyrate was turned to caproate with the increase of ethanol.

353 When ethanol concentration was further increased to 46 g/L, little VFA production
354 was observed, indicating that *C. kluyveri* was inhibited. Lonkar et al. (2016) also
355 observed no chain elongation when ethanol concentration was over 40 g/L. Different
356 from the results obtained in 3.2, no propionate was detected in all the test groups,

357 which may because of the absence of hydrogen.

358

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

360

361 **4. Conclusions**

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

372

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511 **Legends**

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

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

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

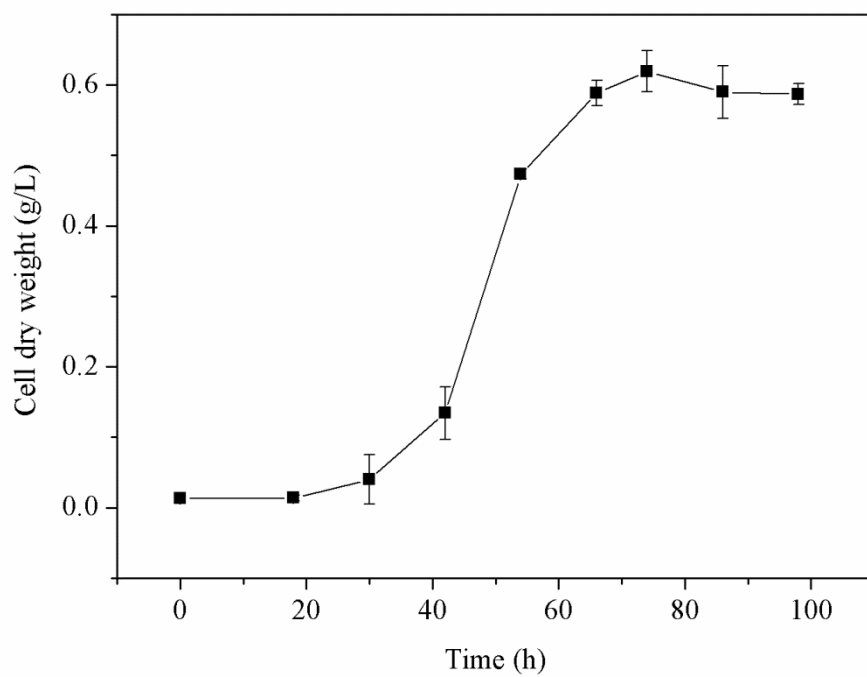
516 Fig. 4 Change of hydrogen content along the fermentation process in different batches

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

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

520 Table 1 Experimental set up of the different batch tests.

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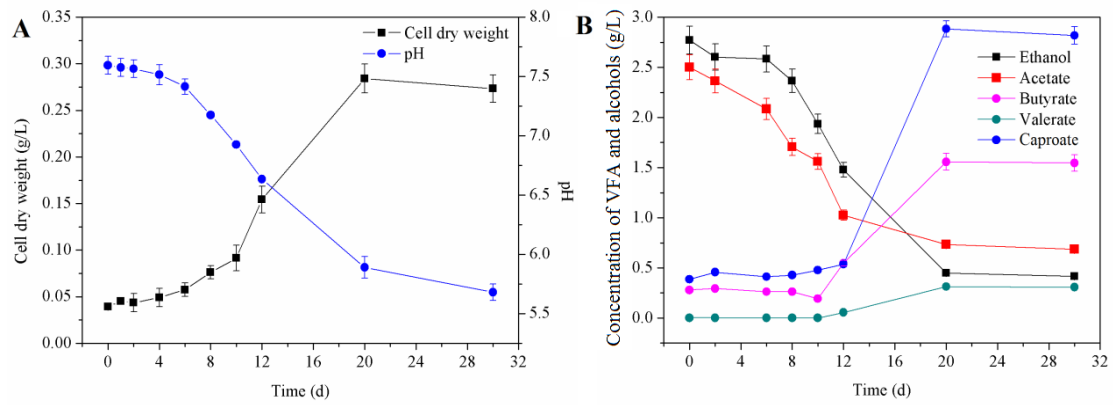
524 Fig. 1

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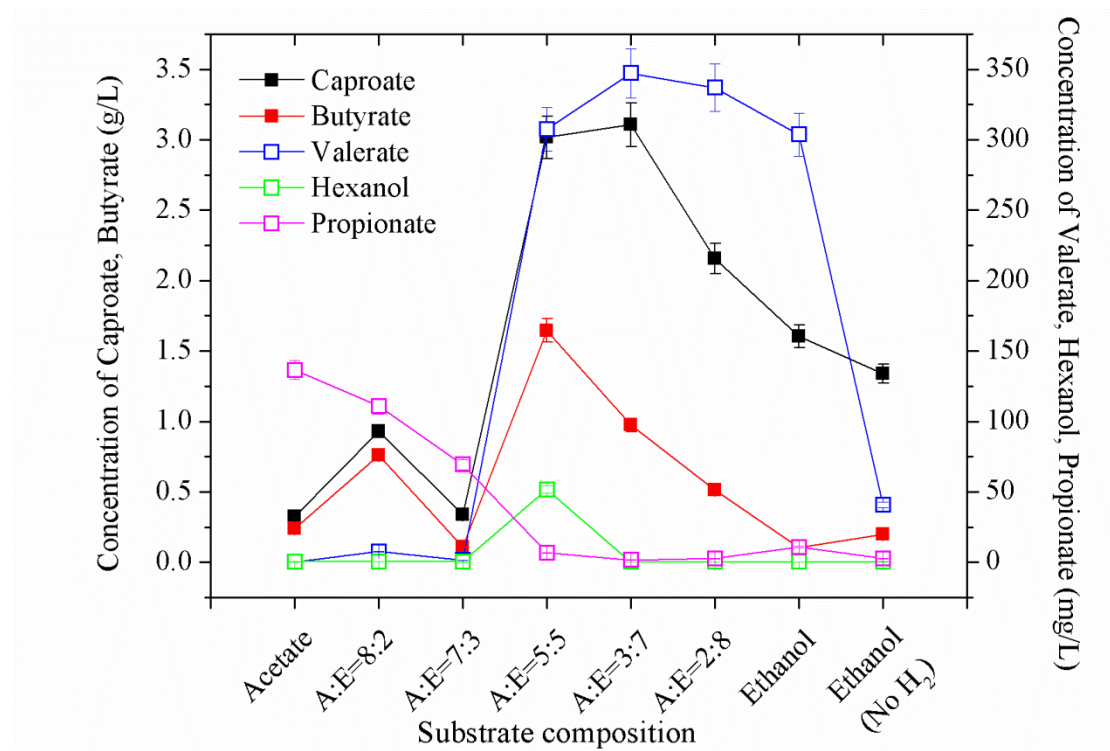
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531 Fig. 2

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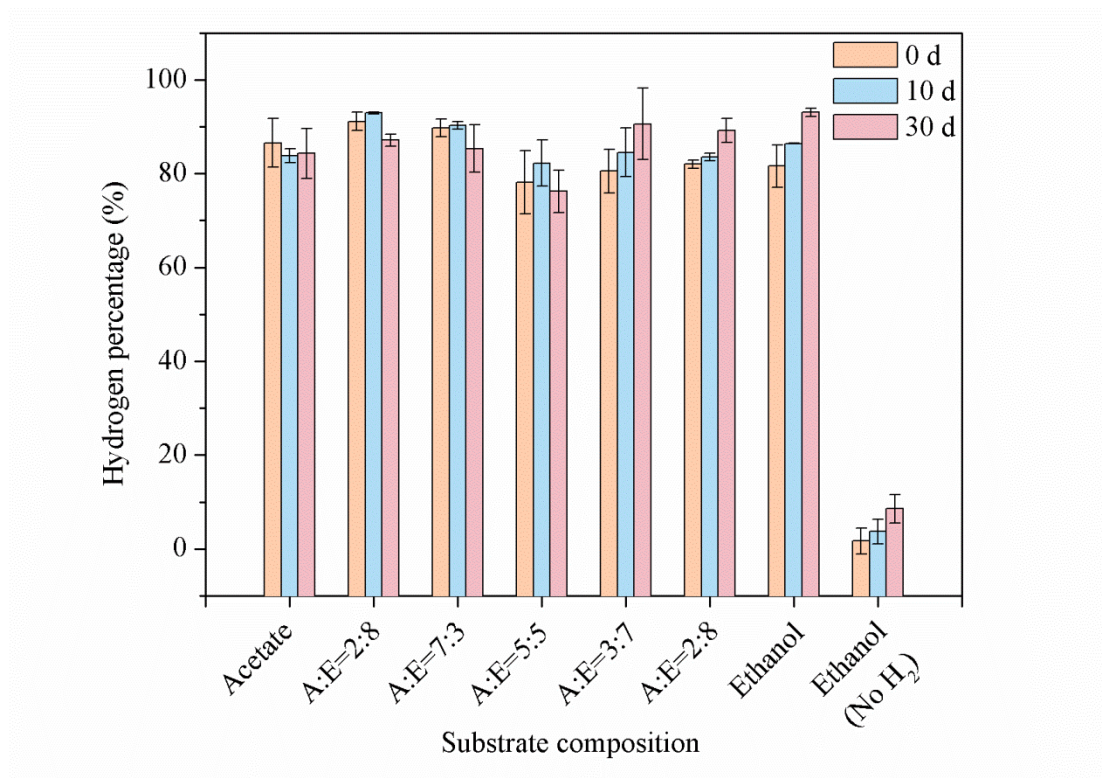
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536 Fig. 3

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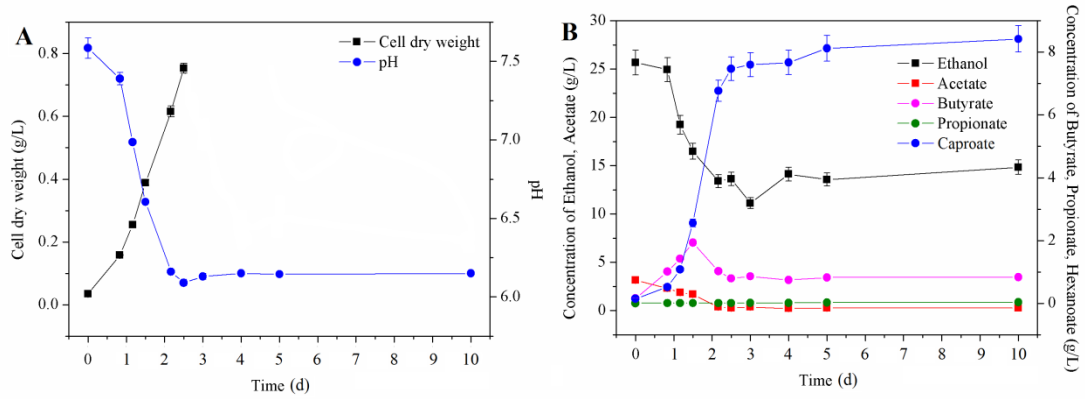
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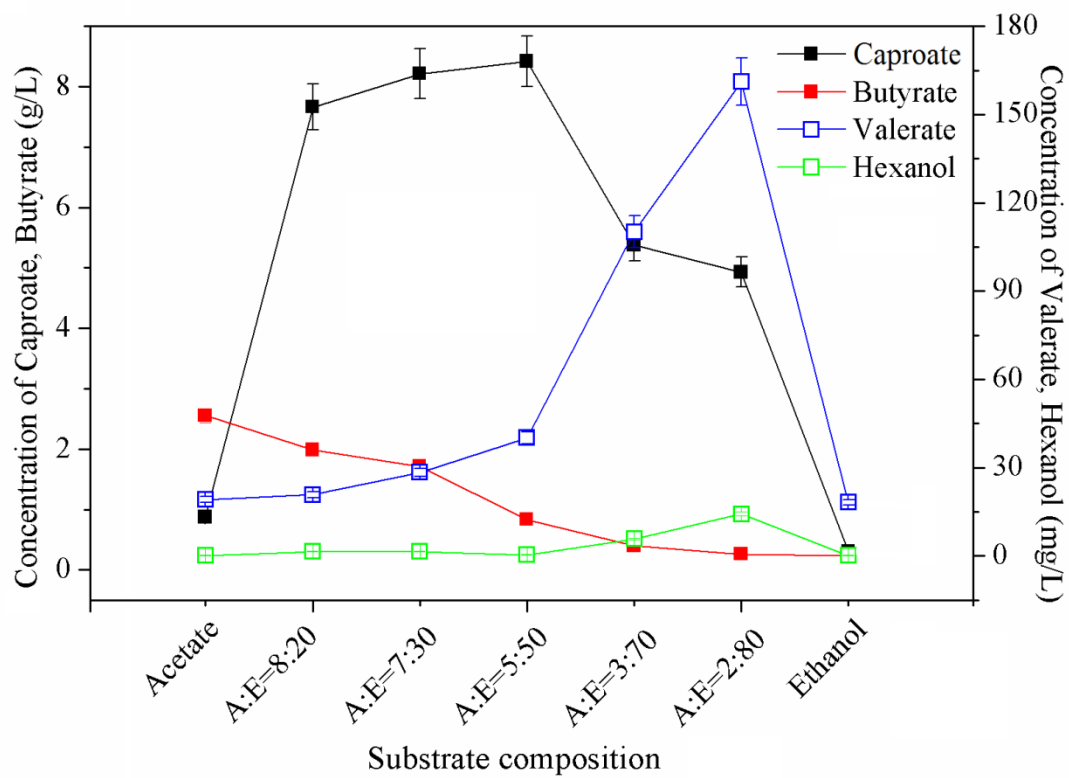
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548 Fig. 5

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553 Fig. 6

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556 **Table 1 Composition of carbon source and addition of hydrogen in different batch**
 557 **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

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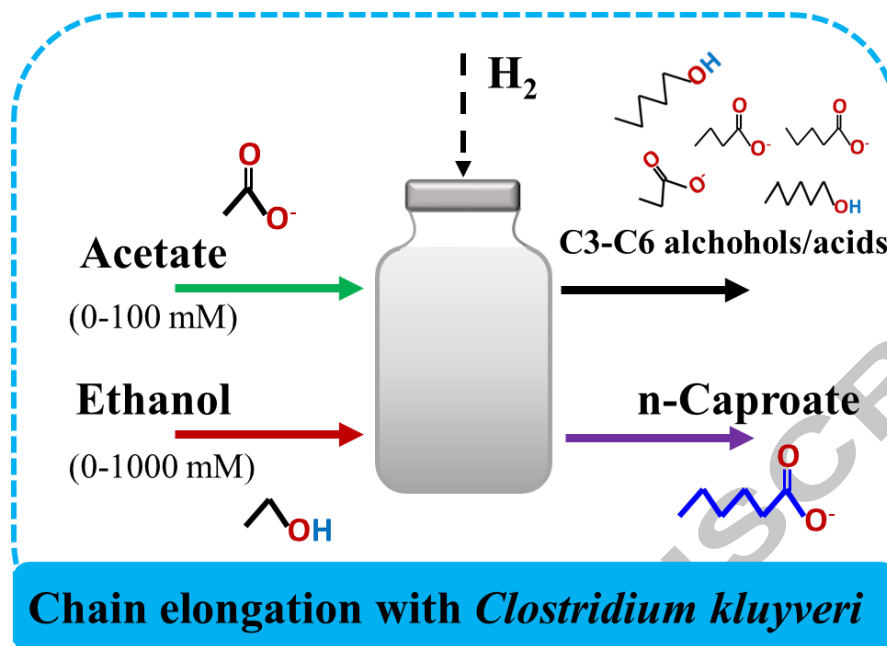
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562 **Highlights**

- 563 • The composition and yield of VFA was influenced by acetate/ethanol ratio.
- 564 • Ethanol was used in priority as electron donor than hydrogen.
- 565 • High carbon source concentration enhanced caproate production.
- 566 • Ethanol concentration over 700 mM inhibited biosynthesis process.
- 567
- 568 • Highest caproate concentration was achieved at acetate/ethanol ratio 1:10
- 569

ACCEPTED MANUSCRIPT



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