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1 **Enhancement of bioenergy production from organic wastes**
2 **by two-stage anaerobic hydrogen and methane production**
3 **process**

4
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16 **Abstract:**

17 The present study investigated a two-stage anaerobic hydrogen and methane process
18 for increasing bioenergy production from organic wastes. A two-stage process with
19 hydraulic retention time (HRT) 3d for hydrogen reactor and 12d for methane reactor,
20 obtained 11% higher energy compared to a single-stage methanogenic process (HRT
21 15d) under organic loading rate (OLR) 3 gVS/(L·d). The two-stage process was still
22 stable when the OLR was increased to 4.5 gVS/(L·d), while the single-stage process
23 failed. The study further revealed that by changing the $HRT_{\text{hydrogen}}:HRT_{\text{methane}}$ ratio of
24 the two-stage process from 3:12 to 1:14, 6.7%, more energy could be obtained.
25 Microbial community analysis indicated the dominant bacterial species were different in
26 the hydrogen reactors (*Thermoanaerobacterium thermosaccharolyticum*-like species)
27 and methane reactors (*Clostridium thermocellum*-like species). The changes of
28 substrates and HRT did not change the dominant species. The archaeal community
29 structures in methane reactors were similar both in single- and two- stage reactors, with
30 acetoclastic methanogens *Methanosarcina acetivorans*-like organisms as the dominant
31 species.

32

33 **Key words:** anaerobic digestion, hydrogen, methane, two-stage process

34

35 **1. Introduction**

36 Hydrogen produced from biomass is renewable energy carrier. Among the various
37 hydrogen production methods, dark fermentation of organic wastes seems to be the
38 most promising and environmentally friendly method. The feasibility of such method
39 has been demonstrated in several studies (Cai et al., 2004; Liu et al., 2006). However,
40 the main obstacles in such process are the lower hydrogen yield ($<4 \text{ mol H}_2/\text{mol}$
41 Glucose) and higher residual organic concentration in the effluent (Xie et al., 2008).
42 The effluents of the dark fermentation process contain mainly acetate, propionate,
43 butyrate etc., which should be further utilized to increase the total energy recovery
44 efficiency.

45 Combined hydrogen and methane production in a two-stage process is a concept
46 which has been developed in recent years (Kyazze et al., 2007; Liu et al., 2006; Ueno
47 et al., 2007). It is similar with the traditional two-phase process that separates
48 hydrolysis/acidogenesis and methanogenesis, and optimizes each process separately,
49 leading to a larger overall reaction rate and biogas yield (Fox and Pohland, 1994). The
50 main difference is that hydrogen is retrieved in the first stage of the two-stage process
51 for hydrogen and methane production. The co-production of hydrogen and methane is
52 more promising from an energy perspective. Liu et al. (2006) has demonstrated that
53 more methane could be obtained by two-stage hydrogen and methane process. Also,
54 the mixture of hydrogen and methane has many advantages than methane alone,
55 which could improve the efficiency of the methane combustion motors and decrease
56 the emissions of CO_2 and CO (Akansu et al., 2004). Several studies have been
57 conducted to investigate the hydrogen and methane production in the two-stage
58 process. However, they mainly focused on the optimization of hydrogen and methane
59 reactors individually (Antonopoulou et al., 2008; Venetsaneas et al., 2009). It is

60 necessary to optimize the whole system for higher total energy production. In addition,
61 the mechanisms involved in the two-stage process and the microbial community
62 structures have not been investigated and clarified, which is crucial for better
63 understanding of the process.

64 Concerns about instability of fossil fuels supply, limits on fossil fuel reserves and
65 not least environmental pollutions and climate changes, have brought new lights in
66 utilization of biomass in biorefinery concepts, where biomass is used as feedstock
67 instead of fossil fuels for production bio-based fuels, chemicals, solvents etc. by
68 biological conversion processes. We have proposed a novel biorefinery concept based
69 on rapeseed plant (Luo et al., 2010a), where the oil seed is used for biodiesel
70 production and the straw is used for bioethanol production. From this process several
71 effluent sub-streams are generated, which need to be utilized for full utilization of the
72 organic matter. Rapeseed cake and glycerol are the by-products in the biodiesel
73 process, and the search for proper disposal methods is still going on (Thamsiriroj and
74 Murphy, 2010). Stillage is the wastewater from bioethanol production process and it
75 contains high concentrations of degradable organic pollutants. The utilization of the
76 above three sub-streams for bioenergy production is necessary from environmental
77 protection and sustainability viewpoints.

78 Therefore, in the present study we investigated and compared different
79 configurations of two-stage process for hydrogen and methane production from the
80 above organic streams and studied the role of the hydrogen reactor in the whole
81 system. Single-stage process for methane production was operated as control. Finally,
82 the microbial communities in different reactors and operation conditions were
83 identified.

84

85 **2. Material and methods**

86 *2.1. Feedstocks and inoculum*

87 The stillage used in this study was obtained from an ethanol plant in Lithuania.
88 Rapeseed cake and glycerol waste from the biodiesel production process were
89 obtained from a local company (Emmelev). The samples were stored at -20 °C. The
90 substrates were thawed and kept at 4 °C for 2-3 days before usage. 24 g cake and 2 ml
91 glycerol was added to 1 L stillage based on the biorefinery concept described in (Luo
92 et al., 2010a). The characteristics of the three wastes and their mixture are shown in
93 Table 1. Thermophilic anaerobic digested manure (Biogas plant, Snertinge, Denmark)
94 was used as inoculum for both hydrogen and methane production.

95 *2.2. Reactor set-up and operations*

96 Two-stage (hydrogen and methane) operation was compared with single-stage
97 methane operation. The hydrogen reactor (H) was a 2 L continuously stirred tank
98 reactor (CSTR) with working volume 1.2 L, while the methane reactors (M) was 4.5 L
99 CSTR with working volume 3.5 L. The configurations of all the reactors were similar
100 and described in Boe et al. (2009). All reactors were stirred four times (3 min for each
101 time) per hour throughout the experiment by motor mixer with a timer. The substrates
102 were fed to all the reactors four times per day using peristaltic pump with timer
103 control. Before feeding, 8 g/L NaHCO₃ was added to the stillage or mixture to adjust
104 the pH to around 6. The two-stage process was tested at two different distributions of
105 HRT between hydrogen and methane reactors. The first HRT distribution tested was
106 3:12 i.e. the HRT for the hydrogen reactor was 3 days (H3) and the HRT for the
107 methane reactor was 12 days (M12), while the second HRT distribution was 1:14, i.e.
108 1 day HRT for the hydrogen reactor (H1) and 14 days HRT for the methane reactor
109 (M14). A single-stage methane reactor was operated at HRT of 15 days (M15). All

110 experiments were conducted at 55 °C. The operation data of the reactors were shown
111 in Table 2.

112 For the first two-stage experiment, the HRT distribution of 3:12 was tested. The
113 reactor H3 was initially filled with 200 ml inoculum, 500 ml stillage and diluted by
114 water to final volume 1.2 L. The initial pH of the mixture was adjusted to 6 by NaOH.
115 After the hydrogen production ceased, the reactor was fed semi-continuously. For
116 M12 and M15, the reactors were initially filled with 3.2 L inoculum and 300 ml
117 stillage. After the methane production ceased, the reactors were also fed
118 semi-continuously. The effluent of H3 was fed to M12. Initially, H3 and M15 were
119 fed with only raw stillage to get a successful start-up at relatively low OLR. After
120 steady-states were achieved, the mixture was fed to the reactors (From day 46 to day
121 118). The steady-state in this study was defined as a stable biogas production with
122 daily variation of lower than 10 %.

123 From day 75 to day 126, the second two-stage experiment with the same total HRT
124 15 d, but HRT distribution of 1:14 between hydrogen and methane reactors was
125 started. The reactors were the same as those used in the experiment with HRT
126 distribution of 3:12, but with different feeding flow rates. The inocula for H1 and
127 M14 were from the effluents of H3 and M12, respectively. The reactors were directly
128 fed with the mixture of stillage, cake and glycerol.

129 *2.3. Specific methanogenic activity (SMA) tests*

130 Batch experiments for estimation of the specific methanogenic activity (SMA) on a
131 specific substrate were carried out when steady-states were achieved in the methane
132 reactors. 40 mL basal anaerobic (BA) medium (Karakashev et al., 2005) was
133 dispensed anaerobically in 100 mL serum bottles. The media were supplemented with
134 different substrates-acetate (20 mM), propionate (10 mM), butyrate (10 mM),

135 hydrogen/carbon dioxide (80/20) under 1 atm, and glucose (10 mM). After addition of
136 vitamin solution and $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ as a reducing agent the medium was inoculated with
137 10 mL fresh samples from each reactor and incubated in respective temperature of
138 inoculums. Bottles with BA medium and inocula only, but without substrates, were
139 used as controls (blanks). All the tests were prepared in duplicates. The SMA was
140 calculated as the initial, linear methane accumulation rate divided by the biomass VS
141 content in each series.

142 *2.4. Microbial community analysis*

143 Bacterial communities in both hydrogen and methane reactors at steady-states were
144 analyzed. Genomic DNA extraction, PCR-DGGE and sequencing were made as
145 previously described (Zhao et al., 2009). Archaeal communities in methane reactors at
146 steady-states were also analyzed. The procedure was similar with bacterial community
147 analysis. The only differences were the PCR primers and amplification procedures.
148 For the first amplification, primers 1492-r and 109-f were used and the thermal
149 cycling program was as follows: 94 °C for 2 min, 35 cycles of three steps: 94 °C for 1
150 min, 51 °C for 1 min, and 72 °C for 1 min, followed by a final step at 72 °C for 10
151 min; For the second amplification, primers 515-r and 109(T)-f were used and the
152 thermal cycling program was as follows: 94 °C for 3 min, 34 cycles of three steps:
153 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min, followed by a final step at
154 72 °C for 10 min. All bands from DGGE were sequenced and identified by comparing
155 the 16S rRNA gene sequences with DNA sequences in the National Centre for
156 Biotechnology Information (NCBI) database using the BLAST algorithm.

157 *2.5. Analytical methods*

158 Total and soluble chemical oxygen demand (TCOD and SCOD), Total solids (TS),
159 volatile solids (VS), ash content, suspended solids (SS), volatile suspended solids

160 (VSS), ammonia and total nitrogen were determined according to the Standard
161 Methods (APHA, 1995). SCOD samples were filtered through glass fiber paper (U90
162 mm, GF50, Schleicher & Schuell). Lipid extraction was carried out by Soxhlet
163 Method (APHA, 1995). Protein and carbohydrate were calculated according to
164 (Kaparaju et al., 2009). The concentrations of ethanol, acetate, butyrate, propionate
165 were determined by gas chromatograph (GC) (Hewlett Packard, HP5890 series II)
166 equipped with a flame ionization detector and HP FFAP column (30 m×0.53 mm×1.0
167 µm). Hydrogen was analyzed by GC-TCD fitted with a 4.5 m×3 mm s-m stainless
168 column packed with Molsieve SA (10/80). Methane was analyzed with GC-TCD
169 fitted with parallel column of 1.1 m×3/16 “Molsieve 137 and 0.7 m× 1/4” chromosorb
170 108. Detailed information about the operation conditions of above GC and HPLC was
171 described in (Luo et al. 2010a). Analysis of variance (ANOVA) at 0.05 level was used
172 to analyze the data.

173

174 **3. Results and discussion**

175 *3.1. Reactor Performances*

176 The two-stage (H3+M12) and single-stage reactors (M15) were started up at the
177 same time. The monitoring profiles of hydrogen, methane, pH, volatile fatty acids
178 (VFA) are shown in Fig 1 and Fig 2, and the overall performances of the reactors at
179 steady-states are summarized in Table 3. Initially (day 1 to 45), the reactors were fed
180 with stillage alone. For the two-stage process, hydrogen was produced immediately
181 and the hydrogen reactor stabilized after about 7 days (Fig 2 A). The hydrogen yield
182 was 69 ml-H₂/gVS, which was comparable with 76 ml-H₂/gVS from cassava stillage
183 in our previous study (Luo et al., 2010b). The methane production rate increased
184 initially slowly, while rapid increase was found after about 6 days (Fig 2 C). After 15

185 days, the methane production was relatively stable with methane production rate
186 around 1300 ml-CH₄/(L·d). Similar trend for methane production was observed in
187 single-stage process (M15). However, the methane production rate in M15 was about
188 990 ml-CH₄/(L·d), 24 % lower than that in M12. The methane yield in M12 was
189 calculated as 348 ml-CH₄/gVS, which was significantly ($p=0.008<0.05$) higher than
190 329 ml-CH₄/gVS in M15. Liu et al. (2006) also investigated two-stage (hydrogen and
191 methane) and single-stage (methane) processes for treatment of household solid waste
192 and found 21 % enhancement of methane yield in the two-stage process. In their study
193 the HRT for methane reactors in both systems were 15 d. In our study, the
194 enhancement was only about 5.7 %, which could be due to the shorter HRT (12 days)
195 in the two-stage process. Considering the additional hydrogen production, the total
196 energy recovery (Table 3) in the two-stage process could be 11 % higher than that in
197 single-stage process. The higher energy recovery in the two-stage process was also
198 consistent with the lower TCOD and VS concentration in the effluent (Table 3). VFA
199 and ethanol were detected in both M12 and M15 with propionate as the dominant
200 metabolite, which indicated the incomplete removal of intermediate metabolites.

201 The addition of cake and glycerol from day 44 did not lead to the increase of
202 hydrogen production compared to stillage alone (Fig 2 A). The hydrogen yield of the
203 mixture was only 48 ml-H₂/gVS. For M12, the methane production rate increased
204 from about 1300 ml-CH₄/(L·d) to 1800 ml-CH₄/(L·d) and the methane yield for the
205 mixture was about 320 ml-CH₄/gVS. The results indicated that cake and glycerol
206 could successfully be utilized for methane production. Rapeseed cake is
207 lignocellulosic material (Egues et al., 2010) and the carbohydrate was not easily
208 accessible for hydrogen production at the short HRT (3 d) applied, but the longer HRT
209 (12 d) in subsequent methane reactor led to the solubilization of organics for methane

210 production. Though studies have demonstrated the feasibility of hydrogen production
211 from glycerol (Selembo et al., 2009), the hydrogen yield was very low (0.28
212 mol-H₂/mol glycerol). The pure glycerol contained in the glycerol waste in our study
213 was only 33 %, and the glycerol concentration in the mixture (2 ml/L) was also low,
214 which could not lead to measurable increase of hydrogen production (only 15
215 ml-H₂/(L·d)). For the single-stage reactor M15, the methane production ceased after
216 about 30 days due to the low pH (<6), resulted from the accumulation of VFA
217 especially acetate and butyrate. This could be attributed to the increase of OLR (from
218 to 3 gVS/(L·d) to 4.5 gVS/(L·d)) by changing the reactor influent from stillage to the
219 mixture of stillage, cake and glycerol. The results further demonstrated that the
220 two-stage process could withstand higher OLR than single-stage process. In M12,
221 propionate was still the dominant metabolite. The acetate concentration increased
222 from 6.3 mM (stillage as substrate) to 19.3 mM (mixture as substrate), but it did not
223 inhibit the methane production.

224 From day 77, another configuration of two-stage process (H1+M14) was started up.
225 The HRT in the hydrogen reactor was reduced to 1 d, while that for the methane
226 reactor was increased to 14 d. Both reactors reached steady-state quickly because the
227 inocula were acclimated to the substrate (Fig 3). The hydrogen yield (40 ml-H₂/gVS)
228 in H1 was 17 % (p=0.006<0.05) lower than that in H3, while the methane yield in
229 M14 (344 ml-CH₄/gVS) was 7.5 % (p=0.005<0.05) higher than that in M12. In our
230 study, the hydrogen production was mainly associated with butyrate production (Fig 3
231 B), which is in accordance with previous studies focusing on thermophilic hydrogen
232 production (Akutsu et al., 2009; Ueno et al., 2007). The decreased hydrogen yield in
233 H1 was coincident with decreased butyrate concentration compared to H3. The total
234 energy recovery in H1+M14 was 12.7 KJ/gVS, which was 6.7 % (p=0.01<0.05)

235 higher compared to H3+M12. It is worth noticing that the acetate concentration in
236 M14 decreased to 8.7 mM, and was significantly lower than that in M12. However,
237 the propionate concentration was still at the same level. The different HRT
238 distribution in hydrogen and methane reactors was shown to significantly affect the
239 production of hydrogen and methane, as well as the total energy recovery. Under the
240 same total HRT, the short HRT in the hydrogen reactor was enough to maintain the
241 stability of the two-stage system, while the longer HRT in the methane reactor would
242 lead to the improved performance of the two-stage system. It is the first time to reveal
243 the importance of HRT distribution between hydrogen and methane reactors on total
244 energy production.

245 In all cases, the energy from hydrogen in the two-stage process accounted for lower
246 than 6% of the total energy recovery (Table 3). The results were consistent with Zhu
247 et al. (2008), who studied the hydrogen and methane production from potato waste
248 and found only about 5 % of the energy was from hydrogen. Theoretically, in the
249 two-stage process, 1 mol glucose could be converted to 4mol hydrogen and 2mol
250 methane ($C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$; $2CH_3COOH \rightarrow 2CH_4 + 2CO_2$)
251 (Xie et al., 2008), which means the energy from hydrogen could be accounted for
252 37.6% of the total energy recovery. Nevertheless, 4 mol- H_2 /mol glucose can not be
253 achieved in practice, considering production of several other metabolites than acetate,
254 such as, butyrate and propionate as well as production of cell biomass (Ueno et al.,
255 2007). In addition, the actual organic wastes may also contain protein and lipids
256 besides carbohydrate, which are not suitable substrates for hydrogen production, but
257 are good for methane production. In our study, the dominant metabolite for hydrogen
258 production was butyrate (Table 3). Additionally, the substrate contains certain
259 amounts of protein and lipids besides carbohydrates (Table 1) which finally led to the

260 lower energy recovery as hydrogen compared to the theoretically calculated value.
261 Though the contribution of hydrogen to total energy is minor, it was crucial to
262 maintain the stability of the subsequent methane reactor. Our results also revealed that
263 optimization of hydrogen and methane reactor individually is not proper since
264 methane is the main energy carrier. By appropriate adjustment of reactor
265 configuration of the two-stage process (i.e. different HRT distribution between
266 hydrogen and methane reactor), the total energy in the system could be enhanced.
267 Therefore, further study should be focused on the optimization of the total energy
268 production in the two-stage system and pilot-scale reactors should be investigated to
269 speed up the application of two-stage process. It needs to be pointed out that though
270 the two-stage process could obtain more energy and achieve higher OLR compared
271 with single-stage process, the operation and control of such process is complicated
272 which should be carefully considered before industrial application.

273 For traditional two-phase anaerobic process, the higher biogas production was
274 attributed to enhanced hydrolysis of the substrate in acidogenic reactor and improved
275 activity of methanogens in the methanogenic reactor (Fox and Poland, 1994).
276 However, it seems that the improved performance of two-stage process in our study
277 was not due to the enhanced hydrolysis of substrate, because there was no significant
278 difference ($p=0.74>0.05$) between effluent VSS in both single-stage and two-stage
279 processes (Stillage as substrate) (Table 3). The short HRT (3d) in hydrogen reactor
280 may be not enough to significantly enhance the hydrolysis of the whole process, but it
281 is suitable for hydrogen production. The reason for higher biogas production in
282 two-stage process should be attributed to the enhanced methanogenic activities in the
283 methane reactors, which was demonstrated by SMA tests in subsequent section.
284 Another possible reason for the improved biogas production was due to a serial

285 operation minimizing the loss of relatively “fresh feed” out of the reactor due to
286 “short-circuiting”, occurring in single-stage fully mixed reactors.

287

288 3.2. SMA

289 The SMA results of the sludge biomass from different methane reactors on various
290 substrates are shown in Table 4. The degradation rates for glucose, acetate, and
291 butyrate in M12 of the two-stage process were much higher than that in M15 when
292 stillage was used as substrate. This could be the reason for the better performance of
293 the two-stage process. When mixture was used as substrate, the degradation rates for
294 glucose, acetate, and butyrate in M12 of the two-stage processes still maintained at a
295 high level, which demonstrated that the higher OLR did not inhibit the biogas process.
296 For M14 (mixture as substrate), the degradation rate for acetate was much higher than
297 that for M12. The result was consistent with the lower acetate concentration and
298 higher energy recovery in M14. Conclusively, the hydrogen reactors played an
299 important role to maintain a stable and efficient gasification process. Our results are
300 consistent with previous investigations of two-phase process, where it was concluded
301 that the separation of acidogenesis and methanogenesis favored the methanogenesis in
302 the second-phase (Fox and Pohland, 1994). In our study the improved performance of
303 the two-stage system was more attributed to the enhanced methanogenic activities and
304 not to enhanced hydrolytic/acidogenic activity. On the contrary, previous
305 investigations on two-phase process focused on optimizing the conditions for
306 hydrolysis/acidogenesis in the first stage and not in hydrogen production (Elefsiniotis
307 et al., 1996; He et al., 2005).

308 The SMA tests also showed that the degradation rates for propionate were very low
309 in all cases, which was consistent with the high propionate concentrations in all the

310 methane reactors. Low propionate degradation rate has been attributed to high organic
311 loading, VFA inhibition and lack of macro- and micro-nutrients in the substrates
312 (Cresson et al., 2006; Kida et al., 1993; van Lier et al., 1993). Anaerobic digestion in
313 single-stage CSTR with OLR between 2.5 and 5 gVS/(L·d) have been reported to
314 work stably, without propionate accumulation (Liu et al., 2006; Zhu et al., 2008). In
315 our study, the OLR was initially 3 gVS/(L·d) and subsequently increased to 4.5
316 gVS/(L·d), which was within the range for good propionate degradation. Furthermore,
317 acetate and butyrate concentrations in our study were not high enough to inhibit
318 degradation of propionate (Van Lier et al., 1993). A possible explanation for
319 accumulation of propionate could be the lack of some macro- and micro-nutrients in
320 the substrate. Agler et al. (2008) reported that VFA accumulation was observed even
321 when the OLR was only around 1.2 gVS/(L·d) when using anaerobic sequencing
322 batch reactor for the treatment of stillage. Addition of Co (20 mg/L) resulted in
323 decrease of VFA. Moreover, the OLR could be increased to as high as 7.5 gVS/(L·d)
324 without the process to be disturbed. We also analyzed for the metal ions in the stillage
325 and mixture, and found both Co was below detection limits (<7 ug/L), which might be
326 the reason for accumulation of propionate. Though the propionate concentration was
327 higher in our study, it did not apparently affect the process stability and the methane
328 yield was still very high (>300 ml-CH₄/gVS). Similarly, Wiegant et al. (1985) found
329 high propionate concentration (27-80 mM) under thermophilic condition, but the
330 anaerobic process was still stable. Moreover, the effluent quality did not deteriorate
331 with increasing loading rates from 17 to 98 kgCOD/(m³·d) in UASB.

Comment [r1]: I think van Lier is written with small v and not capital V. Please check.

332

333 3.3. Microbial communities

334 DGGE was conducted to analyze the microbial communities in all the reactors and

335 the sequencing results were shown in Table 5. The bacterial communities in both
336 hydrogen and methane reactors were shown in Fig 4. For hydrogen reactors (Lanes A,
337 D, F), the bacterial communities included members affiliated within one phyla
338 Firmicutes, and two unaffiliated bands (4 and 6). The change of substrate from stillage
339 to mixture in H3 led to the appearance of another two weak bands 4 and 6 besides the
340 dominant band 9. The decrease of HRT from 3d to 1d led to the significant shift of
341 dominant bands from band 9 to bands 6, 8 and 11. It seems that HRT was an
342 important parameter determining the relative composition of the microbial
343 communities in the hydrogen reactor. Nevertheless, *Thermoanaerobacterium*
344 *thermosaccharolyticum* was always the dominant species (band 8 and 9) even with
345 different substrates and HRT, indicating this bacterium is robust and can grow well in
346 a wide range of environmental conditions. *T. thermosaccharolyticum* can use glucose,
347 starch, and sucrose for hydrogen production and the optimal growth was in the range
348 pH 5-6 under thermophilic condition (O-Thong et al., 2008). *T.*
349 *thermosaccharolyticum* was also reported as the dominant species in thermophilic
350 hydrogen reactors from other study (Ahn et al., 2005).

351 For methane reactors, the phylogenetic affiliations of bacterial community
352 converged within three phyla, Firmicutes, Proteobacteria, Actinobacteria, which were
353 more diverse than the communities in hydrogen reactors. When stillage was used as
354 substrate, the dominant species in M12 was only band 10, while bands 1 and 2 were
355 also dominant species besides band 10 in M15 (Lanes B and C). It is obvious the
356 bacterial communities were different in the methane reactors of two-stage and
357 single-stage process, which also could explain the different performances of the two
358 different systems. Band 1 showed 96 % similarity to uncultured *gamma*
359 *proteobacterium* and band 2 showed 95 % similarity to uncultured *pseudomonas sp.*,

360 both of which were isolated from anaerobic activated sludge (unpublished data). Band
361 10 was related to *Clostridium thermocellum*, which could utilize carbohydrates to
362 produce various metabolites (acetate, ethanol et al.) (Nochur et al., 1992). Band 10
363 was also dominant in M 12 and M 14 when mixture was used (Lanes E and G).
364 Moreover, band 3 was closely related to *Propionibacterium sp.* (97 %), and this
365 bacteria could utilized carbohydrate for propionate production (Schuppert et al., 1992).
366 The higher propionate concentrations in all the methane reactors may be attributed to
367 the above microorganism.

368 For methane reactors, the archaeal DGGE bands were similar at all conditions (Fig
369 5), which indicated archaeal community structures were not obviously affected by the
370 changes of substrates and HRT. Although the relative dominance of microorganisms
371 did not change, the concentration of microorganisms and their activities might have
372 been altered, since DGGE is only a qualitative method. Therefore, the estimated
373 increase of SMA of acetoclastic methanogenesis, could have been due to increase of
374 acetoclastic biomass and activity in the reactors, and not to change of
375 microorganism-types. The dominant band 3 showed 96 % similarity to
376 *Methanosarcina acetivorans str.* , which belonged to acetoclastic methanogens
377 (Karakashev et al., 2005). Band 4 was also related to *Methanosarcina* species.
378 *Methanosarcina* species were reported to be dominant at high acetate concentration
379 (>1.2 mM) (Chu et al., 2010), and the results were consistent with the high acetate
380 concentrations in all the methane reactors (Table 3). Band 1 and 2 were related to
381 *Methanoculleus* species, which were responsible for hydrogenotrophic
382 methanogenesis (Shin et al., 2010). The activities of hydrogenotrophic methanogens
383 were also demonstrated by SMA tests and all the sludge biomass from different
384 methane reactors exhibited obvious degradation rate of hydrogen (>20

385 ml-CH₄/(gVS·d)).

386

387 **4. Conclusions**

388 The two-stage hydrogen and methane process could obtain 11% more energy
389 compared to single-stage process under OLR of 3 gVS/(L·d). The increase of OLR to
390 4.5 gVS/(L·d) led to the break down of the single-stage process, while the two-stage
391 process could work stably. The study also revealed that by proper adjustment of HRT
392 distribution between hydrogen and methane reactors, more energy could be obtained.
393 Microbial community analysis showed the dominant bacteria were always related to *T.*
394 *thermosaccharolyticum* in hydrogen reactors and *C. thermocellum* in methane reactors.
395 The acetotrophic methanogens *Methanosarcina acetivorans*-like organisms were the
396 dominant archaeal species in methane reactors.

397

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517 **Figure caption:**

518 Fig 1 Profiles of methane production, pH and VFA of single-stage process (M15)

519 Fig 2 Profiles of gas production, pH and VFA in hydrogen reactor (A, B) and methane
520 reactor (C, D) of two-stage process (H3+M12)

521 Fig 3 Profiles of gas production, pH and VFA in hydrogen reactor (A, B) and methane
522 reactor (C, D) of two-stage process (H1+M14)

523 Fig 4 DGGE bands of bacterial communities. A, H3 (stillage); B, M12 (stillage); C,

524 M15 (stillage); D, H3 (mixture); E, M12 (mixture); F, H1 (mixture); G, M14

525 (Mixture).

526 Fig 5 DGGE bands of archaeal communities.

527 Table 1 Characterization of substrates
 528

	Stillage	Cake	Glycerol	Mixture
pH	3.9±0.1	/	7±0.1	4.2±0.1
TS (%)	4.75±0.15	85.6±1.55	/	6.85±0.05
VS (%)	4.5±0.11	79.6±1.28	/	6.82±0.03
COD (g/L)	61.9±1.8	/	1638±103	97.3±2.1
SCOD (g/L)	20.8±1.9	/	/	29.8±1.2
VFA (g/L)	0.15±0.08	/	/	0.08±0.01
TSS(g/L)	35.4±1.2	/	/	54.8±1.3
VSS(g/L)	34±1.6	/	/	54±2.1
Total nitrogen (g/L)	1.44±0.06	30.6±0.85 ^a	0.23±0.01	2.16±0.05
Ammonia (g/L)	0.27±0.05	1.4±0.05 ^a	N.D	0.27±0.03
Carbohydrate (g/L)	30	580 ^a	/	48.9
Lipid (g/L)	7.5±1.2	35±1.2 ^a	51±2.5	8±0.6
Protein (g/L)	7.2±0.8	181±5.02 ^a	1.42±0.03	11.3±0.8

529 “/”, not detected
 530 “N.D”, not detectable
 531 “a” Value expressed in g/kg

Table 2 Reactor operation data

Parameter	Single-stage	Two-stage		Two-stage	
	M15	H3	M12	H1	M14
HRT	15	3	12	1	14
working volume, L	3.5	1.2	3.5	1.2	3.5
feed rate, mL/d	233	400	292	1200	250

Table 3 Summary of reactor performances at steady-states

	One-stage	Two-stage	Two-stage	Two-stage
Substrate	Stillage	Stillage	Mixture	Mixture
Hydrogen reactor		H3	H3	H1
HRT(d)	/	3	3	1
Hydrogen yield (ml/gVS)	/	69±6.3	48±5.5	40±4.7
pH	/	5.2±0.1	5.2±0.1	5.3±0.1
Ethanol (mM)	/	17.7±0.8	18.6±1.2	10.1±1.2
Acetate (mM)	/	22.1±1.8	22.8±1.3	17.7±1.3
Propionate (mM)	/	0.7±0.2	0.3±0.1	0.4±0.1
Butyrate (mM)	/	63.7±2.0	64.8±2.4	53.7±1.4
Valerate (mM)	/	0.2±0.1	0.1±0.1	0.2±0.1
SCOD (g/L)	/	23.5±2.2	33.6±3.4	32.5±2.8
TCOD (g/L)	/	57.5±4.7	92.6±7.6	93.8±5.9
NH ₃ -N (mg/L)	/	310±50	360±75	350±62
VSS (g/L)	/	29±1.8	47.7±3.1	51.5±2.6
Energy (KJ/gVS)	/	0.7±0.07	0.5±0.06	0.4±0.05
Methane reactor	M15	M12	M12	M14
HRT	15	12	12	14
Methane yield (ml/gVS)	329±13.7	348±14.2	320±14.5	344±19.5
pH	7.8±0.1	8.0±0.1	7.9±0.1	8.0±0.1
Ethanol(mM)	0.2±0.1	0.3±0.1	0.1±0.1	0.1±0.1
Acetate(mM)	5.1±0.3	6.3±0.3	19.3±1.7	8.7±0.7
Propionate(mM)	36.4±1.6	31.5±2.3	28.5±1.2	28.9±1.6
Butyrate(mM)	1.6±0.3	1.5±0.2	4.6±0.6	3.3±0.5
Valerate(mM)	1.5±0.2	7.5±0.4	4.7±0.2	4.3±0.5
SCOD(g/L)	13.5±2.1	8.3±1.5	12.5±2.8	11.3±1.8
TCOD(g/L)	21.6±1.8	16.2±3.2	35.2±3.3	29.5±2.1
NH ₄ ⁺ -N(mg/L)	1158±320	1135±110	1432±250	1590±370
VSS(g/L)	8±1.8	8.6±2.3	19.5±1.5	18.5±1.9
Energy (KJ/gVS)	11.8±0.49	12.4±0.51	11.4±0.52	12.3±0.69
Total energy (KJ/gVS)	11.8±0.49	13.1±0.55	11.9±0.53	12.7±0.72

Table 4 SMA of sludge biomass from methane reactors (ml-CH₄/(gVS·d))

Substrate	Single-stage (Stillage, M15)	Two-stage (Stillage, H3+M12)	Two-stage (Mixture, H3+M12)	Two-stage (Mixture, H1+M14)
Glucose	57.7±1.6	72.2±1.1	64.6±4.0	70±3.5
Acetate	45.3±2.0	55.1±3.8	63.1±2.9	72±4.6
Propionate	6.7±0.9	5.7±2.5	8.8±3.3	6.1±2.8
Butyrate	31.5±2.0	45.5±1.9	46.1±1.7	41±3.2
Hydrogen	28.8±2.2	22.4±5.7	24.3±2.7	27±2.5

Table 5 DGGE 16S rRNA gene band identifications

DGGE band	Closest match	Identity(%)	Phyla	Accession no.
<i>Bacteria</i>				
1	<i>Uncultured gamma proteobacterium</i>	96	Proteobacteria	HQ219810
2	<i>Uncultured Pseudomonas sp.</i>	95	Proteobacteria	HQ219811
3	<i>Propionibacterium sp.</i>	97	Actinobacteria	HQ219812
4	<i>Uncultured bacterium</i>	93	Unaffiliate	HQ219813
5	<i>Streptococcus thermophilus</i>	93	Firmicutes	HQ219814
6	<i>Uncultured bacterium</i>	96	Unaffiliate	HQ219815
7	<i>Clostridium sp.</i>	94	Firmicutes	HQ219816
8	<i>Thermoanaerobacterium thermosaccharolyticum</i>	100	Firmicutes	HQ219817
9	<i>Thermoanaerobacterium thermosaccharolyticum</i>	95	Firmicutes	HQ219818
10	<i>Clostridium thermocellum</i>	98	Firmicutes	HQ219819
11	<i>Thermoanaerobacterium sp.</i>	100	Firmicutes	HQ219820
<i>Archaea</i>				
1	<i>Methanoculleus thermophilus</i>	96	Euryarchaeota	HQ219821
2	<i>Methanoculleus thermophilus</i>	97	Euryarchaeota	HQ219822
3	<i>Methanosarcina acetivorans str.</i>	96	Euryarchaeota	HQ219823
4	<i>Methanosarcina barkeri</i>	94	Euryarchaeota	HQ219824

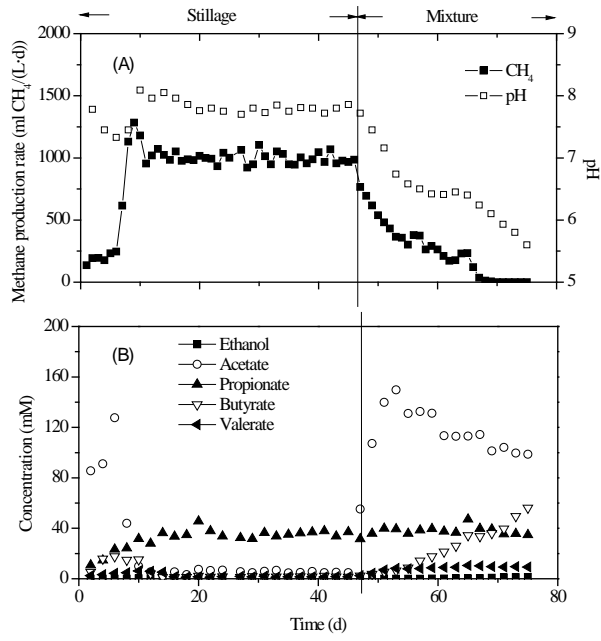


Fig 1 Profiles of methane production, pH and VFA of single-stage process (M15)

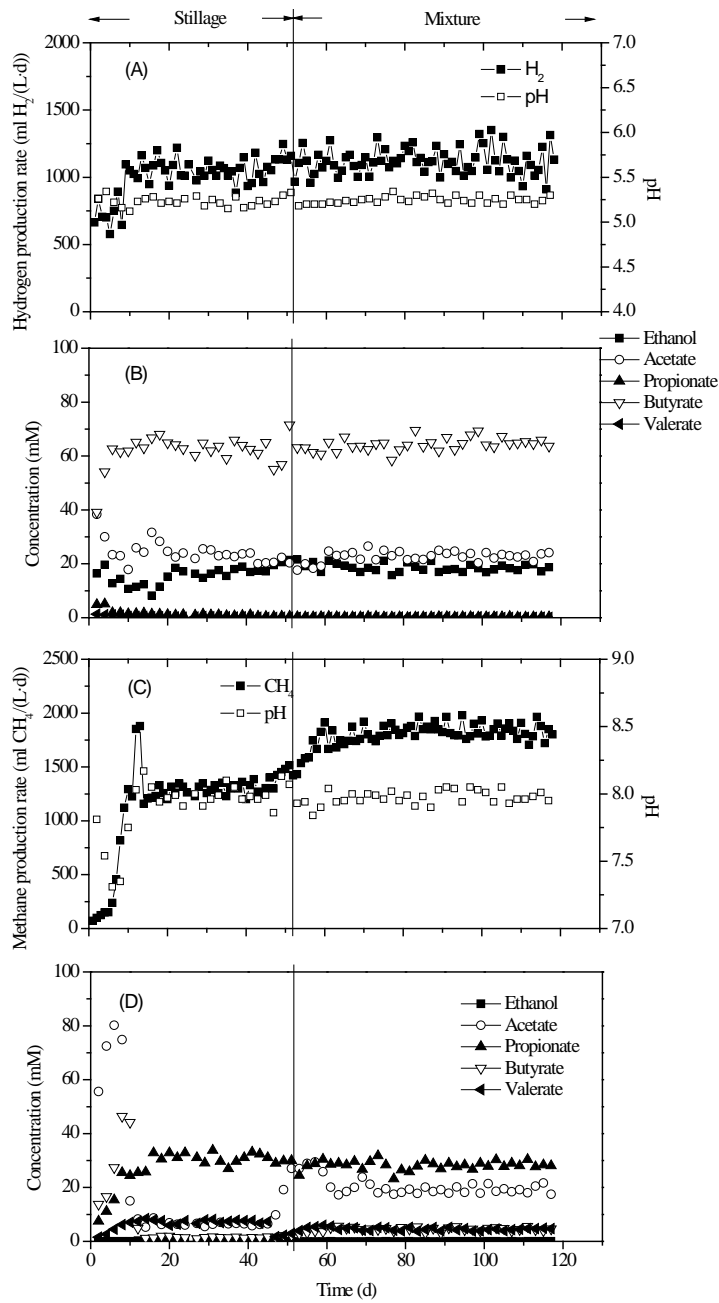


Fig 2 Profiles of gas production, pH and VFA in hydrogen reactor (A, B) and methane reactor (C, D) of two-stage process (H3+M12)

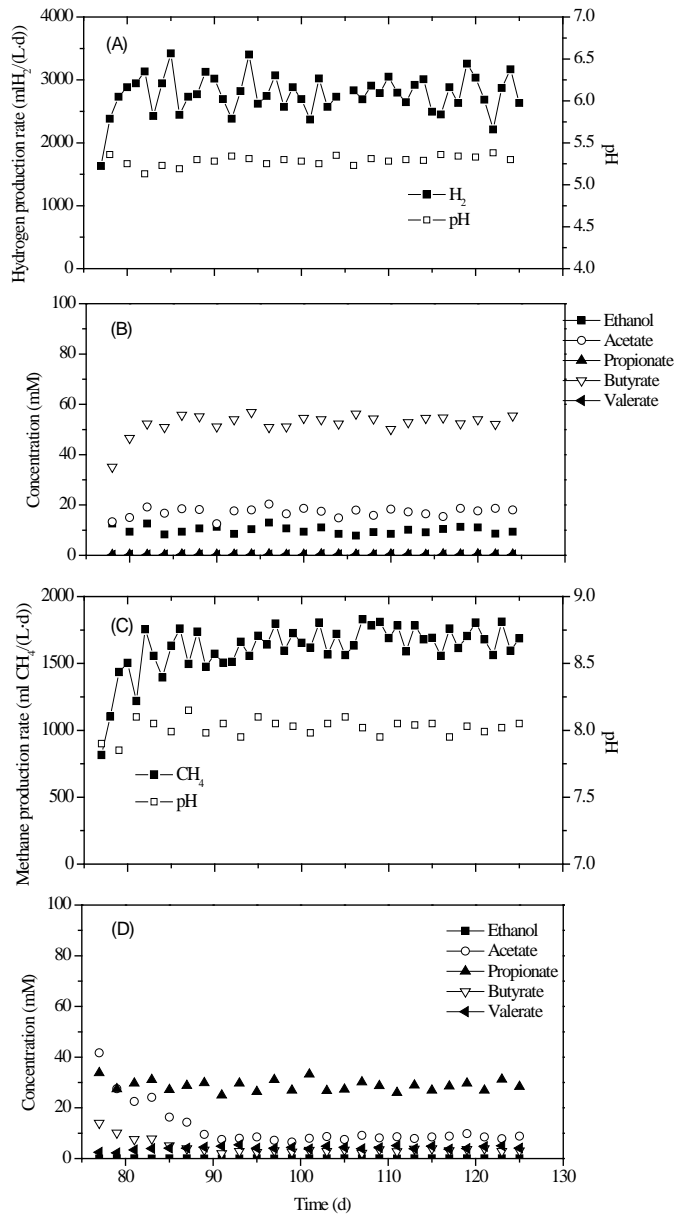


Fig 3 Profiles of gas production, pH and VFA in hydrogen reactor (A, B) and methane reactor (C, D) of two-stage process (H1+M14)

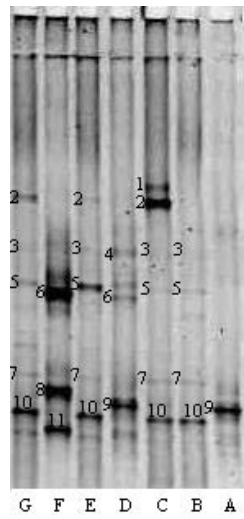


Fig 4 DGGE bands of bacterial communities. A, H3 (stillage); B, M12 (stillage); C, M15 (stillage); D, H3 (mixture); E, M12 (mixture); F, H1 (mixture); G, M14 (Mixture).

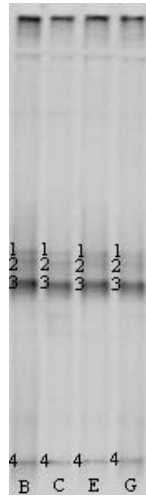


Fig 5 DGGE bands of archaeal communities.