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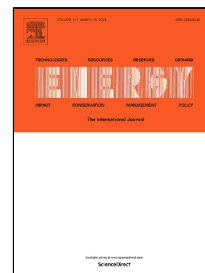
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1 **Assessment of continuous fermentative hydrogen and methane co-production**
2 **using macro- and micro-algae with increasing organic loading rate**

3
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12
13 **Abstract**

14 A two-stage continuous fermentative hydrogen and methane co-production using
15 macro-algae (*Laminaria digitata*) and micro-algae (*Arthrospira platensis*) at a C/N
16 ratio of 20 was established. The hydraulic retention time (HRT) of first-stage H₂
17 reactor was 4 days. The highest specific hydrogen yield of 55.3 mL/g volatile solids
18 (VS) was obtained at an organic loading rate (OLR) of 6.0 gVS/L/d. In the second-
19 stage CH₄ reactor at a short HRT of 12 days, a specific methane yield of 245.0
20 mL/gVS was achieved at a corresponding OLR of 2.0 gVS/L/d. At these loading rates,
21 the two-stage continuous system offered process stability and effected an energy yield
22 of 9.4 kJ/gVS, equivalent to 77.7% of that in an idealised batch system. However,
23 further increases in OLR led to reduced hydrogen and methane yields in both reactors.
24 The process was compared to a one-stage anaerobic co-digestion of algal mixtures at
25 an HRT of 16 days. A remarkably high saline level of 13.3 g/L was recorded and
26 volatile fatty acid accumulation were encountered in the one-stage CH₄ reactor. The
27 two-stage system offered better performances in both energy return and process
28 stability. The gross energy potential of the advanced gaseous biofuels from this algal
29 mixture may reach 213 GJ/ha/yr.

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30 **Keywords:** Macro-algae; micro-algae; two-stage co-fermentation; hydrogen; methane

ACCEPTED MANUSCRIPT

31 1. Introduction

32 In recent years there is an increased interest in producing advanced biofuels from
33 alternative feedstocks. The need to improve energy yields and allay sustainability
34 concerns including land use change of first and second generation biofuels have led to
35 research of algae (both macro and micro) as viable substrates for the production of
36 advanced biofuels. Algal biofuels can overcome the food-or-fuel debate associated
37 with first generation biofuels [1, 2] and do not face the complex conversion processes
38 required for second generation biofuel production [3, 4]. Aquatic algae possess
39 several advantages over terrestrial plants. Firstly, both macro-algae and micro-algae
40 have higher growth rates and biomass productivities as compared to agricultural crops
41 [5-7]. Secondly, the cultivation of algae may not require arable lands or fresh water. A
42 win-win situation can be achieved through coupling algae production with wastewater
43 treatment [8-10]. Thirdly, algae may provide continuous biomass supply throughout
44 the year with optimised cultivation such as CO₂ supplementation using flue gas for
45 micro-algae [11, 12] and efficient preservation such as ensiling for macro-algae [13].

46 Production of liquid biofuels (such as biodiesel and bioethanol) using algae
47 biomass has been extensively explored [14, 15]. However, the parasitic energy
48 demand for the generation of liquid biofuels from raw feedstocks exceeds that in the
49 conversion from substrates to gaseous biofuels such as biohydrogen and biomethane
50 [16-18], leading to comparatively lower overall energy efficiencies. Besides, gaseous
51 biofuels offer more utilisation options, including: compression for vehicles fuels;
52 injection into the existing natural gas grids for use as renewable heat in industry such

53 as breweries [19]; on site electricity generation using internal combustion engines
54 [20]; or increased efficiency through use of biomethane from the gas grid at combined
55 cycle gas turbines.

56 Biological hydrogen production through dark hydrogen fermentation of algae
57 biomass shows advantages over conventional energy-intensive hydrogen-producing
58 methods such as steam methane reforming [21] due to the mild reaction conditions
59 and renewability of the produced hydrogen [22]. However, limited energy conversion
60 restricts its application. An alternative gaseous product biomethane generated through
61 biological anaerobic digestion of algae biomass with better energy output has been
62 analysed in previous studies [15, 23, 24]. Nevertheless, some major bottlenecks still
63 restrict the application of this process. The abundant recalcitrant organics such as
64 polyphenols in macro-algae [5] and triglycerides in micro-algae are not readily
65 digested by the microbes and thereby decrease the biodegradability of biomass [23].
66 In addition, the rigid cell wall structures of algae act as barriers between the
67 intracellular biodegradable contents and anaerobic microbes, hence hindering the
68 degradation and methanogenesis of algae biomass in anaerobic digestion process [24].
69 To tackle this problem, a two-stage process combining hydrogen fermentation and
70 anaerobic digestion can serve as a promising solution. The two-stage set-up separates
71 the process phases and optimises the operational conditions for each. In the first stage
72 of hydrogen fermentation, the anaerobic fermentative bacteria (AFB) favour the pH
73 condition of 5-6 where they can efficiently degrade the large-molecular-weight
74 organics such as carbohydrates and proteins into gaseous hydrogen, carbon dioxide,

75 and liquid soluble metabolic products (such as volatile fatty acids (VFAs), alcohols,
76 and lactic acid) in a short retention time (2-4 days) [22]. Subsequently, the liquid
77 fermentation effluents rich in small-molecular-weight VFAs and alcohols can be
78 readily utilised by the methanogenic organisms in the second stage of anaerobic
79 digestion. Therefore, compared with one-stage anaerobic digestion, the two-stage
80 process presents better energy yields with improved biogas production and
81 significantly shortens the overall retention time with concurrent increase in organic
82 loading rates (OLRs). Yang et al. [25] used lipid-extracted residues of microalgae
83 *Scenedesmus* for two-stage batch fermentative hydrogen and methane co-production
84 and obtained a 22% increase in methane yield and a 27% increase in energy efficiency
85 in contrast to that in one-stage anaerobic digestion. Massanet-Nicolau et al. [26]
86 investigated the two-stage continuous fermentative hydrogen and methane co-
87 production of pelletized grass, which exhibited an overall energy yield of 11.74 kJ/g
88 volatile solids (VS) with an increase of 13.4% compared with one-stage anaerobic
89 digestion. Process stability was maintained whilst the hydraulic retention time (HRT)
90 was greatly shortened from 20 days in the one-stage to 12 days in the two-stage
91 process [26].

92 Apart from relatively limited biodegradability of algae compared with some first
93 generation feedstocks [5], the intrinsic compositional unbalance of certain algae
94 biomass (in particular micro-algae biomass) can impair the anaerobic digestion
95 process [27]. Proteins occupy a large portion of organics in micro-algae, leading to a
96 low C/N ratio in the biomass. The excessive nitrogen is released in the form of

97 ammonia during the degradation of proteins, resulting in severe decrease in the
98 microbial activities of methanogenic microbes [28]. By contrast, some species of
99 macro-algae, such as brown seaweeds *Laminaria digitata* and *Saccharina latissima*,
100 contain rich carbohydrates and have a high C/N ratio when harvested at optimum
101 times [5]. This can in certain cases lead to limited nitrogen supply for the basic
102 metabolisms of AFB in hydrogen fermentation and the methanogens in anaerobic
103 digestion [29]. The optimum C/N ratio was suggested to be 20-30 for algal feedstocks
104 [21, 30]. Thus, adjusting the C/N ratio by mixing nitrogen-rich micro-algae and
105 carbon-rich macro-algae as co-substrates offers an excellent strategy to improve the
106 process performances of both hydrogen fermentation and anaerobic digestion. Xia et
107 al. [29] mixed micro-algae *Arthrospira platensis* and macro-algae *L. digitata* for batch
108 fermentative hydrogen production and achieved an optimal H₂ yield of 85.0 mL/gVS
109 at a C/N ratio of 26.2. A study on the continuous one-stage anaerobic digestion of
110 mixed *A. platensis* and *L. digitata* at a C/N ratio of 25 was conducted and the highest
111 specific methane yield (SMY) of 273.9 mL/gVS was recorded at an OLR of 3.0
112 gVS/L/d and an HRT of 28 days [27]. Although many micro-algae species thrive in
113 tropical and sub-tropical waters while macro-algae are commonly found in temperate
114 sea, the micro-algae cultivation in temperate regions using seawater and flue gas from
115 coal-fired power plants provides the possibility of harvesting micro- and macro-algae
116 biomass in the same place [2, 5, 12].

117 The authors previously conducted a two-stage batch fermentative hydrogen and
118 methane co-production using co-substrates of macro-algae (*L. digitata*) and micro-

119 algae (*Chlorella pyrenoidosa* and *Nannochloropsis oceanica*) [31]. The micro-algae
120 biomass supplied nitrogen to balance the C/N ratio of the algal mixtures. Co-
121 fermentation facilitated the hydrolysis and acidogenesis of the algal co-substrates and
122 further boosted the energy conversion in anaerobic digestion. Although the batch co-
123 fermentation provided some innovative findings, these experimental configurations
124 have significant limitations. Batch systems allow sufficient guaranteed retention
125 times, efficient mixing and anaerobic conditions; they also allow an optimum
126 inoculum to substrate VS ratio of 2:1 which minimises inhibitory effects such as
127 accumulation of volatile fatty acids and ammonia. Batch assays have limited
128 replicability compared with likely industrial applications. In the majority of
129 commercial industrial applications, the loading of reactor is continuous. As such it is
130 necessary to undertake continuous laboratory experiments to assess the impact of
131 higher OLRs and shorter HRTs for a prosperous and stable fermentation process.
132 Economics dictate the need for high processing capability and biofuel outputs for
133 minimum size of reactor system. Therefore, continuous two-stage laboratory co-
134 fermentation is essential to address long term optimised operational conditions.
135 Nevertheless, to date, long term continuous two-stage co-fermentation of micro- and
136 macro-algae biomass remains uninvestigated in literature. This paper will address this
137 knowledge gap in the state of the art through the following objectives:

- 138 (1) Assess co-generation of hydrogen and methane using the mixture of macro-
139 algae (*L. digitata*) and micro-algae (*A. platensis*) at the optimal C/N ratio of
140 20 with increasing OLRs.

- 141 (2) Evaluate the effects of different OLRs and HRTs on the specific hydrogen
142 yields (SHYs), the acidification yields in first-stage dark hydrogen
143 fermentation and the SMYs in second-stage anaerobic digestion.
- 144 (3) Compare the performances of two-stage and one-stage systems on the overall
145 energy conversion and process stability.
- 146 (4) Estimate the gross energy potential of this advanced gaseous biofuel system.

147

148 **2. Materials and methods**

149 **2.1 Algal biomass and inocula**

150 The macro-algae *L. digitata* was naturally grown in the open sea and collected in
151 September in West Cork, Ireland. The harvested *L. digitata* was washed with tap
152 water to remove attached sands and other impurities, and then cut to small particles
153 (4-5 mm) by a mincer (Buffalo Heavy Duty Mincer CD400). The micro-algae powder
154 of *A. platensis* was purchased from Bluegreen Life Foundation Inc. (Lewes, DE,
155 USA). Both macro- and micro-algal samples were cryopreserved at -20 °C before the
156 experiment.

157 The hydrogen inoculum used in biohydrogen potential (BHP) test and continuous
158 hydrogen reactor was taken from the anaerobic sludge of an Irish farm digester. The
159 original sludge was heated at 100 °C in an autoclave (Sanyo MLS-3780, Japan) for 30
160 min to inactivate methanogens and subsequently acclimatized 3 times (3 days each
161 time) using a modified culture medium to activate the spore-forming hydrogenogenic
162 bacteria. The compositions of the modified medium were detailed in our previous

163 study [31].

164 The inoculum used in the biomethane potential (BMP) test and continuous
165 digestion reactors was obtained from the digestate of an existing laboratory scale
166 seaweed anaerobic digester. The methane inoculum was degassed at a temperature of
167 37 °C for 7 days before the experiment.

168

169 **2.2 Biohydrogen and biomethane potential tests**

170 The two-stage batch BHP and BMP tests on the mixture of *L. digitata* and *A.*
171 *platensis* were conducted in triplicate in an AMPTS II system (Bioprocess Control,
172 Sweden).

173 In the BHP test, 3 g VS of the algal substrate were added to each glass bottle and
174 then the liquor volume was adjusted to 270 mL using distilled water. Subsequently,
175 30 mL of hydrogen inoculum was added into each bottle to make the total working
176 volume 300 mL. The VS portions of the two algal biomass in each bottle were
177 calculated to effect a C/N ratio of 20: 2.82 gVS of *L. digitata* mixed with 0.18 gVS of
178 *A. platensis*. The initial pH was adjusted to 6.00 ± 0.05 with 1 M NaOH and 1 M HCl
179 solutions. All bottles were sealed with rubber stoppers and purged with N₂ for 5 min
180 to maintain anaerobic conditions, and then placed in a water bath at a temperature of
181 37 °C for 4 days. Stirrers which were set to switch between on and off for 60 s periods
182 with a mixing speed of 60 rpm were applied to the bottles. Carbon dioxide in the
183 produced gas was absorbed by 80 mL of 3 M NaOH solution and then the hydrogen
184 gas flow was recorded by a gas tipping device based on water displacement. The

185 recorded hydrogen gas volumes were automatically normalised to standard
186 temperature and pressure (STP) and zero moisture content by the AMPST II system.

187 After the BHP test, the effluent in each bottle was analysed and then prepared for
188 subsequent BMP test. The pH values of effluents were adjusted to 8.00 ± 0.05 with 1
189 M NaOH and then inoculated with methane inoculum at the inoculum to substrate VS
190 ratio of 2:1. The total working volume of each bottle was 400 mL and the BMP test
191 ran for 26 days so that the two-stage batch BHP and BMP tests duration reached 30
192 days. All the other BMP test settings were the same as those in the BHP test. A
193 control group with just blank inocula (no substrates) was established and all the
194 hydrogen and methane volumes produced from experimental groups were corrected
195 for the ones produced from control group.

196

197 **2.3 Set-up and operation of continuous reactors**

198 Four lab-scale (5 L) continuously stirred tank reactors (CSTR), which comprised
199 of one H₂ reactor and three CH₄ reactors, were used for the continuous fermentation
200 trials as shown in Fig. 1. The H₂ reactor and CH₄ reactors A and B comprised the two-
201 stage fermentation systems. The CH₄ reactor C acted as a one-stage fermentation
202 system as a comparison to the two-stage system. The working volumes of H₂ reactor
203 and CH₄ reactors were 3 L and 4 L, respectively. The temperature of the reactors was
204 maintained at 37 ± 1 °C using a temperature controller unit. The volume of the
205 produced biogas from each reactor was measured using a wet tip gas meter which was
206 connected to an automated data acquisition system. The reactor configuration has

207 been detailed in previous studies [27, 32].

208 The HRT of the H₂ reactor was set to 4 days. The HRTs of CH₄ reactors A and B
209 were set to 12 days and 24 days, respectively. The HRT of the one-stage CH₄ reactor
210 C was set to 16 days to match the overall HRT of the first two-stage system
211 comprising of the H₂ reactor and the CH₄ reactor A. In a similar fashion, the overall
212 HRT of the second two-stage system comprising of the H₂ reactor and CH₄ reactor B
213 was set to 28 days to match the one in a previous study that investigated the one-stage
214 co-digestion of *L. digitata* and *A. platensis* for methane production [27].

215 The OLR of the H₂ reactor was increased from 3.0 to 12.0 gVS/L/d with an
216 increment of 3.0 gVS/L/d each time. This was achieved by diluting the algal biomass
217 with a calculated volume of water to keep the HRT unchanged. Every time after
218 feeding, the pH value in H₂ reactor was adjusted to ca. 5.5 using 1 M NaOH solution
219 to ensure the pH did not drop to a level to inhibit hydrogen-producing microbes. The
220 effluent from the H₂ reactor was divided into three parts: the first one as the feedstock
221 for CH₄ reactor A, the second one as the feedstock for CH₄ reactor B, and the third
222 one for analyses. The OLR of CH₄ reactor A ranged from 1.0 to 4.0 gVS/L/d with an
223 increment of 1.0 gVS/L/d each time, whilst that of CH₄ reactor B increased from 0.5
224 to 2.0 gVS/L/d with an increment of 0.5 gVS/L/d each time. The OLR of the CH₄
225 reactor C (in the single stage system) started from 1.0 gVS/L/d with an increment of
226 1.0 gVS/L/d until reactor failure was observed. Each OLR of each reactor was
227 maintained constant for 48 days, which equates to two HRTs of CH₄ reactor C, which
228 had the longest retention time.

229 2.4 Analytical methods

230 Total solids (TS) and VS contents of *L. digitata*, *A. platensis*, and inocula were
231 determined using Standard Methods 2540 G [33]. The pH value was measured using a
232 pH meter (Jenway 3510, UK). The ratio of VFAs to total alkalinity (FOS/TAC) was
233 determined based on a two points titration method using 0.1 N H₂SO₄ with end points
234 of pH 5.0 and pH 4.4 [34]. Carbon, hydrogen, and nitrogen contents were determined
235 by an elemental analyser (Exeter Analytical CE 440, UK) and oxygen was calculated
236 as the remaining content of VS. Soluble chemical oxygen demand (sCOD) and total
237 ammoniacal nitrogen (TAN) were measured using Hach Lange cuvette tests (LCK
238 914 and LCK 303, respectively) and evaluated on a DR3900 Hach Lange
239 Spectrophotometer. Salinity of effluents was determined on a VWR hand held C0310
240 monitor (VWR international, USA).

241 The composition of biogas (H₂, CO₂, O₂, N₂, and CH₄) produced in CSTR
242 reactors was determined using a gas chromatograph (GC, Hewlett Packard HP6890,
243 USA) equipped with a Hayesep R packed column and a thermal conductivity detector.
244 The compositions of VFAs in the effluents were determined using a GC (Hewlett
245 Packard HP6890, USA) equipped with a Nukol fused silica capillary column and a
246 flame ionisation detector [32].

247

248 2.5 Calculations

249 The energy values of *L. digitata* and *A. platensis* were calculated using the
250 weight percentages of C, H, N, and O on the basis of the modified Dulong Formula as

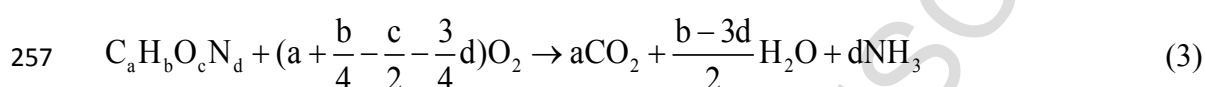
251 shown in Eq. (1) [35]:

$$252 \text{ Energy value of algal biomass (kJ/kg)}=337C+1419(H-0.125O)+23.26N \quad (1)$$

253 The energy conversion efficiency (ECE) was calculated based on Eq. (2) [36].

$$254 \text{ ECE}=\frac{\text{Energy value of H}_2 + \text{Energy value of CH}_4}{\text{Original energy value of algal biomass}} \times 100\% \quad (2)$$

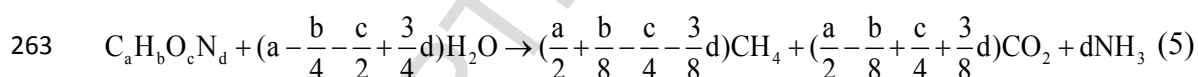
255 The total chemical oxygen demand (tCOD) of algal biomass was calculated
256 based on the element compositions using Eq. (3) [32]:



258 The acidification yield in the H₂ reactor is defined as the percentage of the COD
259 from VFAs to sCOD as shown in Eq. (4) [32]:

$$260 \text{ Acidification yield}=\frac{\text{COD}_{\text{VFAs}}}{\text{sCOD}_{\text{increase}}} \times 100\% \quad (4)$$

261 The theoretical calculation of biomethane yield was based on the Buswell
262 equation as shown in Eq. (5) [32]:



264

265 3. Results and discussion

266 3.1 Characteristics of algal biomass

267 Table 1 presents the characteristics of *L. digitata* and *A. platensis* biomass. The
268 macro-algae *L. digitata* was harvested from natural environments in shallow coastal
269 waters, resulting in a lower VS/TS ratio as compare to the artificially cultivated
270 micro-algae *A. platensis* which avoided the significant salt accumulation from

271 seawater. The harvest timing of September coincided with the peak carbohydrate
272 accumulation in *L. digitata* biomass [5], leading to a high C/N ratio of 26.47. By
273 contrast, the rich proteins in *A. platensis* contributed to the high nitrogen content. This
274 also provided the possibility of mixing the two algal substrates at an appropriate C/N
275 ratio of 20. Moreover, *A. platensis* biomass exhibited higher energy content and
276 theoretical biomethane potential on the basis of elemental composition, despite
277 potential antagonistic effects of recalcitrant organic components on the
278 biodegradability [27]. *L. digitata* biomass is rich in carbohydrates, which generate 20
279 times higher hydrogen-producing potential than proteins and lipids [40] and as such
280 serve as the major components utilised by the AFB for biohydrogen production. *A.*
281 *platensis* is rich in proteins and can supply essential nitrogen sources for the
282 anaerobes in both H₂ and CH₄ reactors to maintain effective metabolism [29]. The
283 lipid contents are relatively low in both algal species and are not readily utilised by
284 the AFB for hydrogen production. The lipids, however, can be slowly degraded and
285 further converted to biomethane in the second-stage anaerobic digestion with a longer
286 retention time [22].

287

288 **3.2 Batch biohydrogen and biomethane potential tests**

289 After the sequential 4-day BHP and 26-day BMP tests using the mixed *L.*
290 *digitata* and *A. platensis* biomass, a BHP yield of 94.6 mL H₂/gVS and a BMP yield
291 of 309.3 mL CH₄/gVS were recorded (Fig. 2). The BHP yield exceeds the result (60.5
292 mL H₂/gVS) obtained in a previous study using algal mixture of *L. digitata* and *A.*

293 *platensis* at a C/N ratio of 16.5 [29], indicating the C/N ratio of 20 is preferred during
294 the batch hydrogen fermentation of this specific algal mixture. Moreover, the BHP
295 yield is close to the findings (94.5-97.0 H₂ mL/gVS) of our previous study on batch
296 hydrogen co-fermentation of macro-algae (*L. digitata*) and micro-algae (*Chlorella*
297 *pyrenoidosa* and *Nannochloropsis oceanica*).

298 After hydrogen fermentation, the VFA compositions in the hydrogenogenic
299 effluent were as follows: 0.64 g/L of acetic acid, 0.02 g/L of propionic acid, 0.02 g/L
300 of isobutyric acid, 0.97 g/L of butyric acid, 0.03 g/L of isovaleric acid, and 0.01 g/L
301 of valeric acid. The acetic and butyric acids accounted for 95.1% of the total VFAs,
302 indicating that the predominant metabolic pathways of the AFB during hydrogen
303 fermentation were acetic and butyric routes [22]. As shown in Fig. 2b. during
304 subsequent BMP test, the soluble VFAs that are readily utilised by methanogens
305 contributed to the first peak of biomethane production rate at 6 days, whereas the
306 solid remnants continued to be hydrolysed and resulted in the second peak of
307 biomethane production rate at 12 days. The BMP yield matches that from the one-
308 stage batch anaerobic co-digestion of *L. digitata* and *A. platensis* (311.5 mL
309 CH₄/gVS) achieved by [27]. Although no significant enhancement of BMP yield was
310 obtained, the two-stage batch co-fermentation of *L. digitata* and *A. platensis* secured
311 an overall energy yield of 12.1 kJ/gVS that is 8.5% higher than that from the one-
312 stage biomethane production [27].

313

314 3.3 Continuous fermentation performances with increasing OLRs

315 The performance characteristics of all four reactors of the two-stage and one-
316 stage systems over increasing OLRs are summarised in Table 2. The first HRT at each
317 OLR in each reactor was deemed as the acclimatisation period for anaerobic
318 microbes, thus the data in Table 2 are displayed as mean values over the post-first
319 HRT duration of each OLR. Throughout the entire experiment, the TAN
320 concentrations of all CH₄ reactors stayed low, indicating that no ammonia inhibition
321 occurred.

322 3.3.1 Performance of H₂ reactor

324 Fig. 3 shows the SHYs of the H₂ reactor with increasing OLRs; Fig 4a shows the
325 compositions of VFAs. At the initial OLR of 3.0 gVS/L/d, the SHYs were quite
326 limited. However, the acidification yield reached 87.5%, indicating a large portion of
327 mixed *L. digitata* and *A. platensis* were utilised by the AFB to maintain basic
328 metabolisms. Thus, the low mean SHY (14.3 mL/gVS) and the high acidification
329 yield at this low OLR indicated that the AFB in H₂ reactor were underfed to some
330 extent. When the OLR increased from 3.0 to 6.0 gVS/L/d, the SHYs drastically
331 increased. Although the SHYs fluctuated between 40.5 and 72.0 mL/gVS over this
332 OLR, an average of 55.3 mL/gVS was achieved, which equates to 58.5% of the BHP
333 yield in the batch trial. As the sCOD of 14.2 g/L at this OLR (6.0 gVS/L/d) was over
334 2-fold of that (7.0 g/L) at the initial OLR (3.0 gVS/L/d), it could be assumed that the

335 hydrolysis of mixed algal substrates was even a little bit more efficient. The tVFA
336 also increased to 5254 mg/L, corresponding to an acidification yield of 63.0%.
337 Similarly, the salinity increased by 55.6%, illustrating that this OLR provided
338 excessive biomass supply for the basic metabolisms of AFB and hence more algal
339 substrates were degraded and utilised for hydrogen production.

340 When the OLR was further lifted from 6.0 to 9.0 gVS/L/d, a sharp drop in
341 hydrogen production was recorded. The mean SHY of 20.4 mL/gVS was 63.1% lower
342 than that at the OLR of 6.0 gVS/L/d. This result was attributed to the accumulation of
343 large quantities of VFAs that inhibited the hydrogen-producing pathways of AFB in
344 the H₂ reactor. The increased loading of algal substrates resulted in sCOD and tVFA
345 values higher by 29.6% and 26.1% in the liquid phase, respectively, whereas the
346 remaining VS in the H₂ reactor (at 9.0 gVS/L/d) increased by 57.5%. As the increase
347 in remaining VS exceeded the increase in sCOD and tVFA, it was assumed that H₂
348 reactor was overfed and hydrolysis and acidification of loaded algal substrates were
349 limited to some extent. With the OLR further rising to 12.0 gVS/L/d, the average
350 SHY marginally declined to 19.0 mL/gVS. Although the sCOD slightly increased, the
351 tVFA unexpectedly decreased a little bit, leading to a lower acidification yield as
352 compared to that at the OLR of 9.0 gVS/L/d. This also indicated that more algal
353 substrates were fermented through ethanol and lactic acid producing pathways. This
354 was probably ascribed to the enhanced fluctuations of pH values at higher OLRs.
355 With the loading increasing, soluble acidic metabolites accumulated and hence the pH
356 drop became more severe between each feed. The lower pH facilitated the shift of

357 acetic and butyric routes to ethanol and lactic acid producing pathways in the H₂
358 reactor [24, 32, 41].

359 These results suggested that the optimum OLR for continuous biohydrogen
360 production through co-fermentation of macro-algae *L. digitata* and micro-algae *A.*
361 *platensis* was 6.0 gVS/L/d in the H₂ reactor. The insufficient biomass supply at lower
362 OLR failed to provide essential feedstock for the AFB to produce hydrogen, whereas
363 the overfeeding of algae at higher OLRs resulted in the accumulation of VFAs which
364 in turn suppressed the hydrogen-producing metabolisms.

365

366 3.3.2 Performance of CH₄ reactors A and B

367 The SMYs of CH₄ reactors A and B of the two-stage system and the variation
368 trends of tVFA and FOS/TAC values over increasing OLRs are illustrated in Fig. 3
369 and Fig. 5, respectively. At the initial OLR of 1.0 gVS/L/d, CH₄ reactor A performed
370 best with an average SMY of 265.5 mL/gVS which accounted for 85.8% of the BMP
371 value in the batch trial. The sCOD and tVFA were low at 0.6 g/L and 354 mg/L,
372 respectively, indicating that most of the soluble metabolites produced via first-stage
373 dark hydrogen fermentation were utilised by the microbes in CH₄ reactor A. The
374 FOS/TAC value was low (0.22) as well. When the OLR increased to 2.0 gVS/L/d, the
375 average SMY slightly decreased to 245.0 mL/gVS, signifying 79.2% of the BMP
376 yield. The low FOS/TAC value of 0.17 ensured the process stability of second-stage
377 anaerobic digestion. Under the conditions of higher sCOD and tVFA inputs from

378 effluents of the H₂ reactor, the sCOD and tVFA values of CH₄ reactor A remained
379 almost as low as those at the previous OLR of 1.0 gVS/L/d, resulting in even higher
380 sCOD and tVFA destruction efficiencies (93.7% and 93.3%, respectively). The
381 continuous increase of OLR from 2.0 to 3.0 gVS/L/d further led to a 9.4% drop in
382 SMY. Although the FOS/TAC value remained within a suitable range, both the VFAs
383 and sCOD increased. The average tVFA value of 877 mg/L was not high, however,
384 the variation trend shown in Fig. 5 implied that the accumulation of VFAs was in
385 progress. Especially as shown in Fig. 4b, the content of propionic acid in CH₄ reactor
386 A significantly increased at 3.0 gVS/L/d as compared to the lower loading rates. The
387 accumulation of propionic acid in the digester is always deemed as an indicator of
388 impending anaerobic digestion failure [42, 43]. At the maximum OLR of 4.0
389 gVS/L/d, a notable reduction in SMY was recorded: the SMY of 174.0 mL/gVS was
390 lower than that at 3.0 gVS/L/d by 24.1% and only equivalent to 65.5% of the highest
391 one obtained at 1.0 gVS/L/d. The sCOD and tVFA further accumulated in CH₄
392 reactor A. The average FOS/TAC value increased to 0.27 and the variation trend
393 shown in Fig. 4 suggested that the FOS/TAC of CH₄ reactor A was rising towards the
394 threshold value. Fig. 4b shows that the propionic acid concentration further increased
395 to 775 mg/L and almost all the iso-acids were higher, illustrating that the process
396 instability of CH₄ reactor A caused by the overloading of mixed algal biomass was in
397 progress [42]. The struggling of CH₄ reactor A at higher OLRs could be associated
398 with the inability of the microbial community to acclimatise to such a high loading in
399 a short HRT of 12 days. This may have resulted in washout of microbial community.

400 Since CH₄ reactors A and B shared the same feedstock origin (effluent from H₂
401 reactor), the 2-fold HRT of CH₄ reactor B led to lower OLRs which equates to half of
402 those of CH₄ reactor A. The FOS/TAC values remained low (0.17-0.19) throughout
403 the entire continuous experiments, indicating that a more stable second-stage
404 anaerobic digestion process was ensured by the longer HRT and lower OLRs of CH₄
405 reactor B as compared to CH₄ reactor A. Although the SMYs were marginally lower
406 than the highest one obtained in CH₄ reactor A, the average values in CH₄ reactor B
407 were less affected by the increasing OLR from 0.5 to 2.0 gVS/L/d and remained
408 within a reasonable range of 223.8-242.5 mL/gVS signifying 72.4-78.4% of the BMP
409 value and 46.7-50.6% of the theoretical methane yield. The sCOD and tVFA stayed
410 low over increasing OLRs, leading to the high sCOD (88.6-95.1%) and tVFA (92.2-
411 95.6%) destruction efficiencies. However, the highest average sCOD (2.2 g/L) and
412 tVFA (551 mg/L) recorded at the maximum OLR of 2.0 gVS/L/d were both higher
413 than those in CH₄ reactor A at the same OLR. This was caused by the feedstock
414 sourced from the effluent of the H₂ reactor at various OLRs. At an OLR of 2.0
415 gVS/L/d, the feedstock loaded into CH₄ reactor B was obtained from the effluent of
416 the H₂ reactor at an OLR of 12.0 gVS/L/d, whilst the one loaded into CH₄ reactor B
417 was originated from the effluent of the H₂ reactor at an OLR of 6.0 gVS/L/d. The
418 sCOD and tVFA values of the former was markedly higher than the latter, resulting in
419 a comparatively more severe impact on the second-stage anaerobic digestion process.
420 Nonetheless, Fig. 4c reveals that no accumulation of propionic acid or iso-acids in
421 CH₄ reactor B were observed at an OLR of 2.0 gVS/L/d, demonstrating that no

422 inhibition of methanogens or anaerobic digestion process failure was evident.

423 Overall, considering SMY, treating capacity, and process stability, an OLR of
424 2.0 gVS/L/d was shown to be optimal for CH₄ reactor A at a fixed HRT of 12 days.

425

426 3.3.3 Performance of CH₄ reactor C

427 The SMYs of CH₄ reactor C of the one-stage system are shown in Fig. 3. With
428 the OLR increasing from 1.0 to 3.0 gVS/L/d, the average SMYs gradually decreased
429 from 204.5 to 72.2 mL/gVS. As shown in Fig. 5, the VFAs accumulated and the
430 FOS/TAC values rose along with the increasing OLR, indicating that the buffer
431 capacity in the CH₄ reactor C was strongly negatively correlated with OLR in this
432 one-stage system. At the initial OLR of 1.0 gVS/L/d, the tVFA already reached 1287
433 mg/L and the VFA composition in Fig. 4d revealed that propionic acid accounted for
434 65.6% of the tVFA. This phenomenon of propionic acid accumulation was similar to
435 that obtained in the CH₄ reactor A at the maximum OLR of 4.0 gVS/L/d, signifying
436 that the process instability of one-stage anaerobic co-digestion was triggered. When
437 the OLR rose to 2.0 gVS/L/d, a remarkable surge in VFAs was noted: the tVFA
438 concentration of 6593 mg/L was even close to that in the H₂ reactor at 9.0 gVS/L/d. It
439 was assumed that the methanogens in CH₄ reactor C suffered severe inhibition under
440 such acidic condition. When the OLR further increased to 3.0 gVS/L/d, the sCOD
441 increased by 110.7%, whereas the tVFA slightly decreased instead, indicating that the
442 acidification process was impaired even though the hydrolysis was efficient. In

443 addition, the enhancements of propionic, butyric, and longer-chain acids and little
444 accumulation of acetic acid were recorded in Fig. 4d. These results suggested that the
445 microbial community was highly affected: the activity of acetogens and methanogens
446 were inhibited to a great extent. Furthermore, the salinity in CH₄ reactor C amounted
447 to 13.3 g/kg, which was far higher than the highest ones obtained in CH₄ reactors B
448 and C during the entire experiment. Although small concentrations of sodium ions
449 (100-350 mg/L) are supposed to be essential for the maintenance of healthy
450 metabolism of the microbes in anaerobic digesters [44], the enhanced osmotic
451 pressure caused by the remarkably high salinity can inhibit microbial activity and
452 even lead to dehydration of microbes [23]. Luo et al. [45] investigated the effects of
453 saline adaptation on anaerobic digestion of sludge and observed that salinity levels
454 higher than 8.7 g/kg impaired the methane production. On the other hand, Tabassum
455 et al. [46] demonstrated acclimatisation to salinity levels of the order of 14 g/L in
456 mono-digestion of farm cultivated *S. latissima* at an OLR of 4.0 kgVS/m³/d. The high
457 salinity levels recorded here of 13.3g/kg at an OLR of 3.0 gVS/L/d will have some
458 inhibitory effects on the microbial consortium in CH₄ reactor C. Although the gas
459 production did not thoroughly stop, the failure of CH₄ reactor C was inevitable.

460 In a previous study, [27] conducted continuous one-stage anaerobic co-digestion
461 of *L. digitata* and *A. platensis* based on a C/N ratio of 25 at a long HRT of 28 days. A
462 high OLR of 4.0 gVS/L/d was shown to be tolerable for the CH₄ reactor and an SMY
463 of 259.6 mL/gVS was recorded. Despite the different seed inocula and minor
464 variation in C/N ratios, the significant reduction in HRT (28 days as compared to 16

465 days here) was assumed to be the key influencing factor between these two one-stage
466 systems. It is suggested that an HRT of 16 days did not supply sufficient time for
467 acclimatisation and enrichment of the microbial consortium in the CH₄ reactor C and
468 led to washout of microbes, accumulation of VFAs, and inhibition of methanogenesis.

469

470 **3.4 Comparisons between two-stage and one-stage fermentation performances**

471 The two-stage system comprising of the H₂ reactor and the CH₄ reactor A and
472 the one-stage system of CH₄ reactor C shared comparable operational parameters such
473 as overall HRT (16 days), OLR, temperature (37 ± 1 °C), and initial seed inoculum
474 for methane production. At an OLR of 6.0 gVS/L/d, the highest average SHY of 55.3
475 mL/gVS, which equates to 58.5% of the BHP yield in batch trial, was obtained in the
476 first-stage dark hydrogen fermentation. In the second-stage anaerobic digestion, the
477 average SMY of 245.0 mL/gVS equivalent to 79.2% of the BMP value was achieved
478 in CH₄ reactor A at a corresponding OLR of 2.0 gVS/L/d, and process stability was
479 secured. The two-stage system effected an energy yield of 9.4 kJ/gVS and the ECE
480 amounted to 51.0%. The energy yield of the continuous two-stage system was 22.3%
481 lower than the batch trial. This is expected due to the disadvantages of shorter
482 retention time (16 days in two-stage versus 30 days for batch) and the larger reactor
483 with less efficient mixing conditions. By contrast, in the one-stage system, the CH₄
484 reactor C recorded its highest SMY of only 204.5 mL/gVS at the initial OLR of 1.0
485 gVS/L/d. The energy yield and ECE were lower at 7.3 kJ/gVS and 39.8%,
486 respectively. Even at this low OLR, a certain degree of VFA accumulation was

487 observed. When the OLR rose to 3.0 gVS/L/d, the process instability of one-stage
488 anaerobic co-digestion of *L. digitata* and *A. platensis* became more obvious.
489 Therefore, the two-stage system prevailed in both energy production from mixed algal
490 feedstock and treating capacity as compared to one-stage system at a fixed HRT of 16
491 days. Even if the energy content in produced hydrogen was nearly negligible, the
492 first-stage dark hydrogen fermentation would serve as an optimised hydrolysis and
493 acidification method pretreating the mixed algal feedstock. Similar results were
494 reported by [26, 32] utilising grass and food waste in continuous two-stage systems.
495 To sum up, the technical feasibility of two-stage co-fermentation of *L. digitata* and *A.*
496 *platensis* biomass has been proven, and several operational parameters have been
497 assessed via this 32-week long experimentation, thus mitigating the gaps between the
498 fundamental innovations obtained by the small-scale batch co-fermentation and the
499 potential commercial deployment of algal biofuel systems in future.

500 Although positive results on two-stage continuous hydrogen and methane co-
501 production using mixed *L. digitata* and *A. platensis* have been achieved in this study,
502 some issues are still noteworthy. The C/N ratio was adjusted to 20 in the mixture of
503 macro- and micro-algae, however, the TAN levels stayed low in all four reactors
504 throughout the entire continuous experiment, indicating that the hydrolysis or
505 degradation of nitrogen-rich micro-algae biomass may have been somewhat limited,
506 especially in a short HRT of 16 days. This was probably ascribed to limited
507 degradation of untreated *A. platensis* due to its recalcitrant cell wall structures. The
508 slow or limited utilisation of micro-algae biomass further restricted the

509 fermentation/digestion process and also explained why the longer HRT in CH₄ reactor
510 B and in the previous study [27] could ensure a more stable process. Therefore, to
511 overcome this drawback, pretreatment of micro-algae and even macro-algae to
512 facilitate the solubilisation and hydrolysis of feedstock is a promising option for a
513 stable continuous fermentation/digestion process in future study.

514

515 **3.5 Comparison between results of this study and relevant literature**

516 To the best of our knowledge, most of the studies on biohydrogen and
517 biomethane production from either macro- or micro-algae biomass were conducted in
518 batch trials [23, 30]. The data on long term continuous fermentation of algae are
519 relatively limited. A comparison between the results of continuous fermentative
520 gaseous biofuel production from algal biomass and other co-substrates in this study
521 and the state of the art in the literatures is summarised in Table 3. Tabassum et al. [46]
522 found that a mixed feedstock of 66.6% macro-algae (*L. digitata* or *S. latissima*) and
523 33.3% dairy slurry was optimal to obtain a maximum biomethane production
524 efficiency during continuous anaerobic co-digestion. The energy yields (9.0-9.3
525 kJ/gVS) were close to that obtained in this study. Allen et al. [47] suggested for the
526 green macro-algae (*Ulva lactuca*) that the optimal mixture in long term continuous
527 digestion would be 25% macro-algae and 75% dairy slurry; this resulted in an SMY
528 of 170 mL/gVS, equivalent to 95% of the BMP value. These differences are attributed
529 to the significant variation in biological characteristics of different macro-algal
530 species. The green seaweed *U. lactuca* typically has a C/N ratio below 10 and as such

531 needs to be co-digested with a carbohydrate-rich co-substrate to increase the C/N ratio
532 for better digestibility. The carbohydrate-rich brown seaweeds *L. digitata* and *S.*
533 *latissima* have high C/N ratios (>25) when they are ripest in late summer [46].
534 Similarly, the protein-rich Taihu blue algae with a low C/N ratio of 6.1 resulted in an
535 SMY of 160 mL/gVS, whereas the mixture of Taihu blue algae and carbohydrate-rich
536 corn straw with a C/N ratio of 20 resulted in an increase in SMY of 46% [48].
537 Herrmann et al. [27] also used micro-algae *A. platensis* as a nitrogen-rich additive to
538 macro-algae *L. digitata* for adjusting the C/N to 25. Compared with the results
539 obtained in the one-stage reactor in this study, the longer HRT (28 days) allowed a
540 higher OLR (4.0 gVS/L/d) with a stable process and a higher SMY. All the above
541 studies were conducted in a one-stage system; only one previous study investigated
542 two-stage continuous fermentation of macro-algae *L. digitata* [49]. The two-stage
543 fermentation system outperformed one-stage system with a higher energy yield in a
544 shorter overall HRT [49]. This finding was consistent with the output of this study.
545 The optimal HRT, OLR, and biofuel yield varied between the studies due to different
546 experimental configurations, different sources of inocula, and different algal
547 feedstocks. However, the results showed similarities in C/N ratios, and the
548 improvements in energy return and process stability.

549

550 **3.6 Gross energy potential from algal mixture**

551 In this study, the major component in the algal mixture is macro-algae *L.*
552 *digitata*, which accounts for 94% of the VS. The co-substrate micro-algae may be

553 considered as a nitrogen-rich additive. Therefore, the gross energy potential from this
554 mixed algal feedstock is heavily associated with the *L. digitata* biomass resource.
555 Nonetheless, the definite data on the annual yields of seaweed per hectare are not
556 available because of a series of variations, such as algal species, locations, harvesting
557 times, etc [44]. According to a latest report of International Energy Agency
558 Bioenergy, the yields of *L. digitata* cultivated using advanced textiles in open sea
559 reached 16 kg/m², equivalent to 160 tons wet weight per hectare per year (t
560 wwt/ha/yr) [50]. Under this scenario, based on the energy yield of 9.4 kJ/gVS in the
561 two-stage continuous co-fermentation system, the gross energy potential is calculated
562 to be 213 GJ/ha/yr. This value is comparable with the gross energy yields of
563 biomethane from terrestrial crops, such as maize (217 GJ/ha/yr), fodder beet (250
564 GJ/ha/yr), and grass (163 GJ/ha/yr) [51]. The advantages of algae cultivation, are that
565 as an advanced third generation biofuel there is no requirement for arable land, the
566 fuel is outside the food-or-fuel debate, and it is an attractive process for countries with
567 long coastlines [44]. For example, in China, Shandong Province is one of the biggest
568 mariculture bases, and macro-algae is one of the major products [52]. Meanwhile, a
569 modern microalgal cultivation plant equipped with large raceway ponds has been
570 constructed in Penglai City, Shandong Province. Seawater is used as basic culture
571 solution, and flue gas from a coal-fired power plant is used as the CO₂ source [53,
572 54]. These examples in the literature indicate that both macro-algae and micro-algae
573 can be grown in the same place, making the combined use of the macro- and micro-
574 algae reasonable and feasible. In addition, integrated multi-trophic aquaculture

575 (coupling seaweed production with fish farms) captures nutrients from fish excrement
576 enhancing seaweed growth and water quality [5], and leading to promotion of
577 industrial scale advanced gaseous biofuel production from algal biomass.

578

579 **4. Conclusions**

580 A continuous two-stage system involving dark hydrogen fermentation and
581 anaerobic fermentation of mixed macro-algae and micro-algae at a C/N ratio of 20
582 was shown to be feasible with an overall ECE of 51.0%. The short HRT (16 days)
583 allowed an efficient fermentation process in the H₂ reactor at 6.0 gVS/L/d and a stable
584 digestion process in the CH₄ reactor at a corresponding OLR of 2.0 gVS/L/d. In
585 contrast to the one-stage system, the first-stage dark hydrogen fermentation in the
586 two-stage system optimised hydrolysis and acidification of algal mixtures, hence
587 facilitating improved methane production and process stability in second-stage
588 anaerobic digestion. The gross energy potential of 213 GJ/ha/yr makes this algal
589 mixture comparable with terrestrial crops in gaseous biofuel production while
590 removing any land use implications.

591

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601

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757 **List of figures and tables:**

758 **Fig. 1** Schematic of continuous fermentation system (**Note: Fig. 1 is a 1.5-column**
759 **fitting image.**)

760 **Fig. 2** Two-stage batch biohydrogen and biomethane co-production from mixed *L.*
761 *digitata* and *A. platensis* biomass at a C/N ratio of 20: (a) biohydrogen
762 production and (b) biomethane production (**Note: Fig. 2 is a one-column**
763 **fitting image.**)

764 **Fig. 3** Specific hydrogen yields of H₂ reactor and specific methane yields of CH₄
765 reactors A, B, and C with increasing organic loading rates in continuous two-
766 stage and one-stage systems (**Note: Fig. 3 is a 1.5-column fitting image.**)

767 **Fig. 4** Compositions of VFAs with increasing organic loading rates in (a) H₂ reactor,
768 (b) CH₄ reactor A of two-stage system, (c) CH₄ reactor B of two-stage system,
769 and (d) CH₄ reactor C of one-stage system (**Note: The sub-figures in Fig. 4**
770 **can be listed in one column, or combined as a 2-column image.**)

771 **Fig. 5** Concentrations of total VFAs and the FOS/TAC values in CH₄ reactors A, B,
772 and C during continuous anaerobic digestion (**Note: Fig. 5 is a 1.5-column**
773 **fitting image.**)

774 **Table 1** Characteristics of algal biomass

775 **Table 2** Summary of results from two-stage and one-stage co-fermentation of *L.*
776 *digitata* and *A. platensis* (mean values of post-first HRT for each OLR)

777 **Table 3** Comparison between the results in this study and relevant literatures on
778 continuous fermentative gaseous biofuel production from algal biomass

779

Table 1 Characteristics of algal biomass

Parameter	<i>Laminaria digitata</i>	<i>Arthrospira platensis</i>	Mixed <i>Laminaria digitata</i> and <i>Arthrospira platensis</i>
Proximate analysis			
Moisture (wt%)	81.87	6.40	81.16
TS (wt%)	18.13	93.60	18.84
VS (wt%)	13.31	86.77	14.01
VS/TS (%)	73.44	92.70	74.34
Ultimate analysis			
C (TS%)	36.08	49.27	36.70
H (TS%)	4.67	6.58	4.76
O (TS%)	31.32	25.48	1.84
N (TS%)	1.36	11.38	31.05
C/N ratio	26.47	4.33	20.00
Biological analysis			
Proteins (TS%)	7.32 ^a	71.13 ^a	10.32
Lipids (TS%)	0.92 ^b	5.00 ^c	1.11
Carbohydrates (TS%)	65.20 ^d	16.57 ^d	62.91
Energy value (kJ/gVS)	18.1	23.4	18.4
tCOD (gCOD/gVS)	1.36	1.50	1.37
Theoretical biomethane yield (mL/gVS)	476.3	525.2	479.2

780 a: The contents of proteins are calculated by multiplying the nitrogen contents by a
 781 factor of 5.38 for brown seaweeds [37] and 6.25 for microalgae [38].

782 b: The lipid content of *Laminaria sp.* is suggested to be 0.92% of the dry weight by
 783 Sánchez-Machado et al. [39].

784 c: The lipid content of *Arthrospira sp.* is suggested to be 5% of the dry weight by
 785 Dismukes et al. [6].

786 d: It is assumed that the sum of proteins, lipids, carbohydrates equates to the VS of
 787 algal biomass.

788

Table 2 Summary of results from two-stage and one-stage co-fermentation of *L. digitata* and *A. platensis* (mean values of post-first HRT for each OLR)

789

	H ₂ reactor				CH ₄ reactor A				CH ₄ reactor B				CH ₄ reactor C		
HRT (days)	4				12				24				16		
OLR (gVS/L/d)	3.0	6.0	9.0	12.0	1.0	2.0	3.0	4.0	0.5	1.0	1.5	2.0	1.0	2.0	3.0
SHY (mL/gVS)	14.3	55.3	20.4	19.0	/	/	/	/	/	/	/	/	/	/	/
SMY (mL/gVS)	/	/	/	/	265.5	245.0	229.1	174.0	242.5	228.9	223.8	236.5	204.5	134.8	72.2
FOS/TAC	/	/	/	/	0.22	0.17	0.21	0.27	0.19	0.17	0.17	0.17	0.61	1.03	1.68
TAN (mg/L)	7	2	4	5	216	148	251	269	281	197	290	279	95	43	158
TS (g/kg)	14.3	23.8	37.9	45.3	11.8	12.8	18.9	23.9	17.3	13.3	19.4	23.5	12.2	26.3	47.6
VS (g/kg)	9.4	15.3	24.1	29.5	5.6	5.4	6.7	9.3	8.7	5.4	7.3	8.0	6.7	12.0	22.5
sCOD (g/L)	7.0	14.2	18.4	21.5	0.6	0.9	2.3	5.2	0.8	0.7	1.4	2.2	2.5	10.3	21.7
tVFA (mg/L)	3776	5254	6626	6587	354	349	877	1365	243	287	279	551	1287	6593	5982
COD _{VFA} (g/L)	6.2	8.9	11.4	11.5	0.6	0.6	1.4	2.1	0.4	0.5	0.5	0.9	2.1	8.9	10.5
Acidification yield (%)	87.5	63.0	62.2	53.5	/	/	/	/	/	/	/	/	/	/	/
Salinity (g/kg)	3.6	5.6	6.5	5.8	4.6	6.4	8.1	5.6	6.6	6.5	8.1	7.7	4.5	9.7	13.3
Energy yield (kJ/gVS)	0.2	0.6	0.2	0.2	9.5	8.8	8.2	6.2	8.7	8.2	8.0	8.5	7.3	4.8	2.6
ECE (%)	0.8	3.3	1.2	1.1	51.7	47.7	44.6	33.9	47.2	44.6	43.6	46.0	39.8	26.2	14.1

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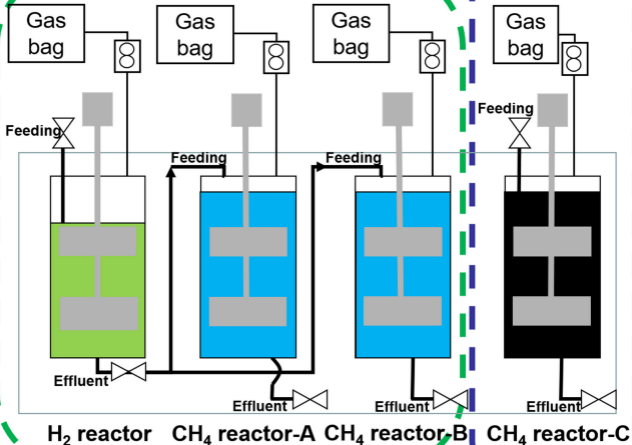
Table 3 Comparison between the results in this study and relevant literatures on continuous fermentative gaseous biofuel production from algal biomass

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Algal species	Co-substrate	Fermentation type	HRT (d)	OLR (gVS/L/d)	SHY (mL/gVS)	SMY (mL/gVS)	C/N ratio	Energy yield (kJ/gVS)	Reference
<i>Laminaria digitata</i>	Dairy slurry	One-stage CH ₄ fermentation	18	4.0	/	261	23.4	9.3	[46]
<i>Saccharina latissima</i>			13	4.0	/	252	15.7	9.0	
<i>Ulva lactuca</i>	Dairy slurry	One-stage CH ₄ fermentation	42	2.5	/	170	16.6	6.1	[47]
Taihu blue algae	/	One-stage CH ₄ fermentation	10	6.0	/	160	6.1	5.7	[48]
	Corn straw			6.0	/	234	20	8.4	
<i>Laminaria digitata</i>	<i>Arthrospira platensis</i>	One-stage CH ₄ fermentation	28	4.0	/	259.6	25	9.3	[27]
<i>Laminaria digitata</i>	/	One-stage CH ₄ fermentation	24	2.4	/	221		7.9	
		Two-stage H ₂ + CH ₄ fermentation	4 (H ₂) + 14 (CH ₄)	12 (H ₂) + 3.43 (CH ₄)	26	234	27.3	8.7	[49]
		One-stage CH ₄ fermentation	16	1.0	/	204.5		7.3	
<i>Laminaria digitata</i>	<i>Arthrospira platensis</i>	Two-stage H ₂ + CH ₄ fermentation	4 (H ₂) + 12 (CH ₄)	6.0 (H ₂) + 2.0 (CH ₄)	55.3	245.0	20	9.4	This study
		Two-stage H ₂ + CH ₄ fermentation	4 (H ₂) + 24 (CH ₄)	12.0 (H ₂) + 2.0 (CH ₄)	19.0	236.5		8.7	

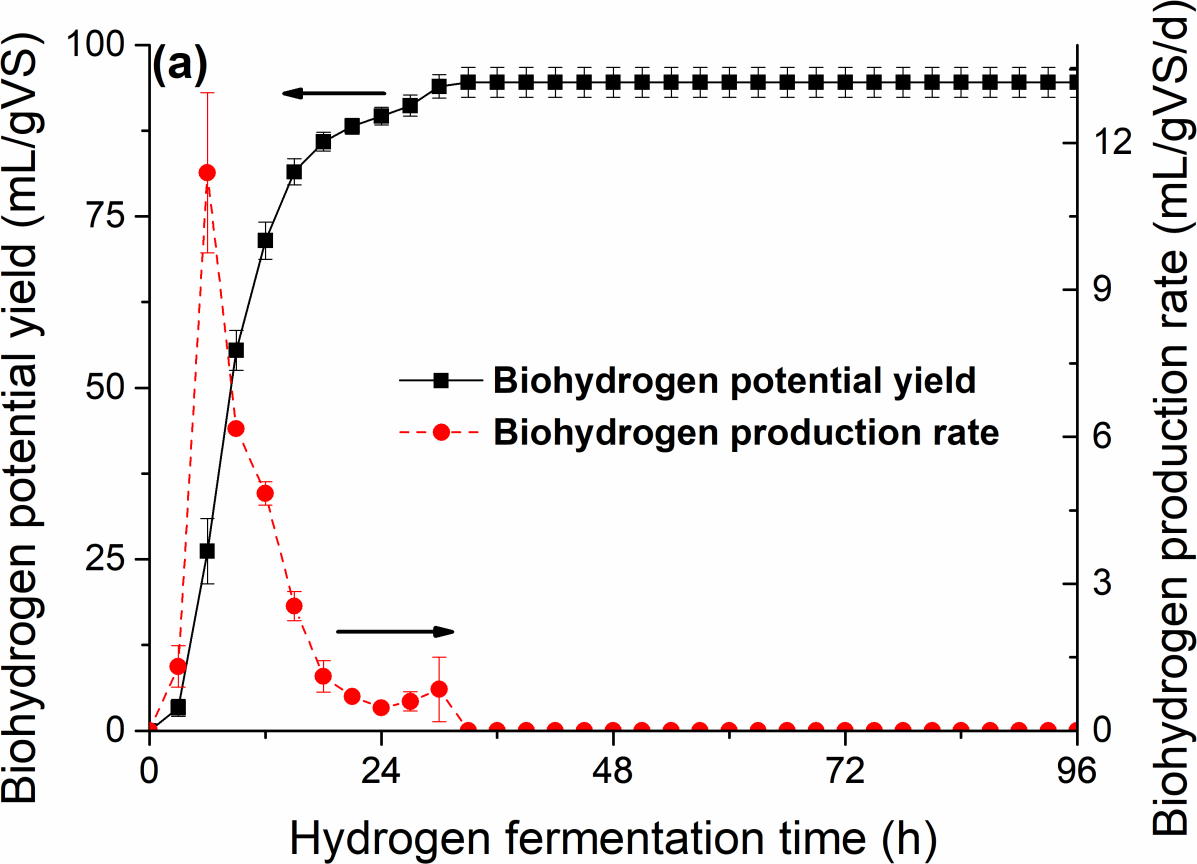
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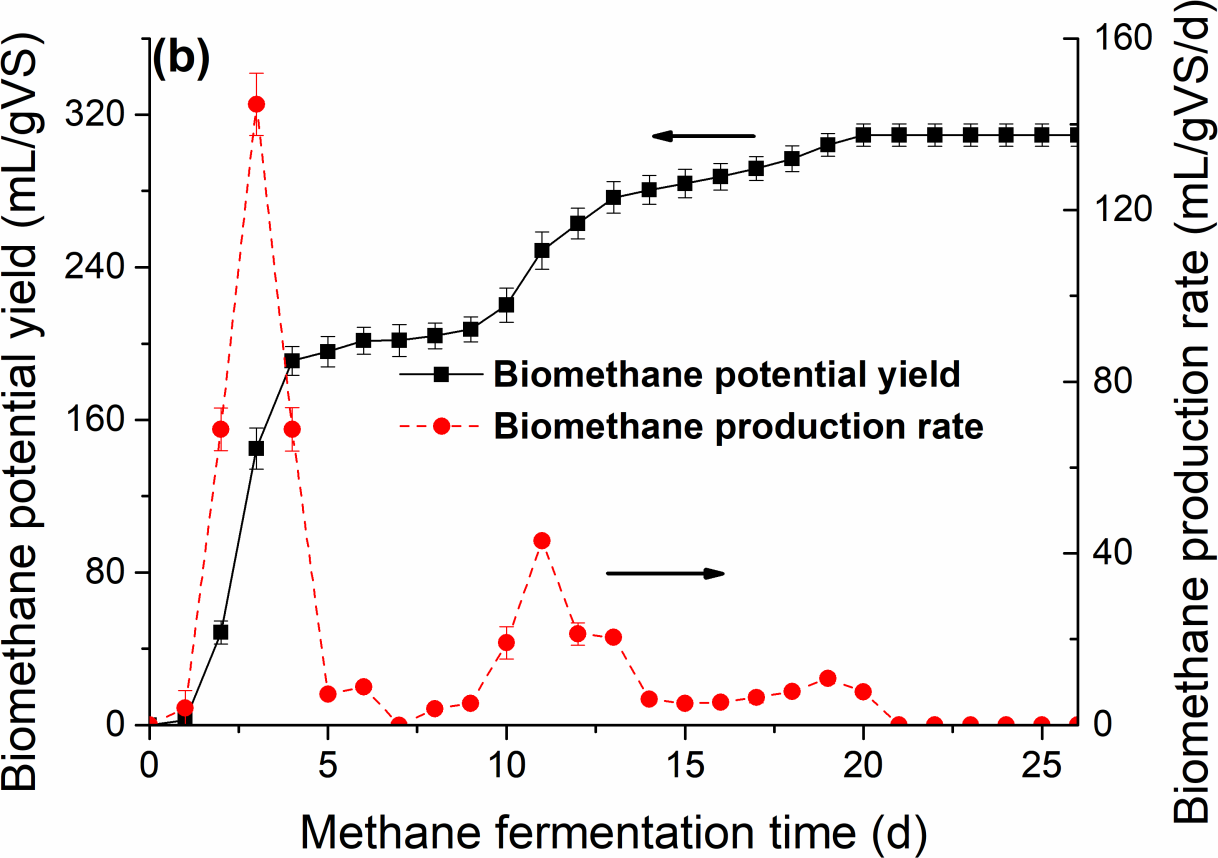
- Two-stage continuous co-fermentation of macro- and micro-algae was investigated.
- Optimum H₂ production was observed at an organic loading rate (OLR) of 6.0 gVS/L/d.
- Second-stage CH₄ production was stable at a corresponding OLR of 2.0 gVS/L/d.
- The two-stage system gave an energy yield of 9.4 kJ/gVS at a retention time of 16 d.
- Gross energy potential of this algal mixture may reach 213 GJ/ha/yr.

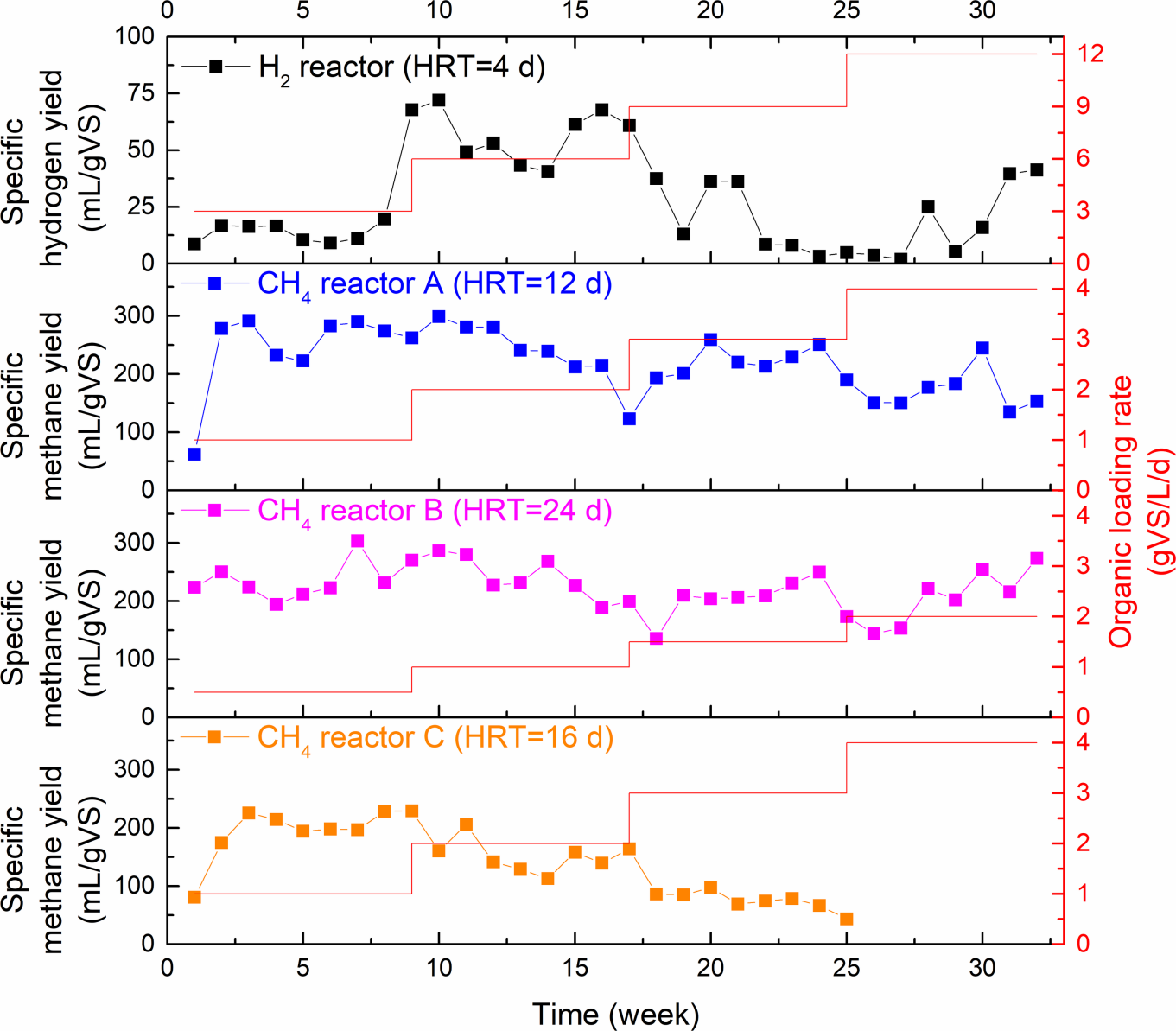


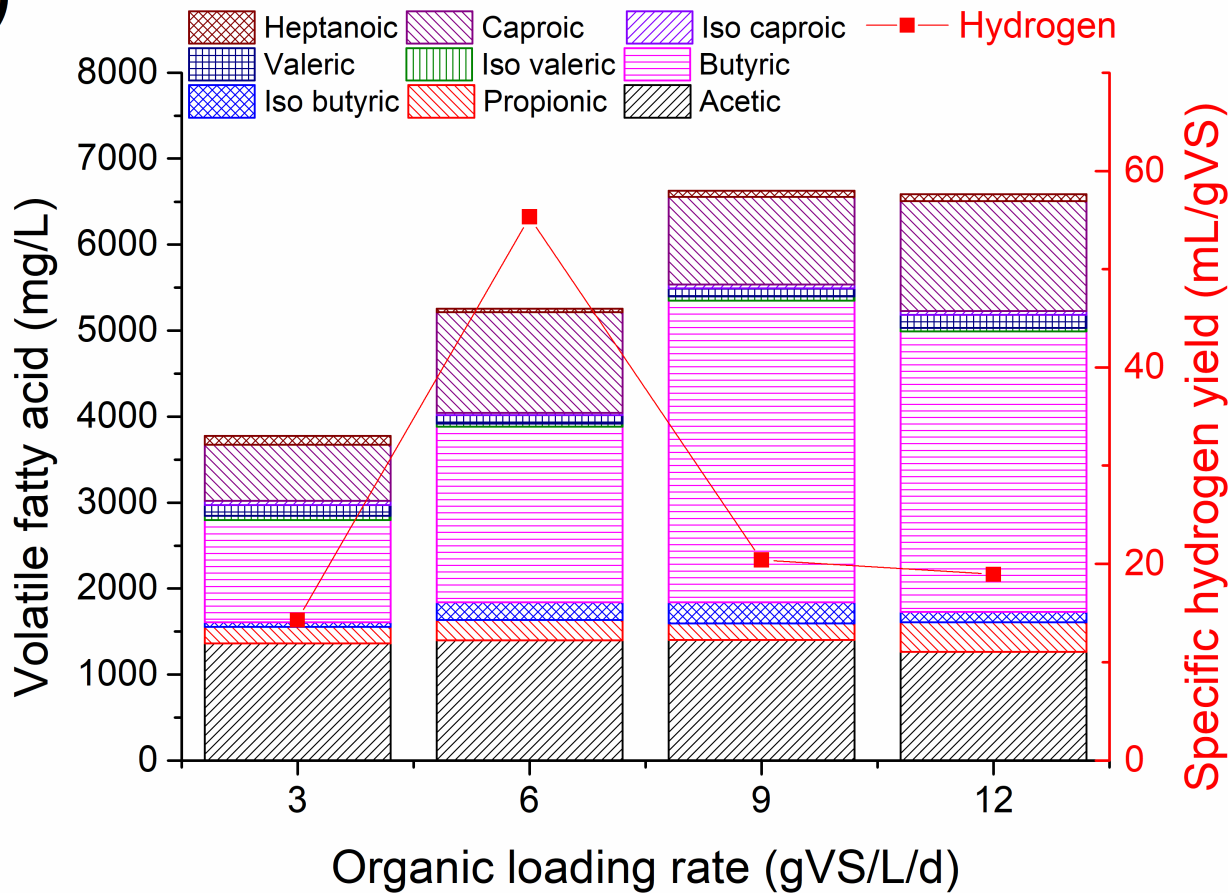
Two-stage process

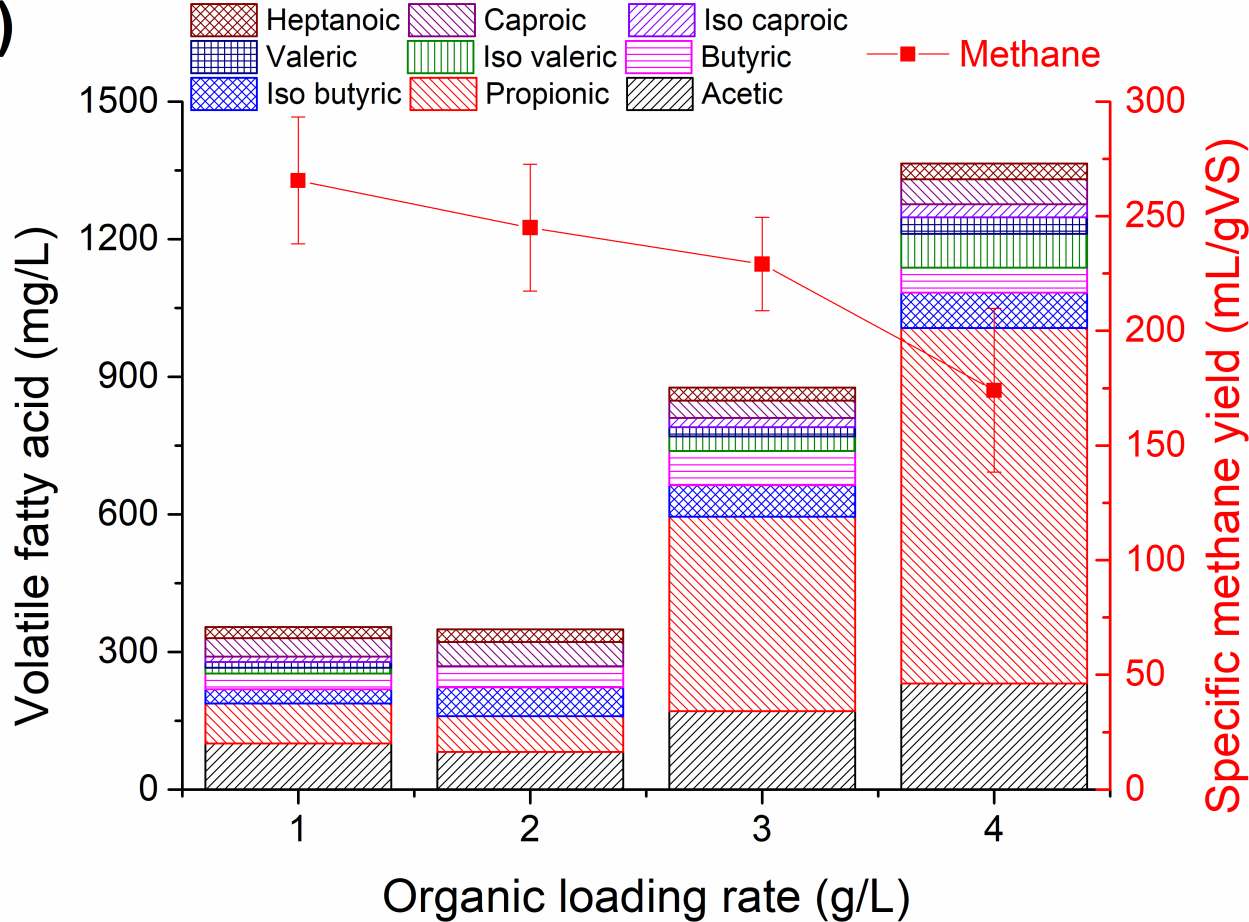
One-stage process

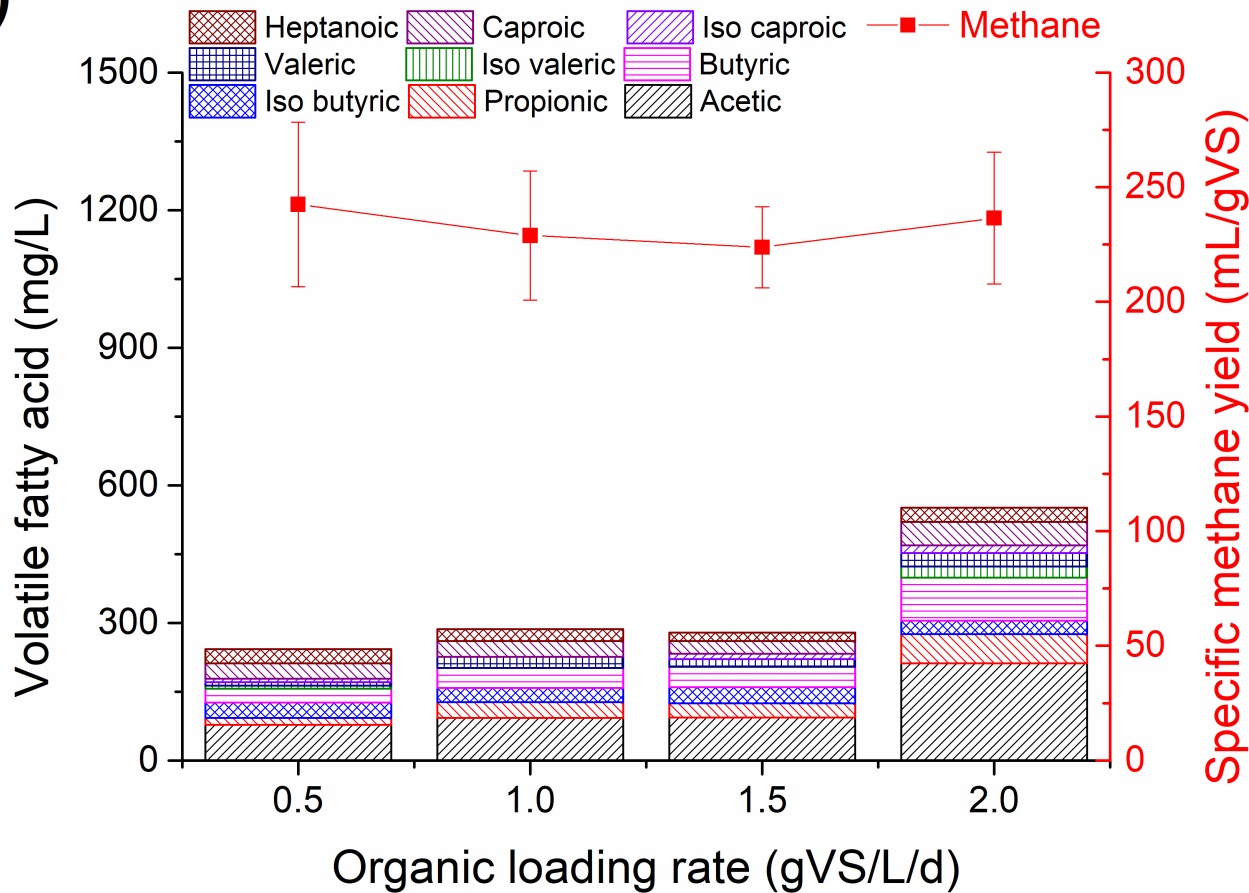






(a)

(b)

(c)

(d)