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1 **Improving fermentative hydrogen and methane**
2 **production from an algal bloom through**
3 **hydrothermal/steam acid pretreatment**

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11 **Abstract**

12 Algal blooms can be harvested as renewable biomass waste for gaseous biofuel
13 production. However, the rigid cell structure of raw algae may hinder efficient
14 microbial conversion for production of biohydrogen and biomethane. To improve the
15 energy conversion efficiency, biomass from an algal bloom in Dianchi Lake was
16 subjected to a hydrothermal/steam acid pretreatment prior to sequential dark hydrogen
17 fermentation and anaerobic digestion. Results from X-ray diffraction and Fourier
18 transform infrared spectroscopy suggest that hydrothermal acid pretreatment leads to

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19 stronger damage of the amorphous structure (including hemicellulose and amorphous
20 cellulose) due to the acid pretreatment, as evidenced by the higher crystallinity index.
21 Scanning electron microscopy analysis showed that smaller fragments (~ 5 mm) and
22 wider cell gaps (~ 1 μm) on algal cell surfaces occurred after pretreatment. In
23 comparison to steam acid pretreatment, hydrothermal acid pretreatment resulted in a
24 maximum energy conversion efficiency of 44.1% as well as production of 24.96 mL
25 H₂/g total volatile solids (TVS) and 299.88 mL CH₄/g TVS.

26

27 **Keywords:** Dianchi Lake algal bloom; hydrogen; methane; hydrothermal
28 pretreatment.

29

30 **1. Introduction**

31 Dianchi Lake, the largest freshwater lake in Yunnan Province, China, suffers
32 from annual algal bloom outbreaks. Until December 2017, the water quality of
33 Dianchi Lake was still in a eutrophic state. During the summer of 2017, more than
34 100 tons of algal biomass were salvaged every day, causing great harm to the
35 ecological environment. However, this large amount of algae can also be used as a
36 potential feedstock for fermentative biofuel production, as demonstrated by many
37 studies on biohydrogen production from algal biomass [1-4].

38 Different algal species, such as *Chlorella* and *Arthrospira*, have been assessed
39 for their biohydrogen potential through dark fermentation [5-7]. To further improve

40 the energy recovery from raw algae, various pretreatment methods, such as steam,
41 ultrasound and microwave treatments, have been developed [8-11]. In addition, the
42 dark fermentation process has been optimized to overcome the inhibitory effects of
43 fermentative intermediates (such as acetic acid) on hydrogen yield [12-15]. Apart
44 from hydrogen production during fermentation, a large amount of volatile fatty acids
45 are generated and remain as unutilized energy. Previous studies have demonstrated
46 that subsequent photo fermentation or anaerobic digestion could increase the biofuel
47 yield and energy conversion efficiency from microalgal biomass [16-18].

48 Researchers have utilized biomass harvested from an algal bloom in Taihu Lake
49 to produce hydrogen, yielding 1.1 kJ of hydrogen per gram of dry biomass weight
50 (g-TVS) [19]. However, the energy conversion efficiency was very low during the
51 one-stage dark hydrogen fermentation of the biomass. In the study, “one-stage” refers
52 to the process of dark hydrogen fermentation or anaerobic digestion and “two-stage”
53 refers to the combined process of dark hydrogen fermentation and anaerobic
54 digestion. In another study, the Taihu Lake algal bloom biomass pretreated with
55 acid-domesticated hydrogenogens resulted in a 47.0% increase in the energy
56 conversion efficiency by cogenerating 256.7 mL/g-TVS hydrogen and 253.5
57 mL/g-TVS methane in a three-stage process that utilized dark-fermentation,
58 photofermentation, and methanogenesis [20]. It is noteworthy that, although hydrogen
59 production was improved, the input light energy was not considered when calculating
60 the energy conversion efficiency.

61 Zhong found that anaerobic digestion of Taihu Lake algae was feasible in

62 laboratory-scale anaerobic reactors [21]. These reactors performed well at an OLR of
63 $2.00 \text{ gVSL}^{-1}\text{d}^{-1}$ for methane production with a VS removal of 50% at an HRT of 10
64 days; however, the rate-limiting step was acetate and propionate degradation. There
65 were also many studies on the co-digestion of the Taihu Lake algal bloom biomass
66 and kitchen wastes [22] or swine manure [23]. The feasibility of adjusting the C/N
67 with co-digestion of Taihu algae and other biomass to increase biogas production was
68 demonstrated. However how to increase the production of hydrogen and methane of
69 algal bloom alone was not considered.

70 To date, there are few studies on the utilization of Dianchi Lake algal bloom
71 biomass for biogas production. Furthermore, an effective pretreatment method to
72 improve biofuel yield remains unclear as unprocessed microalgae are not considered
73 to be the best substrate for biogas production [24]. Additionally, the energy
74 conversion efficiency of single stage hydrogen or methane generation is not high and
75 acetate degradation limits the fermentation rate. Moreover, the degradation effect of
76 microalgal biomass after pretreatment should be quantitatively represented.

77 In this study, biomass harvested from a Dianchi Lake algal bloom was used as
78 the feedstock for fermentation. This biomass, which was mainly composed of
79 *Microcystis*, is of a different composition than that of Taihu Lake. Hydrothermal
80 /steam acid pretreatment was examined to improve the hydrolysis efficiency of the
81 algal biomass. The physicochemical properties of the algal biomass before and after
82 pretreatment were comparatively assessed using X-Ray diffraction (XRD), Fourier
83 transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM).

84 The biomass, which was pretreated under different conditions, was subjected to
85 two-stage hydrogen and methane co-generation in an effort to improve the overall
86 energy conversion efficiency.

87 **2. Materials and methods**

88 **2.1. Substrates and characterization**

89 The substrate used in the fermentation experiments was the algal bloom biomass
90 harvested from Dianchi Lake (Kunming City, Yunnan Province, China).
91 Morphological analysis revealed that *Microcystis wesenbergii* and *Microcystis*
92 *aeruginosa* were the dominant species, accounting for 40%-80% of the algal bloom
93 biomass in the lake. The harvested algal bloom biomass was processed for further
94 experimentation via air-floatation, drying and grinding. The raw substrates were
95 cryopreserved at -20°C before use. The moisture content of the biomass was
96 measured by drying the samples in oven at 100°C until the total mass was constant.
97 The contents of TVS and ash were determined by heating at 450°C for 2 h. The total
98 carbohydrates, lipids and heating value were determined by methods described in our
99 previous study [25]. The microcosmic structure of the Dianchi Lake algae was
100 observed using XRD (X-ray diffractometer, Rigaku MiniFlex 600), FTIR (gas
101 chromatograph- Fourier infrared spectrometer, SGE, Agilent 6890, Nicolet 5700) and
102 SEM (tabletop microscope, TM-1000, HITACHI).

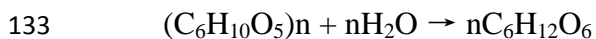
103 **2.2. Algal biomass pretreatment**

104 Many studies have demonstrated that for a variety of algal biomass, the optimal
105 pretreatment temperature in microwave, steam and other pretreatment methods was
106 found in the range of 135°C to 140°C, and the optimal pretreatment time was 15 min
107 to 20 min [26, 27]. When the pretreatment temperature was lower than 135°C or the
108 pretreatment time was shorter than 15 min, the damages to the recalcitrant
109 components in biomass were insufficient, and as a result the large molecular
110 polysaccharides such as cellulose could not be fully degraded into small molecular
111 reducing sugars. When the pretreatment temperature was higher than 140°C or the
112 pretreatment time was longer than 20 min, the Maillard reaction between reducing
113 sugar and protein took place, resulting in decreased yield of reducing sugar and
114 fermentative biogas production. There were also many studies showing that for a
115 variety of algal biomass, the optimal concentration of acid (such as sulfuric acid) in
116 pretreatments was 1%-2% [28, 29]. After the fermentation experiments using Dianchi
117 Lake algal biomass as the feedstock, it was found that the pretreatment with 1%
118 sulfuric acid had insufficient strength for cellulosic composition degradation.
119 Therefore, the sulfuric acid concentration of 2% was selected for our pretreatment
120 experiments.

121 Two pretreatment methods for the Dianchi Lake algal bloom biomass were
122 conducted: (1) Hydrothermal heating with a dilute acid, referred to as the
123 hydrothermal pretreatment, was conducted in a hydrothermal reactor (Parr Instrument
124 4500, USA). In brief, 5 g of algal biomass and 100 mL of dilute H₂SO₄ (2% v/v) were

125 added to a 250 mL hydrothermal reactor. The mixture was then heated to 135°C for
126 15 min. (2) Steam heating with dilute acid, referred to as the steam pretreatment, was
127 conducted in an autoclave (Sanyo MLS-3780, Japan). Briefly, 5 g of algal biomass
128 and 100 mL of dilute H₂SO₄ (2% v/v) were added to a 417 mL glass fermentation
129 bottle. The mixture was then heated in an autoclave to 135°C for 15 min.

130 The reducing sugar content after pretreatment was determined by the 3-5
131 dinitrosalicylic acid method, as described in a previous study [27]. The theoretical
132 maximum of the reducing sugar yield was calculated using the following formula:



134 The ratio of reducing sugar to the theoretical value (%) was defined as the weight
135 ratio of the reducing sugars (g) after pretreatment and hydrolysis to the theoretical
136 reducing sugar products (g) of Dianchi Lake algae.

137 **2.3. Inocula**

138 Inoculum for dark hydrogen fermentation was obtained from anaerobic digestion
139 sludge collected from a biogas plant in Huzhou, Zhejiang Province, China. The
140 original sludge contained a variety of microbes, including hydrogen-producing
141 bacteria and methanogens. To inactivate the methanogens, the sludge was heated at
142 100°C for 30 min in an autoclave. The sludge was then cultured to enrich the
143 abundance of hydrogen-producing bacteria. The major species of dark hydrogen
144 fermentation bacteria, as identified by 16S rRNA gene analysis, was *Clostridium*
145 *butyricum* [30].

146 Inoculum for anaerobic digestion was sourced from the same biogas plant in
147 Huzhou, China. The original digestate was degassed in an anaerobic workstation
148 (Whitley DG250, UK) at 35°C for 14 days to ensure depletion of the remaining
149 substrates before the experiment. The major species of methanogenic bacteria, as
150 identified by 16S rRNA gene analysis, were *Methanosarcina* and *Methanothrix* [20].

151 **2.4. Dark fermentation and anaerobic digestion**

152 Many studies have suggested that for microalgae, kitchen waste, cassava residue
153 and other biomass, the substrate concentration in fermentation was generally in the
154 range of 10 g/L-20 g/L [31, 32]. Some researchers set the fermentation concentration
155 of microalgae to 3 g VS [33]. When the substrate concentration was lower than 10 g/L,
156 the organic load was too low to provide sufficient nutrients level for
157 hydrogen-producing microorganisms during fermentation. However, when the
158 substrate concentration was higher than 20 g/L, the organic load might be too high for
159 the growth and metabolism of microorganisms. Therefore, the addition of algal
160 biomass in each fermenter was set to 3 g-TVS, which was equivalent to 10 g-TVS/L.
161 In these reported studies, the inoculum of hydrogen-producing bacteria was generally
162 selected to be 25 mL. The effluents were then inoculated with the methanogenic
163 inoculum based on the TVS ratio of 1:2 (substrate to inoculum).

164 Biohydrogen production via dark hydrogen fermentation was conducted in 417
165 mL glass reactors. The substrates (100 mL of pretreated solution-5 g of Dianchi Lake
166 algal biomass equivalent) and the hydrogen-producing inoculum (25 mL) were added

167 into each reactor. The total liquor volume was adjusted to 300 mL with distilled water.
168 The initial pH was adjusted to 6.0 ± 0.1 with 6 M NaOH and 6 M HCl. The reactors
169 were then sealed using silicone rubber stoppers and purged with nitrogen gas for 8
170 min to maintain an anaerobic environment. The dark hydrogen fermentation
171 experiment was conducted for 72 h in a water bath, which was maintained at 35.0°C .

172 The hydrogen production experiments were conducted in three groups, which
173 were performed in triplicate. Group 1 contained 100 mL algal bloom suspension after
174 hydrothermal pretreatment with dilute acid, 175 mL distilled water and 25 mL
175 hydrogen-producing inoculum. Group 2 contained 100 mL algal bloom suspension
176 after steam pretreatment with dilute acid, 175 mL distilled water and 25 mL
177 hydrogen-producing inoculum. Group 3 contained 5 g algal bloom suspension without
178 pretreatment, 275 mL distilled water and 25 mL hydrogen-producing inoculum. After
179 dark hydrogen fermentation, the effluents were adjusted to $\text{pH } 8.0 \pm 0.1$ using 6 M
180 NaOH and then inoculated with the methanogenic inoculum according to the TVS
181 ratio of 1:2 (substrate to inoculum). The total working volume of each reactor during
182 anaerobic digestion was 300 mL, including 120 mL effluents from dark fermentation,
183 150 mL methanogenic inoculum and 30 mL distilled water.

184 The reactors were then sealed, purged with nitrogen gas for 8 min to ensure
185 anaerobic environment, and maintained at 35.0°C . The anaerobic digestion
186 experiments were carried out for 25 days. For each group, the experimental conditions
187 of anaerobic digestion without dark hydrogen fermentation were separately used as
188 the control groups.

189 **2.5. Analytical methods and calculations**

190 Hydrogen and methane concentrations were determined using a gas
191 chromatography (GC) system equipped with a thermal conductivity detector
192 (GC-TCD; Agilent 7820A, USA). Hydrogen and methane yields were calculated from
193 the amount and composition of the headspace gas and the total volume. The soluble
194 metabolic product (SMP) compositions were determined using a GC system equipped
195 with a flame ionization detector (GC-FID; Agilent 7820A, USA).

196 Origin 8.0 software was used to fit the modified Gompertz equation (as shown
197 below) and the hydrogen production kinetic parameters were obtained [34, 35].

$$198 \quad H = H_m \exp\{-\exp[R_m e(\lambda - t)/H_m + 1]\}$$

199 where H is the hydrogen yield (mL/g-TVS); H_m is the maximum hydrogen yield
200 potential (mL/g-TVS); R_m is the peak rate of hydrogen production (mL/g-TVS/h); λ is
201 the hydrogen production delay time (h); t is the hydrogen production time (h) and l is
202 the lag-phase time (h).

203 The energy conversion efficiency (ECE) was defined as the ratio of the energy
204 values of hydrogen and methane to the total heating value of the algal biomass [36,
205 37].

206 **3. Results and discussion**

207 **3.1. Characterization of algal biomass before and after** 208 **pretreatment**

209 The TVS accounted for 55% of the total weight of the original algal bloom and
210 the heating value of the algal biomass was 25.17 kJ/g. The dried algal bloom biomass
211 consisted of 36.60% ash, 15.13% carbohydrate, 38.30% protein and 2.54% fat. The
212 main elements detected were C_{ad} (24.09%), H_{ad} (4.93%), N_{ad} (2.59%), O_{ad} (25.26%),
213 and S_{ad} (0.81%). After hydrothermal pretreatment, the dried algal samples consisted
214 of 40.82% ash, 2.06% carbohydrate, 42.22% protein and 2.88% fat. The main
215 elements detected were C_{ad} (27.12%), H_{ad} (5.94%), N_{ad} (2.93%), O_{ad} (25.33%) and S_{ad}
216 (0.91%). In comparison, the dried algal samples after steam pretreatment consisted of
217 39.64% ash, 4.23% carbohydrate, 40.81% protein and 2.69% fat. The main elements
218 detected were C_{ad} (26.06%), H_{ad} (5.14%), N_{ad} (2.79%), O_{ad} (25.88%) and S_{ad} (0.87%).
219 It can be seen that whichever pretreatment method was applied, the proportion of
220 carbohydrates decreased significantly but the proportion of N element increased. This
221 was because after pretreatment, the macromolecular carbohydrates were degraded into
222 small molecular soluble reducing sugars, which were then dissolved in the liquid
223 fraction. As compared to steam pretreatment, hydrothermal pretreatment resulted in a
224 much lower carbohydrate content, which indicated that hydrothermal pretreatment
225 had a stronger effect on carbohydrate degradation. When the reducing sugar content
226 in the liquid fraction was higher, its promotion on hydrogen production and

227 subsequent methanogenesis was more effective.

228 The yield of the reducing sugar from algae without pretreatment was very low at
229 3.6% of the theoretical maximum yield. After hydrothermal or steam pretreatment, the
230 reducing sugar yield reached more than 75% of the theoretical maximum yield. The
231 addition of dilute acid during hydrothermal pretreatment significantly improved the
232 sugar yield to 94.5%. The original cell structure of the algal biomass was very intact
233 and it was difficult for the macromolecular carbohydrates to be hydrolyzed.
234 Hydrothermal and steam pretreatment significantly increased the hydrolysis rate of
235 the algal biomass by destroying cells and hydrolyzing macromolecule sugars.

236 Hydrothermal treatment refers to the reaction processes of degrading, dissolving,
237 oxidizing and synthesizing various substances that exist in high temperature and high
238 pressure water by utilizing the special properties of the water. The fermentation effect
239 of the algal bloom biomass after hydrothermal pretreatment was better than that of the
240 steam pretreatment, which might be due to the following aspects. First, when treated
241 at the same temperature, processing time and acid concentration, the pressure of the
242 hydrothermal pretreatment was higher than that of steam pretreatment due to the
243 smaller volume of the hydrothermal reactor, thereby promoting disruption of cell
244 walls and efflux of cell contents. This could allow for a higher hydrolysis efficiency
245 of macromolecules. Second, the heat transfer of the hydrothermal reaction was via
246 conduction allowing for rapid heating and a better temperature maintenance effect.
247 The heat transfer of the steam reaction was via conduction and convection between
248 the biomass and the steam; therefore, the heating effect was not as fast as the

249 hydrothermal pretreatment. A better heating effect results in a higher hydrolysis
250 efficiency of macromolecular polysaccharides, which is beneficial to the subsequent
251 fermentation experiments.

252 To further reveal the physicochemical changes of the algae after pretreatment,
253 the biomass was examined by XRD, FTIR and SEM. The XRD spectra were used to
254 characterize the ratio of crystalline cellulose and amorphous cellulose of the algal
255 biomass after hydrothermal and steam pretreatment (Fig. 1). In the spectra, $2\theta =$
256 $20\text{--}21^\circ$ represents the region of highly crystalline cellulose and $2\theta = 18^\circ$ represents
257 the region of amorphous cellulose. Since the cellulose is coated with tough lignin, the
258 algal bloom biomass without pretreatment did not show the characteristic peaks. The
259 cellulose crystallinity index (CrI) of the samples could be calculated using Segal's
260 empirical formula.

$$261 \quad \text{CrI} = (I_{21} - I_{18}) / I_{21} \quad [38]$$

262 where I_{21} is the diffraction peak intensity of $2\theta = 21^\circ$ and I_{18} is the diffraction
263 peak intensity of $2\theta = 18^\circ$. The CrI of the non-pretreated algal bloom was 14.3,
264 whereas it was 42.7 for the hydrothermal acid pretreated algal biomass and 33.7 for
265 the steam acid pretreated algal biomass. The higher CrI after hydrothermal or steam
266 pretreatment indicates that the amorphous cellulose was effectively degraded while
267 the crystalline cellulose was more difficult to degrade, thus increasing the CrI.

268 The FTIR spectra were used to characterize the changes in functional groups
269 before and after pretreatment (Fig. 2). The absorption peak at 1430 cm^{-1} notably
270 weakened after the hydrothermal or steam pretreatment. This peak was assigned to

271 CH₂ bending vibrations, which is a characteristic peak of cellulose. This observation
272 is due to the fact that part of the cellulose was degraded after pretreatment. The
273 absorption peaks at 1638 cm⁻¹ and 1558 cm⁻¹ were clearly present before and after the
274 pretreatment. These peaks are assigned to the stretching vibrations of acetyl or
275 carboxylic acid C=O or the stretching vibrations of aromatic C=C, which are the
276 characteristic peaks of lignin. These results show that lignin cannot be effectively
277 degraded by either hydrothermal or steam pretreatment. The absorption peak at 1430
278 cm⁻¹ represents the crystalline cellulose region. The absorption peak at 898 cm⁻¹
279 represents the region of crystalline and amorphous cellulose [39]. By calculating the
280 lateral index (LI) [40], which is the absorption rate A_{1430 cm⁻¹} / A_{898 cm⁻¹}, the
281 proportion of crystalline cellulose in the total cellulose can be analyzed. The LI of the
282 non-pretreated algae was 0.874, while it was 0.985 for the hydrothermal heating
283 pretreated algae and 0.966 for the steam heating pretreated algae. After pretreatment,
284 the algal bloom biomass showed a high proportion of crystalline cellulose,
285 demonstrating that the hydrothermal and steam pretreatments effectively damaged the
286 easily degraded amorphous cellulose. This result was consistent with the XRD and
287 CrI analysis.

288 The microstructural properties of the algae after pretreatment were visualized by
289 SEM (Fig. 3). Before pretreatment, the algae were mainly in the form of irregular
290 clumps (60-90 μm), with the space between the clumps ranging from approximately
291 10 μm to 40 μm. The surface was rough and compact with many tiny particles
292 attached to it. These observations might be due to the loss of moisture from the

293 microalgae cells when exposed to the sun, resulting in formation of a large amount of
294 biomass fragments. Further mechanical grinding resulted in the formation of a rough
295 surface. After the pretreatment of hydrothermal heating (135°C, 15 min) with dilute
296 sulfuric acid (2%), the algal bloom biomass showed a smooth and loose surface with
297 formation of a large amount of fragments of about 5 μm and a large number of gaps
298 ($\sim 1 \mu\text{m}$) between the fragments formed. After the pretreatment of steam heating
299 (135°C, 15 min) with dilute sulfuric acid (2%), the algal bloom biomass also showed
300 a smooth and loose surface with formation of fragments of about 10 μm . The size of
301 the gaps between the fragments was about 1-2 μm . This was due to hydrolysis of the
302 algal bloom biomass in the presence of acid. After hydrothermal or steam heating, the
303 surface structure of the algal cells became fluffy and the gaps between the fragments
304 became wider. However, steam pretreatment was not as effective as hydrothermal
305 pretreatment. This was because the pressure of the autoclave during the steam
306 pretreatment was about 0.2 Mpa, whereas the pressure of the hydrothermal reactor
307 could reach 0.25 Mpa at the same reaction temperature and time. This higher pressure
308 was more likely to cause the formation of more gaps and smaller fragments.

309 **3.2. Hydrogen production through dark-fermentation**

310 Biohydrogen production of algal blooms through dark fermentation is shown in
311 Fig. 4. The dynamic parameters of the dark hydrogen fermentation fitted by the
312 modified Gompertz equation are shown in Table 1. After hydrothermal or steam
313 pretreatment, H_m and R_m increased substantially while λ decreased. Due to the rigid

314 cell structure of the algal 14bloom biomass, the reducing sugars were difficult to
315 release. As a result, the hydrogen yield from the algal biomass without pretreatment
316 was less than 1 mL/g TVS. The hydrogen yield from the algal biomass with
317 hydrothermal pretreatment and dilute acid (24.96 mL/g TVS) was higher than that
318 with steam pretreatment and dilute acid (18.63 mL/g TVS). The peak hydrogen
319 production rate from the algal biomass with hydrothermal acid pretreatment (2.04
320 mL/g TVS/h) was higher than that with the steam acid pretreatment (1.83 mL/g
321 TVS/h). This is because the reducing sugar yield from the algal biomass with
322 hydrothermal pretreatment (94.5%) was higher than that with steam pretreatment
323 (74.8%). The hydrogen yield of this study was much lower compared with the values
324 reported in literature, especially using easily degradable substrates (such as starch and
325 food wastes) [2, 7, 27, 33, 38]. For example, the hydrogen yield of pretreated algal
326 biomass (24.96 mL/g-TVS) was lower than that (147.42 mL/g-TVS) of the food waste
327 biomass. This was due to the fact that the concentration of carbohydrates was low and
328 compared to glucose, lignocellulose was difficult to be degraded. Without enzyme
329 hydrolysis or photo hydrogen fermentation, bio-hydrogen yield from the algal bloom
330 remained low even after the hydrothermal pretreatment.

331 The SMP compositions of the dark fermentation effluents of algal bloom biomass
332 after pretreatment are shown in Fig. 5. The main components of the liquid phase
333 metabolites were acetic acid and butyric acid, which accounted for 88.8%-90.7% of
334 the total soluble metabolites. There were also small amounts of ethanol, propionic
335 acid, isobutyric acid, isovaleric acid, valeric acid and caproic acid. This result is

336 consistent with the fermentation metabolic pathway (i.e., acetate/butyrate pathway) of
337 hydrogen-producing bacteria [41]. After dark fermentation, the total soluble
338 metabolites from the algal biomass without pretreatment was 52.93 ± 10.10 mM. The
339 pretreatment with hydrothermal heating and dilute acid increased the total soluble
340 metabolites to 369.14 ± 33.33 mM, whereas pretreatment with steam heating and
341 dilute acid increased the total soluble metabolites to 310.86 ± 42.62 mM. This result
342 suggests that pretreatment with hydrothermal heating and steam heating can promote
343 the hydrolysis of the algal bloom, thus increasing the utilization of substrates and the
344 fermentation efficiency by hydrogen-producing bacteria. As a result, approximately
345 6-7 times more soluble metabolites can be produced than that without pretreatment.
346 Compared to the steam heating pretreatment, hydrothermal heating pretreatment of
347 the algal bloom biomass showed more efficient hydrolysis and saccharification, which
348 enabled the hydrogen-producing bacteria to efficiently utilize organic components
349 of the hydrolyzed biomass for fermentation, thus greatly promoting the production of
350 soluble liquid phase metabolites.

351 **3.3. Methane production during anaerobic digestion**

352 Second stage anaerobic digestion was employed to further produce methane from
353 the residual hydrogen fermentation solutions. For comparison, one-stage anaerobic
354 digestion of the algal biomass with and without pretreatment was also employed to
355 assess the methane production. The maximum methane production rate occurred
356 within the first two days (Fig. 6). After approximately 25 days, methane yield did not

357 vary substantially; thus, the methanogenic phase was considered to have ended. The
358 two-stage cogeneration of hydrogen and methane generally resulted in a higher
359 methane yield and a peak production rate, as compared to the one-stage anaerobic
360 digestion. The one-stage anaerobic digestion of untreated algal biomass resulted in the
361 lowest methane yield (206.82 mL CH₄ /g TVS) with a peak production rate of 26.91
362 mL CH₄/g TVS/d. The highest methane yield (299.88 mL CH₄ /g TVS) from the
363 two-stage fermentation was achieved with the hydrothermal acid pretreatment.
364 Correspondingly, the peak methane production rate was 49.91 mL CH₄ /g TVS/d. For
365 the one-stage anaerobic digestion, the maximum methane yield (246.13 mL CH₄ /g
366 TVS) and peak production rate (39.27 mL CH₄/g TVS/d) were observed after the
367 hydrothermal pretreatment. Similarly, two-stage fermentation after the steam acid
368 pretreatment led to better methane production, when compared to the one-stage
369 anaerobic digestion. These trends were due to the fact that (1) deep acidification
370 during hydrogen fermentation had an enhanced effect on the degradation of algal cell
371 macromolecules; and (2) formation of intermediate substrates, such as acetic acid and
372 butyric acid, could be easily used by methanogens.

373 It was noted that the hydrothermal acid pretreatment outperforms steam acid
374 pretreatment during two-stage fermentation. This might be due to the following
375 reasons: (1) compared with the steam acid pretreatment, more gaps and smaller
376 fragments of algae were formed after the hydrothermal acid treatment; and (2) the
377 hydrothermal acid pretreatment led to a higher production of total soluble metabolites
378 (369.14 mM) during fermentation than the steam acid pretreatment (310.86 mM).

379 **3.4. Energy conversion efficiency**

380 A comparison of previously published fermentative gaseous biofuel production
381 and energy yields from pretreated algal bloom biomass is shown in Table 2. The
382 energy conversion efficiency was calculated based on the heating values of hydrogen
383 (242 kJ/mol), methane (801 kJ/mol) and algal bloom biomass (25.17 kJ/g TVS). In
384 this study, the energy conversion efficiency of hydrogen production remained low
385 (0.4%), even after hydrothermal or steam pretreatment. However, following methane
386 production from the residual hydrogen fermentation solutions, the energy conversion
387 efficiency significantly increased to 44.10% after hydrothermal pretreatment and
388 39.38% after steam pretreatment. In the study that used the Taihu Lake algal bloom
389 biomass, photo hydrogen fermentation was used to further improve hydrogen
390 production after dark fermentation [20]. Although hydrogen production was improved,
391 the input energy of light was not considered when calculating the energy conversion
392 efficiency. When only hydrogen production by photosynthetic bacteria is considered,
393 the energy conversion efficiency of light energy to hydrogen is only 1%. In this study,
394 the hydrogen yield of pretreated algal biomass was lower than that of the Taihu Lake
395 algal bloom biomass because there was no enzyme pretreatment or photo hydrogen
396 fermentation. However the energy conversion efficiency was almost equal to the
397 three-stage cogeneration of hydrogen and methane from the pretreated algal biomass
398 of Taihu Lake [20].

399 **4. Conclusions**

400 Hydrothermal acid pretreatment of algal bloom biomass from Dianchi Lake
401 could significantly increase the energy conversion efficiency during a two-stage
402 process comprising of dark hydrogen fermentation and anaerobic digestion. XRD and
403 FTIR analysis showed that the cellulose crystallinity index of the algal biomass
404 pretreated with hydrothermal acid was significantly higher than that pretreated with
405 steam acid, thereby suggesting that the hydrothermal acid pretreatment has a stronger
406 degradation effect on hemicellulose and cellulose than the steam acid pretreatment.
407 The energy conversion efficiency of the hydrothermal heating pretreated algal
408 biomass remarkably increased to 44.1% by cogenerating 24.96 mL/g TVS hydrogen
409 and 299.88 mL/g TVS methane during the two-stage process. The effective
410 conversion of algal bloom waste into biofuel demonstrates a promising future for
411 industrial applications.

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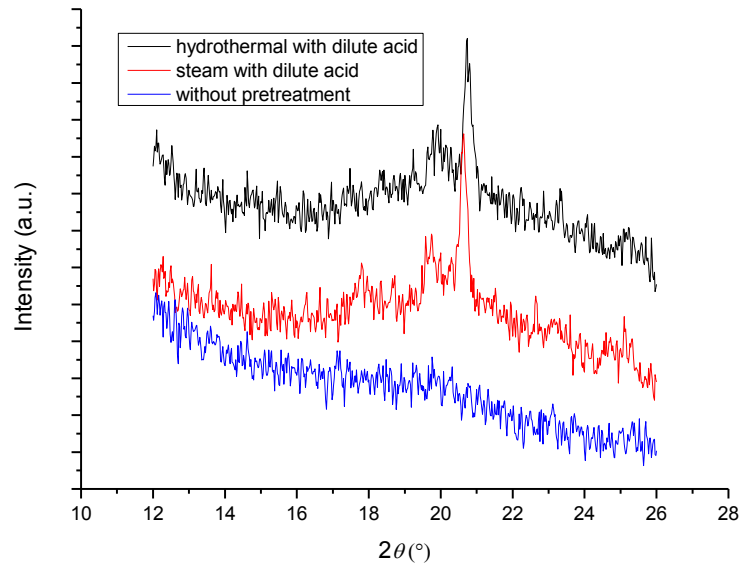
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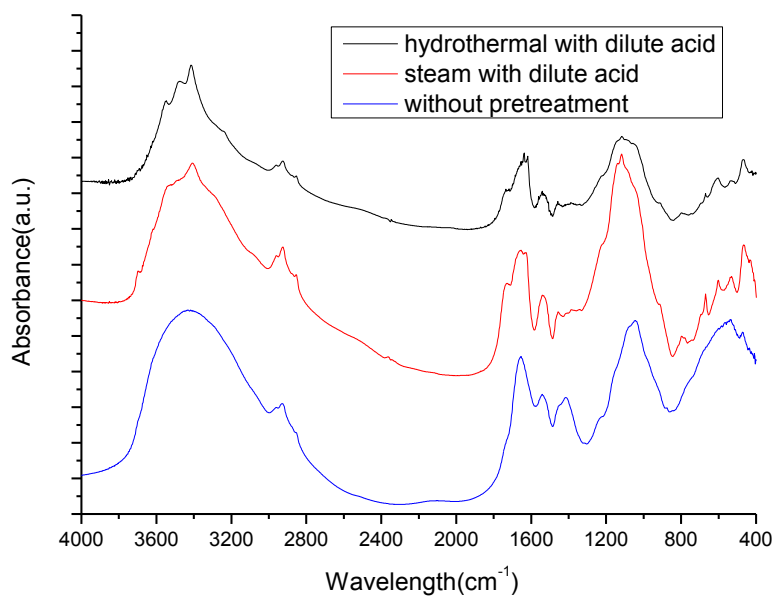
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546 **Fig. 1 XRD spectra of algae biomass pretreated by hydrothermal heating and**

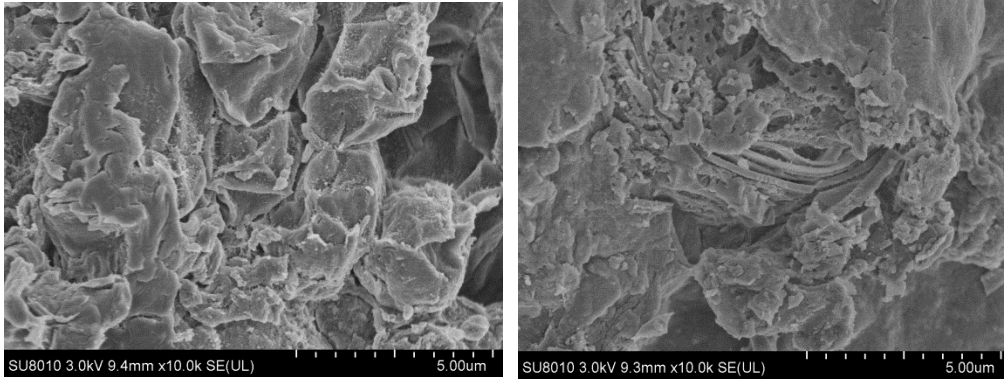
547 **steam heating with dilute acid for 15 min.**



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549 **Fig. 2 FTIR spectra of algae biomass pretreated by hydrothermal heating and**
550 **steam heating with dilute acid for 15 min.**

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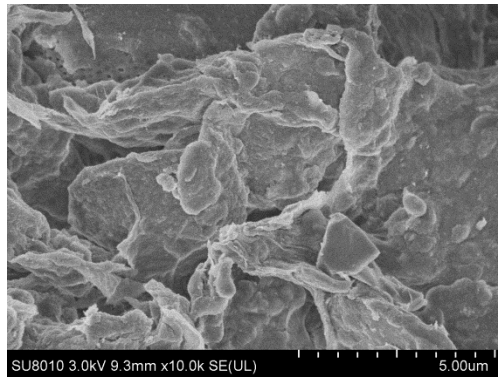
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(a) without pretreatment ($\times 10000$)

(b) hydrothermal with dilute acid ($\times 10000$)



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(c) steam heating with dilute acid ($\times 10000$)

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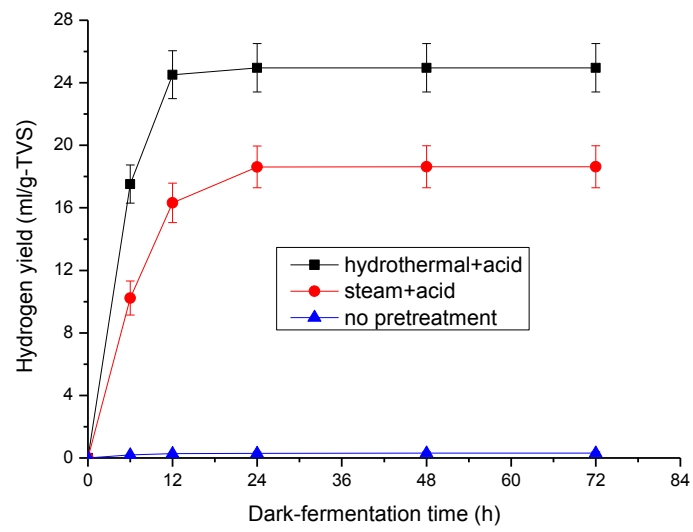
Fig. 3 SEM images of algae biomass pretreated by hydrothermal heating and

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steam heating with dilute acid for 15 min.

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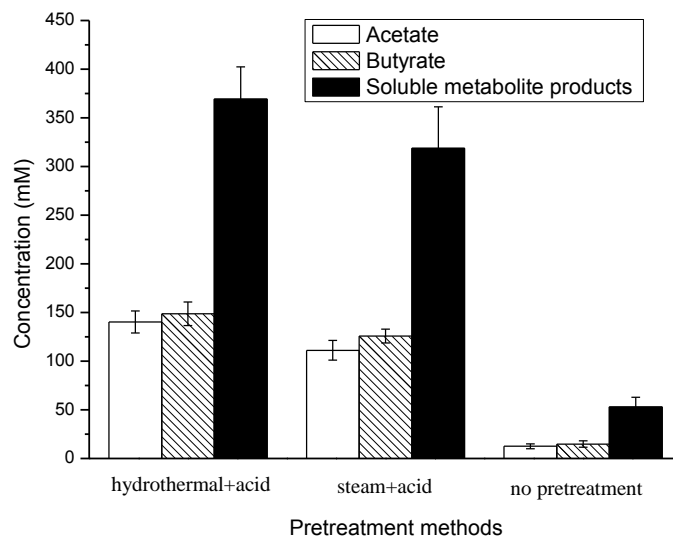


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563 **Fig. 4 Hydrogen production from pretreated algae biomass by dark**
564 **fermentation.**

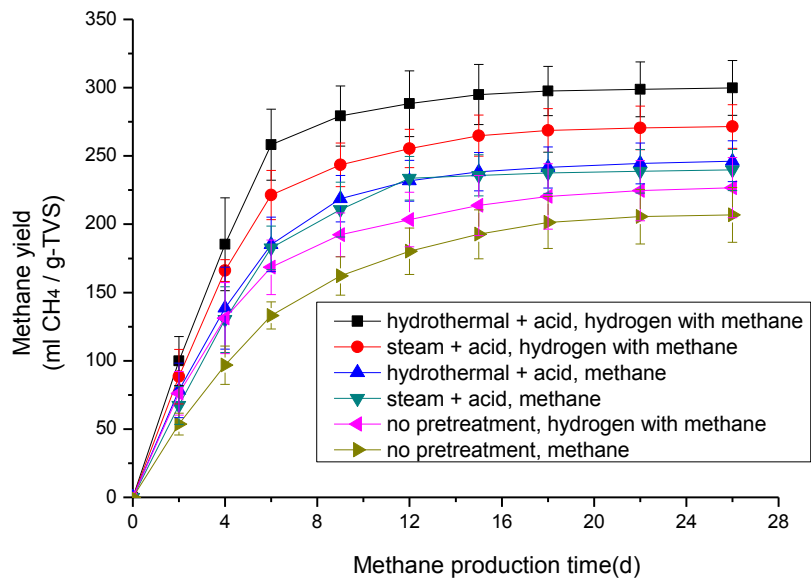
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567 **Fig. 5 Soluble metabolite byproducts from dark hydrogen fermentation of**
 568 **pretreated algae biomass.**

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571

Fig. 6 Methane production from the residues of hydrogen fermentation.

Table 1 Dynamic parameters of hydrogen production in dark fermentation.

Pretreatment method	H ₂ yield (mL/g-TVS)	H ₂ production peak rate (mL/g-TVS/h)	Kinetic model parameters			
			H _m (mL/g-TVS)	R _m (mL/g-TVS/h)	λ(h)	R ²
No pretreatment	0.30	0.03	0.31	0.05	5.08	0.982
Hydrothermal heating +acid	24.96	2.04	24.96	3.05	3.40	0.993
Steam heating +acid	18.63	1.83	18.63	2.46	4.42	0.999

Table 2 Comparison of the energy conversion efficiency of hydrogen and methane cogeneration from pretreated algae biomass in literature.

Feedstock	Carbohydrate content (% of dried biomass)	Feedstock pretreatment	Process	Bacteria	H ₂ yield (mL/g TVS)	CH ₄ yield (mL/g TVS)	Energy yield of only H ₂ (kJ/g TVS)	Energy yield of H ₂ and CH ₄ (kJ/g TVS)	Total energy conversion efficiency (%)	References
Taihu Lake algal bloom	N/A	Alkaline pretreatment	Dark fermentation (H ₂)	Anaerobic granular sludge	105.0	/	1.1	1.1	N/A	[19]
Taihu Lake algal bloom	12	Steam heating with dilute H ₂ SO ₄	Dark fermentation (H ₂) + Photo fermentation (H ₂) + Methanogenesis (CH ₄)	HPB + PSB + MPB	256.74	253.53	2.77	11.84	47.04	[20]
Taihu Lake algal bloom	12	Microwave heating with H ₂ SO ₄	Dark fermentation (H ₂) + Photo fermentation (H ₂) + Methanogenesis (CH ₄)	HPB + PSB + MPB	283.41	166.83	3.06	9.03	35.88	[20]
Dianchi Lake algal bloom	13	Steam heating with dilute H ₂ SO ₄	Dark fermentation (H ₂) + Methanogenesis (CH ₄)	HPB + MPB	18.63	271.51	0.20	9.91	39.38	In this study
Dianchi Lake algal bloom	13	Hydrothermal with dilute H ₂ SO ₄	Dark fermentation (H ₂) + Methanogenesis	HPB + MPB	24.96	299.88	0.28	11.10	44.10	In this study

			(CH ₄)							
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HPB: Hydrogen producing bacteria; PSB: Photosynthetic bacteria; MPB: Methane producing bacteria.