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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 μ m) 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	H_2/g total volatile solids (TVS) and 299.88 mL CH_4/g TVS.
26	
27	Keywords: Dianchi Lake algal bloom; hydrogen; methane; hydrothermal
28	pretreatment.

1. Introduction 30

Dianchi Lake, the largest freshwater lake in Yunnan Province, China, suffers 31 from annual algal bloom outbreaks. Until December 2017, the water quality of 32 33 Dianchi Lake was still in a eutrophic state. During the summer of 2017, more than 100 tons of algal biomass were salvaged every day, causing great harm to the 34 ecological environment. However, this large amount of algae can also be used as a 35 potential feedstock for fermentative biofuel production, as demonstrated by many 36 studies on biohydrogen production from algal biomass [1-4]. 37 Different algal species, such as Chlorella and Arthrospira, have been assessed 38 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

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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).
	4

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

2. Materials and methods

88 2.1. Substrates and characterization

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

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 $\rm H_2SO_4$ (2% v/v) were $_6$

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 H_2SO_4 (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:
133	$(C_6H_{10}O_5)n + nH_2O \rightarrow nC_6H_{12}O_6$
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**

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

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

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

Many studies have suggested that for microalgae, kitchen waste, cassava residue 152 and other biomass, the substrate concentration in fermentation was generally in the 153 range of 10 g/L-20 g/L [31, 32]. Some researchers set the fermentation concentration 154 of microalgae to 3 g VS [33]. When the substrate concentration was lower than 10 g/L, 155 the organic load was too low to provide sufficient nutrients level for 156 157 hydrogen-producing microorganisms during fermentation. However, when the substrate concentration was higher than 20 g/L, the organic load might be too high for 158 the growth and metabolism of microorganisms. Therefore, the addition of algal 159 160 biomass in each fermenter was set to 3 g-TVS, which was equivalent to 10 g-TVS/L. In these reported studies, the inoculum of hydrogen-producing bacteria was generally 161 selected to be 25 mL. The effluents were then inoculated with the methanogenic 162 163 inoculum based on the TVS ratio of 1:2 (substrate to inoculum). Biohydrogen production via dark hydrogen fermentation was conducted in 417 164 mL glass reactors. The substrates (100 mL of pretreated solution-5 g of Dianchi Lake 165 algal biomass equivalent) and the hydrogen-producing inoculum (25 mL) were added 166

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 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.

|

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	$H=H_mexp\{-exp[R_me(\lambda-t)/H_m+1]\}$
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].

|

3. Results and discussion

3.1. Characterization of algal biomass before and after 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

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

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

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

261 $CrI=(I_{21}-I_{am})/I_{21}[38]$

where I_{21} is the diffraction peak intensity of $2\theta = 21^{\circ}$ and I_{am} is the diffraction 262 peak intensity of $2\theta = 18^{\circ}$. The CrI of the non-pretreated algal bloom was 14.3, 263 whereas it was 42.7 for the hydrothermal acid pretreated algal biomass and 33.7 for 264 the steam acid pretreated algal biomass. The higher CrI after hydrothermal or steam 265 pretreatment indicates that the amorphous cellulose was effectively degraded while 266 the crystalline cellulose was more difficult to degrade, thus increasing the CrI. 267 The FTIR spectra were used to characterize the changes in functional groups 268 before and after pretreatment (Fig. 2). The absorption peak at 1430 cm^{-1} notably 269 weakened after the hydrothermal or steam pretreatment. This peak was assigned to 270

271	CH ₂ 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 A1430 cm^{-1} /A898 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 mm), with the space between the clumps ranging from approximately
291	10 μ m to 40 μ m. 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

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

Biohydrogen production of algal blooms through dark fermentation is shown in
Fig. 4. The dynamic parameters of the dark hydrogen fermentation fitted by the
modified Gompertz equation are shown in Table 1. After hydrothermal or steam
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

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

351 3.3. Methane production during anaerobic digestion

Second stage anaerobic digestion was employed to further produce methane from the residual hydrogen fermentation solutions. For comparison, one-stage anaerobic digestion of the algal biomass with and without pretreatment was also employed to assess the methane production. The maximum methane production rate occurred 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 CH ₄ /g TVS) with a peak production rate of 26.91
362	mL CH ₄ /g TVS/d. The highest methane yield (299.88 mL CH ₄ /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 CH ₄ /g TVS/d. For
365	the one-stage anaerobic digestion, the maximum methane yield (246.13 mL CH $_{\rm 4}$ /g
366	TVS) and peak production rate (39.27 mL CH ₄ /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

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hydrothermal acid pretreatment led to a higher production of total soluble metabolites

pretreatment during two-stage fermentation. This might be due to the following

reasons: (1) compared with the steam acid pretreatment, more gaps and smaller

fragments of algae were formed after the hydrothermal acid treatment; and (2) the

(369.14 mM) during fermentation than the steam acid pretreatment (310.86 mM).

379 3.4. Energy conversion efficiency

A comparison of previously published fermentative gaseous biofuel production 380 and energy yields from pretreated algal bloom biomass is shown in Table 2. The 381 energy conversion efficiency was calculated based on the heating values of hydrogen 382 383 (242 kJ/mol), methane (801 kJ/mol) and algal bloom biomass (25.17 kJ/g TVS). In this study, the energy conversion efficiency of hydrogen production remained low 384 (0.4%), even after hydrothermal or steam pretreatment. However, following methane 385 production from the residual hydrogen fermentation solutions, the energy conversion 386 efficiency significantly increased to 44.10% after hydrothermal pretreatment and 387 39.38% after steam pretreatment. In the study that used the Taihu Lake algal bloom 388 389 biomass, photo hydrogen fermentation was used to further improve hydrogen production after dark fermentation [20]. Although hydrogen production was improved, 390 391 the input energy of light was not considered when calculating the energy conversion efficiency. When only hydrogen production by photosynthetic bacteria is considered, 392 the energy conversion efficiency of light energy to hydrogen is only 1%. In this study, 393 the hydrogen yield of pretreated algal biomass was lower than that of the Taihu Lake 394 395 algal bloom biomass because there was no enzyme pretreatment or photo hydrogen fermentation. However the energy conversion efficiency was almost equal to the 396 three-stage cogeneration of hydrogen and methane from the pretreated algal biomass 397 398 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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545

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

547 steam heating with dilute acid for 15 min.





549 Fig. 2 FTIR spectra of algae biomass pretreated by hydrothermal heating and

550 steam heating with dilute acid for 15 min.

551



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

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

steam heating with dilute acid for 15 min.



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



567 Fig. 5 Soluble metabolite byproducts from dark hydrogen fermentation of

pretreated algae biomass.





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

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

|--|

				merature.						
Feedstock	Carbohydrate	Feedstock	Process	Bacteria	H ₂ yield	CH ₄	Energy	Energy yield	Total energy	References
	content	pretreatment			(mL/g	yield	yield	of H_2 and	conversion	
	(% of dried				TVS)	(mL/g	of only H_2	CH ₄	efficiency (%)	
	biomass)					TVS)	(kJ/g TVS)	(kJ/g TVS)		
Taihu Lake	N/A	Alkaline	Dark fermentation	Anaerobic	105.0	/	1.1	1.1	N/A	[19]
algal bloom		pretreatment	(H ₂)	granular						
				sludge						
Taihu Lake	12	Steam heating	Dark fermentation	HPB + PSB +	256.74	253.53	2.77	11.84	47.04	[20]
algal bloom		with dilute	$(H_2) + Photo$	MPB						
		H_2SO_4	fermentation (H_2) +							
			Methanogenesis							
			(CH ₄)							
Taihu Lake	12	Microwave	Dark fermentation	HPB + PSB +	283.41	166.83	3.06	9.03	35.88	[20]
algal bloom		heating with	$(H_2) + Photo$	MPB						
		H_2SO_4	fermentation (H_2) +							
			Methanogenesis							
			(CH ₄)							
Dianchi Lake	13	Steam heating	Dark fermentation	HPB + MPB	18.63	271.51	0.20	9.91	39.38	In this study
algal bloom		with dilute	(H ₂) +							
		H_2SO_4	Methanogenesis							
			(CH ₄)							
Dianchi Lake	13	Hydrothermal	Dark fermentation	HPB + MPB	24.96	299.88	0.28	11.10	44.10	In this study
algal bloom		with dilute	(H ₂) +							
		H_2SO_4	Methanogenesis							

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

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HPB: Hydrogen producing bacteria; PSB: Photosynthetic bacteria; MPB: Methane producing bacteria.