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1 Can acid pre-treatment enhance biohydrogen and biomethane production 2 from grass silage in single-stage and two-stage fermentation processes?

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7

8 **Abstract**

9 Grass silage is an excellent feedstock for biofuel production, however, the recalcitrant
10 cellulosic structure may limit its biodegradability. In this study, the effect of acid pre-
11 treatment with mild thermal treatment conditions on biohydrogen and biomethane production
12 from grass silage was assessed through single-stage (CH₄) and two-stage (H₂ + CH₄)
13 fermentation. Microstructural characterisation showed that pre-treatment significantly
14 reduced the recalcitrance and enlarged the specific area of grass silage. The optimal pre-
15 treatment with 2% H₂SO₄ at 135 °C for 15 min achieved a total reducing sugar yield of
16 333.79 mg/g volatile solid (VS) of grass silage. The pre-treated silage led to a hydrogen yield
17 of 68.26 ml/g VS in the first stage hydrogen fermentation, a 3-fold increase compared to
18 untreated silage. The production of volatile fatty acids accordingly increased by 29.2%. In the
19 second stage anaerobic digestion, untreated silage achieved the highest biomethane yield of
20 392.84 ml/g VS, with a corresponding highest total energy conversion efficiency of 83.5%.
21 Due to a lower biomethane yield, the pre-treated silage presented a decreased total energy
22 efficiency of 68.4%. In comparison, single-stage anaerobic digestion showed lower energy

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23 conversion efficiencies of 49.7% and 54.2% for the pre-treated and untreated silage,
24 respectively. Despite the slight decrease in CH₄ yield, the pre-treatment led to decreased
25 energy consumption for the operation of anaerobic digestion processes due to the shorter
26 digestion duration.

27 **Keywords:** Grass silage; acid pre-treatment; dark fermentation; anaerobic digestion;
28 biohydrogen; biomethane.

29

30 **1. Introduction**

31 **1.1 Grass silage as a resource for biofuel production**

32 Considering the increase in global energy consumption and environmental degradation, there
33 is a pressing need to accelerate the development of renewable energy. The Europe Union (EU)
34 has 2030 binding targets of 32% renewable energy and 14% renewable energy in transport
35 [1]. However, as of 2017 renewable energy share was 17.5% in gross energy consumption
36 and 7.6% in renewable energy in transport; this suggests EU states have significant work to
37 do to ensure compliance with these binding targets. Advanced biofuels (such as biohydrogen
38 and biomethane) produced through fermentative methods have the potential to contribute to
39 achieving the renewable energy targets in a cost-effective way, especially to decarbonizing
40 the transportation sector, and more particularly to haulage and coaches, which are not readily
41 amenable to electrification.

42

43 In an Irish context, grass is the dominant crop, accounting for over 80% of utilizable
44 agricultural land. It is estimated that grass silage has the potential to produce about 35.0 PJ of
45 biogas in 2035 in excess of livestock requirements, equivalent to 22% of natural gas supply in
46 2015 in Ireland [2]. Grass silage has a high moisture content, high carbohydrates content, and

47 a balanced carbon to nitrogen ratio (C/N), and as such is well suited for biohydrogen and
48 biogas production through dark fermentation and anaerobic digestion [3].

49

50 **1.2 Pre-treatment of grass for biohydrogen and biomethane production**

51 Despite the abundant quantities and the potential utilization, the rigid lignocellulosic structure
52 of grass makes it resistant to microbial metabolism, resulting in a sub optimal production of
53 biohydrogen and biomethane in fermentative processes. The reported specific methane yield
54 in single-stage anaerobic digestion of grass silage ranges from 270 to 432 ml/g VS [4-6],
55 decreasing with the increase in fibre components. The digestibility of grass silage highly
56 depends on the content of lignin, hemicellulose and cellulose. For the late cut grass with
57 lower digestibility, pre-treatment is an effective method to enhance the conversion of
58 lignocellulosic components. Thermochemical pre-treatment, such as acid / alkaline pre-
59 treatment [7, 8], microwave / ultrasonic combined with acid pre-treatment [9] [10], hot water
60 pre-treatment [11] and steam explosion pre-treatment [12] were investigated and proven to
61 effectively enhance the hydrolysis, sugar recovery, and biogas production of grass. However,
62 harsh conditions in some pre-treatment processes may also cause the degradation of released
63 sugars to furans and organic acids, which may act as inhibitors in the fermentation process
64 [13]. Pre-treatment conditions have to be optimized to enhance hydrolysis and subsequent
65 anaerobic digestion.

66

67 The hydrogen yields in dark fermentation of untreated grass silage were typically between 4.4
68 to 10.3 ml/g dry grass [8] [10] [14]. The energy content in the produced hydrogen accounts
69 for less than 20% of the total energy in the substrate [15]. A combined second-stage
70 anaerobic digestion process has been demonstrated as a promising technology to recover the
71 residual energy as it can further convert the volatile fatty acids (VFAs) produced in the first-

72 stage dark fermentation into methane. When compared to hydrogen production alone, the
73 energy recovery from sugarcane syrup through two-stage hydrogen and methane co-
74 production increased 6–7 fold [16]. Two-stage fermentation of the brown seaweed *Laminaria*
75 *digitata* reduced the hydraulic retention time by 33% whilst improving the energy conversion
76 by 9.8% as compared to single stage anaerobic digestion [17]. However, the optimal pre-
77 treatment conditions for dark fermentation and anaerobic digestion are probably different due
78 to the different microbial community and metabolic pathways. For instance, the optimum pH
79 value and metal ion concentration (such as Na and K ions) differ for acidogenic and
80 methanogenic microorganisms [18, 19]. This indicates the pre-treatment conditions may lead
81 to different effects on single-stage and two-stage fermentation processes.

82

83 **1.3 Objectives**

84 The present study deals with the effect of acid pre-treatment on biohydrogen and biomethane
85 production from grass silage, as ensiled forage crops are one of the most abundant renewable
86 biomass resources in Europe. Acid pre-treatment of lignocellulosic biomass is widely
87 investigated, but the difference in its effect on single-stage (CH_4) and two-stage ($\text{H}_2 + \text{CH}_4$)
88 fermentation has been rarely reported. The research objective is to fill the gap in the literature
89 by 1) optimizing the pre-treatment conditions to maximise reducing sugar yield during
90 hydrolysis, 2) comparing the effects of pre-treatment on the specific biohydrogen and
91 biomethane yields from single-stage and two-stage fermentation, and 3) assessing the energy
92 conversion efficiency and energy consumption for both processes.

93

94 **2. Material and methods**

95 **2.1 Feedstock and inoculum**

96 The grass silage was sourced from late-cut perennial ryegrass. The grass was initially field
97 wilted for 24 h and ensiled for 5 weeks in 1.2 m diameter cylindrical bales wrapped in
98 polyethylene stretch-film [20]. Then the silage was re-wrapped and stored at approximately
99 18–20 °C in our lab. Before use, the silage was dried at 40 °C for 72 h and subsequently
100 ground into fine particles with diameters of 1–2 mm. The silage was then stored at 4 °C until
101 required. It should be noted that in this work we are dealing with dried silage, which differs
102 from wet silage. In the process of drying silage volatilization of the liquid phase causes a
103 significant loss of volatile compounds. The volatility coefficients in the drying process at
104 60 °C were reported as 0.09, 0.55 and 0.99 for lactic acid, volatile fatty acids and alcohol
105 fermentation products, respectively [21]. The biodegradation efficiency of the liquid silage
106 can achieve 92% [22], much higher compared to the conversion efficiency of the solid silage.
107 As such recalcitrance is associated with the solid silage. Thus, it is expected that fermentation
108 of solid silage will present a lower specific H₂ / CH₄ yield than the whole silage and these
109 studies will outline how best to overcome recalcitrance in, and enhance gaseous biofuel
110 yields from, grass silage.

111

112 The seed inoculum for both hydrogen and methane fermentation was sourced from a lab-
113 scale anaerobic digester. To culture the mixed biomethane inoculum for the biomethane
114 potential (BMP) assays, the seed inoculum was fed with cellulose periodically at 37 °C for 7
115 days. The total solid (TS) content and volatile solid (VS) content in the mixed biomethane
116 inoculum were 2.97% and 1.50%, respectively. To isolate the hydrogenogens for
117 biohydrogen potential (BHP) assays, the seed inoculum was firstly heated in the autoclave at
118 100 °C for 30 min to inactivate methanogens and then acclimated with the modified medium

119 three times to activate the spore-forming hydrogenogens. The composition of the modified
120 medium for hydrogenogens acclimatization was detailed in a previous paper [23]. The TS and
121 VS content in the biohydrogen inoculum were 8.89% and 4.70%, respectively.

122

123 **2.2 Acid pre-treatment**

124 Briefly, 2 g dried grass silage was mixed with 100 ml dilute sulphuric acid in conical flasks.
125 The flasks were sealed with filter paper, and placed in an autoclave (Sanyo MLS 3780, Japan)
126 to allow for pre-treatment at different acid concentrations / temperatures / times. The pre-
127 treatment experiments of grass silage were performed in three groups in triplicate. Group 1:
128 variable H₂SO₄ concentration (0.5%, 1%, 2%, 4% w/w) at 135 °C for 15 min; Group 2:
129 variable heating temperature (95, 105, 115, 125, 135 °C) with 2% H₂SO₄ for 15 min; and
130 Group 3: variable heating time (5, 10, 15, 20, 25 min) with 2% H₂SO₄ at 135 °C. After these
131 three groups of experiments, an optimal condition leading to the maximum reducing sugar
132 yield was then determined, which was selected as the pre-treatment condition for the
133 subsequent fermentation experiments.

134

135 **2.3 Fermentation processes**

136 To compare the effect of acid pre-treatment on the biohydrogen and biomethane production
137 from silage, the assays of single-stage BMP for methane production and two-stage BHP-
138 BMP for hydrogen and methane co-production were conducted at mesophilic temperature
139 (37 °C). Fig. 1 illustrates the processes of single-stage and two-stage fermentation. The BHP
140 and BMP assays were conducted in triplicate using the Bioprocess Control systems (AMPTS
141 II, Sweden) equipped with 15 glass bottle fermenters. Two groups of substrates: 1) oven dried
142 untreated grass silage and 2) the solid-liquid mixture containing both the hydrolysate and the

143 solid residue of the pre-treated silage (abbreviated as pre-treated silage hereafter), were
144 subjected to both the single-stage BMP and the two-stage BHP-BMP assays.

145

146 For the single-stage BMP assays, 2 g untreated silage (equivalent to 1.63 g VS) or pre-treated
147 silage derived from 2 g untreated silage was added into each bottle along with 216.80 g
148 biomethane inoculum (at a VS ratio of inoculum to substrate of 2:1). The total working
149 volume in each bottle was made up to 420 ml with deionised water. The initial pH was
150 adjusted to 7.50 ± 0.05 with 1 M NaOH and 1 M HCl solutions. The single-stage BMP assays
151 ran for 30 days.

152

153 For the two-stage BHP-BMP assays, 2 g substrate (equivalent to 1.63 g VS) was added into
154 each bottle. The volume of the substrate in each bottle was adjusted to 180 ml with deionised
155 water. Then 20 ml biohydrogen inoculum was added so that the total working volume was
156 200 ml. The pH was adjusted to 7.0 ± 0.05 with 1 M NaOH and 1 M HCl solutions. After 4
157 days BHP assays, the pH of the effluents was adjusted to 7.50 ± 0.05 and then inoculated
158 with 216.80 g biomethane inoculum for the second-stage BMP assays. The total working
159 volume was made up to 420 ml with deionised water for each bottle. The second-stage BMP
160 assays ran for 26 days to ensure the overall duration of the two-stage fermentation was 30
161 days.

162

163 For both BHP and BMP assays, all the reactors were sealed and purged with N₂ before the
164 assays to ensure an anaerobic environment. A control group consisting of inoculum and
165 deionised water was set up for each trail to minimize the carryover effect of inoculum. The
166 hydrogen, methane and VFAs yields of the experimental groups were corrected by the yields
167 from the control group without substrates.

168 **2.4 Analytical methods**

169 The TS, VS, and ash content in the substrates and inoculum were analysed according to the
170 Standard Methods 2540 G (APHA, 2005). The elemental analysis was conducted using an
171 elemental analyser with a thermal conductivity detector (Exeter Analytical, CE 440 Model).
172 The harshness of the pre-treatment condition was quantified by the severity factor (SF),
173 determined by Eq. 1 [24]:

174
$$SF = \log \left(t \times e^{\frac{T_H - T_R}{14.75}} \right) - pH \quad (\text{Eq. 1})$$

175 where t, T_H, and T_R represent the heating time (min), hydrolysis temperature (°C) and
176 reference temperature (100 °C), respectively. The 3, 5-dinitrosalicylic acid method (DNS
177 method) [25] was employed to measure the total reducing sugar yield in the hydrolysate
178 derived from acid pre-treatment of grass silage. The content of monosaccharides,
179 disaccharides, furfural, and hydroxymethylfurfural in the hydrolysate were quantitatively
180 identified through a High Performance Liquid Chromatography (HPLC) equipped with a
181 Shodex sugar SH-1011 column, a refractive index detector, and a UV detector, with 0.005 M
182 H₂SO₄ at a flow rate of 0.5 mL/min as the mobile phase. The crude protein in grass silage
183 was calculated as 6.25 times the nitrogen content [26]. The content of cellulose,
184 hemicellulose, and lignin in the untreated silage and the solid residue of pre-treated silage
185 was determined according to a standard analysis procedure published by the National
186 Renewable Energy Laboratory [27]. Briefly, the samples were treated with 72% sulphuric
187 acid at 30°C for 1 h. Then the mixture was diluted to 4% sulphuric acid and hydrolysed at
188 121°C for 1 h. After this two-step hydrolysis, the content of glucose and xylose in the derived
189 hydrolysate was measured by an HPLC as described above. The content of cellulose,
190 hemicellulose, and lignin were calculated based on the sugar content in the hydrolysate and
191 the proximate composition of the solid residue. The total amount of glucose and xylose in the
192 hydrolysate from the two-step hydrolysis of untreated grass silage was considered the

193 theoretical value for reducing sugar yield in the pre-treatment process. The ratio of reducing
194 sugar yield in the pre-treatment process against the theoretical value was defined as the
195 hydrolysis efficiency. The concentrations of various VFAs in the effluents were measured
196 using a gas chromatography system (Agilent 7890 A, USA) equipped with the DB-FFAP
197 column (Φ 0.32 mm \times 50 m) and flame ionization detector. The surface morphology of the
198 untreated and pre-treated silage particles was observed using the scanning electron
199 microscope (SEM, Hitachi SU8010, Japan). The specific surface area was determined using
200 the Brunauer-Emmett-Teller (BET) method based on the nitrogen adsorption isotherm
201 obtained on a Micrometrics ASAP 2020 analyser. A Fourier transform infrared (FTIR)
202 spectrometer (Nicolet 5700, USA) was employed to analyse the chemical functional groups
203 in the silage before and after pre-treatment. X-ray diffraction (XRD) experiment on X'Pert
204 PRO was implemented to analyse the crystallinity of cellulose. The crystallinity index (CrI)
205 was calculated according to the Segal Formula [28]:

$$206 \text{ CrI (\%)} = (I_{002} - I_{18}) / I_{002} \times 100 \quad (\text{Eq. 2})$$

207 in which I_{002} is the peak diffraction intensity of crystalline cellulose at $2\theta = 22.0^\circ$ and I_{18} is
208 the diffraction intensity of amorphous cellulose at $2\theta = 18.2^\circ$.

209

210 **2.5 Energy calculations**

211 The energy value of the grass silage was calculated based on the modified Dulong Formula
212 [29]:

$$213 \text{ Energy value of biomass (kJ/kg)} = 337C + 1419 (H - 0.125O) + 23.26N \quad (\text{Eq. 3})$$

214 in which C, H, O, and N represent the weight percentages of each element in total VS. The
215 energy content in hydrogen and methane was defined as the combustion enthalpy of the gas
216 at standard conditions. The energy content in the VFAs was the sum of the combustion
217 enthalpy of each liquid component at standard conditions. Hydrogen energy efficiency was

241 where ρ ($1 \times 10^3 \text{ kg/m}^3$) is the density of substrates and inoculum mixture; V_s (m^3) is the input
 242 volume of the substrate and diluted acid; C ($4.18 \text{ kJ/kg/}^\circ\text{C}$) is the specific heat capacity; T_p
 243 ($^\circ\text{C}$) is the pre-treatment temperature; T_a ($^\circ\text{C}$) is the ambient temperature ($25 \text{ }^\circ\text{C}$); ϕ is the
 244 heat recovery efficiency; T_1 and T_2 (both $37 \text{ }^\circ\text{C}$) are temperatures for the first-stage dark
 245 fermentation and the single-stage / second-stage AD processes; k ($1 \text{ W/m}^2/^\circ\text{C}$) is the heat
 246 transfer coefficient; A_1 and A_2 (m^2) are the surface areas of the dark fermentation reactor and
 247 the AD reactor, respectively; τ_1 and τ_2 (d) are the effective production durations for dark
 248 fermentation and AD, respectively, which are defined as the fermentation durations for
 249 achieving 80% of the total gas (H_2 / CH_4) production. The coefficient (86.4) was used for unit
 250 conversion from W to kJ/d.

251

252 **3. Results and discussion**

253 **3.1 Effect of pre-treatment on reducing sugar and VFA release from silage**

254 Cellulose and hemicellulose can be hydrolysed into glucose and xylose through reactions R1
 255 and R2, respectively [33].



258 The theoretical value for reducing sugar yield was measured as 639.20 mg/g VS based on the
 259 two-step hydrolysis of untreated grass silage. Fig. 2 (a) to (c) show the dependence of
 260 reducing sugar yield and the hydrolysis efficiency on the sulphuric acid concentration,
 261 treatment temperature, and heating time, respectively. With the increase in acid concentration
 262 reducing sugar yield increased up to 2% and then fell. With the increase in treatment
 263 temperature reducing sugar yield increased up to $135 \text{ }^\circ\text{C}$. With the increase in heating time,
 264 the reducing sugar yield decreased beyond 15 minutes duration. Fig. 2 (d) shows the change

265 of reducing sugar yield with the SF. Within the investigated SF ranging from 0.6 to 2.1, the
266 optimum SF for the highest reducing sugar yield was 1.79. The optimum SF for dilute acid
267 pre-treatment of lignocellulosic biomass such as rice husk was reported within the range of
268 1.7 to 2.0 [24, 34]. Further increasing the SF beyond the optimal range will decrease sugar
269 recovery due to denaturation of sugars, which may necessitate additional treatment to remove
270 inhibitory by-products. Reducing sugar yield reached the highest value of 333.79 mg/g VS
271 corresponding to the highest hydrolysis efficiency of 52.2% with the optimal sulphuric acid
272 concentration of 2% w/w, temperature of 135 °C, and heating time of 15 min.

273

274 The main monosaccharides and disaccharides released at the optimal condition were then
275 identified as xylose, arabinose, glucose and cellobiose, as shown in Fig. 3. The total amount
276 of these sugars was 282.16 mg/g VS, in which xylose and arabinose accounted for 86.8%. It
277 has been observed that the degradation of hemicellulose is more preferable than that of
278 cellulose in mild acidic conditions [8, 35-37]. The presence of a larger amount of xylose in
279 the hydrolysate indicated that the hemicellulose fraction of grass silage was effectively
280 hydrolysed during acid pre-treatment, which was then proved by the compositional analysis
281 of the solid residue. In addition, acetic acid (59.2 mg/g VS) and propionic acid (4.4 mg/g VS)
282 were also generated during the pre-treatment. No hydroxymethylfurfural or furfural was
283 detected in the hydrolysates after pre-treatment at the optimal condition, supposed to be
284 due to the mild treatment temperature and short contact time [38, 39].

285

286 **3.2 Effects of pre-treatment on the properties and microstructures of grass silage**

287 The SEM images in Fig. S1 (see the supplementary material) show the surface morphological
288 changes of grass silage after pre-treatment. A rougher surface with more cracks was observed

289 after pre-treatment. The BET analysis showed that the specific surface area increased from
290 1.6 to 2.4 m²/g. The erosion of the compact surface and the increase in specific surface area
291 indicated the degradation of some structural components, allowing for improved bio-
292 accessibility.

293

294 Table 1 presents the characteristics of the untreated and pre-treated silage. The content of
295 cellulose, hemicellulose, and lignin in the untreated silage was 31.3%, 15.1%, and 27.9%,
296 respectively. After pre-treatment under the optimal condition, the remaining cellulose,
297 hemicellulose, and lignin content in the solid residue accounted for 37.6%, 0.0%, and 57.0%
298 of the dry mass, respectively. Hemicellulose was completely decomposed, whereas 44.7% of
299 cellulose and 6.6% of lignin were removed during the pre-treatment. Láinez et al. also
300 observed a complete hemicellulose hydrolysis and its efficient conversion into xylose when
301 applying dilute sulphuric acid pre-treatment on lignocellulosic biomass of *Agave salmiana*
302 leaves [40]. The complete removal of the hemicellulose fraction leaves the remaining lignin
303 as the primary barrier for cellulose accessibility. The crude protein content in the untreated
304 silage was 9.4% and decreased to 3.6% in the solid residue of the pre-treated grass silage. In
305 the pre-treatment process, proteins were converted to soluble compounds such as peptides
306 and amino acids [12, 41], which led to an increased C/N ratio in the solid residue.

307

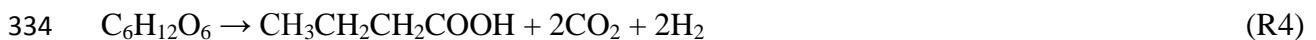
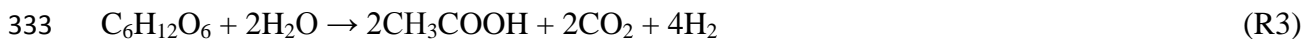
308 The changes in structural arrangement of the molecules in the pre-treated silage could be
309 evaluated by the FTIR spectra shown in Fig. S2 (see the supplementary material). There was
310 no significant change of the adsorption peak at 3448 cm⁻¹ band and the adsorption peak at
311 2950 cm⁻¹ band, which represented the O–H stretching of the hydrogen bonds and the C-H
312 stretching within methylene in the cellulose, respectively [34]. The pre-treated silage residue
313 presented an increase trend in the adsorption peaks at 2860, 1720, and 1251 cm⁻¹, which were

314 associated with lignin. This was ascribed to the fact that the acid pre-treatment removed
 315 larger amount of cellulose and hemicellulose, thus increased the proportional lignin content
 316 in the solid residue (Table 1). The enhanced adsorption at the bands of 1160 cm⁻¹, 1110 cm⁻¹,
 317 1060 cm⁻¹, and 895 cm⁻¹ [42] suggested that the cellulose content in pre-treated silage
 318 increased because the hemicellulose fraction was reduced. The ratio of crystalline cellulose to
 319 amorphous cellulose at 1110 cm⁻¹/895 cm⁻¹ reduced from 8.62 to 3.47 and the ratio of
 320 crystalline to amorphous cellulose at 1430 cm⁻¹/895 cm⁻¹ reduced from 4.77 to 0.87, which
 321 indicated a decreasing share of crystalline cellulose after the pre-treatment [42, 43]. The XRD
 322 analysis confirmed that cellulose crystallinity index of untreated silage was 32% and
 323 decreased to 27% after pre-treatment (see Fig. S3 in the supplementary material). The
 324 increase in amorphous cellulose in the pre-treated sample would reduce the cellulose
 325 recalcitrance, thus facilitating the utilisation by microbes during dark fermentation.

326

327 **3.3 Biohydrogen and VFA production in the first-stage dark fermentation**

328 The cellulose and hemicellulose have been broken down to reducing sugars in the hydrolysis
 329 step. During the acidogenesis step, the monosaccharides are converted to gaseous metabolic
 330 products (such as H₂ and CO₂) and soluble metabolic products (VFAs and alcohols) through
 331 acidogenic microorganisms [44]. The fermentation pathways of glucose and xylose to VFAs
 332 and hydrogen can be expressed by reactions R3 to R6 [45]:



337 It has been demonstrated that 5-C sugar (such as xylose) is more difficult to be used as
338 compared to 6-C sugar (such as glucose). For example, the peak time of hydrogen production
339 from xylose (48 h) was twice as long as that from glucose (24 h) [45].

340

341 The cumulative H₂ yields in the 4-day dark fermentation are shown in Fig. 4 (a). Limited
342 hydrogen (17.47 ml/g) was produced from untreated silage owing to the recalcitrant structure
343 of silage solids. Hydrogen yield was improved by 3 fold reaching 68.26 ml/g VS after pre-
344 treatment. This result was in line with previous studies on fermentative hydrogen production
345 from silage, in which the maximum hydrogen yield of 72.21 mL/g dry silage was achieved
346 with 4% HCl pre-treatment [8] and a yield of 53 mL/g dry silage was achieved with 1% HCl
347 acid pre-treatment [46]. The specific hydrogen yield achieved with acid pre-treatment in this
348 study was higher compared to those with other pre-treatments, such as the yields of 42.2
349 mL/g dry silage with the combined ultrasound and acid pre-treatment [10], 32 mL/g dry
350 silage with ionizing radiation pre-treatment [46], and 6.7–34.5 mL/g VS with alkaline pre-
351 treatment [47]. Sivagurunathan et al. also found that H₂SO₄ pre-treatment method had a much
352 more significant effect on the improvement of biohydrogen production from *Gelidium*
353 *amansii* compared to other acid pre-treatment methods [48].

354

355 As shown in Fig. 4 (b), the production rates of hydrogen peaked within 24 hours after the
356 start-up. The peak production rate from untreated silage was 1.0 ml/g VS/h, while the peak
357 rate doubled after pre-treatment. The enhancement of hydrogen yield and production rate was
358 attributed to the solubilisation of carbohydrates in the silage and provision of more accessible
359 structure for the microbes after pre-treatment.

360

361 Fig. 5 illustrates the changes of VFAs distribution during the dark fermentation. The
 362 metabolites included mainly acetic acid, propionic acid, and butyric acid, with small amounts
 363 of iso-butyric acid, iso-valeric acid, valeric acid, and caproic acid. The total VFAs in the
 364 effluents were measured as 839.3 (equivalent to 103.2 mg/g VS) and 1084.1 mg/L
 365 (equivalent to 133.3 mg/g VS) produced from untreated and pre-treated silage, respectively.
 366 The total energy contents in the VFAs from untreated and pre-treated silage were 2.15 and
 367 2.20 kJ/g VS, respectively. The concentration of acetic acid was predominant and gradually
 368 increased during the fermentation process, indicating an acetic acid type fermentation. At the
 369 end of untreated silage fermentation, the share of acetic and butyric acids in the VFAs was
 370 59.0% and the share of propionic and iso-valeric acids was 17.5%. The formation of
 371 propionic, iso-valeric and caproic acids during fermentation was characterised as hydrogen
 372 consuming pathway; for example, the production of 1 mole propionic acid requires 1 mole
 373 hydrogen ($C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$) [49]. This would lead to a much
 374 lower experimental hydrogen yield than the theoretical values. Acetic and butyric acids
 375 accounted for 98.2% of total VFAs produced from pre-treated silage, indicating a more
 376 efficient fermentation pathway for hydrogen production after pre-treatment.

377

378 **3.4 Biomethane production from both single- and two-stage fermentation**

379 During the final methanogenesis step, acetic acid can be directly utilized by acetoclastic
 380 methanogens to produce methane through R7. Butyric acid can be first oxidized to acetic acid
 381 through R8, and then converted to methane. The produced CO₂ and H₂ can be consumed by
 382 hydrogenotrophic methanogens to produce methane through R9.





386

387 Based on the elemental analysis, the theoretical methane potential of the untreated silage was
388 499 mL CH₄/g VS. According to the results of the single-stage BMP assays displayed in Fig.
389 6, the cumulative methane yield from the untreated silage was 261.00 mL CH₄/g VS,
390 corresponding to 52.3% of the theoretical value. The bioconversion of the silage to methane
391 was slightly lower compared to the corresponding value of 62% found by Tsapekos [50], but
392 in accordance with the 53% biodegradable index of late first cut grass silage reported in our
393 previous paper [22]. The low biodegradable index of the silage could be attributed to the
394 increase in fibre components with an advancing harvest date [51]. Acid pre-treatment was
395 expected to enhance the biomethane yield from silage by solubilizing hemicellulose.
396 However, the specific methane yield from pre-treated silage was 237.10 ml/g VS, accounting
397 for 47.5% of the theoretical yield. Similar inhibition effects caused by diluted H₂SO₄ or
398 NaOH pre-treatment were reported by Venturin [42] and Pakarinen [14]. On one hand, acid
399 pre-treatment could break down the recalcitrant structure of the biomass to accelerate the
400 hydrolysis process and release water soluble sugars. For this reason, the peak methane
401 production rate slightly increased from 64.0 to 66.5 ml/g VS/d after pre-treatment. The
402 methane production rate of pre-treated silage peaked on the first day of the single-stage
403 anaerobic digestion duration, a day before that of the untreated silage. On the other hand,
404 inhibitors such as hydroxymethylfurfural and furfural may form through the degradation of
405 glucose or through reactions of the intermediate products of the pre-treatment, which is
406 unfavourable to the fermentation [52, 53]. Another reason for the reduced methane yield is
407 sodium inhibition caused by the extra addition of NaOH for neutralizing acidity at the start-
408 up. In this single-stage anaerobic digestion of pre-treated silage, the pre-treatment condition
409 could result in an extra Na⁺ concentration of 4.37 g/L, much higher than the reported

410 beneficial sodium concentration 100–200 mg/L for the growth of mesophilic anaerobes [54].
411 Sodium cation had been reported to cause moderate inhibition at 3.5–5.5 g/L [55]. A negative
412 linear relationship between specific methane yield and Na⁺ concentration during pre-
413 treatment was also obtained by Kang [56].

414

415 The two-stage process resulted in a methane yield of 392.84 ml/g VS from untreated silage,
416 an increase of 50% compared to the single-stage process (Fig. 7a). The methane yield from
417 pre-treated silage increased by 28% and achieved 304.39 ml/g VS. The methane production
418 rates in the second-stage anaerobic digestion kept increasing until peaked on the fourth day
419 (Fig. 7b). The peak methane production rate of untreated silage in the second-stage anaerobic
420 digestion was 79.9 ml/g VS/d, an increase of 25% compared to the single-stage process. The
421 peak methane production rate of pre-treated silage achieved 71.5 ml/g VS/d in the second
422 stage, an increase of 7% compared to the single-stage process. The higher methane yields and
423 peak production rates in the two-stage process were attributed to the enhanced hydrolysis of
424 the solid substrates and VFA production in the former dark fermentation stage.

425

426 **3.5 Energy conversion efficiency and consumption**

427 The theoretical total energy conversion efficiency, defined as the ratio of energy content in
428 the gaseous biofuel products (H₂ + CH₄) to the energy content in the substrate, can be
429 calculated according to the simplified reactions representing the processes [57]; glucose is
430 used to exemplify here. The maximum total energy conversion efficiency of glucose to CH₄
431 in a single-stage anaerobic digestion is calculated as 94.78% based on the global reaction R10;
432 and in the two-stage fermentation is 103.80% based on the global reaction R11. This
433 indicates that two-stage process favours energy recovery from gaseous biofuels production.





436

437 In this study, due to the low biodegradability of the dried silage solids, the total energy
438 conversion efficiency of the single-stage anaerobic digestion was lower than that of the two-
439 stage process, as shown in Table 2. In single-stage fermentation, untreated silage exhibited an
440 efficiency of 54.7%, while pre-treated silage showed a decreased efficiency of 49.7% due to
441 Na^+ inhibition. Owing to the enhanced hydrogen and methane yields, the total energy
442 conversion efficiencies of the two-stage processes were enhanced to 83.5% and 68.4% for the
443 untreated and pre-treated silage, respectively. In the two-stage process, pre-treatment
444 significantly enhanced hydrogen yield, but the energy efficiency decreased due to the low
445 biomethane yield from the second stage anaerobic digestion process. The energy content in
446 hydrogen only accounted for 4.6% of the total energy value in the biomass. In the untreated
447 silage case, the hydrogen energy accounted for 1.2% of the total energy in the biomass.

448

449 As shown in Table 3, the energy consumption calculated based on the batch experimental
450 data presented a higher value compared to the larger-scale AD process [58], as the small-
451 scale batch reactors resulted in a great heat loss during the fermentation processes.

452 Nonetheless, the comparison in this study was still of great use to help distinguish different
453 fermentation processes from the perspective of energy consumption. With heat recovery from
454 the pre-treatment, the energy consumed for the pre-treatment operation accounted for a small
455 part of the total energy consumption. Most of the energy was consumed in the operation of
456 the AD processes. In both single-stage and two-stage fermentation processes, the pre-
457 treatment saved energy input for the operation of AD processes due to the shorter effective
458 production durations. In two-stage fermentation processes of both untreated and pre-treated
459 grass silage, the increment of hydrogen and methane yields was not sufficient to cover the

460 increment of energy consumption for the process operation due to the prolonged effective
461 production durations.

462

463 From these results, it can be concluded that the optimal acid pre-treatment process (2%
464 H₂SO₄, 135°C, 15 minutes) is a promising method to remove hemicellulose, release reducing
465 sugars from grass silage and enhance H₂ and VFA yields and production rate in dark
466 fermentation. In contrast, acid pre-treatment at the optimal condition slightly inhibited CH₄
467 yield in anaerobic digestion, possibly due to the increased Na⁺ concentration. This
468 phenomenon indicated that the single indicator of reducing sugar yield may not be sufficient
469 for evaluating the effect of pre-treatment. Further studies may identify the inhibitors and
470 optimise the pre-treatment process towards a maximum BMP target rather than a maximum
471 reducing sugar yield. Despite the slight decrease in CH₄ yield, the acid pre-treatment
472 positively reduced the energy consumed for operating the AD process. This was ascribed to
473 the fact that it accelerated the hydrolysis of biomass and resulted in a shorter digestion
474 duration. The increase in production rate has the potential to increase methane production in
475 the continuous digesters, especially when a shorter retention time is applied. However, acid
476 pre-treatment at elevated temperatures introduces extra costs, including for acid, pH buffering
477 agent, heating, and labour; this is the main bottleneck in implementing acid pre-treatment in
478 AD plants. Acid pre-treatment process should be designed in a way that the increment in
479 methane production can provide enough energy for pre-treatment requirements and cover the
480 increased operation costs.

481

482 **4. Conclusions**

483 This study demonstrated that two-stage (H₂ + CH₄) digestion of grass silage could lead to
484 higher biofuel yields than single-stage (CH₄) digestion. By applying acid pre-treatment, the

485 optimal condition resulted in the highest hydrogen yield of 68.26 ml/g VS in the first stage
486 hydrogen fermentation (a 3-fold increase compared to untreated silage). However, in the
487 second stage anaerobic digestion, the pre-treated silage showed a 22.5% decrease in
488 biomethane production, leading to a decreased total energy efficiency of 68.4% as compared
489 to 83.5% for untreated silage. In comparison, single-stage anaerobic digestion showed lower
490 energy conversion efficiencies of 49.7% and 54.2% for the pre-treated and untreated silage,
491 respectively. Despite the slight decrease in CH₄ yield, the acid pre-treatment reduced the
492 energy consumption for the operation of the anaerobic digestion process due to a shorter
493 digestion duration.

494

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502

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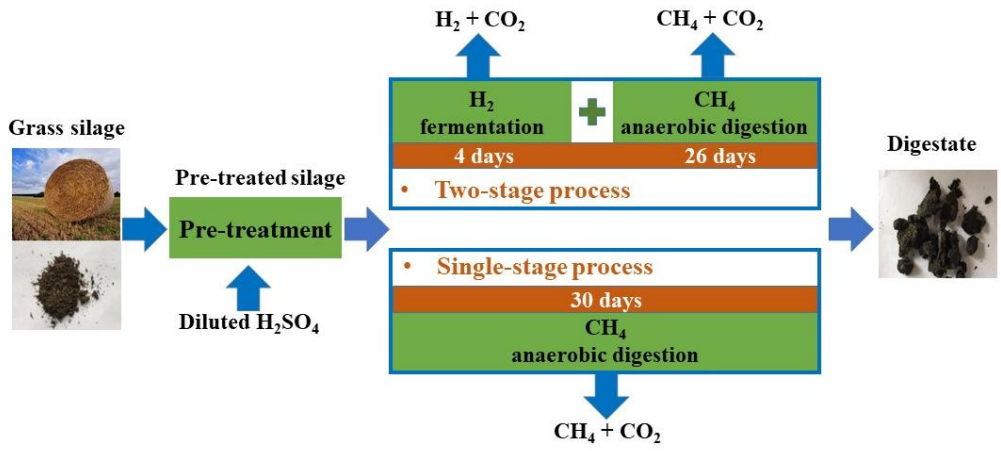
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17

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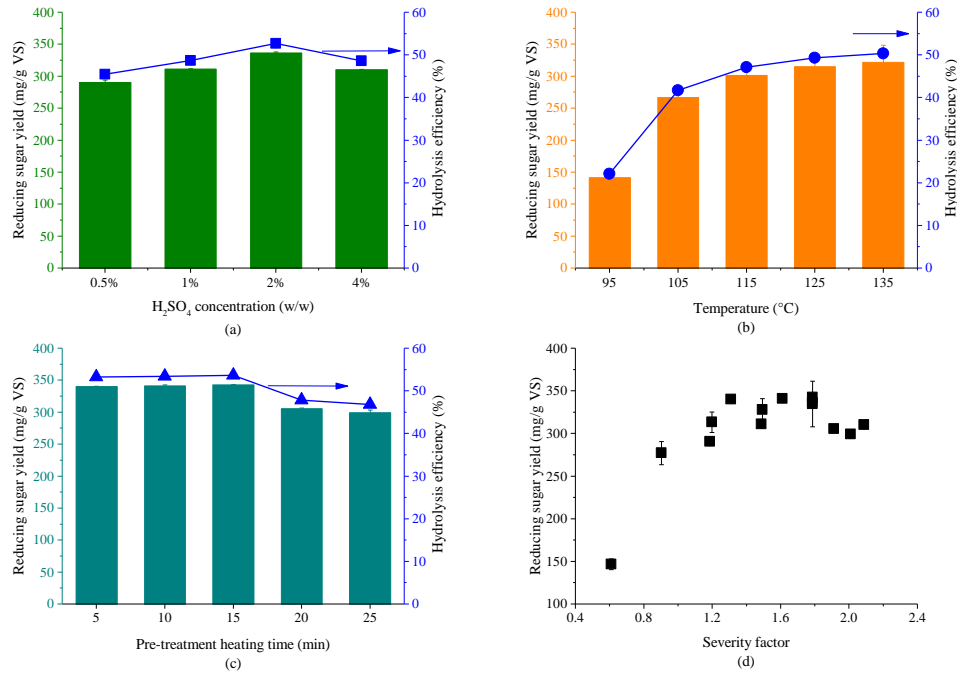
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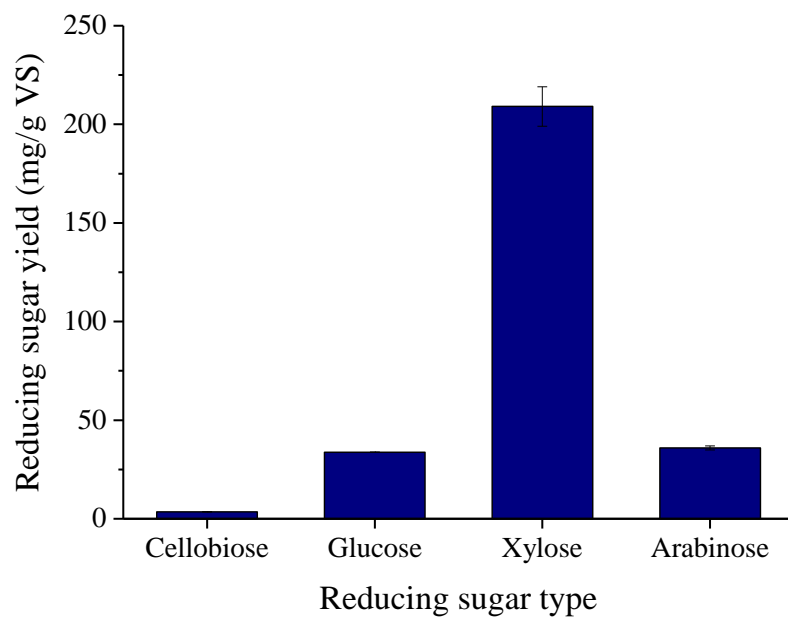
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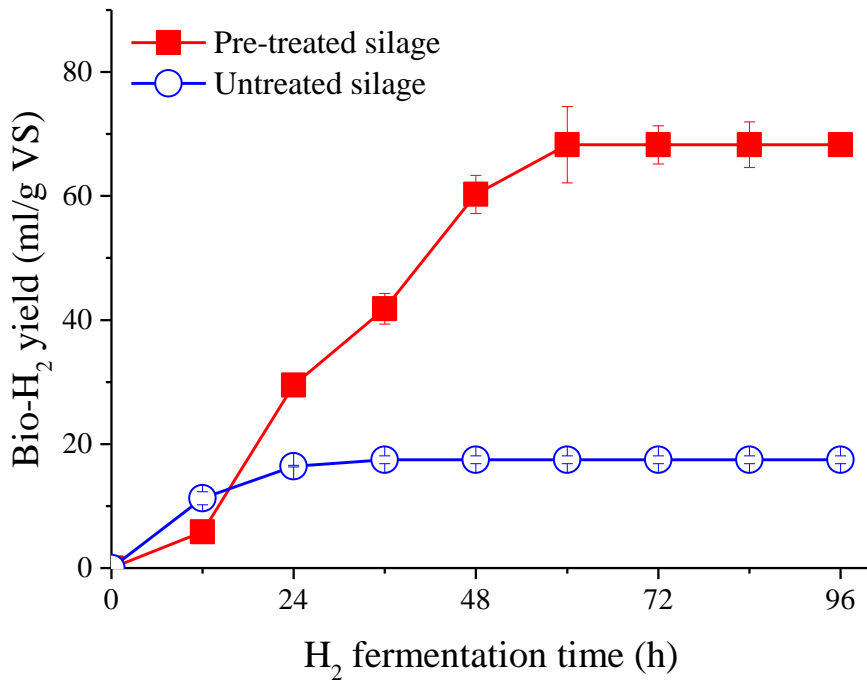
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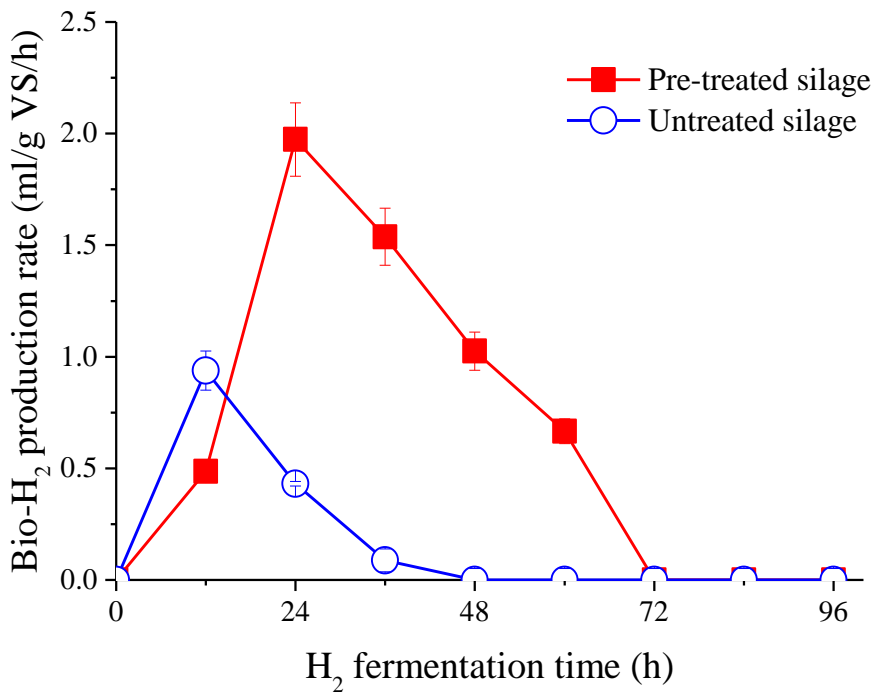
33 Fig. 3. The contents of reducing sugar in the hydrolysate under the optimal pre-treatment

34 condition.



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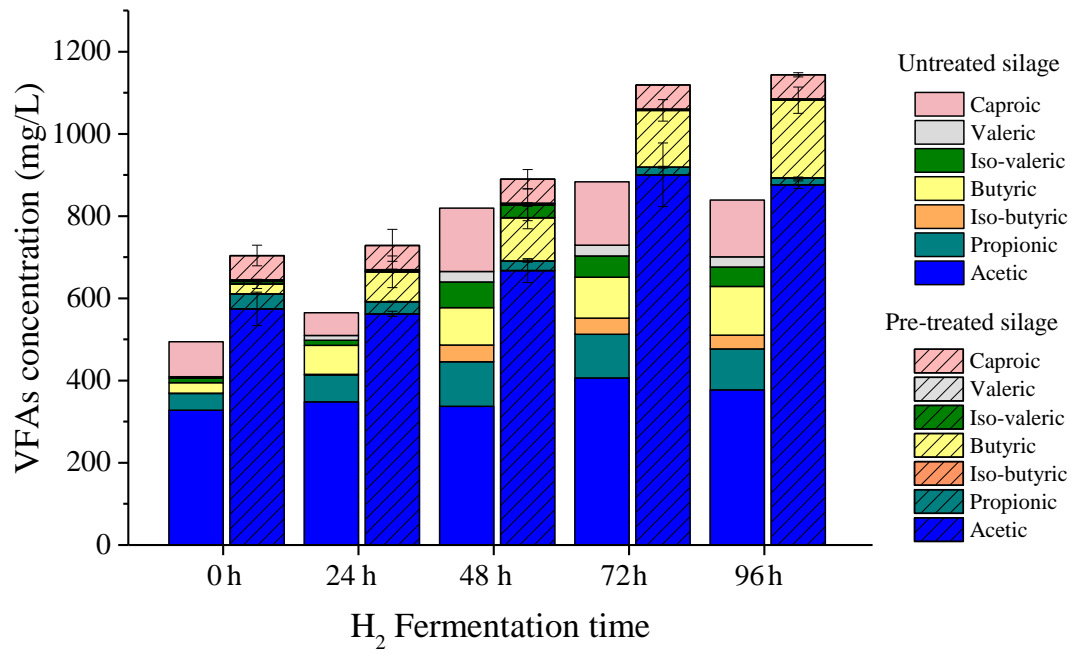
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(b)

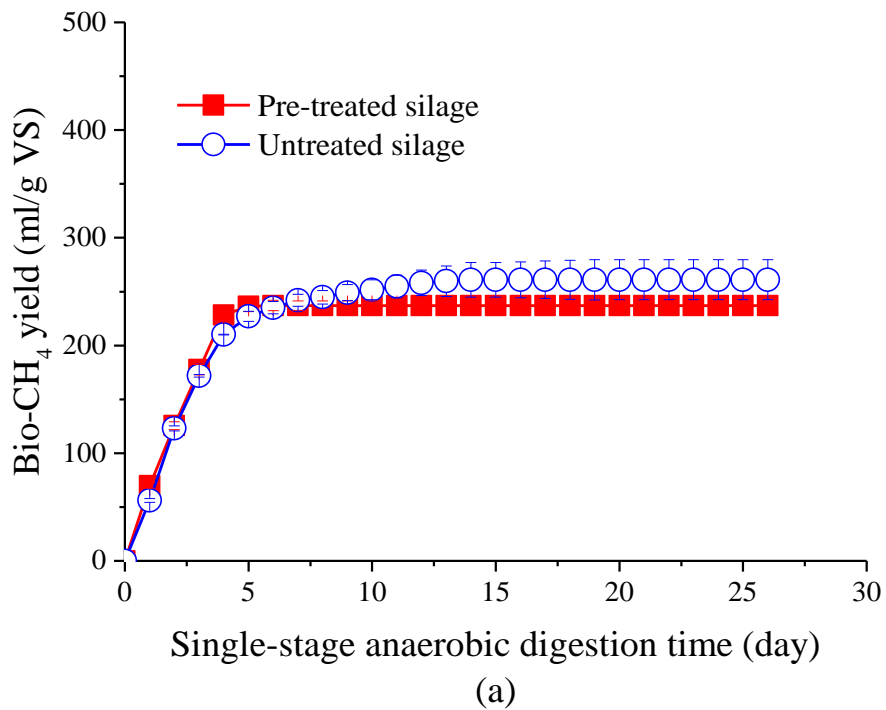
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37 Fig. 4. (a) Biohydrogen yield and (b) biohydrogen production rate in the first-stage dark
38 fermentation.

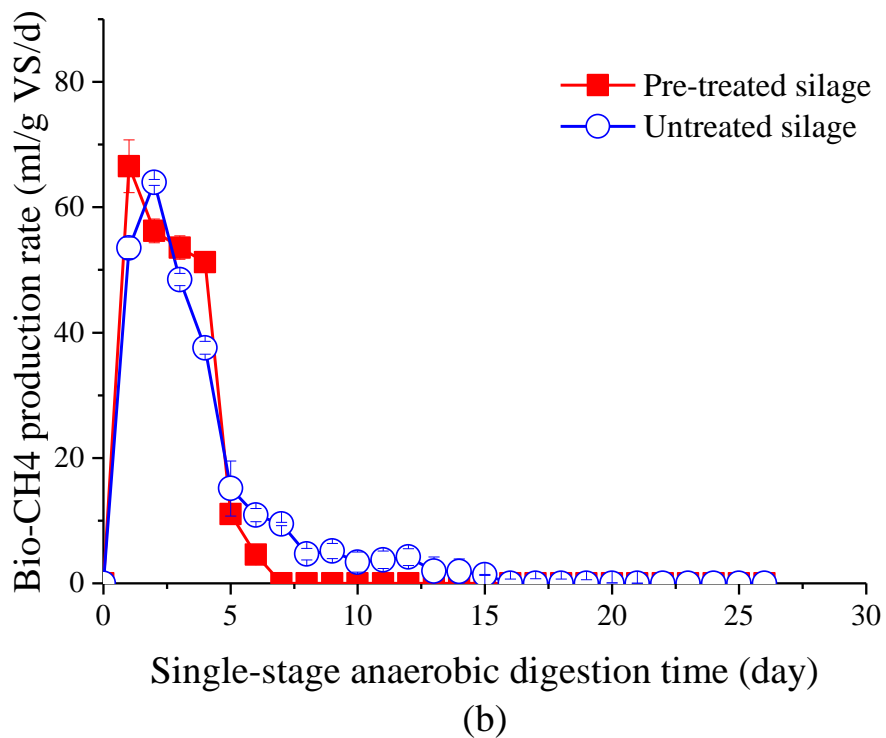


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41 Fig. 5. Concentration of the volatile fatty acids (VFAs) during the first-stage dark
42 fermentation.

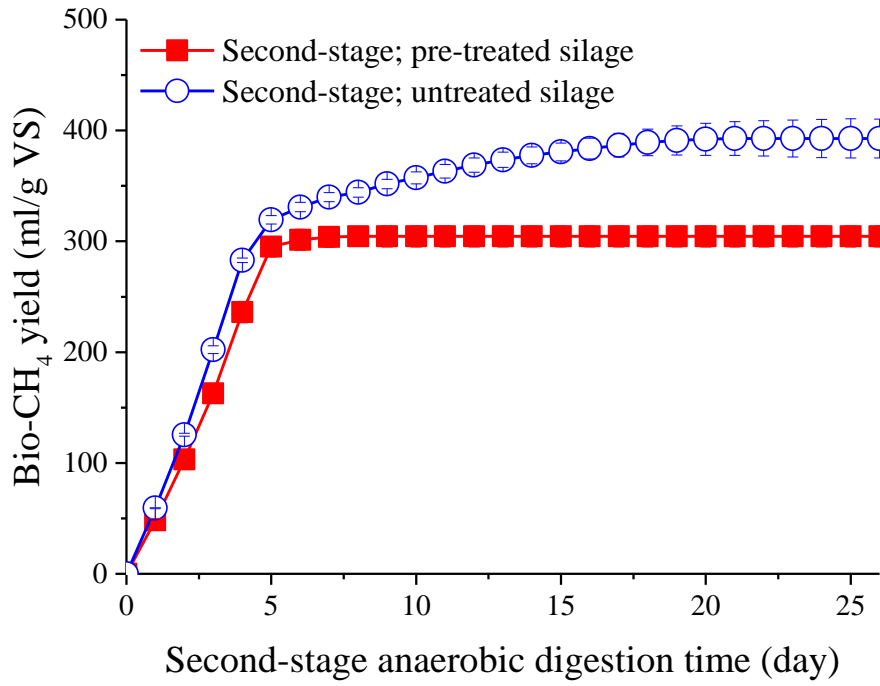


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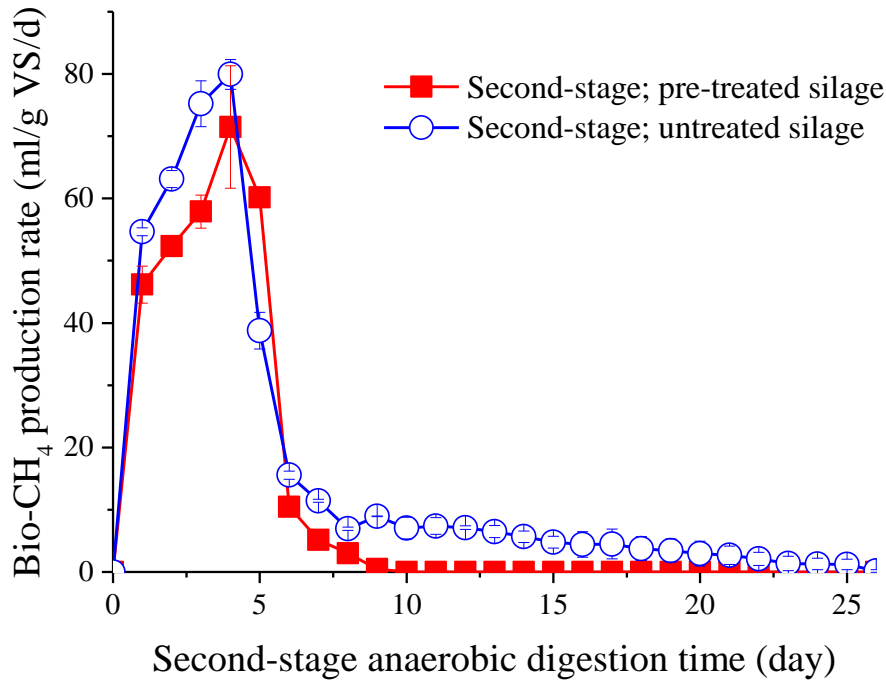
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45 Fig. 6. (a) Biomethane yield and (b) biomethane production rate in the single-stage anaerobic
 46 digestion.



(a)

47



(b)

48

49 Fig. 7. (a) Biomethane yield and (b) biomethane production rate in the second-stage

50 anaerobic digestion.

51 Table 1 Compositional characteristics of grass silage.

	Untreated silage	Pre-treated silage (solid residue)
Pre-treatment condition	None	2% H ₂ SO ₄ , 135 °C, 15 min
Solid recovery (% TS)	100	50.20±0.06
Proximate analysis (wt %)		
TS	91.1±1.3	55.5±0.5
VS	81.3±0.1	51.1±0.4
VS/TS	89.1±1.0	92.0±0.1
Ash/TS	10.9±0.1	8.1±0.1
Ultimate analysis (% VS)		
Carbon	50.5±0.2	39.7±0.3
Hydrogen	6.5±0.0	3.8±0.0
Oxygen	41.3±0.1	55.9±0.2
Nitrogen	1.7±0.2	0.6±0.1
C/N mass ratio	29.7	62.2
Biological analysis (% TS)		
Cellulose	31.3±0.5	37.6±0.6
Hemicellulose	15.1±1.0	0.0±0.0
Lignin	27.9±3.0	57.0±0.1
Crude protein	9.4±0.9	3.6±0.3
Energy value (kJ/g VS)	18.9	
Theoretical biomethane yield (ml/g VS)	499	

52

53 Table 2 Energy conversion efficiency of single-stage anaerobic digestion (AD) and two-stage
 54 dark fermentation + AD.

Process	Substrate	H ₂ yield (ml/g VS)	CH ₄ yield (ml/g VS)	Biodegradabili ty index	H ₂ energy efficienc y	Total Energy efficiency
Single-stage AD	Pre-treated silage	/	237.10	47.5%	/	49.7%
	Untreated silage	/	261.00	52.3%	/	54.7%
Two-stage dark fermentation + AD	Pre-treated silage	68.26	304.39	61.0%	4.6%	68.4%
	Untreated silage	17.47	392.84	78.7%	1.2%	83.5%

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56 Table 3 Energy consumption for the operation of single-stage and two-stage fermentation
 57 processes with / without pre-treatment.

Process	Substrate	Effective production duration (day)		Energy consumption in different processes (kJ/g VS)			Total energy consumption Q_{cons} (kJ/g VS)
		Dark fermentation	AD	Pre-treatment with heat recovery	Dark fermentation		
					Dark	AD	
Single-stage AD	Pre-treated silage	0	3.2	5.58	0	246.82	252.40
	Untreated silage	0	4.0	0	0	304.72	304.72
Two-stage dark fermentation + AD	Pre-treated silage	1.7	4.0	5.58	91.16	304.72	401.46
	Untreated silage	0.8	5.0	0	39.07	380.90	419.97

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