

Title	Can acid pre-treatment enhance biohydrogen and biomethane production from grass silage in single-stage and two-stage fermentation processes?
Authors	Deng, Chen;Lin, Richen;Cheng, Jun;Murphy, Jerry D.
Publication date	2019-05-23
Original Citation	Deng, C., Lin, R., Cheng, J. and Murphy, J. D. (2019) 'Can acid pre-treatment enhance biohydrogen and biomethane production from grass silage in single-stage and two-stage fermentation processes?', Energy Conversion and Management, 195, pp. 738-747. doi: 10.1016/j.enconman.2019.05.044
Type of publication	Article (peer-reviewed)
Link to publisher's version	http://www.sciencedirect.com/science/article/pii/S0196890419305990 - 10.1016/j.enconman.2019.05.044
Rights	© 2019, Elsevier Ltd. All rights reserved. This manuscript version is made available under the CC BY-NC-ND 4.0 license. - https://creativecommons.org/licenses/by-nc-nd/4.0/
Download date	2026-08-26 08:40:51
Item downloaded from	https://hdl.handle.net/10468/8025

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

Chen Deng ^{a, b}, Richen Lin ^{a, b*}, Jun Cheng ^c, Jerry D Murphy ^{a, b}

^a MaREI Centre, Environmental Research Institute, University College Cork, Cork, Ireland

^b School of Engineering, University College Cork, Cork, Ireland

^c State Key Laboratory of Clean Energy Utilization, Zhejiang University, Hangzhou, China

Abstract

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

* Corresponding author: Dr. Richen Lin, MaREI Centre, Environmental Research Institute, University College Cork, Ireland. Tel.: +353 (0)21 490 1948. Email: richen.lin@ucc.ie

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

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

1. Introduction

1.1 Grass silage as a resource for biofuel production

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

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

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

1.2 Pre-treatment of grass for biohydrogen and biomethane production

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

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

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

1.3 Objectives

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

2. Material and methods

2.1 Feedstock and inoculum

The grass silage was sourced from late-cut perennial ryegrass. The grass was initially field wilted for 24 h and ensiled for 5 weeks in 1.2 m diameter cylindrical bales wrapped in polyethylene stretch-film [20]. Then the silage was re-wrapped and stored at approximately 18–20 °C in our lab. Before use, the silage was dried at 40 °C for 72 h and subsequently ground into fine particles with diameters of 1–2 mm. The silage was then stored at 4 °C until required. It should be noted that in this work we are dealing with dried silage, which differs from wet silage. In the process of drying silage volatilization of the liquid phase causes a significant loss of volatile compounds. The volatility coefficients in the drying process at 60 °C were reported as 0.09, 0.55 and 0.99 for lactic acid, volatile fatty acids and alcohol fermentation products, respectively [21]. The biodegradation efficiency of the liquid silage can achieve 92% [22], much higher compared to the conversion efficiency of the solid silage. As such recalcitrance is associated with the solid silage. Thus, it is expected that fermentation of solid silage will present a lower specific H_2 / CH_4 yield than the whole silage and these studies will outline how best to overcome recalcitrance in, and enhance gaseous biofuel yields from, grass silage.

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

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

2.2 Acid pre-treatment

Briefly, 2 g dried grass silage was mixed with 100 ml dilute sulphuric acid in conical flasks. The flasks were sealed with filter paper, and placed in an autoclave (Sanyo MLS 3780, Japan) to allow for pre-treatment at different acid concentrations / temperatures / times. The pre-treatment experiments of grass silage were performed in three groups in triplicate. Group 1: variable H_2SO_4 concentration (0.5%, 1%, 2%, 4% w/w) at 135 °C for 15 min; Group 2: variable heating temperature (95, 105, 115, 125, 135 °C) with 2% H_2SO_4 for 15 min; and Group 3: variable heating time (5, 10, 15, 20, 25 min) with 2% H_2SO_4 at 135 °C. After these three groups of experiments, an optimal condition leading to the maximum reducing sugar yield was then determined, which was selected as the pre-treatment condition for the subsequent fermentation experiments.

2.3 Fermentation processes

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

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

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

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

For both BHP and BMP assays, all the reactors were sealed and purged with N_2 before the assays to ensure an anaerobic environment. A control group consisting of inoculum and deionised water was set up for each trail to minimize the carryover effect of inoculum. The hydrogen, methane and VFAs yields of the experimental groups were corrected by the yields from the control group without substrates.

2.4 Analytical methods

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

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

where t , T_H , and T_R represent the heating time (min), hydrolysis temperature (°C) and reference temperature (100 °C), respectively. The 3, 5-dinitrosalicylic acid method (DNS method) [25] was employed to measure the total reducing sugar yield in the hydrolysate derived from acid pre-treatment of grass silage. The content of monosaccharides, disaccharides, furfural, and hydroxymethylfurfural in the hydrolysate were quantitatively identified through a High Performance Liquid Chromatography (HPLC) equipped with a Shodex sugar SH-1011 column, a refractive index detector, and a UV detector, with 0.005 M H_2SO_4 at a flow rate of 0.5 mL/min as the mobile phase. The crude protein in grass silage was calculated as 6.25 times the nitrogen content [26]. The content of cellulose, hemicellulose, and lignin in the untreated silage and the solid residue of pre-treated silage was determined according to a standard analysis procedure published by the National Renewable Energy Laboratory [27]. Briefly, the samples were treated with 72% sulphuric acid at 30°C for 1 h. Then the mixture was diluted to 4% sulphuric acid and hydrolysed at 121°C for 1 h. After this two-step hydrolysis, the content of glucose and xylose in the derived hydrolysate was measured by an HPLC as described above. The content of cellulose, hemicellulose, and lignin were calculated based on the sugar content in the hydrolysate and the proximate composition of the solid residue. The total amount of glucose and xylose in the hydrolysate from the two-step hydrolysis of untreated grass silage was considered the

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

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

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

2.5 Energy calculations

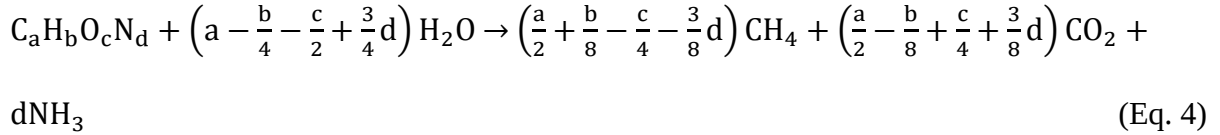
The energy value of the grass silage was calculated based on the modified Dulong Formula [29]:

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

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

defined as the ratio of energy content in hydrogen to the total energy in the biomass. Total energy conversion efficiency was defined as the ratio of the total energy content in the produced hydrogen and methane to the energy value in the biomass substrate.

Theoretical methane yield was calculated according to the Buswell Equation Eq. 4 [30]:



The biodegradability index (BI) was defined as the ratio of methane yield in the BMP assay to the theoretical methane yield.

Energy consumed for the operation of different processes with / without pre-treatment was analysed. In order to simplify the calculations, the following assumptions were made [31, 32]: 1) the specific heat capacity and density of the mixed substrates and inoculum were similar to those of water; 2) the ambient temperature was constant; 3) the autoclave was made of insulation materials and the heat loss during pre-treatment was negligible; 4) the heat consumed for the pre-treatment could be recovered for the operation of fermentation processes through a heat exchanger with an estimated heat recovery efficiency of 85%; 5) heat loss through the digester wall was taken into account and the heat transfer coefficient (k) was assumed as 1 W/m²/°C; 6) the surface area of the digester wall was calculated from the working volume, considering a diameter of 0.1 m in this study. The total energy consumed for the operation of pre-treatment and fermentation processes (Q_{cons}) was calculated according to Equation 5.

$$Q_{\text{cons}} = \rho V_s C (T_p - T_a) - \phi \rho V_s C (T_p - T_1) + 86.4 k A_1 \tau_1 (T_1 - T_a) + 86.4 k A_2 \tau_2 (T_2 - T_a) \quad (\text{Eq. 5})$$

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

3. Results and discussion

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

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



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

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

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

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

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

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

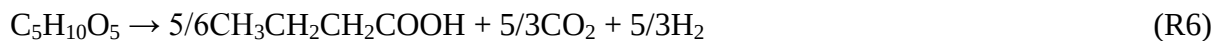
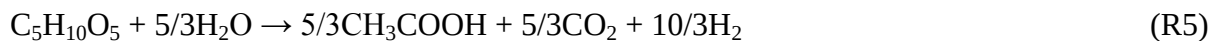
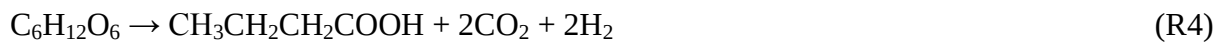
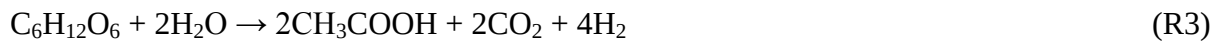
Table 1 presents the characteristics of the untreated and pre-treated silage. The content of cellulose, hemicellulose, and lignin in the untreated silage was 31.3%, 15.1%, and 27.9%, respectively. After pre-treatment under the optimal condition, the remaining cellulose, hemicellulose, and lignin content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

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

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

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

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



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

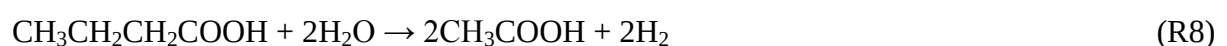
The cumulative H₂ yields in the 4-day dark fermentation are shown in Fig. 4 (a). Limited hydrogen (17.47 ml/g) was produced from untreated silage owing to the recalcitrant structure of silage solids. Hydrogen yield was improved by 3 fold reaching 68.26 ml/g VS after pre-treatment. This result was in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

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

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

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

During the final methanogenesis step, acetic acid can be directly utilized by acetoclastic methanogens to produce methane through R7. Butyric acid can be first oxidized to acetic acid through R8, and then converted to methane. The produced CO_2 and H_2 can be consumed by 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

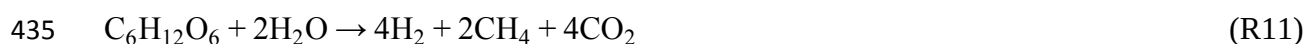
beneficial sodium concentration 100–200 mg/L for the growth of mesophilic anaerobes [54]. Sodium cation had been reported to cause moderate inhibition at 3.5–5.5 g/L [55]. A negative linear relationship between specific methane yield and Na^+ concentration during pre-treatment was also obtained by Kang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

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

3.5 Energy conversion efficiency and consumption

The theoretical total energy conversion efficiency, defined as the ratio of energy content in the gaseous biofuel products ($\text{H}_2 + \text{CH}_4$) to the energy content in the substrate, can be calculated according to the simplified reactions representing the processes [57]; glucose is used to exemplify here. The maximum total energy conversion efficiency of glucose to CH_4 in a single-stage anaerobic digestion is calculated as 94.78% based on the global reaction R10; and in the two-stage fermentation is 103.80% based on the global reaction R11. This 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

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

From these results, it can be concluded that the optimal acid pre-treatment process (2% H_2SO_4 , 135°C, 15 minutes) is a promising method to remove hemicellulose, release reducing sugars from grass silage and enhance H_2 and VFA yields and production rate in dark fermentation. In contrast, acid pre-treatment at the optimal condition slightly inhibited CH_4 yield in anaerobic digestion, possibly due to the increased Na^+ concentration. This phenomenon indicated that the single indicator of reducing sugar yield may not be sufficient for evaluating the effect of pre-treatment. Further studies may identify the inhibitors and optimise the pre-treatment process towards a maximum BMP target rather than a maximum reducing sugar yield. Despite the slight decrease in CH_4 yield, the acid pre-treatment positively reduced the energy consumed for operating the AD process. This was ascribed to the fact that it accelerated the hydrolysis of biomass and resulted in a shorter digestion duration. The increase in production rate has the potential to increase methane production in the continuous digesters, especially when a shorter retention time is applied. However, acid pre-treatment at elevated temperatures introduces extra costs, including for acid, pH buffering agent, heating, and labour; this is the main bottleneck in implementing acid pre-treatment in AD plants. Acid pre-treatment process should be designed in a way that the increment in methane production can provide enough energy for pre-treatment requirements and cover the increased operation costs.

4. Conclusions

This study demonstrated that two-stage ($\text{H}_2 + \text{CH}_4$) digestion of grass silage could lead to higher biofuel yields than single-stage (CH_4) digestion. By applying acid pre-treatment, the

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

Acknowledgements

This study was funded by Science Foundation Ireland (SFI) through the Centre for Marine and Renewable Energy (MaREI) under Grant No. 12/RC/2302 and 16/SP/3829. This work was also supported by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant (No. 797259) and the Environmental Protection Agency – Ireland (EPA 2018-RE-MS-13). Industrial co-funding from Gas Networks Ireland through the Gas Innovation Group is also gratefully acknowledged.

References

- [1] DIRECTIVE (EU) 2018/2001 on the promotion of the use of energy from renewable sources (Renewable Energy Directive 2018/2001).
- [2] SEAI. SEAI Assessment of Cost and Benefits of Biogas and Biomethane in Ireland. [https://www.seai.ie/resources/publications/Assessment of Cost and Benefits of Biogas and Biomethane in Ireland](https://www.seai.ie/resources/publications/Assessment%20of%20Cost%20and%20Benefits%20of%20Biogas%20and%20Biomethane%20in%20Ireland). (2017).

509 [3] R. Bedoić, L. Čuček, B. Ćosić, D. Krajnc, G. Smoljanić, Z. Kravanja, et al. Green
510 biomass to biogas – A study on anaerobic digestion of residue grass. *Journal of Cleaner*
511 *Production*. 213 (2019) 700-9.

512 [4] A. Lehtomaki, S. Huttunen, T.M. Lehtinen, J.A. Rintala. Anaerobic digestion of grass
513 silage in batch leach bed processes for methane production. *Bioresour Technol*. 99 (2008)
514 3267-78.

515 [5] M. Seppala, T. Paavola, A. Lehtomaki, J. Rintala. Biogas production from boreal
516 herbaceous grasses--specific methane yield and methane yield per hectare. *Bioresour Technol*.
517 100 (2009) 2952-8.

518 [6] H. Himanshu, J.D. Murphy, J. Grant, P. O'Kiely. Antagonistic effects on biogas and
519 methane output when co-digesting cattle and pig slurries with grass silage in in vitro batch
520 anaerobic digestion. *Biomass and Bioenergy*. 109 (2018) 190-8.

521 [7] S. Sarto, R. Hildayati, I. Syaichurrozi. Effect of chemical pretreatment using sulfuric acid
522 on biogas production from water hyacinth and kinetics. *Renewable Energy*. 132 (2019) 335-
523 50.

524 [8] M. Cui, J. Shen. Effects of acid and alkaline pretreatments on the biohydrogen production
525 from grass by anaerobic dark fermentation. *International Journal of Hydrogen Energy*. 37
526 (2012) 1120-4.

527 [9] S. Irmak, B. Meryemoglu, A. Sandip, J. Subbiah, R.B. Mitchell, G. Sarath. Microwave
528 pretreatment effects on switchgrass and miscanthus solubilization in subcritical water and
529 hydrolysate utilization for hydrogen production. *Biomass and Bioenergy*. 108 (2018) 48-54.

530 [10] G. Yang, J. Wang. Ultrasound combined with dilute acid pretreatment of grass for
531 improvement of fermentative hydrogen production. *Bioresour Technol*. 275 (2018) 10-8.

532 [11] Q. Yu, J. Liu, X. Zhuang, Z. Yuan, W. Wang, W. Qi, et al. Liquid hot water
533 pretreatment of energy grasses and its influence of physico-chemical changes on enzymatic
534 digestibility. *Bioresour Technol.* 199 (2016) 265-70.

535 [12] P. Niemi, V. Pihlajaniemi, M. Rinne, M. Siika-aho. Production of sugars from grass
536 silage after steam explosion or soaking in aqueous ammonia. *Industrial Crops and Products.*
537 98 (2017) 93-9.

538 [13] S.K. Bhatia, S.-H. Kim, J.-J. Yoon, Y.-H. Yang. Current status and strategies for second
539 generation biofuel production using microbial systems. *Energy Conversion and Management.*
540 148 (2017) 1142-56.

541 [14] O.M. Pakarinen, H.P. Tähti, J.A. Rintala. One-stage H₂ and CH₄ and two-stage H₂+CH₄
542 production from grass silage and from solid and liquid fractions of NaOH pre-treated grass
543 silage. *Biomass and Bioenergy.* 33 (2009) 1419-27.

544 [15] M. Cooney, N. Maynard, C. Cannizzaro, J. Benemann. Two-phase anaerobic digestion
545 for production of hydrogen-methane mixtures. *Bioresour Technol.* 98 (2007) 2641-51.

546 [16] C. Nualsri, A. Reungsang, P. Plangklang. Biochemical hydrogen and methane potential
547 of sugarcane syrup using a two-stage anaerobic fermentation process. *Industrial Crops and*
548 *Products.* 82 (2016) 88-99.

549 [17] A.J. Guneratnam, A. Xia, J.D. Murphy. Comparative study of single- and two-stage
550 fermentation of the brown seaweed *Laminaria digitata*. *Energy Conversion and Management.*
551 148 (2017) 405-12.

552 [18] P. Tsapekos, M. Alvarado-Morales, P.G. Kougias, K. Konstantopoulos, I. Angelidaki.
553 Co-digestion of municipal waste biopulp with marine macroalgae focusing on sodium
554 inhibition. *Energy Conversion and Management.* 180 (2019) 931-7.

555 [19] M.J. Lee, T.H. Kim, B. Min, S.J. Hwang. Sodium (Na^+) concentration effects on
556 metabolic pathway and estimation of ATP use in dark fermentation hydrogen production
557 through stoichiometric analysis. *J Environ Manage.* 108 (2012) 22-6.

558 [20] D.M. Wall, E. Allen, B. Straccialini, P. O'Kiely, J.D. Murphy. The effect of trace
559 element addition to mono-digestion of grass silage at high organic loading rates. *Bioresour*
560 *Technol.* 172 (2014) 349-55.

561 [21] R.S.M. M.G. Poter. The volatility of components of grass silage on oven drying and the
562 inter- relationship between dry- matter content estimated by different analytical methods.
563 *Grass and Forage Science.* 56 (2001) 405-11.

564 [22] E. Allen, D.M. Wall, C. Herrmann, J.D. Murphy. A detailed assessment of resource of
565 biomethane from first, second and third generation substrates. *Renewable Energy.* 87 (2016)
566 656-65.

567 [23] L. Ding, J. Cheng, A. Xia, A. Jacob, M. Voelklein, J.D. Murphy. Co-generation of
568 biohydrogen and biomethane through two-stage batch co-fermentation of macro- and micro-
569 algal biomass. *Bioresour Technol.* 218 (2016) 224-31.

570 [24] R.R. Gonzales, P. Sivagurunathan, S.-H. Kim. Effect of severity on dilute acid
571 pretreatment of lignocellulosic biomass and the following hydrogen fermentation.
572 *International Journal of Hydrogen Energy.* 41 (2016) 21678-84.

573 [25] G.L. MILLER. Use of dinitrosalicylic acid reagent for determination of reducing sugar.
574 *Analytical Chemistry.* 31 (1959) 426-8.

575 [26] C. Herrmann, C. Idler, M. Heiermann. Biogas crops grown in energy crop rotations:
576 Linking chemical composition and methane production characteristics. *Bioresour Technol.*
577 206 (2016) 23-35.

578 [27] B.H. A. Sluiter, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton. Determination of sugars
 579 byproducts and degradation products in liquid fraction process samples. Laboratory analytical
 580 procedure, National Renewable Energy Laboratory NREL/TP-510-42623 (2008).

581 [28] J.J.C. L. Segal, A.E. Martin, Jr, C.M. Conrad. An Empirical Method for Estimating the
 582 Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. Textile
 583 Research Journal. 29 (1959) 786-94.

584 [29] N.E.K.J.D.M. Abdul-Sattar Nizami. Review of the Integrated Process for the Production
 585 of Grass Biomethane. Environ Sci Technol. 43 (2009) 8496-508.

586 [30] M.R. Tabassum, A. Xia, J.D. Murphy. Biomethane production from various segments of
 587 brown seaweed. Energy Conversion and Management. 174 (2018) 855-62.

588 [31] W. Huang, Z. Zhao, T. Yuan, W. Huang, Z. Lei, Z. Zhang. Low-temperature
 589 hydrothermal pretreatment followed by dry anaerobic digestion: A sustainable strategy for
 590 manure waste management regarding energy recovery and nutrients availability. Waste
 591 Manag. 70 (2017) 255-62.

592 [32] T. Yuan, Y. Cheng, Z. Zhang, Z. Lei, K. Shimizu. Comparative study on hydrothermal
 593 treatment as pre- and post-treatment of anaerobic digestion of primary sludge: Focus on
 594 energy balance, resources transformation and sludge dewaterability. Applied Energy. 239
 595 (2019) 171-80.

596 [33] L. Ding, J. Cheng, L. Yue, J. Liu, L. Zhang, J. Zhou, et al. Fermentative hydrogen and
 597 methane co-production from pretreated *Spartina anglica* biomass with optimal
 598 saccharification effect under acid/alkali-assisted steam/microwave heating and enzymolysis.
 599 Energy Conversion and Management. 127 (2016) 554-60.

600 [34] T.C. Hsu, G.L. Guo, W.H. Chen, W.S. Hwang. Effect of dilute acid pretreatment of rice
 601 straw on structural properties and enzymatic hydrolysis. Bioresour Technol. 101 (2010)
 602 4907-13.

603 [35] M. Kim, B.-C. Kim, K. Nam, Y. Choi. Effect of pretreatment solutions and conditions
 604 on decomposition and anaerobic digestion of lignocellulosic biomass in rice straw.
 605 Biochemical Engineering Journal. 140 (2018) 108-14.

606 [36] W. Li, H. Khalid, Z. Zhu, R. Zhang, G. Liu, C. Chen, et al. Methane production through
 607 anaerobic digestion: Participation and digestion characteristics of cellulose, hemicellulose
 608 and lignin. Applied Energy. 226 (2018) 1219-28.

609 [37] D. Sahoo, S.B. Ummalyma, A.K. Okram, A. Pandey, M. Sankar, R.K. Sukumaran.
 610 Effect of dilute acid pretreatment of wild rice grass (*Zizania latifolia*) from Loktak Lake for
 611 enzymatic hydrolysis. Bioresour Technol. 253 (2018) 252-5.

612 [38] P. Mahmoodi, K. Karimi, M.J. Taherzadeh. Efficient conversion of municipal solid
 613 waste to biofuel by simultaneous dilute-acid hydrolysis of starch and pretreatment of
 614 lignocelluloses. Energy Conversion and Management. 166 (2018) 569-78.

615 [39] G. Kumar, B. Sen, C.Y. Lin. Pretreatment and hydrolysis methods for recovery of
 616 fermentable sugars from de-oiled *Jatropha* waste. Bioresour Technol. 145 (2013) 275-9.

617 [40] M. Láinez, H.A. Ruiz, A.A. Castro-Luna, S. Martínez-Hernández. Release of simple
 618 sugars from lignocellulosic biomass of *Agave salmiana* leaves subject to sequential
 619 pretreatment and enzymatic saccharification. Biomass and Bioenergy. 118 (2018) 133-40.

620 [41] S.A. Neshat, M. Mohammadi, G.D. Najafpour, P. Lahijani. Anaerobic co-digestion of
 621 animal manures and lignocellulosic residues as a potent approach for sustainable biogas
 622 production. Renewable and Sustainable Energy Reviews. 79 (2017) 308-22.

623 [42] B. Venturin, A. Frumi Camargo, T. Scapini, J. Mulinari, C. Bonatto, S. Bazoti, et al.
 624 Effect of pretreatments on corn stalk chemical properties for biogas production purposes.
 625 Bioresour Technol. 266 (2018) 116-24.

626 [43] G. Di Girolamo, M. Grigatti, L. Bertin, C. Ciavatta, L. Barbanti. Enhanced substrate
 627 degradation and methane yield with maleic acid pre-treatments in biomass crops and residues.
 628 Biomass and Bioenergy. 85 (2016) 306-12.

629 [44] M.R. Tabassum, A. Xia, J.D. Murphy. Potential of seaweed as a feedstock for renewable
 630 gaseous fuel production in Ireland. Renewable and Sustainable Energy Reviews. 68 (2017)
 631 136-46.

632 [45] A. Xia, J. Cheng, L. Ding, R. Lin, W. Song, H. Su, et al. Substrate consumption and
 633 hydrogen production via co-fermentation of monomers derived from carbohydrates and
 634 proteins in biomass wastes. Applied Energy. 139 (2015) 9-16.

635 [46] G. Yang, J. Wang. Pretreatment of grass waste using combined ionizing radiation-acid
 636 treatment for enhancing fermentative hydrogen production. Bioresour Technol. 255 (2018) 7-
 637 15.

638 [47] J. Massanet-Nicolau, R. Dinsdale, A. Guwy, G. Shipley. Utilising biohydrogen to
 639 increase methane production, energy yields and process efficiency via two stage anaerobic
 640 digestion of grass. Bioresour Technol. 189 (2015) 379-83.

641 [48] P. Sivagurunathan, G. Kumar, T. Kobayashi, K. Xu, S.-H. Kim. Effects of various dilute
 642 acid pretreatments on the biochemical hydrogen production potential of marine macroalgal
 643 biomass. International Journal of Hydrogen Energy. 42 (2017) 27600-6.

644 [49] L.T. Angenent, K. Karim, M.H. Al-Dahhan, B.A. Wrenn, R. Domiguez-Espinosa.
 645 Production of bioenergy and biochemicals from industrial and agricultural wastewater.
 646 Trends Biotechnol. 22 (2004) 477-85.

647 [50] P. Tsapekos, P.G. Kougias, I. Angelidaki. Biogas production from ensiled meadow grass;
 648 effect of mechanical pretreatments and rapid determination of substrate biodegradability via
 649 physicochemical methods. Bioresour Technol. 182 (2015) 329-35.

- [51] J. McEniry, P. O'Kiely. Anaerobic methane production from five common grassland species at sequential stages of maturity. *Bioresour Technol.* 127 (2013) 143-50.
- [52] G. Kumar, H.-C. Cheon, S.-H. Kim. Effects of 5-hydromethylfurfural, levulinic acid and formic acid, pretreatment byproducts of biomass, on fermentative H₂ production from glucose and galactose. *International Journal of Hydrogen Energy.* 39 (2014) 16885-90.
- [53] S. Bolado-Rodriguez, C. Toquero, J. Martin-Juarez, R. Travaini, P.A. Garcia-Encina. Effect of thermal, acid, alkaline and alkaline-peroxide pretreatments on the biochemical methane potential and kinetics of the anaerobic digestion of wheat straw and sugarcane bagasse. *Bioresour Technol.* 201 (2016) 182-90.
- [54] Y. Chen, J.J. Cheng, K.S. Creamer. Inhibition of anaerobic digestion process: a review. *Bioresour Technol.* 99 (2008) 4044-64.
- [55] P.L.M. Irwin J. Kugelman. Cation toxicity and stimulation in anaerobic waste treatment. *Water Pollution Control Federation.* 37 (1965) 76-116.
- [56] X. Kang, Y. Sun, L. Li, X. Kong, Z. Yuan. Improving methane production from anaerobic digestion of Pennisetum Hybrid by alkaline pretreatment. *Bioresour Technol.* 255 (2018) 205-12.
- [57] A. Xia, C. Herrmann, J.D. Murphy. How do we optimize third-generation algal biofuels? *Biofuels, Bioproducts and Biorefining.* 9 (2015) 358-67.
- [58] S.-F. Fu, K.-Q. Chen, R. Zhu, W.-X. Sun, H. Zou, R.-B. Guo. Improved anaerobic digestion performance of *Miscanthus floridulus* by different pretreatment methods and preliminary economic analysis. *Energy Conversion and Management.* 159 (2018) 121-8.

1 **List of Figures and Tables**

2 Fig. 1. Schematic diagram of single-stage and two-stage fermentation processes with pre-
3 treatment.

4 Fig. 2. Dependence of reducing sugar yield and hydrolysis efficiency on: (a) the
5 concentration of sulphuric acid, (b) the pre-treatment temperature, (c) the pre-treatment
6 heating time, and (d) the severity factor.

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

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

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

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

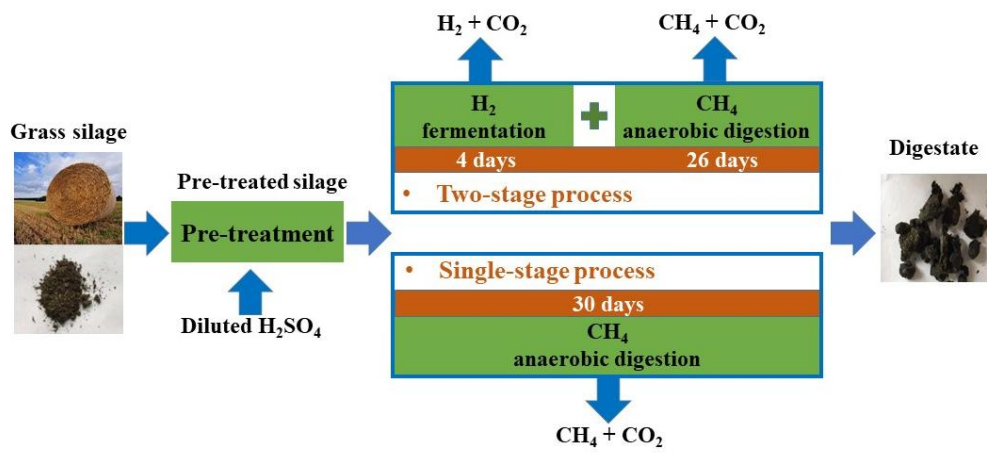
15 Fig. 7. (a) Biomethane yield and (b) biomethane production rate in the second-stage
16 anaerobic digestion.

17

18 Table 1 Compositional characteristics of grass silage.

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

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



25 Fig. 1. Schematic diagram of single-stage and two-stage fermentation processes with pre-
 26 treatment.

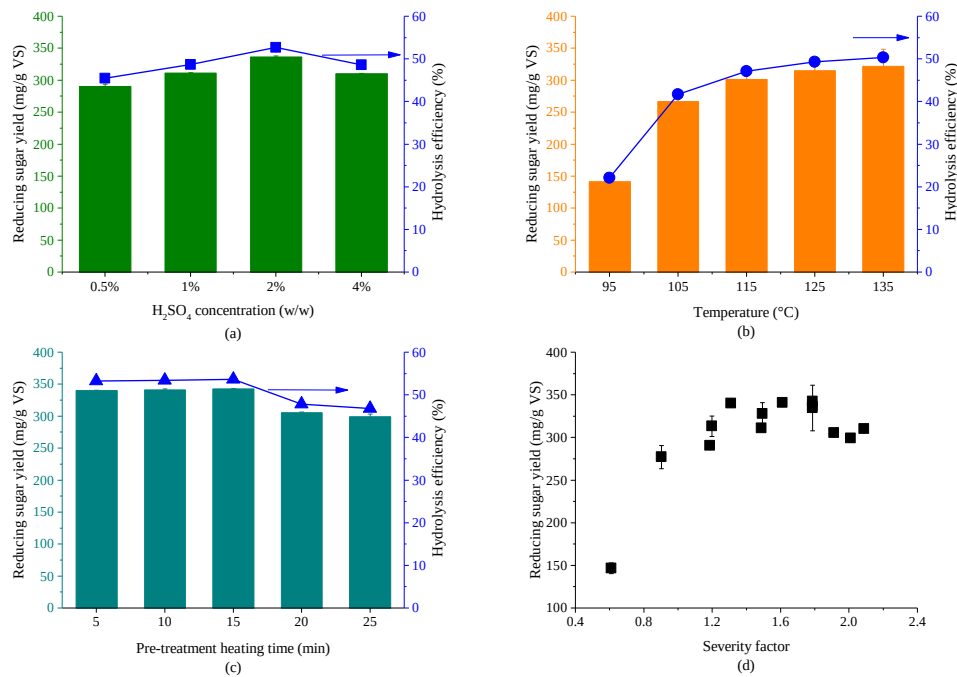
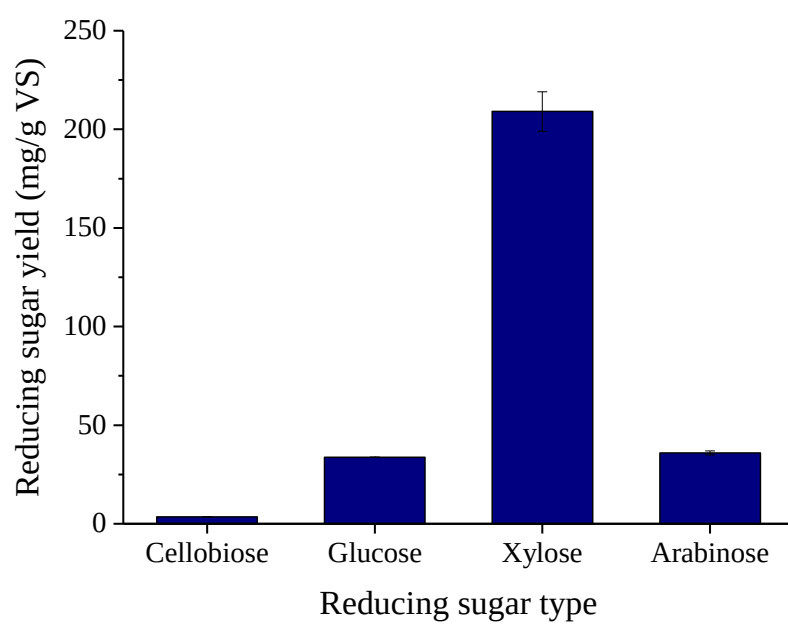


Fig. 2. Dependence of reducing sugar yield and hydrolysis efficiency on: (a) the concentration of sulphuric acid, (b) the pre-treatment temperature, (c) the pre-treatment heating time, and (d) the severity factor.



32

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

34 condition.

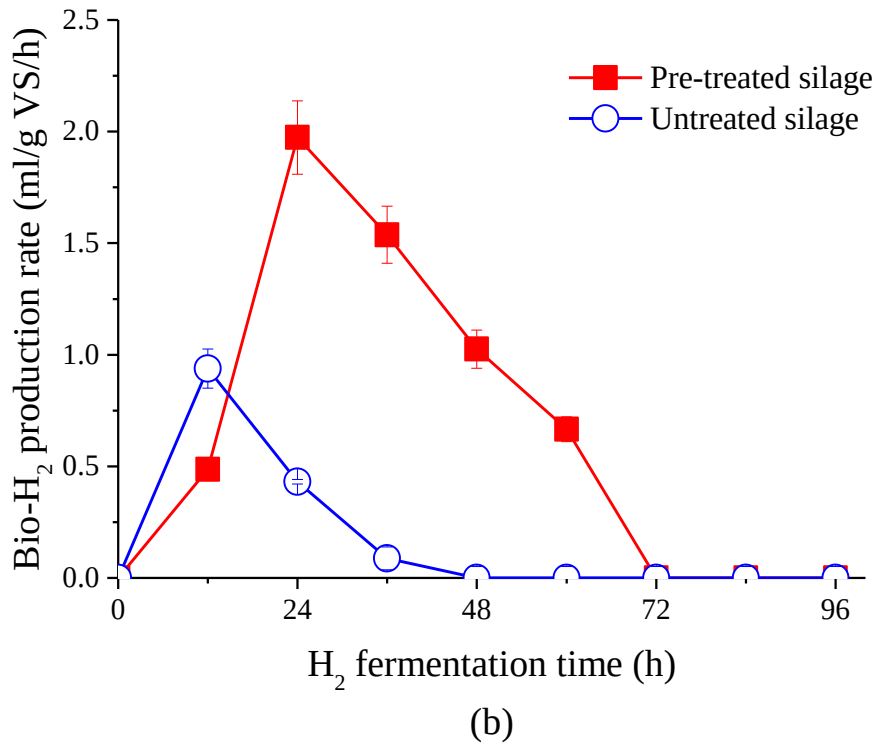
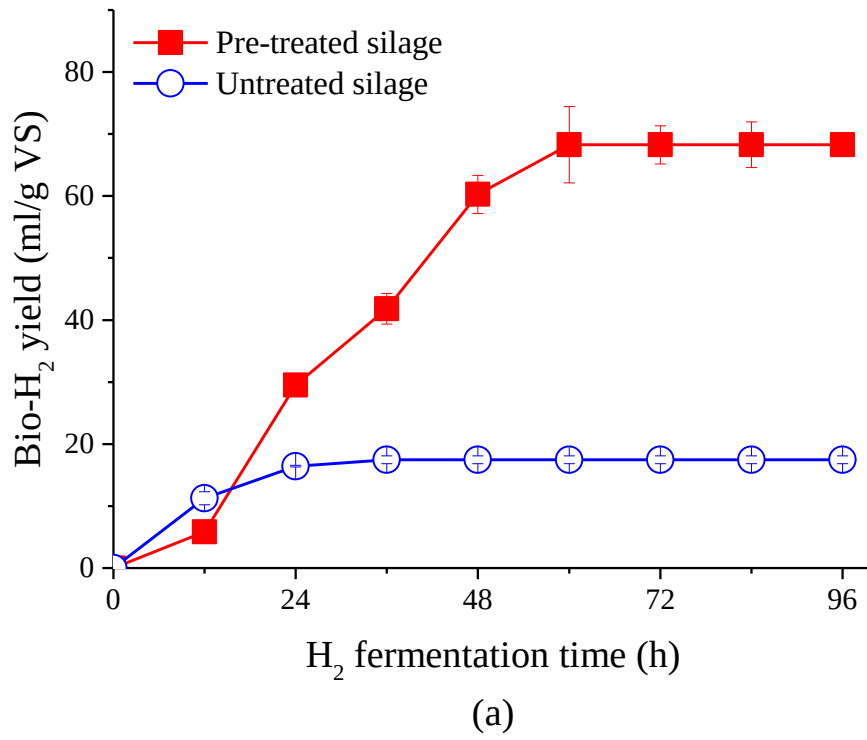
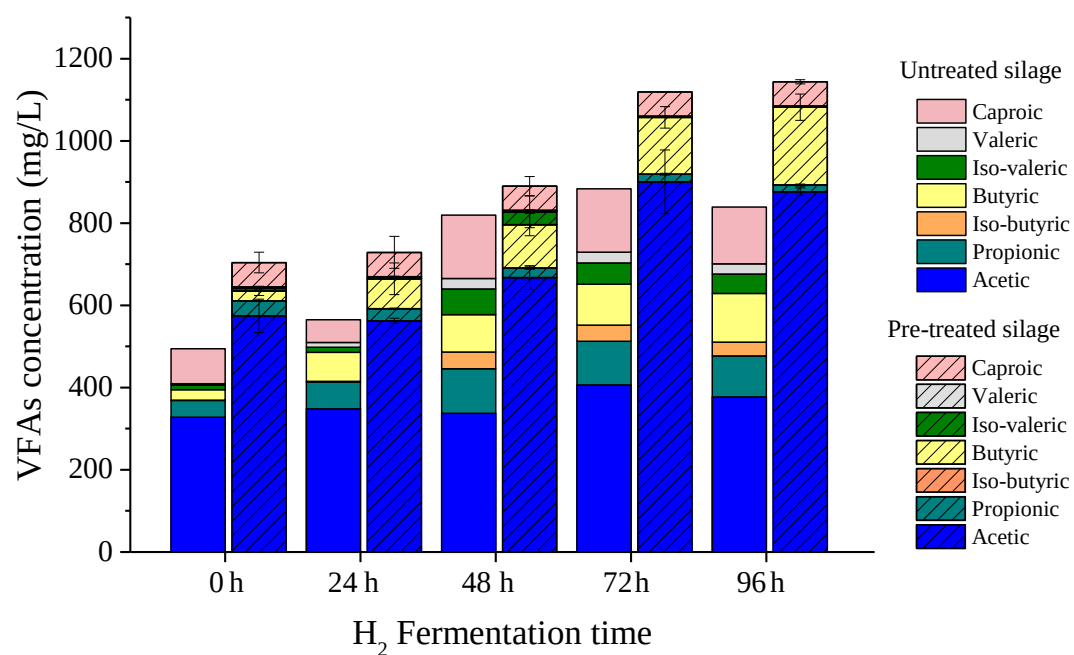
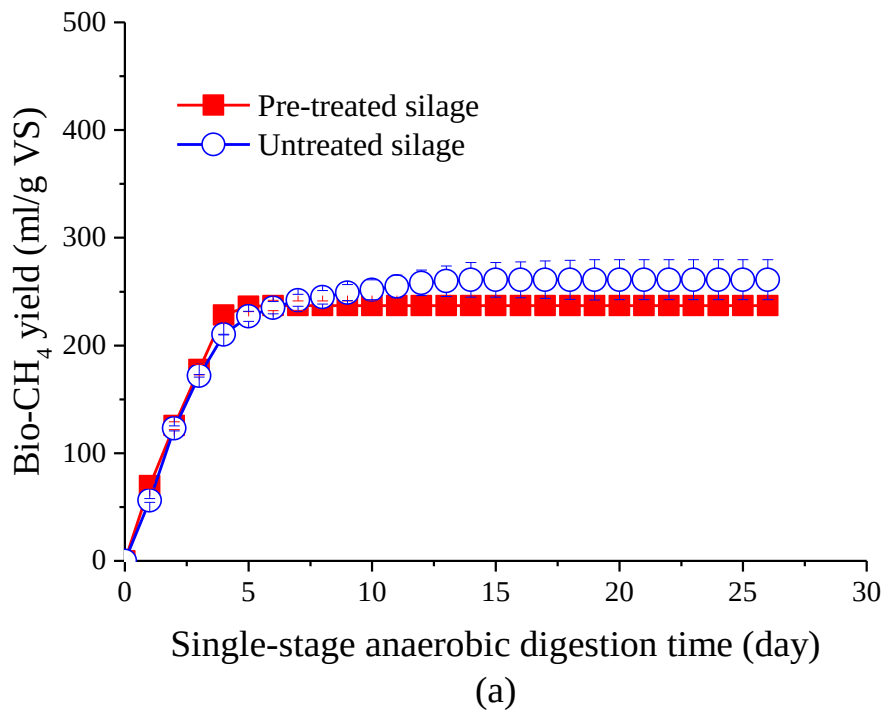


Fig. 4. (a) Biohydrogen yield and (b) biohydrogen production rate in the first-stage dark fermentation.

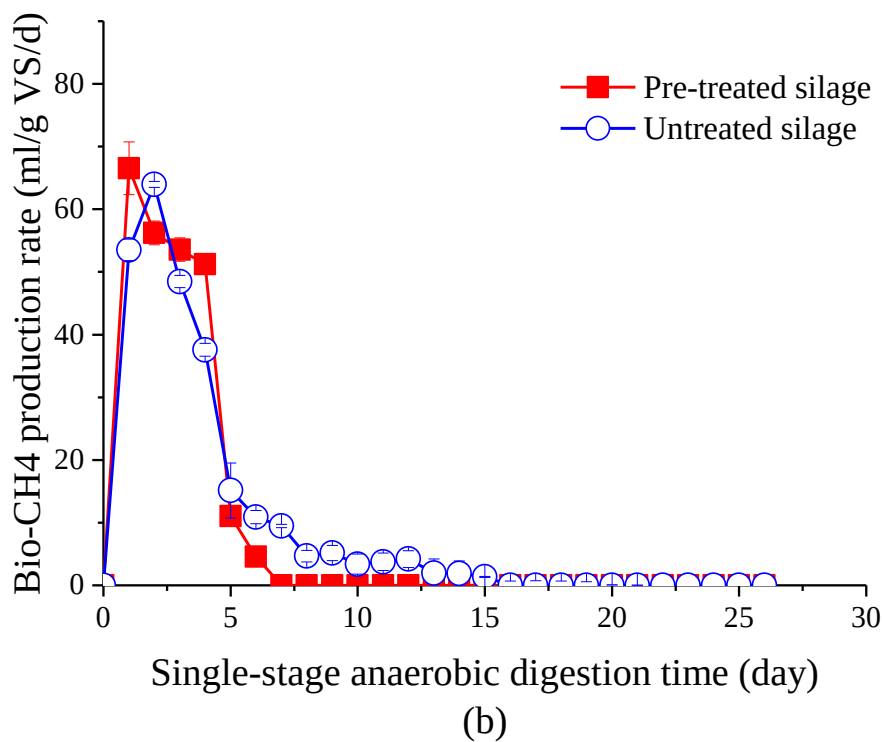


39
40

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



43



44

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

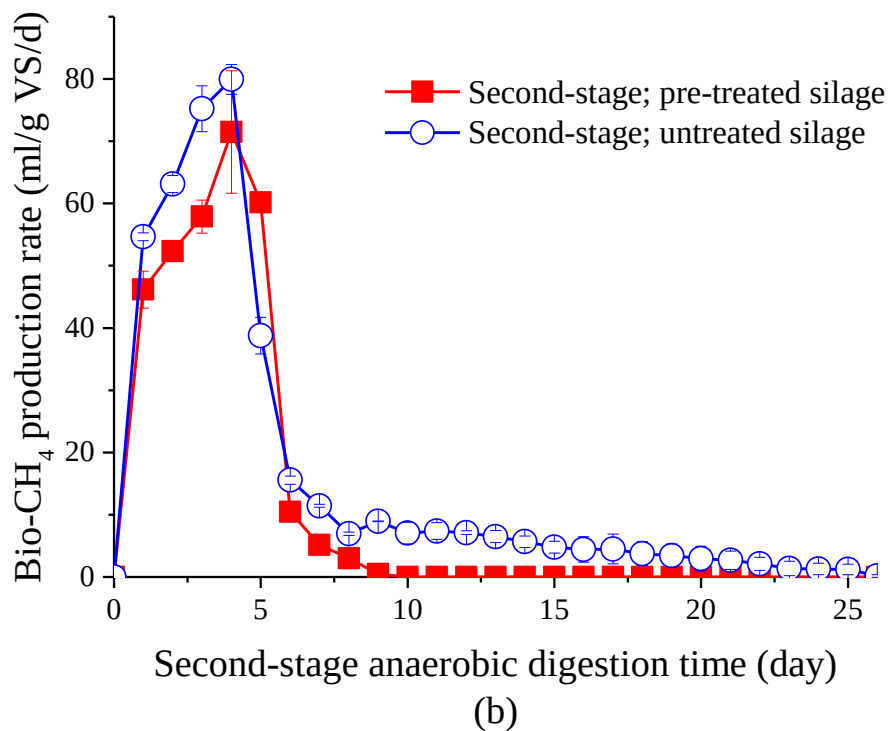
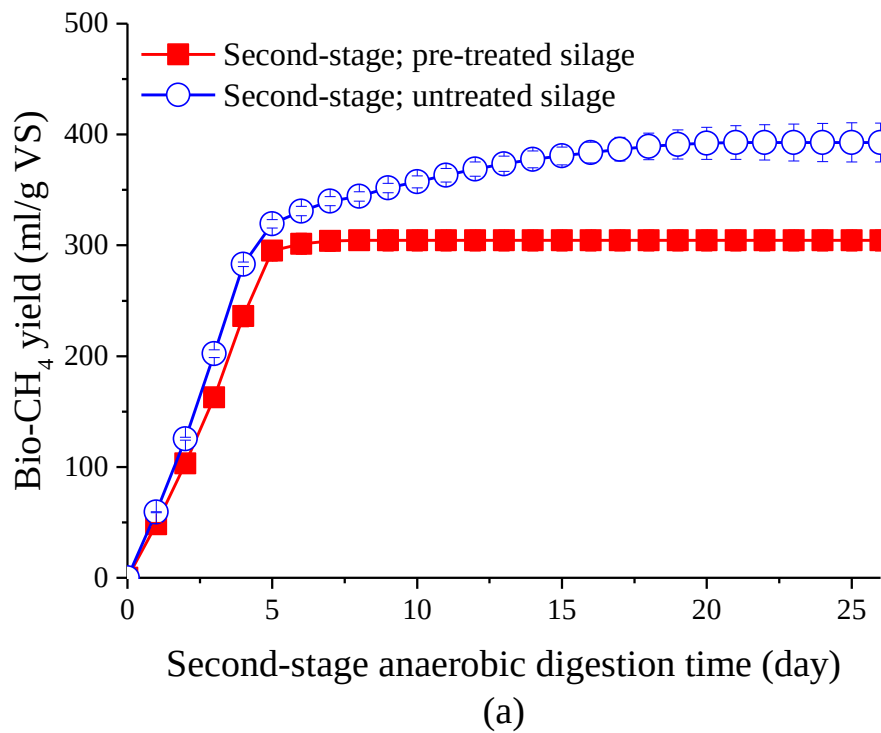


Fig. 7. (a) Biomethane yield and (b) biomethane production rate in the second-stage anaerobic digestion.

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)	Biodegradability index	H ₂ energy efficiency	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%

55

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
		Dark		Pre-treatment		Dark fermentation	Q _{cons} (kJ/g VS)
		fermentatio n	AD	with heat recovery	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

58