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Improving biohydrogen and biomethane co-production via two-stage dark fermentation and anaerobic digestion of the pretreated seaweed *Laminaria digitata*

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Abstract

The marine macro-algae *Laminaria digitata* is an abundant brown seaweed, which may be used as a feedstock for gaseous biofuel production via sequential dark fermentation and anaerobic digestion. Various methods, including hydrothermal pretreatment (HTP), hydrothermal dilute acid pretreatment (HTDAP), enzymolysis, and combinations thereof, were employed to depolymerize *L. digitata*, and assess the effects on biohydrogen and biomethane yields. Scanning electron microscopic images revealed that the intact and smooth structure of the seaweed was severely damaged;

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some micro pores and debris were generated after HTP (140 °C for 20 min), whilst the undegraded components remained as filamentous structures. The complex carbohydrate polymers in *L. digitata* constrained the catalytic effects of glucoamylase, leading to limited increase in the yield of carbohydrate monomers. With the aid of H_2SO_4 (1 v/v%) in HTP, depolymerization of biomass and its further conversion to carbohydrate monomers were significantly improved. The yield of total carbohydrate monomers after HTDAP (0.564 g/gVS) was 3.5-fold that in raw biomass; this led to an increase of 60.8% in biohydrogen yield (57.4 mL/gVS) in the first-stage dark fermentation. However, the generation of byproducts such as hydroxymethylfurfural under such harsh conditions impaired the second-stage anaerobic digestion of hydrogenogenic effluent, resulting in a 25.9% decrease in biomethane yield. HTP was considered the optimum pretreatment improving energy conversion efficiency from seaweed to gaseous biofuels by 26.7% as compared to that of the unpretreated *L. digitata*.

Keywords: *Laminaria digitata*; cascading bioenergy conversion; dark fermentation; biohydrogen and biomethane; hydrothermal pretreatment; carbohydrate monomer.

1. Introduction

Marine macro-algae, also known as seaweed, assimilates CO_2 and nutrients in seawater through photosynthesis, and quickly accumulates biomass (Laurens, 2017). Typical seaweeds include brown algae (e.g., *Laminaria digitata*, *Saccharina latissima*), green algae (e.g., *Ulva lactuca*, *Ulva prolifera*), and red algae (e.g., *Palmaria palmata*, *Gracilaria confervoides*) (Murphy et al., 2015). Seaweed can be naturally harvested on seashores and in shallow waters, especially in areas suffering from eutrophication with associated availability of nutrients such as nitrogen and potassium (Aitken et al., 2014). However, according to a latest report published by the Food and Agriculture Organization (FAO) of the United Nations (FAO, 2018), wild harvest only accounted for 3.3 % of the total seaweed production globally (30.4 million tons) in 2015. About 96.7% of the seaweed production was from artificial cultivation, and this harvest is growing year on year. Artificial cultivation usually involves growing seaweed on suspended ropes with separation to allow boat travel between lines for harvest. By using advanced textiles in the form of mats (in lieu of ropes), a seaweed yield up to 200 t/ha/yr could be expected (Murphy et al., 2015). When coupled with marine aquaculture industries, seaweed cultivation can assimilate nutrient pollutants discharged from fish farms into biomass, thus simultaneously reducing water eutrophication and providing increased and sustainable biomass supply (Tabassum et al., 2017). Assimilation of CO_2 during seaweed cultivation and the perceived sustainability of seaweed can lead to improved commercial viability (Pechsiri et al., 2016). The use of seaweed as food depends on aspects such as heavy

metal content, which is influenced by whether the seaweed is naturally collected or artificially cultivated or associated with eutrophication or generated from aquaculture wastes. The possibility of contamination by heavy metals is one of the reasons why the wild seaweed harvest remains at a constantly low level of 1.06-1.29 million tons from 2006 to 2015 (FAO, 2018). Another parameter to consider is the fact that seaweed is widely used as a food in Asia, but not in Europe. The European Commission Renewable Energy Directive has established a target to increase the contribution of advanced biofuels, the definition of which allows for biohydrogen and biomethane derived from seaweeds, to at least 3.6% in the transport sector by 2030, whilst the limited maximum share of food-based biofuels is set up as 3.8% (European Commission, 2017). Indeed, the aforementioned carbon sequestration by seaweed can allow for the production of a sustainable advanced gaseous transport fuel that meets the stringent criteria (Czyrnek-Delêtre et al., 2017). Therefore, seaweed can find a niche in applications of advanced biofuel and bioenergy production (Ertem et al., 2017). Unharvested wild seaweeds have large resources (Mac Monagail et al., 2017) but limited application for food industries due to heavy metal contamination. This can lead to opportunities for biofuel production especially for cast seaweed of nuisance value on beaches. However, the environmental impact of large-scale natural harvest of kelp forests would have influenced the perception of a clean sustainable biofuel. Cultivated seaweeds are not associated with heavy metal contamination and indeed may be used to clean coastal waters, whilst generating sustainable biofuels in a circular bioeconomy system (Seghetta et al., 2017). According to a report published

by the International Energy Agency (IEA) Bioenergy (Laurens, 2017), the potential gross bioenergy in the form of biogas produced from harvested seaweeds can be over 300 GJ/ha/yr, higher than that from typical terrestrial crops such as maize, fodder beet, and grass. For the specific seaweed species *L. digitata* used in this study, the gross energy yield was estimated to range from 38 to 96 GJ/ha/yr (Tabassum et al., 2017).

As compared to terrestrial lignocellulosic biomass, seaweeds are rich in polysaccharides, contain no lignin, and have little cellulose content, making the biomass amenable for biogas production through anaerobic digestion (Laurens, 2017). Nonetheless, some large-molecular-weight carbohydrate polymers (e.g., alginate, fucoidan, laminarin, and agar) in seaweed cannot be readily degraded into monomers such as glucose, galactose, and mannose (Guneratnam et al., 2017). The recalcitrance of some carbohydrate polymers and the unavailability of enough readily utilized carbohydrate monomers in seaweeds could result in quite limited biodegradability during anaerobic digestion (Allen et al., 2015; Murphy et al., 2015). Therefore, pretreatments to depolymerize seaweed biomass for improving subsequent gaseous biofuel production are required (Sudhakar et al., 2019). Liu and Wang (2014) pretreated *Laminaria japonica* using steam heating at 121 °C for 30 min, increasing the fermentative biohydrogen yield from 10.0 to 66.7 mL/g total solids (TS). Sivagurunathan et al. (2017) further combined steam heating and dilute acid pretreatments to improve the solubilization of *Gelidium amansii*, increasing the biohydrogen yield by 92.6%. Apart from biohydrogen production, investigations on pretreatments to improve biomethane production via anaerobic digestion have also

been reported. Barbot et al. (2014; 2015) pretreated macro-algae biomass using 0.2 M HCl at 80 °C for 2-24 h, resulting in 39-140% increases in biomethane yields. Herrmann et al. (2015) ensiled seaweed for 90 days and found that 10-28% of the biomass was released as liquid effluent enriched with soluble products such as lactic acid; subsequent anaerobic digestion of the ensiled seaweed secured increases of up to 28% in biomethane yields as compared to that of the raw biomass. The long retention time of seaweed ensiling primarily serves as a storage method to preserve the biomethane potential as opposed to an immediate pretreatment process.

Although various pretreatments to improve anaerobic fermentation or digestion have been reported, investigation of the effects on biohydrogen and biomethane co-production through two-stage process is still limited to date. Costa et al. (2015) steam-heated *Sargassum* sp. (121 °C, 15 min) to obtain the highest biohydrogen and biomethane yields of 91.3 and 541 mL/g volatile solids (VS), respectively. However, the pretreatment effects on the compositional and structural changes of seaweed were not elucidated. This information is essential for further understanding the pretreatment mechanisms and offering directional optimizations. To fill in this knowledge gap in the state of the art, typical methods including hydrothermal pretreatment (HTP), hydrothermal dilute acid pretreatment (HTDAP), enzymolysis, and combinations thereof were employed in this paper to pretreat the seaweed *L. digitata*. As an ecofriendly pretreatment method in which water at high temperature and pressure is used as a reaction medium with excellent solvent properties (Ruiz et al., 2015), HTP has been reported to efficiently penetrate and fractionate biomass, including food

waste (Ding et al., 2017b), sewage sludge (Yuan et al., 2019), and seaweed (Lin et al., 2019), thus resulting in higher biomass degradability and biogas production. The high temperature weakens H-bonding in water, leading to the autoionization of water into H_3O^+ acting as catalysts and OH^- (Ruiz et al., 2013). Due to the complexity of seaweed biomass, dilute acid catalyst reported to improve the thermal pretreatment efficiency (Barbot et al., 2014; Sivagurunathan et al., 2017) was incorporated with HTP to evaluate the influence on subsequent biogas production. Similarly, enzymatic hydrolysis of *L. digitata* was also assessed.

The ambition of this paper is to assess the feasibility of HTP of the seaweed *L. digitata* prior to two-stage dark fermentation and anaerobic digestion in order to produce a cleaner and more sustainable advanced gaseous biofuel. The objectives are to: (1) analyze the biomass depolymerization caused by pretreatments based on ultrastructural and compositional changes; (2) assess the biohydrogen and biomethane co-production from pretreated *L. digitata* through two-stage dark fermentation and anaerobic digestion; and (3) evaluate the energy balance of cascading HTP of *L. digitata* and the subsequent two-stage process.

2. Materials and methods

2.1 Feedstock and inocula

The feedstock *L. digitata* was collected from shallow waters off the West Cork coast in Ireland in September. The collected *L. digitata* was washed by tap water to remove impurities, cut into small pieces, dried in an oven at 105 °C, and ground into

powder. *L. digitata* powder was then cryo-stored at -20 °C before experimentation. The VS content of *L. digitata* powder was 79.7% of TS. The elemental composition of *L. digitata* was as follows (% of VS): carbon, 45.73; hydrogen, 6.22; nitrogen, 1.62; and oxygen, 46.43. The C/N ratio was 28.19. The energy content was calculated as 16032 J/gVS. The inoculum used in dark fermentation originated from sludge from an industrial digester treating swine slurry in Huzhou, China. The separation and enrichment processes were detailed in a previous study (Xia et al., 2015). The seed inoculum used for anaerobic digestion originated from the liquid digestate of an industrial digester treating food waste in Hangzhou, China. The seed inoculum was kept at an anaerobic workstation (Whitley DG250, UK) for 7 days to degas before experimentation. The TS and VS of seed inoculum before inoculation were 3.85 wt% and 1.93 wt%, respectively.

2.2 Pretreatment

Fig. 1 outlines the schematic of experiments including pretreatments of seaweed *L. digitata*, first-stage dark fermentation for biohydrogen production, and second-stage anaerobic digestion for biomethane production.

2.2.1 Hydrothermal pretreatment

HTP of *L. digitata* was conducted in a 500-mL batch reactor (Parr 4500, USA). In each batch, *L. digitata* powder equivalent to 3 gVS was added to the reactor. Deionized water was then added to make the total working volume 200 mL. The

reactor filled with *L. digitata* and water was sealed, heated to 140 °C, and then maintained at 140 °C for 20 min. A mechanical stirrer operated at 500 rpm ensured the biomass was evenly heated. Under HTDAP, deionized water was replaced with 1 v/v% H_2SO_4, while other parameters were maintained as for HTP. After pretreatment, the reactor was cooled to ambient temperature and the *L. digitata* mixture was taken out for subsequent analyses and experiments.

2.2.2 Enzymolysis

Volumes of 200 mL of *L. digitata* mixtures were transferred to a 250-mL Erlenmeyer flask. The *L. digitata* mixtures included as aforementioned: HTP; HTDAP; and without pretreatment (mixture of raw biomass and deionized water at the same ratios as for pretreated mixtures). The pH of mixtures was then adjusted to 4.5 using 6 M NaOH. Subsequently, glucoamylase (1,4-α-D-glucan glucohydrolase, from *Aspergillus niger*, ~70 U/mg, Sigma-Aldrich, USA) and $CaCl_2$ were both added at a ratio of 0.05 g:gVS. Each flask was sealed using a glass stopper and parafilm and placed in an oscillator at 62 °C and 150 rpm for 24 h.

2.3 Experimental set-up of two-stage process

Two-stage biohydrogen and biomethane co-production of *L. digitata* biomass, including the first-stage dark fermentation and the second-stage anaerobic digestion, was performed using AMPTSII systems (Bioprocess Control, Sweden). Six experimental groups of *L. digitata* biomass (unpretreated, HTP, HTDAP,

enzymolysis, HTP + enzymolysis, HTDAP + enzymolysis) each in triplicate were prepared for the first-stage dark fermentation. In brief, 200 mL of pretreated *L. digitata* mixture (3 gVS of dried biomass, the rest deionized water for the unpretreated *L. digitata*) was added into each bottle. Subsequently, deionized water was added to make the volume 270 mL, and pH of each bottle was adjusted to 6.0 ± 0.1 using 6 M NaOH. Then 30 mL of acclimatized biohydrogen inoculum was added to make the total working volume 300 mL and the *L. digitata* biomass concentration 10 gVS/L. The bottles filled with biomass and inoculum were purged with N_2 for 10 min to maintain anaerobic, sealed with rubber stoppers, and placed in a water bath at 35 °C for 3 days. The mechanical stirrers were set on/off every 60 seconds at 60 rpm in experiments. Besides, a group of blanks in triplicate with only 270 mL of deionized water and 30 mL of inoculum in each bottle were also operated. The biohydrogen and carbon dioxide produced in the experimental groups were corrected by offsetting that produced from the inoculum in the blank control.

After dark fermentation, a part of the effluents were sampled for further analyses and the residual effluents were then inoculated with the degassed seed inoculum for the second-stage anaerobic digestion. The working volume in each bottle was 300 mL calculated according to the inoculum to feedstock VS ratio of 2:1. Subsequently, each inoculated bottle was purged with N_2 for 10 min to maintain anaerobic, sealed with a rubber stopper, and placed in a water bath at 35 °C for 21 days. The stirrer was set on the same operation mode as that in dark fermentation. The effects of the carryover of biohydrogen inoculum on second-stage anaerobic digestion were offset by correcting

the biomethane volumes for that produced from the blank control.

2.4 Analytical methods

TS and VS of *L. digitata* biomass and seed inocula were determined according to Standard Methods 2540G (APHA, 1999). The elemental composition (C, H, and N) of *L. digitata* was determined using an elemental analyzer (Exeter Analytical CE 440, UK), and the O was assumed to be the remaining VS. The soluble chemical oxygen demand (COD) was tested using Hach vials (Hach 2125915C, USA) and determined on a portable spectrophotometer (Hach DR890, USA). A field scanning electron microscope (SEM; SU8010, Hitachi, Japan) was employed to record the ultrastructural changes of *L. digitata* after pretreatments.

The biohydrogen and biomethane volumes were recorded using the AMPTSII systems. Every 12 h, the biogas before entering the carbon dioxide removal bottles filled with 3 M NaOH was sampled using a gas-tight syringe (Hamilton, Switzerland), and the biogas composition (H_2, CH_4, and CO_2) was determined using a gas chromatography (GC) system (Agilent 7820A, USA) equipped with a thermal conductivity detector and a 5A column (Φ 3 mm × 3 m; Agilent, USA). Volatile fatty acids (VFAs) including acetic acid (HAc), propionic acid (HPr), iso-butyric acid (Iso-HBu), butyric acid (HBu), iso-valeric acid (Iso-HVa), valeric acid (HVa), and caproic acid (HCa) were determined on another GC (Agilent 7820A, USA) equipped with a flame ionization detector and a DB-FFAP column (Ding et al., 2017b). Carbohydrate monomers including glucose, galactose, fucose, and mannitol were

determined on a high-performance liquid chromatography (HPLC) system (Agilent 1200, USA) using a refractive index detector and an Aminex HPX-87P column (Bio-Rad, USA) at 80 °C with H_2O as mobile phase at 0.6 mL/min. Furfural and hydroxymethylfurfural (5-HMF) were determined using the same HPLC equipped with an ultraviolet detector at 278 nm and an Aminex HPX-87H column (Bio-Rad, USA) at 65 °C with 0.005 N H_2SO_4 as mobile phase at 0.5 mL/min. All the trials and measurements were conducted in triplicate, and the results were presented as mean ± standard deviation.

2.5 Calculations

The energy content of *L. digitata* was calculated based on the elemental composition according to the modified Dulong formula in Eq. (1) (Lin et al., 2019).

$$\text{Energy content of } L.\ digitata = 337\text{C} + 1419\left(\text{H} - 0.125\text{O}\right) + 23.26\text{N} \quad (1)$$

The increase in energy yield was calculated according to Eq. (2).

$$\text{Increase in energy yield} = \frac{\text{Energy yield of pretreated seaweed - Energy yield of unpretreated seaweed}}{\text{Energy yield of unpretreated seaweed}} \times 100\% \quad (2)$$

To assess the proximate energy balance in future industrial applications, some assumptions were made as follows: (1) the heat during the cooling of *L. digitata* mixtures after HTP was recovered using heat exchangers with an efficiency (η) of 85% (Fakheri, 2006; Yuan et al., 2019) in a continuous mode; (2) the specific heat capacity (C) and density of *L. digitata* mixtures (ρ) were assumed as 4.18 kJ/(kg·°C) and 1 kg/L, respectively, similar to water; and (3) the initial *L. digitata* mixtures temperature

and the ambient temperature (T_a) were assumed as 25 °C. The additional energy requirements of mixing and pumping *L. digitata* mixtures were neglected for simplification. Therefore, the energy input of HTP (Q_{HTP}, kJ/kgVS) was calculated according to Eq. (3).

$$Q_{HTP} = \rho VC(T_{HTP}-T_a) - \eta\rho VC(T_{HTP}-T_{df}) \quad (3)$$

where V is the working volume per kg VS of *L. digitata* during HTP process, L/kgVS; T_{HTP} is the HTP temperature, °C; and T_{df} is the dark fermentation temperature, 35 °C.

The theoretical total COD of *L. digitata* calculated based on the elemental composition as shown in Eq. (4) (Ding et al., 2017b) is 1.22 gCOD/gVS.

$$C_aH_bO_cN_d + (a + \frac{b}{4} - \frac{c}{2} - \frac{3}{4}d)O_2 \rightarrow aCO_2 + \frac{b-3d}{2}H_2O + dNH_3 \quad (4)$$

The solubilization yield of *L. digitata* is defined as the ratio of soluble COD to total COD. The acidification yield of *L. digitata* after first-stage dark fermentation is defined as the ratio of COD of total VFAs to soluble COD.

The significances of differences between biohydrogen yield, biomethane yield, soluble COD, carbohydrate monomer yield, 5-HMF yield, VFA concentration, and energy conversion efficiency (ECE) means were examined through the analysis of variance (ANOVA) on Origin 9.0 using Tukey tests ($P < 0.05$).

3. Results and discussions

3.1 Ultrastructural changes of *L. digitata* after pretreatments

The SEM images in Fig. 2 exhibit the ultrastructural changes of *L. digitata* biomass after pretreatments. The structure of unpretreated *L. digitata* was intact and

the surface was smooth (Fig. 2a). After HTP, micro-pores with varied sizes, small pieces of debris, and some filaments were generated (Fig. 2b). The large-molecular-weight organic materials were depolymerized under HTP, falling off from the original intact structure and leaving micro-pores and small debris. Meanwhile, components difficult to degrade remained as filamentous structures. This phenomenon became much more obvious after HTDAP: more micro-pores, small debris, and filaments were generated, whilst intact and smooth surfaces were scarce (Fig. 2c). The existence of H_2SO_4 under HTP significantly enhanced the biomass depolymerization.

As shown in Fig. 2d, the full piece structure of *L. digitata* still remained after enzymolysis, whilst some micro-pores in the surface was observed. This was attributed to the hydrolysis of large-molecular-weight glucans catalyzed by glucoamylase. Due to the specificity of glucoamylase versus the complexity of carbohydrate polymers in *L. digitata*, the areas and components hydrolyzed were limited. Combining HTP and enzymolysis, more filaments were observed (Fig. 2e) as compared to HTP (Fig. 2b). HTP damaged the intact biomass structure, creating more areas available for glucoamylase to contact and more glucans to be hydrolyzed. Employing HTDAP and enzymolysis (Fig. 2f) increased the filamentous structures and created larger pores than HTP and enzymolysis (Fig. 2e), demonstrating the facilitation of biomass depolymerization and degradation caused by H_2SO_4 in the pretreatment.

3.2 Compositional changes of *L. digitata* after pretreatments

Table 1 presents the composition of *L. digitata* mixtures before and after pretreatments. The solubilization yields of pretreated *L. digitata* increased by 21.8-79.9% than the unpretreated, highlighting that all these pretreatments improved the biomass depolymerization and hydrolysis. HTP significantly increased the soluble COD of *L. digitata* by 57.6%. However, the slight increase (15.4%) in total carbohydrate monomers was much lower than that in soluble COD. This elucidated that HTP could efficiently degrade the solid carbohydrate polymers into soluble polysaccharides and oligosaccharides, whilst their further conversion to monomers was limited without the aid of certain catalysts such as H_2SO_4 during HTDAP. Therefore, after HTDAP, although the solubilization yield increase was limited, the yield of total carbohydrate monomers markedly increased to 0.564 g/gVS, 3-fold that after HTP. Of particular note, the glucose content significantly increased to 0.329 g/gVS from 0.015 g/gVS in the unpretreated biomass. This illustrated that H_2SO_4 can significantly facilitate the further conversion of depolymerized soluble polysaccharides from *L. digitata* biomass into small-molecular-weight carbohydrate monomers under hydrothermal conditions.

Glucoamylase improved the hydrolysis of large-molecular-weight glucans in *L. digitata*. However, the solubilization yield only increased by 21.8% and total carbohydrate monomers by 14.2% as compared to the unpretreated biomass. Most of the increased carbohydrate monomers were glucoses, because 1,4-α-D-glucan glucohydrolase specifically catalyzes the hydrolysis of terminal 1,4-α-D-glucose

residues successively from non-reducing ends of the chains with the release of glucose molecules. Although *L. digitata*, as a brown alga, is rich in carbohydrates, the complexity of these specific carbohydrates (e.g., alginate, fucoidan, laminarin) (Guneratnam et al., 2017) limited the catalysis effects of glucoamylase whose specificity is mainly for glucans. Similarly, the improvements of total carbohydrate monomers induced by enzymolysis after HTP and HTDAP were both limited. Fucose, a hexose and the sub-unit of large-molecular-weight polysaccharide fucoidan in *L. digitata*, was only found at a limited level in the mixtures after HTDAP and HTDAP followed by enzymolysis. It could be deduced that fucoidan was not easily hydrolyzed under HTP until H_2SO_4 was added as a catalyst. Different from glucose, mannitol almost remained constant after pretreatments. This implied that mannitol already exists at a certain level in *L. digitata* as a typical carbohydrate monomer.

The changes in solubilization yields and carbohydrate monomers of *L. digitata* coincided with the ultrastructural changes shown in Fig. 2. This indicated that HTDAP efficiently depolymerized the intact and compact *L. digitata* biomass, hence releasing carbohydrate monomers. Although certain improvements on biomass hydrolysis using glucoamylase were observed, the complex composition of carbohydrates limited its effects. Under harsh pretreatment conditions (e.g., high temperature, acidic/alkaline solutions), some soluble reducing sugars derived from carbohydrate polymers could be further degraded into furfural and 5-HMF, which would exhibit severe inhibition on subsequent anaerobic fermentation and digestion processes (Monlau et al., 2014). Under HTP (140 °C, 20 min), some hexoses derived

from polysaccharides were dehydrated into 5-HMF. With the addition of H_2SO_4, more hexoses such as glucose were generated, whilst the further degradation of these hexoses contributed to higher 5-HMF. However, furfural, a typical furan aldehyde derived from pentoses such as xyloses, was not detected. This was because most of the carbohydrate monomers generated from *L. digitata* were hexoses; no pentoses were detected in this study.

3.3 First-stage dark fermentation of *L. digitata*

3.3.1 Biohydrogen production

Fig. 3 shows the biohydrogen production of *L. digitata* via the first-stage dark fermentation. Due to the lowest carbohydrate monomers, unpretreated *L. digitata* exhibited the lowest biohydrogen yield of 35.7 ± 0.3 mL/gVS. The intact structure of unpretreated *L. digitata* and the undegraded polysaccharides were difficult to utilize by microbes in dark fermentation. After separate HTP and enzymolysis pretreatments, the large-molecular-weight carbohydrate polymers in *L. digitata* were degraded into soluble small-molecular-weight products including carbohydrate monomers, leading to increased biohydrogen yields of 44.8 ± 2.2 mL/gVS and 44.0 ± 1.2 mL/gVS, respectively. The combination of HTP and enzymolysis further enhanced the biohydrogen yield to 55.2 ± 1.1 mL/gVS.

The yield of total carbohydrate monomers in *L. digitata* after HTDAP reached 0.564 g/gVS, 3.5-fold that of the unpretreated biomass. Further enzymolysis increased the total carbohydrate monomers to 0.593 g/gVS. Nonetheless, the increases in

biohydrogen yields after these two pretreatments were only 60.8-62.7% as compared to the unpretreated, significantly lower than that in total carbohydrate monomers. The rationale for this was postulated as follows. Firstly, apart from carbohydrate monomers, some other soluble oligosaccharides and disaccharides derived from the pretreated biomass were also readily hydrolyzed into reducing sugars and then utilized by microbes to produce biohydrogen. Secondly, the byproducts generated under harsh HTDAP conditions, such as 5-HMF, would cause DNA damage and inhibit several enzymes of the glycolysis pathway, thus inhibiting microbial cell growth and decreasing biohydrogen production during dark fermentation (Monlau et al., 2014).

3.3.2 Hydrogenogenic effluents

The composition of VFAs in the effluents after first-stage dark fermentation is shown in Table 2. Since the primary strain in the inoculum was *Clostridium butyricum* (Xia et al., 2013), acetic and butyric acids dominated the VFA composition in the effluents due to the dominance of the acetic and butyric metabolic pathways. The percentages of acetic and butyric acids (91.5-94.5%) in the VFAs in this study were much higher than that in our previous studies using micro-algae biomass (Ding et al., 2017a; Ding et al., 2016). This was attributed to the high C/N ratio of *L. digitata*. The amino acids derived from micro-algal proteins would be hydrolyzed to large amounts of propionic and iso-valeric acids (Cheng et al., 2015), thus lowering the content of acetic and butyric acids in the fermentation effluents. Notably, the contents of

propionic acid in the effluents of *L. digitata* pretreated with HTDAP and HTDAP followed by enzymolysis were significantly higher than that from other groups. This implied that H_2SO_4 boosted the degradation of proteins, thus generating soluble peptides and amino acids which could be utilized by microbes during dark fermentation.

As shown in Table 2, the acidification yield of unpretreated *L. digitata* was higher than that pretreated with HTP and HTDAP. A trend of decreasing acidification yield along with harsher pretreatment conditions was observed. HTP and HTDAP not only generated more soluble carbohydrates, but also degraded other components such as poly-phenols of *L. digitata* which are not readily utilized by the hydrogenogens (Tabassum et al., 2017). By contrast, the acidification yields of *L. digitata* pretreated with enzymolysis were higher, indicating that the soluble small-molecular-weight carbohydrates obtained after enzymolysis were easily utilized via dark fermentation.

3.3.3 Carbon conversion ratios

L. digitata biomass was the sole feedstock in the experiments, and the effects of remaining organics in the hydrogen inoculum were corrected for the blank control group. Hence, the carbon in the products of first-stage dark fermentation was considered from *L. digitata* only. The carbon conversion ratio (CCR) was calculated based on Eq. (5), and the results are shown in Fig. 4.

$$\text{Carbon conversion ratio} = \frac{\text{Carbon in VFAs} + \text{Carbon in } CO_2}{\text{Carbon in } \textit{L. digitata} \text{ biomass}} \times 100\% \quad (5)$$

The lowest CCR of 30.8% was obtained from unpretreated *L. digitata*, while all

pretreatments enhanced the CCRs. The CCRs of *L. digitata* after HTP and HTDAP were 46.4% and 49.5%, respectively. The highest CCR of 55.2% was from the *L. digitata* pretreated with HTDAP followed by enzymolysis. However, as compared with that of pure chemicals such as amino acids (Cheng et al., 2015; Xia et al., 2013) and carbohydrate monomers (Fang and Liu, 2002; Xia et al., 2015), the CCRs of *L. digitata* were still quite limited. This could be explained by the following reasons. Firstly, pure small-molecular-weight chemicals are easier for microbes to directly utilize during dark fermentation, whilst the complexity of organic matters in *L. digitata* impaired this process. Secondly, a large part of carbon still remained in the undegraded biomass remnants and some other byproducts (e.g., ethanol, lactic acid, long-chain fatty acids), which could probably be utilized in the second-stage anaerobic digestion.

3.4 Second-stage anaerobic digestion of hydrogenogenic effluents

3.4.1 Biomethane production

Biomethane production of the hydrogenogenic effluents of *L. digitata* biomass in the second-stage anaerobic digestion is shown in Fig. 5. The biomethane yield of the hydrogenogenic effluents of unpretreated *L. digitata* was 222.6 ± 23.8 mL/gVS. The cumulative biomethane yields of those pretreated by HTP, enzymolysis, and HTP followed by enzymolysis were 282.2 ± 2.0 mL/gVS, 271.0 ± 2.7 mL/gVS, and 294.2 ± 3.9 mL/gVS, which were higher than that from the unpretreated one by 26.8%, 21.7%, and 32.2%, respectively. Unexpectedly, the two groups pretreated with the aid

of H_2SO_4 exhibited the lowest biomethane yields of 159.4-164.9 mL/gVS, which were 25.9-28.4% lower than the unpretreated one. This could be attributed to the severity of pretreatment condition. The addition of H_2SO_4 not only accelerated the depolymerization and hydrolysis of *L. digitata* biomass, but also enhanced the further degradation of soluble components, thus generating digestion inhibitor 5-HMF. Besides, Maillard reactions between sugars and amino acids may have happened under HTDAP, resulting in the generation of melanoidins (Wang and Li, 2015) and the reduction of digestible substrates. Similarly, this also explained why the rates of increases in the biohydrogen yields of *L. digitata* pretreated with HTDAP and HTDAP followed by enzymolysis were much lower than that in the carbohydrate monomers. Nonetheless, the markedly higher carbohydrate monomers in these two groups still ensured high biohydrogen yields and CCRs during dark fermentation.

The soluble COD and the corresponding removal efficiencies in the effluents of *L. digitata* biomass before and after second-stage anaerobic digestion are compared in Fig. 6. The soluble COD removal efficiency of unpretreated *L. digitata* after anaerobic digestion was 79.1%, while the ones pretreated by HTP, enzymolysis, and HTP followed by enzymolysis increased to 87.0%, 84.5%, and 91.0%, respectively. By contrast, the two groups pretreated with H_2SO_4 shared the lowest soluble COD removal efficiency of 55.4%, corresponding to a 30% reduction as compared to the unpretreated seaweed. Although the soluble COD of these two groups before anaerobic digestion were high, the methanogens were not able to further utilize the byproducts or inhibitors generated and reduce the COD. These results clearly

demonstrated that the variation trends of soluble COD removal efficiencies coincided with that of the biomethane yields under these different pretreatments.

3.4.2 Energy conversion efficiencies

Fig. 7 presents the ECEs from *L. digitata* to biohydrogen and biomethane after two-stage process. Although the biohydrogen yields of pretreated *L. digitata* significantly exceeded that of raw biomass, the ECE in the biohydrogen accounted only for 3.0-3.9%. Most of the bioenergy remained in the hydrogenogenic effluents as soluble degradation products and undegraded solid remnants. The energy yields of *L. digitata* pretreated by HTP, enzymolysis, and HTP followed by enzymolysis reached 10.2-11.1 kJ/gVS, corresponding to ECEs of 63.4-69.3%. By contrast, due to the inhibition of the second-stage anaerobic digestion, the ECEs from *L. digitata* pretreated with H_2SO_4 were 39.5-40.7%, even lower than that from raw seaweed by 21.9-24.2%. The addition of glucoamylase would lead to a more complex pretreatment process with increased cost at a commercial scale, whilst its contribution to bioenergy yields would be quite limited. Therefore, HTP without H_2SO_4/glucoamylase was considered the optimum pretreatment to boost the energy conversion from *L. digitata* biomass to gaseous biofuels in this study.

Table 3 outlines the comparison of gaseous biofuel production results of pretreated marine macro-algae biomass between literature and this study. Due to the different conditions including algal species, sea conditions, and harvest time, the gaseous biofuel production and energy yields varied greatly. Fermentative

biohydrogen production correlates significantly with the contents of reducing sugars in the biomass, thus pretreatments that helped release more reducing sugars improved the biohydrogen yields (Liu and Wang, 2014; Sivagurunathan et al., 2017). Although pretreatments facilitated significant improvements on biohydrogen yields, the soluble byproducts and undegraded solid remnants in the effluents retained most of the energy, leading to low energy yields after one-stage dark fermentation. By contrast, one-stage anaerobic digestion was considered the most direct way to generate bioenergy in the form of biomethane from seaweed biomass; the energy yields were also much higher than that obtained from one-stage fermentative biohydrogen production (Barbot et al., 2015). Costa et al. (2015) and Lin et al. (2019) further assessed the feasibility of facilitating energy conversion via biohydrogen and biomethane co-production of pretreated seaweed biomass. In the present study, the merits of higher biohydrogen yields and increased ECE were simultaneously achieved through combining HTP of seaweed and two-stage biohydrogen and biomethane co-production. As compared to other pretreatments, HTP did not require addition of non-reusable catalysts (e.g., strong liquid acids) or long treatment retention times. The HTP process could be deemed as a more powerful feedstock sterilization than conventional pasteurization (70°C for 1 h), which is usually required to prevent pathogen contamination (Abbasi et al., 2012).

3.5 Energy balance assessment

As aforementioned, an increase in energy yield of 2229.5 kJ/kgVS (measured in

terms of biohydrogen and biomethane production from *L. digitata* after HTP) was obtained as compared to the unpretreated seaweed. However, the HTP process necessitates high external energy input, which reduces the merits of increased gaseous biofuel production. Whether this increased energy yield could offset the additional energy input to the HTP process is critical for future industrial applications. According to Eq. (3), the energy input of HTP is dependent on two major factors: the working volume per kg VS of *L. digitata* and the HTP temperature. Based on the HTP treatment volume of 66.7 L/kgVS and the HTP temperature of 140 °C in this study, Q_{HTP} was calculated as 7176 kJ/kgVS, which significantly exceeded the energy yield increase. This result clearly indicated that at the current high HTP working volume (66.7 L/kgVS) or low treatment density, the net energy gain by adding HTP was negative. Nonetheless, a positive energy balance could be achieved by lowering the working volume below 20.7 L/kgVS (or increasing the treatment density from 1.5 VS% to over 4.8 VS%) while maintaining the other conditions. This demonstrated that increasing the treatment density could markedly reduce the energy input per unit mass of *L. digitata*, which was in line with the findings in previous studies (Passos and Ferrer, 2015; Yuan et al., 2019). Passos and Ferrer (2015) assessed the energy balance of anaerobic digestion of microalgae biomass after HTP (130 °C for 15 min), and found that increasing the treatment density of microalgae biomass from 2.3 to 7.4 TS% yielded a neutral energy balance; the energy gained through pre-treatment was equivalent to the energy used in pre-treatment. Yuan et al. (2019) used hydrothermally pretreated primary sludge (130 °C for 30 min) for anaerobic digestion and calculated

that a positive energy balance could be achieved when the TS of primary sludge input was over 2.2%. In this study, the treatment density (1.5 VS%) of *L. digitata* was quite low. It could be reasonably speculated that increasing the treatment density to over 4.8 VS% would not significantly alleviate the HTP effects on *L. digitata* biomass. The typical VS content of raw *L. digitata* biomass (10-16 wt%) (Allen et al., 2015; Herrmann et al., 2015) indicated that there is still a high possibility of increasing the treatment density. Alternatively, reducing the HTP temperature could also decrease Q_{HTP}.

In this study, the addition of dilute H_2SO_4 as a catalyst in HTP was proven to facilitate biomass depolymerization and improve first-stage dark fermentation, but it led to deterioration in efficiency of the second-stage anaerobic digestion. A topic for future research is proposed in developing reusable solid acid catalysts, which can simultaneously facilitate biomass degradation and minimize inhibitory effects caused by over-release of byproducts. Such a catalyst could optimize HTP effects on biogas production and may lead to reduction in HTP temperature and retention time required, thus decreasing the energy input.

Although gaining a positive energy balance through improving advanced gaseous biofuel production of hydrothermally pretreated *L. digitata* seems promising, the capital costs and additional energy and labor requirements associated with the HTP reactor and ancillary facilities (e.g., pumps, pipelines, heat exchangers, etc.) need to be taken into account for future industrial applications at a commercial scale.

4. Conclusion

To facilitate degradation of the seaweed *L. digitata* and improve two-stage fermentative biohydrogen and biomethane co-production, various pretreatments were employed to depolymerize the seaweed. HTP (140 °C for 20 min) was considered the optimum pretreatment. Due to the excellent solvent properties of water at high temperature, HTP damaged the original intact algal structures and generated micro-pores and debris, thus enhancing the solubilization of biomass and facilitating the degradation of carbohydrate polymers. The addition of dilute H_2SO_4 during HTP contributed to a higher yield of carbohydrate monomers but generated more inhibitive byproducts. After pretreatment of *L. digitata*, the two-stage dark fermentation and anaerobic digestion process secured biohydrogen and biomethane yields of 44.8 and 282.2 mL/gVS, respectively, corresponding to a total energy yield of 10.6 kJ/gVS, 26.7% higher than the unpretreated biomass; a better soluble COD removal was achieved as well. Considering the external energy input into the HTP process, a positive energy balance could be still expected if the treatment density of *L. digitata* was raised from 1.5 VS% in this study to over 4.8 VS%. For a future commercial process, research is needed to optimize the energy balance and optimization may be achieved through reusable solid acid catalysts, which may reduce HTP temperature and retention time.

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Figures and Tables

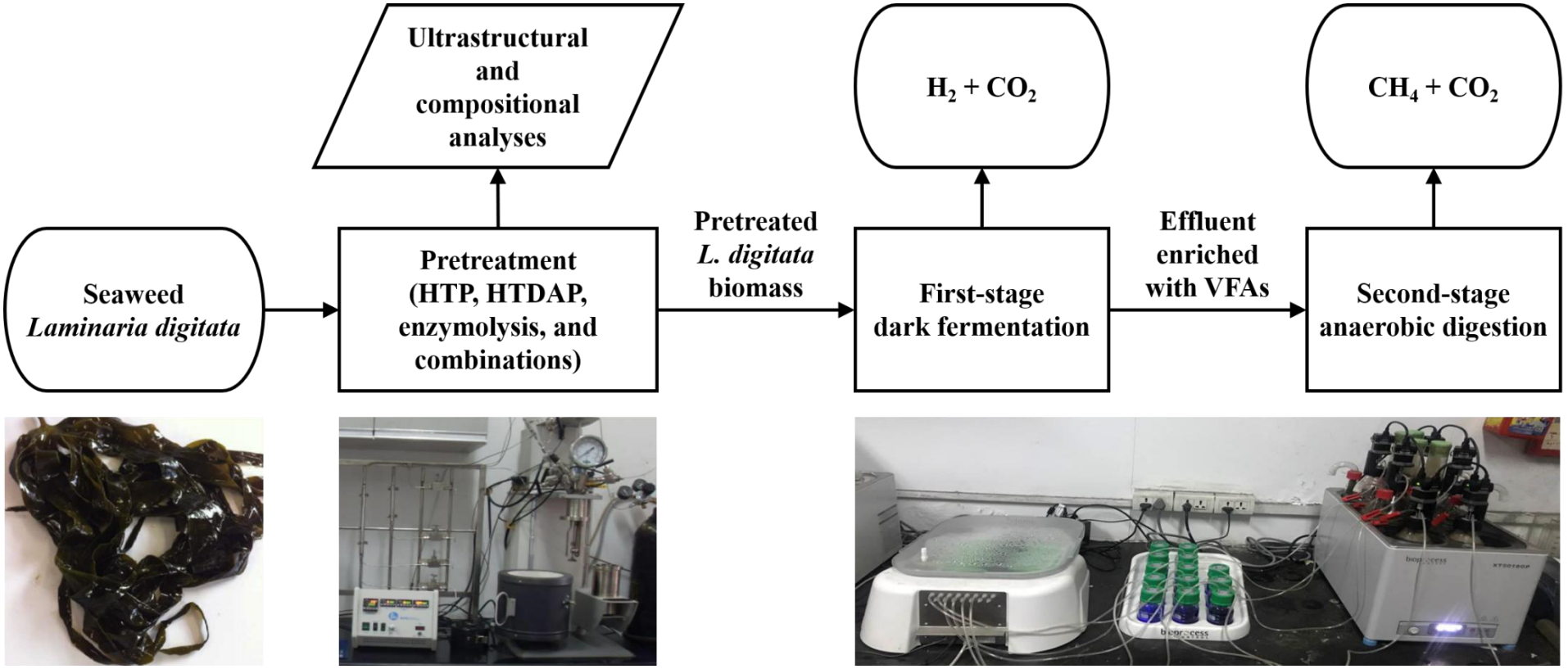

Fig. 1 Experimental schematic of *L. digitata* pretreatments and two-stage dark hydrogen fermentation and anaerobic digestion

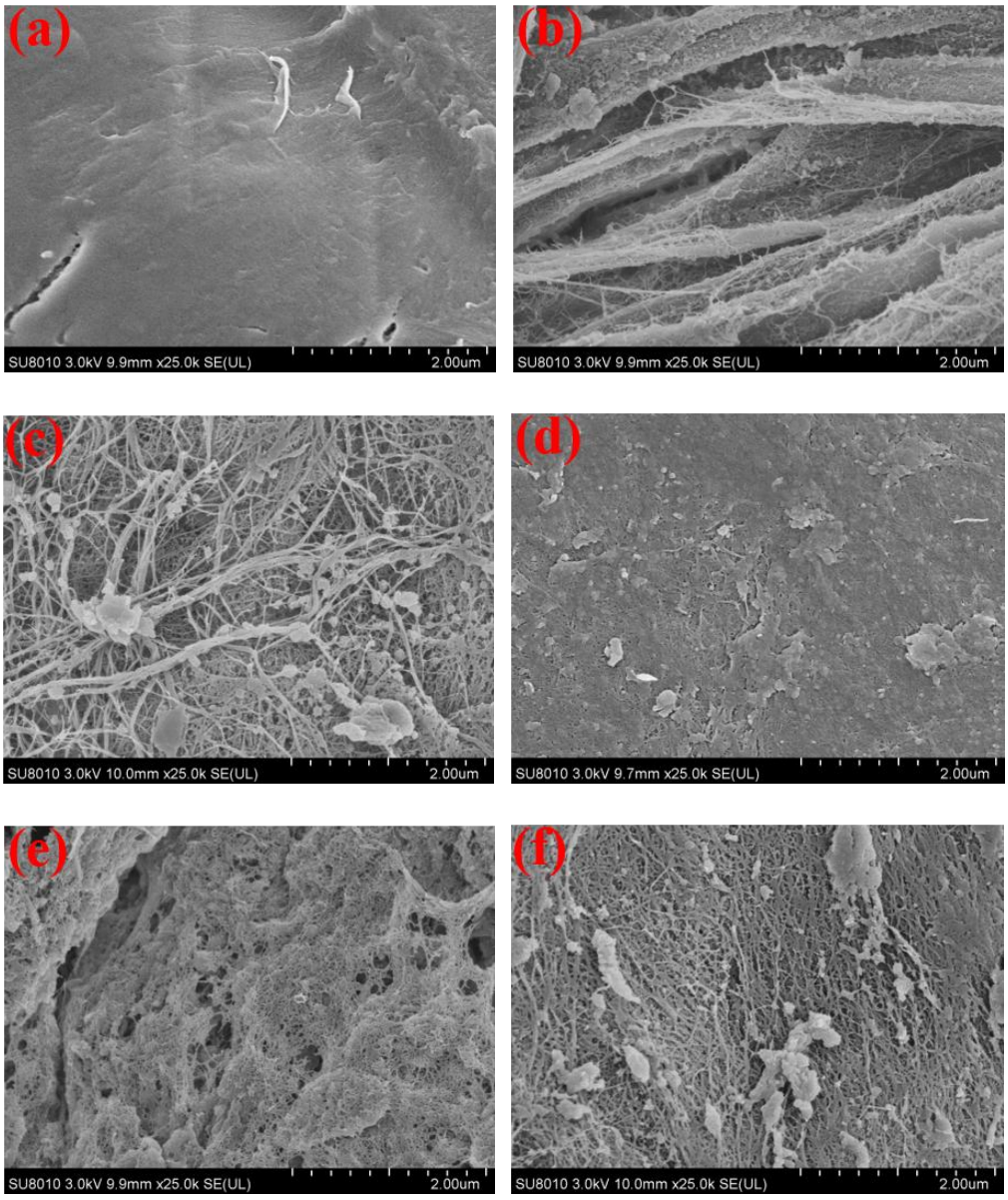

Fig. 2 SEM images of *L. digitata* (at 25,000×) before and after different pretreatments: (a) before pretreatment; (b) after HTP; (c) after HTDAP; (d) after enzymolysis; (e) after HTP + enzymolysis; and (f) after HTDAP + enzymolysis.

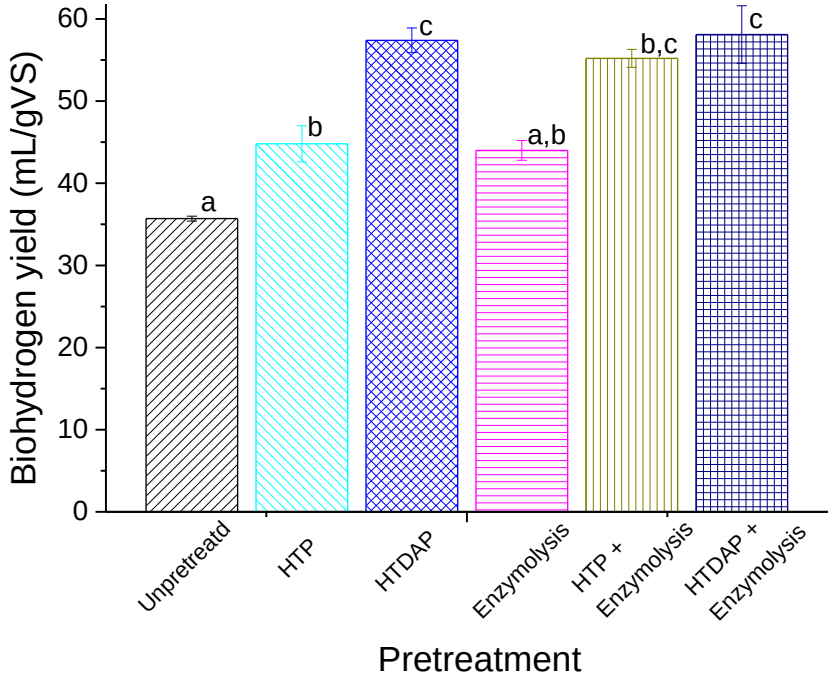

Fig. 3 Biohydrogen production of *L. digitata* biomass through first-stage dark fermentation. Different letters (abc) indicate significant differences between biohydrogen yield means ($P < 0.05$).

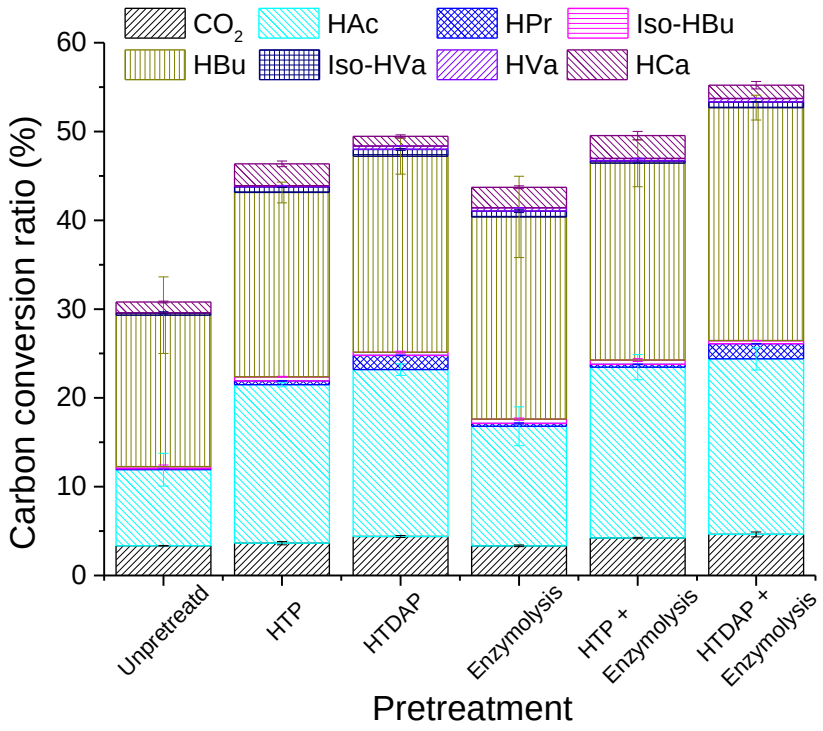

Fig. 4 Carbon conversion of *L. digitata* biomass to carbon dioxide and VFAs through first-stage dark fermentation.

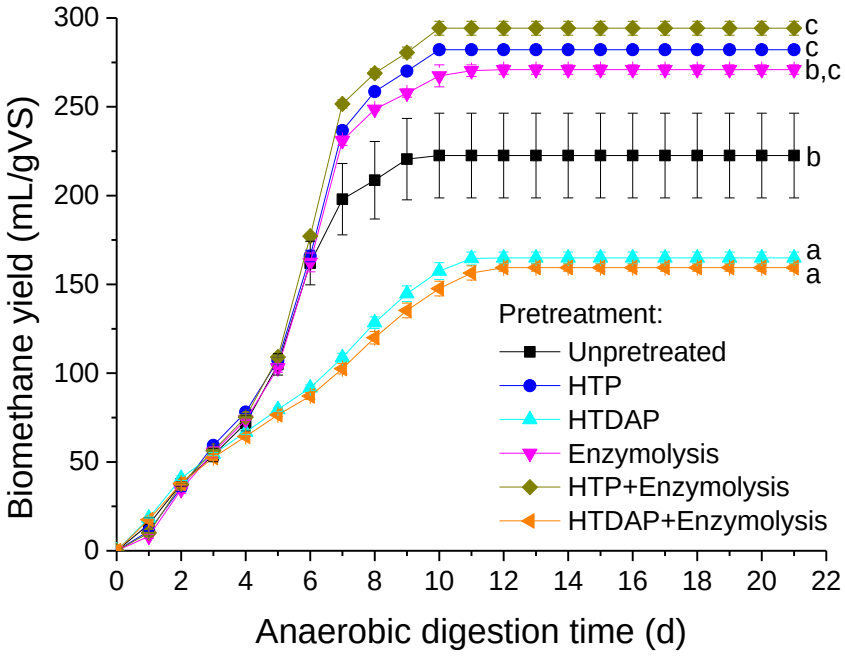

Fig. 5 Biomethane production of hydrogenogenic effluents of *L. digitata* biomass in second-stage anaerobic digestion. Different letters (abc) indicate significant differences between biomethane yield means ($P < 0.05$).

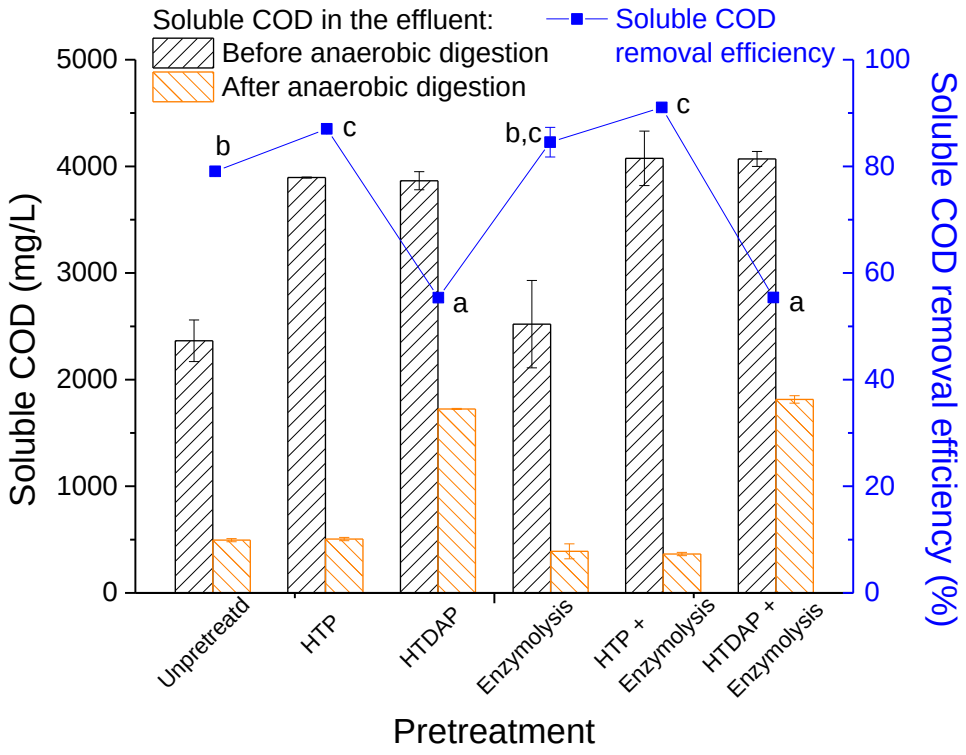

Fig. 6 Soluble COD and removal efficiencies in the effluents of *L. digitata* biomass before and after second-stage anaerobic digestion. Different letters (abc) indicate significant differences between soluble COD removal efficiency means ($P < 0.05$).

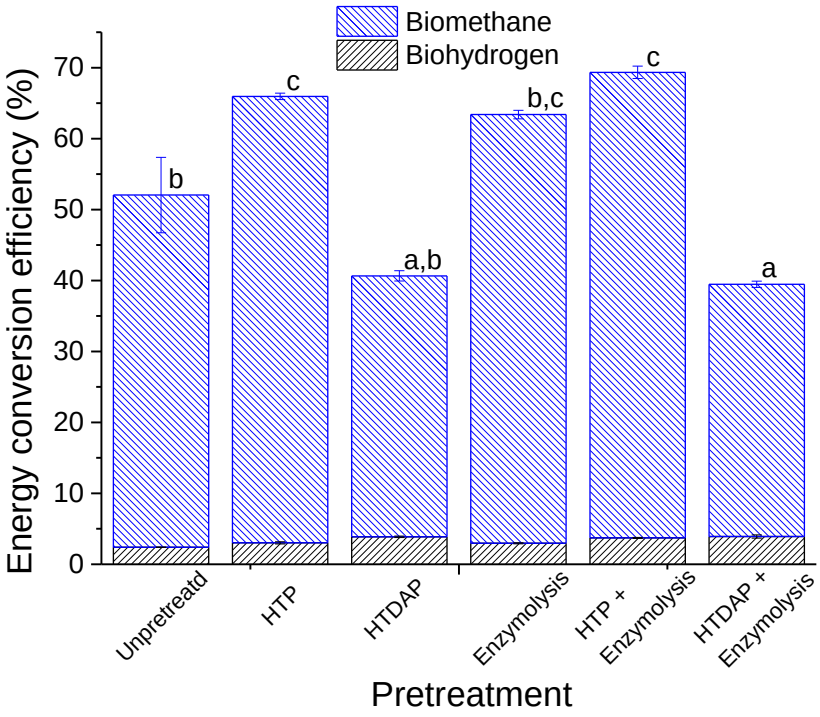

Fig. 7 Energy conversion efficiency from *L. digitata* biomass to biohydrogen and biomethane after two-stage dark fermentation and anaerobic digestion. Different letters (ab) indicate significant differences between energy conversion efficiency means ($P < 0.05$).

Table 1 Composition of *L. digitata* biomass before and after different pretreatments

Pretreatment	Unpretreated	HTP	HTDAP	Enzymolysis	HTP + enzymolysis	HTDAP + enzymolysis
Soluble COD (g/gVS)	0.556[a]	0.876[c]	0.949[cd]	0.677[b]	0.896[c]	1.000[d]
Solubilization yield (%)	45.4	71.5	77.5	55.2	73.2	81.7
Glucose (g/gVS)	0.015[a]	0.016[a]	0.329[b]	0.033[a]	0.044[a]	0.332[b]
Galactose (g/gVS)	0.018[a]	0.022[a]	0.054[b]	0.016[a]	0.023[a]	0.058[b]
Fucose (g/gVS)	/	/	0.034[a]	/	/	0.059[a]
Mannitol (g/gVS)	0.129[a]	0.149[a]	0.147[a]	0.136[a]	0.142[a]	0.144[a]
Total carbohydrate monomers (g/gVS)	0.162[a]	0.187[a]	0.564[b]	0.185[a]	0.209[a]	0.593[b]
Furfural (g/gVS)	/	/	/	/	/	/
5-HMF (g/gVS)	/	0.002[a]	0.005[b]	/	0.002[a]	0.005[b]

Note: Different letters (abcd) indicate significant differences between soluble COD, carbohydrate monomer yield, and 5-HMF yield means ($P < 0.05$).

Table 2 Composition of VFAs in the effluents of *L. digitata* biomass after first-stage dark fermentation

Pretreatment	VFA concentration (g/L)								COD of total VFAs (gCOD/L)	Acidification yield (%)	pH
	HAc	HPr	Iso-HBu	HBu	Iso-HVa	HVa	HCa	Total VFAs			
Unpretreated	0.98^{a}	0.01^{a}	0.02^{a}	1.43^{a}	0.02^{a}	0.00^{a}	0.09^{a}	2.55^{a}	3.94	70.8	5.40
HTP	2.04^{b}	0.04^{ab}	0.04^{a}	1.74^{a}	0.05^{a}	0.01^{b}	0.18^{a}	4.10^{ab}	5.99	68.4	5.28
HTDAP	2.15^{b}	0.15^{c}	0.03^{a}	1.85^{a}	0.06^{a}	0.03^{c}	0.08^{a}	4.35^{ab}	6.30	66.4	5.46
Enzymolysis	1.54^{ab}	0.03^{a}	0.04^{a}	1.91^{a}	0.05^{a}	0.03^{c}	0.17^{a}	3.17^{ab}	5.77	85.3	5.34
HTP + Enzymolysis	2.20^{b}	0.03^{ab}	0.04^{a}	1.86^{a}	0.02^{a}	0.02^{b}	0.19^{a}	4.36^{ab}	6.35	70.8	5.29
HTDAP + Enzymolysis	2.26^{b}	0.16^{c}	0.03^{a}	2.10^{a}	0.05^{a}	0.03^{bc}	0.11^{a}	4.74^{b}	6.93	69.3	5.45

Note: Different letters (abc) indicate significant differences between VFA concentration means ($P < 0.05$).

Table 3 Comparison of gaseous biofuel production using pretreated macro-algae biomass

Macro-algae	Pretreatment	Fermentation type	Biohydrogen yield (mL/gVS)	Biomethane yield (mL/gVS)	Energy yield (kJ/gVS)	Increase in energy yield (%)	Reference
Gelidium amansii	Steam-heating with dilute acid (121 °C for 30 min, 1% H_2SO_4)	One-stage dark fermentation	68.6	/	0.7	92.6	(Sivagurunathan et al., 2017)
Laminaria japonica	Steam-heating (121 °C for 30 min)	One-stage dark fermentation	66.7	/	0.7	667	(Liu & Wang, 2014)
Biomass mixture of beach macro-algae	Thermal acidic (80 °C for 2 h, 0.2 M HCl)	One-stage anaerobic digestion	/	121	4.3	39	(Barbot et al., 2015)
Ascophyllum nodosum	Ensiling (90 d)	One-stage anaerobic digestion	/	239.3	8.6	28.9	(Herrmann et al., 2015)
Sargassum sp.	Steam-heating (121 °C for 15 min)	Two-stage dark fermentation and anaerobic digestion	91	541	20.3	/	(Costa et al., 2015)
Saccharina latissima	HTP (140 °C for 30 min)	Two-stage dark fermentation and anaerobic digestion	10.7	345.1	12.5	22.5	(Lin et al., 2019)
L. digitata	HTP (140 °C for 20 min)	Two-stage dark fermentation and anaerobic digestion	44.8	282.2	10.6	26.7	This study

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author Contribution Statement

Lingkan Ding: Conceptualization, Investigation, Formal analysis, Writing-Original Draft;
Jun Cheng: Conceptualization, Writing-Reviewing and Editing, Funding acquisition, Supervision;
Richen Lin: Conceptualization, Resources, Writing-Reviewing and Editing;
Chen Deng: Resources, Writing-Reviewing and Editing;
Junhu Zhou: Project Administration, Funding acquisition;
Jerry D. Murphy: Writing-Reviewing and Editing, Funding acquisition, Supervision.