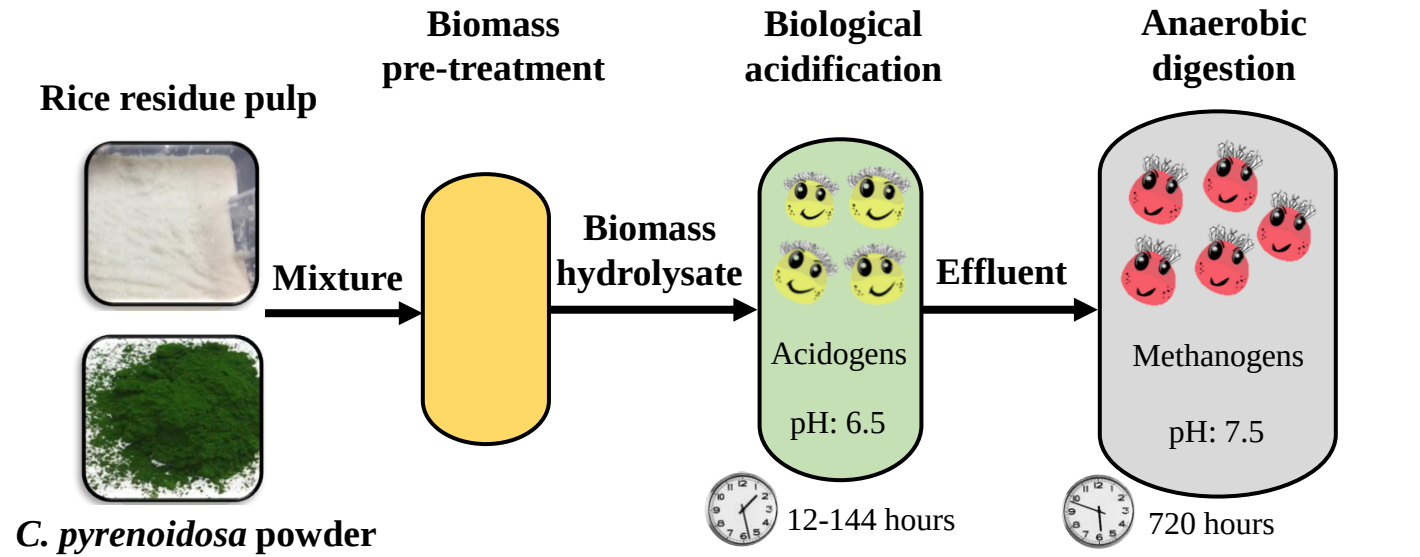
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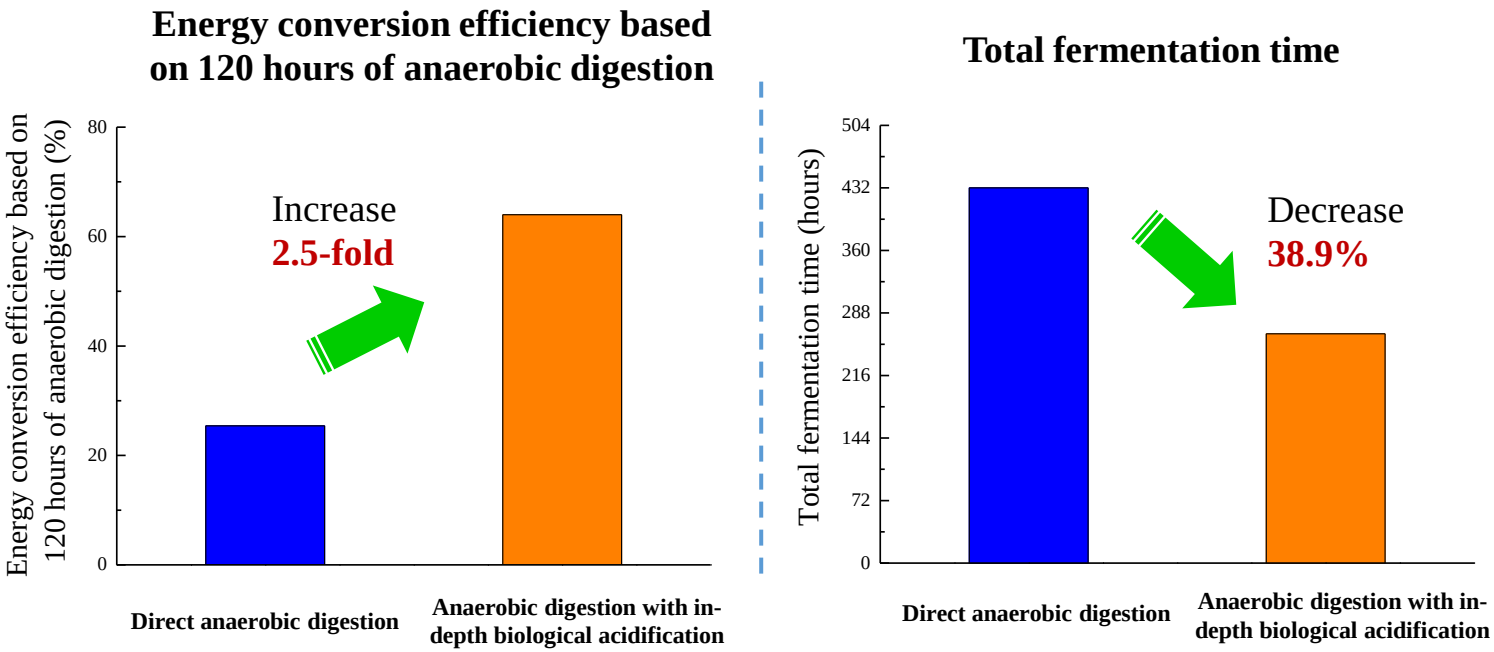
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Graphical Abstract (for review)





Highlights

- Synergistic effects of pre-treatment and bio-acidification were assessed.
- Hydrothermal acid pre-treatment was benefical for biological acidification.
- Increasing bio-acidification time increased acetic acid production.
- Bio-acidification decreased lag-phase time whilst improving methane production.
- 144 hours bio-acidification achieved maximum energy conversion efficiency of 64%.

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Effects of pre-treatment and biological acidification on fermentative hydrogen and methane co-production

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Abstract

A sequential two-stage process comprising biological acidification followed by anaerobic digestion was proposed to enhance gaseous biofuel production from the mixture of rice residue and microalgae with thermo-chemicial hydrolysis. The maximum specific hydrogen yield of 223.1 ± 8.8 mL/g volatile solids (VS) and production rate of 10.4 ± 0.4 mL/g VS/h were achieved from hydrothermal acid pre-treated biomass during biological acidification. Increase in hydraulic retention time of biological acidification from 12 to 144 hours significantly affected the distribution of solubilised metabolic products and led to improve biological acidification rates (BARs) from 15.5% to 78.5%. Compared with single stage anaerobic digestion, the first stage acidification phase led to reductions in the lag-phase time and peak time of anaerobic digestion in such a two-stage process. The maximum specific methane production rate of 2.2 ± 0.03 mL/g VS/h was achieved with a deep acidification of 144 hours yielding a BAR of 78.5%. Increasing the length of time in biological acidification from 12 to 144 hours contributed to improve energy conversion efficiency of 25.4%–64% after 120 hours of anaerobic digestion. These results demonstrate that biological acidification is feasible to improve bioenergy recovery in two-stage fermentation.

Keyword: Fermentation; Hydraulic retention time; Biological acidification; Biomethane; Algae; Food waste.

1 Introduction

Biofuels, such as biogas, biodiesel and bioethanol, are alternative renewable options for carbon intensive transport fossil fuels; they can significantly reduce greenhouse gas emissions, improve air quality and increase the security of energy supply [1-4]. Compared with liquid biofuels, gaseous biofuels (such as biogas) generally have more advantages [5-7], especially in the energy conversion efficiency, greenhouse gas emissions, and the convenience of distribution system. Biogas can be used as an energy source for various applications including heat, electricity, and vehicle fuel [8]. Additionally, biogas can substitute for all natural gas applications when injected to the gas grid [2].

Anaerobic digestion is a well-established bioconversion technology, in which various types of biomass and organic wastes can be converted to biogas by anaerobic microorganisms at relatively low temperature (35–55 °C) and ambient pressure [9,10]. This technology comprises four stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) all of which require interaction between different types of microorganisms [6,11]. However, the microorganisms in each stage have different metabolic pathways and activities, and can be disturbed by environmental stresses and undesirable process factors (such as pH, temperature, and retention time). As such, the environment and the process variables need to be optimised to ensure a high efficiency for biogas production in anaerobic digestion.

Previous studies found that the growth rate of acidogens at the acidogenesis stage was far superior than that of methanogens at the methanogenesis stage, especially

when easily degradable feedstock (such as food waste) was used as fermentation substrate [12]. Based on this, the production capacity of volatile fatty acids (VFAs) by acidogens would exceed the ability of methanogens to process the VFAs [12]. Excessive accumulation of VFAs can cause a significant drop of pH, thereby hindering the fermentative methane production [13]. A separate process of biological acidification prior to anaerobic digestion is considered as one of the effective methods to avoid this imbalance between VFA production and methanogenic consumption. And not only that, hydrogen produced via biological acidification would become another key renewable energy product. The addition of hydrogen to methane can overcome several disadvantages of pure methane in engines, such as high ignition temperature, slow burning speed and narrow flammability range [6].

Notably, the biochemical conversion efficiency and biological stability in such a two-stage process are affected by the biological acidification rate (BAR) of feedstocks [14,15]. This can be controlled by adapting the hydraulic retention time (HRT) of biological acidification. A short HRT generally results in a low BAR with a low VFA production rate, corresponding to a low hydrogen yield [6,16]. Also, the insufficient substrates of VFAs for methanogens in anaerobic digestion would further lead to a low methane yield. A long HRT favours the complete degradation of feedstocks and produces a high concentration of VFA. Even so, the composition and concentration of soluble metabolic products (SMPs), such as alcohols and VFAs, differ with different HRTs [17]. The performance of the subsequent methane producing stage is influenced by these SMPs. For instances, the bioactivity of methanogens is inhibited at a

propionic acid concentration of 900 mg/L, while the inhibitory concentrations of ethanol, acetic acid, and butyric acid were higher than 1800 mg/L [18].

Additionally, the biological acidification process is also closely related to the physicochemical properties of feedstocks and the intensities of pre-treatment [19-22]. For easily degradable biomass, a relatively low HRT is sufficient for acidogens to achieve the high hydrolysis and acidification efficiencies, subsequently resulting in significant increases in hydrogen and methane yields [6]. However, to ensure an effective biological acidification of recalcitrant feedstocks, the HRT has to be sufficiently high [6]. Since the release and hydrolysis of high-molecular intracellular organic matters for recalcitrant feedstocks can be enhanced by the application of pre-treatment, this technology is generally used to decrease the HRT of biological acidification and improve the subsequent fermentation performance [19-22]. It should be noted that the side reactions, such as the self-decomposition of sugars (or amino acids) and Maillard reactions between sugars and amino acids, would also occur during thermo-chemical pre-treatment of biomass, especially for mixtures of carbohydrate-rich and protein-rich biomass [23]. Some of toxic by-products produced from these adverse reactions, such as furans and phenols, may inhibit the bioactivity of enzymes and damage the structures of DNA, further impeding the fermentation pathway during biological acidification and subsequent anaerobic digestion [24,25].

Overall, the gap in the state of the art is the study of synergistic effects of pre-treatment and biological acidification at various HRTs on anaerobic digestion, especially for the substrates with significantly different physicochemical properties. In

this study, the easily degradable rice residue (RR) with high content of carbohydrates and the slowly degradable microalgae *Chlorella pyrenoidosa* (CP) with high content of proteins were hydrothermally pre-treated, and then the mixture was used as the co-fermentation feedstock. The objectives of the present study are to:

- Analyse the impacts of various HRTs on biological acidification.
- Evaluate the synergistic effects of pre-treatment and biological acidification on subsequent anaerobic digestion.
- Assess the bioenergy recovery characteristics of gaseous biofuel production from co-fermentation of algae and rice residues.

2 Materials and methods

2.1. Substrates and inocula

RR was collected from a dining hall in Chongqing University, China. To remove the attached greases, the RR was washed thoroughly using deionized water. Subsequently, the treated RR was blended into pulp using a blender. RR pulp was loaded into zip-lock bags and then stored at −20 °C before use. CP powder was purchased from Yantai Hairong Biotechnology Co., Ltd., China. The purchased CP powder was stored at room temperature in a dry environment before use. The characteristics of RR pulp and CP powder are shown in Table 1.

The inocula for biological acidification were collected from a rural domestic biogas digester in China. The raw sludge was heated at 100 °C for 30 min in an autoclave (Boxun YXQ-LS-SII, China). After heating treatment, the acidogens survived through forming spores, while the methanogens were deactivated. To revive

and enrich the acidogens, the heat-treated sludge was acclimatized 3 times (72 hours for each time interval) using a modified culture medium at 35 °C under an anaerobic environment [26]. Total solids (TS) and volatile solids (VS) of the activated acidogens were 116.9 and 73.1 g/kg fresh weight, respectively. The inocula for anaerobic digestion were collected from the same biogas digester. The raw sludge was filtered by a 2-mm sieve to remove large particles, and subsequently acclimatized 3 times (168 hours for each time interval) using cellulose (1.5 g/L) at 35 °C under an anaerobic environment. TS and VS of the activated methanogens were 58.2 and 27.1 g/kg fresh weight, respectively.

2.2. Pre-treatment of mixed biomass

The hydrothermal (140 °C, 10 min) and hydrothermal acid pre-treatment (140 °C, 10 min, 1% (v/v) H_2SO_4) were carried out in triplicate in a 70 mL reaction kettle (Taikang QN-WCGF, China) [27]. The working volume of this reaction kettle was 50 mL. The mixed raw RR and CP (2.5 g TS) were pre-treated at a VS ratio of RR to CP of 25 [27], corresponding to a C/N molar ratio of 31.3. After hydrothermal acid pre-treatment, the pH values of biomass hydrolysates were adjusted to 7.5 ± 0.1 using 3 M NaOH and HCl solutions. The hydrolysates composed of solubilised matters together with solid residues were loaded into 50 mL centrifuge tubes and then stored at −20 °C before being used as fermentation substrates. Mixed RR and CP without pre-treatment was set as the control group.

2.3. Biological acidification

The experimental design details of biological acidification and subsequent anaerobic

digestion as well as direct anaerobic digestion are shown in Fig 1. Biological acidification was performed in triplicate in 500 mL glass fermenters with an effective working volume of 300 mL each. The mixed RR and CP hydrolysates including solubilised matters and solid residues (containing 5 g TS) were used in biological acidification. A certain amount of deionized water was added to each fermenter to maintain an overall volume of 270 mL, and then the fermenters were inoculated with 30 mL of activated acidogens. The initial pH values were adjusted to 6.5 ± 0.1 using 3 M NaOH and HCl solutions [28,29]. All the fermenters were sealed with rubber stoppers, and nitrogen was purged for 5 min to ensure an anaerobic environment. The biological acidification process was operated in a thermostatic water bath at 35.0 ± 0.5 °C under various HRTs (12–144 hours). During biological acidification, the pH values were adjusted to 6.5 ± 0.1 using 3 M NaOH and HCl solutions at predetermined time intervals (6 or 12 hours). The produced gases were released from the headspace of fermenters, collected in graduated gas collectors [30], and then recorded at each predetermined time interval [27]. A blank group only containing inocula and a control group using raw mixed biomass with no pre-treatment as substrate were also operated under the same experimental procedure.

2.4. Anaerobic digestion

Anaerobic digestion was performed in triplicate in the AMPTS II system (Bioprocess Control, Sweden) [31]. The biological acidification effluents (BAEs) including supernatants and solid residues were transferred to 500 mL glass fermenters and then used as substrate in anaerobic digestion. Each fermenter with a working volume of

410 mL contained 150 mL of BAEs and 260 mL of methanogens, corresponding to a substrate to inoculum VS ratio of 1:2 [32,33]. The initial pH values were adjusted to 7.5 ± 0.1 using 3 M NaOH and HCl solutions. All the fermenters were sealed with rubber stoppers, purged with nitrogen for 5 min, and then placed in a water bath at 35.0 ± 0.5 °C for 720 hours. During anaerobic digestion, the pH values were without control. A blank group only containing inocula and a control group (direct anaerobic digestion) separately using raw, hydrothermal pre-treated, and hydrothermal acid pre-treated mixed biomass, and cellulose as substrate were also operated under the same experimental procedure.

Notably, the AMPTS II system cannot analyse the composition of the produced gases. To measure the yields of hydrogen and methane produced during the initial 72 hours of anaerobic digestion, the produced gases contained hydrogen, methane, and carbon dioxide were firstly collected in the graduated gas collectors and then recorded at every 12 hours, as previously discussed. Differently, this process had no pH adjustment. After the initial 72 hours of anaerobic digestion, the produced gases would contain only methane and carbon dioxide. The fermentation reactors could be connected to the carobon dioxide adsorption unit in the AMPTS II system and then the yield of methane could be automatically recorded and measured by a build-in tipping device [31].

2.5. Analytical methods

The carbohydrates, reducing sugars, proteins, lipids, TS, and VS were measured as described in the previous studies [34,35]. A spectrophotometer (Hach DR3900, USA)

coupled with a heating digestion unit (Hach DRB200, USA) was used to analyse the chemical oxygen demand (COD) and total ammonia nitrogen (TAN) [27]. An elemental analyser (Elementar Vario MACRO cube, Germany) was applied to measure the C, H, N, and S contents [36]; the remaining VS content was determined as O [36,37]. The pH values were measured by a portable pH meter (METTLER F2, Switzerland). Hydrogen, methane, and carbon dioxide were determined by a gas chromatograph (GC) (Thermo Trace 1300, USA) equipped with a micro-packed column (ShinCarbon ST Columns, 2 m, OD 1/16, ID 1.0 mm, Mesh 100/120) and a thermal conductivity detector (TCD) [25,36]. The contents of SMPs including ethanol, acetic acid, propionic acid, butyric acid, valeric acid, and caproic acid were analysed using another GC (Agilent 7890B, USA) equipped with a polar capillary column (Agilent DB-FFAP Column, 30 m × 0.25 mm × 0.25 μm) and a flame ionization detector (FID) [36,38]. All experimental trials and measurements were conducted in triplicate, and the results were expressed as the average ± standard deviation.

2.6. Calculations

The BAR (%) was calculated using the ratio of the total COD weight of SMPs to the total COD weight of initial mixed biomass. The specific hydrogen yield (SHY) and methane yield (SMY) (mL/g VS) were calculated based on the volume of total gases (normalized to zero moisture content, standard temperature of 0 °C and pressure of 1 atm) and content of hydrogen and methane both in the gas collector and fermenter headspace at each time interval [27]. Thereafter, the SHY and SMY were simulated using a modified Gompertz equation [39]; the kinetic parameters including the

maximum production potential (H_m, mL/g VS), peak rate (R_m, mL/g VS/h), peak time (T_m, h), and lag-phase time (λ, h) were calculated through the Origin software.

The higher heating values of hydrogen and methane were determined as 286 and 889 kJ/mol, respectively [29,36]. The higher heating values (kJ/g VS) of RR and CP were calculated using the Mendeleev formula, Eq. (1). Where, C, H, O, and S represent the VS percentage of each element in the initial biomass, respectively. Additionally, the energy recovery characteristics were quantitatively analysed by energy conversion efficiency (ECE) and energy conversion percentage (ECP_{120}). The ECE (%) and ECP_{120} (%) were calculated based on the Eqs. (2) – (3).

$$\text{Heating value (kJ/g VS)} = 0.33858C + 1.254H - 0.10868(O - S) \quad (1)$$

$$\text{ECE (\%)} = \frac{\text{Total energy value (kJ) of the produced hydrogen and methane}}{\text{Total energy value (kJ) of initial mixed biomass}} \times 100 \quad (2)$$

$$ECP_{120}\ (\%) = \frac{\text{ECE (\%) based on 120 hours of anaerobic digestion}}{\text{Total ECE (\%) based on the complete anaerobic digestion}} \times 100 \quad (3)$$

3 Results and Discussion

3.1. Comparison of organic matter solubilisation from different pre-treatments

The effects of hydrothermal and hydrothermal acid pre-treatment were analysed in terms of solubilised COD, carbohydrates, proteins, and reducing sugars, as shown in Fig. 2. The initial concentration of solubilised carbohydrates and proteins derived from raw biomass were only 4.27 ± 0.18 and 0.43 ± 0.02 g/L, respectively, corresponding to 6.63 ± 0.76 g solubilised COD/L. Meanwhile, the low reducing sugar concentration of 0.86 ± 0.01 g/L indicated that most of solubilised carbohydrates presented in the form of starch, which is the main polysaccharide in RR and CP [40].

After hydrothermal pre-treatment, carbohydrates (28.77 ± 0.89 g/L) and proteins (1.87 ± 0.32 g/L) in the soluble phase increased 5.7-fold and 3.3-fold, respectively. This illustrated that RR and CP cells could be effectively disrupted by hydrothermal pre-treatment. Nevertheless, the reducing sugar concentration (1.87 ± 0.24 g/L) only constituted 6.5% of solubilised carbohydrates. The hydrolysis of starch into reducing sugars such as maltose and glucose is due to the fracture of glycosidic bonds. Without adding any chemicals, the glycosidic bonds of starch are more difficult to damage under a relatively low reaction time (10 min) and temperature (140 °C) [27].

When raw biomass was subjected to hydrothermal acid pre-treatment, the reducing sugar concentration (45.53 ± 3.3 g/L) significantly increased 52.2-fold and 23.3-fold, respectively, compared with raw and hydrothermal pre-treated biomass. Unexpectedly, this concentration was more than the total concentration of solubilised carbohydrates (42.06 ± 2.6 g/L). Previous studies found that monosaccharides such as galactose and glucose could further break down into various by-products under severe pre-treatment conditions in the presence of acids [41-43]; some of these by-products may present reduction properties, which caused the high detected reducing sugar concentration [27]. Additionally, monosaccharide can react with amino acids, which is named as the Maillard reaction [44,45]. The Maillard products may also possess the reducing groups such as free aldehyde and ketone groups [27]. Thus, excess reducing sugars detected in the hydrolysates could be caused by the formation of these reducing substances.

3.2. Effects of different pre-treatments on biological acidification

3.2.1 Degradation of organic matters

The degradation effects of solubilised organic matters before and after biological acidification are shown in Table 2. When hydrothermal and hydrothermal acid pre-treated biomass were separately fermented for 144 hours, the concentrations of solubilised carbohydrates were significantly decreased from 9.59–14.02 g/L to 0.32–0.47 g/L. Whereas, the changes in the concentrations of solubilised proteins (from 0.62–1.92 g/L to 0.4–0.56 g/L) were not obvious. This indicated that carbohydrates were more readily utilised by acidogens than proteins. Previous studies found that amino acids derived from proteolysis were not suitable substrates for hydrogen production during biological acidification; the negligible SHYs were generally ranged from 0.2 to 16.2 mL/g VS [46,47]. Therefore, hydrogen could be mainly produced from the fermentation of carbohydrates. Additionally, the low final concentration of solubilised carbohydrates from raw biomass (0.16 g/L) indicated that organics in the solid substance would be also hydrolysed and utilized by acidogens.

3.2.2 Biohydrogen production

Fig. 3 depicts the effects of pre-treatment methods on fermentative hydrogen production during biological acidification. When raw RR and CP were used as mixed substrate, the maximum SHY and specific hydrogen production rate (SHPR) were only 100.7 ± 6.6 mL/g VS and 2.7 ± 0.3 mL/g VS/h, respectively. Since the organic matters in the raw biomass were tightly surrounded by the intact biomass cell wall, the low concentrations of solubilised carbohydrates (1.42 ± 0.07 g/L) and proteins (0.14 ± 0.00 g/L) were insufficient to maintain the bioactivity and growth of acidogens [36],

as shown in Table 2. Additionally, high-molecular polysaccharides were difficultly to hydrolyse and utilise by acidogens [48]. A low reducing sugar concentration (0.29 ± 0.01 g/L) before biological acidification resulted in low SHY and SHPR.

When hydrothermal pre-treated biomass was used as substrate, the maximum SHY and SHPR increased to 169.5 ± 25.0 mL/g VS and 5.8 ± 0.4 mL/g VS/h, respectively. This could be attributed to the improvements in the damage to the biomass structures and the release of intracellular carbohydrates (9.59 ± 0.28 g/L) and proteins (0.62 ± 0.09 g/L). However, the low hydrolysis efficiency of polysaccharides during hydrothermal pre-treatment also resulted in low reducing sugar concentration (0.62 ± 0.11 g/L), which still resulted in an unsatisfactory level of hydrogen production during biological acidification.

When hydrothermal acid pre-treatment was applied to hydrolyse raw biomass, the maximum SHY and SHPR significantly increased to 223.1 ± 8.8 mL/g VS and 10.4 ± 0.4 mL/g VS/h respectively, which shows 2.2-fold and 3.8-fold increases compared with the values obtained from raw biomass. This was due to the highest concentrations of solubilised organic matters especially reducing sugars (15.18 ± 0.23 g/L) before biological acidification. Meanwhile, the increase of available carbon and nitrogen sources effectively promoted the biomass degradation by acidogens [49,50].

The kinetic parameters of fermentative hydrogen production derived from the modified Gompertz model are presented in Table 3. The highest SHY potential of 223.7 mL/g VS and SHPR of 10.8 mL/g VS/h were obtained from hydrothermal acid pre-treated biomass. Compared with raw biomass without/with hydrothermal

pre-treatment, the high amounts of easily degradable low-molecular organic matters obtained from hydrothermal acid pre-treatment also effectively shortened the lag-phase time (decreased from 6.7 to 4.2 hours) and peak time (decreased from 21.9 to 11.8 hours) of biological acidification.

3.2.3 Production of soluble metabolite products

As shown in Table 2, the SMPs in the effluents of biological acidification contained abundant butyric acid (6.18–9.51 g COD/L) and acetic acid (2.62–3.86 g COD/L), and a small quantity of propionic acid (0.27–1.54 g COD/L), caproic acid (0.99–1.11 g COD/L), ethanol (0.22–0.39 g COD/L) and valeric acid (0.12–0.2 g COD/L). In this case, acidogens mainly conducted acetic acid and butyric acid pathways. The total SMP concentration obtained from raw, hydrothermal pre-treated, and hydrothermal acid pre-treated biomass were 11.75 ± 0.35, 13.16 ± 0.58 and 15.19 ± 0.14 g COD/L, respectively, corresponding to the BARs of 60.7 ± 1.2%, 68.0 ± 2.0% and 78.5 ± 1.2%. Compared with hydrothermal and hydrothermal acid pre-treatment, the concentration of propionic acid (1.54 ± 0.01 g COD/L) from raw biomass without pre-treatment was 1.2-fold and 4.7-fold higher, respectively. Since the production of propionic acid is considered as an unfavourable hydrogen consuming pathway [51], such a phenomenon may be another explanation for the relatively lower SHY and SHPR obtained from raw biomass.

The concentrations of TAN derived from amino acid hydrolysis increased to the range of 40.3–204.8 mg/L. But such a concentration is still below the suggested threshold level (4000 mg/L) that negatively affects the bioactivity of acidogens [13].

This further confirmed that co-fermentation of RR and CP could effectively mitigate the inhibitory effects of TAN accumulation during biological acidification.

3.3. Effects of hydraulic retention time on biological acidification

The hydrothermal acid pre-treated biomass was used as fermentation substrate to assess the effects of various HRTs on biological acidification. As shown in Fig. 3 and Table 2, when the HRT was set as 12 hours, the final concentration of solubilised carbohydrates and proteins were still as high as 9.5 ± 1.53 and 0.88 ± 0.09 g/L, which are 67.8 ± 3.5% and 45.8 ± 2.1% of the initial concentration, respectively. Meanwhile, a low BAR of 15.5 ± 0.6% and a low SHY of 87.2 ± 0.8 mL/g VS were obtained from converting biomass to SMPs (3.0 ± 0.34 g COD/L). Rathbun et al. and Xia et al. found that acidogens required a few hours lag-time (3–6 hours) to develop the ability to degrade specific organic matters [52,53]. Such a short HRT of 12 hours in biological acidification was insufficient for complete biomass degradation, as well as high SMP and hydrogen production.

When the HRT was increased to 48 hours, the concentration of solubilised carbohydrates significantly decreased from 9.5 to 1.04 g/L, thereby leading to a high SHY of 222.4 ± 8.9 mL/g VS, a high SMP production of 14.47 ± 0.13 g COD/L and a high BAR of 74.7 ± 1.4%. However, the change in the concentration of solubilised proteins was not obvious (from 0.88 to 0.62 g/L). During the adaptation period (0–12 hours), a certain amount of proteins (1.04 g/L) were hydrolysed and then used as the nitrogen sources to sustain the growth and reproduction of acidogens. After the bioactivity of acidogens was effectively improved (12–48 hours), monosaccharide

consumption by acidogens generally preceded amino acid consumption [46], as previously discussed. The conservative increases of TAN concentration ranged from 13.5 ± 1.1 to 42.7 ± 3.0 mg/L confirmed such an explanation.

The non-obvious decrease trend for the concentration of solubilised proteins (from 0.62 to 0.6 g/L) was not disappeared when the HRT was increased to 72 hours. Differently, since monosaccharide was largely exhausted, the utilisation of amino acids derived from proteolysis was greatly enhanced, resulting in an obvious increase in TAN (120.1 ± 6.9 mg/L). Even so, due to the low-effective fermentative hydrogen production of amino acids [46,47], the SHY (222.4 ± 8.9 mL/g VS), the SMP production (15.03 ± 0.32 g COD/L), and the BAR (77.6 ± 0.8%) were similar to these obtained after 48 hours fermentation. This also indicated that hydrogen production via biological acidification had been completed during the period of 48–72 hours.

When a long HRT of 144 hours was applied to acidize biomass, the final TAN concentration further increased to 204.8 ± 11.6 mg/L. Although a relative in-depth acidification has no significant effects on improving the SHY (223.1 ± 8.8 mL/g VS), the SMP production (15.19 ± 0.14 g COD/L), and the BAR (78.5 ± 1.2%), the distribution of SMPs was altered. To be specific, at the end of rapid biological acidification period (about 24 hours), the concentrations of propionic acid, valeric acid, and caproic acid were only 0.05 ± 0.01, 0.01 ± 0.00, and 0.11 ± 0.01 g COD/L, respectively. However, after biological acidification of 144 hours, their final cumulative concentrations increased to 0.27 ± 0.01, 0.12 ± 0.00, and 1.08 ± 0.04 g COD/L, respectively. Hydrogen could be consumed during the production of these

three types of VFAs [6,54]. This may be one reason for the non-obvious increases in SHY. Some hydrogen and carbon dioxide could be also converted to acetic acid via the homoacetogenic pathway, thereby resulting in the increase in acetic acid concentration (3.86 ± 0.19 g COD/L). Additionally, the decrease in butyric acid concentration (9.51 ± 0.19 g COD/L) also suggested that a long HRT of biological acidification may lead to a mutual transformation of different SMPs.

Noblecourt et al. reported that the process of hydrogen production via biological acidification would obviously decrease when the mass concentration of VFAs exceeded 12.5 g/L [55]. In this study, the COD concentration of VFAs obtained from 12–144 hours fermentation were in the range of 2.74–14.85 g COD/L (Table 2), corresponding to the mass concentration of 1.78–9.58 g/L. These values are much lower than the threshold level. Besides, the pH values for biological acidification were all adjusted to 6.5 ± 0.1 at each predetermined time interval (6 or 12 hours). Therefore, the inhibitory effects caused by the pH drop and VFA accumulation would be fully mitigated during the whole period of biological acidification.

3.4. Methane production during anaerobic digestion

3.4.1 Direct anaerobic digestion

When cellulose was used as substrate for direct anaerobic digestion, the maximum SMY was 311.5 ± 2.2 mL/g VS. This result suggested a healthy inoculum condition, and the bioactivity of methanogens for converting cellulose to methane was already within the acceptable range [31]. Fig. 4a shows the SMYs obtained from raw, hydrothermal pre-treated, and hydrothermal acid pre-treated biomass during direct

anaerobic digestion. Based on raw biomass, the maximum SMY of 220.0 ± 0.2 mL/g VS was relatively low due to the presence of unbroken biomass cell structures. After hydrothermal pre-treatment, the maximum SMY increased to 272.4 ± 0.8 mL/g VS, indicating that the application of pre-treatment could also improve the performance of anaerobic digestion [56]. Whereas, a severe pre-treatment such as the use of dilute acid led to an obvious decrease in SMY (255.7 ± 1.2 mL/g), which was a totally different trend to hydrogen production during biological acidification. This was attributed to the combined action of anaerobic mixed microflora and special fermentation substrate.

The anaerobic mixed microflora used in methane production contained various types of microorganism such as acidogens and methanogens. Since starch could be effectively hydrolysed into reducing sugars under hydrothermal acid pre-treatment, the easily degradable substrates especially RR (more than 90% of starch) were rapidly degraded by acidogens, resulting in the accumulation of VFAs and decrease of pH (5.03 ± 0.51) at the early stage of direct anaerobic digestion (0–48 hours). Thus, the growth of methanogens would be inhibited [6], thereby achieving a relatively low SMY.

In fact, such negative effects also occurred during direct anaerobic digestion from raw and hydrothermal pre-treated biomass, which was directly reflected through the fluctuation of specific methane production rates (SMPRs), as shown in Fig. 4b. On the first 24 hours of direct anaerobic digestion, the SMPRs were as high as 1.5–2.0 mL/g VS/h, suggesting efficient substrate degradation and methane production. Su et

al. reported that acidogens generally grew faster than methanogens [12]. As previously discussed, the VFAs produced by acidogens were more than the VFAs consumed by methanogens. The excessive accumulation of VFAs resulted in a significant drop of pH and a rapid decrease of SMPRs (only 0.01–0.12 mL/g VS/h) in the next 72 hours. After an adaptation period of 216–312 hours, the accumulated VFAs were gradually consumed, and the SMPRs climbed back to 1.9–2.2 mL/g VS/h with the recovery of methanogens.

In direct anaerobic digestion, the produced hydrogen in the acidogenesis process could not be completely consumed by methanogens at the initial stage of fermentation (0–72 hours). As a result, a significant amount of hydrogen was generated from raw, hydrothermal pre-treated, and hydrothermal acid pre-treated mixed biomass with maximum SHYs of 56.1 ± 2.3, 106.4 ± 4.1, and 141.1 ± 5.6 mL/g VS, respectively.

As shown in Table 3, the fitting coefficients from fermentative methane production via direct anaerobic digestion using the modified Gompertz equation were in the range of 0.9379 to 0.9797, which are lower than the values from anaerobic digestion with biological acidification. The unstable methane production caused by the accumulation of VFAs generated a big difference between the kinetic fitting parameters and the experimental data.

3.4.2 Effects of pre-treatments on anaerobic digestion after biological acidification

Fig. 5 shows the effects of pre-treatments on anaerobic digestion after biological acidification. The BAEs derived from 144 hours of biological acidification with optimal adjusted pH values of around 7.5 were used as substrate in the subsequent

anaerobic digestion process. Since most of the organic matters especially carbohydrates were degraded through biological acidification with sufficient duration, the inhibition phenomenon disappeared and no hydrogen was produced.

Based on the BAEs of raw mixed biomass, the maximum SMY and SMPR were 206.4 ± 2.8 mL/g VS and 2.8 ± 0.1 mL/g VS/h, respectively. When the mixed biomass was hydrothermally pre-treated, the maximum SMY and SMPR increased to 223.1 ± 0.8 mL/g VS and 3.2 ± 0.1 mL/g VS/h, respectively. This could be attributed to the fact that the BAEs of hydrothermal pre-treated biomass contained more SMPs (13.16 ± 0.58 g COD/L) as compared with the BAEs of raw biomass (11.75 ± 0.35 g COD/L), as shown in Table 2. Generally, the performance of anaerobic digestion was positively related with the concentration of SMPs (alcohols and VFAs) in the BAEs [23]. SMPs are considered as favourable substrates for fermentative methane production.

Notably, when raw mixed biomass was hydrothermally pre-treated by diluted acid, the concentration of SMPs in the BAEs further increased to 15.19 ± 0.14 g COD/L, while the maximum SMY and SMPR reduced to 183.7 ± 1.4 mL/g VS and 2.2 ± 0.03 mL/g VS/h, respectively. This may be explained by the toxic by-products such as furan derivatives, phenols and the Maillard products typically derived from thermochemical pre-treatment [23]. Overall, hydrothermal pre-treatment under acid catalysis is an effective method to improve hydrogen production during biological acidification, whereas the subsequent methane fermentation of their BAEs may not perform as optimally as desired.

As shown in Table 3, anaerobic digestion from various BAEs (144 hours of

biological acidification) could be accurately described by the modified Gompertz equation with high fitting coefficients (R^2 ranged from 0.9888 to 0.9926). The highest SMY potential of 224.1 mL/g VS and SMPR of 3.3 mL/g VS/h were obtained from the BAEs of hydrothermal pre-treated biomass. Compared with direct anaerobic digestion, the two-stage process comprising biological acidification and anaerobic digestion could effectively avoid inhibition caused by VFA accumulation, thereby significantly reducing the lag-phase time (decreased from 64.9–121.3 hours to 21.0–23.1 hours) and peak time (decreased from 170.7–211.1 hours to 46.1–48.3 hours) of anaerobic digestion. On account of this, the whole fermentation period of anaerobic digestion in two-stage process obviously decreased to 60–168 hours, around only 1/3 of direct anaerobic digestion (Figs. 4–5).

3.4.3 Effects of biological acidification time on anaerobic digestion

The BAEs of hydrothermal acid pre-treated biomass were used as substrate to assess the effects of biological acidification time on subsequent anaerobic digestion, as shown in Fig. 6. When a short HRT of 12 hours was set for biological acidification (BAR: 15.5 ± 0.6%), incomplete degradation of carbohydrates (Table 2: 9.5 ± 1.53 g/L of residual carbohydrates) led to VFA accumulation and pH decrease at the early stage of anaerobic digestion. For this reason, the maximum SMY of 132.3 ± 8.7 mL/g VS and SMPR of 1.8 ± 0.1 mL/g VS/h were relatively low. Meanwhile, small amounts of hydrogen of 40.0 ± 2.3 mL/g VS was observed in anaerobic digestion.

When the HRT of biological acidification was increased to 24 hours (BAR: 41.0 ± 1.2%), most of the carbohydrates were degraded (Table 2: 2.61 ± 0.58 g/L of

residual carbohydrates), thereby mitigating the above inhibition in anaerobic digestion. Whilst, no hydrogen was produced in anaerobic digestion due to the low concentration of carbohydrates in the BAEs. Nevertheless, it was still considered as an unstable fermentative methane production process. The maximum SMY slightly increased to 147.0 ± 6.1 mL/g VS, while the maximum SMPR decreased to 1.5 ± 0.1 mL/g VS/h.

When the HRT was increased to 48 hours (BAR: 74.7 ± 1.4%), the maximum SMY and SMPR significantly increased to 203.1 ± 1.0 mL/g VS and 2.0 ± 0.0 mL/g VS/h, respectively. This suggested that an appropriate improvement in biomass acidification degree could effectively enhance methane production from the BAEs. Since biological acidification had been completed during the period of 48–72 hours, further increasing the HRT of biological acidification to 72 hours (BAR: 77.6 ± 0.8%) resulted in slight changes in the maximum SMY (192.0 ± 1.8 mL/g VS) and SMPR (1.9 ± 0.1 mL/g VS/h).

It should be noted that in this work a long HRT (144 hours) of biological acidification with a very small increase of BAR (78.5 ± 1.2%) as compared to 72 hours would lead to a lower maximum SMY of 183.7 ± 1.4 mL/g VS. This unfavourable phenomenon may be caused by excessive consumption of total carbon sources during biological acidification. Some of the carbon sources may be converted to carbon dioxide and released from the fermenters, which could not be reused in subsequent anaerobic digestion. Interestingly, the maximum SMPR increased to 2.2 ± 0.03 mL/g VS/h (from 1.9 ± 0.1 mL/g VS/h). This result may be attributed to the

increase in acetic acid concentration (3.86 ± 0.19 g COD/L), as shown in Table 2. It is known that acetic acid can be directly utilised by acetotrophic methanogens, whereas other SMPs (such as ethanol, propionic acid, and butyric acid) should be firstly degraded to acetic acid and then utilised to produce methane in methanogenesis [57]. As previously discussed, a relative in-depth acidification (144 hours) could promote the further degradation of amino acids, the conversion of butyric acid to acetic acid, and the process of homoacetogenesis, thereby resulting in a high concentration of acetic acid and a high SMPR during anaerobic digestion.

As shown in Table 3, anaerobic digestion combined with biological acidification (with HRTs from 12 to 144 hours) also could be accurately described by the modified Gompertz equation with high fitting coefficients (R^2 ranged from 0.9811 to 0.9952). The highest SMY potential of 204.1 mL/g VS was obtained after 48 hours of biological acidification. A relative in-depth biomass acidification of 144 hours showed the highest SMPR of 2.5 mL/g VS/h. These results were consistent with the experimental data. Compared with direct anaerobic digestion, different degrees of biomass acidification (15.5%–78.5%) all decreased the lag-phase time (from 64.9–121.3 hours to 21.0–53.6 hours) and peak time (from 170.7–211.1 hours to 48.3–81.4 hours) of anaerobic digestion in two-stage process. Furthermore, in terms of the lag-phase time and peak time, the most significant enhancement was observed after 144 hours of biological acidification with a highest BAR of 78.5 ± 1.2%.

3.5 Comparison of energy conversion efficiencies and energy conversion percentages

The ECEs were calculated based on 120 hours of anaerobic digestion with various

HRT for biological acidification (0–144 hours) by using hydrothermal acid pre-treated biomass as substrate. As shown in Fig. 7(a). The ECEs from hydrogen were in the range of 10.3%–18.0%, which was still insufficiently high for industrial applications. The maximum hydrogen ECE of 18.0 ± 0.7% was achieved after 48 hours of biological acidification with a BAR of 74.7 ± 1.4% (Table 2). Further increasing the HRT of biological acidification had almost no effect on improving the energy conversion from hydrogen due to the non-obvious enhancement of hydrogen production (Fig. 3).

By combining methane production, the ECEs significantly increased to 25.4%–64.0%. This growing trend was positively related to the acidification degree of biomass. For direct anaerobic digestion without biological acidification, the methane fermentation period was generally quite long (nearly 480 hours), as shown in Fig. 4. In this case, when the ECEs were calculated based on 120 hours of anaerobic digestion, the achievable maximum ECE was only 25.4 ± 0.3% due to the low SMY of 55.6 ± 1.6 mL/g VS. However, when a relative in-depth biological acidification of 144 hours with a highest BAR of 78.5 ± 1.2% (Table 2) was applied prior to anaerobic digestion, the methane production could be accomplished quickly. The maximum SMY of 183.7 ± 1.4 mL/g VS (Fig. 6) had been achieved at the calculated period of anaerobic digestion of 120 hours, thereby resulting in the maximum ECE of 64.0 ± 1.0% from hydrogen and methane.

Compared with the ECEs obtained from 120 hours of anaerobic digestion (25.4%–64.0%), the total ECEs based on the complete fermentation process were in

the range of 43.5%–75.6%. Notably, the ratio of the two parameters was defined as the ECP_{120} (see Equation 3), which could be used to assess the effects of biological acidification on the required time to achieve the maximum total ECE. As shown in Fig. 7(b), the ECP_{120} from direct anaerobic digestion was only 33.5 ± 0.4%, indicating that raw biomass without biological acidification could not be rapidly degraded to produce methane. Once raw biomass underwent biological acidification, even only 12 hours of biological acidification with a low BAR of 15.5 ± 0.6% (Table 2), the corresponding ECP_{120} (84.3 ± 0.9%) still increased 1.5-fold. However, by increasing the HRT of biological acidification to 72 hours (BAR: 77.6 ± 0.8%), the increase of ECP_{120} slightly increased from 84.3 ± 0.9% to 89.6 ± 1.2%.

Such a phenomenon was caused by the presence of high amounts of easily degradable organic matters in the mixed biomass of RR and CP (i.e., starch). Since these substrates could be readily used by acidogens, a short-term biological acidification (12–72 hours) was sufficient to enhance subsequent methane production via anaerobic digestion. Although the BAR of 78.5 ± 1.2% only slightly increased during a relative in-depth biological acidification of 144 hours duration, the distribution of SMPs revealed obvious differences (Table 2). As previously discussed, the high concentration of acetic acid would finally realize a rapid methane production with a maximum SMPR of 2.2 ± 0.03 mL/g VS/h (Fig. 6), thereby leading to a maximum ECP_{120} of 99.8 ± 0.1%. Overall, an in-depth biological acidification could effectively accelerate the subsequent fermentative methane production, and achieve the maximum potential of energy conversion over a short time frame corresponding to

a small volume and lower capital investment.

4 Conclusion

The biological acidification rates (60.7%–78.5%) would maximally increase 1.3-fold with enhancing the intensity of biomass pre-treatment, thereby achieving the maximum specific hydrogen yield of 223.1 ± 8.8 mL/g VS and production rate of 10.4 ± 0.4 mL/g VS/h. Increasing the hydraulic retention time of biological acidification (12–144 hours) had significant effects on improving biological acidification rates (15.5%–78.5%). Meanwhile, such a phenomenon could further affect the performance of subsequent fermentative methane production. A relative in-depth biological acidification of 144 hours resulted in the minimum lag-phase time and peak time of anaerobic digestion. The whole anaerobic digestion period (120 hours) decreased by 70% with a maximum methane production rate of 2.2 ± 0.03 mL/g VS/h. Moreover, the energy conversion efficiency based on 120 hours of anaerobic digestion combined with an in-depth biological acidification was 64.0 ± 1.0%, which shows 2.5-fold increase compared with direct anaerobic digestion.

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Fig. 2 Organic matter solubilisation under different pre-treatments.

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Fig. 7 Energy conversion efficiencies and energy conversion percentages during hydrogen and methane co-production: (a) Eenergy conversion efficiencies; (b) Energy conversion percentages. Substrate used in anaerobic digestion was the biological acidification effluents of hydrothermal acid pre-treated mixed biomass.

Table 1 Characteristics of rice residue pulp and *Chlorella pyrenoidosa* powder.

Parameters	Rice residue pulp	*Chlorella pyrenoidosa* powder
Proximate analysis		
Moisture (wt%)	81.84 ± 0.01	6.26 ± 0.24
TS (wt%)	18.16 ± 0.01	93.74 ± 0.24
VS (wt%)	18.03 ± 0.01	78.91 ± 0.34
VS/TS (%)	99.28 ± 0.02	84.18 ± 0.37
Ultimate analysis		
C (VS%)	43.07 ± 0.21	50.62 ± 0.17
H (VS%)	5.09 ± 0.09	6.23 ± 0.31
O (VS%)	50.04 ± 0.42	33.42 ± 0.15
N (VS%)	1.31 ± 0.01	9.31 ± 0.07
S (VS%)	0.49 ± 0.03	1.28 ± 0.04
C/N molar ratio	38.4 ± 0.13	6.35 ± 0.05
Energy value (kJ/g VS)	15.58 ± 0.52	21.46 ± 0.37
tCOD (mg/g VS)	1167.91 ± 56.33	1429.44 ± 47.18
tCarbohydrates (mg/g VS)	917.21 ± 45.57	332.98 ± 26.23
tProteins (mg/g VS)	128.12 ± 17.86	504.04 ± 32.14
tLipids (mg/g VS)	12.69 ± 2.78	169.41 ± 14.69
sCOD (mg/g VS)	118.65 ± 5.64	152.65 ± 12.74
sCarbohydrates (mg/g VS)	87.53 ± 13.65	64.62 ± 11.54
sProteins (mg/g VS)	6.65 ± 1.01	40.75 ± 0.97
Reducing sugars (mg/g VS)	14.41 ± 0.76	3.68 ± 0.24
TAN (mg/g VS)	/	1.07 ± 0.04

The abbreviation referred to total (t) and solubilised (s) matters;

TS: Total solids; VS: Volatile solids; COD: Chemical oxygen demand;

TAN: Total ammonia nitrogen;

Table 2 Degradation of organic matters and production of soluble metabolic products during biological acidification.

Description	Pre-treatment methods						
	Without	Hydrothermal	Hydrothermal acid				
Acidification time (hours)	144	144	12	24	48	72	144
sCarbohydrate (g/L) [a]	1.42 ± 0.07	9.59 ± 0.28	14.02 ± 0.13	14.02 ± 0.13	14.02 ± 0.13	14.02 ± 0.13	14.02 ± 0.13
sCarbohydrate (g/L) [b]	0.16 ± 0.02	0.32 ± 0.01	9.5 ± 1.53	2.61 ± 0.58	1.04 ± 0.22	0.55 ± 0.09	0.47 ± 0.11
sProtein (g/L) [a]	0.14 ± 0.00	0.62 ± 0.09	1.92 ± 0.09	1.92 ± 0.09	1.92 ± 0.09	1.92 ± 0.09	1.92 ± 0.09
sProtein (g/L) [b]	0.12 ± 0.02	0.40 ± 0.13	0.88 ± 0.09	0.72 ± 0.24	0.62 ± 0.18	0.6 ± 0.11	0.56 ± 0.05
Reducing sugar (g/L) [a]	0.29 ± 0.01	0.62 ± 0.11	15.18 ± 0.23	15.18 ± 0.23	15.18 ± 0.23	15.18 ± 0.23	15.18 ± 0.23
Reducing sugar (g/L) [b]	0.04 ± 0.00	0.03 ± 0.01	8.68 ± 1.33	2.43 ± 0.21	1.01 ± 0.03	0.64 ± 0.01	0.04 ± 0.00
TAN (mg/L) [b]	40.32 ± 2.35	122.8 ± 8.56	13.5 ± 1.05	27.8 ± 2.31	42.7 ± 3.02	120.1 ± 6.93	204.8 ± 11.64
Total SMPs (g COD/L) [c]	11.75 ± 0.35	13.16 ± 0.58	3.0 ± 0.34	7.94 ± 0.32	14.47 ± 0.13	15.03 ± 0.32	15.19 ± 0.14
Ethanol (g COD/L)	0.22 ± 0.03	0.39 ± 0.05	0.26 ± 0.01	0.28 ± 0.03	0.3 ± 0.03	0.3 ± 0.03	0.34 ± 0.02
Acetic acid (g COD/L)	2.62 ± 0.15	3.39 ± 0.24	0.72 ± 0.08	1.6 ± 0.14	3.33 ± 0.21	3.49 ± 0.18	3.86 ± 0.19
Propionic acid (g COD/L)	1.54 ± 0.01	0.69 ± 0.14	0.03 ± 0.01	0.05 ± 0.01	0.09 ± 0.01	0.19 ± 0.01	0.27 ± 0.01
Butyric acid (g COD/L)	6.18 ± 0.4	7.38 ± 0.54	1.9 ± 0.16	5.88 ± 0.49	10.12 ± 0.28	10.38 ± 0.41	9.51 ± 0.19
Valeric acid (g COD/L)	0.2 ± 0.01	0.19 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.05 ± 0.01	0.12 ± 0.00
Caproic acid (g COD/L)	0.99 ± 0.07	1.11 ± 0.14	0.08 ± 0.03	0.11 ± 0.01	0.61 ± 0.03	0.62 ± 0.02	1.08 ± 0.04
BAR (%)	60.7 ± 1.23	68.0 ± 2.03	15.47 ± 0.57	40.99 ± 1.22	74.7 ± 1.35	77.64 ± 0.77	78.46 ± 1.17

TAN: Total ammonia nitrogen; SMPs: Soluble metabolic products; BAR: Biological acidification rate; COD: Chemical oxygen demand.

[a] Before fermentation; [b] After fermentation; [c] The concentrations of SMPs were based on the unit of "g COD/L" according to the references [58,59].

Table 3 Kinetic parameters of hydrogen production during biological acidification and methane production during anaerobic digestion.

Processes	Substrates	Pre-treatment methods	Acidification time (hours)	Kinetic model parameters				
				H_m (mL/g VS)	R_m (mL/g VS/h)	λ (h)	T_m (h)	R^2
Biological acidification	Raw mixed biomass	/	144	101.61	2.50	6.67	21.62	0.9952
		Hydrothermal	144	173.35	4.20	6.70	21.89	0.9876
		Hydrothermal acid	144	223.68	10.78	4.17	11.80	0.9985
Anaerobic digestion	Raw mixed biomass	/	0	229.53	0.94	121.28	211.12	0.9379
		Hydrothermal	0	276.76	1.44	111.94	182.65	0.9797
		Hydrothermal acid	0	267.61	0.93	64.85	170.72	0.9535
	BAEs [a]	/	144	206.96	3.10	21.50	46.06	0.9897
	BAEs [b]	/	144	224.07	3.30	23.11	48.09	0.9888
	BAEs [c]	/	12	132.93	1.76	53.61	81.40	0.9920
		/	24	148.14	1.40	28.64	67.57	0.9811
		/	48	204.12	1.86	27.30	67.68	0.9952
		/	72	193.16	2.05	29.00	63.67	0.9897
		/	144	184.43	2.48	20.97	48.33	0.9926

BAEs: Biological acidification effluents. [a] BAEs of raw mixed biomass. [b] BAEs of hydrothermal pre-treated mixed biomass. [c] BAEs of hydrothermal acid pre-treated mixed biomass.

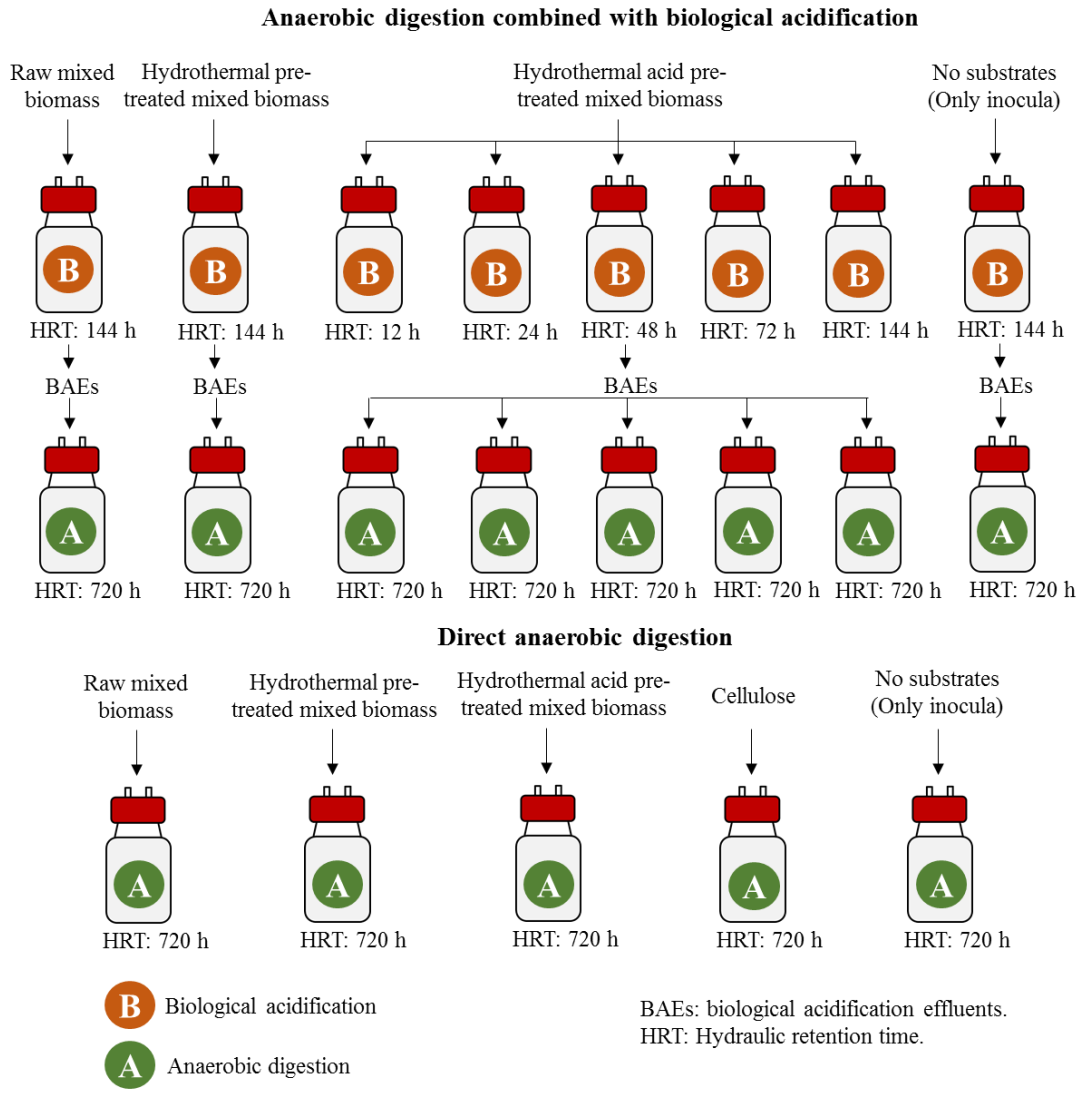


Fig 1 Design of experiment including for anaerobic digestion combined with biological acidification and direct anaerobic digestion.

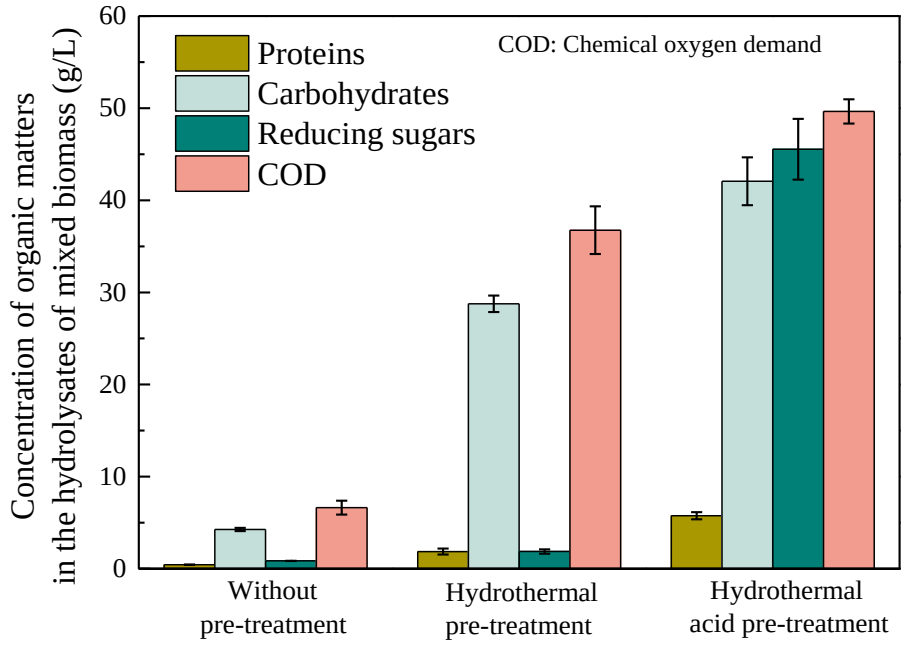


Fig. 2 Organic matter solubilisation under different pre-treatments.

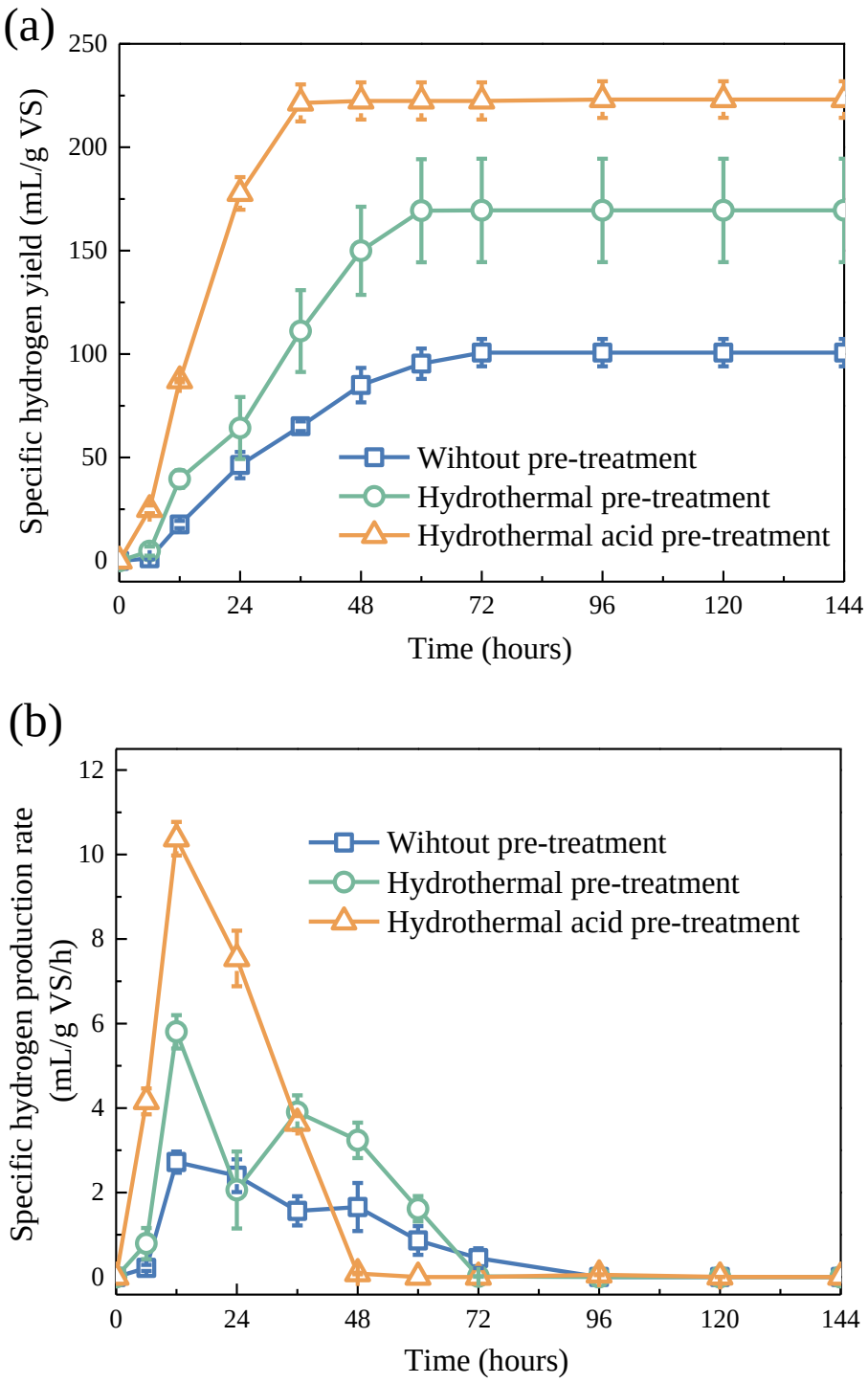


Fig. 3 Effects of different pre-treatments on hydrogen production during biological acidification: (a) Specific hydrogen yield; (b) Specific hydrogen production rate.

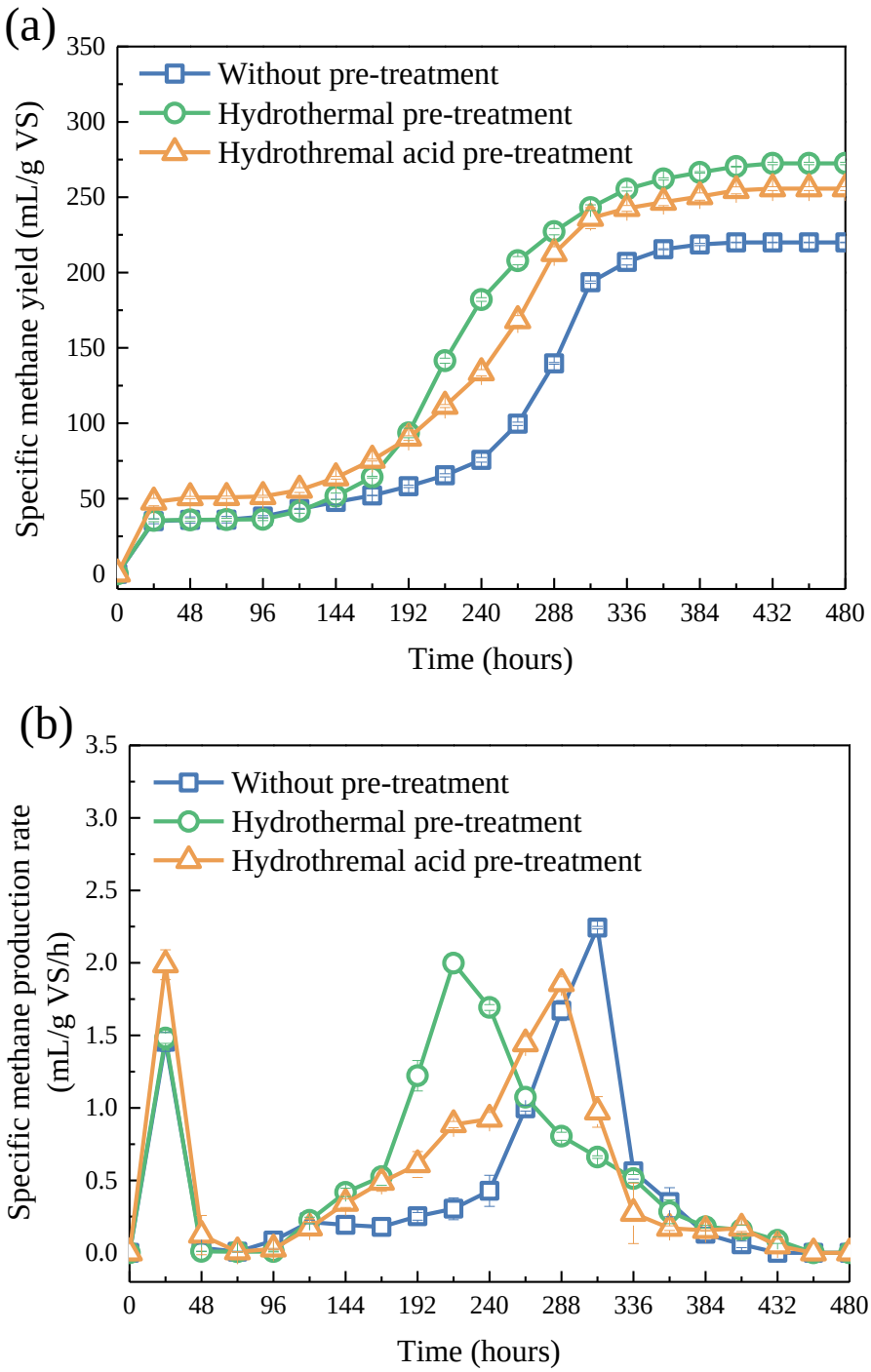


Fig. 4 Methane production during direct anaerobic digestion from raw, hydrothermal pre-treated, and hydrothermal acid pre-treated mixed biomass: (a) Specific methane yield; (b) Specific methane production rate.

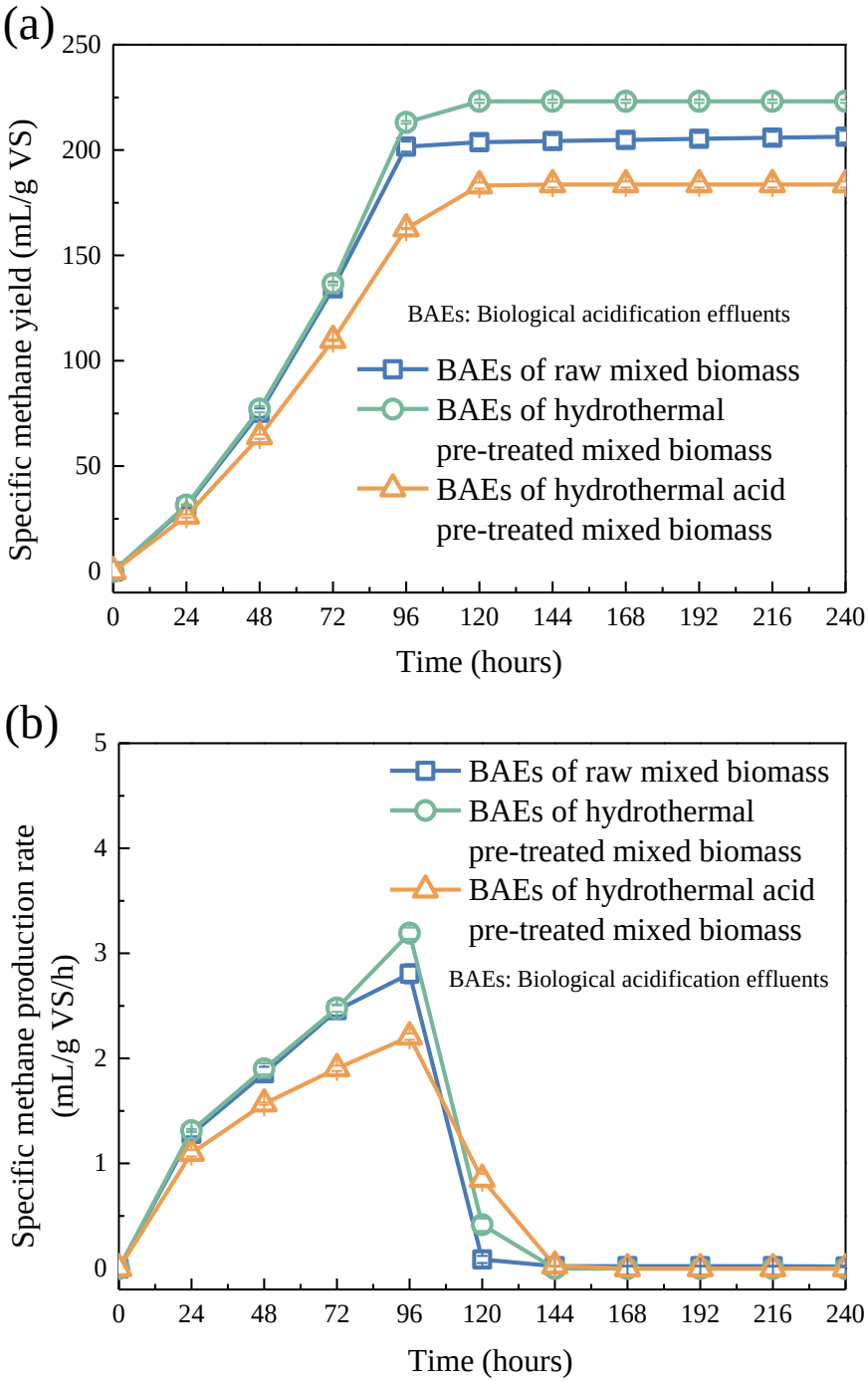


Fig. 5 Methane production during anaerobic digestion from biological acidification effluents: (a) Specific methane yield; (b) Specific methane production rate. Hydraulic retention time of biological acidification was set as 144 hours.

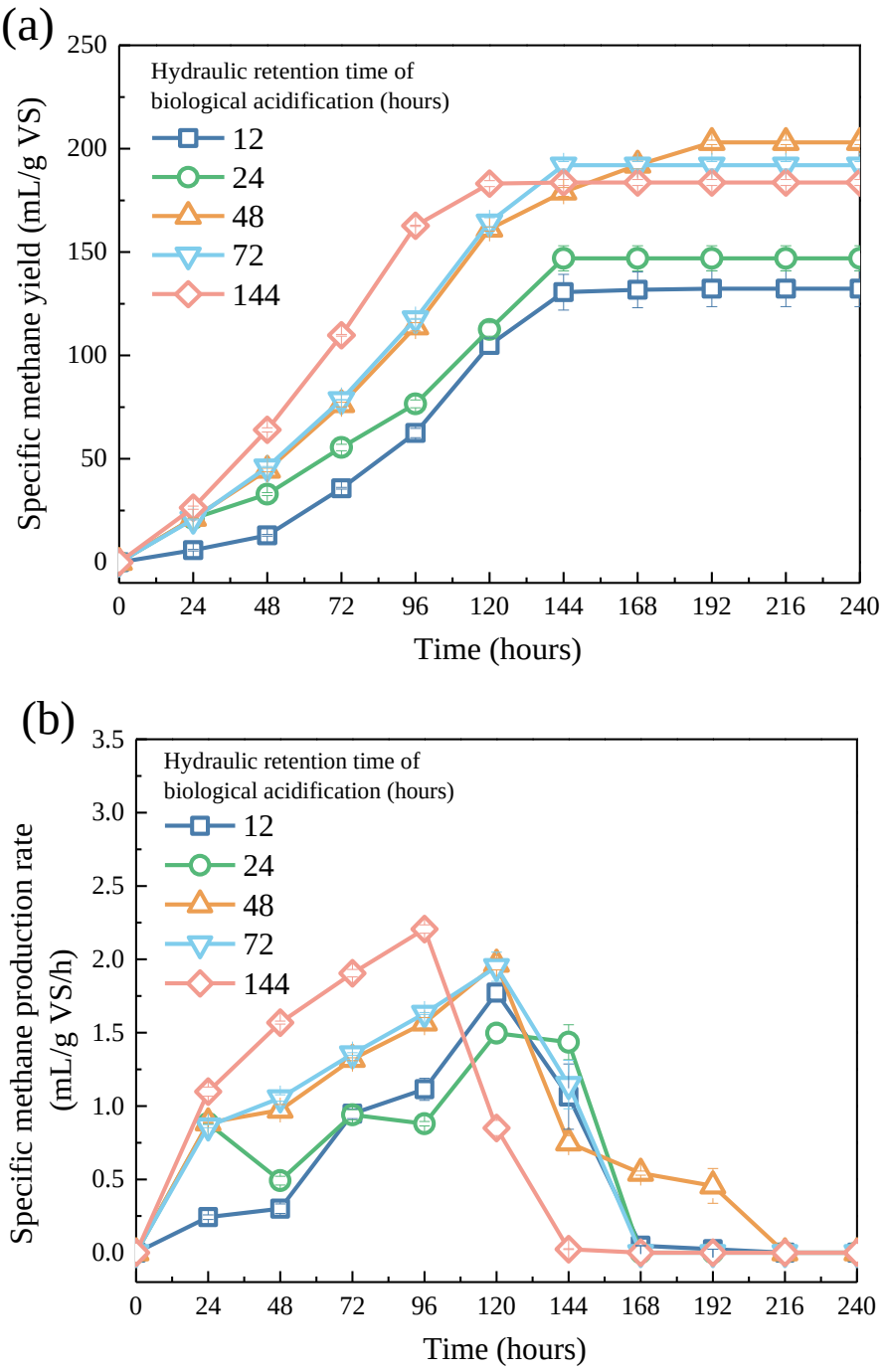


Fig. 6 Effects of biomass acidification time on anaerobic digestion: (a) Specific methane yield; (b) Specific methane production rate. Substrate used in anaerobic digestion was the biological acidification effluents of hydrothermal acid pre-treated mixed biomass.

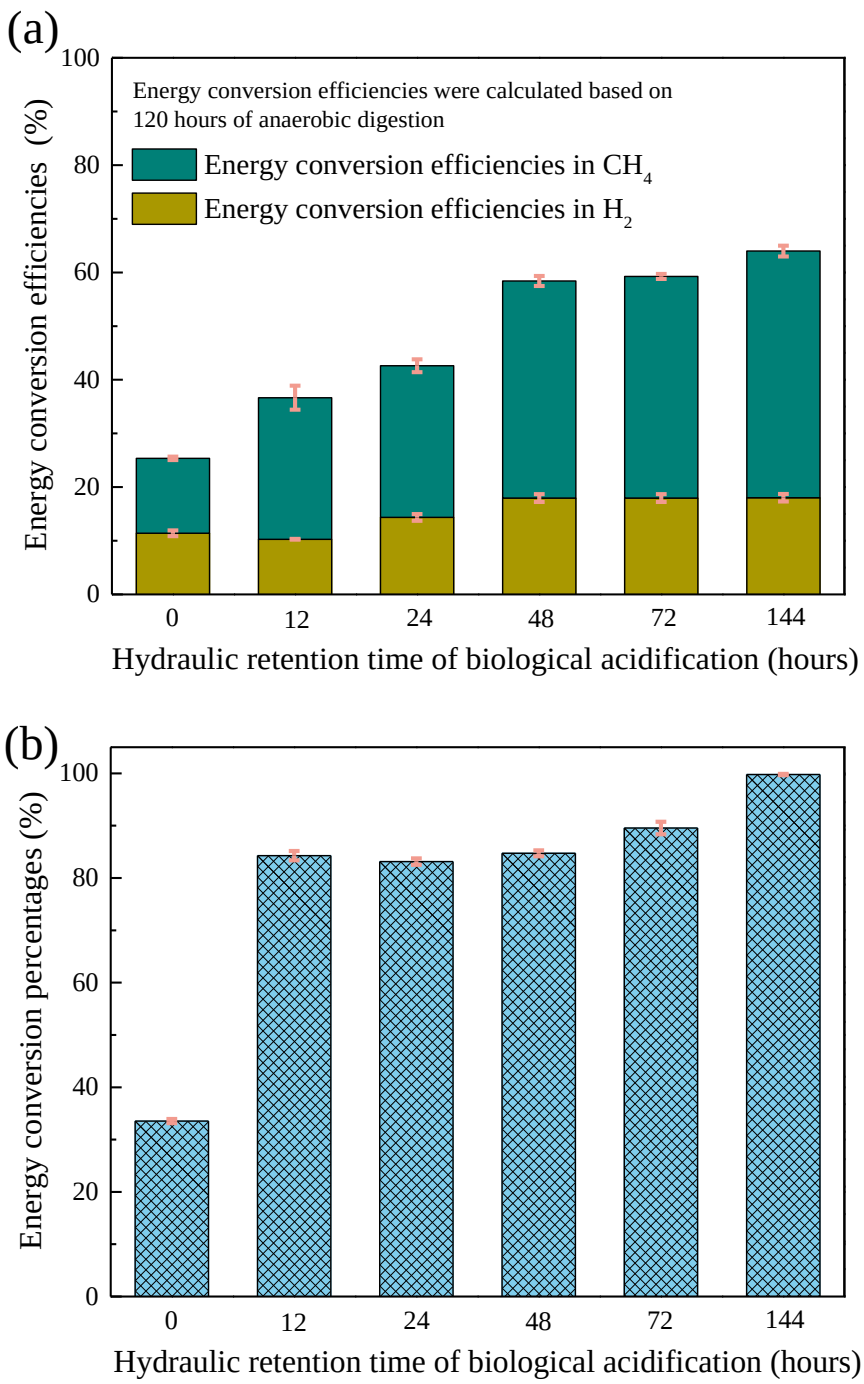


Fig. 7 Energy conversion efficiencies and energy conversion percentages during hydrogen and methane co-production: (a) Eenergy conversion efficiencies; (b) Energy conversion percentages. Substrate used in anaerobic digestion was the biological acidification effluents of hydrothermal acid pre-treated mixed biomass.