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Variations in the substrate composition and microbial community structure in the anaerobic fermentation process using the green algae *Enteromorpha prolifera*

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Abstract

Enteromorpha prolifera is a nutrient-rich green alga and abound in the Yellow Sea and the Bohai Sea of China. In this study, *E. prolifera* was anaerobically digested for biogas production. The variations of chemical compositions and microbial community structure as well as the physical structure of *E. prolifera* in anaerobic digestion process were investigated. This is the first report of multiple ways to deeply analysis the process of *E. prolifera* anaerobic digestion. Results from the present work showed that the biogas obtained from *E. prolifera* anaerobic digestion could achieve 409.7 $mL \cdot g^{-1}$ TS with an average methane concentration of 53.2%, and the VFAs content in substrate played a vital role for driving the biogas production of flora. Moreover, *S1* of *Thermotogaceae* and *Cenarchaeum*, the dominant bacteria and archaea in digestion flora, respectively, played important roles in degrading *E. prolifera*, acidizing slurry, and providing methanogenic substrate for methanogens.

Keywords: *Enteromorpha prolifera*, Anaerobic digestion, High-throughput sequencing

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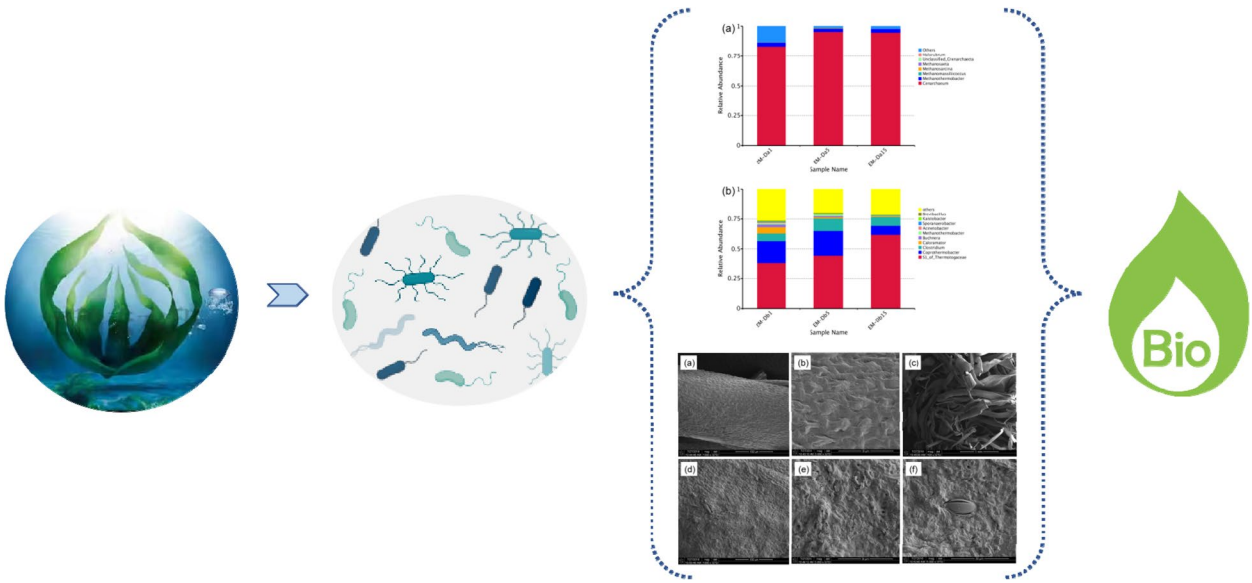
Graphical Abstract



Introduction

Enteromorpha prolifera, belonging to the phylum Chlorophyta, class Chlorophyceae, order Ulvales, and genus *Enteromorpha*, is mainly composed of polysaccharides and crude fibre. Due to its rich nutritional value and unique flavor, *E. prolifera* gets the increasing attention of the public and has been widely studied as potential food and medicine (Du et al. 2018; Kim et al. 2011). Despite its advantages, *E. prolifera* has attracted special attention because of the continuous formation of large-scale "green tide" in the Qingdao of China's Yellow Sea since 2008, which resulted in a negative effect on ocean ecology, economic loss and CO_2 burden (Gobler 2020; Liu et al. 2012; Zhao & Ruan 2011). Fortunately, the high content of carbohydrates and immense biomass of *E. prolifera*, give it a chance to be utilized as a special feedstock for biofuel (Li et al. 2011; Magdalena et al. 2017). Moreover, since the industrial revolution, the development of human society and the availability of energy have led to a significant increase in energy. Renewable energy in the form of biofuels seems to be one of the most effective solutions (Saleh et al. 2017). Over the past few decades, there has been considerable research on algal biomass as a source of liquid and gaseous biofuels, such as biodiesel (Wang et al. 2013), algae power generation (Aziz 2016), and methane (Saratale et al. 2018).

Algae is a photoautotroph that converts light energy into chemical energy. It creates a permanently renewable pure energy source for industrial and human consumption (Barry et al. 2015), and is a promising feedstock option for anaerobic digestion with less threat to the food market than other organic solid wastes (Zhang et al. 2021). Among the various processes for converting algae into biofuel, anaerobic digestion is a simple, feasible and low-cost approach. This can achieve excellent degradation of organic substrates in biomass while harvesting biomass energy (methane) and providing organic fertilizer (Montingelli et al. 2015). Algae anaerobic digestion for obtaining biogas could be traced back to the 1950s, a previous study showed that each pound of algal volatile matter introduced into a digester would yield approximately 8.0 cu ft of gas, of which approximately 2.5 cu ft will be CO_2, and 5.0 cu ft will be hydrogen, nitrogen, and other gases (Golueke et al. 1957). With the development of the society and economy, the use of algae to produce biogas has increasingly become popular in the world.

Theoretically, the biomethane yield of microalgae could be achieved by 0.48–0.80 L CH_4 g^{-1} volatile solids (VS) (González-Fernández et al. 2012; Zamalloa et al. 2011). Previous studies demonstrated that biogas yield of anaerobic digestion was affected by several factors, such as component of substrate, pre-treatment, and microflora structure. Algae is a protein-rich substrate, therefore, co-digestion of algae with other nutrients-rich biomaterials (i.e., sludge, various food wastes, and fertilizers) can significantly enhance the biogas yield of algae (Álvarez et al. 2020; Astals et al. 2015; Solé-Bundó et al. 2017, 2019). Pre-treatment is an efficient way to release the

nutrients from plant tissue. However, too fast and excessive nutrients in substrate will result in fast acidizing of slurry, which may inhibit the growth of methanogens (Zhang et al. 2018). Therefore, pre-treatment methods should cater for the process of plant tissue eroded by microflora which could effectively improve the nutrient availability. The information of microflora structure changes in the fermentation process is important feedback for further optimizing the biogas fermentation procedure. Previous study showed that more attention should be given to the microflora structure, and fermentation mechanism studied through bioinformatics and high throughput sequencing technologies to fundamentally break through the technical bottleneck of biomethane fermented from algae compared with the fermentation techniques (Wu et al. 2016). Based on the above, a comprehensive study, focusing on the changes in the chemical composition of the substrate and the variations of microflora during substrate degradation, should be done. This can help us to understand the fundamentals of the microscopic process of fermentation, help to increase the output of biogas, and optimize the fermentation process.

In the present study, *E. prolifera* was used to produce biomethane by anaerobic fermentation. The daily biomethane yield was measured to evaluate the potential of *E. prolifera* utilized as bio-fuel material. Besides, the chemical composition changes of substrate were determined for giving a detailed understanding of the *E. prolifera* digestion process. In addition, to know the dynamic changes of the microflora structure in anaerobic fermentation, high-throughput sequencing technology was used to obtain the microflora information. This study brings an in-depth understanding of the *Enteromorpha* fermentation process through combining these chemical and biological analysis and lays a theoretical basis for improving the effect of biogas fermentation of *E. prolifera* biologically.

Materials and methods

Pre-treatment processes of E. prolifera

Anaerobic fermentation slurry was collected from hot springs sludge in Yongtai, Fujian province, China. A highly efficient *E. prolifera* digestion flora was obtained using the enrichment cultivation technique, and named as EM-D. The enrichment culture was operated at 55 °C in a lab-scale anaerobic reactor for a week with the *E. prolifera* as mono-substrate. The total solid (TS) of the fermentation fluid was 1.43% in the enrichment culture process. The wet *E. prolifera* harvested in August 2015 was purchased from Fujian Haixing Health Food Co. Ltd. (Fuqing, China). As the wet *E. prolifera* contains a lot of salt, it is important to do some pre-treatments to make it more suitable for fermentation. In this study, the wet *E. prolifera* was treated with the following three steps: (a) per kilogram of wet *E. prolifera* was washed with a litre of tap water 3 times; (b) dried in 65 °C for 24 h; and (c) once dried, *E. prolifera* was cut into pieces of 10–15 mm in length. Characteristics of the substrate are shown in Table 1.

Experimental setup and fermentation process

Five gram of dried *E. prolifera* was anaerobic digestion as single substrate (Fig. 1). A variable size flask with the maximum volume of 560 mL was used as anaerobic digestion reactor. The fermentation fluid consisted of 300 mL inoculum and 50 mL water, mixed with the substrate, the pH value of the fermentation liquid was adjusted to 7.2, and nitrogen was aerated to reduce the concentration of oxygen and exhausted the air of the bottle. The fermentation process was operated at 55 ± 1 °C in a constant temperature incubator for 16 days. Each batch contained 16 reaction bottles. The pH value, the concentration of methane and the gas production rate were measured every 24 h. Biogas residues, slurry and cells were collected on days 2, 4, 6, 8, 10, 12, 14, and 16 in the period. Biogas manure was filtrated by 4-layer gauze, centrifuged at 12,000 g for 5 min, and finally, enclosed in the cells and intestine with tubes to divide the different parts of the biogas fermentative fluid.

Collection of community DNA, amplification, and sequencing

The bacterial and archaeal communities in the biogas reactor that used *E. prolifera* as mono-substrate could be investigated into three phases which were artificially

Table 1 The characteristics of substrates

Material	Ash content	Crude Fat	Salt content	Crude carbohydrate	Crude fibre	Crude protein	Total solid
Wet *E. prolifera*[a]	37.01%	0.2%	2.1%	2.78%	16.7%	16.3%	-
Dry *E. prolifera*	9.44%	0.7%	-[b]	10.05%	5.2%	30.2%	84.99%

[a] The wet *E. prolifera* was dried in 65 °C for 24 h before measured. [b] not detected

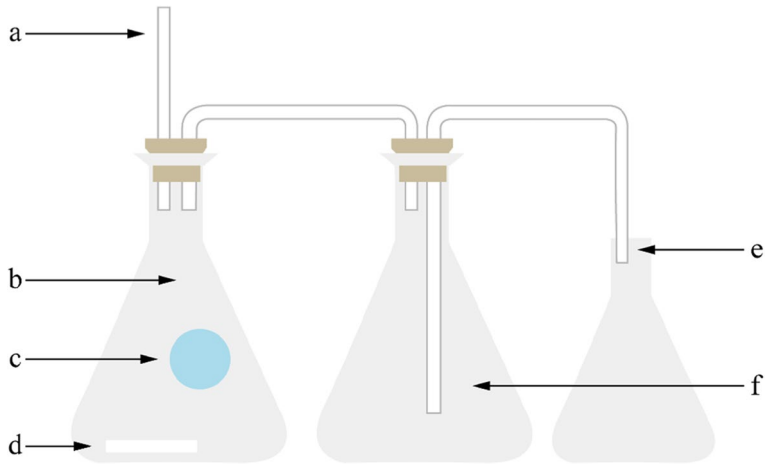


Fig. 1 Experimental set up for *E. prolifera* anaerobic digestion. **a** Nitrogen inlet; **b** Variable size water ball with rubber as outer package; **c** Fermentation broth; **d** Magnetic stirrer; **e** Water; **f** Drainage outlet

separated according to the features of anaerobic digestion: (a) *Acidification phase*, the highest acid content stage in fermentation, which was observed on the second day in this study; (b) *Methanogenesis phase*, the most gas production rate phase-in anaerobic digestion, which was the 5th day in this study; and (c) *Degenerating phase*, which was the 15th day in the fermentation process, where a low degradation efficiency of the substrate and poor ability to produce acid and biogas was seen. The bacterial cells were collected from these three separate culture phases. Total community DNA was extracted from 250 mg of sludge sediment using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). Extracted genomic DNA contents were diluted to 1 ng/μL with sterile water for community analyses based on the 16 S rRNA (V4 region). The diluted DNA from three separate steps of anaerobic digestion was used as template and was cloned with Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs). Specific primers with Barcode were used in the amplification process based on the sequenced region. The following primers were used to amplify the bacteria target fragment: F515 5′-CACGGTCGKCGGCGC CATT-3′ and R806 5′-GGACTACHVGG GTWTCT AAT-3′. The amplification of the archaea target fragment was in two procedures: firstly, F340 5′-CCCTAYGGG GYGCASCAG-3′ and R1000 5′-GGCCATGCA CYW CYTCTC-3′, secondly, F349: 5′-GYGCASCAGKCGM-GAAW-3′. PCR products were detected by agarose gel electrophoresis with an agarose gel concentration of 2%. Samples were equivalent mixed according to PCR product concentration, and then detected by agarose gel electrophoresis again. The aim strips were collected from a gel extraction kit (Qiagen). The 16 S rDNA libraries of the bacteria and archaea community were constructed with TruSeq® DNA PCR-Free Sample Preparation Kit and quantified by Qubit and QPCR. The sequencing of 16 S rRNA PCR products from the 3 samples was performed on the Illumina HiSeq2500 platform. The V4 hyper-variable region was sequenced by overlapping using the PE250 sequencing strategy. For quality control purposes, all of the sequences that contained ambiguous reads (N) and any sequences that contained mismatches in the forward or reverse primers were removed. Downstream analysis was performed with QIIME 1.7.02 (Caporaso et al. 2010) using the following setup and data preparation methods: (a) sequence quality filtering step with barcode correction and sample splitting of de-nosied reads; (b) *de-novo* picking of Operational Taxonomic Units (OTUs) with UCLUST similarity of 97%, taxonomy assignment with RDP Classifier confidence score (Wang et al. 2007) of 0.8 based on GreenGenes database, representative sequence alignment by PyNAST, removal of chimaeras (PCR artefacts) by ChimeraSlayer Software (Haas et al. 2011).

Analytical methods

Volatile fatty acids (VFAs) were analyzed using an HPLC (Agilent 1100, USA) equipped with a UV detector reported by Xu et al. (2014). UV detector was set at the wavelength of 210 nm, and a ZORBAX SB-A column (300.0 × 7.8 mm, Biorad, USA) was used for separating samples with the column temperature of 30 °C. The mobile phase consisted of 0.5% acetonitrile and 99.5% KH_2PO_4 (0.02 mol/L), at a flow rate of 0.5 mL/min. Total organic carbon (TOC) was monitored with a TOC analyzer (Elementar, Germany). Crude carbohydrate was determined by the phenol sulphuric acid method (Dubois et al. 2002). Organic nitrogen (ON), protein were

determined using Kjeldahl procedure according to AOAC 990.03. Crude fibre (CF) were determined based on the methods outlined in AOAC 954.02. Ash content was determined by decomposing sample (100 mg) at 500 °C in a muffle furnace (STUART, UK) for 16 h following the dry ashing procedure. Crude fat was determined by Soxhlet extraction with petroleum ether after hydrolysis with hydrochloric acid. Biogas composition, i.e., the content of CO_2, CH_4, N_2, and O_2, was analysed with a gas chromatograph (GC-2010-Shimadzu) equipped with a thermal conductivity detector and two columns (Molsieve 5 A 50 m × 0.53 mm for N_2, O_2, and CH_4 and Porabond Q 50 m × 0.53 mm for CO_2). The salt content was measured using a salinity meter (Salinity Meter 5051, Shanghai Sanxin, China), by adding 10 g *E. prolifera* into 100 mL de ionized water, dissolving the salt in the water. The surface images of the original and fermented *E. prolifera* were examined by scanning electron microscope (SEM) (Model S-4800, Hitachi, Tokyo, Japan). Before imaging, samples were coated with platinum using a sputter coater to capture their surfaces. The images were captured with the magnification at the range of 100–5000.

Statistical analysis

All experiments were independently executed in triplicate, unless otherwise stated. Data was analyzed using a one-way ANOVA performed by the SPSS 22.0 software (IBM Corp., USA). Significant differences between the means were identified using Duncan's multiple range tests. Statistical data have a significant difference when $p < 0.05$.

Results and discussion

Chemical changes in biogas fermentation

After 16 days of digestion, the biogas and methane yield of *E. prolifera* were 409.7 $mL \cdot g^{-1}$ TS and 217.9 $mL \cdot g^{-1}$ TS, respectively (Fig. 2a). The biogas production lasted 16 days with two biogas production peaks at the 5th and

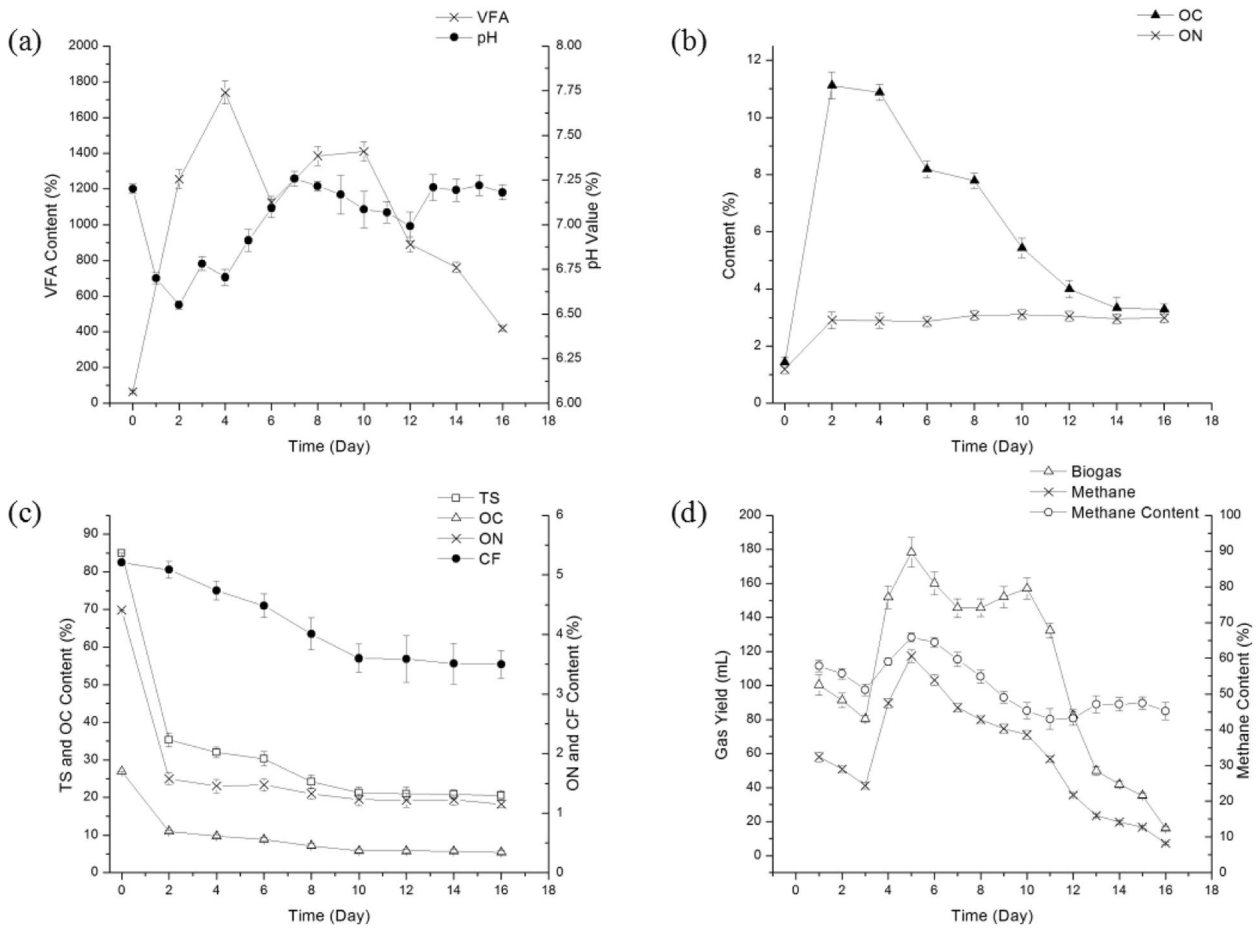


Fig. 2 Variations of typical chemical properties in biogas fermentation process. **a** VFAs content substrate and pH value of substrate; **b** OC and ON content in slurry; **c** TS, OC, ON and CF content in residue; **d** biogas, methane and methane content in reactor. The definition of abbreviations: VFAs, volatile fatty acids; OC, organic carbon; ON, organic nitrogen; TS, total solid; CF, crude fire

10th day, and it decreased rapidly after 11th days. Interestingly, trend of gas production was like that of VFAs, suggesting VFAs content was closely related to the generation of biogas. Previous study has shown that VFAs was important substrate to methanogens for producing biogas (Tampio et al. 2019). Thus, it could explain why the changes of VFA content (Fig. 2a) were always one day earlier than that of biogas production (Fig. 2d). In the case of the VFAs content, before 5th day, it kept in a relatively higher level in the earlier 5 days (Fig. 2a). It could be attributed to the acidification caused by acid-forming bacteria in slurry. The VFAs content raised rapidly in the first two day with the decreased pH value of the substrate which achieved the lowest pH value of 6.55 on the second day. However, till the 4th day, the peak value of VFAs content was 1740 $mg \cdot L^{-1}$ which was not in agreement with the time of the lowest pH value. This unconformity between pH value and VFAs content could be attributed to the fact that hydrolytic bacteria hold efficient ability to degrade carbohydrate and protein into VFAs at the early stage of fermentation (Fig. 2a and c). The methanogenic bacterium was sensitive to the acidic condition in new anaerobic fermenters and possessed low ability to convert VFAs into biogas. This phenomenon agreed with the previous works (Chen et al. 2018). As a results, the ON and OC were accumulated in the slurry from 1st day to 4th day (Fig. 2b). Sufficient substrates, VFAs, and optimum pH provided suitable growth conditions to methanogens which generated large quantities of biogas from the 4th day until the 11th day. With the peak value of biogas yield occurring on the 5th day, the OC and VFAs content rapidly reduced which caused a rise in the trend of the pH value. On the 5th day, the biogas yield, methane yield and methane content were 178.2 mL, 117.4 mL and 65.8%, respectively. After the 12th day, the reduced biogas yield coincided with lower OC and VFAs levels. On the last day of the fermentation process, the remaining VFAs, OC and accumulated ON were 420 $mg \cdot L^{-1}$, 3.29 $mg \cdot L^{-1}$, and 3.00%, respectively whereas, the biogas yield, methane yield and methane concentration were 16 mL, 7.4 mL and 45.3%, respectively.

Biogas generation was accompanied by the degradation of organic components in the substrate (Fig. 2c). Compared to the variation of CF in anaerobic digestion, the degradation of organic carbon (OC), total solid (TS) and organic nitrogen (ON) were significant in the first two days, which indicated that carbohydrates, proteins and lipids were preferentially degraded than fibre. Indeed, previous work has demonstrated that sugar, protein, and fat have the priority to be used in anaerobic biogas fermentation (Wang et al. 2017). Due to high protein content (Table 1), these effects will enhance the buffering capacity of the digester before CF degradation when *E. prolifera* was taken as substrate of anaerobic digestion. On the 2nd day, the TS, OC, and ON were 35.3%, 11.0%, and 1.6%, and reached 20.5%, 5.4%, and 1.2% at the end of the fermentation period, respectively. These demonstrated that the degradation of OC, TS and ON mainly occurred in the first two days. Rapid degradation of CF occurred between the 6th and 10th day as TS suffered a second round of rapid decrease, and the remaining CF on the 16th day was 3.5%. From these aspects, it demonstrated that different nutrients in *E. prolifera* were degraded in different time, thus caused two peaks emerged both in VFAs and biogas production during anaerobic digestion process (Fig. 2a and d).

The data demonstrated that *E. prolifera* anaerobic digestion was related to each component rather than occurring independently. The faster rate of degradation of protein and carbohydrate in comparison to that of cellulose resulted in the acidification of biogas slurry. However, the cellulose seemed to affect the fermentation period, and provide a nutrient source for microflora in the later fermentation stage.

The fermentation process is continuous and indivisible. However, in the actual process, the main factors, including the change of active microbes and nutrition changes in substrate, affected the fermentation. Therefore, the process could be artificially divided into the following stages: (a) acidogenic stage, (b) gasification stage and (c) degenerating stage. In the present study, the acidogenic stage ranged from 0 to 3 day, and was accompanied with the fastest degradation rate of substrate; the gasification stage appeared from 5 to 12 day, and the gas production peak was emerged at the fifth day; finally, degenerating stage began on the 15th day with a significant reduction of biogas yield, and the degradation of biogas residue tended to stagnate.

Methanogenic community structure analysis based on high-throughput sequencing of microflora composition and temporal dynamics

The sequencing of 16 S rRNA PCR products from the 3 samples was performed on the Illumina HiSeq2500 platform. The V4 hyper-variable region was sequenced by overlapping using the PE250 sequencing strategy. For quality control, all the sequences that contained ambiguous reads (N) and any sequences that contained mismatches in the forward or reverse primers were removed. Based on the sequencing results of EM-D communities, the 3 samples (obtained from anaerobic reactors on days 1, 5, and 15, respectively) showed a distinct microbial community structure, with *Thermotogae* being the dominant bacterial class for all samples while *Thaumarchaeota* was a major phylum for the archaeal class (Fig. 3).

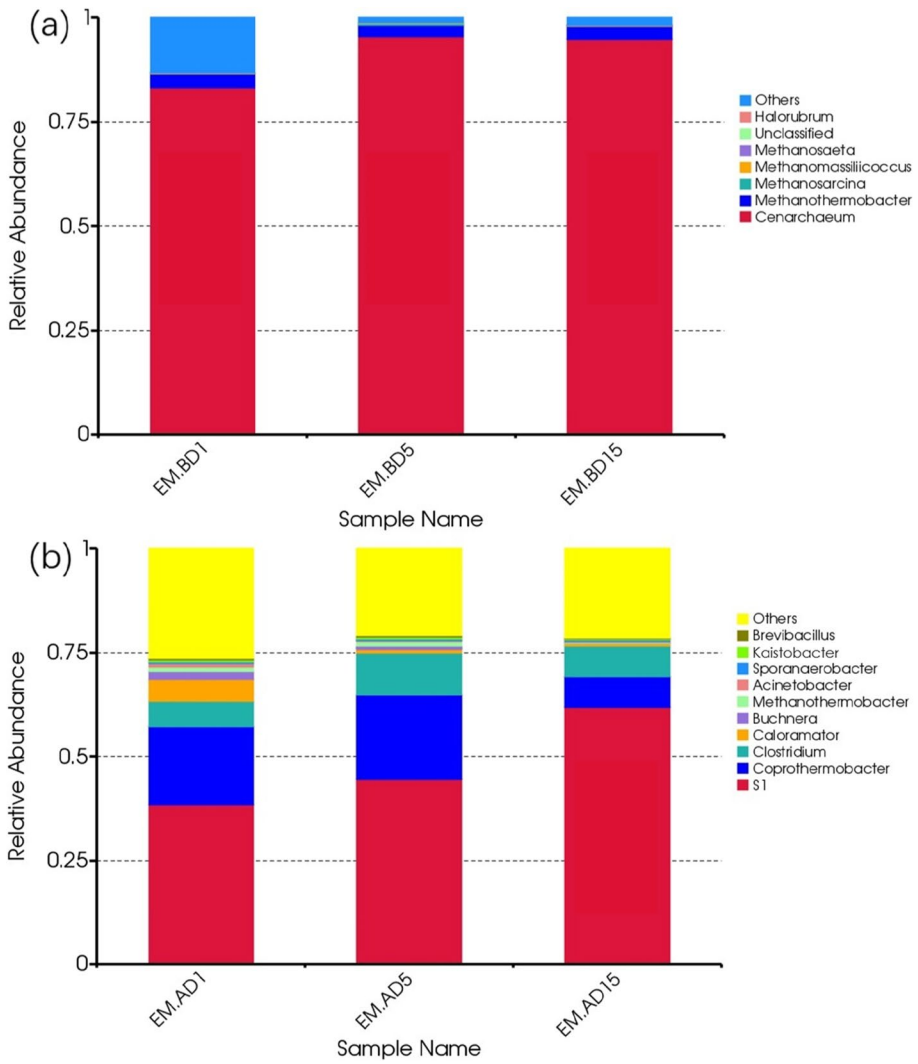


Fig. 3 The relative abundance histogram of sequencing results in *E. prolifera* fermentation flora. EM-Da means the archaea of EM-D, EM-Db means the bacteria of EM-D, the number of the sample name means the acquisition time in fermentation

A total of nine phyla were detected in the sample of biogas slurry. *Firmicutes*, *Thermotogae*, and *Proteobacteria* were found at high percentages. In contrast, *Euryarchaeota*, *Bacteroidetes*, *Acidobacteria*, *Chloroflexi*, and *Planctomycetes* appeared at low percentages. *Firmicutes, Thermotogae*, and *Proteobacteria* were distributed widely among all samples. The microflora varied significantly during the fermentation process, for example, the percentage of *Firmicutes* was 44.9% in EM-D on day 1 which increase to 48.7% on day 5, followed by a decrease to 33.2% on day 15. *Thermotogae* increased in relative abundance within the anaerobic digester, which was 38.4%, 44.8% and 62.1% on days 1, 5, and 15, respectively. The proportion of *Proteobacteria* rapidly decreased during the anaerobic fermentation process with the amounts being 11.8%, 3.8%, and 2.1% on daya 1, 5, and 15, respectively. These changes could be attributed the adaptation of the microflora to the digester environment. Interestingly, *Euryarchaeota*, with methanogenic bacteria, made up a very low proportion with the percentage of 1.1%, 1.3%, and 0.3% at day 1, 5 and 15, respectively.

EM-D showed unique microbial diversity at the genus level. The structure of the microflora community varied with the different fermentation stages. An overview of the results obtained is shown in Table 2. The top 10 genera of *Bacteria* phylum in the community were *S1* of *Thermotogaceae*, *Coprothermobacter*, *Clostridium*, *Caloramator*, *Buchnera*, *Methanothermobacter*, *Acinetobacter*, *Sporanaerobacter*, *Kaistobacter*, and *Brevibacillus.* Since the 16 S rRNA gene V4 region sequence of the

Table 2 Diversity estimates for bacteria and archaea from 16 S rDNA gene clone libraries constructed from biogas reactor fed with *E. prolifera*

Library	Taxonomy (Order, family, genus)	Abundance (a)[x], (b)[y], (c)[z]	Metabolic features	Reference
Bacteria	*Thermotogales; Thermotogaceae; S1*	38.35, 44.64, 61.82	Degraded carbohydrates and peptides, produced H_2 and acetic acid, sulfur-reducing in broth	Briones et al. 2009; Hao & Wang 2014
	Thermoanaerobacterales; Thermodesulfobiaceae; Coprothermobacter	18.35, 20.48, 7.43	Degraded sugar or protein; produced H_2, CO_2 and acetic acid	Bernard & Jean-Louis 2015; Sasaki et al. 2011
	Clostridiales; Clostridiaceae; Clostridium;	6.24, 10.31, 7.30	Degraded cellulose, sugar and protein; produced H_2, CO_2 and organic acid	Hahnke et al. 2014; Jeon et al. 2015
	Clostridiales; Clostridiaceae; Caloramator;	5.30, 0.98, 0.71	Degraded glucose, xylose, disaccharide; produced acetate, isobutyrate, isovalerate, valerate, lactate, H_2, CO_2 and ethanol.	Crespo et al. 2012
	Clostridiales; Clostridiaceae; Sporanaerobacter;	0.44, 0.47, 0.39	Degraded glucose to acetate, H_2, and CO_2	Hernandez-Eugenio et al. 2015
	others	30.25, 21.84, 22.05	Substrate hydrolysis	Qiu et al. 2014
Archaea	*Cenarchaeales; Cenarchaeaceae; Cenarchaeum*	83.22, 95.45, 94.74	Degraded substrate; played a vital role of nitrogen and sulfur cycle with ammoxidation	Hallam et al. 2006
	Methanobacteriales; Methanobacteriaceae; Methanothermobacter	3.36, 2.83, 3.28	Converted hydrogen and/or format carbon dioxide to methane	Seifert et al. 2014
	Unclassified; Unclassified; Methanomassiliicoccus	0.19, 0.21, 0.15	Converted hydrogen and carbon dioxide to methane	Dridi et al. 2012
	Methanosarcinales; Methanosarcinaceae; Methanosarcina	0.07, 0.36, 0.06	Converted acetate, hydrogen and carbon dioxide to methane; methylotrophs	Vrieze et al. 2012
	Methanosarcinales; Methanosaetaceae; Methanosaeta	0.06, < 0.01, < 0.01	Converted acetate to methane and carbon dioxide	Smith & Ingram-Smith 2007

[x] Ranking of relative abundance within the *Acidification phase*. [y]Ranking of relative abundance within the *Methanogenesis phase*. [z]Ranking of relative abundance within the *Degenerating phase*

Methanothermobacter is like that of the *Bacteria* phylum, it can be detected by PCR amplification and sequencing in the *Bacteria* phylum.

Among the samples, *S1* of *Thermotogaceae* was the most dominant genus. *Thermotogaceae* has a symbiotic relationship with methanogens, and it could degrade carbohydrates and peptides to acetic acid which has a great contribution to the acidification of the substrate during the biogas fermentation, especially in the later stage of fermentation (Hao & Wang 2014). *Thermotogaceae* could also regulate the concentration of sulphur and reduce the inhibition of H_2S on methanogenisis, resulting in high COD removal and methane production. As a result, the proportion of *Thermotogaceae* had been continuously increased. A previous study showed that *Coprothermobacter* could be found in the anaerobic methane fermentation with cow dung (Tandishabo et al. 2012). *Coprothermobacter* could grow well in high protein substrate and degraded proteins to H_2, CO_2 and acetic acid. Meanwhile, hydrogen was an important electron transfer between *Coprothermobacter* and methane- producing bacteria in the anaerobic reactor. Therefore, along with the reduction of protein content, *Coprothermobacter* possessed a high percentage on day 1 and day 5 but had a relatively lower ratio on day 15 in the anaerobic digestion process. *Clostridium*, possessing the ability to hydrolyse cellulose, sugar, and proteins into organic acids and alcohols, plays an important role in the fermentation of *E. prolifera* (Braga et al. 2020). *Clostridium* was the third largest genus in EM-D. It could be related to the CF content of *E. prolifera*, which was not high compared to the terrestrial plant. The percentage of *Clostridium was* 6.2%, 10.3%, and 7.3% at day 1, 5 and 15, respectively. It could explain why the CF of the substrate was rapidly degraded between the 6th and 10th day. *Caloramator* had a proportion of 5.30%, 0.98% and 0.71% at day 1, 5, and 15, respectively. It is an obligate anaerobic and gram-positive bacterium that can convert proteins and sugars to acetic acid, formic acid, branched-chain fatty acid, and H_2 with suitable growth conditions of 55 ℃ and pH of 7.0 ~ 7.5 (Rubiano-Labrador et al. 2012). The suitable pH value of 7.6 and the presence of low content of inhibitory factors, like ethanol, formic acid, acetic acid, etc., in the reactor, resulted in a high abundance of *Caloramator* in three samples on the first day. However, the increased content of inhibitory factors and the reduction of pH value led

to a decrease in the abundance of the *Caloramator*. This showed that *Caloramator* played a role in organic matter degradation combined with other microorganisms in the early stage of methane fermentation.

In the case of the *Archaea* phylum, the genera of *Cenarchaeum, Methanothermobacter, Methanosarcina, Methanomassiliicoccus, Methanosaeta*, and *Halorubrum* had been detected in EM-D. Some other unclassified archaebacteria were also detected in EM-D, indicating that many previously uncovered species could be related to the degradation of *E. Prolifera*. *Cenarchaeum* was the dominant archaebacteria of EM-D. The abundance of *Cenarchaeum* was 83.22%, 94.45% and 94.74%, respectively, in the tested samples. *Cenarchaeum* is widely distributed in every corner of the earth, including water, seawater and soil (Schleper et al. 1997). *Cenarchaeum symbiosum*, the only Mexico marine sponge symbiotic archaea, is a species of *Cenarchaeum*, which had a proportion of more than 65% in marine sponges (Preston et al. 1996). Glycolytic pathway and TCA cycle were the basal metabolisms of *Cenarchaeum symbiosum*, which could offer electron acceptor and substrate to methanogen. The current reports of *Cenarchaeum* are limited, especially in the high-temperature fermentation of biomass gas. This study could also show the importance of the *Cenarchaeum symbiosum* in the biogas fermentation process. Previous study has shown that many archaebacteria could obtain enough energy for sustain metabolism by converting H_2S, S, and other sulphide oxidation to sulphuric acid, and archaebacteria would assimilate carbon dioxide and sulphur particles in this process. Furthermore, *E. prolifera* is sulphur-rich substrate. Accordingly, *Cenarchaeum* may give a great contribution to the sulphur cycle and nitrogen cycle in the reactor. *Methanothermobacter*, *Methanosarcina*, *Methanomassiliicoccus and Methanosaeta* are dominant methanogens with *Methanothermobacter* being the most prevalent methanogenic genus in the reactor. The abundance of methanogens was in a dynamic balance with the proportion of 4% in the archaea due to the competition for nutrients. Among the methanogens, *Methanothermobacter*, *Methanosarcina* and *Methanomassiliicoccus* could conduct the hydrogenotrophic methanogens genesis pathway to synthesize methane from H_2 and CO_2. However, *Methanosaeta*, a kind of acetoclastic methanogen and highly dependent on acetate, accounted for less than 0.06% of EM-D. The probable cause for this could be the large percentage of hydrogen bacteria, like *Thermotogaceae*, *Coprothermobacter*, *Clostridium*, and *Caloramator*, which offered enough H_2 and CO_2.

Methanothermobacter was the dominant methanogen in the fermentation with the proportions of 3.4%, 2.8% and 3.3% respectively. *Cysteine*, an accelerating growth factor of *Methanothermobacter*, was abundant in *E. prolifera*, thereby affecting the methanogens composition. The preceding results showed that microbial degradation accounted for the vast proportion of the fermentation process, while methanogenic accounted for only a small part. The degradation microbials, such as *S1* of *Thermotogaceae*, *Coprothermobacter* and *Caloramator* converted sugar or protein to organic acids, H_2, and CO_2. *Clostridium*, as the major fibre degradation microorganism, was increased and played a vital role in the continuity of biogas generation in the later fermentation stage. *Cenarchaeum* as a major member of the bacteria group, contributed to the degradation of raw materials, along with making a significant contribution to the nitrogen and sulphur cycle in the reactor. Most of the microbes in the community could produce H_2 and CO_2, resulting in *Methanothermobacter* becoming the main methanogen.

Morphology changes of E. prolidera in fermentation process

The unfermented *E. prolifera* exhibited a cylindrical surface morphology with regular bump all over the smooth algae (Fig. 4a and b). However, after fermentation, the tube-shaped structure o*f E. prolifera* was broken into slices or section (Fig. 4c). In addition, the regular bump disappeared from algae surface which shaped irregularly concave and convex with small holes (Fig. 4d and e). These holes were generated by microorganism which broke the algae cells. And microorganism embedded in algae surface could be observed from SEM image (Fig. 4f). From the above results, in the anaerobic fermentation process, microbes firstly attached to the surface of *E. prolifera* and damaged the cell wall. Then, microbes obtained nutrients from the epidermis of algae, and gradually eroded the deeper tissue cells of *E. prolifera*. Eventually, the tube-shaped structure of *E. prolifera* was totally breakdown and emerged many holes. It indicated that proper pretreatments for breaking *E. Prolifera* cell wall may be efficiency ways to enhance the effect of biogas digestion.

Conclusion

E. prolifera was a kind of carbohydrate-rich fast-growing green algae, which is responsible for "green tide" in eutrophicated sea area. *E. prolifera* was used as substrate for anaerobic fermentation, and dynamic process of *E. prolifera* digestion was deeply investigated by combining chemistry, biology and physics in this study. Batch experiments showed that digestion of *E. prolifera* generated great mass of biogas, more than half of which was methane. This study revealed that anaerobic digestion process of *E. prolifera* could be artificially divided into three stages accompanying with replacement of active microbes in community. In the case of substrate

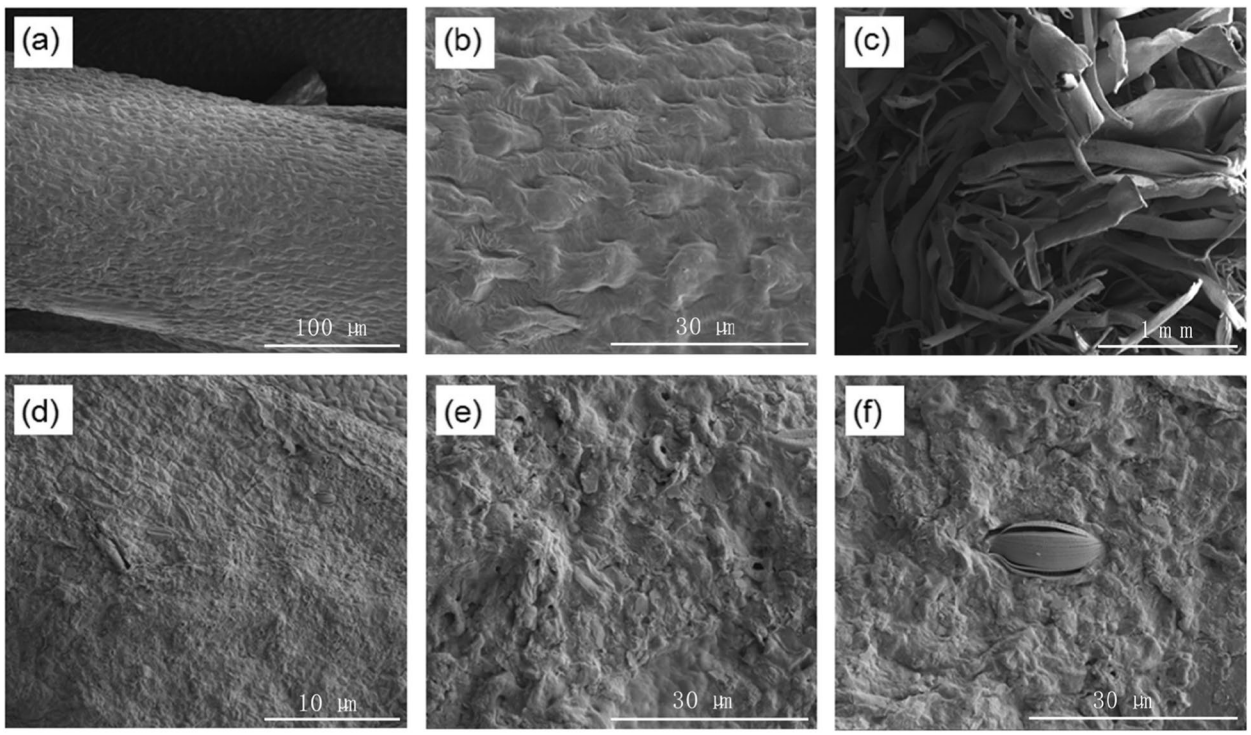


Fig. 4 SEM photographs of *E. prolifera* in biogas fermentation

degradation Most total solid, including carbohydrate, proteins and fibre, were degraded at first two days, and the digestion of sugar and protein was prior than fibre. High-throughput 16 S rRNA sequencing of community from the fermentation showed that *Cenarchaeum* and *S1* of *Thermotogaceae* were predominant archaea and bacteria, and *Methanothermobacter* was the major methanogen caused by hydrogen-bacteria-rich structure of the community. SEM images of this study suggested that microbes damaged the epidermis of *E. prolifera* and gradually embedded into algae body, then broke the tube-shaped structure of *E. prolifera* during anaerobic fermentation. Although the interactions in microflora were not exactly explicated by genetic level or metabolic pathway map which will be explored in further study, this paper gave an important contribution to the field of *E. prolifera* biogas fermentation. Moreover, this study gives strong evidence to verify the possibility of algae utilized as raw material for biogas fermentation.

Abbreviations

VS: Volatile solids; HRT: Hydraulic retention time; WH: Water hyacinth; SD: Swine dung; TS: Total solid; OTUs: Operational Taxonomic Units; VFAs: Volatile fatty acids; ON: Organic nitrogen; OC: Organic carbon; CF: Crude fibre.

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Authors' contributions

Chao Ai and Aijun Tong performed the experiment and wrote the article. Jiahui Wen, Ruoxin Chen and Yajun Huang wrote and edited the article. Chao Zhao provided Conceptualization, Resources, Writing- Review & Editing, Visualization, Supervision, Funding acquisition. All authors read and approved the final manuscript.

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors hereby declare no conflict of interest.

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