Effects of organic loading rate on biogas production from macroalgae: Performance and microbial community structure

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Selected highlights
• Effects of organic loading rate on anaerobic digestion of macroalgae were studied.
• The growing organic loading rate in an appropriate range could improve biogas yield.
• The overrange organic loading rate could cause system instability.
• Unfavorable VFAs concentration, pH and salinity might be main causes for instability.
• Several bacterial and archaeal phyla altered with growing OLR apparently.

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Abstract
Macroalgae biomass has been considered as a promising feedstock for biogas production. In order to improve the efficiency of anaerobic digestion (AD) of macroalgae, semi-continuous fermentation was conducted to examine the effects of organic loading rate (OLR) on biogas production from Macrocystis pyrifer. Results showed that, under OLRs of 1.37, 2.74, 4.12 and 6.85 kg VSSubstrate/(m3 d), the average unit biogas yields were 438.9, 477.3, 480.1 and 188.7 mL/(g VSSubstrate d), respectively. It indicated that biogas production was promoted by the increased OLR in an appropriate range while inhibited by the OLR beyond the appropriate range. The investigation on physical-chemical parameters revealed that unfavorable VFAs concentration, pH and salinity might be the main causes for system failure due to the overrange OLR, while the total phenols failed to reach the inhibitory concentration. Microbial community analysis demonstrated that several bacterial and archaeal phyla altered with increase in OLR apparently.

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1. Introduction

The global economy is claiming for renewable resources to satisfy the continuous growing energy consumption and demand (Goyal et al., 2008; Steinberger and Roberts, 2010). The marine macroalgae biomass has been considered as a promising alternative feedstock for biofuels and biomaterials production due to its advantages, including high growth rate and no utilization of cultivated land (Konda et al., 2015; Ward et al., 2014). Biogas production via anaerobic digestion (AD) has been proved to be a feasible method to utilize macroalgae (Débowski et al., 2013; Nkemka and Murto, 2010). Quite a few studies conducted AD processes of macroalgae and obtained decent biogas yields. Fan et al. treated marine sediments from littoral and sublittoral location as inocula for anaerobic fermentation of M. pyrifer, obtained biogas yields of 383.1 ± 9.7 and 282.4 ± 13.3 mL/g VSSubstrate, respectively (Fan et al., 2015). 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pretreatment has increased to 605 ± 4 L/kg VSsubstrate via co-digestion of two-phase olive mill solid residue increased more than because of the severe accumulation of VFAs (Li et al., 2015). Rincón et al. 2015) and 28 known bacterial phyla

* methanoseta
* meta-analysis approach, which assigned microorganisms to
* rial phyla involve in other stages which provide substrates for
* methane). Microorganisms involved in these stages were divided
* into archaeal phyla and bacterial phyla. The archaeal phyla mainly
* transformed into various organic acids (acetic, propionic acid,
* forms. During acidogenesis, the products from hydrolysis are
* were functioned by specialized microorganisms. In the first step
* methane (hydrolysis), complex macromolecules are cleaved into simpler
* participated in methanogenesis stage immediately, while the bacte-
* phyla involve in other stages which provide substrates for
* for behaviors of AD process.

The organic loading rate (OLR), a critical operational parameter
* affects biogas production significantly. Review of literatures
* increased OLR beyond the range, the biogas yield decreased dras-
* for behaviors of AD process.

An anaerobic digestion (AD), a multistage process, comprises of
* hydrolysis, acidogenesis, acetogenesis and methanogenesis, which
* were functioned by specialized microorganisms. In the first step
* complex macromolecules are cleaved into simpler forms. During acidogenesis, the products from hydrolysis are
* the fer-
* mented products are oxidized into acetate, CO2 and H2. In the
* last stage (methanogenesis, the key step for methane production),
* microorganisms converted acetate, CO2 and H2 into biogas (mainly
* methane). Microorganisms involved in these stages were divided
* into archaeal phyla and bacterial phyla. The archaeal phyla mainly
* participate in methanogenesis stage immediately, while the bacte-
* rial phyla involve in other stages which provide substrates for
* methane production (Goswami et al., 2016). Nelson et al. analyzed the microbial diversity observed in anaerobic digesters using a
* meta-analysis approach, which assigned microorganisms to
* archaeal phyla (mainly methanoseta and the uncharacterized
* WSA2 group) and 28 known bacterial phyla, respectively (Nelson et al., 2011). Chojnacka et al. investigated the characterization of
* methane-producing microbial community which processed acidic
* effluent from molasses fermentation, and revealed that Bacteria was dominated by Firmicutes, Bacteroidetes, Proteobacteria, Cloaci-
* monetes and Spirochetes, while in the domain Archaea, Methanomi-
* crobiales, Methanomassiliicoccales and Methanosarcinales were
* predominant (Chojnacka et al., 2015). Notably, these microorganisms participating in each phase are interdependent during AD process. Consequently, the stability of the process might breakdown easily when one of the four stages is out of balance (Nelson et al., 2011). These microorganisms differ in nutritional requirement, growth kinetics and environmental tolerance, as a result, their abundance and behavior vary with external factors, including volatile fatty acids (VFAs), pH, salinity and phenols (Yi et al., 2014). The VFAs above 20.00 g/L and the pH value below 6.5 could severely inhibit methane productivity of methanogens (Babel et al., 2004; Zhai et al., 2015). High salt concentration was proved toxic on methanogens (Marquez et al., 2013). Moen et al. mentioned that high concentration of polyphenols could probably have toxic effects on microorganisms which involve in anaerobic digestion (Moen et al., 1997). These physical-chemical factors could affect the distribution of microorganisms, accordingly, the performance of AD system (Yi et al., 2014). Therefore, it could be speculated that the variations of microbial community, which were caused by changing external conditions, were the underlying reasons for behaviors of AD process.

The organic loading rate (OLR), a critical operational parameter
* which represents the biological conversion capacity of AD system, affects biogas production significantly. Review of literatures showed that, the AD was inefficient at lower OLR, and was improved with increase of OLR at an appropriate range, while increase of OLR beyond the range, the biogas yield decreased drastically and the failure of the system occurred (Li et al., 2015; Rincón et al., 2008). Li et al. carried out continuous batch experiments (40 L) to investigate the effects of OLR on the anaerobic mesophilic co-digestion of rice straw and pig manure. Results showed that the maximum volumetric biogas production rate (VBP) was improved with increased OLR which was in the range of 3.00–8.00 kg VSsubstrate/(m3·d), and the maximum VBP of 3.45 m3/(m3·d) was obtained at OLR of 8.00 kg VSsubstrate/(m3·d), while the VBP decreased dramatically at OLR of 12.00 kg VSsubstrate/(m3·d) because of the severe accumulation of VFAs (Li et al., 2015). Rincón et al. mentioned that methane productivity of one-stage anaerobic digestion of two-phase olive mill solid residue increased more than 40% when OLR ranged from 1.50 to 9.20 kg VSsubstrate/(m3·d), while further increase of OLR caused reactor destabilization and process failure (Rincón et al., 2008). Chandra et al. argued that a higher OLR could reduce the size of digester and capital cost of digestion (Chandra et al., 2011). Consequently, a suitable OLR have to be chosen to optimize the AD process (Montingellia et al., 2015).

Notably, the variations of microbial community in AD system with changing OLR were rarely analyzed. Yi et al. examined the performances and microbial communities of AD process treating food waste as substrate under varying total solids (TS, 5.00–20.00%), and suggested that the methane yield increased with elevated TS, meanwhile, the abundance of different microbial groups varied apparently (Yi et al., 2014). Therefore, laboratory tests for effects of OLR on structure of microbial community in AD system make it possible to gain better insights into the relationship between microbial groups and fermentation performance with changed OLR, and even helpful to optimize the OLR of AD process.

In this work, semi-continuous fermentation reactor treating sewage sludge as inoculum was carried out to digest M. pyrifera under different OLRS for biogas production. The objectives were (1) to investigate the effects of OLR on biogas production, (2) to look into the variations of VFAs concentration, pH, salinity and total phenols concentration, (3) to examine the distribution of underlying microbial communities in AD system for analyzing the regularity of their variations.

2. Methods

2.1. Substrate and inoculum

The substrate used in this study was M. pyrifera biomass, which was purchased from Gather Great Ocean Algae Industry Group, Qingdao, China. The total solid (TS) was 25.81% and the volatile solid (VS) was 62.99% (based on TS). Proportions of protein, carbohydrate and lipid were 20.00%, 12.90% and 26.50% (based on TS), respectively.

Inoculum used in this study was sewage sludge obtained from a 20L size of semi-continuous stirred tank reactor, which treated M. pyrifera biomass as substrate and inoculated with the sewage sludge collected from Tuandaow Sewage Treatment Plant, Qingdao, China. The TS was 14.66% and the VS was 59.44% (based on TS). The pH value of sludge was 6.9.

2.2. Semi-continuous AD process

Anaerobic digestion of M. pyrifera was carried out in a 20 L semi-

* continuous stirred tank reactor. The working volume was 16 L. Temperature was maintained at 37 °C. The hydraulic retention time (HRT) was 15d, reactor stirring for 2 min with a time interval of 10 min. Four OLRS of 1.37, 2.74, 4.12 and 6.85 kg VSsubstrate/(m3·d) were set. The initial OLR was 1.37 kg VSsubstrate/(m3·d), after system operated stably for a certain period, the OLR was raised sequentially. The biogas yield and methane concentration were measured daily. The VFAs concentrations, pH value, salinity and total phenols concentration were tested periodically.

2.3. Microbial community structure analysis

Sample Q1, Q2 and Q3 contained 10 mL of slurries were col-

* Sample Q1, Q2 and Q3 contained 10 mL of slurries were collected from AD system on the basis of stable operation under OLRS of 1.37, 2.74 and 4.12 kg VSsubstrate/(m3·d), respectively. Samples were stored at –80 °C in a refrigerator before use. The DNA extraction, PCR amplification, 16s rRNA library sequencing and analysis were conducted at GENEWIZ, Inc. (Beijing, China), which was in compliance with the reported methods (Fan et al., 2015).
2.4. Batch AD with different salinity

Batch AD of M. pyrifera biomass was conducted in triplicate in 300 ml bottles. Each bottle was added with 200 mL of anaerobic slurry, which was collected from semi-continuous stirred tank reactor when it operated stably under OLR of 4.12 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d). Seven groups were carried out and their salinity were adjusted to 38‰, 41‰, 48‰, 56‰, 63‰, 70‰, 80‰ by NaCl, respectively. The methane yield was tested daily.

2.5. Analytical methods

TS and VS were analyzed according to the APHA (APHA, 2005). Elemental analysis was estimated by an elemental analyzer (Vario EL III). Carbohydrate and lipid contents of M. pyrifera biomass were determined by the phenol sulfuric acid method (Safi et al., 2013). The protein content was tested based on the total elemental nitrogen measurement using the conversion factor 6.35 (He et al., 2016). Biogas volume was measured by a wet gas flow meter (LMF-1, Changchun, China). Methane concentration was tested by gas chromatograph (SP6890, Ruihong, Shandong, China). VFAs concentrations were measured by gas chromatograph (450GC, Varian, America). The pH value and salinity were determined by a pH meter (B-212, HORIBA) and a refractometer (HB-211), respectively. Total phenol concentration was measured by a ultraviolet spectrophotometer (UV2000, Younike, Shanghai, China).

3. Results and discussion

3.1. Biogas production during AD with increased OLR

Biogas yields during AD process of M. pyrifera in a semi-continuous stirred tank reactor were depicted in Fig. 1(a). When OLRs were 1.37, 2.74 and 4.12 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the average volumetric biogas productions (AVBP) were 0.60, 1.31 and 1.98 L/(L d), respectively, while the AVBP dramatically declined to 1.29 L/(L d) at OLR of 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d). The average unit biogas productions (AUBP) were 438.9, 477.3 and 480.1 mL/(g VS\textsubscript{substrate}) at OLRs of 1.37, 2.74 and 4.12 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), respectively, whereas, further increased OLR of 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d) lead to decrease of AUBP to 188.7 mL/(g VS\textsubscript{substrate}). Results indicated that biogas production was improved with increased OLR which were below 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), while inhibited severely at OLR of 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d).

Methane concentrations during AD process of M. pyrifera were shown in Fig. 1(b). At OLRs of 1.37, 2.74 and 4.12 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the methane concentration varied between 50.00% and 60.00% when biogas production was stable. At OLR of 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the methane concentration declined radically and dropped to 1.43% on the 130th day. With OLRs below 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the biogas yield was improved with increased OLR (Fig. 1(a)), and the methane concentration of biogas was stable (Fig. 1(b)), which indicated that methane yield was raised synchronously. Under OLR of 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the biogas yield and the methane concentration were decreased simultaneously (Fig.1), which revealed a drastic decline of methane production.

The increased OLR brought more substrate into AD system and enhanced the concentration of substrate, which improved biogas/methane productions. However, when OLR further increased to a high level, the overloading substrate occurred, consequently, the AD process turned to be instable and the biogas/methane productions were inhibited.

3.2. The variations of VFAs and pH value with increased OLR

The concentrations of total volatile fatty acid (TVFA) during AD process of M. pyrifera were shown in Fig. 2(a). At OLRs of 1.37 and 2.74 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the TVFA concentrations were less than 1.00 g/L. Then the TVFA increased markedly (below 10 g/L) at OLR of 4.12 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d). When OLR was raised to 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the TVFA concentration went up sharply and reached the maximum value of 26.28 g/L. Babel et al. mentioned that the VFAs in AD system were generated by hydrolysis/acidogenesis and consumed by methanogenesis subsequently, accordingly, the concentration of TVFA stabilized at an appropriate value when system was steady (Babel et al., 2004). Additionally, Li et al. pointed out that hydrolysis/acidogenesis occur quickly than methanogenesis (Li et al., 2015). In this work, the increased OLR caused enhancement of substrate concentration, which made hydrolysis/acidogenesis occur quickly and generate a great deal of VFAs. When OLR varied in an appropriate range, the VFAs could be digested by methanogenesis soon. While at the overrange OLR, the VFAs failed to be consumed by methanogenesis in time, which led to an excessive accumulation of VFAs. The VFAs above 20.00 g/L could inhibit methane productivity of methanogens (Babel et al., 2004). As depicted in Fig. 2(a), under OLR of 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the TVFA concentration rose drastically and over 20.00 g/L after the 117th day. Accordingly, biogas and methane productions were ceased by excessive accumulation of VFAs at over range of OLR.

The concentrations of VFAs during AD process at OLR of 4.12 and 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d) were depicted in Fig. 2(b). When OLR was 4.12 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the concentrations of acetic acid, butyric acid and propionic acid maintained at low values. With OLR of 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the concentrations of acetic acid and butyric acid increased sharply to the maximum values of 19.82 g/L and 10.67 g/L, respectively, while the propionic acid were stable and varied between 1.48 g/L and 2.63 g/L. Dogan et al. argued that the concentrations of 4.00, 2.00 and 6.00 g/L were the beginning of acetic acid, propionic acid and butyric acid inhibition respectively on methane production (Dogan et al., 2005). Fotidis et al. recommended that the methane productivity of methanogens decreased by 50% when concentrations of acetic acid, butyric acid and propionic acid were more than 13.00, 15.00 and 3.50 g/L, respectively (Fotidis et al., 2013). Results indicated that the over range of OLR made the concentrations of acetic acid and butyric acid rose sharply and caused excessive accumulation, which restrained biogas and methane productivities of AD system, consequently, biogas and methane yields decreased.

The pH values during AD process of M. pyrifera in a semi-continuous stirred tank reactor at OLRs of 4.12 and 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d) were shown in Fig. 2(c). At OLR of 4.12 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the pH value varied in the range of 7.7–8.2. When OLR was further raised to 6.85 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the pH value decreased dramatically to 5.3, which showed that a drastic acidification occurred in AD system. Review of reports indicated that the pH value of 6.5–7.5 was the optimal range for growth of methanogens, and when it was below 6.5, the growth and methane productivity of methanogens were severely inhibited (Zhai et al., 2015; Zhang et al., 2015; Kim et al., 2003). In this work, the over range of OLR let to overloaded VFAs accumulation and a severe decline of pH value, which might be the inhibitory factors for biogas and methane productions.

3.3. Salinity

3.3.1. The variation of salinity during AD process with increased OLR

The salinity of AD system during AD process of M. pyrifera were shown in Fig. 3(a). When OLRs were 1.37, 2.74 and 4.12 kg VS\textsubscript{substrate}/(m\textsuperscript{3} d), the salinity rose slowly (below 38‰) with increased
OLR. When OLR was raised to 6.85 kg VS_{substrate}/(m^3 d), the salinity continued to rise slowly in the initial period, then went up drastically and reached the maximum value of 63‰. Apparently, the salinity grew with increased OLR. Due to the macroalgae is a kind of marine plant, the macroalgae biomass possessed the bulk of intracellular and extracellular salts (Marquez et al., 2013). With the increased OLR, an increasing amount of macroalgae biomass was digested, and an increasing number of salts dissolved into AD system, which accounted for rising of salinity (Marquez et al., 2013). It could be speculated that the overrange OLR could let to a soared salinity, which might have a negative impact on methane production.

3.3.2. Effect of salinity on methane yield during batch AD process

Batch AD of M. pyrifera was carried out to examine the effect of salinity on biogas yield. The cumulative methane yields of seven groups with different salinity were depicted in Fig. 3(b). The maximum value of 211.04 mL/g VS_{substrate} was obtained at salinity of 38‰, and the cumulative methane yield decreased a little at salinity of 41‰, while it reduced drastically at salinity above 48‰. Apparently, the cumulative methane yield declined with elevated salinity. Marquez et al. argued that the salinity above 42‰ could inhibit methanogenic activity and methane production of AD system treating sea wrack as substrate (Marquez et al., 2013). According to Fig. 3(a), under OLR of 6.85 kg VS_{substrate}/(m^3 d), the salinity of AD system was more than 42‰ during middle-late period. Results demonstrated that, at the over range of OLR, the over-loaded substrate brought a high salinity, which could be an inhibitory factor on methane production.

3.4. The variation of total phenols concentration during AD process with increased OLR

The concentrations of total phenols during AD system of M. pyrifera at OLRs of 4.12 and 6.85 kg VS_{substrate}/(m^3 d) were shown in Fig. 4. The concentration of total phenols rose slowly with increased OLR, eventually reached the maximum value of 195.67 mg/L on the 122th day. Phenols were suggested to be generated during anaerobic decomposition of macroalgae, and it could inhibit biogas production with concentration above 300.00 mg/L.
3.5. Analysis of microbial communities

3.5.1. Variations of functional Bacteria with increased OLR

3.5.1.1. Major phyla. The distribution of several kinds of Bacteria at the phylum level in each sample was depicted in Fig. 5(a). The populations of bacterial phyla varied apparently with increased OLR. Most of the sequences were affiliated with five major groups: Bacteroidetes, Firmicutes, Synergistetes, Proteobacteria and Spirochaetes.

The phylum Bacteroidetes accounted for 29.9537%, 32.3972%, and 38.7922% in sample Q1, Q2 and Q3, respectively, which was the most prevalent group among the Bacteria identified in all samples (Fig. 5(a)). The phylum Bacteroidetes was shown to be a kind of proteolytic bacteria, which probably involved in the hydrolysis/acidogenesis stages and degraded various proteins into VFAs, succinate and NH3 (Riviére et al., 2009). The macroalgae was reported to be a kind of protein-rich biomass (Ghadiryanfara et al., 2016), and the protein content of substrate (the M. pyrifera biomass) used in this work was 20.00% (based on TS). The prevalence of phylum Bacteroidetes during AD process might be in accordance with the high protein content of macroalgae biomass. Additionally, increased OLR brought a higher protein-input to AD system, which could result in the enhancement of Bacteroidetes. With increase of substrate, the Bacteroidetes bred rapidly, consequently, the VFAs were largely generated and accumulated in system.

The phylum Firmicutes was the second major bacterial group among all samples. It was assigned to be 17.9955%, 19.1752% and 37.1104% in sample Q1, Q2 and Q3, respectively (Fig. 5(a)). With increased OLR, the Firmicutes was selectively enriched. When OLR varied from 2.74 kg VSsubstrate/(m$^3$-d) to 4.12 kg VSsubstrate/(m$^3$-d), the population of Firmicutes increased sharply and almost doubled. The phylum Firmicutes was well-known to be a kind of acetogenic bacteria which degraded VFAs and produced acetic acid (Yi et al., 2014). When OLR were 1.37 and 2.74 kg VSsubstrate/(m$^3$-d), the TVFA concentrations maintained at low values (below 0.40 g/L). While under OLR of 4.12 kg VSsubstrate/(m$^3$-d), the TVFA concentration rose to a high level and reached the maximum value of 9.19 g/L on the 96th day (Fig. 2(a)). It implied that a VFAs accumulation occurred. The increased OLR enlarged the concentration of substrate, which accelerated the VFAs production. The enriched VFAs favored growth and reproduction of Firmicutes, accordingly, the abundance of Firmicutes soared.

In sample Q1, the phylum Synergistetes was the third major bacterial group which appeared to be 7.7010%, while accounted for 10.1647% and 6.8280% in sample Q2 and Q3, respectively (Fig. 5(a)). Several reports suggested that Synergistetes used to be a minor phylum in AD system (Yi et al., 2014; Nelson et al., 2011; Guo et al., 2015), however, in this work, it appeared to be a major phylum. The phylum Synergistetes plays a critical role in acidogenesis phase and is capable of protein degradation (Nelson et al., 2011). Accordingly, the prevalence of Synergistetes was possibly resulted from the high protein content in macroalgae substrate. Interestingly, the proportion of Synergistetes was higher in sample Q2 compared to that for sample Q1, whereas it decreased to the lowest value in sample Q3. It indicated that, under OLRs of 1.37 and 2.74 kg VSsubstrate/(m$^3$-d), Synergistetes could be enhanced with increased concentrations of substrates which brought by increased OLR. However, at OLR of 4.12 kg VSsubstrate/(m$^3$-d), Synergistetes might be possibly more sensitive to environmental change, including sharp increases of VFAs concentrations and salinity, which accounted for the decrease of its abundance.

The phylum Proteobacteria occurred with a proportions of 6.7786%, 4.5863%, 1.9693% in sample Q1, Q2 and Q3, respectively (Fig. 5(a)). When OLR ranged from 1.37 to 2.74 kg VSsubstrate/(m$^3$-d), the abundance of Proteobacteria increased, afterwards, it declined drastically at OLR of 4.12 kg VSsubstrate/(m$^3$-d). Proteobacteria is likely to be ubiquitous in nearly all AD processes (Ariesyady et al., 1997). Results showed that with increased OLR, numerous phenols were produced from digestion of substrate. However, the maximum value of total phenols concentration in AD system (195.67 mg/L) was less than 300.00 mg/L. Accordingly, it was speculated that phenols had no obvious inhibitory effect on biogas production at OLR of 6.85 kg VSsubstrate/(m$^3$-d).
This phylum contains several genera which participate in different phases of AD process in several, including *Comamonadaceae* (acetlastic), *Parabacteroides* (saccharolytic), *Smithella* (propionate oxidation). These genera might not only involve in the hydrolysis of organic wastes but also consume propionate, butyrate, and acetate (Guo et al., 2015; Rademacher et al., 2012).

The phylum *Spirochaetes* took 5.6753%, 10.2539%, 8.9885% of sample Q1, Q2 and Q3, respectively. When OLR turned to 2.74 kg VSSubstrate/(m³d), *Spirochaetes* was enriched in AD process, whereas it reduced slightly at OLR of 4.12 kg VSSubstrate/(m³d). This phylum was stated to be capable of fermenting carbohydrates or amino acids into acetate, H₂ and CO₂, which were the main substrates for methane production (Klocke et al., 2007). Similar to *Synergistetes*, the abundance of phyla *Proteobacteria* and *Spirochaetes* increased when OLR was raised to 2.74 kg VSSubstrate/(m³d), while it declined at OLR of 4.12 kg VSSubstrate/(m³d). The decrease of their abundance under the high OLR might be due to the weaker tolerance to change in physical-chemical factors.

### 3.5.1.2. Minor phyla

Several minor bacterial groups were detected in AD process, including *Chloroflexi*, *Thermotogae*, *Actinobacteria*, *Planctomycetes*, *Tenericutes*. These phyla appeared to be low abundance in AD system, but varied apparently with increased OLR (Fig. 5a).

The phylum *Chloroflexi* was classified with 1.3422%, 4.7487% and 1.0554% in sample Q1, Q2 and Q3, respectively. The proportion of *Chloroflexi* increased to the maximum value when OLR raised to 2.74 kg VSSubstrate/(m³d), then it decreased to the lowest value at OLR of 4.12 kg VSSubstrate/(m³d). *Chloroflexi* involves in hydrolysis phase and decomposes easily degrading organic carbon (including glucose and soluble microbial products) into acetic and H₂ (Yi et al., 2014). When OLR was raised to 2.74 kg VSSubstrate/(m³d), the *Chloroflexi* was enriched due to the increased concentration of substrate. In contrast, the further increase of OLR brought unfavorable physical–chemical conditions in AD system, which might possibly result in reduction of *Chloroflexi*.

The phylum *Thermotogae* accounted for 0.8846%, 2.3307% and 0.7889% in sample Q1, Q2 and Q3, respectively. *Thermotogae* is a kind of thermophilic sugar consumer which ferments oligosaccharide, monosaccharide, glycerol and pyruvate into acetic acid, lactic acid, CO₂, H₂ and ethanol, besides, some species also degrades cellulose and keratolin (Rademacher et al., 2012). The cause for the variation of *Thermotogae* with increased OLR might be analogous to *Chloroflexi*. When OLR was slightly raised, more carbohydrates were brought to AD system, which enhanced the abundance of *Thermotogae*. However, when OLR was further raised to a higher level of 4.12 kg VSSubstrate/(m³d), untunable conditions emerged in system, which was unfavorable to *Thermotogae*. 

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**Fig. 3.** The variation of salinity and the methane production of batch AD: a, the salinities during semi-continuous AD process with increased OLR; b, the cumulative methane yields from batch AD process at different salinities. — stands for the salinity.
The phylum *Planctomycetes* occupied 0.8077%, 1.2099%, 0.2936% in sample Q1, Q2 and Q3, respectively. *Planctomycetes* was essential for ammonia removal in AD process (Ariesyady et al., 2007).

The phylum *Actinobacteria* represented 0.3217%, 0.2156%, 0.2394% in sample Q1, Q2 and Q3, respectively, which was reported to be a multifunctional bacterial group. The suborder *Propionibacterinae* is defined as a propionate producer.

The abundance of phylum *Tenericutes* in sample Q1 and Q2 were 0.0461% and 0.1580%, respectively, while was undeveloped in sample Q3. *Tenericutes* was found to be related with lignin degradation (Boucias et al., 2013), however, the macroalgae biomass is nearly free of lignin (Roesijadi et al., 2010), which could account for the low abundance of *Tenericutes*.

### 3.5.2. Variations of functional Archaea with increased OLR

The proportions of several kinds of *Archaea* at the phylum level in each sample were depicted in Fig. 5(b). Three functional archaeal phyla which were *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales* varied considerably depending on the OLR.

The phylum *Methanobacteriales* was the most major methanogens among the AD system, which was identified with 0.0461%, 0.0502% and 0.0723% in sample Q1, Q2 and Q3, respectively (Fig. 5(b)). This phylum was selectively enhanced with the increased OLR. *Methanobacteriales* was suggested to be a kind of hydrogen-utilizing methanogens, and it shows high methane productivity with H$_2$/CO$_2$ as substrate (Niu et al., 2015). When OLR was elevated, a mass of substrate was fermented and converted to H$_2$/CO$_2$ by bacterial groups, which was beneficial to the reproduction and growth of *Methanobacteriales*. Additionally, the predominance of *Methanobacteriales* inferred that the hydrogenotrophic methanogens pathway was mainly utilized for methane production in AD system of macroalgae (Rademacher et al., 2012).

The subdominant archaeal phylum was *Methanosarcinales*, which was revealed to be 0.0272%, 0.0465% and 0.0301% in sample Q1, Q2 and Q3, respectively (Fig. 5(b)). When OLR was raised to 2.74 kg VS$_{\text{substrate}}$/m$^3$ d$^{-1}$, the abundance of *Methanosarcinales* increased to the highest level, while it declined under OLR of 4.12 kg VS$_{\text{substrate}}$/m$^3$ d$^{-1}$. *Methanosarcinales* was reported to be a strict acetoclastic pathway carrier. The OTUs number of the genus *Methanosaeta* in sample Q1, Q2 and Q3 were 8, 25 and 16 reads, respectively (Table 1). It was suggested to be a utilizer of algae substrates from freshwater (Ellis et al., 2012). The acetic acid concentration above 92.8 mM and the TVFA concentration above 7.10 g/L could restrict *Methanosaeta* (Rademacher et al., 2012). Summarily, *Methanosaeta* was unable to cope with high salinity and VFAs concentrations. Under OLR of 4.12 kg VS$_{\text{substrate}}$/m$^3$ d$^{-1}$, the acetic acid concentration (Fig. 2(a)), TVFA concentration (Fig. 2(b)) and salinity (Fig. 3(a)) went up sharply with the digestion of substrate, which was unfavorable to *Methanosaeta*.

The phylum *Methanomicrobiales* was appeared to be 0.0035%, 0.0019% and 0.0181% among sample Q1, Q2 and Q3, respectively (Fig. 5(b)). *Methanomicrobiales* was a kind of hydrogen-utilizing *Methanobacteriales* (Yi et al., 2014). However, *Methanomicrobiales* was less detected in all samples compared to *Methanobacteriales*, which implied that the macroalgae substrate used in this work favored apparent prevalence of *Methanobacteriales*.

The OTUs reads of the phylum *Halobacteriales* were 133, 117 and 820 in sample Q1, Q2 and Q3, respectively (Table 1). *Halobacteriales* was reported to be a kind of *Archaea* with high salt tolerance. In the AD system of macroalgae, the salinity went up with the increased OLR (Fig. 3(a)), which could account for the enhancement of *Halobacteriales* (Najjari et al., 2015).

It is worth mentioning that, when system operated stably, the sample Q1, Q2 and Q3 were collected from AD system at OLRs of 1.37, 2.74 and 4.12 kg VS$_{\text{substrate}}$/m$^3$ d$^{-1}$, respectively, for microbial community analysis. The further increased OLR of 6.85 kg VS$_{\text{substrate}}$/m$^3$ d$^{-1}$ stood for the concentration of total phenols.
strate/(m³ d) caused the drastic decrease of biogas yield and system failure (Fig. 1). During this period, it was imprecise to treat one point as sampling time which reflected the natures of microbial community of the whole phase. Additionally, due to the stable production failed to occurred at OLR of 6.85 kg VSsubstrate/(m³ d), it was improper to compare its microbial community with three previous phases which operated stably. Collectively, the microbial community analysis at OLR of 6.85 kg VSsubstrate/(m³ d) has not been chosen for comparison. The further study is under investigation, which focuses on the variations of microbial community at the overrange OLR. Several time points have been selected out for sampling, which could reflect the details and changes of microbial community during the system instability.

4. Conclusions

The OLR was demonstrated to affect the performance and structure of microbial community of AD process of macroalgae. The

![Figure 5](image_url)  
**Fig. 5.** The relative abundance of functional microbial groups at phylum level: a, bacterial groups; b, archaeal groups. Q1 stands for OLR of 1.37 kg VSsubstrate/(m³ d), Q2 stands for 2.74 kg VSsubstrate/(m³ d), Q3 stands for 4.12 kg VSsubstrate/(m³ d).

**Table 1**  
The abundance of the genus Methanosaeta and the phylum Halobacteriales.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>The number of reads</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1</td>
<td>Q2</td>
</tr>
<tr>
<td>k_Archaea; p_Euryarchaeota; c_Methanomicrobia; o_Methanosarcinales; f_Methanosaetaceae; g_Methanosaeta</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>k_Archaea; p_Euryarchaeota; c_Halobacteria; o_Halobacteriales; f_Deep_Sea_Hydrothermal_Vent_Gp_6(DHVEG-6); g_Candidatus; s_Parvarchaeum</td>
<td>133</td>
<td>117</td>
</tr>
</tbody>
</table>
appropriate increased OLR favored the biogas production, while excess OLR restrained it and caused system failure. With increased OLR, the physical-chemical parameters of AD system, including VFAs concentration, pH value, salinity and total phenols concentration, ranged apparently. They influenced the performance of AD system. Notably, the unfavorable VFAs concentration, pH value and salinity might be the main causes of system failure due to the overrange OLR. Microbial analysis revealed the increased OLR influenced the structure and behavior of microbial consortia significantly.

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