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Effect of inoculum/substrate ratio on methane yield and orthophosphate release from anaerobic digestion of *Microcystis* spp.

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ABSTRACT

A batch anaerobic test was conducted to evaluate the effects of inoculum/substrate ratios (ISRs) on the methane yield and orthophosphate release from the anaerobic digestion of *Microcystis* spp. The results demonstrated an obvious influence on methane yield and orthophosphate release by ISR. The maximum methane yield decreased from 140.48 to 94.42 mL/g VS when the ISR decreased from 2.0 to 0.5. The highest maximum methane yield calculated from Ørskov equation was 153.66 mL/g VS at ISR value of 1.0. The values of pH, ammonia and volatile fatty acids (VFAs) corroborated the appropriate stability of this anaerobic process.

1. Introduction

Over the last few decades, *microcystis* (cyanobacteria) bloom has been the most pressing problem of lakes and reservoirs in China, well in line with the global trend [1]. To date, more than 60% of lakes in China have been eutrophicated and suffered from harmful algae blooms, which are more likely to have higher treatment costs, greater difficulties to meet the standard for drinking water [2,3]. To minimize this threat, more frequently, algae were skimmed from lakes to prevent algae from getting into drinking-water plants. The efforts are taken to use the garnered algae, such as used as fertilizer, mixed with waste to be composted, dehydrated and mixed in poultry feed, or just storing in garbage dumps [4,5]. However, the last solution is only a transfer of pollution [6]. It would be of great significance if a new technology could be found to change algae from wastes into valuable products [7].

Anaerobic digestion is a process where in oxygen-free environment bacteria decompose organic matter and produce biogas primarily containing methane and carbon dioxide. The process not only reduces organic pollution, but also provides a new source of energy. Some works have been done to evaluate the conversion of algae, in the broad sense of the term (including cyanophyceae), into methane by anaerobic digestion, but few on cyanophyceae (cyanobacteria) themselves. Golueke et al. [8] published the first study on anaerobic digestion of micro-algae (*Scenedesmus* spp. and *Chlorella* spp.). And then, an integrated process associating the production of micro-algae in an open pond for the treatment of sewage water with the recovery of energy from the algae by anaerobic digestion was proposed [9]. The anaerobic digestion of macro-algae has also been reported, including giant brown kelp *Macrocystis pyrifera* [10], red marine alga *Gracilaria ceae* [11], and green marine alga *Ulva* sp. [12–14]. For the cyanobacteria, they were principally used as the feedstock of anaerobic digestion in mixture with micro-algae or phytoflagellates, at the time of experiments where the following cultures have been successfully used: culture of *Scenedesmus* sp. alone or together with either *Spirulina* sp., *Euglena* sp., *Microactinum* sp., *Melosira* sp. or *Oscillatoria* sp. [15]. From these studies, it can be concluded that algae are good feedstock for the anaerobic digestion process, because of high conversion rates and obtained efficiencies.

During anaerobic digestion of algae, assimilable phosphorus may release from the cells. Previous studies indicate that phosphorus is a key factor causing eutrophication of fresh water lakes and reservoirs [16,17]. And it is now generally accepted that phosphorus inputs must be decreased to mitigate the eutrophication of lakes and reservoirs [18]. The new European Union Water Framework Directive requires widespread control of phosphorus inputs to rivers to sustain/improve riverine ecology [19]. So the concentration of assimilable phosphorus must be reduced in anaerobic digestion effluents before final disposal. As orthophosphate

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Table 1
Characteristics of the inoculum and samples.

Constituent	Inoculum	Substrate
Total solids, TS (%)	7.74	0.87
Volatile solids, VS (%-TS)	60.80	92.14
Total carbon, TC (mg/g TS)	22.12	4.27
Total nitrogen, TN (mg/g TS)	0.70	0.13
Total phosphorus, TP (mg/g TS)	0.03	0.01

constitutes the assimilable part of phosphorus, it must particularly be paid attention to it. However, in this specific case of orthophosphate release during the anaerobic digestion of algae, available literature is, at present, particularly scarce [20].

Therefore, this research studied the performances of anaerobic digestion of *Microcystis* spp., which was the dominant species of eutrophicated Taihu Lake, and particularly on the influence of the inoculum/substrate ratios (ISRs) on the methane yield in a batch assay. In addition, orthophosphate release was also tested.

2. Materials and methods

2.1. Substrate

The substrate used in the experiment was the mixtures of algae bloom and lake water. The mixtures were collected from Meiliang Bay, Taihu Lake (120°30′N, 31°27′ E), where the occurrence of heavy cyanobacterial blooms in warm seasons has increased in frequency and intensity in recent years [21]. The *Microcystis* spp. was the dominant species in the mixtures (>99%). The mixtures were stored at 4 °C before further use. Characteristics of the samples were shown in Table 1.

2.2. Inoculum

Actively digested dairy cattle manure slurry from an 800 m^3 size biogas plant (Jimo, Qingdao, China) operating at 32 °C, with a 25 days retention time, were filtered and used as the inoculum. Characteristics of the inoculum were also shown in Table 1.

2.3. Anaerobic digestion tests

The experiments were carried out in 250 mL serum bottles with rubber caps of appropriate size. Three different inoculum substrate ratios (based on VS ratio) in this study were 2.0, 1.0 and 0.5, which were achieved by keeping a constant inoculum concentration (3 g VS/L) and varying the substrate concentration. And finally, the bottles were filled up to 120 mL with distilled water and flushed with a mixture of 80% N_2 and 20% CO_2 to maintain the proper pH and anaerobic conditions. The bottles were kept at 35 °C in a temperature controlled water-bath and inverted twice per day manually. A control without substrate was also performed. All the experiments were carried out in duplicate and the results were expressed as means.

2.4. Maximum methane yield

The methane volumes were corrected by subtracting the mean methane volume of the control and were converted to standard temperature and pressure (STP, 0 °C and 760 mm Hg). Methane yields were calculated by dividing the corrected methane volume by the weight of substance VS added to each bottle. The degradation of each sample was assumed to follow a first order rate of decay. Thus, the yield of methane was calculated using the Ørskov equation [22]:

$$B = B_0(1 - \exp(-kt))$$

where B (mL CH_4/g VS) is the cumulative methane yield at time t and t is time in days. B_0 (mL CH_4/g VS) is assumed to the maximum methane yield and k (per day) is estimated by taking the reciprocal of the time from the start of the experiment until when B equaled $0.632B_0$.

2.5. Analytical methods

The volume of biogas was measured by U-type pressure gauge daily. The volume was corrected to dry gas at STP. Methane concentration in the biogas was analyzed by a gas chromatograph (SP 6890, Shandong Lunan Instruments), equipped with Porapak Q stainless steel column (180 cm long, 3 mm outer diameter) and a thermal conductivity detector (TCD). The injector, detector and oven temperatures were 120, 150 and 50 °C, respectively. A 0.2 mL gas sample was injected into the chromatograph using nitrogen as the carrier gas. Total solids (TS), volatile solids (VS), pH were determined according to the standard methods [23]. Orthophosphate and volatile fatty acids (VFAs) were measured after filtering of samples through a 0.45 μm glass microfiber filter. COD were measured with COD analysis systems (Lianhua Technology Company, China) composed of a spectrophotometer (5B-3), COD reactor (5B-1), COD digestion reagent according to the procedures from the manufacturer. Orthophosphate was measured by the UV–visible spectrophotometer (Hitachi U3010, Hitachi Company, Japan) after converting the phosphoric anions to molybdenum blue [23]. VFAs were analyzed by a gas chromatograph (SP 6890, Shandong Lunan Instruments), equipped with Innowax column (30 m × Ø0.25 mm × 0.25 μm) and flame ionization detectors (FID). The operating temperatures were injection port 220 °C, detector 250 °C, and oven 120–150 °C (20 °C/min).

3. Results and discussion

3.1. Biogas productions and methane yields

The experiments lasted for 30 days. Fig. 1 shows the cumulative biogas production of *Microcystis* spp. as a function of time at different ISRs. As can be seen, the biogas production increased as the ISR value decreased. The cumulative biogas production after 30 days of digestion for the ISR value of 2.0, 1.0 and 0.5 were 70.38, 126.57 and 153.51 mL, respectively. The average daily cumulative biogas of ISR value of 2.0, 1.0 and 0.5 amounted to 2.35, 4.22 and 5.12 mL, respectively. The percentage of methane in total biogas volume was increased as the ISR decreased. The percentages of methane in the biogas were 35.92%, 38.74% and 45.19% with ISR value of 2.0, 1.0 and 0.5, respectively. Raposo et al. [24] published that the substrates amount contributed substantially in increasing the amount of methane in the biogas.

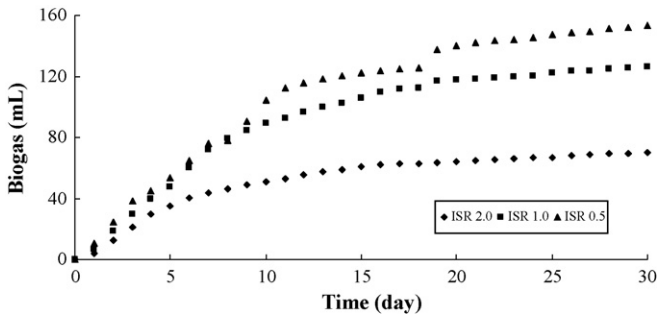

Fig. 1. Variation of the cumulative biogas production with digestion time at different ISRs.

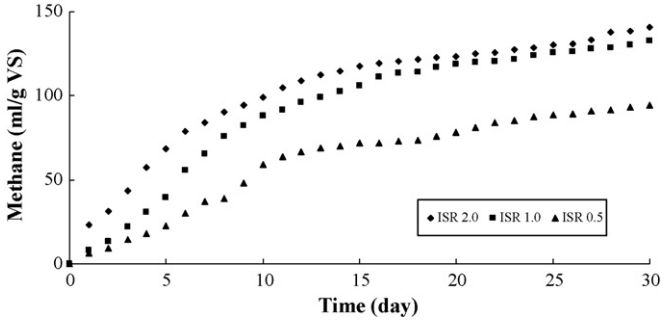

Fig. 2. Variation of the cumulative methane yield with digestion time at different ISRs.

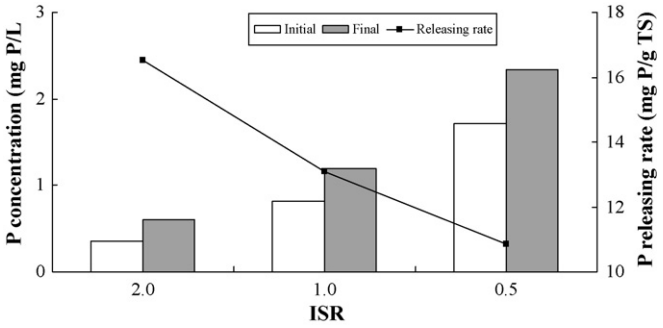

Fig. 3. Variation of orthophosphate concentrations and release rate with ISRs.

Table 2
Experimental and maximum methane yields in the anaerobic digestion[a].

ISR	CMY[b] (mL/g VS)	B_0 (mL/g VS)[c]	k (per day)	R^2
2.0	140.48	145.87(1.10)	0.134(0.004)	0.995
1.0	132.44	153.66(3.31)	0.086(0.005)	0.987
0.5	94.42	111.50(4.63)	0.064(0.005)	0.982

[a] All the value are expressed at standard temperature and pressure.
[b] CMY: cumulative methane yield in this experiment.
[c] Results are the average with standard deviation (shown in parentheses).

Fig. 2 shows the cumulative methane yield as a function of digestion time at different ISRs. The cumulative methane yield was calculated by dividing the methane production by the weight (in VS) of substrate added for each ISR. As can be seen from Fig. 2, the cumulative methane yield decreased from 140.48 to 94.42 mL/g VS when the ISR decreased from 2.0 to 0.5. The same conclusion was previously achieved by other researchers using different substrates [24–27]. The cumulative methane yield in this study was higher than that found in the batch experiments with non-washed *Ulva* sp. carried out by Briand and Morand [12] who reported a yield of 110 mL/g VS. Golueke et al. [8] studied the batch anaerobic digestion of *Secnedesmus* spp. and *Chlorella* spp. collected from stabilization lagoon with the TS in reactor of 8–9%. The methane yield of their study was 541 mL/g VS, which is higher than that of this test.

Table 2 summarizes the experimental results of cumulative methane yields at different ISRs, as well as the maximum methane yields and kinetic constant according to Ørskov equation. The results showed that it was possible to make good descriptions of the experimental data using Ørskov equation. The highest methane yield in the anaerobic digestion came from the ISR value of 2.0 (140.48 mL/g VS), which was close to the maximum methane yield predicted by the Ørskov equation (145.87 mL/g VS). The lowest maximum methane yield of 111.50 mL/g VS presented at the ISR value of 0.5, and the highest B_0 value (153.66 mL/g VS) corresponded to ISR value of 1.0, which was probably influenced by the sharp change in biogas composition in the first few days of the test [27]. Therefore, the ISR can be seen as an essential factor to influence the ultimate methane yield in the batch anaerobic digestion of *Microcystis* spp.

3.2. Process stability

As can be seen from Table 3, all final pH values ranged from 6.91 to 7.26, and the highest value corresponded to the ISR value of 0.5. These final pH values were compatible with the normal growth of anaerobic microorganisms. Ammonia could mainly influence the anaerobic digestion by affecting acetate-utilizing methanogenic Archaea, hydrogen-utilizing methanogens and syntrophic bacteria. Though the inhibitory concentrations of ammonia are different due to different experiments, 1.7–5 g total ammonia-N/L, corresponding to 0.4–1 g NH_3-ammonia/L are the most acceptable concentrations inhibiting the anaerobic digestion [28]. From Table 3, the final ammonia concentrations increased from 26.14 mg ammonia-N/L to 75.70 mg ammonia-N/L as the ISR value decreased from 2.0 to 0.5. This suggested that the initial and final ammonia are too little to inhibit the anaerobic digestion. Numerous observations related to anaerobic digestion suggest that volatile fatty acids (VFAs), as one of the most important parameters for the accurate control of anaerobic digestion, have a direct correlation with the digester performance. In this experiment, only n-butyric acid (n-HBu) has been detected at the initial stage of the experiment in all the samples. The initial values of VFAs were increased with the amount of algae added. And there are no VFAs detected after 30 days of digestion at different ISRs. According to Lin and Hu, both forms of butyric acid could be degraded in anaerobic digestion [29]. Hence, no VFAs detected probably means that the anaerobic digestion process was complete.

3.3. Orthophosphate release

Orthophosphate, the most stable form of phosphate, is sometimes referred as reactive phosphorus because it is the form which could be used directly by algae and plants [30,31]. Fig. 3 shows the initial and final concentrations of orthophosphate and the release rate during anaerobic digestion of algae. The concentrations were expressed by subtracting the mean orthophosphate concentrations of the control. It was shown that the initial concentrations of orthophosphate were 0.36, 0.81 and 1.71 mg P/L with the ISR value of 2.0, 1.0 and 0.5, respectively. And the final concentrations of orthophosphate increased from 0.60 to 2.34 mg P/L when the ISR decreased from 2.0 to 0.5. All the final concentrations of

Table 3
Performance data during the anaerobic process of *Microcystis* spp.

ISR	pH		Ammonia (mg ammonia-N/L)		VFAs (mg/L)	
	Initial	Final	Initial	Final	Initial	Final
2.0	7.23	6.98	82.07	26.14	122.16	ND
1.0	7.22	7.16	120.55	44.32	239.38	ND
0.5	7.26	7.23	198.17	75.70	325.62	ND

ND: not detected.

orthophosphate were higher than that of initial ones at the determined ISR. The orthophosphate release rate, which was calculated by dividing final mass of orthophosphate subtracting initial mass in a determined ISR by added TS of substrate, was defined in order to facilitate the comparison. As can be seen from Fig. 3, the orthophosphate release rate decreased from 16.53 to 10.85 mg P/g TS with the ISR decreasing from 2.0 to 0.5. Hence, the release of orthophosphate is obviously influenced by the ISR. A possible reason for the increase of orthophosphate is that the intracellular phosphorus could release into the solution followed by the decomposition of algae cells. The decomposition could be accelerated by the added of anaerobic microorganisms. On the other hand, under anaerobic conditions, organic substrates (such as VFAs) are consumed and subsequently stored as poly-β-hydroxybutyrate in anaerobic microorganisms, while the reducing equivalents needed are provided by the degradation of internally stored glycogen. The energy for this process is obtained partly from the degradation of glycogen but mostly from the hydrolysis of the intracellular stored polyphosphate, resulting in an orthophosphate release into the solution [32]. Orthophosphate content, therefore, the potential for phosphorus precipitation, increased after anaerobic digestion of *Microcystis* spp. The concentration of other ions such as ammonium, potassium and magnesium also increases in the digester according to previous researcher [33]. Thus, recovery and reuse of the phosphorus from the anaerobically digested effluent in the form of struvite by precipitation is sustainable and economical.

3.4. Stoichiometry of anaerobic digestion of algae

According to Redfield [34], the often-used representation of algae is $C_{106}H_{263}O_{110}N_{16}P$. The anaerobic digestion of algae could occur through the reaction as shown below [35]:

$$C_{106}H_{263}O_{110}N_{16}P \rightarrow 53CO_2 + 53CH_4 + 16NH_3 + H_3PO_4 \quad (1)$$

According to the stoichiometry, 0.19, 0.37 and 0.75 g COD and 1.7×10^{-3}, 3.4×10^{-3} and 6.8×10^{-3} g P should be generated in this experiment with the ISR value of 2.0, 1.0 and 0.5, respectively. Based on the experimental data collected during the anaerobic digestion, the COD productions were 0.12, 0.31 and 0.58 g with the ISR value of 2.0, 1.0 and 0.5, respectively. And the H_3PO_4 productions were 0.9×10^{-3}, 1.4×10^{-3} and 2.3×10^{-3} g P along with the ISR value of 2.0, 1.0 and 0.5, respectively. A comparison of the experimental data reveals that the COD and H_3PO_4 productions were less than that expected from the stoichiometry of reaction (1). Although the yield of anaerobic bacteria is low, a fraction of COD and phosphorus are used for cell synthesis and maintenances [36]. In addition other reactions might have occurred simultaneously, which needs a further study.

4. Conclusions

This study demonstrated that *Microcystis* spp. is a potential substrate for anaerobic digestion. In batch anaerobic digestion carried out at different ISRs, the ultimate methane yield decreased from 140.48 to 94.42 mL/g VS when the ISR decreased from 2.0 to 0.5, which showed an obvious influence on methane yield. It was possible to describe the experimental data using Ørskov equation. All the values of pH, ammonia and VFAs were suitable for the anaerobic digestion corroborating the appropriate stability of this anaerobic process. The results also showed that, whatever the ISR, the final concentrations of orthophosphate increased, in relation to initial ones. Hence, the concentrations of phosphorus in the effluents of digested *Microcystis* spp. should be recovered or treated to minimize the threat of eutrophication.

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