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ORIGINAL ARTICLE

# Start-up performance and granular sludge features of an improved external circulating anaerobic reactor for algae-laden water treatment

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**KEYWORDS**

Algae-laden water;
Microbial structure;
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**Abstract** The microbial characteristics of granular sludge during the rapid start of an enhanced external circulating anaerobic reactor were studied to improve algae-laden water treatment efficiency. Results showed that algae laden water was effectively removed after about 35 d, and the removal rates of chemical oxygen demand (COD) and algal toxin were around 85% and 92%, respectively. Simultaneously, the gas generation rate was around 380 mL/gCOD. The microbial community structure in the granular sludge of the reactor was complicated, and dominated by coccus and filamentous bacteria. *Methanosphaera*, *Methanolinea*, *Thermogymnomonas*, *Methanoregula*, *Methanomethylovorans*, and *Methanosaeta* were the major microorganisms in the granular sludge. The activities of protease and coenzyme $F_{420}$ were high in the granular sludge. The intermittent stirring device and the reverse-flow system were further found to overcome the disadvantage of the floating and crusting of cyanobacteria inside the reactor. Meanwhile, the effect of mass transfer inside the reactor can be accelerated to help give the reactor a rapid start.


## 1. Introduction

Lake eutrophication and cyanobacterial bloom are some of the most critical environmental problems faced by the entire world. Even when exogenous input decreases, endogenous nutrients that accumulate during lake sedimentation over time can also support the phenomenon of cyanobacterial bloom in Lake Taihu (Eleuterio and Batista, 2010). In South China, timely refloatation and collection are effective methods to reduce fast cyanobacterial growth (Yan et al., 2010; Cai et al., 2012). The algae-laden water collected from Taihu Lake (the water content of the mixture of cyanobacteria and water is

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generally around 99%) was deposited in the depression of the lakeside without immediate and effective treatment (Yu et al., 2014). Simultaneously, algae from the contaminated water died and released a significant amount of algal toxin, nitrogen, and phosphorus after collection, and so must be treated promptly (Duy et al., 2000; Chen and Xie, 2005; Codd et al., 2005; Liu et al., 2011). Thus, appropriate storage and treatment of algae-laden water is an important measure for the treatment of secondary pollution caused by algal disruption.

Algae-laden water is a typical high-density organic wastewater after natural decomposition. Anaerobic biological treatment is the most effective method for the treatment of high-density organic wastewater, and the biomass energy can be obtained after the process of degradation (Yen and Brune, 2007). The traditional anaerobic reaction limits the application of practical engineering for wastewater treatment. Algae-laden water is composed of Cyanobacteria, which floats and crusts in the anaerobic reactor, and reduces the effectiveness of treatment in the reactor (Agusti and Phlips, 1992; Bonnet and Poulin, 2002). Algae-laden water treatment becomes limited by the traditional anaerobic reactor. Thus, a highly efficient reactor needs to be developed. The modified parameters could accelerate the start-up of the anaerobic reactor. The enhanced external circulating anaerobic reactor is based on the upflow anaerobic sludge blanket reactor, whereas the circulation can be formed outside the reactor using the technology of reverse flow of sludge water (Zhao et al., 2008). External circulation can increase the upflow rate in the reaction area, decrease the burden of solid–liquid separation of three-phase separator, enhance the contact between the sewage and the granular sludge, and improve the effectiveness of treatment in the reactor.

During the anaerobic process of the cyanobacteria, low-efficiency hydrolysis rate delays the time of digestion of cyanobacteria. The protease activity involved in anaerobic digestion and the response to anaerobic digestion have been well documented (Aoki, 1995). The purpose of these studies is to investigate the relationship between microbial community and nutrient removal efficiency. However, the relationship between protease activity and cyanobacteria treatment was reported rarely. Based on the enzyme activity of the granular sludge, the capacity of the granular sludge and methane production caused by cyanobacteria digestion could be explored further. The activities of protease and coenzyme $F_{420}$ could reflect the operation condition of the anaerobic granular sludge.

The main objective of the present study is to investigate the critical factors of granular sludge formation in the improved enhanced external circulating anaerobic reactor. Rapid start-up of anaerobic fermentation was discussed during the treatment of the algae-laden water in Lake Taihu, and the mechanisms of methane production were discussed. Enzyme activity throughout the start-up of enhanced external circulating anaerobic reactor was studied using protease and coenzyme $F_{420}$.

## 2. Materials and methods

### 2.1. Devices

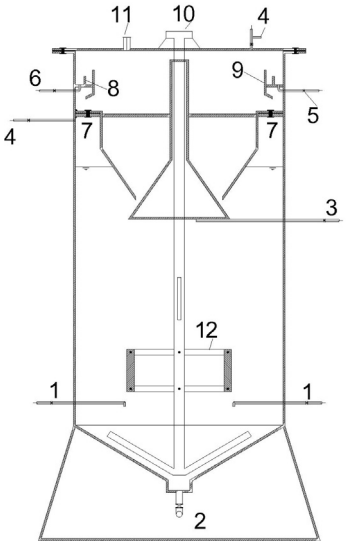
The experiment was conducted in the Taihu Research Base of Southeast University in Fudu Port of Zhoutie County of Yixing in Jiangsu Province. The reactor used in the experiment was made by UPVC with 0.4 m diameter and 3 m height, and the effective volume was 370 L. Automatic stirring device was provided, and the automatic heating device was maintained at a temperature range of 3035 °C. The structure diagram of the reactor is shown in Fig. 1.

### 2.2. Wastewater characteristic

The algae-laden water used in the experiment was collected from the piling pool for the cyanobacteria refloating station of Fudu Port of Zhoutie County of Yixing. The algae-laden water was decomposed naturally in the compiling pool for 5–7 d. The algae-laden water contained chemical oxygen demand (COD) of 30006000 mg/L, suspended solids (SS) of 365–1002 mg/L, total nitrogen (TN) of 55–125 mg/L, total phosphorus (TP) of 12–46 mg/L and had a pH of 5.8–7.0. The algae water displayed brownish yellow.

### 2.3. Experimental operational conditions and procedures

The enhanced external circulating anaerobic reactor was inoculated with activated sludge collected from the Yixing Qingyuan Sewage Plant in Jiangsu Province. In the beginning of the experiment, the inoculated sludge was accounted for 1/4 of the effective volume, and was mixed with algae-laden water which was accounted for 3/4 of the effective volume. The ratio of mixed liquor volatile suspended solid (MLVSS) to mixed liquor suspended solid (MLSS) was 0.75 in the inoculated sludge. The sludge concentration was 28.5 gMLVSS/L. The continuous input was conducted in the experiment with a flow rate of 37 L/d, and a reflux ratio of 200%. Intermittent agitation was used to enhance mixing of cyanobacteria and sludge. The duration of the stirring process was 10 min with a rate of 30 r/min every 6 h. Pig stool was added into the reactor in the first 2 days. Low-load start was adopted and three stages were carried out to start up the reactor. The stages were as follows: (1) loading of COD was conducted at 550 mg/L for the first 10 days; (2) loading of COD in the intermediate stage was




1. inlet pipe;
2. sludge pipe;
3. return pipe;
4. gas collection pipe;
5. outlet pipe;
6. sampling pipe;
7. gas chamber;
8. effluent weir;
9. mudguard;
10. churning motor;
11. preformed hole;
12. stirring paddle;

**Figure 1** Schematic diagram of the enhanced external circulating anaerobic reactor.

800–2000 mg/L from the 10th to the 30th day; and (3) loading of COD was 2000–3000 mg/L after the 10th day.

### 2.4. Analytical methods

Analysis of COD, TN, TP, MLVSS, SS, and pH was conducted in accordance with standard methods (China, 2004). The amount of collected gas was determined by the wet corrosion gas meter (Changchun Automobile Filter Co., Ltd.), whereas the content of methane in the sewage gas was determined using the gas chromatographic method (Zhu et al., 2008). Sludge samples were taken from the reactor after the operation of 35 d and the image of sludge was examined by scanning electron microscopy (SEM) with model JSM 5600LV, Shimazu, JAP. At first, sludge samples were prepared at 25 °C for 4 h with 2.5% (w/v) glutaraldehyde in Sorenson phosphate buffer, and then dehydrated through a graded series of water–ethanol mixtures (10%, 25%, 50%, 75%, 90%, and 100%), finally, brought to equilibrium in each mixture for 10 min and dried by the frozen drying method before sputter coating with gold particles (Rong et al., 2011). The protease was analyzed using the McDonald Chen spectrophotometry, and the coenzyme $F_{420}$ was analyzed using the spectrophotometry (Whitmore et al., 1986; Whiteley et al., 2002). The concentrations of total microcystins-LR (TMC-LR) and extracellular microcystins-LR (EMC-LR) were monitored by acetone extraction spectrophotometer (Ji et al., 2009).

The predominant methanogens in the sludge samples were determined using denaturing gradient gel electrophoresis (DGGE) (Arooj et al., 2007). Total DNA from mixed culture was extracted using the phenol–chloroform method and estimated by 1% agarose gel electrophoresis (Venkata Mohan et al., 2010). Total extracted genomic DNA was used as a template to amplify the V4-5 variable region on 16S rRNA by PCR (Yu, 2014). The nucleotide sequences of the universal primers were as follows: primer F787, 5′- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CGATTAGATACCCSBGTAGTCC -3′; R1059, 5′-GCCATGCACCWCCTCT -3′. PCR amplification was performed in an automated thermal cycler (Bio-rad, USA). Initial denaturation was performed at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, and annealing at 56 °C for 1 min. Each annealing step was followed by an extension at 72 °C for 1 min, except for the final extension step, which lasted for 7 min. PCR products were analyzed using electrophoresis on a 1.5% (w/v) agarose gel for 30 min at 120 V with ethidium bromide in 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0). Images of the gel were recorded to determine the size, purity, and concentration of the DNA using the gene genius bioimaging system.

DGGE analysis was performed using the DCode Universal Mutation Detection system (Bio-Rad, USA). PCR samples were directly analyzed on 8.0% (w/v) polyacrylamide gels in 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) with a denaturing gradient that ranged from 30% to 60% (100% denaturant is 7 M urea and 40% v/v formamide). The gradient gel was cast with a gradient delivery system. Electrophoresis was performed at a constant voltage of 180 V at 60 °C. After 4 h of electrophoresis, the gel was stained with ethidium bromide for 30 min before being visualized on a UVI transilluminator (U-3010 Hitachi).

Bands in the gel were excised and rinsed in 1 mL deionized water. The gel was crushed in 20 μL sterile deionized water and incubated at 30 °C for 1 h. The DNA extract was used as template in PCR amplification as described above. The PCR products were sequenced on an ABI LR-377 DNA sequence. The sequences of successfully amplified DNA fragments were then compared with GenBank database using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## 3. Results and discussion

### 3.1. COD removal of the reactor

The concentrations of COD at influent and effluent in the reactor and the COD removal efficiency in the start-up are shown in Fig. 2. The removal rate of COD was increased with the extension of time during the period of the reactor. In the initial stage (1–10 d), the removal rate of COD was relatively slow at a level of around 40%. Meanwhile, fluctuation was relatively large, mainly because the inoculated sludge needed a period of adaptation in the new environment. During the intermediate stage (10–30 d), the concentration of the influent COD was increased continuously, and the organic loading increased to 0.08–0.2 kgCOD/(d $m^3$). Granular sludge was formed gradually, and the removal rate of COD increased from 72% to 85%. In the stable stage (30–50 d), organic loading was increased to 2000–3000 mg/L. The concentration of effluent COD was decreased to 500 mg/L, and the corresponding removal rate was maintained at 85%.

The suitable stirring could overcome the drawbacks of cyanobacteria floating and crusting in the reactor. At the same time, the reflux system could accelerate the mass transfer to promote the formation of granular sludge in the reactor so as to contribute the start-up of the reactor faster. These beneficial effects were the main reasons why the improved reactor was able to treat effectively the algae-laden water.

### 3.2. Gas production

The gas production rate under organic loading rate of reactor in start-up is shown in Fig.3. Gas production was stabilized with the increase of organic loading rate in the start-up of the reactor. Gas production rate of the reactor was within the range of 280–410 mL/gCOD. At the start of the reaction (1–10 d), the fluctuation rate of the gas production was high. During the stable stage, the gas production rate was maintained at around 380 mL/gCOD. Cyanobacteria were treated effectively in the reactor, and biofuel was produced stably.

Gas chromatography detection of methane content showed that the content of methane in the sewage gas was around 67–75% from 30 to 50 d. Hence, the content of methane was relatively higher, and the reactor could realize the utilization of the cyanobacterium source.

### 3.3. Algal toxin removal in the start stage of the reactor

The genus of microcystis, anabaena, Oscillatoria, and nostoc belonging to the cyanobacteria can produce the secondary metabolite of microcystins. However, the microcystic toxins are highly toxic compounds with stable structure. Thus, it

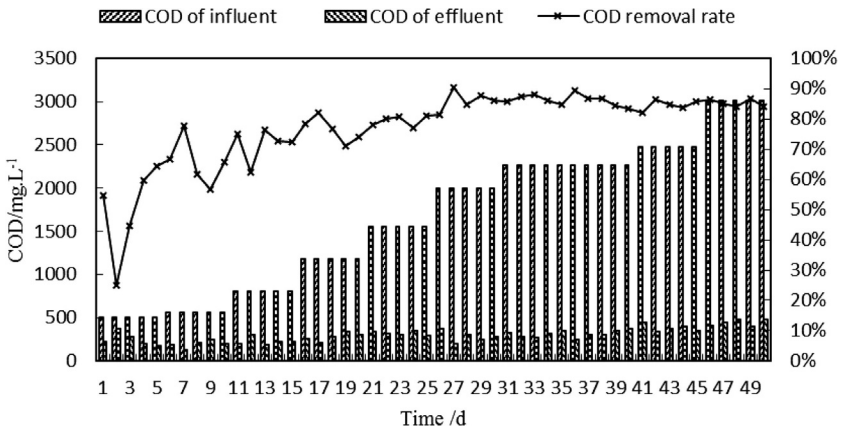



**Figure 2** COD concentration and removal of the reactor in start-up.

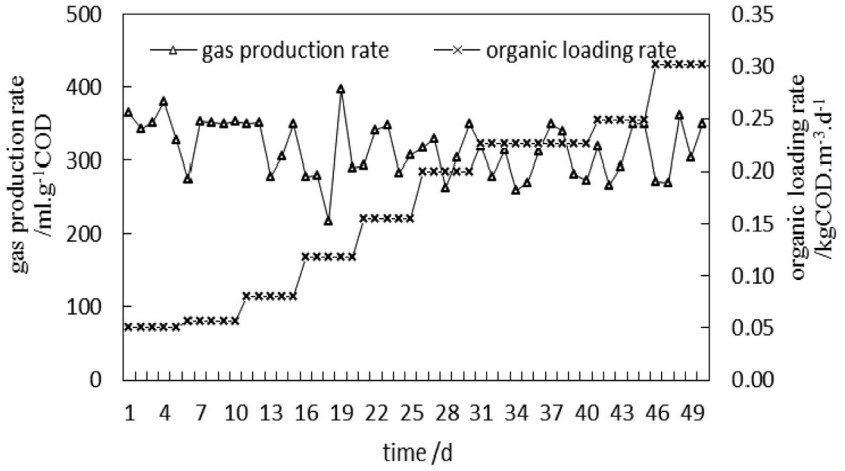



**Figure 3** Gas production rate under organic loading rate of reactor in start-up.

becomes the potential harmful substance in the water environment. The degradation of TMC-LR and EMC-LR in the reactor after 35 d was measured. The concentration of TMC-LR and EMC-LR decreased from 453 and 263 μg/L to 32 and 20 μg/L respectively. Corresponding removal rates of TMC-LR and EMC-LR were 92.9% and 92.3% respectively. The results of this experiment showed that the reactor removed both TMC-LR and EMC-LR well. Granular sludge could adsorb and retain more algae in the algae-laden water. In the other words, granular sludge increased the hydraulic retention time of the algae, so more algae can be removed in the reactor. A large number of granular sludge of the reactor could intercept and absorb the algal toxins effectively after the successful start.

### *3.4. Biological features of the granular sludge*

The efficiency of the reactor mainly relied on the quantity and activity of the microorganism in the reactor. Biological features of the granular sludge could reflect gas producing capacity directly. Photograph of the SEM was used to detect biological features of the granular sludge. As shown in Fig. 4, a large amount of granular sludge existed in the system after the successful start-up of the reactor. SEM photograph demonstrated that a large amount of dense zoogloea was found in the granular sludge of the reactor. The biofacies was complicated and diversified, and methanococcus constituted the main framework of the zoogloea. The biofacies included the bacillus and filamentous bacteria. The reactor used in the experiment was single-phase equipment. The population of the microorganism was rich. Bacillus, coccus, syntrophic acetogen, and methane bacteria coexisted after hydrolyzed fermentation (Xie et al., 2011). A number of cavities was found on the surface of the granular sludge that provided the channel of the overflow of the sewage gas (Xu et al., 2003).

On the 35th day, the total DNA of the granular sludge in the reactor was extracted and purified. It was amplified using PCR. Subsequently, DGGE analysis was conducted to obtain the fingerprint of the archaebacteria in the granular sludge. As shown in Fig. 5, seven main stripes in the DGGE fingerprint were cut, recovered, and sequenced. The sequence was included in the Genbank database for comparison, and the results of sequencing are shown in Table 1. As shown in Table 1, the dominant methanogenic archaea was extremely significant in the granular sludge of the reactor. All of the sequences from the archaeal libraries matched with methanogens of the phylum Euryarchaeota and could be classified into

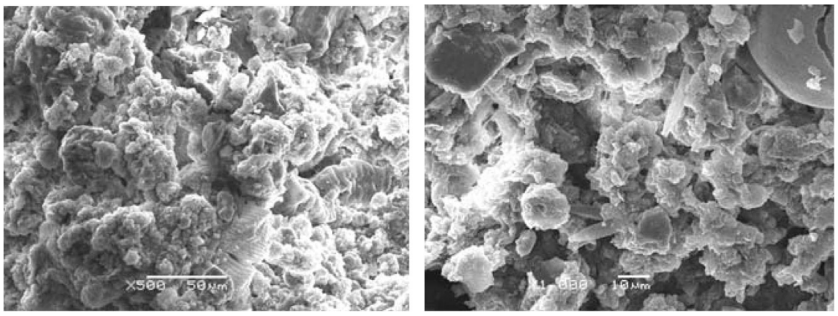


**Figure 4** Scanning electron micrographs of sludge surface.

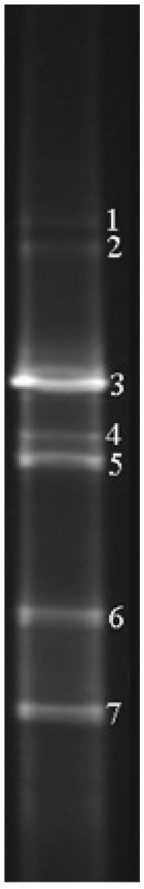



**Figure 5** DGGE fingerprint of the granular sludge on days 35.

**Table 1** Identity of genomic sequences in dominant DGGE bands by sequencing and BLAST analysis.

| Band | Nearest species and taxon | Phylogenetic affiliation to family or genus level | Similarity (%) | Accession No. |
|---|---|---|---|---|
| Ad1 | Uncultured archaeon | *Methanosphaera* | 90.9 | AJ576239 |
| Ad2 | Methanolinea tarda | *Methanolinea* | 96.4 | AB162774 |
| | Uncultured Methanolinea sp. | *Methanolinea* | 97.3 | AB434764 |
| Ad3 | Uncultured Thermoplasmatales archaeon | *Thermogymnomonas* | 90.6 | EU731598 |
| Ad4 | Uncultured Methanoregula sp. | *Methanoregula* | 96.2 | JF789587 |
| Ad5 | Uncultured euryarchaeota | *Methanomethylovorans* | 95.1 | DQ676261 |
| Ad6 | Methanosaeta concilii | *Methanosaeta* | 94.7 | X16932 |
| | Uncultured archaeon | *Methanosaeta* | 94.7 | AY570656 |
| Ad7 | Uncultured euryarchaeota | *Methanosaeta* | 95.8 | AB175352 |
| | Uncultured archaeon | *Methanosaeta* | 97 | AJ576230 |

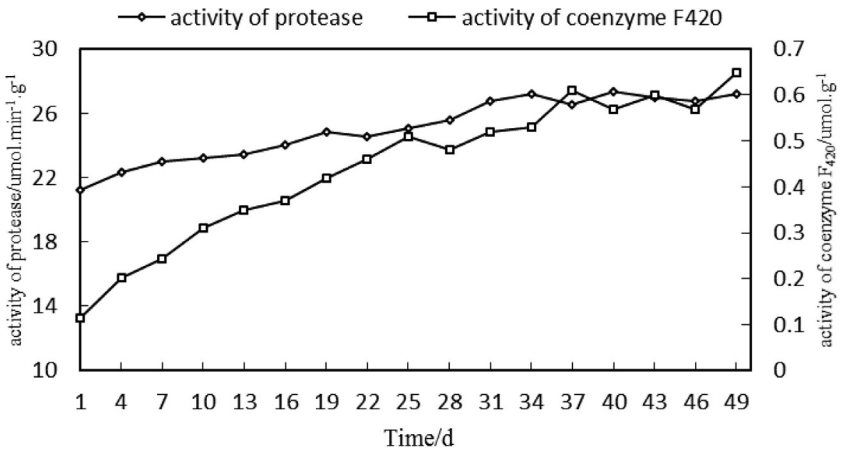



**Figure 6** Variation of activity protease and enzyme $F_{420}$.

six genera: *Methanosphaera*, *Methanolinea*, *Thermogymnomonas*, *Methanoregula*, *Methanomethylovorans*, and *Methanosaeta*. Ad2 was closely related to *Methanolinea* species with 97.3% similarities, and Ad7 was closely related to *Methanosaeta* species with 97% similarities. It was indicated that the genus might be the same (Wang et al., 2009). The *Methanosaeta concilii* in the archae bacteria from the granular sludge was the dominant flora (Xie et al., 2011). However, similarities Ad1 and Ad3 in the Genbank database were below 91%, and they might be the unknown archae bacteria, such as *Methanosphaera* and *Thermogymnomonas*.

### 3.5. Analysis of enzyme activity

In the anaerobic reactor, cyanobacteria digestion and methane production relied on different enzymes produced by the anaerobic microorganism of the reactor. The activity of protease was measured to represent the activity of the enzyme during the process of hydrolysis (Ma et al., 2011). The content of coenzyme $F_{420}$ was used to represent the activity of methane production (Chen et al., 2007).

The regularity of the enzyme activity in the granular sludge during the start-up process is shown in Fig. 6. The concentrations of protease activity and enzyme $F_{420}$ were increased with the extension of time. In the stable stage, the concentration of protease increased to 26.2–27.3 μmol/(min g). Protease generated by the microorganism could hydrolyze the cell wall of cyanobacteria effectively. Hydrolytic bacteria produced a significant number of enzymes for the hydrolysis of cyanobacteria, and their hydrolysis ability was increased with the increase of protease concentration.

Coenzyme $F_{420}$ was unique for methane producing bacteria, which was used as the carrier for the low potential electron transfer. Thus, coenzyme $F_{420}$ played an important role in the formation of methane (Ranalli et al., 1996). The change of coenzyme $F_{420}$ concentration is shown in Fig. 6. The concentration of coenzyme $F_{420}$ in the granular sludge was increased with extension of time. During the start-up stage, the coenzyme $F_{420}$ concentration was similar to the change in concentration of protease activity, and the concentration of coenzyme $F_{420}$ increased from 0.1 μmol/g to 0.52–0.65 μmol/g. The content of coenzyme $F_{420}$ in the granular sludge was related to that of the methane producing bacteria. Methane producing bacteria generated coenzyme $F_{420}$ under suitable substrate and environment. Thus, coenzyme $F_{420}$ was increased with the operation of the reactor system during the anaerobic fermentation process.

## 4. Conclusions

The improvement of the reactor and the supplement of the stirring device promoted the decrease of floating and crusting caused by cyanobacteria in the anaerobic reactor. The algae-laden water could be successfully treated around 35 d by supplementing inoculating sludge running and improving the load by steps in the improved reactor. The granular sludge was formed after around 35 d of operation in the reactor.

The supplementation of the intermittent stirring system in the reactor can effectively overcome crusting caused by the floating of cyanobacteria and increasing the gas production. Meanwhile, the stirring system can facilitate the contact between the microorganism and the cyanobacteria to enhance the effect of mass transfer and shorten the start time of reactor. After the successful start, the structure of the microorganism in the granular sludge of the reactor became complicated, and was major in coccus and filamentous bacteria. *Methanosphaera*, *Methanolinea*, *Thermogymnomonas*, *Methanoregula*, *Methanomethylovorans*, and *Methanosaeta* were the dominant archaeal species in the reactor. The activity of the protease and coenzyme $F_{420}$ in the granular sludge was strong.

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