# Effect of Methylene Blue on the Properties and Microbial Community of Anaerobic Granular Sludge

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Basic dyes, which are widely used in industry, can cause serious damage to the environment if they are discharged to waterways. If directly discharged into water, they can cause serious damage to the environment due to their deep color and low degradation rate. The results showed that the average chemical oxygen demand removal rate from the wastewater was greater than 60%, while the methylene blue removal rate was greater than 90%. Anaerobic granular sludge can remove methylene blue and organic matter simultaneously. *Candidatus Cloacimonetes* was not detected when the methylene blue concentration was 0 mg/L, 2 mg/L, or 4 mg/L. When the methylene blue concentration was 6 mg/L, 8 mg/L, and 10 mg/L, more *Candidatus Cloacimonetes* was detected in the sludge as the concentration of methylene blue increased. The *Candidatus Cloacimonetes* content was found to be 1.02%, 1.08%, and 2.11% in these samples, respectively.

*Keywords: Methylene blue; Wastewater treatment; Anaerobic granular sludge*

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## INTRODUCTION

Dye wastewater is one of the main types of industrial wastewater. Due to its deep color, non-degradability, and complex composition, dye wastewater can cause serious pollution if discharged directly into a body of water. In addition, some dye precursors and dye degradation products are carcinogens and mutagens (Manu and Chaudhari 2002). Methylene blue is a representative water-soluble azo dye that forms quaternary ammonium cations in aqueous solutions, has a high chroma, and cause serious environmental pollution (Yao *et al.* 2010). Azo and reactive dyes are electron-deficient in nature, and this property makes them less susceptible to oxidative catabolism (Knackmuss 1996). Thus, dye wastewater is difficult to degrade. Currently, the main methods for treating dye wastewater include adsorption, membrane separation, electrochemical reaction, advanced oxidation, anaerobic biodegradation, and aerobic biodegradation. Adsorption is widely used due to its low energy consumption, inability to introduce new pollutants and ability to separate and concentrate organic pollutants in wastewater (Liu *et al*. 2005; Hameed and El-Khaiary 2008; Sun *et al*. 2010). However, the adsorption materials currently used also have the disadvantages of poor processing efficiency, cumbersome preparation, and difficulty of separation from wastewater (Lv 2015). Therefore, discovery of highly efficient dyestuff adsorption has become a key to solving the dye wastewater treatment problem.

Anaerobic granular sludge contains many pores and cavities on its surface, giving it biosorption properties. Not only does it have the advantage of being a common

biosorbent, but it also exhibits good sedimentation performance (He 1998), meaning anaerobic granular sludge plays an important role in the wastewater treatment process. In this study, anaerobic granular sludge was used as a biosorbent to remove methylene blue from wastewater. Firstly, the effect of methylene blue on the removal of organic pollutants with anaerobic granular sludge was studied. Secondly, a feasibility analysis of the removal of methylene blue from wastewater by anaerobic granular sludge was performed. The effects of methylene blue on the physicochemical properties of anaerobic granular sludge and the interaction between methylene blue and the microbial community in anaerobic granular sludge were studied in depth.

## EXPERIMENTAL

### Materials

Anaerobic granular sludge was taken from an anaerobic fermentation tank located at a paper mill in Guangxi, China, and its particle size was determined to be approximately 1 mm using an ultra-fast, intelligent, particle size distribution meter (Mastersizer 3000, Malvern, Worcestershire, UK). This experiment used artificially-simulated wastewater with a chemical oxygen demand (COD) of approximately 500 mg/L. Its specific composition was as follows: glucose (472 mg/L), $NH_4Cl$ (47.8 mg/L), $KH_2PO_4$ (10.96 mg/L), $FeSO_4$ (39 mg/L), $CaCl_2$ (30 mg/L), $MgSO_4 \cdot 7H_2O$ (49 mg/L), and $NaHCO_3$ (300 mg/L). The pH value was approximately 7.

### Methods

Batch anaerobic digestion was used in this study. The anaerobic reaction was performed in 250 mL brine bottles. Each group contained 25 g of granular sludge. Methylene blue dye was added until the concentration reached 0 mg/L, 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L, and 10 mg/L, labelled A, B, C, D, E, and F, respectively. Two parallel experiments were performed for each group. A reaction device was placed in a thermostatic water bath at 38 °C ± 1 °C. Each anaerobic bottle was filled with a stream of nitrogen for 8 min to remove the oxygen and ensure an anaerobic environment within the bottle. Each day of the study, changes in the COD and methylene blue concentration in the incoming and outgoing flows were analyzed. Following the experiment, the physical properties of the anaerobic granular sludge were analyzed using a Mastersizer 3000. In addition, the apparent structure and microbial community of the granular sludge were analyzed with scanning electron microscopy (SEM).

*COD and methylene blue analysis*

The COD was determined using potassium dichromate titration (National Environmental Protection Agency 1989), and the concentration of methylene blue was measured with a spectrophotometer (Agilent 8453, Agilent Technologies, California, USA) with the wavelength set at 665 nm.

*Particle size distribution analysis*

The particle size distribution of the sludge was measured using a Malvern laser particle size distribution analyzer (Mastersizer 3000) with a particle refractive index of 2.42. Deionized water was used as a dispersant and its refractive index was 1.330.

Activated sludge was slowly poured into a beaker containing 800 mL of deionized water. Measurement began when the background value reached approximately 10%.

*SEM analysis*

SEM analysis was performed using the method described below (Tay *et al*. 2001; Lenz *et al*. 2008). Two grams of sludge were placed into a weighing bottle and rinsed three times with phosphate buffer solution with a pH of 7.4. The washed granular sludge was submerged in 2.5% glutaraldehyde for 4 h, and then a phosphate buffer was used to wash the granular sludge three times at 10 min intervals. For dehydration, 50%, 70%, 80%, and 90% ethanol were used sequentially, with each series interval lasting approximately 30 min. The sample was then lyophilized. The surface of the sample was sprayed with a 1500-nm thick gold film and the gold-sprayed sample was analyzed by SEM (Hitachi SU8220, Hitachi, Tokyo, Japan).

*High-throughput sequencing*

DNA from the anaerobic granular sludge was sequenced. Total DNA was extracted using an EZNA® soil kit (Omega Bio-tek, Norcross, GA, US). The DNA concentration and purity were determined using a NanoDrop 2000. Agarose gel electrophoresis on a 1% gel was used to determine the quality of the extracted DNA. The V3-V4 variable region was amplified by polymerase chain reaction (PCR) using the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR product was recovered using a 2% agarose gel, purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), eluted with Tris-HCl, and then visualized with 2% agarose electrophoresis. Quantitative detection was performed using a QuantiFluorTM-ST (Promega, Madison, WI, USA). The purified amplified fragments were constructed into a library of PE2*300 according to the standard protocol for the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

## RESULTS AND DISCUSSION

### Anaerobic Granular Sludge Removal of COD from the Wastewater

The continuous reaction displayed in Fig. 1 shows that the removal efficiency of COD in each reactor increased rapidly in the beginning and then stabilized. During the initial phase of the reaction, the COD removal efficiency from the wastewater was approximately 60%, which indicated that the decontamination efficiency of the granular sludge was not remarkable. This was because the methylene blue that was added to the wastewater is toxic to the granular sludge and the microbial activity of the granular sludge was reduced, which affected the decontamination performance of the sludge. The granular sludge at this stage had not yet adapted to the wastewater environment. Granular sludge has a low ability to remove organics from wastewater, therefore the COD removal efficiency lower. In the middle of the reaction, it can be seen that the COD removal efficiency shows a rapid increase. With the adaptation of the sludge to methylene blue and enrichment of the dominant bacteria, the sludge performance gradually improved, and the sludge's decontamination ability also improved. That is, the COD removal efficiency increased. In the later stage of the experiment, it can be seen that with the continuous reaction, the COD removal efficiency tends to be stable and then has a

downward trend. This is because the performance of the granular sludge is gradually weakened as the time increases for the granular sludge to treat the wastewater. Figure 1 also shows that as the reaction progressed, the COD removal efficiency in the reactor with a high methylene blue concentration was also high, due to the redox mediator properties of methylene blue, which caused the biodegradation efficiency to increase (Ong *et al*. 2005).

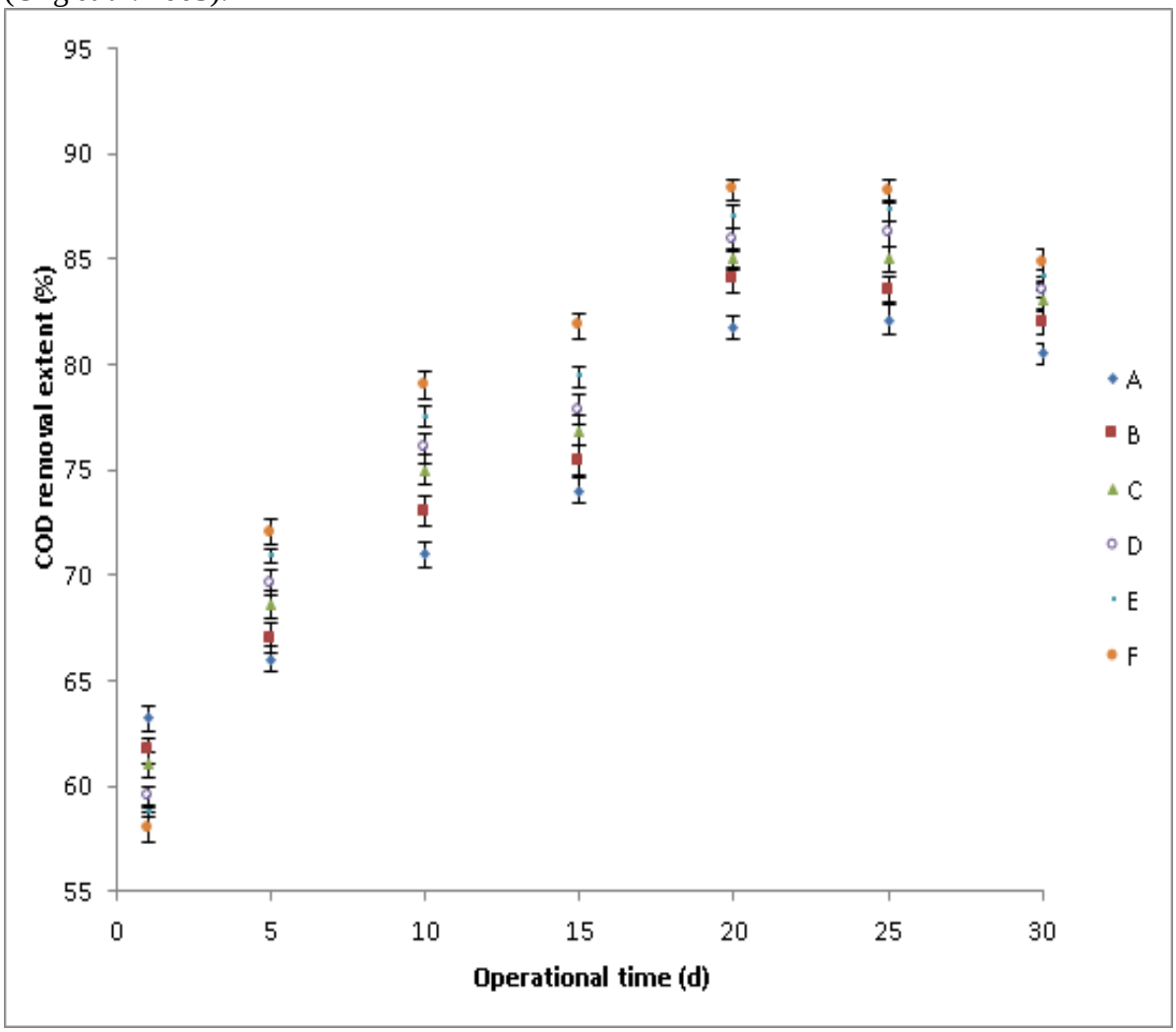



**Fig. 1.** COD removal extent (%) for methylene blue concentrations of 0 mg/L (A), 2 mg/L (B), 4 mg/L (C), 6 mg/L (D), 8 mg/L (E), and 10 mg/L (F)

## Anaerobic Granular Sludge Degradation of the Methylene Blue in Wastewater

To more intuitively describe the removal rate of the methylene blue color from the wastewater, the average color removal rate was calculated over 30 d for each sample. Figure 2 indicates that the methylene blue removal extent was approximately 90%, meaning that the anaerobic granular sludge was able to substantially decolorize methylene blue in the wastewater. Decolorization in an anaerobic sludge environment is the result of a combination of biological and abiotic reactions. The microorganisms in the anaerobic granular sludge release electrons due to oxidative decomposition of the substrate glucose and the released electrons are absorbed by methylene blue, which is then reduced. Methylene blue is colorless in the reduced state (Ong *et al*. 2005). Because

the reaction uses glucose (electron-donating compound) as a substrate, the electrons generated by its oxidation are used to form reducing cofactors (FAD, FMN, and NADH), which can stimulate the reduction of azo dyes and result in a higher decolorization efficiency (Wuhrmann *et al*. 1980; Carliell *et al*. 1995; Brás *et al*. 2001). Azo dyes can be directly reduced by reaction with a large number of biologically-derived reducing agents and biological reactions, and indirectly by enzyme-catalyzed reactions and reductase cofactors (van der Zee *et al*. 2003).

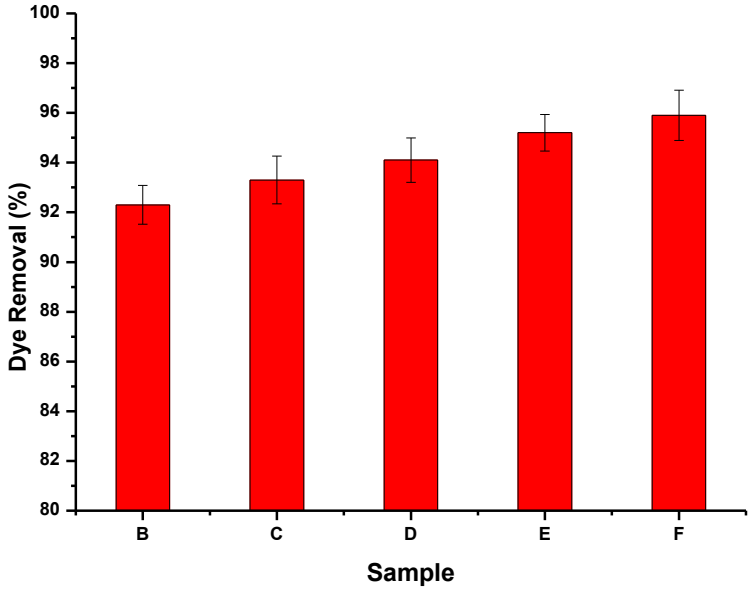



**Fig. 2.** Color removal extent for methylene blue concentrations of 2 mg/L (B), 4 mg/L (C), 6 mg/L (D), 8 mg/L (E), and 10 mg/L (F)

## Particle Size Distribution in the Granular Sludge

Figure 3 shows that the particle size of the granular sludge was mainly distributed between 500 μm and 1300 μm. The average particle size was approximately 1000 μm, and the sample that had not been treated with methylene blue-containing wastewater had the highest percentage of particles of this range of size. The proportion of small particles in the granular sludge was high when the granular sludge did not contain methylene blue wastewater. Granular sludge from samples containing methylene blue wastewater generally had a higher percentage of particles within the 1300 to 1500 μm size range than the sample without methylene blue. The proportion of large-sized particles in the granular sludge was high in samples containing methylene blue, suggesting that methylene blue promoted sludge granulation. This was due to the adaptation of sludge to methylene blue. Also, enrichment of the dominant bacteria caused sludge performance to gradually improve. Macroscopically, the ability of the granular sludge to treat wastewater increased and the microscopic manifestations were an increase in the particle size of the granular sludge.

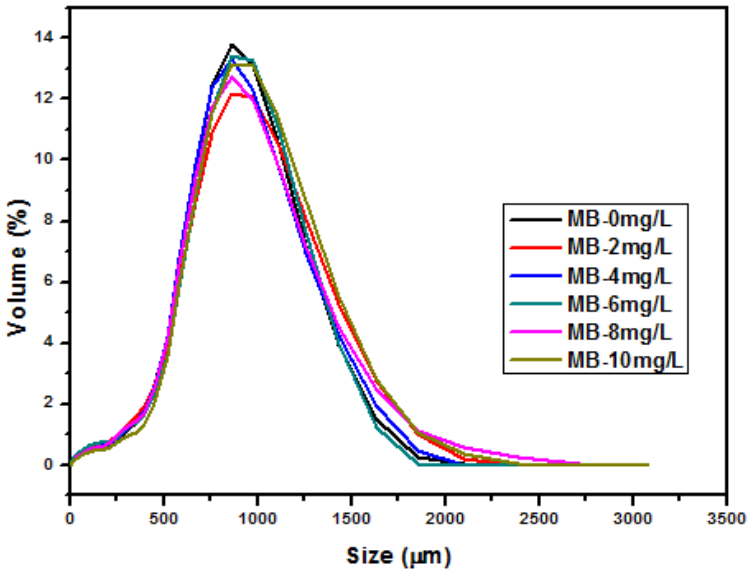
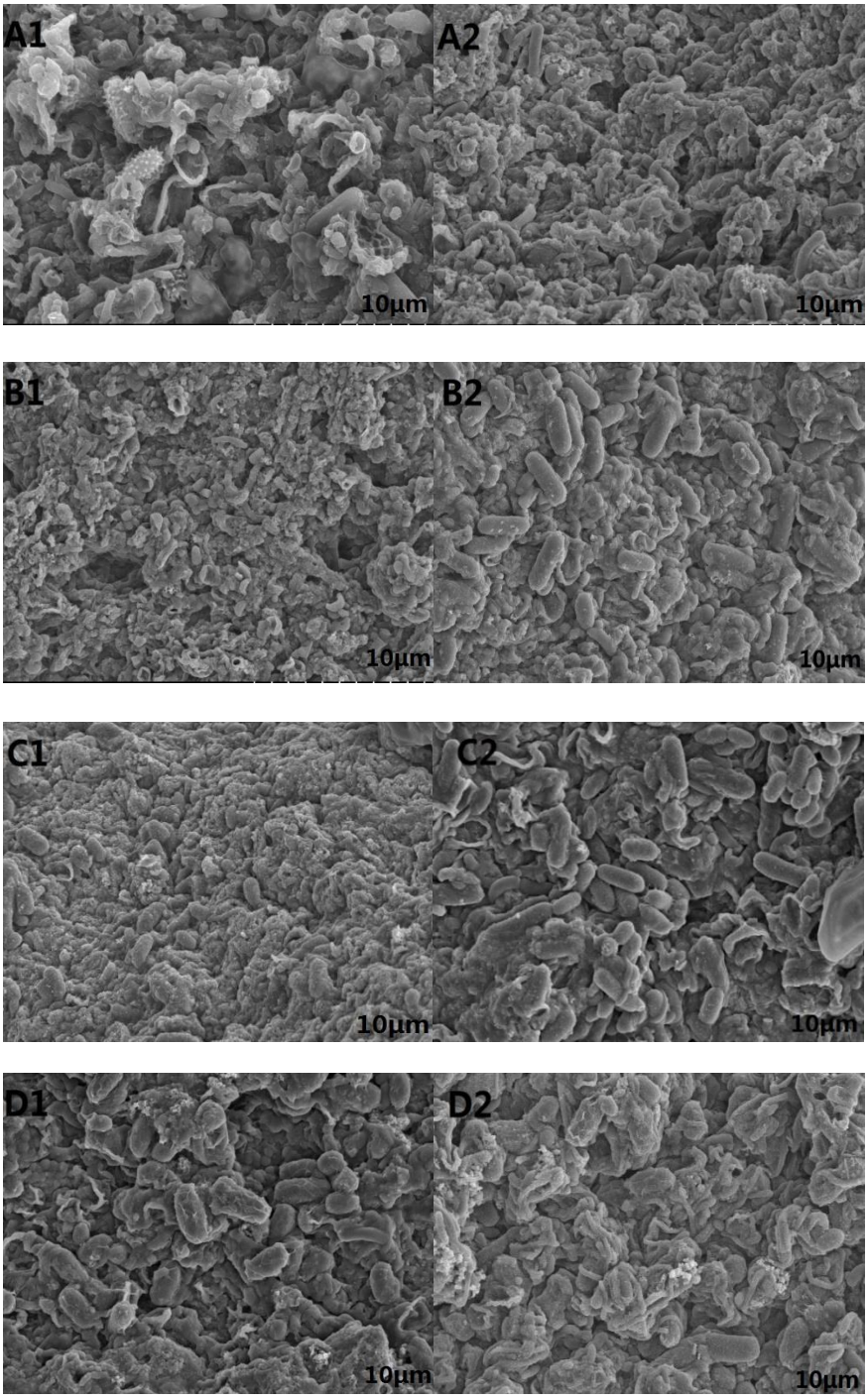



**Fig. 3.** Particle size distribution analysis of the granular sludge

## SEM Analysis of the Anaerobic Granular Sludge

Figures 4B1, 4C1, 4D1, 4E1, and 4F1 show that there were fewer microbial cells on the surface of the granular sludge that had been incubated for 5 d. There were few microorganisms in the granular sludge that had been used to directly treat the methylene blue wastewater by absorbing methylene blue. When methylene blue was added to the wastewater, it was responsible for anaerobic particulate pollution. Mud microbes cause a certain toxicity, and strains that could not adapt to this toxicity were removed with the water. In other words, there were fewer microorganisms to detect in the SEM image and the microorganisms that are resistant to methylene blue were also poorly active at this time, resulting in a low sludge removal efficiency. From B2, C2, D2, E2, and F2, it can be seen that the adaptation of sludge to methylene blue and the enrichment of dominant bacteria caused sludge performance to gradually improve, and the sludge decontamination ability was also improved. Figure 5 shows a comparison of COD removal extent on days 5 and 30. It can be seen that the COD removal extent on day 30 was greater than on day 5, indicating that as the reaction proceeds, the activity of the microorganisms gradually increases. This also means that the COD removal efficiency increased. Figure 5 shows that there were many small pores on the surface of the granular sludge. These pores ensure the transfer of nutrients inside the sludge, which is also beneficial for discharging gases, such as $CH_4$ and CO, from the interior of the sludge.


A1
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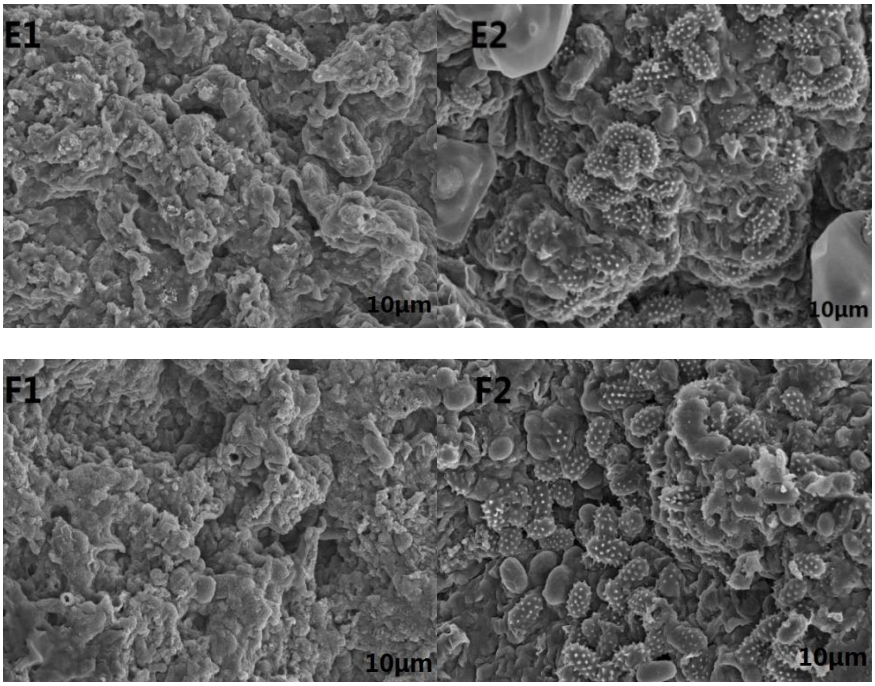


**Fig. 4.** A1, B1, C1, D1, E1, and F1 are the SEM images for samples A, B, C, D, E, and F, respectively, when the reaction was performed for 5 d; A2, B2, C2, D2, E2, and F2 show the SEM images for samples A, B, C, D, E, and F, respectively, at the end of the reaction.

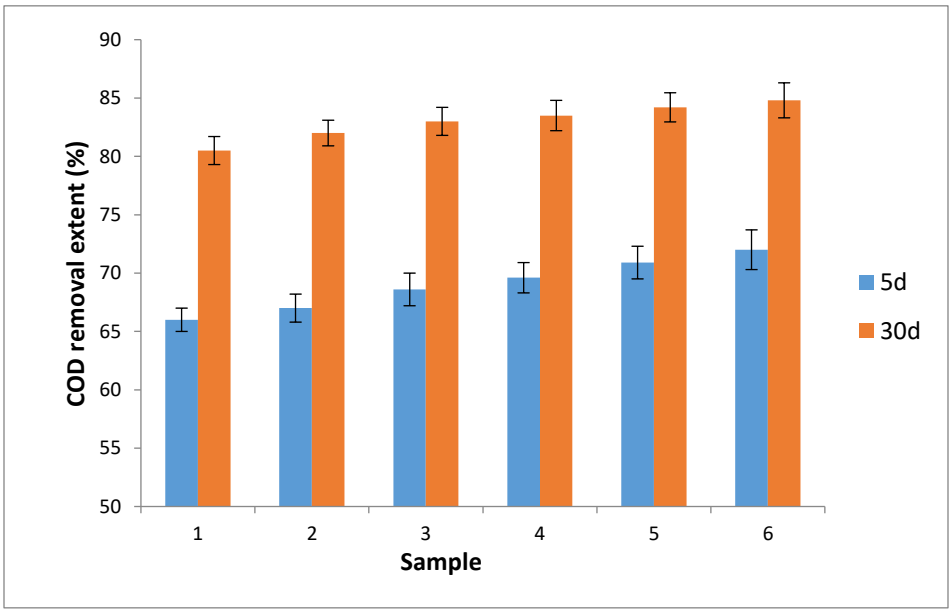


**Fig. 5.** COD removal rates for samples A, B, C, D, E, and F on day 5 and day 30 of the reaction

## Sludge Microbial Diversity Analysis

At the 97% classification level, alpha diversity is used to analyze the species diversity in a single sample (Beazley *et al*. 2012), mainly using the Chao, Ace, Shannon, Simpson, and Coverage indices. The Chao and Ace indices are used to measure community abundance. The greater the index, the higher the degree of community richness. Table 1 shows that the Chao and Ace indices for the six groups were in the following order: F > B > D > A > E > C. The Simpson index indicates the species

diversity of a community and a smaller Simpson index indicates a higher species diversity. The size order of the Simpson index for the six groups was as follows: B > E > D > F > C > A. The coverage index represents the coverage of a single sample library and when the coverage index is high, then the probability that the sequences in the sample are reliable is also high. Table 1 shows that the coverage values were all above 0.99, indicating that the data was highly reliable. The Chao, Ace, and Simpson indices indicated that the microbial diversity and richness of the six sludge groups were high.

**Table 1.** Alpha-diversity Indices of the Bacterial Communities

| Sample | Chao | Ace | Shannon | Simpson | Coverage |
|---|---|---|---|---|---|
| **A** | 395 | 398 | 4.23 | 0.0314 | 0.9992 |
| **B** | 403 | 399 | 4.14 | 0.0416 | 0.9988 |
| **C** | 393 | 393 | 4.18 | 0.0330 | 0.9987 |
| **D** | 396 | 398 | 4.13 | 0.0397 | 0.9985 |
| **E** | 392 | 399 | 4.16 | 0.0407 | 0.9988 |
| **F** | 423 | 417 | 4.15 | 0.0360 | 0.9986 |

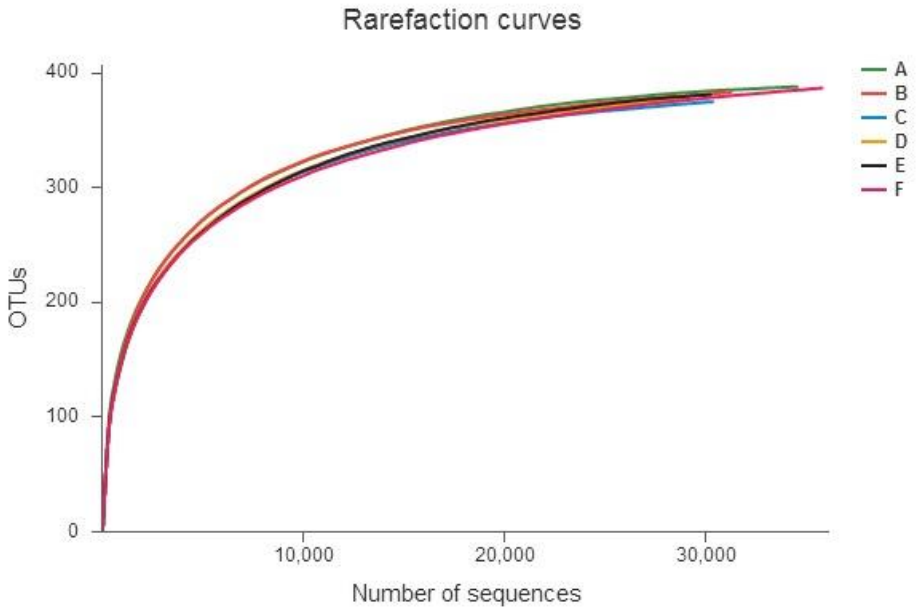


**Fig. 6.** Rarefaction curves of the bacterial communities

Figure 6 shows the rarefaction curve for each sample. Each curve tended to be flat and saturated, indicating that the sequencing depth covered most of the bacterial species within in the sample. It was determined that the microorganisms in the six sludge sample groups could be detected completely by high-throughput sequencing.

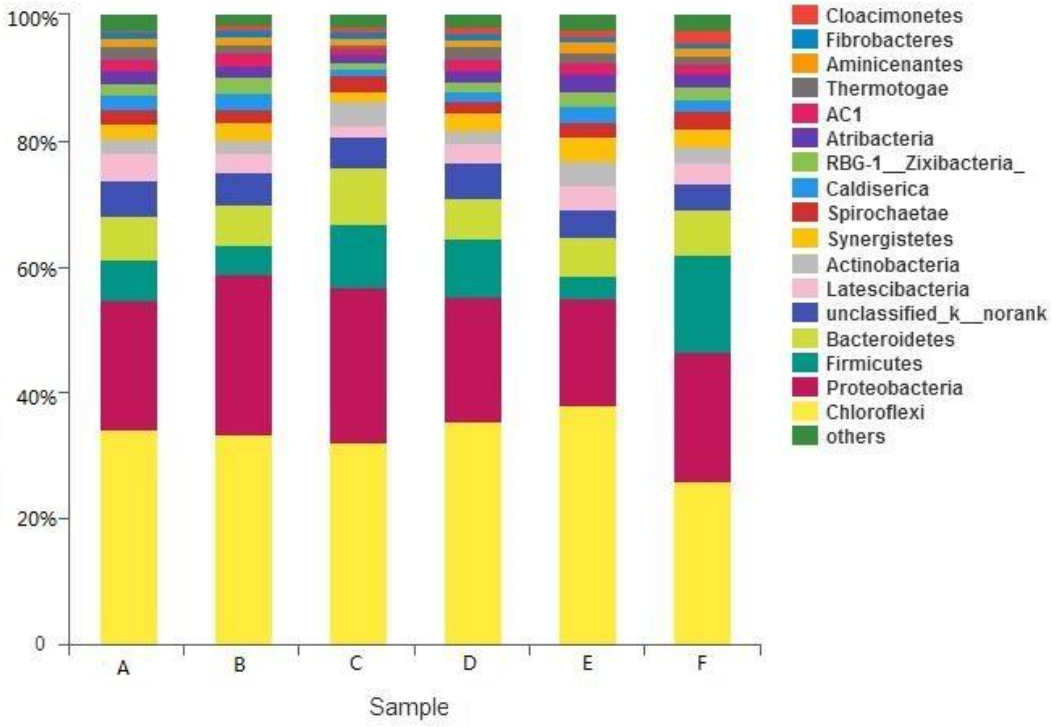


**Fig. 7.** Bacterial composition of the sludge samples with respect to the phylum levels

The bacterial community compositions at the phylum level of the sludge samples are shown in Fig. 7. A total of 18 bacterial phyla in the six sludge sample groups were identified, which indicated that the bacterial community structures had a high diversity at the phylum level. At the phylum level, the main bacterial groups were Chloroflexi, Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. When the methylene blue concentration was lower, *Candidatus Cloacimonetes* was not detected in the A, B, and C reactors. As the methylene blue concentration increased above 4 mg/L, more *Candidatus Cloacimonetes* bacteria were detected in the sludge. *Candidatus Cloacimonetes* accounted for 1.02%, 1.08%, and 2.11% of the bacteria in the D, E, and F samples, respectively. Chloroflexi bacteria are facultative bacteria. In anaerobic sludge reactors, Chloroflexi are the dominant bacteria (Rivière *et al*. 2009). Studies have shown that Chloroflexi play a supporting role in anaerobic ammoniacal sludge. Additionally, Chloroflexi play an important role in the granulation process of granular sludge (Björnsson *et al*. 2002; Yao *et al*. 2017). The main role of Proteobacteria is to degrade the organic matter in wastewater, and at the same time they play a role in nitrogen and phosphorus removal during the treatment of anaerobic wastewater (Miura *et al*. 2007). Synergistetes bacteria are capable of amino acid fermentation (Chojnacka *et al*. 2015).

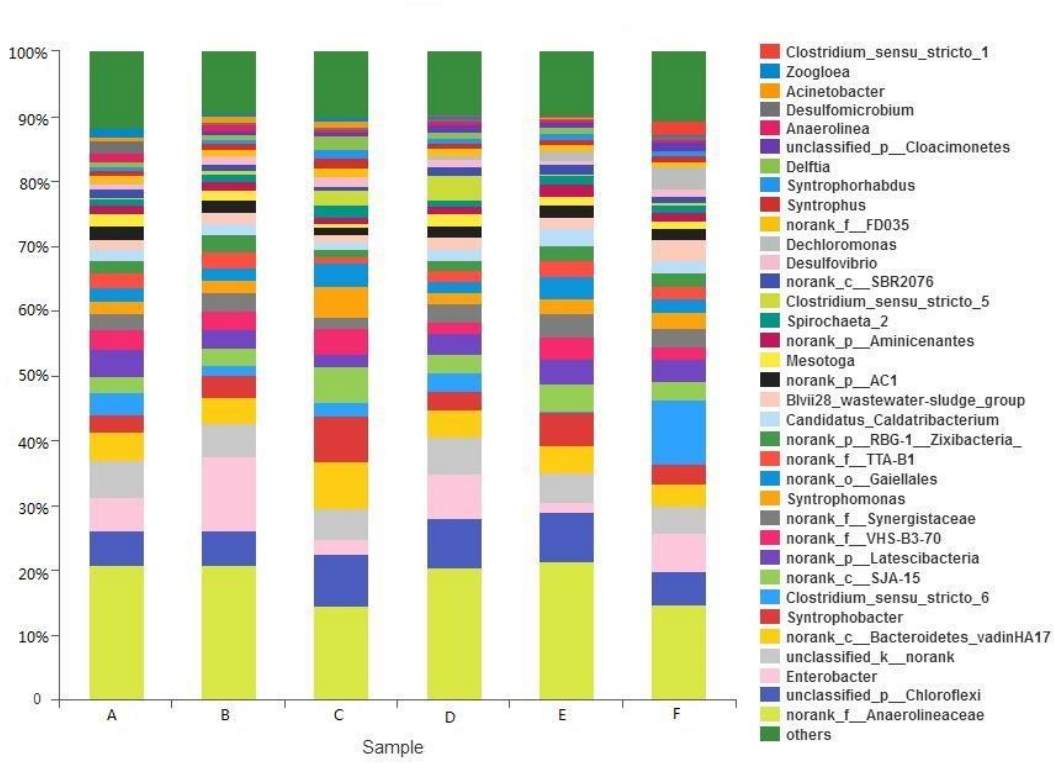


**Fig. 8.** Bacterial composition of the sludge samples with respect to the genus levels

The compositions of the microorganisms in each sample at the genus level are shown in Fig. 8. The main bacterial genera were *Anaerolineaceae*, *Chloroflexi*, *Enterobacter*, *Bacteroidetes*, *Syntrophobacter*, and *Latescibacteria*. *Anaerolineaceae* bacteria provide organic acids for the decomposition of methanogens by acetic acid, and *Enterobacter* are highly efficient degrading bacteria and are the dominant bacteria used for the treatment of phenol-containing wastewater. *Enterobacter* are commonly used together with *Eichhornia crassipes* in wastewater treatment in China (Wu and Zheng 1993). *Bacteroidetes* are the dominant bacteria in wastewater treatment systems (Wagner and Loy 2002). *Syntrophobacter* is a genus of hydrogen-producing acetogenic bacteria that oxidatively decompose higher fatty acids and alcohols into acetic acid and $H_2$ (Lu *et al.* 2017). *Desulfovibrio* are sulfate-reducing bacteria that use organic material on the surface of metals as a carbon source to reduce sulfates to hydrogen sulfide and accelerate metal pipeline corrosion (Lu *et al*. 2017). Clostridia can convert lactate and acetate into butyrate and hydrogen (Chojnacka *et al*. 2015). Studies have shown that *Clostridium* are key bacteria for anaerobic sludge fermentation (Zheng *et al*. 2013).

In the treatment of methylene blue wastewater with granular sludge, the concentration of methylene blue in the wastewater can be measured by spectrophotometer because methylene blue has an absorption peak at 665 nm. By measuring the concentration of methylene blue in the influent and effluent water, the effect of granular sludge treatment on wastewater containing methylene blue was characterized. Spectrophotometers also play an important role in the study of granular sludge treatment of wastewater containing heavy metal ions. By measuring the content of heavy metal ions in wastewater, the effect of granular sludge treatment is also described. A spectrophotometer can also be combined with other testing instruments or high sensitivity reagent to improve the detection efficiency (Zou and Lu 2013). In the future,

this group will continue to study the kinetics and thermodynamics of methylene blue adsorption from granular sludge using a spectrophotometer.

## CONCLUSIONS

1. In this study, methylene blue was used as the target pollutant, and glucose was used as the metabolic substrate. Through one cycle of operation, granular sludge was used to decontaminate and adsorb methylene blue and the average COD removal efficiency was more than 70%. The methylene blue removal efficiency was approximately 90%.
2. The results of the particle size distribution analysis showed that the addition of methylene blue to the wastewater contributed to the formation of large-sized sludge particles to some extent.
3. The SEM results showed that there were many pores on the surface of the granular sludge, and the granular sludge had a microbial community formed by the interweaving of various bacteria. In the initial stage of the reaction, there were fewer microorganisms on the surface of the sludge due to the toxicity of the methylene blue. As the reaction progressed, the dominant bacterial species continued to increase in number and accumulate. The microbial activity on the surface of the sludge increased, and sludge decontamination performance also increased.
4. At the phylum classification level, it was found that the number of microorganisms in the *Candidatus Cloacimonetes* phylum increased with an increase in the methylene blue concentration. When the methylene blue concentration was low, *Candidatus Cloacimonetes* bacteria were not detected in the A, B, and C reactors. When the methylene blue concentration was greater than 4 mg/L, more *Candidatus Cloacimonetes* bacteria were detected in the sludge. *Candidatus Cloacimonetes* accounted for 1.02%, 1.08%, and 2.11% of the bacteria in the D, E, and F samples, respectively.

## ACKNOWLEDGEMENTS

The authors are grateful to the National Science Foundation of China (No. 31660182), the Nature Science Foundation of Guangxi (Nos. 2015GXNSFBA139042; and 2017GXNSFAA198200), and the Dean Project of Guangxi Key Laboratory of Clean Pulp & Papermaking, and Pollution Control (No. ZR201607).

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Article submitted: March 21, 2018; Peer review completed: May 13, 2018; Revisions accepted: June 14, 2018; Published: June 18, 2018.
DOI: 10.15376/biores.13.3.6033-6046