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C058 and Other Functional Microorganisms Promote the Synthesis of Extracellular Polymer Substances in Mycelium Biofloc

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Abstract: The mycelium biofloc bioaugmented by *Cordyceps* strain C058 effectively purifies water, which may be related to the synthesis of extracellular polymer substances. To verify this conjecture, we analyzed the changes in extracellular polymer substances content in the mycelium biofloc under various hydraulic retention times (36 h, 18 h, and 11 h). The microstructure and microflora composition were analyzed using a scanning electron microscope and high-throughput sequencing. The ordinary biofloc without bioaugmentation was taken as a control. The results showed that under the above hydraulic retention time, the extracellular polymer substances contents of the mycelium biofloc were 51.20, 55.89, and 33.84 mg/g, respectively, higher than that of the ordinary biofloc (14.58, 15.72, and 18.19 mg/g). The protein content or the polysaccharide content also followed the same trend. Meanwhile, the sedimentation performance of the mycelium biofloc was better than that of the ordinary biofloc, attributed to the content of the extracellular polymer substances. It is worth noting that C058 is the main biofloc content, which promotes the synthesis of extracellular polymer substances in the mycelium biofloc. Other functional microorganisms in the mycelium biofloc were *Janthinobacterium*, *Phormidium*, *Leptolyngbya*, *Hymenobacter*, and *Spirotrichea*, which also promote the synthesis of extracellular polymer substances.

Keywords: C058; mycelium biofloc; extracellular polymeric substances; microcosmic structure; microflora composition

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1. Introduction

Extracellular polymeric substances (EPS) are sticky polymers secreted by microorganisms through metabolism and cell autolysis [1]. They contain proteins (PN), polysaccharides (PS), humic acid, and nucleic acids [2,3]. PN and PS are the main EPS constituents, accounting for about 70–80% of the total EPS [4]. Because EPS promote adhesion and aggregation between microbial cells [5], and their content can affect the properties of flocs, such as flocculation capacity, settling performance, surface charge, and microbial community structure [6–8], EPS are key substances that determine the physical, chemical, and biological properties of flocs, and thus the water purification performance. Biofloc technology (BFT) is widely used in aquacultural tailwater purification. Zoogloea and filamentous bacteria are the core of ordinary biofloc (OBF). They adhere to bacteria, fungi, algae, protozoa, and organic polymers, forming structurally diverse flocs using secreted extracellular polymer substances [9]. OBF removes ammonium nitrogen, total nitrogen, and total phosphorus from water through microbial assimilation and nitrification [10] and removes organic matter from water through biological adsorption [11]. Recent studies have shown that the

bioaugmented biofloc efficiently purifies water, but the mechanism is not clearly described, in particular, the EPS synthesis [12–14].

Cordyceps sp. belongs to phylum Ascomycota, class Sordariomycetes, order Hypocreales, and family Clavicipitaceae. Its ascospores germinate into and belong to large filamentous mycelium. Numerous studies have shown that fungi can efficiently remove contaminants in wastewater [15–18]. Our previous study showed that C058 and its bioaugmented biofloc named mycelium biofloc (MBF) effectively purified water [19,20]; however, the mechanism underlying this process is unclear. Zhang [21] used a mycelium ball as a biomass carrier to load Pseudomonas stutzeri T13, and found that the total nitrogen removal rate in the SBR reactor was about 10% higher than in the traditional activated sludge reactor, and the protein content in EPS secreted by mycelium ball is high, which enhances the solid–liquid separation performance of the system by improving the hydrophobicity of activated sludge. *Cordyceps* secretes large amounts of PS, PN, and other macromolecules [22,23]. Hence, we speculate that the water purification effect of MBF may be related to its EPS synthesis. To validate this hypothesis, we analyzed the changes in EPS content, the microstructure, and the microflora composition in MBF under various hydraulic retention times (HRT) using a scanning electron microscope (SEM) and high-throughput sequencing. The OBF without bioaugmentation was taken as a control. The findings of this study provide a theoretical basis for the water purification effect of MBF.

2. Results and Discussion

2.1. Changes in EPS Content in the Biofloc

Figure 1 shows that the EPS content was higher in the experimental reactor than that of the control reactor under various HRTs, which may be attributed to the C058 in the experimental reactor. C058 secretes a large amount of PS and PN [19], generating higher EPS content in MBF of 51.20, 55.89, and 33.84 mg/g under the above HRTs. Morgan et al. [24] proposed that the activity of functional microorganisms affects the EPS content. Accordingly, the EPS contents were different in the two reactors [25]. There was lower EPS content in OBF (14.58, 15.72, and 18.19 mg/g under the above HRTs). Meanwhile, It can be seen from Figure 1 that the PN content or PS content also followed the same trend. In addition, the flow rate influenced the HRT and thus the shear forces. Appropriated shear force is essential for optimal EPS secretion in aerobic granular sludge, but excessive shear force destabilizes the sludge [26]. This may explain the increase in EPS content in both reactors during the first and second operation stages (Figure 1). Filamentous bacteria are intolerant to shear forces, and HRT 11 h may cause autolysis of filamentous bacteria with a decrease in EPS content in MBF.

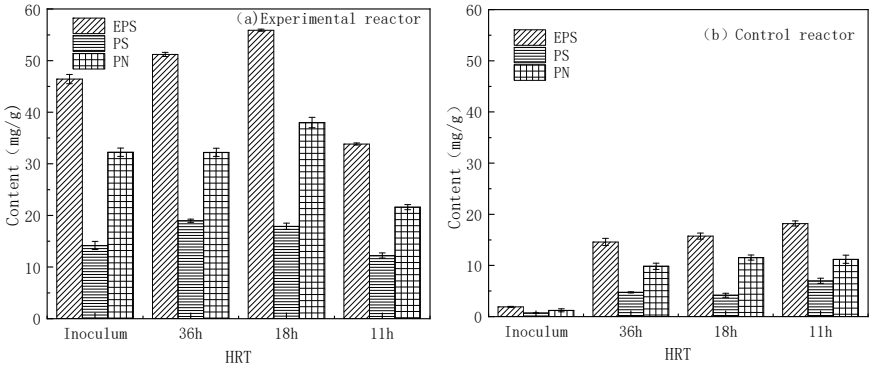

Figure 1. Contents of EPS and its components in biofloc from the two reactors under various HRTs.

2.2. Sludge Volume Index (SVI) Changes of Biofloc

The settling performance of flocs can indirectly reflect the content and composition of EPS. It can be seen from Figure 2 that the SVI was lower in the experimental reactor than that in the control reactor under various HRTs, and all the SVI in the experimental

reactor were lower than 150 mL/g, implying that the settling performance of MBF occurred within a given favorable range. That of the control group was above 150 mL/g under various HRTs. The OBF was loose and was detrimental to sedimentation [27]; thus, the settling performance of MBF was better than that of OBF. Meanwhile, there was a positive correlation between EPS and SVI in the experimental reactor, consistent with a previous study [28]. Li et al. [29] found that microorganisms can secrete EPS to enhance flocculation and sedimentation. In the present study, the sedimentation performance of MBF was better than that of OBF, which indirectly proved that the EPS content of MBF was higher than that of OBF. In addition, the settling performance of flocs reflects the composition of EPS. Previous studies have shown that PN possesses hydrophobic groups, which improve the hydrophobicity of the cells and enhance the mutual attraction between bacteria, promoting their sedimentation [30,31]. In this study, the PN content of the experimental reactor was higher than that of the control reactor under various HRTs (Figure 1).

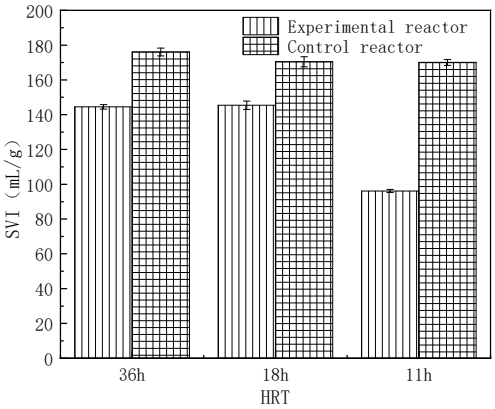

Figure 2. SVI changes of biofloc in both reactors under various HRTs.

2.3. Changes in the Appearance of Biofloc

The appearance of MBF and OBF were analyzed through a visual examination using an SEM. The morphological structure of C058 is shown in Figure 3. C058 is filamentous and supercoiled. The bioflocs in both reactors under various HRTs are shown in Figure 4. As shown in Figure 4a,c,e, bacteria aggregates on the MBF surface increased over time, forming a symbiotic structure with C058. Although MBF was constructed by fungi and bacteria together, fungi were still the dominant microorganism. As shown in Figure 4b,d,f, the OBF mainly comprised zoogloea, whose size decreased with time. It revealed that the shortened HRT strengthened the shear force and minimized the zoogloea size.

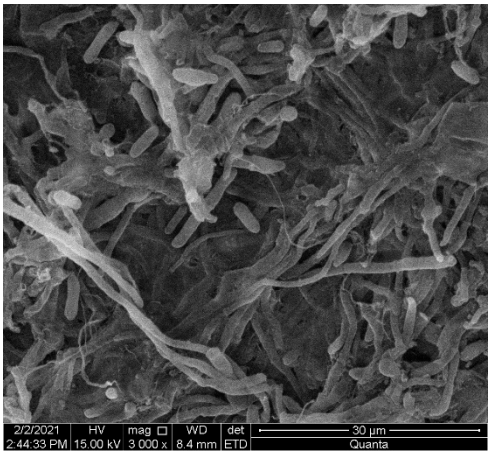

Figure 3. The morphological structure of *Cordyceps* strain C058.

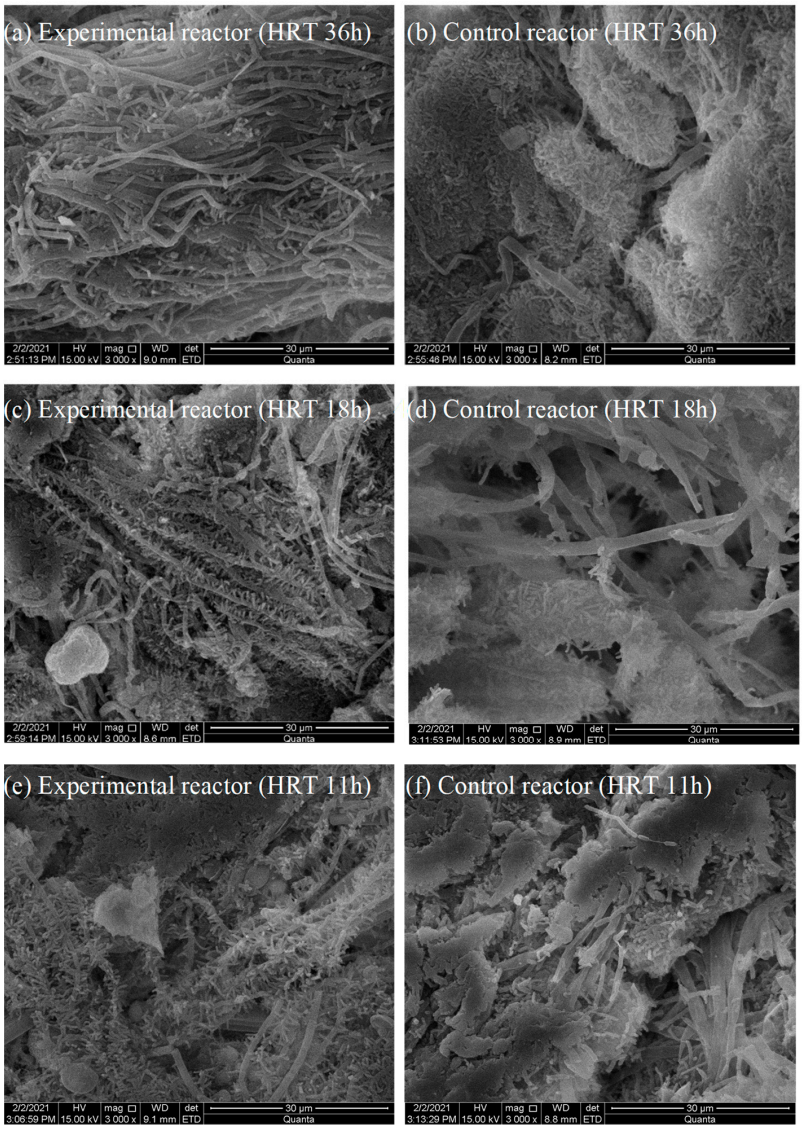

Figure 4. SEM of biofloc from both reactors under various HRTs.

2.4. Changes in the Microbial Community Structure in Biofloc

During the operation of both reactors, the biofloc was sampled when it had achieved stable operation under various HRTs. A total of eight samples were collected and included CKCB (inoculum of the experimental reactor), CB-1 (sample of the experimental reactor at HRT 36 h), CB-2 (sample of the experimental reactor at HRT 18 h), CB-3 (sample of the experimental reactor at HRT 11 h), CKWB (inoculum of the control reactor), BF-1 (sample of the control reactor at HRT 36 h), BF-2 (sample of the control reactor at HRT 18 h), BF-3 (sample of the control reactor at HRT 11 h).

The microbial community structure in biofloc was determined using 16S rRNA and 18S rRNA gene sequencing, and the sequencing process was completed in Guangdong Meilikang Biotechnology Co., Ltd., Guangzhou, China.

2.4.1. Changes in Prokaryote in Biofloc

(1) The diversity index of prokaryote

The diversity of the microbial community, including the species diversity and relative abundance of each group, was described based on the α-diversity index. We found that the Chao1 index in the experimental reactor negatively correlated with the HRT, and was at the lowest level (1906.67) at HRT 11 h. The change of the Chao1 index in the control

reactor was the same as in the experimental reactor, and the lowest Chao1 index was 1489.73 at HRT 11 h (Table 1). The change of HRT had less of an effect on the bacteria abundance in the experimental reactor than in the control reactor. Meanwhile, the Shannon index and Simpson index also followed the same trend. Hence, the species diversity of microorganisms was higher in the experimental reactor than in the control reactor, implying that the microbial community in the experimental reactor was more stable.

Table 1. The alpha diversity index of biofloc from both reactors based on the 16S rRNA sequences.

	Chao1	Shannon	Simpson
CKCB	1701.59	6.16	0.969
CB-1	2868.92	8.42	0.988
CB-2	2720.18	8.27	0.987
CB-3	1906.67	6.16	0.942
CKWB	2477.06	7.80	0.988
BF-1	2242.12	7.82	0.981
BF-2	2059.89	6.67	0.915
BF-3	1489.73	3.97	0.745

(2) The relative abundance of prokaryotes at the phylum level

It can be seen from Figure 5 that the most dominant phylum in the experimental reactor at the first operation stage was Thermi, followed by Proteobacteria and Cyanobacteria. At HRT 36 h, Cyanobacteria became the most dominant phylum, with an abundance of 38.05%, followed by Proteobacteria (27.99%) and Bacteroidetes (13.34%). At HRT 18 h, the abundance of Proteobacteria increased to 53.34%, making it the most dominant phylum, followed by Cyanobacteria and Bacteroidetes. At HRT 11 h, the abundance of Proteobacteria was 37.35%, and it was still the most dominant phylum, followed by Cyanobacteria and Firmicutes. In the control reactor, the most dominant phylum at the first operation stage was Proteobacteria, followed by Actinobacteria and Bacteroidetes. The abundance of Proteobacteria decreased gradually from 27.21% (HRT 18 h) to 14.59% (HRT 11 h) with the shorting of HRT, and it was no longer the most dominant bacteria. The abundance of Actinobacteria also displayed the same trend. Instead, Thermi became the most dominant phylum at HRT 11 h; therefore, MBF was dominant by Proteobacteria. Proteobacteria plays an indispensable role in the material cycle, including nitrogen transformation in the water [32,33], enabling nitrogen removal in aquacultural wastewater [34]. Meanwhile, the abundance of Bacteroidetes and Cyanobacteria was also higher in the experimental reactor than in the control reactor under various HRTs. Bacteroidetes had the particularity of using nitrogen compounds for growth and Cyanobacteria had nitrogen fixation capacity [32,35]. These bacteria can promote the synthesis of EPS in MBF.

(3) The relative abundance of prokaryotes at the genus level

It can be seen from Figure 6 that the abundance of *Janthinobacterium* belonging to Proteobacteria in the experimental reactor reached 20.74% at HRT 11 h, significantly higher than that in the control reactor (0.68%). This may explain the effective water purification capability of MBF at short HRT, as reported in our previous study [18]. Other studies have shown that *Janthinobacterium* has nitrogen removal capacity in the water. Yang et al. [36] found that *Janthinobacterium* sp. M-11 still exhibited nitrite removal capacity under low temperature, and the removal rate of nitrite and nitrate even reached 93% and 98%, respectively, under anaerobic conditions, which was beneficial for the treatment of eutrophic water. Meanwhile, as dominant genera, the abundance of *Phormidium* and *Leptolyngbya* belonging to Cyanobacteria was higher in the experimental reactor than in the control reactor under various HRTs. Other studies have shown that *Phormidium* and *Leptolyngbya* had excellent nitrogen fixation activity and PN and PS production capacity. Through the $^{15}N_2$ gas tracer method combined with the stable isotope nucleic acid probe technique (DNA-stable isotope probing), it was confirmed that *Leptolyngbya* was one of the most important nitrogen fixation microorganisms in paddy soil [37]. Bar et al. [38] found that *Phormidium* secretes PS for flocculation. Kim et al. [39,40] found that *Leptolyngbya* sp. KIOST-1 produces a

large amount of PN. In addition, the abundance of *Hymenobacter* belonging to Bacteroides was higher in the experimental reactor than in the control reactor under various HRTs. *Hymenobacter* possesses nitrogen fixation capacity [41]; therefore, in addition to C058, many bacteria that can synthesize EPS were present in the experimental reactor, which promoted the synthesis of EPS in MBF, enhancing the water purification efficiency of the system.

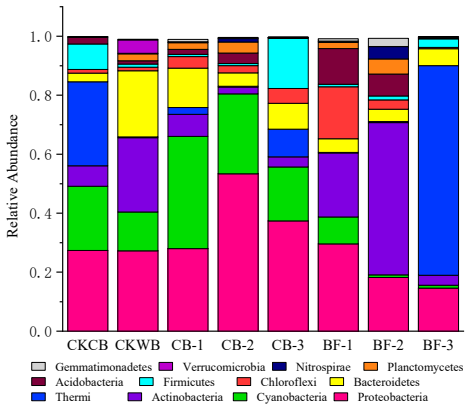

Figure 5. Composition of dominant prokaryote phyla.

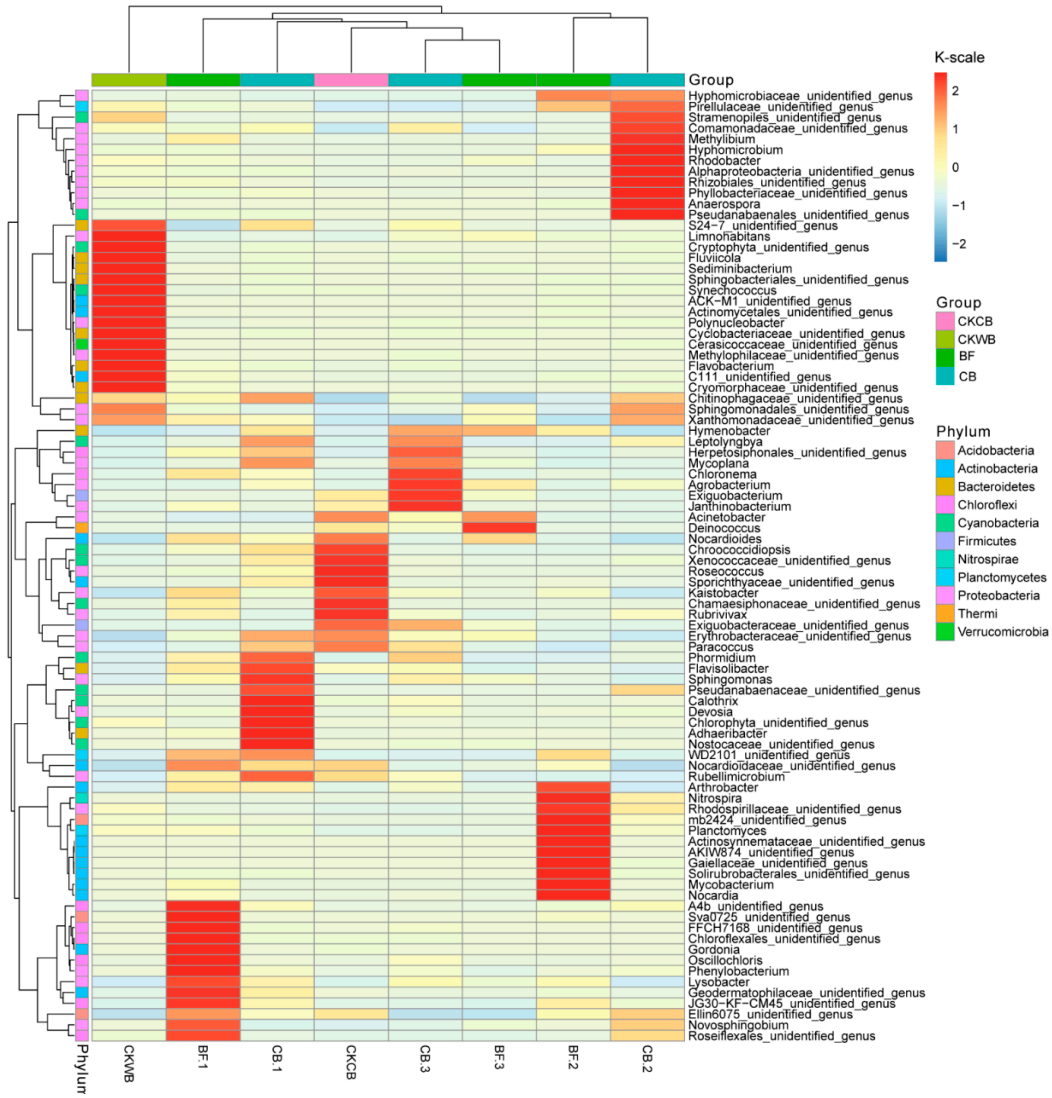

Figure 6. Heatmap diagram for the dominant prokaryote genera.

2.4.2. Changes in Eukaryotes in Biofloc

(1) The diversity index of eukaryotes

The microflora abundance in the experimental reactor was on the rise on the whole with the shortening of HRT, but the control reactor displayed an opposite trend (Table 2). Compared with the control reactor, the microflora diversity was lower in the experimental reactor, probably because C058 filamentous fungi dominated the experimental reactor, and competition limited the growth of the other eukaryotes.

Table 2. The alpha diversity index of biofloc from both reactors based on the 18S rRNA sequences.

	Chao1	Shannon	Simpson
CKCB	290.25	4.43	0.92
CB-1	387.69	2.94	0.63
CB-2	259.12	2.21	0.52
CB-3	393.45	3.17	0.75
CKWB	492.11	4.06	0.87
BF-1	481.27	4.93	0.93
BF-2	372.07	4.16	0.88
BF-3	353.57	3.75	0.86

(2) The relative abundance of eukaryotes at the phylum level

It can be seen from Figure 7 that the most abundant eukaryote was Opisthokonta at the first operation stage of the experimental reactor, achieving an abundance of 46.72%. In the latter stages, SAR became the most abundant eukaryote, at 88.36% (HRT 36 h), 86.19% (HRT 18 h), and 90.49% (HRT 11 h), respectively; however, the proportion of SAR in the control reactor declined. Studies have shown that protozoa caused flocs formation by excreting gelatinous mucus before ingesting the bacteria [42]. After the formation of the floc, ciliated protozoa become the main protozoan group, and the secretions during its predation further promote the accumulation of microorganisms in the sludge [43]. Hence, the abundance of SAR remained higher in the experimental reactor, which promoted the flocs' formation and EPS synthesis.

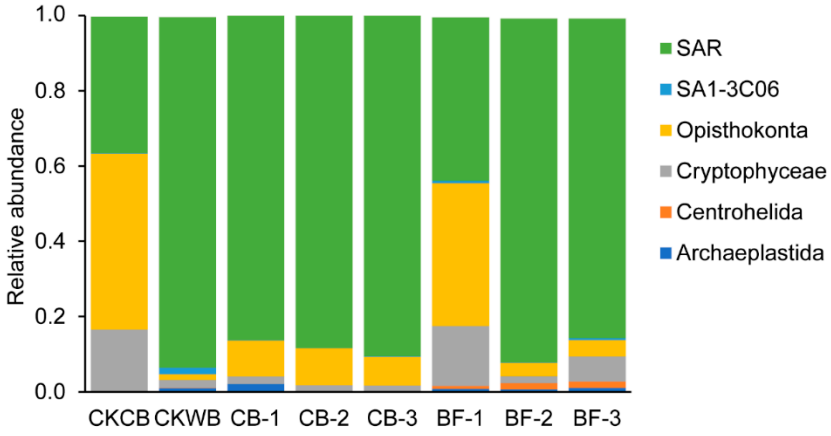

Figure 7. Composition of dominant eukaryote phyla.

(3) The relative abundance of eukaryotes at the genus level

It can be seen from Figure 8 that *Spirotrichea* was the most dominant group in both reactors, but their abundance changed with time. The abundance of *Spirotrichea* in the experimental reactor increased from 18.67% (inoculum) to 79.02% (HRT 36 h), 84.67% (HRT 18 h), and 62.26% (HRT 11 h), but decreased in the control reactor from 70.64% (inoculum) to 21.24% (HRT 36 h), 40.82% (HRT 18 h), and 56.42% (HRT 11 h). *Spirotrichea* belonging to Ciliophora is a complex and diverse group of ciliates. It is an indispensable

part of the microfood ring and is the main contributor to the energy cycle. Its abundance in marine water was negatively correlated with the N and P contents [44–46]. Ciliates stimulate the formation and adhesion of bacteria colonies, which are efficient for nutrient uptake [47]; Ciliates appeared on the surface of aerobic granular sludge and secreted the viscous substances that can absorb suspended particles and bacteria, and the dead remains were used as the skeleton to form granular sludge [48]; therefore, the increase in the abundance of *Spirotrichea* in the experimental reactor promoted flocs' formation, and thus improved the efficiency of water purification.

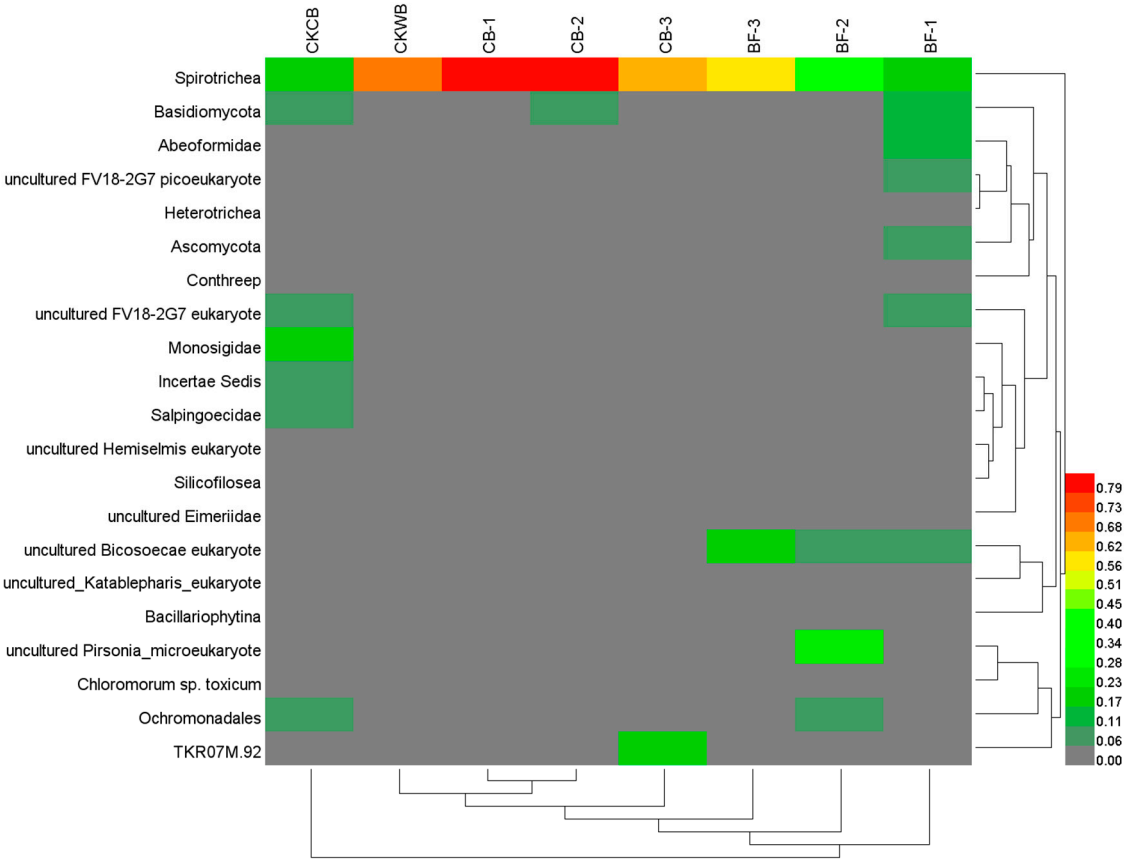

Figure 8. Heatmap diagram for the dominant eukaryote genera.

3. Materials and Methods

3.1. Experimental Materials

The Cordyceps strain C058 was donated by the Medicinal Fungi Research Group of the Institute of Microbiology, Guangdong Academy of Sciences. The formula of the C8 medium was referred to by Li et al. [22]. The formula of the simulated wastewater was referred to by Schryver and Verstraete [49]. The size and structure of the baffled reactor were described by Yang [20].

3.2. Experimental Methods

3.2.1. Culture and Collection of C058

The mycelium was taken from the C058 slope and inoculated into a C8 culture medium. After a large number of new mycelium balls were grown out at 28 °C, 150 rpm in a rotating incubator, they were collected by centrifugation under the condition of 2000 rpm, 10 min, and were washed three times with sterilized water. The washed mycelium balls were used for inoculation.

3.2.2. Operation of the Reactors

The experiment was carried out in two sets of identical baffled reactors. After filling with the simulated wastewater, one reactor, as an experimental reactor, was inoculated with 1% of C058 and 1% of Pearl River water (23°06′37.9″ N, 113°16′51.6″ E) to form mycelium biofloc (MBF), and the other reactor as a control reactor was only inoculated with 1% of Pearl River water to form OBF. Here, Pearl River is chosen as a typical natural water body that is rich in natural microflora. The reactors were operated at room temperature and contained above 5 mg/L of dissolved oxygen by continuous aeration.

After a consideration of the effect of HRT on the EPS content [26], the reactor was operated under three stages: HRT 36 h (v = 4.7 L/h), 18 h (v = 9.2 L/h) and 11 h (v = 15.3 L/h). The first operation stage was HRT 36 h. When the reactor became stable, sampling for latter index determination was carried out. Then, the influent velocity was changed to control the HRT of 18 h for a second operation stage; so did the third operation stage at HRT 11 h.

3.3. Analysis Method

3.3.1. Extraction and Determination of EPS

The EPS was extracted using the centrifugal heating method [50]. In this study, the main components of EPS, PN, and PS, were used to characterize EPS. The PN content was determined using Coomassie brilliant blue spectrophotometry, and the PS content was determined using phenol-sulfuric acid spectrophotometry [51].

3.3.2. Determination of SVI

Sludge settling velocity in 30 min (SV_{30}) and mixed liquid suspended solids (MLSS) content were determined according to "Monitoring of Water and waste Water" [52], and SVI = SV_{30}/MLSS.

3.3.3. SEM Analysis

Samples were dried (Tousimis Autosamdri-815, American) and sprayed with gold (EMS 150T, American) before the electron microscope scanning (Tungsten Filament SEM Q25, American). The specific operation was referred to by Shen et al. [53].

3.3.4. Analysis of Microbial Community Structure

(1) The 16S rRNA sequence analysis

Total DNA was extracted using the soil strong DNA extraction kit (DNeasy PowerSoil Kit, QIAGEN, Hilden, Germany). The DNA was diluted to 10 ng/μL for PCR amplification. According to Tamaki [54], the V4-V5 hypervariable region of the 16S rRNA gene of the experimental sample was amplified by primers 515F(5′-GTGYCAGCMGCCGCGGTA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′). Reaction system: every 25 μL PCR reaction solution contains 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (Transgen, Beijing, China), 1.0 μM primers, 0.25U Ex Taq (TaKaRa, Beijing, China), and 10 ng DNA template. The PCR reaction procedure was pre-denatured at 94 °C for 3 min, then extended for 10 min at 72 °C after 30 cycles of conventional amplification (denatured at 94 °C for 40 s, annealing at 56 °C for 60 s, extension at 72 °C for 60 s). The same sample was amplified twice, and the two PCR products obtained from the same sample were mixed. After 1.2% agarose gel electrophoresis, the gel was cut and purified by sanPrep DNA gel recovery kit (Raw engineering, China). After all the purified DNA was mixed in the same amount, PE250 sequencing was carried out using the Illumina Miseq platform [55].

The original sequencing data were spliced using the FLASH1.2.8 software and screened using the QIIME1.9.0 software after splicing [29,56,57], using the Uchime program [58] to detect and remove chimera sequences, then using the QIIME1.9.0 software to divide OTUs (Operational Taxonomic Units) according to 97% similarity of sequences, and using the RDP classifier [46] to annotate each species.

(2) The 18S rRNA sequence analysis

The 18S rRNA was extracted as 16s rRNA. According to Lejzerowicz et al. [59], the V4 hypervariable region of 18s rRNA gene of the experimental sample was amplified by primer TAReuk454WD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and primer TAReukREV3 (5′-ACTTTCGTTCTTGATYRA-3′). PCR reaction system: every 25 μL PCR reaction solution contains 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (Transgen, China), 1.0 μM primers, 0.25 U TransFast Taq DNA polymerase (Transgen, China), and 10 ng DNA template. The PCR reaction procedure was the same as that of 16S rRNA. After the completion of the PCR reaction, two PCR products amplified from the same sample were mixed, and the gel was cut using 1.2% agarose gel electrophoresis and purified by the AxyPrep DNA gel recovery kit (Axygen, Hangzhou, China). After all the purified DNA was mixed in the same amount, PE250 sequencing was carried out using the Illumina Miseq platform [29].

The original sequencing data were analyzed as 16S rRNA.

3.4. Data Processing

Microsoft Excel 2019 was used for data processing and analysis, and Origin 2018 was used for drawing.

4. Conclusions

(1) The EPS contents in MBF were 51.20 mg/g (HRT 36 h), 55.89 mg/g (HRT 18 h), and 33.84 mg/g (HRT 11 h), respectively, higher than the EPS content of OBF under the corresponding HRTs. PN content or PS content also followed the same trend.
(2) The sedimentation performance of MBF was better than that of OBF, attributed to higher EPS PN contents.
(3) MBF was constructed by fungi and bacteria together, and C058 was the main component, promoting the synthesis of EPS.
(4) Compared with OBF, MBF bioaugmented by *Cordyceps* strain C058 had higher diversity and abundance of microorganisms, realizing a more stable operation of the experimental reactor. More importantly, C058 promoted the growth of some functional bacteria, including *Janthinobacterium* belonging to Proteobacteria, *Phormidium* and *Leptolyngbya* belonging to Cyanobacteria, and *Hymenobacter* belonging to Bacteroides, which participate in nitrogen fixation and PN and PS production, promoting the EPS synthesis. In addition, C058 also promoted the growth of *Spirotrichea* belonging to Ciliophora, which benefited floc formation and enhanced the water purification.

5. Patents

Chinese patent: A MPBR reactor suitable for sewage purification, CN212833060U. Applicant: Zhongkai University of Agriculture and Engineering. Inventor: Yiyong Li, Yongcong Yang, Kangchun Peng, Baoe Wang, Xueqin Tao, Chong Lin, Zexiang Lei, Jianjun Du.

Author Contributions: Conceptualization, Y.L. and B.W.; methodology, Y.L. and Y.Y.; software, W.L. (Wanyi Luo); validation, W.L. (Wen Liu), Z.L., X.T. and B.W.; formal analysis, W.L. (Wanyi Luo); data curation, B.W.; writing—original draft preparation, W.L. (Wanyi Luo); writing—review and editing, Y.L.; supervision, B.W.; funding acquisition, Y.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to confidentiality issues.

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Conflicts of Interest: The authors declare no conflict of interest.

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