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Microbiological Mechanisms for Nitrogen Removal Using Anaerobic Fermentation Liquid from Spent Mushroom Substrates as a Carbon Source

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Abstract: In wastewater treatment, a low C/N ratio highly inhibits the bioremoval of nitrogen, and commercial external carbon sources are widely used. In order to obtain an economical substitute, fermentation broth of spent mushroom substrates (SMS) was employed here as a carbon source for denitrification in a sequencing batch reactor (SBR). During the domestication process, the SMS fermentation broth-feeding treatment presented comparable nitrogen removal ability (74.44%) with a commercial carbon source group (77.99%). *Rhodobacter*, *Lactobacillus* and *Pseudomonas* were the dominant bacteria in the fermentation broth, and *Saccharomycetales Gymnopilus dilepis* was the dominant fungi. At the early domestication stage, the relatively high concentration of fermentation broth led to a much lower abundance of typical nitrate reductase genes than the control group. Furthermore, extracellular polymeric substance (EPS) formation was observed in the broth-feeding sample. The microbial structure dynamic was investigated, which showed a high influent effect when 20% fermentation broth was added. As domestication proceeded, similar dominant species in the control and broth-feeding treatments were observed. Overall, SMS fermentation broth can be used as a promising substitute to replace a costly commercial carbon source.

Keywords: nitrogen removal; agricultural waste; additional carbon source; SBR reactor

Citation: Chen, R.; Zhang, W.; Bi, X.; Jin, Y.; Yang, Y. Microbiological Mechanisms for Nitrogen Removal Using Anaerobic Fermentation Liquid from Spent Mushroom Substrates as a Carbon Source. *Water* **2023**, *15*, 3530. https://doi.org/10.3390/w15203530

Academic Editors: Kai He, Jing Wei, Zhishui Liang and Yuanfeng Qi

Received: 14 September 2023
Revised: 1 October 2023
Accepted: 8 October 2023
Published: 10 October 2023

1. Introduction

Effective removal of nitrogen (N) is one of the most important targets in domestic wastewater treatment plants, and biological denitrification plays a significant role in this process. The denitrifying microbes can be divided into heterotrophic and autotrophic, with heterotrophic denitrifying bacteria being the most common denitrifying bacteria in nature as well as in activated sludge [1]. Thus, heterotrophic denitrification has been widely applied due to its low treatment cost and high efficiency [2]. However, it has already been reported that sufficient carbon sources must be supplied to ensure complete denitrification [3], while the current domestic sewage generally has a low carbon–nitrogen ratio (C/N). Consequently, an additional carbon source is necessary to increase the denitrification rate and completeness when the C/N is too low [4–7]. As yet, organic compounds such as acetate, alcohol, and glucose have been used as commercial external carbon sources, but their relatively high cost is unfavorable. Thus, it is significant to seek alternatives with lower costs. Agricultural wastes are considered an important resource and could be used as external carbon sources with high economic benefits [8,9].

Edible mushrooms are an important food resource, and their cultivation process could generate large amounts of solid waste (spent mushroom substrates (SMS)), leading to a formidable challenge for disposal management [10–12]. Besides the various recycling methods for SMS [13–19], we have previously proven that the acid hydrolysates of SMS

can act as an external carbon source for the treatment of low C/N ratio wastewater using a sequencing batch reactor (SBR) [20]. However, the acid pretreatment of SMS proceeds at a high temperature with additional sulfur acid, and a more economical and convenient method should be proposed. Fermentation is a favorable option for extracting carbon sources from various biodegradable biomasses; the fermentation liquid of sludge [21] food waste [22] has already been reported as an excellent external carbon source. However, the effect of SMS fermentation liquid as an external carbon source on domestic wastewater denitrification has not been investigated. Moreover, as a biological production, using fermentation liquid would consequently introduce various different microbes into the activated sludge and probably lead to a change in the micro-community and the denitrifying function. However, there are few reports about the microbial community dynamic of activated sludge influenced by the fermentation liquid carbon source, especially the important but unstable acclimatization stage.

Thus, in this research, we used the anaerobic fermentation liquid from a spent mushroom substrate, comprising *Hypsizygus marmoreus* as the carbon source, for denitrification by the SBR process. The main objectives are: (1) to investigate the effect of SMS anaerobic fermentation liquid on the SBR nitrogen removal performance and physicochemical property of activated sludge during the acclimatization process; (2) to clarify the microbiological mechanisms of activated sludge acclimatization. Our research can provide valuable information on using SMS fermentation as an external carbon source to enhance the removal of nitrogen in wastewater.

2. Material and Methods

2.1. Preparation of SMS Fermentation Liquid

The SMS of *Hypsizygus marmoreus* was used as the raw material and was obtained from Gutian, Fujian, PR China. The SMS was dried in air and then ground to a particle size of 800 μm and was stored at 4 °C in airtight containers for further use. Fermentation liquid was prepared as follows. Briefly, 250 g ground SMS sample and 1 L tap water were mixed and added into an anaerobic fermentation cylinder (2 L) for natural fermentation at room temperature. The pressure of the cylinder was adjusted by gas emission in the first 3 days. After 14 days, the fermentation mixture was filtered with a 4-layer gauze, and then the filtrate was centrifuged at 12,000 rpm. The supernatant was stored at 4 °C for further investigation. The specific components of the fermentation liquid were subject to analysis by the Shanghai WEIPU Testing Technology Group Co., Ltd. (Shanghai, China).

2.2. SBR Device and Operation

An SBR device with 6 L working volume was employed, where the temperature was held at 30 °C and 5 effective aerobic denitrifying bacteria (*Enterobacter asburiae*, *Pseudomonas putida*, *Bacillus* sp. K5, *Acinetobacter* sp.TX5, and *Stenotrophomonas* sp.) kept in our laboratory were added into the device. Each bacteria was harvested at the late log phase and adjusted to OD_{600} = 1.0, then they were mixed with an equal volume ratio, and the total mixed bacteria solution added into each device was 300 mL (5% volume ratio). Synthetic wastewater was prepared for the treatment. In the control group, the synthetic wastewater contained $FeSO_4 \cdot 7H_2O$ (0.1 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), NH_4Cl (0.4 g/L), KH_2PO_4 (0.5 g/L), $Na_2HPO_4 \cdot 12H_2O$ (1 g/L), sodium citrate (1.0 g/L), glucose (1.0 g/L), and trace element solution (2 mL/L), where glucose and ammonium chloride were used as the carbon source and nitrogen source, respectively. The per liter trace element solution contained EDTA 50.0 g, $ZnSO_4$ 2.2 g, $CaCl_2$ 5.5 g, $MnCl_2 \cdot 4H_2O$ 5.06 g, $FeSO_4$ $7H_2O$ 1.57 g, and $CoCl_2$ 1.61 g.

Regarding the test treatment, the influent was composed of 95% synthetic wastewater and 5% SMS fermentation liquid initially (calculated as the COD ratio), which was gradually increased during the operation. The SRB was started up with a 40 rpm rotation speed, and each react cycle time was about 8 h; the detailed operation parameters are shown in Table 1. The sludge was sampled at 10-day intervals, and the sampling time was fixed

at the first operation cycle. The separated sludge was mixed with an equal volume of 60% glycerin and stored at −80 °C for further 16S rRNA and ITS sequencing by Sangon Biotech (Shanghai) Co., Ltd. Ammonium nitrogen (NH^{4+}-N), nitrite nitrogen (NO^{2-}-N), nitrate nitrogen (NO^{3-}-N), and COD, were measured by the standard methods [23].

Table 1. Operating parameters of the reactor.

Operation Time (d)	C/N Ratio	Aeration Rate (m^3/h)	Aeration Time (h)	Settling Time (min)	Fermentation Liquid Amount
1–5	13	20	6.5	30	5%
6–10	6.5	20	7	20	10%
11–15	6.5	20	7	10	15%
16–20	6.5	20	7	10	20%
21–25	6.5	20	7	10	25%
26–30	6.5	20	7	10	30%

2.3. High through Sequencing

The total microbial DNA of the fermentation liquid was extracted with a soil microbial DNA extraction kit according to the manufacturer's instructions (OMEGA). The V3-V4 16s rRNA and ITS1-ITS2 ITS regions were amplified with general primers, as shown in Table 2, and the sequencing was carried out by the Biomarker Biotechnology Company (Beijing, China). The data was analyzed as in our previous report [20].

Table 2. Primers for high through sequencing.

Sequencing Type	Forward Primer		Reverse Primer	
16S rDNA V3-V4	341F	ACTCCTACGGGAGGCAGCAG	805R	GACTACHVGGGTATCTAATCC
ITS ITS1-ITS2	ITS1	CTTGGTCATTTAGAGGAAGTAA	ITS2	GCTGCGTTCTTCATCGATGC

2.4. Functional Gene Quantification

An absolute quantitative polymerase chain reaction (qPCR) was employed to quantify the typical nitrite reductase genes *NirS* and *NirK* in activated sludge samples. The qPCR system and standard curve were prepared as in our previous report [20].

2.5. Extracellular Polymeric Substance Extraction and Determination

In order to investigate the effect of fermentation liquid on extracellular polymeric substances (EPS), briefly, 50 mL of sludge was collected and settled for 30 min. Then, the supernatant was discarded, and 50 mL of deionized water was added to resuspend the sludge. The sludge was separated by centrifugation at 8000 rpm for 15 min and then resuspended with 10 mL deionized water, which was heated at 65 °C for 15 min. Then, the sample was centrifuged at 12,000 rpm for 30 min, and the supernatant was stored at 4 °C for further determination. Extracellular protein (PN) and polysaccharides (PS) were determined by the Coomassie Brilliant Blue method and anthrone–sulfuric acid colorimetry [24].

2.6. Analytical Techniques and Statistical Analysis

An ultraviolet-visible spectrophotometer (UV-1801) was used to determine the ammonium nitrogen (NH^{4+}-N), nitrite nitrogen (NO^{2-}-N), nitrate nitrogen (NO^{3-}-N), and COD concentrations. The water content in the fermentation liquid was analyzed with a Karl Fischer volumetric titrator (KSQL-310S), and the total protein and total polysaccharide was determined with an ultraviolet–visible spectrophotometer. High-performance liquid chromatography (HPLC, Thermo Fisher U3000, Waltham, MA, USA) equipped with a UV

detector was employed to analyze the small molecular acids, where 20 mM NaH_2PO_4 solution (80%) and acetonitrile (20%) were used as the mobile phase. For the quantification of free amino acid concentrations, liquid chromatography coupled with a triple four-pole tandem mass spectrometry (LC-MS, AB5000, SCIEX, San Jose, CA, USA) was employed. The mobile phase consisted of 0.1% formic acid solution and acetonitrile, and the temperature of the ion source was set at 450 °C with a multi-reaction monitoring mode. In order to quantify the mineral elements in the fermentation broth, inductively coupled plasma emission spectrometry (ICP-OES, ICAP 7400 Radial, Thermo Fisher, USA) was used. The fermentation broth samples were diluted (5-, 10-, 20-fold) with 5% HNO_3 solution before analysis. The significant differences were evaluated with SPSS software (Version 18.0, IBM, Armonk, NY, USA), and a 0.05 threshold value was set for the *p*-value. The standard error of the triplicate samples was calculated using Excel software (Version 2019, Microsoft, Redmond, WA, USA).

3. Results and Discussion

3.1. Composition and Microbial Community of Fermentation Broth

The components of fermentation liquid are shown in Table 3. The concentration of COD in the fermentation liquid was 20,000 mg/L, and the pH was 6.19, which is proper for microbes. As well as water, the main components of total protein and total polysaccharide showed similar contents, ranging from 0.4% to 0.7% and 0.5% to 0.8%, respectively. The small molecular acids in the fermentation liquid were analyzed, and acetic acid (0.33 mg/kg), glycolic acid (0.07 mg/kg), and lactic acid (0.16 mg/kg) were detected. There were 11 kinds of free amino acids detected (883.1 mg/kg total), which ranged from 12.3 to 205.1 mg/kg. It is well known that these compounds can be utilized by microbes. The fermentation liquid also contained a high concentration of mineral elements, which reached 3027.5 mg/kg, and K, Ca, and Mg accounted for about 95% of the total amount. Fe and Mg are important for the formation and stabilization of most enzymes and has also been reported for nitrite reductase [25]. Cu is important for the construction of both Cu-containing nitrite reductase (Cu-NIR) and nitrous oxide reductase [26,27]. Thus, it is suggested that influent carbon sources of fermentation broth meet the requirement well for denitrification by microbes.

Table 3. Detail of the fermentation liquid composition.

Index	Method/Instrument	Value
COD	Potassium dichromate method	20,000 mg/L
pH	pH meter	6.19
Water	Karl Fischer	96.5–98.5%
Total protein	UV-vis	0.4–0.7%
Total sugar	UV-vis	0.5–0.8%
Acetic acid	HPLC	0.33 mg/kg
Lactate	HPLC	0.07 mg/kg
Glycollic acid	HPLC	0.16 mg/kg
L-alanine	LC-MS	205.1 mg/kg
L-leucine	LC-MS	186.2 mg/kg
L-valine	LC-MS	117.8 mg/kg
L-isoleucine	LC-MS	116.3 mg/kg
L-threonine	LC-MS	79.4 mg/kg
L-proline	LC-MS	31.8 mg/kg
L-phenylalanine	LC-MS	73.5 mg/kg
L-methionine	LC-MS	25.5 mg/kg
L-aspartate	LC-MS	21.5 mg/kg
L-glycine	LC-MS	13.7 mg/kg
L-tryptophan	LC-MS	12.3 mg/kg
K	ICP-OES	1356.0 mg/kg
Ca	ICP-OES	974.0 mg/kg
Mg	ICP-OES	541.7 mg/kg

Table 3. *Cont.*

Index	Method/Instrument	Value
Na	ICP-OES	66.7 mg/kg
Si	ICP-OES	43.8 mg/kg
Cu	ICP-OES	21.9 mg/kg
Fe	ICP-OES	16.2 mg/kg
Al	ICP-OES	7.2 mg/kg

Figure 1a shows the microbial community of the fermentation broth. In terms of bacteria, it was found that the dominant bacteria phyla were identified as *Proteobateria*, *Actinobateria*, *Firmicytes*, and *Bacteroidetes*. Twelve genera with high relative abundance (>1%) were identified as *Rhodobacter*, *Pseudomonas*, *Stenotrophomonas*, *Sphingobacterium*, *Enterococcus*, *Acinetobacter*, *Brevundimonas*, *Bifidobacterium*, *Klebsiella*, *Gemmobacter* and *Herminiimonas*, respectively. The microbes from the fermentation process should also play a vital role in the COD utilization and nitrogen removal. For instance, *Lactobacillus*, *Rhodobacter*, *Pseudomonas*, and *Sphingobacterium* were the dominant bacterial genera (>10%) in the fermentation broth, which were reported by the fermentation function with diverse substrates [28–30], partly contributing to the high COD utilization.

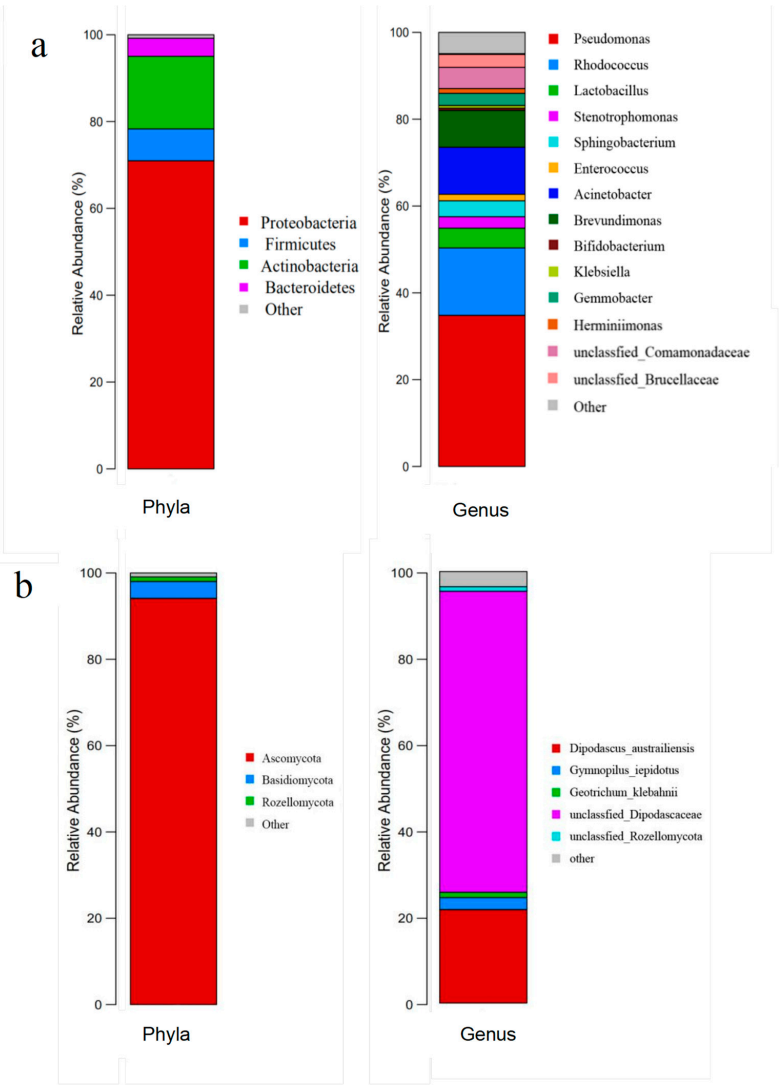

Figure 1. Relative abundance of dominant species in fermentation broth; (**a**,**b**) stands for the bacteria and fungi at phyla and genus level, respectively.

In terms of fungi, three phyla account for more than 94.9%, which were identified as *Ascomycota*, *Basidiomycota*, and *Rozellomycota,* respectively (Figure 1b), and five dominant species were identified as unclassified *Dipodascaceae* (61.76%), *Dipodascus australiensis* (21.67%), *Geotrichum klebahnii* (1.24%), *Gymnopilus lepidotus* (2.75%), and unclassified *Rozellomycota* (1.06%), respectively. These fungi were almost saprophytic, which could promote the degradation of cellulose and lignin in SMS.

3.2. Denitrification Performance and COD Removal

To investigate the effect of the fermentation broth on activated sludge formation and nitrogen removal ability, the SBR reactor was operated for about 30 days (Figures 2 and 3). The average influent NH_4^+-N concentrations of the control group and fermentation broth-added treatment were 88.20 and 80.95 mg/L, while the corresponding effluent concentrations were 19.74 and 20.63 mg/L, respectively. The average nitrogen removal rates between the control group (77.99%) and the fermentation broth-added treatment (74.44%) showed no significant difference (p = 0.155).

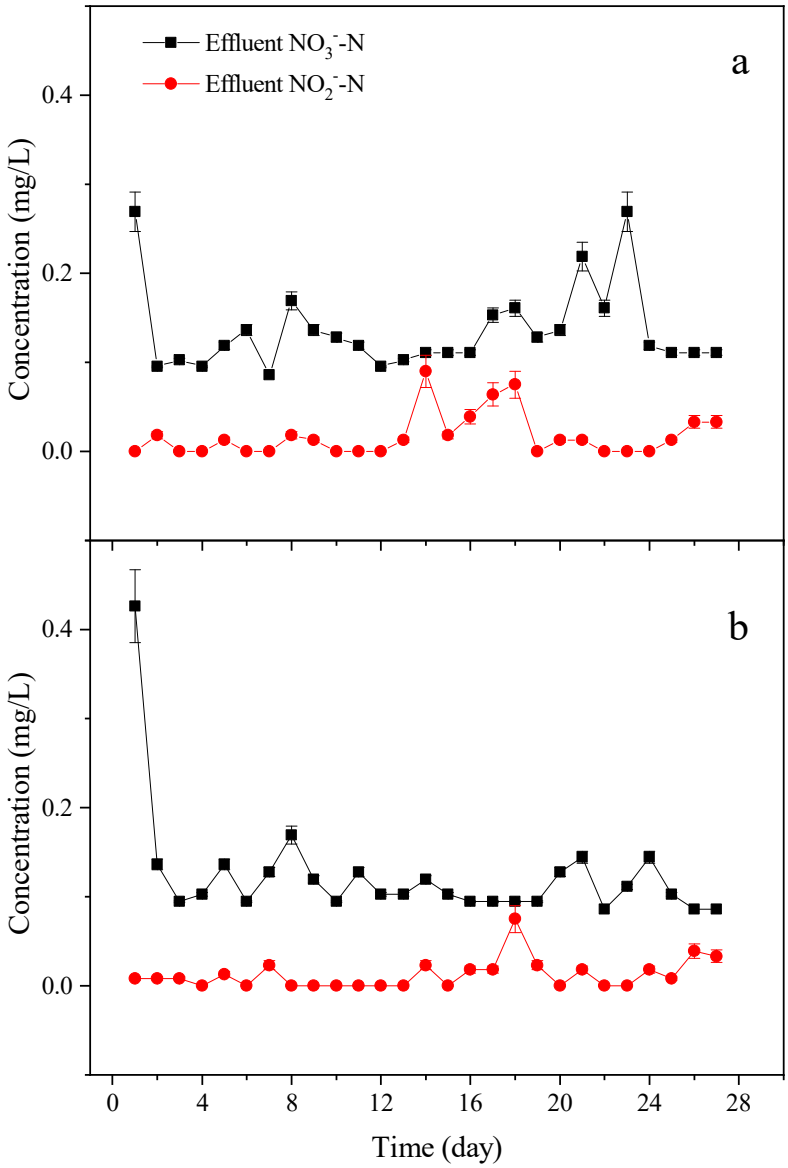

Figure 2. Nitrogen removal performance in the SBR reactors, (**a**) fermentation broth treated; (**b**) control group treated with glucose.

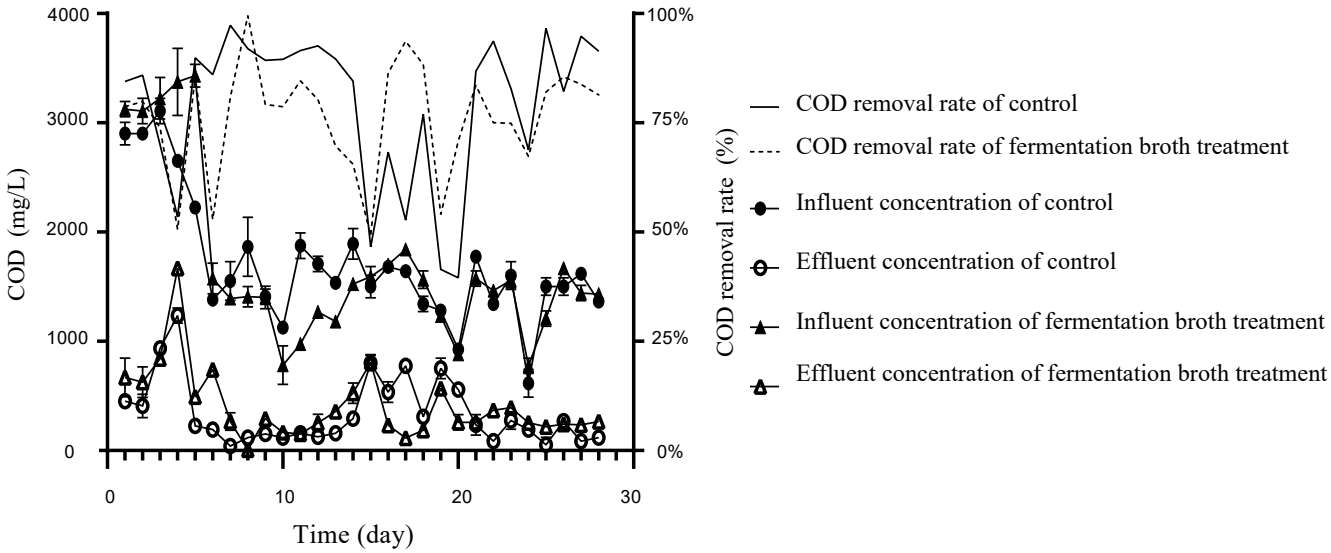

Figure 3. Variation of the COD concentration in two SBR systems.

Figure 2 shows the variation of NO_2^--N and NO_3^--N. Almost no accumulation of the two N species was found; the highest NO_2^--N concentration was lower than 0.09 mg/L in both of the two reactors. The maximum effluent NO_3^--N concentrations present on the first day were 0.426 and 0.269 mg/L in the control and fermentation broth-added treatments, respectively. As the operation proceeded, the NO_3^--N concentration in the control group was below 0.2 mg/L, which was lower than 0.269 mg/L in the treatment group. The low effluent NO_2^--N and NO_3^--N concentrations in the overall operation process also indicated the relatively high activity of nitrate reductase and nitrite reductase in the activated sludge.

The variation of the COD in the two reactors was also determined (Figure 3). In the first 5 days, a relatively high carbon source (C/N ratio = 13) was applied in order to promote the formation of flocculent-activated sludge. After that, the COD was adjusted to a normal level (about 1700 mg/L). The average effluent COD of the fermentation broth-added treatment (408.6 mg/L) was higher than the control group (344.6 mg/L), but the corresponding average COD removal rates showed no significant difference (p = 0.3268). Thus, it can be said the fermentation broth showed no negative effects on the COD and nitrogen removal of the SBR process.

3.3. Extracellular Polymeric Substance Composition

Hydrophilic PSs are an important part of a EPS, and their concentration variation in activated sludge was determined (Figure 4a). It can be found that PS concentrations in both reactors showed a similar increasing tendency to arrive at the first peak value on the 15th day. Fluctuation was observed after day 17 for the two groups, and the fermentation broth-added group presented its highest PS concentration (1288.77 mg/L) on day 21, while the PS in the control group ranged from 500 to 1000 mg/L. The average PS concentration in the fermentation broth-added treatment was 697.3 mg/L, which was significantly higher than the control group (p = 0.0219). The similar tendency of the PSs in the two reactors can be ascribed to both glucose and carbohydrate in the fermentation broth and favor PS synthesis [31,32].

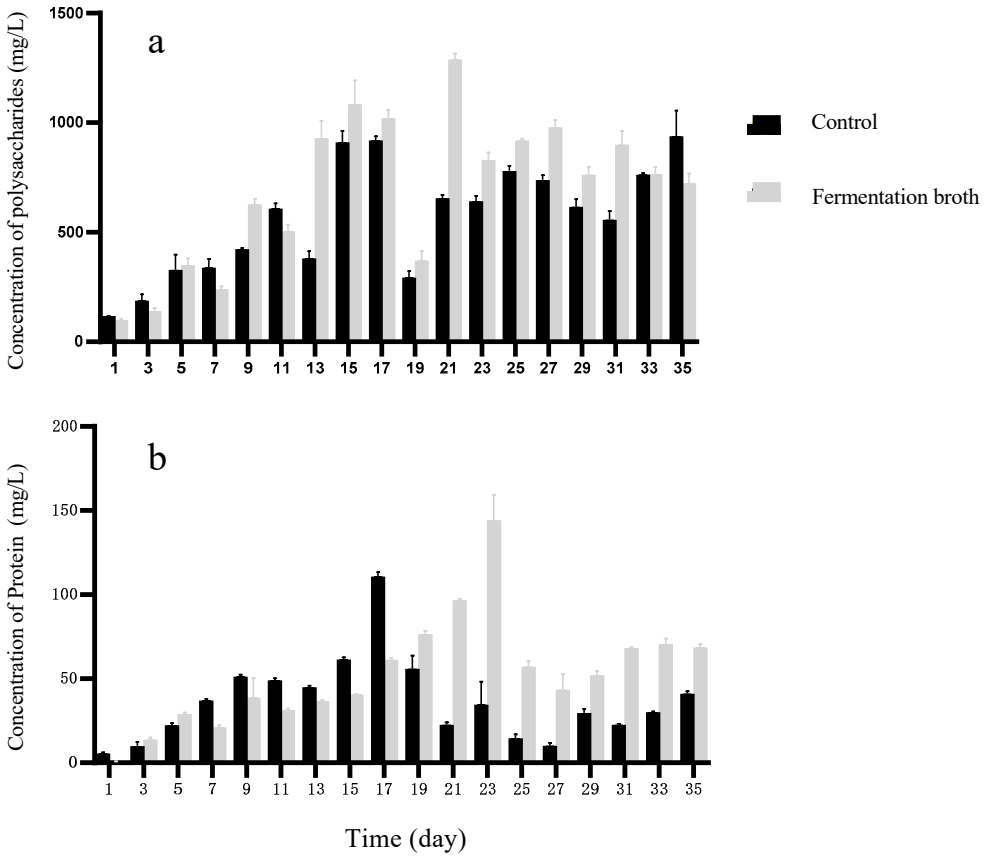

Figure 4. Concentration of PS (**a**) and PN (**b**) from activated sludge.

Figure 4b shows the variation of the PN concentration. Initially, PN in the control group increased as the operation proceeded and reached 110.90 mg/L on day 17, and then decreased to 50 mg/L after 5 days. In the treatment group, PN increased to a peak value (144.47 mg/L) on day 23, which was 30% higher than control. After the peak, PN in the fermentation broth-added reactor was maintained at a relatively high level, ranging from 50 to 60 mg/L. The higher PN concentration in the control group within the first 17 days may be ascribed to the easy degradation of glucose. It has been reported that an easily biodegradable organic carbon source leads to higher expression of biological activity [33]. The significantly higher PN concentration of the broth-added treatment in the further operation period may be ascribed to the gradually complete construction of the consortia. As a result, the abundant amino acids can be utilized to form PN, and the average PN concentrations were 52.59 mg/L in the treatment group, which was significantly higher than in the control (p = 0.0001).

It can be found that PS and NP reach a peak value and then start declining. This phenomenon can be ascribed to the influent COD concentration in both of the two groups decreased on day 17 and the starvation shock can reduce the bacterial metabolism, leading to the termination of the flocs bacteria in the sludge [34]. Thus, activated sludge became unstable and the EPS may present a relatively high degradable property. It is well known that EPS released by bacteria highly influenced the microbial aggregation, which were mainly determined by the carbon source here. According to our results, the addition of fermentation broth could promote the production of EPS, which is a benefit for the granulation of sludge. In a previous report, it was found that adding organic reject water increased the extracellular proteins/polysaccharides ratio of activated sludge, leading to higher adsorption and degradation of organic compounds [28]. Moreover, it has also been proven that over-production of EPS is a common self-protective strategy towards various external stresses. Thus, the higher EPS production in the fermentation-added treatment

can be partly ascribed to the large amount of Cu^{2+} and Mg^{2+}. A higher EPS may also offer the activated sludge a better resistance ability toward environmental impact.

3.4. Absolute Abundance of Denitrification Functional Genes

Two typical nitrite reductases, *NirK* and *NirS*, were determined in activated sludge samples on day 10, which presented the most different beta diversity index compared with the original sample. As shown in Table 4, both the copies of *NirK* and *NirS* were lower than the control group, and the total nitrite reductase copies in the fermentation broth-added sample were only 25.27% of that in the control, indicating that adding the SMS fermentation broth showed a negative effect on the nitrogen removal function at an early stage. However, this negative effect can be removed since the whole nitrogen removal efficiency was comparable to the control group, which can be ascribed to the adaption of the microbial community.

Table 4. Nitrite reductase gene copies in day 10 samples.

Treatment	*NirS* (Average Copies)	*NirK* (Average Copies)
Control	26,316.472	15,039.141
Fermentation broth	6724.9544	3912.6366

3.5. Community Dynamics of Activated Sludge

Obviously, the nitrogen removal ability was recovered after domestication for 30 days, and investigation into the microbial structure dynamic could obtain a deep insight into the mechanism. Figure 5 presents the relative species abundance variation in the activated sludge at the phyla level. The predominant phyla were *Proteobacteria*, *Candidatus saccharibacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobateria*, and *Verrucomicrobia*. Among the six phyla, *Proteobacteria*, *Candidatus saccharibacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobateria* were familiar bacteria in wastewater treatment plants with functions such as denitrification, dephosphorization, and biodegradation [35–38]. In terms of the control group, *Proteobateria* was dominant in the initial sample and sludge obtained from day 10. The abundance of *Candidatus saccharibacteria*, *Bacteroidetes*, and *Firmicutes* increased in the samples from both of the two reactors on day 10, while the *Proteobacteria* abundance decreased. It has been reported that *Candidatus saccharibacteria* prefers to enrich with complex carbon sources [39]. As domestication proceeded, *Candidatus saccharibacteria* showed obvious differences in the control and fermentation broth-added treatments and was much higher in the fermentation broth-added treatment on day 20. On day 30, *Candidatus saccharibacteria* became the predominant phylum in both two reactors with similar relative abundances. *Bacteroidetes* was the second priority phylum in the tested treatment, and was identified as *Proteobacteria* in the control group.

At the genus level (Figure 6), the predominant genus in the two reactors was identified as *unclassified Enterobacteriaceae* belonging to *Proteobacteria* initially. With the proceeding operation, the abundance of *unclassified Enterobacteriaceae* decreased, and the dominant genus was identified as *Saccharibacteria_ genera_incertae_sedis* at the end of domestication. This can be ascribed to the favorability of metabolizing various refractory pollutants, macromolecular organics, and complex carbon sources [40–42]. It was also found that the genera (>1%) can be divided as four clusters. The cluster composed of *Saccharibacteria_ genera_incertae_sedis*, *Flavobacterium*, unclassified *Rhodobacteraceae*, *Bdellovibrio*, and *Desulfovibrio*, *Trichococcus* occupied a dominant position during the domestication process. While the cluster of unclassified *Enterobacteriaceae*, *Acinetobacter*, *Rhizobium*, *Stenotrophomonas*, and *Comamonas* showed a decreased tendency during the operation. There were eight genera that presented higher abundances in the fermentation broth-added reactor rather than in the control group, identified as unclassified *Propionibacteriaceae*, unclassified *Bacteroidetes*, *Niabella*, unclassified *Sphingobacteriales*, unclassified *Flavobacteriaceae*, *Verrucomicrobium*, *Gemmobacter*, and *Runella*, respectively. These genera could highly contribute to the nitro-

gen removal ability of the sludge in treatment. For example, *Niabella* has been proven to oxidize NH_4^+-N [43], and *Bacteroidetes* can not only decompose complex carbon sources but also show nitrification ability [43,44].

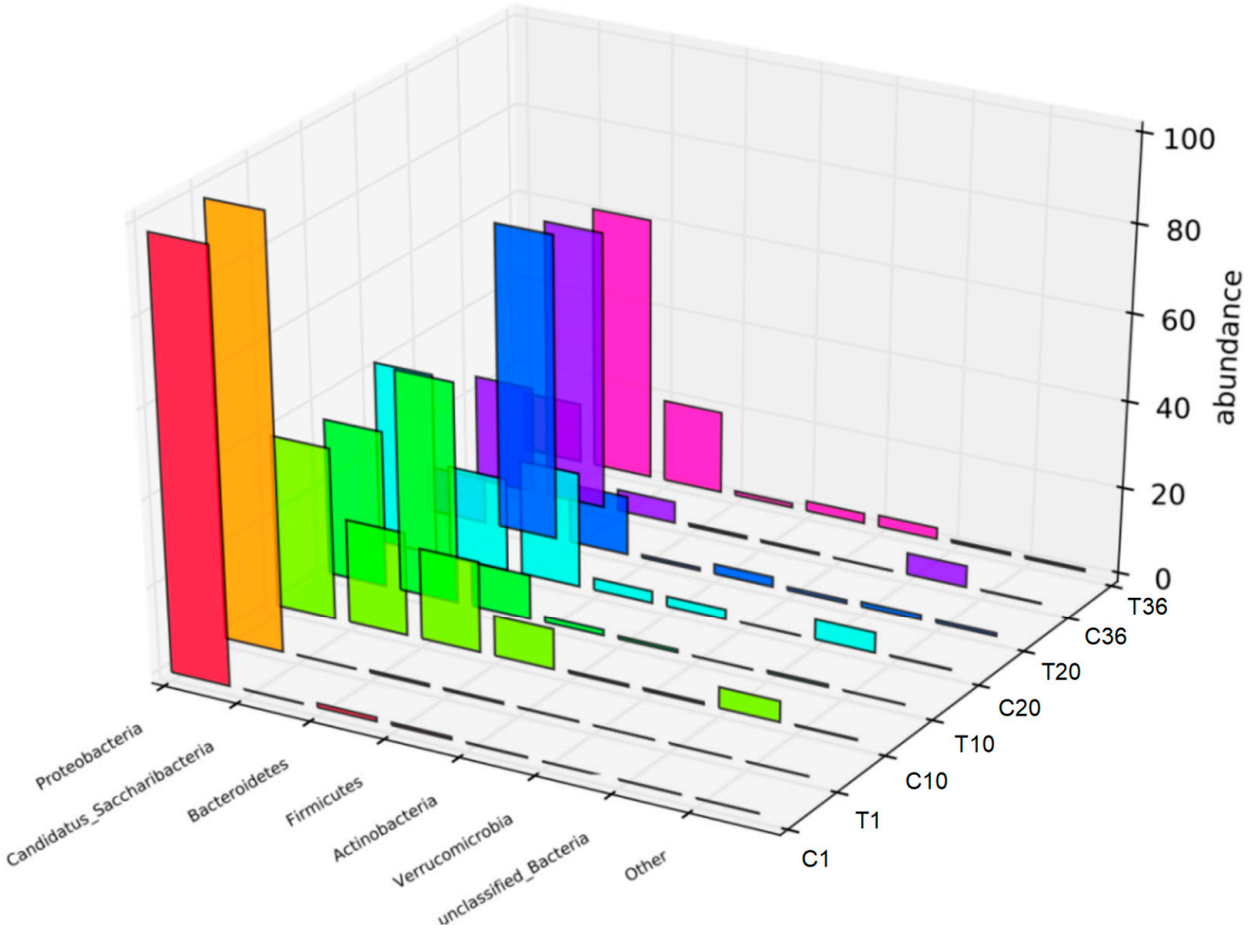

Figure 5. Bacterial diversity in activated sludge on a phyla level. C and T represent the control and broth-added treatments, respectively, and the number stands for the time (day) that the sample was obtained.

The dominant phyla of fungi were identified as *Ascomycota*, *Basidiomycota*, *Rozellomycota*, and unclassified phylum (Figure 7), and *Ascomycota* showed the highest abundance during the domestication process. These fungal phyla were widely identified in wastewater treatment plants [45]. The dynamic of the dominant genera (>1%) during the domestication is shown in Figure 8. Initially, *Aspergillus* occupied the dominant position in the activated sludge, which was replaced by *Dipodascus* and unclassified *Dipodascaceae* in the samples on day 10. It has been reported that *Dipodascaceae* can promote cellulose degradation in sawdust [46], which is beneficial for fermentation. The abundance of *Dipodascus* and unclassified *Dipodascaceae* decreased during the further domestication process, and the final fungal community was assembled with *Meyerozyma* and *Fusarium* as the dominant species. *Fusarium* species, such as *Fusarium solani*, was proven to show independent nitrification and denitrification abilities. It can be said that the fermentation broth showed a less obvious effect on the dominant fungal species.

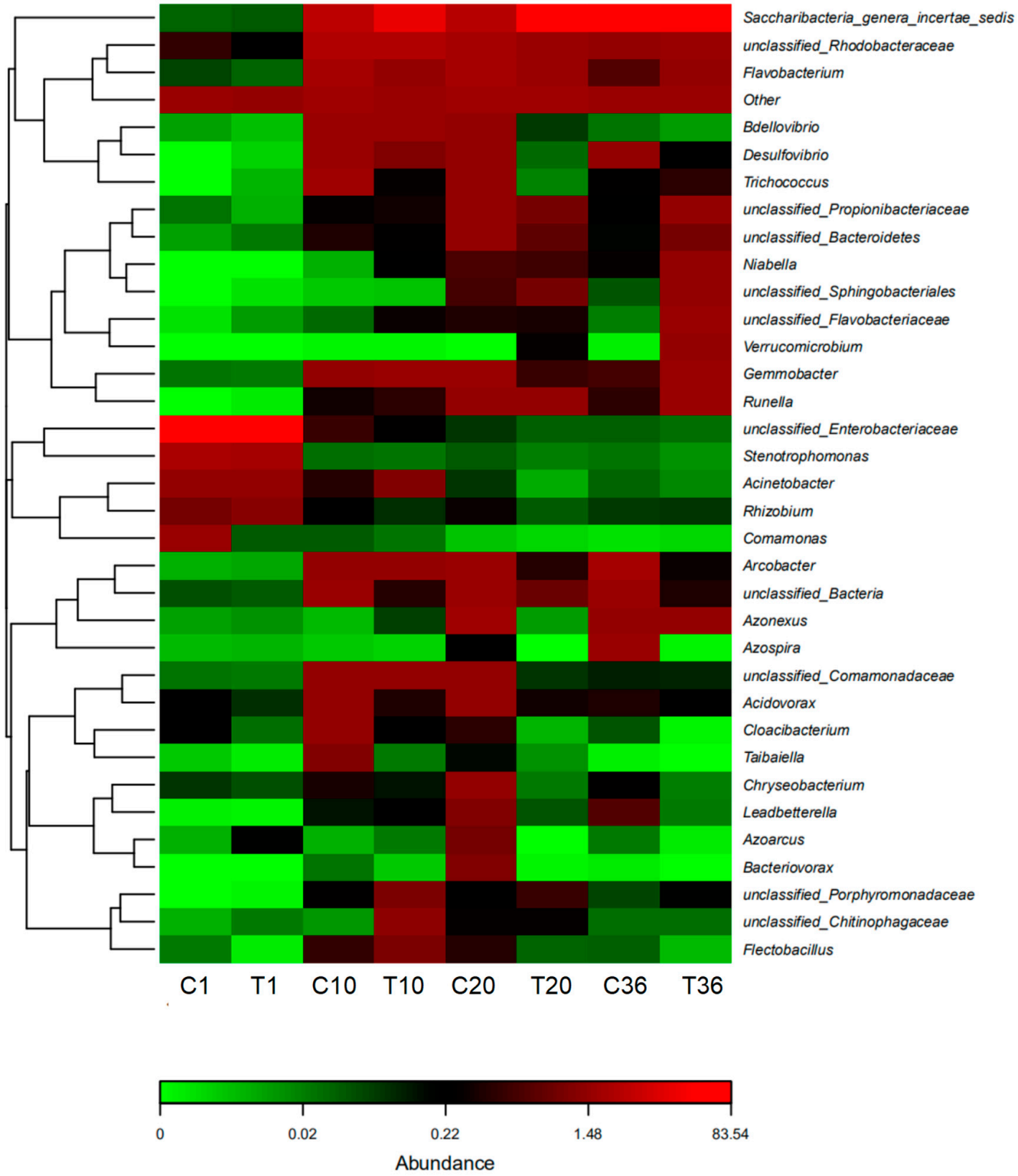

Figure 6. Bacterial diversity in the activated sludge on the genus level. C and T represent the control and broth-added treatment, respectively, and the number stands for the time (day) that the sample was obtained.

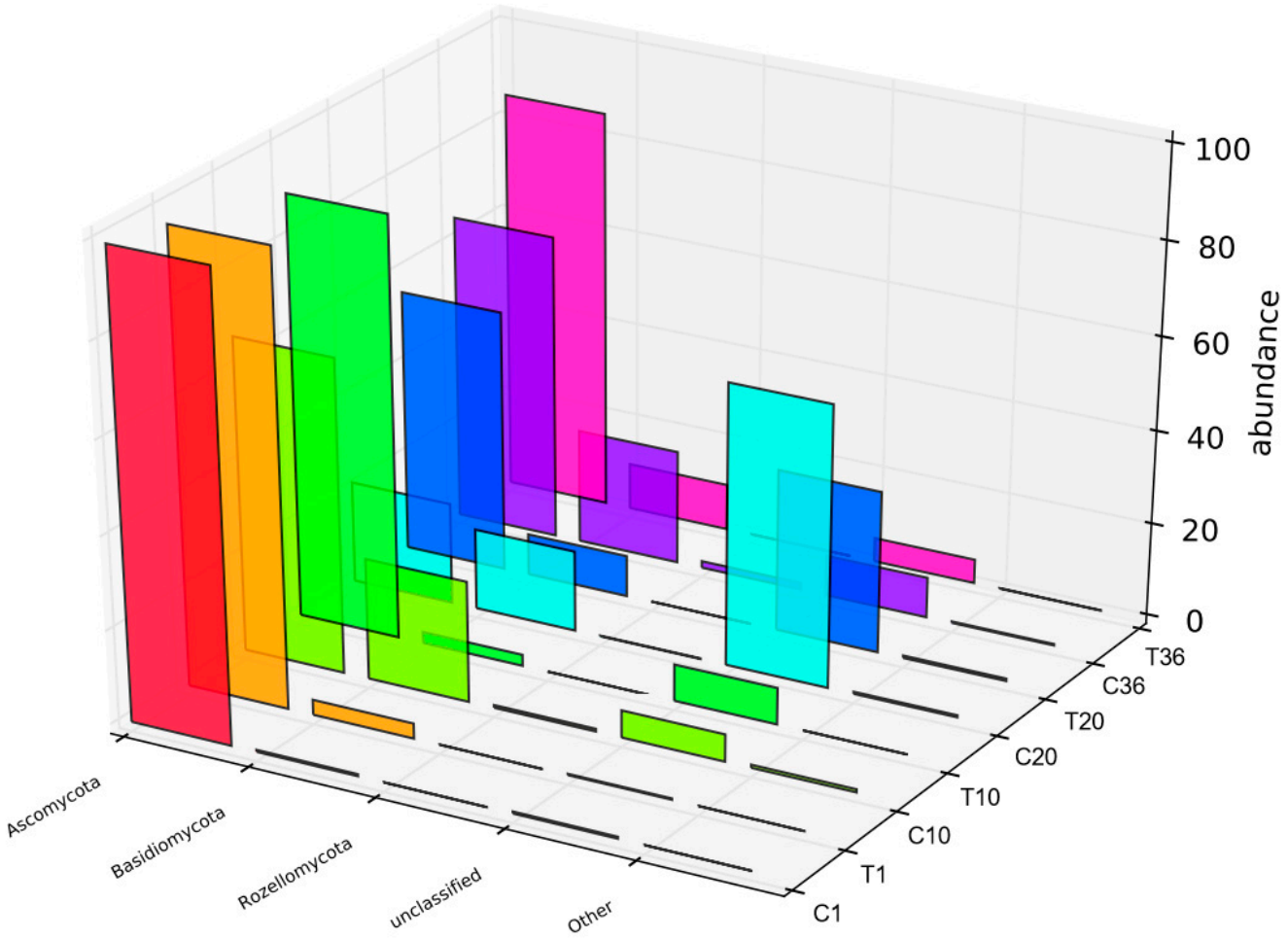

Figure 7. Fungal diversity in the activated sludge on the phyla level. C and T represent the control and broth-added treatment, respectively, and the number stands for the time (day) that the sample was obtained.

3.6. Environmental and Economic Benefits

It is estimated that each kilogram of fresh mushroom would produce approximately 5 kg of SMS [19]; thus, it is not only a challenge but also a huge fortune to recycle such amounts of waste. The economic benefit was evaluated according to the following rough calculation based on the COD concentration. The market price of SMS and glucose was about CNY 90.0 and CNY 1500/ton, respectively. To replace the COD from glucose (calculated as 1000 mg/L) with fermentation broth (20,000 mg/L), 12.5 kg of SMS was required for a 1000 L wastewater treatment. The required material cost was CNY 1.125 and CNY 1.5 for glucose. Besides the economic benefit, SMS recycling also avoids the environmental pollution caused by the incineration of SMS. Thus, using SMS to prepare the fermentation broth for wastewater treatment was eco-friendly and cost-effective.

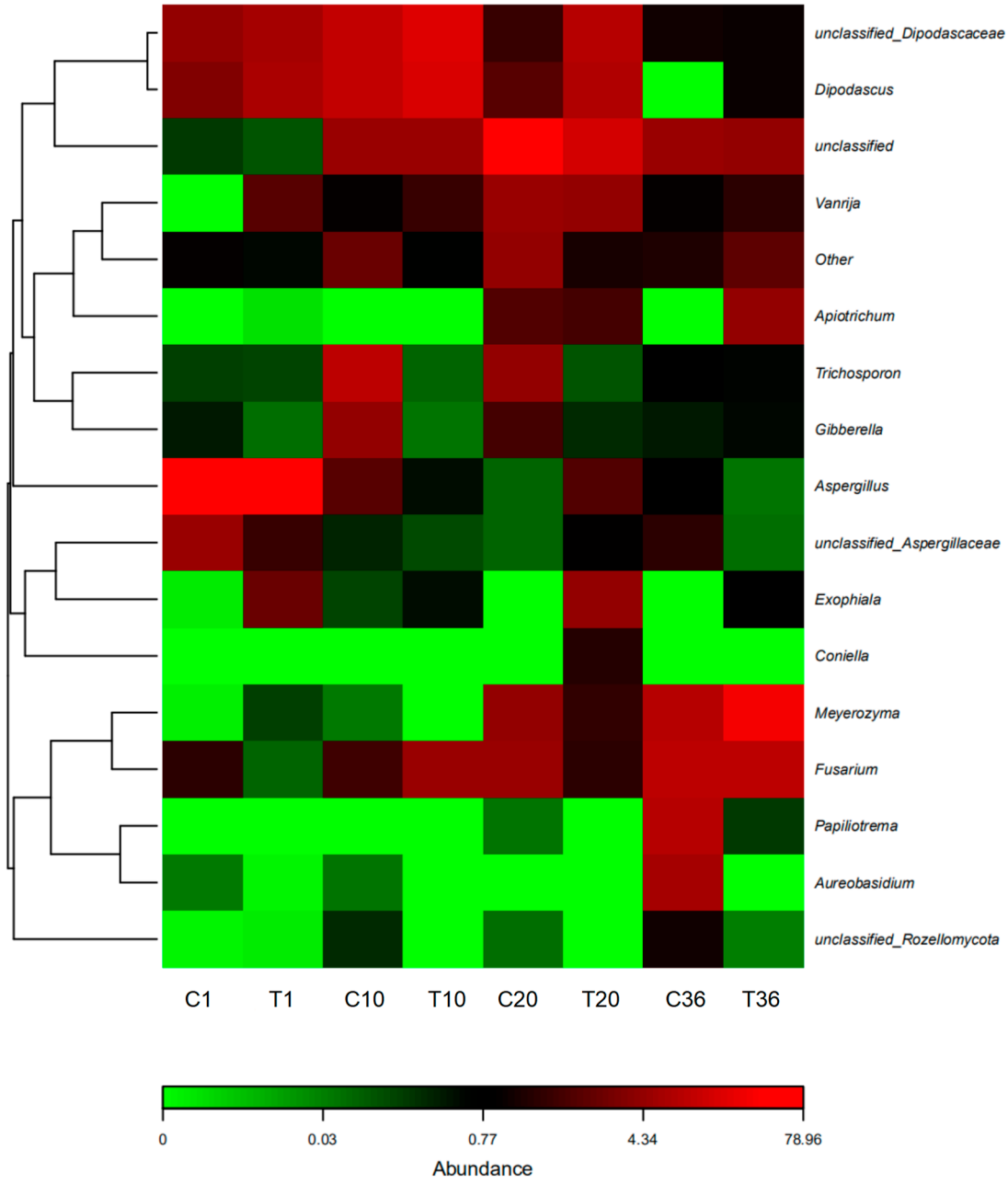

Figure 8. Fungal diversity in the activated sludge on the genus level. C and T represent the control and broth-added treatment, respectively, and the number stands for the time (day) that the sample was obtained.

4. Conclusions

In the present study, we employed SMS fermentation broth as an external carbon source for a nitrogen removal SBR system. The fermentation broth contains various nutrients such as proteins, polysaccharides, and organic acids, as well as mineral elements. In the fermentation broth, the dominant bacteria were *Rhodobacter*, *Lactobacillus*, and *Pseudomonas*, and the dominant fungi were *Saccharomycetales* and *Gymnopilus dilepis*. Compared with commercial external carbon sources, fermentation broth inhibited the nitrogen removal activity at the early stage, but a higher EPS formation and recovery phenomenon of nitrogen removal ability were observed after domestication for as long as 30 days. Microbial communities were highly influenced when 20% fermentation broth was added. As domestication proceeded, similar dominant species in the control and broth-feeding treatments

were observed in both of the two groups. In general, SMS fermentation broth can be used as an external carbon source for nitrogen bioremoval.

Author Contributions: Data curation, W.Z. and X.B.; funding acquisition, R.C. and Y.Y.; investigation, W.Z. and X.B.; resources, Y.Y.; supervision, Y.Y.; writing-original draft, R.C.; writing-review and editing, Y.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the Major Program for Science and Technology Innovation of Wenzhou City (ZG2022038), the Public Research Project of Zhejiang Province (LQ23E090001), the Science and Technology Projects of Wenzhou City (S20220012), and the National Natural Science Foundation of China (42207433).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

Abbreviation

SMS	spent mushroom substrates
C/N	carbon–nitrogen ratio
EPS	extracellular polymeric substance
SBR	sequencing batch reactor
COD	chemical oxygen demand
qPCR	quantitative polymerase chain reaction
PN	extracellular protein
PS	extracellular polysaccharides
UV-vis	ultraviolet–visible spectrophotometer
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatography mass spectrometry
ICP-OES	inductively coupled plasma emission spectrometry

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