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*Original Research*

# Simulated Ammonia Nitrogen Wastewater Treated with Spent Mushroom Compost in a Laboratory Bioreactor

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**Abstract**

The simulated ammonia nitrogen wastewater was treated with spent mushroom compost (SMC) in a laboratory bioreactor (LBR), and shifts in microbial diversity in LBR were conducted by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE). We found that the removal efficiencies of $NH_4^+$-N and COD reached 73.4% and 61.7%, respectively, and that simultaneous nitrification and denitrification (SND) was observed during the process of $NH_4^+$-N removal. In addition, we observed that there were no obvious changes in microbial diversity shifts, and that the predominant four isolates were identified as *Flavobacterium johnsoniae, Sphinbacterium multivorum*, *Comamonas sp.*, and *Rhizobium sp.*, which functioned together and played a critical role in treating simulated ammonia nitrogen wastewater in LBR. Overall, SMC could provide both carbon sources and indigenous functional microorganisms for nitrogen removal, indicating that SMC has potential for wastewater treatment.

**Keywords**: ammonia nitrogen wastewater, spent mushroom compost, laboratory bioreactor, simultaneous nitrification and denitrification, functional bacteria

## Introduction

Spent mushroom compost (SMC) is a waste material that is left after mushroom picking. The mushroom industry is expanding worldwide because of the ever-increasing demand for the protein-rich food, which produces a large amount of SMC. Statistically, world mushroom production has reached a level greater than 25 mt. As the current largest mushroom producer, China has attained more than 20 mt and accounted for more than 80% of the world's mushroom production [1]. It has been reported that producing 1 kg of mushrooms will generate 5 kg of SMC [2], and thus mushroom production is facing a problem of how to treat SMC. The problem has become especially serious in China, as the country's farmers have been intent on enhancing SMC management. Generally, the majority of SMC is disposed of as agricultural waste except for a small amount being used in horticulture, which gives further rise to environmental deterioration.

Undoubtedly, the efficient solution is to increase SMC demand by exploring its new applications. As a matter of

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fact, SMC has been applied in environmental remediation such as heavy metals biosorption [3], acid mine drainage treatment [4], spilled petroleum removal [5], chemically polluted soils remediation [6], metsulfuron methyl degradation [7], and phenolic compounds oxidation [8]. Besides, SMC also has been utilized to produce many kinds of value-added products such as biogas [9], bulk enzymes [10-11], organic fertilizers [12], and energy feedstock [13]. However, using SMC to treat wastewater containing ammonia nitrogen has not been reported. It is well known that the carbon source plays a critical role in nitrogen removal through denitrification or simultaneous nitrification and denitrification (SND) [14-16]. Of these carbon sources, liquid carbon is easily employed but could generate secondary pollution, whereas a solid carbon source is difficult to be employed, but if it works won't cause secondary pollution. As a waste material, SMC comprises not only both liquid and solid carbon sources, but also some other ingredients favorable for nitrogen removal, such as trace elements and indigenous microorganisms. Accordingly, it is possible to use SMC to treat nitrogen wastewater.

The purpose of this study was to evaluate the potential for using SMC to treat ammonia nitrogen wastewater in a laboratory bioreactor (LBR). Some important factors including the ratio of SMC to water, temperature, rotation speed, and pH were assessed. Furthermore, shifts in the microbial community were investigated by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and those predominant functional bacteria were identified. The data from this work may find a new way to use waste to treat waste.

## Materials and Methods

### Pretreating SMC

The SMC of *Hypsizygus marmoreus* was provided by Fuquanxin Edible Fungi Limited Company in the city of Gutian, Fujian, China. The bag of randomly obtained SMC was stripped off and the bud on the surface was picked out. After the mildewed and decayed parts were cut off, SMC was stored at -20ºC.

### Batch Experiments

Some important factors that might affect SMC treatment were investigated through batch experiments, all of which were conducted for five days in a 250 ml of bottle containing 100 ml of cultures at an initial concentration of $NH_4^+$-N ranging from 50 to 100 mg/L. These factors included the ratio of SMC to water, pH, temperature, and rotation speed. After being filled with cultures, the bottle was sealed with a rubber stopper with air permeability, and samples were taken out periodically. Samples were firstly centrifuged for 3 min at 12,000 rpm, and then the resultant supernatants were stored at -20ºC for further analysis.

### Experimental Set-up

Fig. 1 shows the schematic diagram of the laboratory bioreactor for nitrogen removal. This column bioreactor (with a maximum volume of 5 L) was equipped with an aerator, a dissolved oxygen (DO) monitor, and a pH and temperature control system. Simulated ammonia nitrogen wastewater (SANW) was used, the ingredients of which are listed in Table 1. Initial pH, temperature, ratio of SMC to SANW, and airflow rate were controlled at 7.0, 37ºC, 5%, and 0.8 L/min, respectively. Samples were taken out periodically. After being centrifuged for 3 min at 12,000 rpm the resultant supernatants were stored at -20ºC for further analysis.

### DNA Extraction, Polymerase Chain Reaction, and Denaturing Gradient Gel Electrophoresis

1.5 ml of cultures from LBR was centrifuged for 3 min at 12,000 rpm. The pellet was used for DNA extraction with an Easy Pure Genomic DNA Kit (TransGen Biotech, Beijing, China) according to manufacturer's instructions. Extracted DNA was dissolved in the elution buffer provided by the kits and quantified by measuring its absorbance at 260 nm with a spectrophotometer. The 1% agarose gel electrophoresis was used to analyze the quality of the extracted DNA.

In order to analyze the microbial diversity in LBR during the whole experiment, polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) was conducted on the cultures in SBR. The above-extracted DNA was used as a template to amplify the 16s RNA genes. PCR was performed in a Mastercycler gradient (Eppendorf 5331, Germany) using the following primers: GC341F (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G CCT ACG GG A GGC AGC AG-3′) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′). DGGE was carried out in a Universal Mutation Detection System (BIO-RAD DCodeTM, USA). The gel contained a gradient of denaturant ranging from 30% to 60% (100% denaturant is 7 M urea and 40% deionized formamide). DGGE was run at 200 V for 5 h at 60ºC. After electrophoresis, the gel was stained with GoldView II (Solarbio, Beijing, China) for 30 min and viewed with a UV transilluminator (BIO-RAD, Italy).

Table 1. Composition of simulated $NH_4^+$-N wastewater.

| Ingredients | Concentration (mg/L) |
|---|---|
| $NH_4Cl$ | 500 |
| Glucose | 400 |
| $MgSO_4$ | 400 |
| $NaHCO_3$ | 500 |
| $KH_2PO_4$ | 70 |

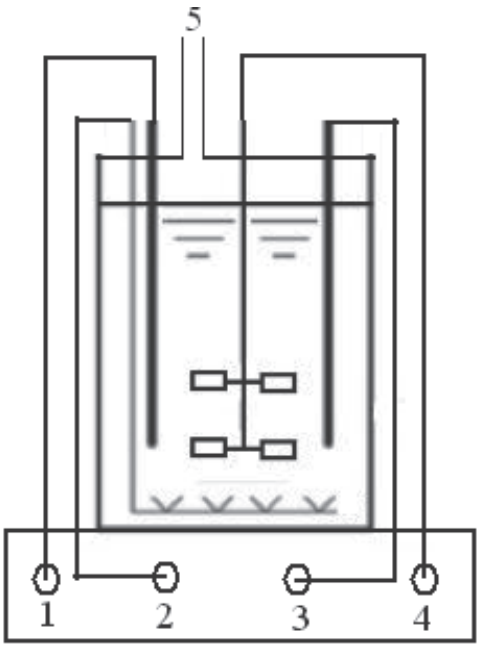


Fig. 1. Schematic diagram of the laboratory bioreactor for nitrogen removal. 1. pH meter, 2. Aerator, 3. Thermometer, 4. Stirrer, 5. Air outlet.

### Sequencing and Phylogenetic Tree Construction

The main strands on DGGE were cut down and then cloned into a pEASY T1 vector (TransGen Biotech, Beijing, China). The resultant vector was transformed into DH5α. After being incubated for about 16 h, clones with the correct insert were sent to Sangon Co., Ltd. for sequencing. The sequences were searched against GenBank using BLAST search option accessible from the homepage at the National Center for Biotechnology Information (*www.ncbi.nlm.nih.gov*). The MEGA 4.0 version software was used to check alignment and construct the the phylogenetic tree.

### Analytical Methods

$NH_4^+$-N, $NO_2^-$-N and COD were determined based on standard procedures as described in the Standard Methods [17].

## Results and Discussion

### Effect of the SMC-to-water Ratio on Nitrogen Removal

Usually there are plenty of various ingredients in SMC. In particular, it includes both solid and solvable carbon sources as described above. The solvable matters, especially some organic matters (expressed as COD), can easily lead to secondary pollution if they can't be consumed completely. Therefore, the ratio of SMC to water in a gradient ranging from 3% to 8% was examined to investigate its effect on nitrogen removal, and results were shown in Fig. 2a. The residual $NH_4^+$-N at 120h under conditions of 3%, 4%, 5%, 6%, 7%, and 8% was 28.6, 25, 23.2,19.1, 11, and 13.7 mg/L, respectively. It seems that the higher the ratio of SMC to water, the more the ammonia nitrogen was removed if SMC was controlled at less than 7%. Once the ratio of SMC to water exceeded that value, the removal efficiency of $NH_4^+$-N was decreased. The following two reasons might be responsible for this result. First of all, a high concentration of SMC contained more organic nitrogen sources that could be utilized by microorganisms to produce more $NH_4^+$-N. In addition, an appropriate COD/N is critical for nitrogen removal, and probably more SMC generated an unsuitable COD/N. However, a certain concentration of $NO_2^-$-N, though not very high, was also detected, achieving 0.27, 0.34, 0.4, 0.5, 0.61, and 0.65 mg/L when the ratio of SMC to water was 3%, 4%, 5%, 6%, 7%, and 8%, respectively. These results suggest that $NH_4^+$-N removal was possibly ascribed to simultaneous nitrification and denitrification (SND), which plays an important role in the nitrogen cycle and has attracted more and more attention [18-20]. Also, it could be seen that the higher the ratio of SMC to water, the more nitrite nitrogen accumulated. On the whole, considering the secondary pollution and nitrite accumulation, 5% SMC was selected to carry out the following experiments.

### Effect of Temperature on Nitrogen Removal by 5% SMC

Fig. 2b shows the effect of temperature on nitrogen removal by 5% SMC. Clearly, the most favorable temperature for nitrogen removal was 37°C, at which point the concentration of $NH_4^+$-N decreased from 111.8 mg/L at 0h to 75.4 mg/L at 120h, whereas other temperatures did not have significant effects on the removal of $NH_4^+$-N. At 37°C, the removal efficiency (RE) of nitrogen was about 32%, which was much lower than that in Fig. 2a. By contrast, as far as $NO_2^-$-N was concerned, the average concentration accumulated was much more than that in Fig. 2a. These phenomena were mainly attributable to the high concentration of initial $NH_4^+$-N, which not only inhibited nitrification but also contributed to nitrite accumulation.

A lot of reports have revealed that temperature was an important factor affecting nitrification and denitrification. For example, *Chelatococcus daeguensis* TAD1 was demonstrated to be an aerobic denitrifier that had the capability of removing 122.7 and 71.7 mg/L $NH_4^+$-N by 18 h at 50 and 30°C, respectively [21]. *Providencia rettgeri* YL was also an aerobic heterotrophic nitrifying-denitrifying bacterium, the ammonium removal of which occurred rapidly with 100% $NH_4^+$-N being removed within 12 h of incubation at temperatures ranging from 20 to 30ºC, whereas there were almost no changes in $NH_4^+$-N removal at 10 and 40°C [22]. In the current study, a different result could be seen that 37ºC was the best temperature for nitrogen removal by 5% SMC.

Probably, this should be ascribed to the various indigenous microorganisms inhabiting SMC.

## Effects of pH on Nitrogen Removal by 5% SMC

In order to evaluate the effect of pH on ammonia nitrogen removal, pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 were selected, and results were displayed in Fig. 2c. With regard to $NH_4^+$-N removal there were almost no obvious changes in the selected pHs except for pH 6.0. At pH 7.5, $NH_4^+$-N was reduced from 56.2 mg/L at 0 h to 18.4 mg/l at 120 h, whereas at pH 6.0 it was reduced from 56.1 mg/L at 0 h to 31.3 mg/L at 120 h, suggesting that acid environment was unfavorable for nitrogen removal by 5% SMC. For nitrite accumulation, all the selected pHs exhibited the same changing trend, and the final concentration of $NO_2^-$-N was only about 0.4 mg/L. However, it was reported that nitrite accumulation ratio (NAR) could be increased from 1.68±1.51 to 35.46±7.86% when increasing the pH values from 7.5 to 8.3 due to the increased free ammonia concentration [23], which is obviously inconsistent with the result obtained in the present study. Interestingly, another report showed that it was advantageous to the treatment of high-strength ammonia animal manure wastewater when the pH was about 8.0 [24]. Probably the above different results could also be attributable to the various microorganisms inhabiting the different experimental environments.

## Effect of Rotation Speed on Nitrogen Removal by 5% SMC

Fig. 2d depicted the time courses of $NH_4^+$-N and $NO_2^-$-N at different rotation speeds (rpm). It can be seen that different rotation speeds exhibited no distinct differences in the removal of $NH_4^+$-N. But the changing trends of nitrite accumulation under conditions of different rotation speeds were unlike $NH_4^+$-N. At 180 rpm, $NO_2^-$-N increased from 0.87 mg/L at 12h to 1.29 mg/L at 48 h, then decreased to 0.9 mg/L at 108 h, while the final $NO_2^-$-N concentration at 150 and 210 rpm was about 1.4 mg/L, indicating that the optimum rotation speed seemed to be 180 rpm and appropriate dissolved oxygen (DO) is critical to nitrogen removal.

## Simulated $NH_4^+$-N Wastewater Treated with 5% SMC in Laboratory Bioreactor

To access the feasibility of applying SMC we employed simulated ammonia nitrogen wastewater that was treated with 5% SMC in LBR. Fig. 3a exhibits the flow chart including DO (%), pH, rotation speed, temperature, and airflow rate in LBR. Fig. 3b displayed the time courses of

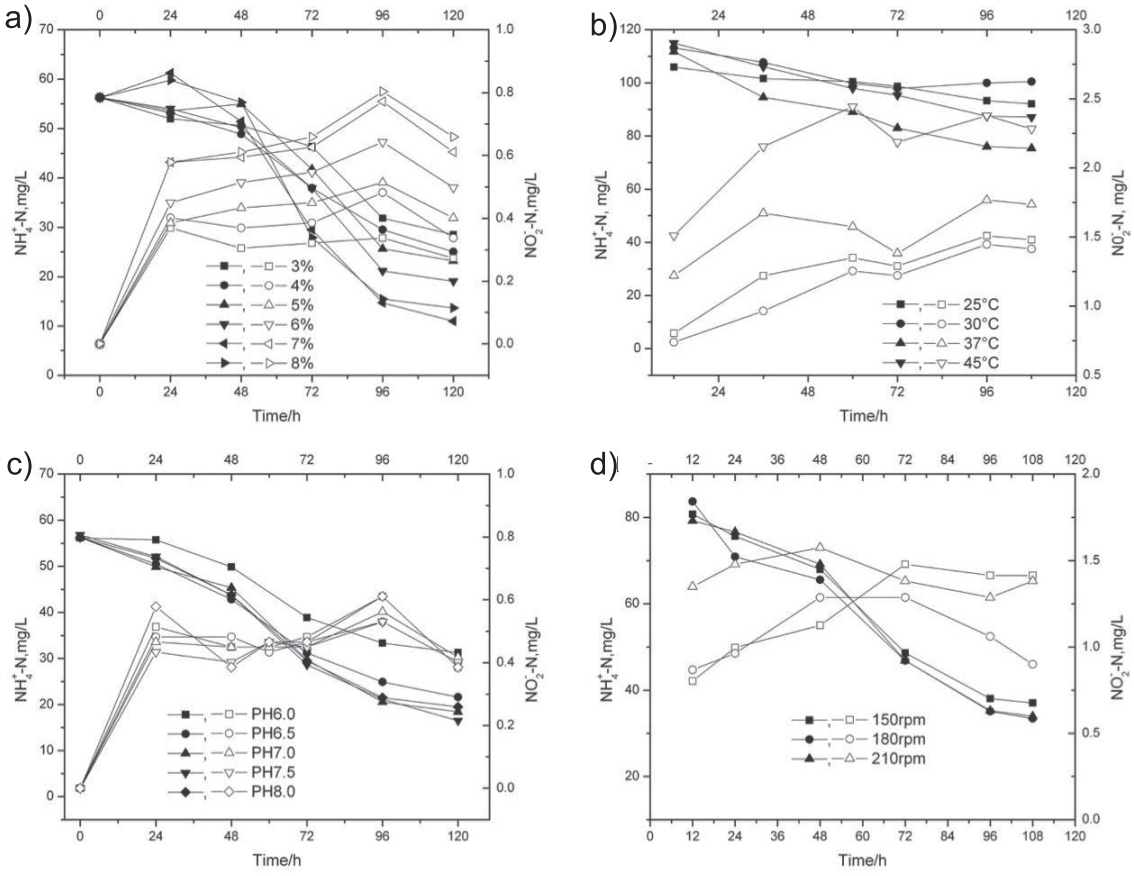


Fig. 2. Effect of the SMC-to-water ratio a), temperature b), pH c), and rpm d) on nitrogen removal; solid and hollow icons represent $NH_4^+$-N and $NO_2^-$-N, respectively.

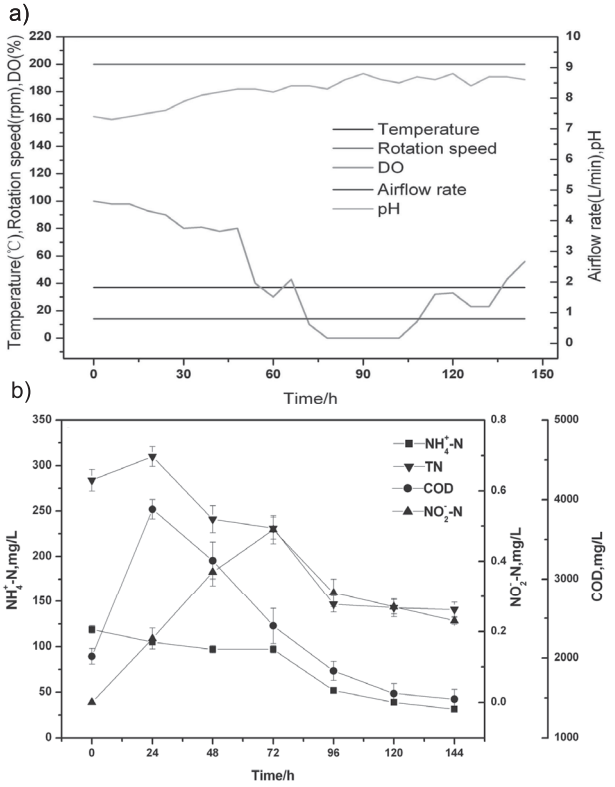


Fig. 3. Pollutant removal with 5% SMC in the LBR: a) Flow chart including DO (%), pH, rotation speed, temperature, and airflow rate; b) Time courses including $NH_4^+$-N, $NO_2^-$N, and COD.

$NH_4^+$-N, $NO_2^-$-N, TN (total nitrogen), and COD. During the first 48h, $NH_4^+$-N concentration lowered from the initial 119 mg/L to 97 mg/L, but there was no change within the following 24h. The possible reason responsible for this was that there were a lot of organic nitrogen sources left by the metabolism of mushrooms, which were then utilized by the indigenous microorganisms to release ammonia. The resultant ammonia counteracted those consumed, and thus $NH_4^+$-N leveled off. Subsequently, $NH_4^+$-N concentration decreased rapidly from 97 mg/L at 72 h to 52 mg/L at 96 h, then to 31.6 mg/L at 144 h with 73.4% of total $NH_4^+$-N being removed. Similar to batch experiments, a peak of nitrite accumulation (though not high) occurred at 72 h, indicating that simultaneous nitrification and denitrification (SND) was also a major way to remove $NH_4^+$-N in the current LBR.

As far as pH was concerned, it rose from an initial 7.4 to 8.5 at 48 h and then remained at that level. In general, nitrification is conducive to pH decreases whereas denitrification is conducive to pH increases, which has been reported by some researchers [25-26]. However, the pH change shown in Fig. 3a was contradictory to what has been reported previously. This is mainly because of complex components and indigenous microorganisms involved in SMC.

Additionally, it is worth noticing the change of COD. As exhibited in the figure, COD increased sharply from 2,021 mg/L at 0 h to 3,880 mg/L at 24 h, then was degraded to 1,485 mg/L at 144 h with about 61.7% of total COD being removed. Two reasons might explain this phenomenon:

1. As discussed above, lots of solvable carbon sources existed in SMC after the mushroom was picked, which included not only nutrients unemployed in the medium but also metabolites by the mushroom. This matter, most of which was combined with the solids, was dissolved slowly with time elapsing, especially in the first 24 h.
2. It is well known that there are a certain number of extracellular enzymes in SMC (such as cellulase, xylanase, and ligninase) that have been purified from SMC by some researchers [11, 27-28]. These enzymes were active and could slowly hydrolyze those solid carbon sources to release solvable carbon sources.

As far as TN was concerned, a trend similar with COD could be seen from Fig. 3b that TN rose from an initial 284 mg/L to 310 mg/L at 24 h, then decreased step by step to 141 mg/L at 144 h with about 50.4% of TN being removed. Also, this phenomenon was attributed to the fact that those matters combined with solids were released progressively during the first 24h.

## Microbial Diversity Shifts in LBR and Isolate Identification

Simulated ammonia nitrogen wastewater treated with 5% SMC was cultured for six days in SBR, and shifts in microbial diversity were examined by PCR-DGGE. As shown in Fig. 4, there were no obvious changes in the whole diversity, except that from the third day on the SU1 strain lessened sharply and almost disappeared in the sixth day, whereas the CD1 strain appeared and always inhabited LBR.

The predominant 4 isolates FU2, RM3, SU1, and CD1 (Fig. 4) were identified using 16s rRNA gene sequencing. Based on comparison of the 16s rRNA gene sequence of the

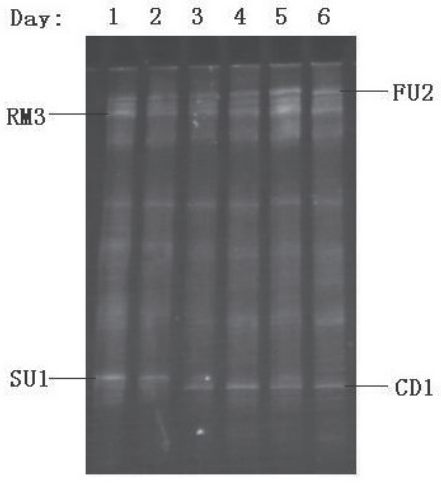


Fig. 4. Shifts in the microbial diversity in SBR by PCR-DGGE. The four main predominant strains (FU2, RM3, SU1, and CD1) were identified using 16s rRNA gene sequencing.

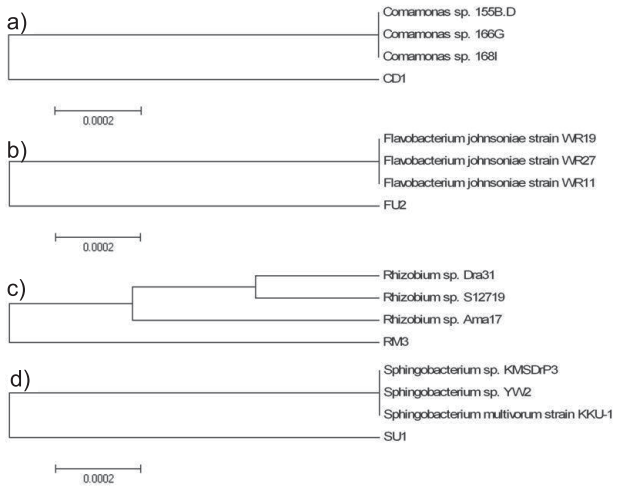


Fig. 5. Phylogenetic trees (neighbor-joining) were constructed based on 16s rRNA sequences and other related sequences. a) relationship of CD1 with related strains, b) relationship of FU2 with related strains, c) relationship of RM3 with related strains, d) relationship of SU1 with related strains.

isolates with that of other bacteria existing in the GenBank database, the identification showed that isolates FU2, SU1, and CD1 shared 99% similarity with *Flavobacterium johnsoniae* WR11, *Sphinbacterium multivorum* KKU-1, and *Comamonas sp.* 168I, respectively, whereas RM3 shared 100% similarity with *Rhizobium sp.* Ama17. The phylogenetic trees based on the 16s rRNA gene sequences of our isolates and other related sequences were constructed, and results are shown in Fig. 5.

*Comamonas sp.* is well known to be an aerobic denitrifier that was able to consume oxygen and nitrate simultaneously with the production of nitrogen and without built-up nitrite [29]. A study on its denitrifying enzymatic system demonstrated that the nitrate and nitrite reductase were active and synthesized under aerobic conditions, and a double of nitrate reductase enzymatic system might exist [30]. When cultured in a continuous chemostat, *Comamonas sp*. could consume the oxygen and produce the nitrogen at both low and high oxygen concentrations, implying that the four denitrifying enzymes in this strain could be synthesized and were active under various aerobic conditions [31]. In the current study, results that there was almost no nitrite accumulation (Fig. 3b) and CD1 predominated from the third day were in good agreement with those reported in the studies mentioned above.

*Rhizobia* are bacteria with the unique ability to establish a $N_2$-fixing symbiosis, but some species were reported to be able to use nitrate to denitrify under certain conditions [32-33]. Although the ability to denitrify by *Flavobacterium johnsoniae* was scarcely reported, it could express the xylanase gene to degrade xylan [34], which further helped to explain why COD rose in the first 24 h (Fig. 3). Likewise, there were almost no reports on the denitrifying ability in *Sphinbacterium multivorum*, but it still has potential to be applied in environmental remediation [35].

## Conclusions

The simulated ammonia nitrogen wastewater was first treated with the spent mushroom compost in a laboratory bioreactor. The results demonstrated that $NH_4^+$-N and COD could be removed simultaneously. Although no obvious shifts in microbial diversity were seen, the predominant four isolates could cooperate with each other during the whole experiments. Overall, results obtained in this study suggested that the spent mushroom compost has the potential to be applied in wastewater treatment.

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