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Original Research

Mixotrophic Denitrification Desulfurization Wastewater Treatment Process: Bioreactor Performance and Analysis of the Microbial Community

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Abstract

The mixotrophic denitrification desulfurization process was adopted in an upflow attached-growth bioreactor for wastewater treatment. The microorganisms in the system were acclimated to remove sulfide, nitrate, nitrite, and organics simultaneously while the contaminants were finally converted to element sulfur, nitrogen gas, and carbon dioxide. The 16S rDNA clone library technique was utilized to investigate the characteristics of the microbial community within this mixotrophic environment. The results showed that *Azoarcus* was the main sulfur-based denitrification desulfurization bacteria and had a relative abundance of 8.0% in this system. It used sulfide as electron donors, with both nitrate and nitrite as eletron acceptors. *Thauera*, *Vulcanibacillus*, and *Paracoccus* were the main heterotrophic denitrification bacteria and had the relative abundances of 29.5%, 9.82%, and 1.78%, respectively. They utilized arganics as electron donors, with nitrate or nitrite as electron acceptors. The high removal efficiencies of contaminants were attributed to the interaction of these two main species.

Keywords: microbial community, mixotrophic, sulfide, sulfur, nitrite

Introduction

The wastewater from chemical plants, fermentation factories, paper mills, and pharmacy industries contained high concentrations of ammonia/ammonium (NH_3/NH_4^+) and sulfate (SO_4^{2-}) [1], which would pollute natural water bodies if discharged directly without proper treatment [2]. The anaerobic treatment method was the common process for SO_4^{2-} removal, in which SO_4^{2-} was reduced to hydrogen sulfide (H_2S) by sulfate reducing bacteria (SRB) under anaerobic environments [3]. However, H_2S was soluble in water and could easily enter the cells of aquatic organisms, which reduced the intracellular redox potential, inactivated the enzyme, and seriously affected the growth metabolism of organisms. It could also cause air pollution if discharged into the atmosphere [4]. And NH_3/NH_4^+ contributed mainly to eutrophication of

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water bodies [5]. In addition, nitrate (NO_3^-), which was the product of the oxidation of NH_3/NH_4^+, would cause human methemoglobinemia if entering the human body [6]. Meanwhile, NO_3^- could be reduced to nitrite (NO_2^-) under anaerobic conditions. These nitrogen compounds had risks associated with toxicity and bad odors [7]. Therefore, an integrated wastewater treatment process was highly demanded for the simultaneous removal of nitrogenous and sulfurous compounds.

Last century, Driscol et al. proposed a biological denitrification process using sulfide (S^{2-}) as an electron donor [8], and some microorganisms were found to use NO_3^- as electron acceptors to oxidize S^{2-} to elemental sulfur (S) [9]. Up to now, the denitrification desulfurization process has been a novel and main method for simultaneous removal of nitrogenous and sulfurous compounds [10-11]. It has been proposed that there might be two main reactions in the denitrification desulfurization process with organic compounds being added to the system [12]: sulfide-based denitrification desulfurization, which used S^{2-} as the electron donor and NO_3^- and NO_2^- as the electron acceptors; and heterotrophic denitrification, which used organics as the electron donors and NO_3^- and NO_2^- as the electron acceptors. As this process is based on the metabolism of microorganisms, the exploration of the sulfide-based denitrification desulfurization functional bacteria have recently become the focus of research.

Cai et al. [13] isolated two strains with the function of denitrification desulfurization from the sludges in a long-time running bioreactor treating nitrogenous and sulfurous compounds. Both strains were affiliated with *Bacillus* and were similar to *Bacillus Hemicellulosilytus* and *Bacillus Halodurans*. Chen et al. enriched and screened out 20 strains on selective media [14]. Among these 20 strains, two strains were able to remove more than 60% of NO_2^- and S^{2-}. They were facultative anaerobic and mixotrophic heterotrophic, and were similar to *Pseudomonas Fluorescens* and *Pseudomonas Eruginosa*. Gevertz et al. isolated two strains of denitrification desulfurization bacteria from the oil fields – both of which were strict chemoautotrophy bacteria [15]. They were similar to *Thiomicrospira Denitrifcans* and *Arcobacter sp.*, respectively. Chen et al. found that the mixed culture (*Pseudomonas Fluorescens* and *Pseudomonas Aeruginosa*) could achieve high removal rates of S^{2-}, NO_2, and NO_3^- [16].

In a word, the recent studies on denitrification desulfurization were mostly focused on system efficiency and effect factors, such as the ratio of carbon to sulfur, the concentrations of contaminants, and sulfide types [17]. The studies on denitrification desulfurization bacteria focused on the isolation and identification of pure strains. However, because the bacteria leading two types of denitrification both used nitrogenous compounds as electron acceptors and the competitive/collaborative relationship between them was not clear, it was necessary to promote a study on the microbial interaction principles and microbial community characteristics.

In this bioreactor, different kinds of contaminants were removed successfully: S^{2-} was predominantly oxidized to S, which could be collected for reuse [18]; the organics were further removed by heterotrophic denitrification; and NO_3^- and NO_2^- were reduced to nitrogen gas (N_2) instead of nitous oxide (N_2O), which could bring pollution to the atmosphere [19]. The object of this work is to explore the microbial community characteristics in the mixotrophic denitrification desulfurization system, to analyze the competitive/collaborative relationship between different functional bacteria, and to provide microbiological information for improving the treatment efficiency of this process.

Methods and Materials

Bioreactors and Inoculation

We used a 3.5 L anaerobic attached-growth reactor in column shape (Fig. 1). The reactor was inoculated with 1.5 L of the sludge collected from an anaerobic continuous stirred tank reactor (CSTR) used to treat NO_3^- and S^{2-} wastewater, giving the biomass a concentration of 16.5 MLVSSg/L. One peristaltic pump was used to feed artificial wastewater from the bottom of the reactor into the system and the other peristaltic pump was used to recirculate water to achieve uniform mixing. On top of the reactor a three-phase separator was used to separate the biogas, the sludge, and the effluent. Biogas was collected by a water-sealing tank. In order to increase the biomass inside the reactor, sponge cubes (8×8×8mm) were applied as attached-growth media, which were washed with distilled water for three times before use. The oxidation-reduction potential (ORP) detector and the pH detector were inserted into the reactor to test ORP and pH. A constant temperature of 30±1ºC inside the reactor was realized via temperature sensor connected with temperature controller (MWZK-02, China) and heating threads bonded around the reactor.

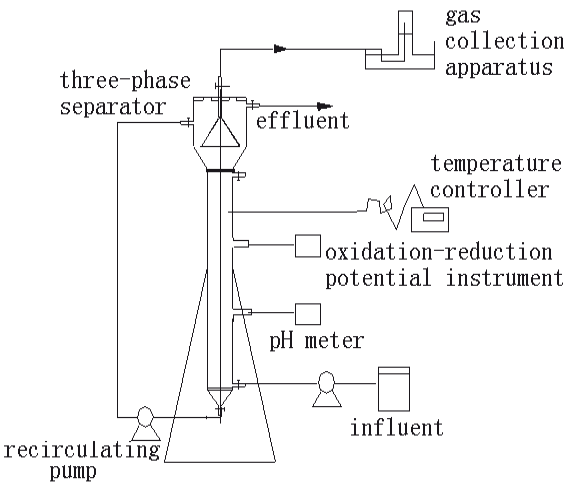

Fig. 1. Schematic view of upflow attached-growth bioreactor.

Chemical Analytical Methods

The liquid sample for analyzing was taken from the bioreactor daily. The sulfide was measured using the methylene blue spectrophotometric method (UV-2550, Japan). To measure SO_4^{2-}, NO_3^-, and NO_2^-, liquid samples were filtrated with a 0.45μm filter and injected into an ion chromatograph (DIONEX ICS 3000, USA) equipped with an inhibitory type conductivity detector and an Ionpac column (AG4A AS4A-SC, 4 mm). The flow rate of carrier liquid was 1.0 mL/min. N_2 and N_2O were analyzed by gas chromatography (Agilent 4890D, USA) equipped with a thermal conductivity detector and a molecular screen column (5Å). The temperatures of column, injector, and detector were 60ºC, 100ºC, and 100ºC, respectively. Measurements for the concentrations of organics (TOC) were taken by a TOC analyzing instrument (TOC-VCPH, Japan). The value of pH was measured by a pHs-3c pH meter (China). All the items mentioned above were analyzed according to APHA [20]. Sulfur was analyzed using a method described by Henshaw [21].

Substrates

Artificial wastewater containing sodium sulfide as electron donor, potassium nitrate and sodium nitrite as electron acceptors, glucose and sodium acetate as organic carbon sources, sodium bicarbonate as an inorganic carbon source, and potassium dihydrogen phosphate as the phosphorus source for bacteria growth were used as the feed to the reactor. The pH was adjusted to 7.0 using 1 mol/L hydrochloric acid. The concentrations of S^{2-}, TOC, NO_3^-, and NO_2^- were 389 mgS^{2-}-S/L, 144 mgC/L, 160 mgNO_3^--N/L, and 160 mgNO_2^--N/L, respectively. HRT decreased stepwise from 6.6 h, 4.1 h, to 3.3 h, and the influent loadings are shown in Table 1.

Biomass Samples Collection

For the purpose of microbial community study, biomass was collected directly from the anaerobic system using a tubing system to extract biomass at different points of the bioreactors. The biomass collected was stored in flasks at -80ºC until further DNA extraction.

DNA Extraction and PCR Amplification

Total DNA extraction of the samples was done using the E.Z.N.A. Soil DNA Kit (Omega Biotec, Norcross, GA, USA, D5625-01) according to the manufacturer's instructions. And the Agarose Gel electrophoresis was done by electrophoresis apparatus (DYCP-31DN, China). The PCR reaction was carried out in a 30 μL reaction volume with 15 μL Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA, M0491), 5 μL reaction buffer, 5 μL high GC buffer, 10 mM dNTP, 10 μM forward and reverse primers, and 1μL template DNA. Thermal cycling consisted of denaturation at 98ºC for 30s, followed by 25-27 cycles of 98ºC for 15s, 50ºC for 30s, 72ºC for 30s, and finally 72ºC for 5 min. The PCR products were analyzed on 2% agarose gel and purified using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA, P7589). The results of agarose electrophoresis are shown in Fig. 2.

Clone Library and Sequencing

The purified PCR products were cloned into T vector using the pUCm-T Vector Cloning Kit (Sangon Biotech, Shanghai, China, B522213), followed by tranformating competent E. coli cells, selecting positive recombination, and purifying it for further identification using a DNA sequencing analyzer (ABI 3730XL, USA). The sequencing results of bases were compared on EzBioCloud (www.ezbiocloud.net/eztaxon) to obtain the most similar strains.

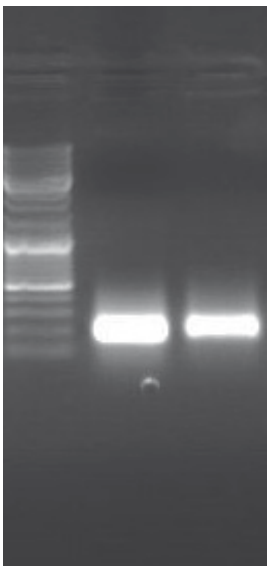

Fig. 2. Agarose gel electrophoretogram of PCR amplification.

Table 1. Influent contaminants.

Day	Nitrate	Nitrite	Sulfide	TOC	HRT
	mgN/(L·d)	mgN/(L·d)	mgS/(L·d)	mgC/(L·d)	h
1-24	579	579	1,407	521	6.6
25-48	928	928	2,254	835	4.1
49-115	1,160	1,160	2,819	1,044	3.3

Results

Simultaneous Removal of Contaminants

The bioreactor was operated for 115 days. The removal of S^{2-}, TOC, NO_3^-, and NO_2^- are shown in Figs 3 and 4. As the inoculation was from a well-sulfide and nitrate-treated anaerobic bioreactor, the removal efficiencies of S^{2-} and NO_3^- were high, at above 99%. However, at each start of HRT changing, TOC removal decreased sharply and later increased gradually to a steady value. Nevertheless, this fluctuation weakened gradually with decreasing HRT. And TOC removal efficiency could reach 87.6% at loading of 1,044 mgC/(L·d). This performance explained that the microorganisms were affected by influent shock load; but after the microorganisms were adapted to this kind of complicated environment, TOC removal efficiency came to be stable. When HRT was maintained at 6.6h, NO_2^- removal efficiency increased gradually from 75.4% to 95.2%. At the initial stage, as NO_2^- might have an inhibition effect on the microorganisms, nitrite removal efficiency was not high. Nevertheless, removal efficiency was enhanced after the microorganisms adapted to the environment.

The high removal efficiencies of contaminants were obtained in the bioreactor, which meant the microorganisms in the bioreactor could remove S^{2-}, NO_3^-, NO_2^-, and organics simultaneously. Therefore, the biofilms were collected from this system for analyzing the microbial community characteristics at this time.

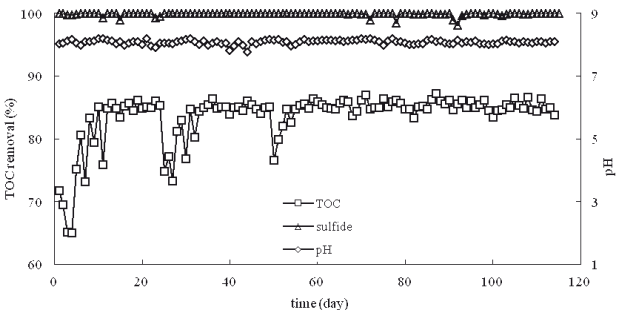

Fig. 3. Removal efficiencies of sulfide and TOC, and pH variation.

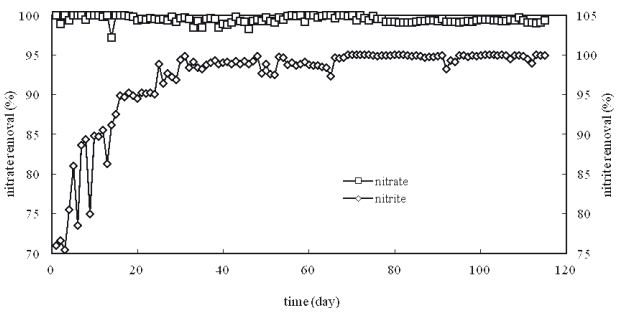

Fig 4. Removal efficiencies of nitrite and nitrate.

Sequencing and Microbial Diversity

The 112 bands' results of pyrosequencing of 16S rDNA gene revealed that this mixotrophic denitrification desulfurization system composed 12 main bacteria that were affiliated with three phyla. The relative abundances were 25.9% *Bacteroidetes*, 17.9% *Firmicutes*, and 44.6% *Proteobacteria* (Table 2).

As the contaminants were removed, the biodiversity not only acclimated but also acted as insurance for denitrification desulfurization system functions against loading changes. The main bacteria for the removal of sulfurous and nitrogenous compounds are listed in Table 3. On the genus level, *Thauera*, *Vulcanibacillus,* and *Paracoccus* were the heterotrphic bacteria for nitrogenous compound removal, whose relative abundances were 29.5%, 9.82%, and 1.78%, respectively. *Azoarcus* was the main simultaneous denitrification desulfurization bacteria in this system, whose relative abundance was 8.0%.

Discussion

The organics disappeared with sulfurous and nitrogenous compounds in the bioreactor. This simultaneous respiratory process could be explained in terms of the microbial diversity present in this system, where it could be possible to find groups of microorganisms simultaneously carrying out the biological reduction of NO_3^- and NO_2^- using glucose and S^{2-} as electron donors.

According to Tables 2 and 3, *Thauera*, *Paracoccus,* and *Vulcanibacillus* were the three main species for heterotrophic denitrification in this system. *Thauera* was in the highest abundance (32 bands), which were *T.phenylacetica*, *T.aminoaromatica*, *T.selenatis*, *T.m echernichensis*, and *T.aromatica*. *Thauera* was gram-negative bacteria, in rod shape with flagllum, facultative anaerobic, using NO_3^- as electron acceptor for denitrification under the anaerobic environment. Its optimum growth temperature was 25-30°C, and the optimum pH was 8. *T.phenylacetica* was in elliptic or short rod shape, and under anaerobic conditions it could reduce NO_3^- and NO_2^- to N_2 with no nitrogen fixation [22]. Its optimum growth temperature and pH were 28°C and 7-7.5, respectively. *T.aminoaromatica* was in short rod shape. Its optimum growth temperature was 28°C, while its optimum growth pH was 7-7.4. It was similar to *T.phenylacetica*, which could reduce NO_3^- or NO_2^- to N_2, and had no nitrogen fixation [22]. *T.selenatis* was found to be able to reduce NO_3^- to N_2O by heterotrophic denitrification process [23]. *T.aromatica* was also found to use organics as carbon sources, and NO_3^- as an electron acceptor in an anaerobic environment. NO_3^- was converted to NO_2^- at first, and then NO_2^- was further reduced to N_2O [24]. However, in this system no N_2O was detected in the biogas, which might be attributed to the degradation function of *T.mechernichensis*. Scholten et al. found that chemoheterotrophic denitrifying bacteria *T.mechernichensis* could use organics as carbon sources to reduce NO_3^- to N_2O and N_2 in an anaerobic

Table 2. Similar strains according to base comparison.

No. of Band	Similar strain	Similarity	Login number	Genus
2, 3, 41, 48, 63, 72, 74, 79, 89, 97, 114	*V.modesticaldus*	99.45%	AM050346	*Vulcanibacillus*
6, 94	*B.alcalophilus*	98.04%	X60603	*Bacillus*
16	*B.aryabhattai*	99.28%	EF114313	
14, 19, 46, 59, 66	*B.luteus*	98.46%	HE996968	
7, 8, 10, 37, 43, 55, 71, 77, 85, 87, 91, 93	*T.aminoaromatica*	99.98%	AMXD01000247	*Thauera*
12, 21, 25, 27, 35, 40, 45, 53, 54, 62, 69, 82, 96, 99, 110, 111, 112	*T.phenylacetica*	99.98%	AMXF01000339	
49	*T.selenatis*	98.37%	Y17591	
24, 88	*T.mechernichensis*	98.35%	Y17590	
109	*T.aromatica*	95.37%	X77118	
9, 47	*P.solventiborans*	99.18%	Y07705	*Paracoccus*
5, 34, 67, 83, 70, 104,108	*A.taiwanensis*	97.12%	GQ389714	*Azoarcus*
26, 38, 60, 68, 84, 116	*G.ferrihydriticua*	97.11%	DQ309326	*Geoalkalibacter*

Table 3. Characteristics of species for sulfur and nitrogen removal.

Genus	Morphological characteristics	Trophic mode
Vulcanibacillus	gram-positive bacteria, rod	strictly anaerobic, chemoheterotrophic, denitrification using NO_3^- as electron acceptor, no fermentation metabolism
Thauera	gram-negative bacteria, rod	facultative anaerobic, chemoheterotrophic, denitrification using denitrification using NO_3^- and NO_2^- as electron acceptors
Paracoccus	gram- negative bacteria, spherical or short rod	facultative anaerobic, chemotrophic heterotrophic or facultative chemoautotrophic, no fermentation metabolism, denitrification using NO_3^- as electron acceptors
Azoarcus	gram-negative, rod	nitrogen fixation, some strains can denitrify, among which *A.taiwanensis* is desulfurization and denitrification bacteria

environment [25]. N_2O was found to be further reduced to N_2 as well. Therefore, *Thauera* was the main heterotrophic denitrifying bacteria in this mixotrophic denitrification desulfurization system, which followed the chemical reactions shown in Equations (1) and (2). The five species of *Thauera* cooperated to reduced NO_3^- and NO_2^- to N_2O, and further converted N_2O to N_2, which could be discharged into the atmosphere.

$$6NO_3^- + 1.25C_6H_{12}O_6 \rightarrow 3N_2 + 4.5H_2O + 6HCO_3^- + 1.5CO_2 \quad (1)$$

$$10NO_2^- + 1.25C_6H_{12}O_6 \rightarrow 5N_2 + 5H_2O + 5HCO_3^- + 2.5CO_3^{2-} \quad (2)$$

The *P.solventiborans* was the only specie of *Paracoccus* discovered in this system, which was isolated from a sludge bed treating natural gas [26]. It was gram-positive at logarithmic phase, gram-negative at stable growth stage, and facultative anaerobic. Its optimum pH was 7-8 and its optimum growth temperature was 30-37ºC. Under the anaerobic environment it could utilize organics as carbon sources, and NO_3^- as the only electron acceptor to generate N_2. The growth of *P.solventiborans* could be promoted if supplied with a low concentration of $S_2O_3^{2-}$, but was inhibited by a high concentration of $S_2O_3^{2}$. It was suspected that *P.solventiborans* had a relationship with sulfur compound removal, which could later be studied further.

Vulcanibacillus was also the chemoheterotrophic denitrifying bacteria in this bioreactor. It was gram-positive bacteria, rod, and strictly anaerobic. The NO_3^- was adopted as the only electron acceptor by *Vulcanibacillus*, and was reduced to NO_2^- rather than N_2. The *V.modesticaldus* was the only species of *Vulcanibacillus* in this system, and it was isolated from the deep-sea hydrotherm with growth pH of 6-8.5 and optimum growth temperature of 37-60ºC [27]. *V.modesticaldus* could reduce NO_3^- to NO_2^- rather than N_2. However, little NO_2^- was found in the effluent of this bioreactor, even though the influent NO_2^- concentration was high at160 mgN/L. The removal of an enormous amount of NO_2^- was discussed as being attributted to the *Thauera* and *Azoarcus* mentioned below, because *Paracoccus* in this system could only accept NO_3^- as electron acceptors.

Azoarcus had the function of nitrogen fixation, among which certain strains had the function of denitrification, such as *A.tolulyticus*, *A.taiwanensis*, and *A.evansii*. In this bioreactor, *A.taiwanensis* was found to have the function of denitrification desulfurization. *A.taiwanensis* was isolated from hot springs, which was gram-negative, in rod shape [28]. It could survive in an environment of 15-40ºC and its optimum growth temperature was 37ºC. It could exist under pH of 6.5-10.5 and the optimum growth pH was 9.0. It was facultative anaerobic and chemolithotrophic. *A.taiwanensis* utilized S^{2-} as electron donor and NO_3^- or NO_2^- as electron acceptors, and finally converted them to S and N_2. Therefore, *A.taiwanensis* was the main denitrification desulfurization functional bacteria, which followed the chemical reactions shown in Equations (3) and (4).

$$5S^{2-}+2NO_3^-+12H^+ \rightarrow 5S+N_2+6H_2O \quad (3)$$

$$3S^{2-}+2NO_2^-+8H^+ \rightarrow 3S+N_2+4H_2O \quad (4)$$

In addition, the alkaliphile *Geoalkalibacter* and *Bacillus* exsited in this system, which was discussed to be the reason for maintaining the stable alkalinity in this complicated environment. According to Fig. 3, the effluent pH was higher than the influent pH and was stable. It was analyzed that the alkaliphile existing in this system stabilized the alkalinity.

Conclusions

The upflow attached-growth bioreactor actualized simultaneous removal of S^{2-}, NO_3^-, NO_2^-, and organics using the mixotrophic denitrification desulfurization process. The microorganisms in this system were collected to study microbial community characteristics. Both sulfur-based denitrification and heterotrophic denitrification were considered to exist in the bioreactor. *Thauera*, *Paracoccus*, and *Vulcanibacillus* were the main heterotrophic denitrifying bacteria. *Thauera* and *Paracoccus* could use NO_3^- and NO_2^- as electron acceptors, while *Vulcanibacillus* could only convert NO_3^- to NO_2^-. The generating NO_2^- was presumed to be further removed by *Thauera*, *Paracoccus,* and *Azoarcus*. *Azoarcus* was the main sulfur-based denitrification bacteria in this system, which utilized S^{2-} as an electron donor and NO_3^- or NO_2^- as electron acceptors. The sulfurous and nitrogenous compounds were finally converted to S and N_2, which would not lead to secondary pollution.

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