1 **Bioaugmentation of activated sludge with elemental sulfur producing strain**
2 ***Thiopseudomonas denitrificans* X2 against nitrate shock load**

3 Wenbo Tan ^a, Cong Huang ^a, Chuan Chen ^a, Bin Liang ^b, Aijie Wang ^{a,b,*}

4 ^a*State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology,*
5 *Harbin 150090, PR China*

6 ^b*Key Laboratory of Environmental Biotechnology, Research Center for Eco-Environmental*
7 *Sciences, Chinese Academy of Sciences, Beijing 100085, PR China*

8 *Corresponding author.

9 E-mail address: waj0578@hit.edu.cn (A. Wang).

10

ABSTRACT

The sulfide and nitrogen compounds in wastewaters are toxic and cause a serious environmental problem. *Thiopseudomonas denitrificans* X2, which is the type species of a novel genus *Thiopseudomonas* was used for bioaugmentation. It oxidized sulfide and acetate with nitrate, and generated elemental sulfur that could be recovered as resource. The generation rate of elemental sulfur was enhanced significantly by the bioaugmentation under the condition of excessive nitrate feeding. The inoculums survived and worked actively in the activated sludge system as the dominant population. *Thiopseudomonas denitrificans* X2 could be applied to wastewater treatment and resource recovery simultaneously.

Keywords: Bioaugmentation; Elemental sulfur; *Thiopseudomonas denitrificans* X2; Resource recovery

1. Introduction

The wastewater generated from industry often contains the contaminants of carbon (C), nitrogen (N) and sulfur (S) compounds. The effluent of oil industry usually contains a high concentration of organic compounds, such as phenol and cresol. In addition, some inorganic compounds (e.g. sulfide and ammonia) are found frequently (Reyes-Avila et al., 2004). The streams of paper mills and tanneries also contain sulfide and nitrate. These contaminants create tremendous challenges for wastewater treatment before discharge.

Ammonia is toxic to fish and other aquatic life because it causes cell death in the central nervous system. Nitrate and other nutrients could cause increasing population of cyanobacteria and then oxygen depletion. They will result in the eutrophication of receiving water body finally. The nitrate in drinking water causes the methemoglobinemia and "blue-baby" syndrome. It also results in childhood diabetes and formation of carcinogenic compounds, e.g. nitrosamines (Show et al., 2013). Sulfide has unpleasant odor and toxicity because it reacts with the iron from cytochromes and then inhibits the respiration (Visser et al., 1997). Sulfide is also a kind of corrosive compound that causes pipeline leakage and economic problems in sewage and petrochemical industry. The chemical oxygen demand (COD) of sulfide in wastewater is also a trouble that should not be ignored.

So far, many biotechnologies have been employed to treat the wastewater containing C, N and S contaminants. In synthetic and oil refining influent treatment, *Thiobacillus denitrificans* and

Thiomicrospira denitrificans were successfully used for simultaneous biological removal of sulfide and nitrate by autotrophic denitrification (Manconi et al., 2006). The sulfide and COD that came from methanogenesis process were oxidized by nitrate generated from the nitrification unit, and then elemental sulfur (S^0) was separated and recovered as resource. This biotechnology has been applied to disposing of the wastewater from pharmaceutical companies (Yuan et al., 2014).

Resource recovery in wastewater treatment process is highly beneficial. Biologically produced S^0 is hydrophilic and can be used as soil fertilizer or fungicide. In contrast to biological S^0 production process, physicochemical technology is costly and energy-intensive due to the consumption of catalysts. Many studies have made it possible to produce and recover S^0 from industrial wastewater adopting different strategies. Some bacterial strains, such as *Thiobacillus denitrificans* and *Chlorobium thiosulfatophilum*, and different configuration of reactors were used to improve the efficiency of sulfur recovery (Henshaw and Zhu, 2001; Huang et al., 2016).

According to standard Gibbs free energy change, sulfide is oxidized by activated sludge with the hypothetical biochemical equations as follows (Chen et al., 2008):

The equations indicate that the concentration of electron acceptor is a crucial factor for the end product of sulfur oxidation. Many studies have evaluated the effect of S/N molar ratio on the oxidation of sulfide and S^0 reclaiming rate (Huang et al., 2015). Nevertheless, in full-scale plant, the influent load is usually fluctuant, and the operating bioreactor is often subject to high load shock which could potentially cause the breakdown of activated sludge system. The newly isolated *Thiopseudomonas denitrificans* X2 is the type species of a novel genus *Thiopseudomonas*, which is affiliated with the family *Pseudomonadaceae* phylogenetically. It is able to oxidize acetate and sulfide using nitrate as electron acceptor (Tan et al., 2015). The end product of sulfide oxidation is S^0 rather than sulfate even with excessive nitrate feeding. Due to the novel physiological properties, strain X2 is a suitable candidate for industrial wastewater treatment and sulfur resource recovery.

In this study, the bioaugmentation of activated sludge for improved generation rate of

elemental sulfur was operated under the condition of high nitrate shock load in up-flow anaerobic sludge blanket (UASB) reactor. The relative abundance of inoculum and the microbial community of activated sludge were analyzed using high-throughput 16S rRNA gene sequencing. The research aims to elucidate the feasibility of bioaugmentation strategies for the S^0 resource recovery during wastewater treatment process.

2. Materials and methods

2.1. Experimental set-up

The control (C-UASB) and bioaugmented (B-UASB) reactors were inoculated with granule sludge that was from the operated bioreactor in our lab. The total and working volume of UASB reactors were 1 L and 0.5 L respectively. The reactors were operated with a hydraulic retention time (HRT) of 12 hours. The total volume of activated sludge for inoculation was 150 mL. The volatile suspended solids (VSS) and suspended solids (SS) were measured following the standard methods (Aktas et al., 2001). The synthetic influent contained the following components (g/L): $Na_2S \cdot 9H_2O$, 1.5; CH_3COONa , 3.28; KNO_3 , 7.474; NH_4Cl , 1.0; $NaHCO_3$, 1.0; KH_2PO_4 , 1.8; $Na_2HPO_4 \cdot 12H_2O$, 3.0; $MgSO_4 \cdot 7H_2O$, 0.1 and trace metal solution (Pfennig and Lippert, 1966). It was stored in an anaerobic tank and transmitted to both reactors by peristaltic pumps. The inoculum of strain X2 was pre-grown in Luria-Bertani medium (NaCl, 10 g/L; tryptone, 10 g/L; yeast extract, 5 g/L) at 30°C for 24 hours. The bacterial count was determined by hemacytometer, and the B-UASB was inoculated with strain X2 to a final concentration of 3.5×10^9 cells/mL. The reactors worked with a HRT of 48 hours for 2 days to promote the colonization of strain X2 in activated sludge.

2.2. Sampling and chemical analysis

The samples of influent and effluent were taken for analysis every 24 hours. For genomic DNA isolation, the samples of activated sludge were collected and frozen at -80 °C immediately. S^{2-} was detected using the standard methylene blue method (Trüper and Schlegel, 1964). The concentrations of CH_3COO^- , NO_3^- , NO_2^- , SO_4^{2-} and $S_2O_3^{2-}$ were determined by ion chromatography (ICS-90A, DIONEX, USA) after filtration through a 0.45- μ m-pore-size filter. The production of elemental sulfur in effluent was calculated according to the equation (de Graaff et al., 2012): $[S^0] = [Influent S^{2-}] + [Influent SO_4^{2-}] + [Influent S_2O_3^{2-}] - [Effluent S^{2-}] - [Effluent SO_4^{2-}] - [Effluent S_2O_3^{2-}]$.

2.3. Microbial community analysis

The genomic DNA of activated sludge was extracted using the E. Z. N. A. Soil DNA Kit (OMEGA, USA) according to the manufacturer's instructions. Fluorometer (Qubit 2.0, Invitrogen, USA) was used to quantify the genomic DNA. Bacterial V3-V4 region of 16S rRNA gene was amplified using the forward primer 341F (5'-CCTACGGGNGGCWGCAG-3') and the reverse primer 805R (5'-GACTACHVGGGTATCTAATCC-3'). The PCR products were purified using Agencourt AMPure XP Kit (Beckman, USA) and then sequenced by sequencing platform (Miseq 2000, Illumina, USA). The raw sequencing data were analyzed by Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010).

2.4. Nucleotide sequence accession numbers

The strain *Thiopseudomonas denitrificans* X2 has been deposited in the center for culture collection under accession number DSM 28679. The 16S rRNA sequences of strain X2 and microbial community have been deposited in GenBank under accession No. KJ567598 and SRP076047 respectively.

3. Results and discussion

3.1. Performance of reactors

The VSS/SS ratio of activated sludge in reactors was 0.6. At steady state, the removal rate of CH_3COO^- and S^{2-} were all 100% in the C- and B-UASB reactors. The corresponding removal rates of NO_3^- were 74% and 64% approximately. The concentration of SO_4^{2-} in the effluent of C-UASB (146mg/L) was higher than that of B-UASB (87mg/L). $\text{S}_2\text{O}_3^{2-}$ was undetected in both reactors within the process of bioaugmentation (Table 1). According to the equation (de Graaff et al., 2012) which was created based on elemental sulfur balance, the generation rate of S^0 in C-UASB was fluctuant between 20% and 37%, and that in B-UASB was between 45% and 70% (Fig. 1). The generation rate of S^0 in B-UASB increased with the bioaugmentation of strain X2 significantly.

The genome sequencing results indicated that strain X2 had flavocytochrome c sulfide dehydrogenase (FccAB), which is capable of catalyzing the oxidation of sulfide to the terminal product of S^0 (Brune, 1995). The enzymes associated with sulfur over-oxidation, such as dissimilatory sulfite reductase (DsrAB), were not found in strain X2 (unpublished results). Therefore, when strain X2 was dominant in the bacterial community of B-UASB, the

concentration of sulfate entered a downtrend, and the generation rate of S^0 increased accordingly.

As defined mainly by carbon and energy metabolism, the sulfur bacteria were subdivided into many physiological types, e.g. chemolithotrophs, facultative chemolithotrophs, chemolithoheterotrophs and chemoorganoheterotrophs. The chemolithoheterotrophs with which strain X2 is affiliated are heterotrophic bacteria that have the ability to generate energy from the oxidation of reduced sulfur compounds (Muyzer et al., 2013). When strain X2 was the dominant population in the activated sludge after bioaugmentation, sulfide turned into a supplemental energy source for metabolism and proliferation. More acetate was assimilated for anabolism rather than catabolized to generate energy for cell activities. In addition, the incomplete oxidation of sulfide provided fewer electrons to the nitrate, so the removal efficiency of nitrate in B-UASB decreased slightly (Table 1).

3.2. Bacterial community analysis of reactors

The bacterial community analysis based on 16S rRNA gene sequencing suggested that a core group of organisms, including *Pseudomonas*, *Azoarcus*, *Arcobacter* and *Thauera*, was responsible for the bulk of denitrification and sulfur oxidation (Fig. 2A). The genera *Azoarcus* and *Thauera* within the *Betaproteobacteria* are most active denitrifiers in the full-scale wastewater treatment plants (WWTPs) environment. The studies have suggested that the genera *Pseudomonas* and *Arcobacter* have the physiological property of denitrification (Thomsen et al., 2007). The genus *Vulcanibacillus* that was isolated from deep-sea hydrothermal vents was rarely identified in WWTPs. It could reduce nitrate to nitrite with carbohydrates, proteinaceous substrates and organic acids as electron donors (L'Haridon et al., 2006).

Some denitrifiers in the core group also have the ability to oxidize reduced sulfur compounds. A *Pseudomonas stutzeri* group consisting of eight strains was able to grow anaerobically with acetate and nitrate, and oxidized thiosulfate to tetrathionate under the same conditions (Sorokin et al., 1999). An organism most closely related to *Arcobacter nitrofigilis* was detected in oil field communities, and it may play an important role in sulfur or nitrogen cycles (Voordouw et al., 1996). In addition, as active denitrifiers, *Thauera* spp. were ubiquitous in the anoxic sulfide-rich incubations. They are also capable of performing nitrate-dependent sulfide oxidation (Cytryn et al., 2005).

The ribosomal database project (RDP) by which the 16S rRNA gene sequencing data was

analyzed does not include the newly isolated and published *Thiopseudomonas denitrificans* X2 at present. In addition, the V3-V4 regions of 16S rRNA gene of *Thiopseudomonas* and *Pseudomonas* share high similarity (>96%). So the 16S rRNA gene sequencing results could not give sufficient information that differentiates genus *Thiopseudomonas* from *Pseudomonas*. They were grouped into the same operational taxonomic unit (OTU). The relative abundance of *Pseudomonas* in B-UASB was 65.4% at day 0 (before the operation of reactors). After lag phase, the generation rate of S^0 reached up to 70% at day 4. The relative abundance decreased to 28.9% at day 10 as a result of biomass loss which was caused by hydraulic flush. Along with biomass loss, the generation rate of S^0 decreased from 70% to 55% in this period. The relative abundance of *Pseudomonas* declined slightly (28.9% to 26.8%) at the end of the bioaugmentation experiment (Fig. 2B), and the generation rate of S^0 decreased from 55% to 45% accordingly (Fig. 1). Acetate was metabolized completely as the most of microbial community members were heterotrophic denitrifiers. Excessive nitrate was also removed efficiently in both reactors (Fig. 1). The number of 16S rRNA sequences of X2 in samples C0 (268/23788 reads), B0 (16319/26557 reads) and B15 (5218/25384 reads) approximately coincided with the relative abundance of *Pseudomonas* above.

There are many factors limiting the application of bioaugmentation, such as the adaptation of the inoculums, competition from indigenous microbial populations, the insufficiency of substrate and grazing by protozoa (Bouchez et al., 2000). The primary factor that has deep influence on bioaugmentation is the survival of introduced strain. Vital to survival consideration are the relative spatial and temporal abundance, and the ability to tolerate the prevailing conditions in target habitats (Thompson et al., 2005). Increasing inoculum size is usually used to avoid wash-out from the reactors. However, excessive inoculation of selective strains could cause the breakdown of structure and function of the indigenous microbial community sometimes. In this study, the inoculation of strain X2 (3.5×10^9 cells/mL) made the relative abundance of *Pseudomonas* increasing to 65.4% initially. Consequently, compared with the C-UASB, the generation rate of S^0 in B-UASB increased significantly. Along with the loss in biomass of inoculums, the generation rate was in the downward trend but still significantly higher than that of the control reactor (Fig. 1). Finally, the relative abundance of *Pseudomonas* was almost not changed between day 10 and day 15 (Fig. 2B), because partial inoculums had got through the acclimation period and colonized the activated sludge successfully. The bioaugmentation did not negatively influence the activated

sludge system and enhanced the performance of reactor efficiently.

4. Conclusion

Thiopseudomonas denitrificans X2, which is the type species of novel genus, was inoculated for bioaugmentation. The generation rate of elemental sulfur in UASB reactor (45% to 70%) was significantly enhanced by bioaugmentation compared with that in the control UASB reactor (20% to 37%). The inoculums colonized activated sludge dominantly with the result that the relative abundance was leveling out at around 19% at the end of bioaugmentation experiment. These results indicated that elemental sulfur producing strain X2 has great potential for carbon-nitrogen-sulfur containing wastewaters treatment and sulfur resource recovery.

Acknowledgements

This research was supported by the National Science Foundation for Distinguished Young Scholars (Grant No. 51225802), National Natural Science Foundation of China (Grant No. 51308147) and National Science Foundation for Young Scientists of China (Grant No. 51408591).

References

1. Aktas, E.S., Imre, S., Ersoy, L., 2001. Characterization and lime treatment of olive mill wastewater. *Water. Res.* 35 (9), 2336-2340.
2. Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Dore, J., Delgenes, P., Moletta, R., Wagner, M., 2000. Ecological study of a bioaugmentation failure. *Environ. Microbiol.* 2 (2), 179-190.
3. Brune, D.C., 1995. Sulfur compounds as photosynthetic electron donors. in: *Anoxygenic photosynthetic bacteria*, Springer, pp. 847-870.
4. Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods.* 7 (5), 335-336.
5. Chen, C., Wang, A., Ren, N., Kan, H., Lee, D.-J., 2008. Biological breakdown of denitrifying sulfide removal process in high-rate expanded granular bed reactor. *Appl. Microbiol. Biotechnol.* 81 (4), 765-770.
6. Cytryn, E., van Rijn, J., Schramm, A., Gieseke, A., de Beer, D., Minz, D., 2005. Identification of bacteria potentially responsible for oxic and anoxic sulfide oxidation in biofilters of a recirculating mariculture system. *Appl. Environ. Microbiol.* 71 (10), 6134-6141.
7. de Graaff, M., Klok, J.B., Bijmans, M.F., Muyzer, G., Janssen, A.J., 2012. Application of a 2-step process for the biological treatment of sulfidic spent caustics. *Water. Res.* 46 (3), 723-730.
8. Henshaw, P.F., Zhu, W., 2001. Biological conversion of hydrogen sulphide to elemental sulphur in a fixed-film continuous flow photo-reactor. *Water. Res.* 35 (15), 3605-3610.
9. Huang, C., Li, Z.-l., Chen, F., Liu, Q., Zhao, Y.-k., Gao, L.-f., Chen, C., Zhou, J.-z., Wang, A.-j., 2016. Efficient regulation of elemental sulfur recovery through optimizing working height of upflow anaerobic sludge blanket reactor during denitrifying sulfide removal process. *Bioresour. Technol.* 200, 1019-1023.
10. Huang, C., Li, Z.-l., Chen, F., Liu, Q., Zhao, Y.-k., Zhou, J.-z., Wang, A.-j., 2015. Microbial

- community structure and function in response to the shift of sulfide/nitrate loading ratio during the denitrifying sulfide removal process. *Bioresour. Technol.* 197, 227-234.
11. L'Haridon, S., Miroshnichenko, M., Kostrikina, N., Tindall, B., Spring, S., Schumann, P., Stackebrandt, E., Bonch-Osmolovskaya, E., Jeannot, C., 2006. *Vulcanibacillus modesticaldus* gen. nov., sp. nov., a strictly anaerobic, nitrate-reducing bacterium from deep-sea hydrothermal vents. *Int. J. Syst. Evol. Microb.* 56 (5), 1047-1053.
 12. Manconi, I., Carucci, A., Lens, P., Rossetti, S., 2006. Simultaneous biological removal of sulphide and nitrate by autotrophic denitrification in an activated sludge system. *Water. Sci. Technol.* 53 (12), 91-99.
 13. Muyzer, G., Kuenen, J.G., Robertson, L.A., 2013. Colorless sulfur bacteria. in: *The Prokaryotes*, Springer, pp. 555-588.
 14. Pfennig, N., Lippert, K.D., 1966. Über das vitamin B₁₂-bedürfnis phototropher Schwefelbakterien. *Arch. Microbiol.* 55 (3), 245-256.
 15. Reyes-Avila, J., Razo-Flores, E.a., Gomez, J., 2004. Simultaneous biological removal of nitrogen, carbon and sulfur by denitrification. *Water. Res.* 38 (14), 3313-3321.
 16. Show, K.-Y., Lee, D.-J., Pan, X., 2013. Simultaneous biological removal of nitrogen–sulfur–carbon: recent advances and challenges. *Biotechnol. Adv.* 31 (4), 409-420.
 17. Sorokin, D.Y., Teske, A., Robertson, L.A., Kuenen, J.G., 1999. Anaerobic oxidation of thiosulfate to tetrathionate by obligately heterotrophic bacteria, belonging to the *Pseudomonas stutzeri* group. *FEMS. Microbiol. Ecol.* 30 (2), 113-123.
 18. Tan, W.-B., Jiang, Z., Chen, C., Yuan, Y., Gao, L.-F., Wang, H.-F., Cheng, J., Li, W.-J., Wang, A.-J., 2015. *Thiopseudomonas denitrificans* gen. nov., sp. nov., isolated from anaerobic activated sludge. *Int. J. Syst. Evol. Microb.* 65 (1), 225-229.
 19. Thompson, I.P., Van Der Gast, C.J., Ciric, L., Singer, A.C., 2005. Bioaugmentation for bioremediation: the challenge of strain selection. *Environ. Microbiol.* 7(7), 909-915.
 20. Thomsen, T.R., Kong, Y., Nielsen, P.H., 2007. Ecophysiology of abundant denitrifying bacteria in activated sludge. *FEMS Microbiol. Ecol.* 60 (3), 370-382.
 21. Trüper, H., Schlegel, H., 1964. Sulphur metabolism in Thiorhodaceae I. Quantitative measurements on growing cells of *Chromatium okenii*. *Anton. Leeuw. Int. J. G.* 30 (1), 225-238.
 22. Visser, J.M., Robertson, L.A., Van Verseveld, H.W., Kuenen, J.G., 1997. Sulfur production by obligately chemolithoautotrophic thiobacillus species. *Appl. Environ. Microbiol.* 63 (6), 2300-2305.
 23. Voordouw, G., Armstrong, S.M., Reimer, M.F., Fouts, B., Telang, A.J., Shen, Y., Gevertz, D., 1996. Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. *Appl. Environ. Microbiol.* 62 (5), 1623-1629.
 24. Yuan, Y., Chen, C., Liang, B., Huang, C., Zhao, Y., Xu, X., Tan, W., Zhou, X., Gao, S., Sun, D., 2014. Fine-tuning key parameters of an integrated reactor system for the simultaneous removal of COD, sulfate and ammonium and elemental sulfur reclamation. *J. Hazard. Mater.* 269, 56-67.

270 **Table 1**271 Simultaneous removal of acetate, nitrate and sulfide in C-UASB and B-UASB reactors^a.

272

Reactors	CH ₃ COO ⁻		NO ₃ ⁻		S ²⁻		S ₂ O ₃ ²⁻	SO ₄ ²⁻
	Influent	Removal	Influent	Removal	Influent	Removal	Effluent	Effluent
	(mg·L ⁻¹)	rate (%)	(mg·L ⁻¹)	rate (%)	(mg·L ⁻¹)	rate (%)	(mg·L ⁻¹)	(mg·L ⁻¹)
C-UASB	2360±80	100.0	5100±96	74±4.2	198±8.2	100.0	0	146±13.9
B-UASB	2360±80	100.0	5100±96	64±5.4	198±8.2	100.0	0	87±13.2

273

274 ^a The data are shown as the mean ± standard deviation from 15 samples.

Fig.1. Generation rate of S^0 in C-UASB and B-UASB reactors.

Fig.2. Bacterial community structure (A) and genera responsible for sulfur oxidation and denitrification (B) in C-UASB and B-UASB reactors.