Bioresource Technology 220 (2016) 647–650

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

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

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HIGHLIGHTS

- The type species of novel genus *Thiopseudomonas* was inoculated for bioaugmentation.
- The C, N and S contaminants were simultaneously removed in UASB reactor.
- The generation rate of S^0 was significantly enhanced by bioaugmentation.

ARTICLE INFO

Article history:
Received 19 July 2016
Received in revised form 23 August 2016
Accepted 24 August 2016
Available online 27 August 2016

Keywords:
Bioaugmentation
Elemental sulfur
Thiopseudomonas denitrificans X2
Resource recovery

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.

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

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http://dx.doi.org/10.1016/j.biortech.2016.08.093
0960-8524/

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):

$$S^{2-} + 1.25CH_3COOH + 3.6NO_3^- + 1.6H^+ \rightarrow SO_4^{2-} + 2.5CO_2 + 1.8N_2 + 2.3H_2O + 2OH^- \quad \Delta G^0 = -2100.1\ \text{kJ/reaction} \quad (1)$$

$$S^{2-} + 1.25CH_3COOH + 2.4NO_3^- + 2.4H^+ \rightarrow S^0 + 2.5CO_2 + 1.2N_2 + 2.7H_2O + 2OH^- \quad \Delta G^0 = -1463.1\ \text{kJ/reaction} \quad (2)$$

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 h. 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_2PO4, 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 h. 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 h 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 h. 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-μm-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′-CCTACGG GNGGCWGCAG-3′) and the reverse primer 805R (5′-GAC TACHVGGGTATCTAATCC-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

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 (146 mg/L) was higher than that of B-UASB (87 mg/L). $S_2O_3^{2-}$ 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