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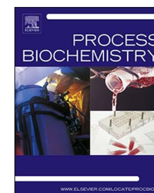

Enrichment and characterization of autotrophic *Thiobacillus* denitrifiers from anaerobic sludge for nitrate removal

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ABSTRACT

An efficient and cost-effective method was established to enrich sulfur-based autotrophic denitrifiers from anaerobic sludge from a municipal wastewater treatment plant. Successful enrichment was achieved using thiosulfate as a substrate after 28 days at 30 °C under anaerobic conditions. Terminal restriction fragment length polymorphism (TRFLP) and high-throughput Illumina sequencing of 16S rRNA genes were applied to investigate the evolution of the microbial community during the enrichment, and observed a significant reduction in the bacterial diversity along the enrichment. Illumina sequencing showed that *Thiobacillus* became the dominant genus in the final enrichment culture, in which autotrophic denitrification rate was 21 mg N_2-N (g VS d)$^{-1}$. In addition, flow-through nanostructured pyrrhotite (NPyr) packed biofilters seeded with the final enrichment culture were evaluated for the autosulfurotrophic denitrification of nitrate-contaminated water. Stable and complete nitrate reduction of 30.24 mg NO_3^--N kg_{NPyr}^{-1} d^{-1} was achieved under a loading rate of 56 mg NO_3^--N (L d)$^{-1}$ in a 30-day trial. Further research is needed to determine the potential of NPyr-packed biofilters to remove nitrogen, and to verify the efficacy of this technology for simultaneous nitrogen and phosphorus removal in general engineering practice.

1. Introduction

Nitrate contamination in waterbodies mainly results from uncontrolled agricultural runoff and wastewater discharges has emerged as a serious issue causing environmental problems and health issues (e.g. methemoglobinemia and carcinogenicity) all over the world [1,2]. Heterotrophic denitrification has traditionally been used for the treatment of nitrate-contaminated waters, such as groundwater, drinking water, and secondary municipal wastewater [3]. Due to the low concentrations of organic carbon present in the wastewater, organic substrates such as methanol must be added to facilitate heterotrophic denitrification [4]. In contrast, autotrophic denitrification does not require organic carbon, thereby reducing operating costs and lowering sludge production [5]. As such, autotrophic denitrification has become an effective alternative to heterotrophic denitrification for the removal of nitrogen (N) from nitrate-contaminated, organic-limited water systems [6]. Biological autotrophic denitrification is an anaerobic process facilitated by autotrophic denitrifiers who can utilize a variety of electron donors, including hydrogen [6,7] and reduced sulfur compounds [8,9], with nitrate as electron acceptor. The reduced sulfur refers to various compounds, including soluble thiosulfate [10,11], insoluble elemental sulfur [12,13] and pyrite (FeS_2) [14–17].

Sulfur-based autotrophic denitrification has been successfully employed to remove nitrate from groundwater, drinking water, and secondary effluent wastewater for over 35 years [10,18]. As a specialized microbial consortium is required for the process, it is necessary to have a convenient, cost-effective and reliable enrichment method to produce a biological seed inoculum. Direct enrichment of autotrophic denitrifiers in wastewater treatment plants is more economical in terms of time and cost in comparison with separately sourcing of denitrifying microorganisms from the market and its subsequent activation. Previous studies demonstrated successful enrichment of sulfur-based

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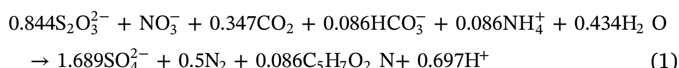
autotrophic denitrifying biomass from anaerobic sludge as seed inoculum for nitrate removal in wastewater [4,12,14,19,20]. However, the main focus of these studies was on the efficiency of nitrate removal, while Pu et al. [14] detected *Sulfurimonas denitrificans* and Zhou et al. [11] found the predominance of *Thiobacillus* in different sulfur-based denitrification systems after treating nitrate-contaminated wastewater. There was no detailed characterization of the enriched microbial community structure. In this study, to evolve a cost-effective method to enrich sulfur based autotrophic denitrifers and characterize the enriched culture for its application in sulfur-based autotrophic denitrification, a simple methodology is proposed to enrich sulfur-based autotrophic denitrifiers from anaerobic sludge separately using thiosulfate or natural pyrite as substrate. 16S rRNA-based methods including terminal restriction fragment length polymorphism (TRFLP) and Illumina sequencing were applied to follow the development of bacterial community over time and to determine the composition of the bacterial community at the end of the enrichment procedure. Sulfur based flow-through biofilters (using nanostructured pyrrhotite (NPyr, $Fe_{1-x}S$, $0 < x < 0.125$) as the sulfur source) were seeded with the final enrichment culture to evaluate feasibility and the denitrifying capacity of the enriched culture for sulfur-based autotrophic denitrification.

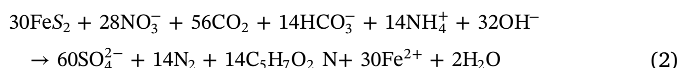
2. Materials and methods

2.1. Enrichment of sulfur-based autotrophic denitrifiers

Anaerobic sludge from Mutton Island Waste Water Treatment Plant in Galway, Ireland, was used as the inoculum for enrichment cultures. Total solids (TS) of the sludge was $25\,g\,L^{-1}$, which was determined using the Standard Methods [21]. Two separate enrichment cultures were prepared using either pyrite (diameter, 250–450 μm) or thiosulfate, as the electron donors. Natural pyrite minerals were collected from Xinqiao Mine of Tongling City, Anhui Province, China. The medium for the thiosulfate enrichment was prepared using deionized water containing ($g\,L^{-1}$): $Na_2S_2O_3{\cdot}5H_2O$, 5; K_2HPO_4, 2; KNO_3, 2; $NaHCO_3$, 1; NH_4Cl, 0.5; $MgCl_2{\cdot}6H_2O$, 0.5; and $FeSO_4{\cdot}7H_2O$, 0.01 [22]. $NaHCO_3$ and $FeSO_4{\cdot}7H_2O$ were filter-sterilized and were added after autoclaving due to their instability during autoclaving. $Na_2S_2O_3{\cdot}5H_2O$ and $FeSO_4{\cdot}7H_2O$ were replaced by pyrite ($2.5\,g\,L^{-1}$) and NaCl ($0.01\,g\,L^{-1}$) in the medium for pyrite fed enrichments. The autotrophic denitrification processes for thiosulfate (Eq. (1)) and pyrite (Eq. (2)) occur according to the following equations [16,23]:



$$0.844S_2O_3^{2-} + NO_3^- + 0.347CO_2 + 0.086HCO_3^- + 0.086NH_4^+ + 0.434H_2O \rightarrow 1.689SO_4^{2-} + 0.5N_2 + 0.086C_5H_7O_2N + 0.697H^+ \quad (1)$$



$$30FeS_2 + 28NO_3^- + 56CO_2 + 14HCO_3^- + 14NH_4^+ + 32OH^- \rightarrow 60SO_4^{2-} + 14N_2 + 14C_5H_7O_2N + 30Fe^{2+} + 2H_2O \quad (2)$$

Enrichment cultures were started with 50 mL anaerobic sludge and 500 mL sterile culture medium. Cultures were flushed with N_2 for 20 min, sealed with rubber stoppers, and incubated at 30 °C. Each stopper had an outlet connected to a syringe to collect gas during denitrification. Enrichment cultures were subcultured when approximately 110 mL of gas was produced, based on the maximum possible gas production from $2\,g\,L^{-1}$ KNO_3 in the medium, according to Eq. (1). Seven subcultures (S1–S7) were performed over the course of the 49 d enrichment based on the enrichment time from previous studies [14,19].

2.2. Microbial community analysis- illumina sequencing and TRFLP fingerprinting

Biomass from the anaerobic seed sludge and from subcultures 1–7 (immediately prior to subculturing), were collected for DNA extraction. Samples were flash-frozen in liquid nitrogen, then stored at −80 °C until required. Samples (6 mL) were defrosted at room temperature and centrifuged at 4000*g* for 2 min to concentrate biomass. DNA was extracted from resulting pellets using a Maxwell 16 Tissue DNA Purification Kit and a Maxwell 16 Research Instrument System (Promega, USA). All DNA extractions were visualized on 1% (w/v) agarose gels containing SYBR Safe under UV light (Thermo Fisher Scientific, UK). Extracted DNA was quantified using the Broad-Range Qubit Assay (Invitrogen, USA). The V4 region of the 16S rRNA genes was polymerase chain reaction (PCR)-amplified using Golay barcoded primers [24] and the KAPA HiFi HotStart PCR Kit. The F515 and R806 primers [24] were used with the following conditions: initial denaturation at 95 °C for 5 min; with 25 cycles of 98 °C for 20 s, 60 °C for 15 s and 72 °C for 40 s; followed by final extension at 72 °C for 1 min. PCR products were gel-purified and quantified using the High-Sensitivity Qubit Assay (Invitrogen, USA). The pooled multiplexed library normalized to 5 ng/ul DNA was sequenced using the Illumina Miseq benchtop sequencer. Data were processed and quality assessed according to the Illumina Amplicons Processing Workflow (http://userweb.eng.gla.ac.uk/umer.ijaz#bioinformatics). The Shannon diversity index H was calculated as follows:

$$H = -\Sigma\,(pi\,\ln(pi))$$

where pi is the proportion of an individual taxonomic unit relative to all sequences analyzed [25].

Bacterial 16S rRNA genes from DNA recovered from subcultures 1–7 were PCR amplified using primers: 63F (labeled with FAM 6-carboxyfluorescein) and 518R as follows: an aliquot of 2 μL DNA was added to respective PCR mixtures of 1 μL of each prime (10 μM), 35.5 μL DEPC H_2O, 10 μL PCR buffer (Bioline), and 0.5 μL My*Taq* polymerase (Bioline). The amplification protocol was: 1 min at 95 °C, and 30 cycles of 15 s at 95 °C, 15 s at 55 °C and 15 s at 72 °C, followed by 10 min at 72 °C. Amplicons were purified using a Wizard SV gel and PCR Clean-Up System (Progema, USA), and quantified using a Qubit Fluorometer (Invitrogen, USA). PCR amplicons were digested using Alu I enzyme (Promega, USA) following the manufacturers instructions. Restriction digestates were typed using an applied biosystems capillary electrophoresis systems. TRFLP profiles were aligned using T-align programme [26] and statistical analyses was performed using Primer 6 [27]. A Bray–Curtis resemblance matrix of square root transformed abundance data was generated and a non-metric multidimensional scaling (NMDS) plot was used to visualize the differences in bacterial community structures between samples.

2.3. Denitrification rate of the final enrichment culture

The autotrophic denitrification rate of subculture 7 (S7) was examined in 60-mL serum vials at 30 °C in triplicate. The vials contained 40 mL culture medium supplemented with varying concentrations of NO_3^--N ($30\,mg\,L^{-1}$, $50\,mg\,L^{-1}$, and $80\,mg\,L^{-1}$). In addition, two sets of negative controls, one without biomass and the other without nitrate, were included to correct for nitrate reduction not associated with autotrophic denitrification. For denitrification rate assay, the culture medium was supplemented with resazurin ($0.001\,g\,L^{-1}$) as a redox indicator. The headspace (20 mL) in each vial was flushed with argon gas for 5 min and autoclaved. Filter-sterilized L-Cysteine was added to each vial to the concentration of $0.5\,g\,L^{-1}$ as an oxygen scavenger. Biomass concentration in the vials was $4.33\,g\,VS\,L^{-1}$. Temporal biogas production in all vials was monitored using a pressure transducer (Model PSI-15, Ireland). The gas was assumed to be $N_{2(g)}$ under nitrate limiting conditions when the g $S_2O_3^{2-}$-S/g NO_3^--N was higher than 3.85 that required by stoichiometry by Eq. (1) [28]. Denitrification rates were calculated from the slope of the cumulative volume of biogas production in the headspace over time and related to the biomass concentration in the vials by Eq. (3).

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