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Short communication

Isolation and characterization of a microorganism involved in sulfide-oxidizing autotrophic denitrification in a vertical fixed-bed reactor

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ABSTRACT

This study evaluates the microbial community involved in autotrophic denitrification by sulfide oxidation, applied in the post-treatment of anaerobic effluent. The enrichment of the bacterial community and autotrophic denitrifying community was performed with biomass samples collected from a vertical fixed bed reactor operated under the following condition: autotrophic nitrate reduction with excess sulfide. The nitrate reduction condition with N/S ratio of 0.8 showed the highest concentration of autotrophic denitrifying microorganisms. The isolated bacterium was identified as *Pseudomonas stutzeri* - like organism. The maximum specific growth rate of the population (μmax) was 0.037 h^{-1}, with doubling time of 18.7 h. The cell yield (Y) was 0.15 gSSV gN^{-1} and denitrification rate was of approximately 0.24 gN gSSV h^{-1}. The data obtained point to the feasibility of applying the isolated species in the autotrophic denitrification process using sulfide as electron donor.

1. Introduction

The removal of nitrogen compounds is particularly important for maintaining the quality of water bodies that receive effluents from wastewater treatment systems. Despite satisfactory results, the biological processes used for nitrogen removal in wastewater treatment still produce certain cost-dependent constraints. Denitrification is commonly performed by a two-stage biological process—an autotrophic nitrification stage, which requires alkalinity and oxygen availability in the medium, followed by a heterotrophic denitrification stage, which depends on an organic carbon and electron source. Then, the treatment costs increase due to the need of alkalinity and oxygen requirements for nitrification and the addition of exogenous organic carbon for denitrification. Thus, research on nutrient removal is often directed toward the development and optimization of processes that would join technical feasibility with reduced operational cost.

The use of anaerobic reactors as the main units for the treatment of domestic sewage has increased in warm climate countries [1]. As the effluents from these reactors have low concentrations of readily biodegradable organic matter, the heterotrophic denitrification stage generally depends on the addition of an exogenous carbon source, hence resulting higher operational costs.

However, denitrification can also be performed by various chemoorganotrophic, lithoautotrophic, phototrophic bacteria and some fungi. Autotrophic denitrification uses the energy released by anaerobic inorganic redox reactions, using hydrogen, reduced sulfur compounds, methane and ammonium (Anammox—anaerobic ammonium oxidation). These processes have been studied in the past years, showing the potentials of such alternative for nitrogen removal of different types of wastewater [2,3].

Effluents from anaerobic reactors treating sewage normally contain inorganic reduced compounds such as ammonium and sulfide. Sulfides are corrosive, toxic, malodorous, and exerts an oxygen demand. The conventional removal of sulfides is performed by physical-chemical processes that include aeration, oxidation and chemical precipitation that use oxidizing agents which entail high costs, in addition to potential problems associated with the chemical compounds released into the environment.

Some chemolithoautotrophic denitrifying bacteria can oxidize inorganic sulfur compounds (HS^-, H_2S, S, $S_2O_3^{2-}$, S_4O_6 or SO_3) as electron donors to reduce nitrogen compounds using inorganic compounds as carbon source (CO_2, HCO_3) [4]. Autotrophic denitrification does not require external organic carbon sources, produces less sludge and catalyzes the oxidation of sulfide and other reduced sulfur compounds (sulfoxidation) in the absence of elemental

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oxygen. Thus, it can be an advantageous and sustainable alternative for denitrification. In wastewaters containing both N and S contaminants the chemolithoautotrophic bacteria oxidizes reduced inorganic sulfur compounds such as sulfide (S^{2-}), elemental sulfur (S_0), thiosulfate ($S_2O_3^{2-}$) or sulfite (SO_3^{2-}), for the reduction of nitrate or nitrite, to promote the simultaneous oxidation of sulfides and denitrification of nitrates or nitrites. The process has been investigated for industrial wastewater treatment [5,6,7] and groundwater contaminated with nitrogen and sulfur compounds [8].

The knowledge about the role of microorganisms associated with physicochemical monitoring tools have provided comprehensive information with respect to the overall performance of treatment facilities and have helped to improve design efficiency and applicability of anaerobic sewage treatment [5,9]. There is a lack of information about the microorganisms and their role in sulfur-driven denitrification in effluents from anaerobic reactors treating domestic sewage.

The facultative autotrophic species *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* [10] and the facultative autotrophic species *Pseudomonas stutzeri* are among the known species able to perform autotrophic oxidation of sulfur compounds coupled to the reduction of nitrogen compounds [11]. The scarcity of studies on microorganisms that can colonize autotrophic denitrifying reactors justifies the importance of this work. Studies on the physiology of autotrophic denitrifying microorganisms can provide valuable information on the enrichment process of reactors with microbial communities of interest. At the best of our knowledge, kinetic studies on autotrophic bacteria isolated from reactors used for the post treatment of anaerobic effluents have not been conducted so far. This study presents and discusses the results of enrichment, isolation, nutritional characterization and growth kinetics of an autotrophic denitrifying microorganism from a reactor used for the post-treatment of domestic sewage anaerobic effluent.

2. Methods

2.1. Fixed bed reactor

The biomass under study was taken from the bench-scale vertical fixed bed with 528 mL. It was composed of three modules: feed chamber at the bottom (56 mm height), support bed for biomass attached growth (22 cm height), and exit chamber at the top (56 mm height). All parts were made of acrylic tubes with 50 mm and 45 mm of external and internal diameter, respectively, connected by flanges with rubber seal. Such flanges supported screens of stainless steel holding the biomass support media between them. The reactors' bed consisted of 0.5 cm polyurethane foam cubic matrices, in which the biomass from a UASB reactor treating poultry slaughterhouse waste water was immobilized according to Moraes et al. [12], Fig. 1. The working volume was 375 mL. After inoculation, the reactor was kept in a chamber at 30 ± 1 °C and operated in upward flow mode at the hydraulic retention time (HRT) of 9 h. Feeding substrate contained nitrate and total dissolved sulfide concentrations were 20 mgN L^{-1} and 60 mgN L^{-1} respectively, maintaining the N/S ratio of 0.8. The final composition (mg L^{-1}) was: KNO_3 (144), KH_2PO_4 (36), NH_4Cl (16), $NaHCO_3$ (2000), $MgCl_2{\cdot}6H_2O$ (28), $CaCl_2{\cdot}2H_2O$ (18).

The biomass samples used in this study were collected from the reactor after 30 days of operation. The solid material containing the autotrophic denitrifying community was detached from the foam and the turbid liquid was used as inoculum in the quantification tests, enrichment and purification of the autotrophic denitrifying culture. To study the bacterial diversity, the liquid detached from the foam was centrifuged for 10 min at 6000 rpm per minute and the pellet was washed with PBS buffer solution (13.7 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4).

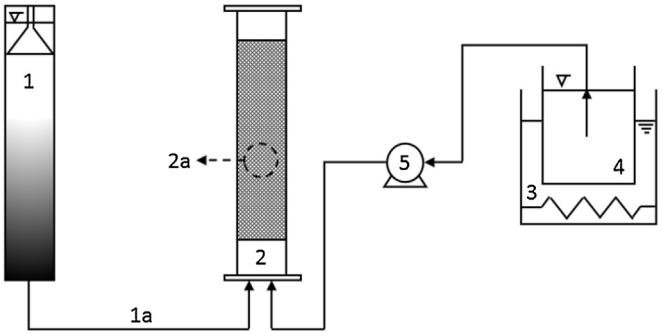

Fig. 1. Simplified layout of the fixed bed reactor: UASB reactor (1); inoculum (1a); fixed bed reactor (2); biomass within foam cubic matrices (2a); thermostatic bath (3); fedding bottle with nitrified substrate and sulfide (4); peristaltic pump (5).

2.2. Microbial characterization

The qualitative characterization of bacterial communities in the inoculum sludge and the biomass detached from the foam was performed by PCR (Polymerase Chain Reaction), followed by DGGE (Denaturing Gradient Gel Electrophoresis).The genomic DNA extraction protocol was performed as described by Griffiths et al. [13]. The 16S rRNA gene fragments were amplified with a primer set for the Domain Bacteria (968F + GC and 1392R) [14] and separated by DGGE [15] using gels containing denaturant gradient of 40% and 60%. The DGGE band profiles were analyzed using the Bionumerics® software program (version 3.5) and the Jaccard similarity coefficient was calculated [16].

The Most Probable Number (MPN) method was used to estimate the density of autotrophic denitrifying populations, according to the methodology described by Eaton et al. [17]. The enrichment and isolation of the autotrophic denitrifying culture in the reactor resulted from the highest positive dilution in the MPN assay, alternating the liquid and solidified culture medium with agar(1.5%). The culture medium described by Zinder et al. [18] was used for enrichment, modified, without carbon source and without ammonium chloride. The medium composition was: K_2HPO_4 (400 mg L^{-1}), $MgCl_2{\cdot}6H_2O$ (1000 mg L^{-1}), $CaCl_2{\cdot}2H_2O$ (100 mg L^{-1}), KNO_3 (144 mg L^{-1}), $Na_2S{\cdot}9H_2O$ (450 mg L^{-1}), $NaHCO_3$ (2000 mg L^{-1}) and micronutrients (100 mg L^{-1}). The atmosphere in the reaction vials was maintained with N_2:CO_2 (70:30%) to preserve the pH. After several transfers of the culture and evidence of denitrification by decay of NO_3^- and sulfate formation, the culture was transferred to a solid medium of the same composition as the liquid medium and with the addition of $Na_2S_2O_3$. The plates were sealed with PVC film to prevent the entry of oxygen and were incubated at 30 °C. The isolated colonies were stained by the Gram stain method. Molecular characterization of purified culture was performed by genomic DNA extraction, amplification of the 16S rRNA gene fragments and the amplified products were subjected to DGGE. The bands corresponding to the autotrophic denitrifying microorganisms in the purified culture were cut out of the gel and the DNA was recovered by soaking the band fragment in ultrapure water for 24 h. The 16S rRNA fragments of the purified culture from the DNA recovered from the cut DGGE band were reamplified with the same set of primers (968F and 1392R) without the GC clamp. After confirming the amplification on agarose gel, the PCR product was purified using the *Illustra GFX™ PCR DNA* and *GEL BAND Purification kit* (*GE*) and sequenced. The sequencing reaction was performed using the BigDye terminator (Applied Biosystem®) with labeled nucleotides in ABI 310