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Characterization of nitrous oxide emissions from a thermophilic denitrifying bacterium *Chelatococcus daeguensis* TAD1 in an aerated sequencing batch reactor

Han Li[a,b], Menglan Pan[a,b], Shaofeng Zhou[a,b], Shaobin Huang[a,b,*], Yongqing Zhang[a,b]

[a] College of Environment and Energy, South China University of Technology, Guangzhou 510006, PR China
[b] Guangdong Provincial Key Laboratory of Atmospheric Environment and Pollution Control, Guangzhou 510006, PR China

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ABSTRACT

This study was conducted to investigate the nitrogen removal efficiency and N_2O emission characteristics of a novel thermophilic aerobic denitrifying bacterium, *Chelatococcus daeguensis* TAD1, under different C/N ratios and pH values in a batch reactor. Nitrogen removal efficiency and N_2O emissions were dramatically influenced by C/N ratio and pH. Moreover, multifactor analysis of variance suggested that these two factors also had significant interaction effects on N_2O emissions. The optimum C/N ratio was determined to be 8 (where pH was set to 7), at which a very high nitrogen removal efficiency (>99%) was achieved. Under these conditions, N_2O emissions were only 34.43 μg/L and the N_2O emission factor was 0.046%, which could offer a promising new microbial resource for N removal and reduction of greenhouse gas emissions during wastewater treatment.

1. Introduction

Although the importance of nitrous oxide (N_2O) has long been recognized, it is often overlooked [1], despite its importance as a potent greenhouse gas with significant impacts on global temperature [2]. Indeed, the 100-year global warming potential of N_2O is about 320 times stronger than that of CO_2 [3] and N_2O is currently the most important ozone-depleting gas [4]. Therefore, even low amounts of N_2O emission are a matter for concern. Accordingly, greater attention should be given to the sources of this gas and measures taken to control its emission [5].

Nitrous oxide is produced via microbial transformations (nitrification and denitrification) of nitrogenous compounds. Denitrification, defined as the stepwise reduction of NO_3^- to N_2 ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), is the most important source of N_2O emissions [6]. Denitrification reactions are catalyzed by nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, while individual reductase enzymes catalyze each step in bacterial denitrification. It has been reported that nitrous oxide reductase is more sensitive to environmental conditions than the other reductases involved in denitrification, and that the potential for release of intermediate N_2O is higher during reactions that use nitrous oxide reductase [7,8]. Traditionally, the denitrifying activity of most denitrifying bacteria is suppressed in insufficiently anaerobic environments and the final step of denitrification (reduction of N_2O to N_2) cannot be catalyzed [9,10]. Current anoxic–aerobic batch reactor systems (A/O) used to enhance shortcut biological nitrogen (N) removal release considerable amounts of N_2O during denitrification. This is because the complete removal of dissolved O_2 is difficult before anaerobic denitrification that follows aerobic nitrification. To overcome this problem, novel aerobic denitrifying bacteria are required to construct aerobic denitrifying processes. Examples of such organisms have been isolated from activated sludge by Baumann et al.; *Paracoccus denitrificans* is an aerobic denitrifier that efficiently reduces NO_3^-, even in the presence of a saturated O_2 concentration [11]. More recent surveys of aerobic denitrifiers have revealed several other novel species [12–15]. In most studies of aerobic denitrifiers, researchers have only described NO_3^- and NO_2^- consumption under aerobic conditions, and studies focusing on denitrified gas (N_2O and N_2) are limited. Moreover, most aerobic denitrifiers are mesophilic bacteria, and few studies have investigated thermophilic microorganisms [16–18]. However, thermophiles generally exhibit higher pollutant removal rates than mesophiles, making them an attractive agent for efficient bioremediation at high temperatures.

* Corresponding author at: College of Environment and Energy, South China University of Technology, Guangzhou 510006, PR China.
E-mail addresses: lihanenjoy@163.com (H. Li), 546157223@qq.com (M. Pan), zsfking@hotmail.com (S. Zhou), chshuang@scut.edu.cn, ldblh727@163.com (S. Huang), zhangyq@scut.edu.cn (Y. Zhang).

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Nitrogen, which is generally present in wastewater in a reduced form such as NH_4^+, is removed during conventional wastewater treatment by two sequential biological processes: nitrification and denitrification. During nitrification, NH_4^+ is converted into NO_2^- or NO_3^-, which is subsequently reduced to N_2 gas during denitrification. Both processes involve N_2O emissions at intermediate stages in the reaction. Considerable amounts of N pollutants removed from treated water are released into the atmosphere as N_2O and N_2 gas when N removal in wastewater treatment facilities is inefficient [19,20]. Therefore, during the last two decades, many studies have investigated N_2O emissions from STPs. The results of these studies have shown that several factors influence N_2O emissions, including dissolved oxygen (DO), influent C/N ratio, carbon (C) source, NO_2^-, and pH [20,21]. Among these factors, the influent C/N ratio is acknowledged as a major factor that can dramatically influence N_2O emissions [22]. Hu et al. [23] and Sun et al. [24] both suggested that a lower influent C/N ratio can cause higher N_2O emissions. Moreover, pH is also considered to be an important factor affecting N_2O emissions, as different pH values affect the growth of denitrifiers and the activity of reductases, which are closely related to the reduction of NO_3^- and N_2O [25].

It is well known that N removal in wastewater treatment plants is essentially based on the activity of nitrifying and denitrifying microorganisms. However, there have been more investigations of N_2O emissions from wastewater treatment plants than of emissions produced by denitrifying microorganisms, and investigations of emissions by thermophilic strains are extremely rare [15,16,26,27]. Essentially, the primary method of controlling the operational parameters and environmental conditions of the denitrification process is to screen efficient bacteria to realize decreased N_2O emissions. Therefore, screening and characterizing natural denitrifiers that produce N_2 by reducing NO_3^- efficiently while emitting low levels of N_2O is necessary.

In this study, we investigated N_2O emissions from a novel, thermophilic aerobic denitrifying bacterium, *Chelatococcus daeguensis* TAD1, which has a considerable denitrification capacity. Moreover, we investigated the influences of pH and the C/N ratio on N_2O emissions from TAD1 in a batch reactor to enhance knowledge of N_2O emissions from this thermophilic aerobic denitrifying bacterium. The results presented herein provide a basic foundation for the application of thermophilic aerobic denitrifying bacteria to engineering processes to control N_2O emissions. Furthermore, detailed and separate characterization of environmental factors will contribute to a better understanding of N_2O emissions by denitrifying bacteria, enabling improved optimization and operation strategies.

2. Materials and methods

2.1. Microbe

The thermophilic aerobic denitrifying bacterium, TAD1, was isolated from the biofilm of a biotrickling filter for the removal of NO [28]. TAD1 is registered at the China General Microbiological Culture Collection Center (CGMCC no. 5226). In addition, the 16S ribosomal DNA gene sequence of TAD1 has been submitted to the DNA Data Bank of Japan, European Molecular Biology Laboratory and GenBank databases under accession no. HM000004. This organism has a high similarity (99%) to that of *C. daeguensis* K106. In this study, TAD1 was stored in 30% glycerol at −20 °C and maintained at 4 °C on agar slants.

2.2. Media

The following growth media were used for TAD1: Luria-Bertani medium (g/L): tryptone, 10.0; yeast extract, 5.0; NaCl, 5.0. Mineral salt medium (g/L): KNO_3, 1.0; Na_2HPO_4, 7.9; KH_2PO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.1; 1 mL of trace elements solution. Trace elements solution (g/L): EDTA, 50.0; $CaCl_2$, 5.5; $MnCl_2{\cdot}4H_2O$, 5.06; $FeSO_4{\cdot}7H_2O$, 5.0; $ZnSO_4$, 2.2; $CoCl_2{\cdot}6H_2O$, 1.61; $CuSO_4{\cdot}5H_2O$, 1.57; $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 1.1. The denitrification medium (DM) was mineral salt medium with sodium succinate as the sole C source and the amount of the C source was adjusted according to the C/N ratio.

2.3. Batch reactor

During our experiments, we used the closed reusable BIOSTAT Aplus (Sartorius Stedim Biotech) bioreactor (Fig. 1). This reactor consists of an autoclavable glass vessel with a maximum working volume of 5 L. The reactor is equipped with a Rushton stirrer, a gas diffuser at the bottom of the reactor, and probes to monitor dissolved oxygen, temperature, and pH. A system controller that can automatically adjust the pH, DO, rotation stirring speed (rpm), and temperature in the reactor, as well as the velocity of the liquid into the glass vessel, was connected to the reactor. A gas outlet was used to allow off-gas measurements.

2.4. Operation strategies for the batch bioreactor and batch tests for N_2O emissions

First, TAD1 was pre-cultured in 50 mL of Luria-Bertani medium for 12 h at 50 °C and 160 rpm, after which 10 mL of this seed medium was inoculated into 100 mL of DM in 300-mL flasks and shaken for 24 h at 50 °C and 160 rpm. Finally, 100 mL of the medium was inoculated into 2 L of DM in the reactor with a stirring speed of 150 rpm for batch culture. A dissolved oxygen (pO_2) saturation between 60% and 100% in the medium was maintained, which was automatically controlled by the system controller of the bioreactor. The pH in the reactor during all batch tests was automatically controlled at ±0.05 of the predesigned set points (see below) by adding 0.5 M HCL or 0.5 M NaOH solutions. All tests were carried out at a temperature of 50 °C. During batch culture, 40-mL aliquots were withdrawn periodically (0, 4, 8, 12, 16, 20, and 24 h) from all batches to analyze the NO_3^- and NO_2^- concentrations. The gas samples were collected every 4 h at the gas outlet for detection as follows: a 100-mL glass syringe was used at the gas outlet to pump the gas from the bioreactor slowly and uniformly, then the sample was immediately injected into a 500-ml vacuum air pocket that was cleaned and vacuumed with high-purity N_2 and then subjected to gas chromatography analysis (see Section 2.5).

Twelve batch tests were performed in the batch rector to investigate the combined effects of C/N and pH on N_2O production, as well as the individual effects of the C/N ratio (where pH was set to 7) or pH (C/N ratio = 8) on the N_2O emissions from TAD1 under batch culture. The C/N ratios in the medium of the batch reactor were adjusted by changing the amount of the C source and maintaining a constant NO_3^- concentration. The detailed experimental conditions of the batch tests are shown in Table 1. Other experimental culture conditions were identical to those described above.

2.5. Analytical methods and calculations

As suggested by Czepiel et al. [29] and Liu et al. [30], the concentration of N_2O was analyzed using an Agilent 7890A gas chromatograph equipped with an electron capture detector. A mixture of Ar (90%) and CH_4 (10%) was used as the equilibrium gas. High-purity N_2 was used as the carrier gas. The N_2 was analyzed by gas chromatography using He applied at a flow rate of 25 mL/min as the carrier and column, analyzer, and vaporizing chamber temperatures of 32 °C, 85 °C, and 75 °C, respectively.

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