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Isolation and nitrogen removal characteristics of an aerobic heterotrophic nitrifying–denitrifying bacterium, *Bacillus subtilis* A1

Xin-Ping Yang, Shi-Mei Wang, De-Wei Zhang, Li-Xiang Zhou *

College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China

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ABSTRACT

Bacterium A1, isolated to enhance nitrogen removal from ammonium-rich wastewater *in situ*, exhibited an amazing ability to convert ammonium to gaseous nitrogen compounds under fully aerobic conditions, while growing autotrophically or heterotrophically. A1 was identified as *Bacillus subtilis* by morphological and physiological characteristics, and phylogenetic analysis of its 16S rDNA gene sequence. Nitrogen removal by A1 was analyzed in relation to the ammonium concentration, presence of organic carbon, carbon source, and carbon-to-nitrogen ratio (C/N). The nitrogen balance during 120 h of autotrophic growth in the presence of 104.12 ± 1.27 mg/L NH_4^+—*N* showed that 20.4 ± 2.7% of NH_4^+—*N* was removed as gaseous nitrogen compounds, and A1 removed 58.4 ± 4.3% of NH_4^+—*N* within 60 h of growth in acetate medium at a C/N of 6. A mean ammonium removal rate of 3.52 mg NH_4^+—*N*/(L h) was achieved in an open wastewater system, indicating great potential of A1 for future full-scale applications.

1. Introduction

The most common, efficient, and cost-effective methods for the removal of nitrogen from municipal and industrial wastewaters involve the use of aerobic autotrophic nitrifiers and anaerobic heterotrophic denitrifiers, which convert nitrogenous compounds to nitrogen gas (N_2) (Chiu et al., 2007; Khardenavis et al., 2007). However, the nitrification process tends to be time-consuming and demands large expanses of space to house separate aerobic and anaerobic tanks (Khin and Annachhatre, 2004). Additionally, autotrophs are sensitive to high loads of ammonium and organic matter (Kim et al., 2006; Kulikowska et al., 2010); thus, their applications are restricted when treating high-strength ammonium wastewater. Recent studies have highlighted the existence of bacteria such as *Microvirgula aerodenitrificans* (Patureau et al., 2001), *Pseudomonas stutzeri* (Su et al., 2001), *Alcaligenes faecalis* (Joo et al., 2007), *P. putida* (Kim et al., 2008), and *Acinetobacter calcoaceticus* (Zhao et al., 2010) that are capable of performing heterotrophic nitrification and have a phenomenal ability to denitrify their nitrification products under aerobic conditions (Gupta and Gupta, 2001; Bernat and Wojnowska-Baryła, 2007). Although these microorganisms have potential applications in future nitrogen biotreatment systems, further research is needed to ensure that this method will be cost-effective and sufficient to meet the demands of a full-scale operation.

There are no selective enrichment or isolation methods for aerobic heterotrophic nitrifying–denitrifying microorganisms (Brierley and Wood, 2001). Media that support growth of these species must contain organic carbon in addition to nitrogen, and this permits the development of many activated sludge microflora that are unable to oxidize or remove nitrogen. In this study, heterotrophic nitrifying–denitrifying bacteria were isolated in the presence of oxygen by combining inorganic ammonium oxidation bacteria enrichment cultures and washed-agar plate procedures.

As presented above, a number of heterotrophic microorganisms have been reported to nitrify and transform many types of nitrogen compounds. These have been intensively studied for use in overcoming problems inherent in the conventional nitrogen removal process. Considering the key role of microorganisms in aerobic heterotrophic nitrification–denitrification activities, recent research has focused mainly on screening for microbes with high nitrogen-removing efficiencies. The progress of heterotrophic nitrifier research in recent years has increased the feasibility of future full-scale applications. However, given the diversity of the reactions, heterotrophic nitrifier activities have been generally lower than those of autotrophic and heterotrophic two-step nitrogen-removing microbes (Béline and Martinez, 2002; Plósz et al., 2003; Elefsiniotis et al., 2004; Kim et al., 2005).

Taking cost-effectiveness into account, a higher heterotrophic nitrification and denitrification activity is still urgently required to meet the demand of high-strength ammonium wastewater treatment, and understanding the characteristics of microorganisms in detail is very important for maintaining highly efficient treatment at all times. Furthermore, the possible microbiological

* Corresponding author. Tel./fax: +86 25 84395160.
E-mail address: lxzhou@njau.edu.cn (L.-X. Zhou).

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nitrogen conversions should be described, and the performance of isolated specific microorganisms in an open wastewater system must be examined.

2. Methods

2.1. Isolation using enrichment cultures

A basal inorganic medium (pH 8.0–8.2) for enrichment and isolation of ammonium oxidation bacteria (AOB) was prepared by dissolving 0.5 g ammonium sulfate, [$(NH_4)_2SO_4$ as a source of reduced N to investigate the acceptability of inorganic N sources for heterotrophic nitrification; Brierley and Wood, 2001], 0.7 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g $CaCl_2{\cdot}2H_2O$ and 1 mL of trace mineral solution in 1 L of distilled water. The trace mineral solution contained (per liter): 2.86 g H_3BO_3, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.08 g $CuSO_4{\cdot}5H_2O$, 2.03 g $MnSO_4{\cdot}4H_2O$, and 1.26 g $Na_2MoO_4{\cdot}2H_2O$ (pH 7.0). The AOB medium was autoclaved for 30 min at 121 °C. Sterile medium (100 mL) in 250-mL conical flasks ($n = 3$) was inoculated with 2 mL of fresh activated sludge from the Jiangxinzhou Municipal Wastewater Treatment Plant (Nanjing, China) and incubated at 28 °C on a rotary shaker at 180 rotations per minute (rpm). Every 7 days, 0.1 mL medium was spot-tested for total oxidized nitrogen (nitrite and nitrate) using the Griess-Ilosvay method; when the tests proved positive, 1 mL of the enrichment cultures was transferred to fresh medium. Purified isolates were obtained by repeated streaking on fresh washed-agar plates (liquid inorganic medium, 2% washed agar as reducing organic matter). The resulting bacterial isolates were tested for their ability to produce nitrite and nitrate by inoculation into 250-mL conical flasks ($n = 3$) containing 100 mL of liquid inorganic medium. After 5 days of incubation at 28 °C on a rotary shaker, the medium was sampled and analyzed colorimetrically for ammonium, total oxidized nitrogen, total nitrogen (TN), and change in pH. The results are expressed relative to non-inoculated, incubated medium. A bacterium with highest nitrogen removal efficiency was obtained and named A1. The strain A1 was suspended in 25% glycerol solution at −80 °C for long-term storage.

2.2. DNA extraction, PCR amplification, and 16S rDNA gene sequence analysis

DNA was extracted from an A1 bacterial suspension (after incubation for 24 h) using an EZ-10 Spin Column genomic DNA isolation kit (Sangon, Shanghai). The 16S rDNA gene was amplified by PCR (PTC200; Bio-Rad, USA) using the primers 5′-AGAGTTTGATCCTGGCTCAG and 5′-TACCTTGTTACGACTT. PCR products were purified with an EZ-10 Spin Column PCR product purification kit (Sangon). Partial sequencing of the 16S rDNA gene was performed by Genscript Corp. (Nanjing). The 16S rDNA sequence of the isolate was compared with those of other microorganisms using the Basic Local Alignment Search Tool program (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

2.3. Shaking-culture experiments

Strain A1 was cultivated for 48 h in basal inorganic medium at 28 °C with shaking at 180 rpm. The bacteria were collected by centrifugation (4 °C, 15 min, 3600*g*) and washed with sterile water; this was repeated three times. Medium (100 mL), adjusted to pH 7.2–7.5 with NaOH (aq) or H_2SO_4 (aq) as neutral conditions are known to favor the growth of A1, and 5 mL of bacterial suspension were placed into 250-mL conical flasks, followed by cultivation at 28 °C and 180 rpm for 120–288 h. During incubation, the cultures were sampled periodically to determine optical density (OD_{600}), pH, and the levels of NH_4^+—*N*, NO_2^-—*N*, NO_3^-—*N*, TN, chemical oxygen demand (COD), and dissolved oxygen (DO).

2.4. Influence of ammonium concentration on autotrophic nitrification/aerobic denitrification

The influence of the ammonium concentration on the nitrifying capacity of strain A1 was assessed in basal inorganic medium. Initial NH_4^+—*N* concentrations were adjusted to 105.58 ± 11.01, 257.23 ± 1.97, 536.21 ± 3.15, and 1014.17 ± 39.32 mg/L using $(NH_4)_2SO_4$. The culture conditions were as described above.

2.5. Influence of carbon source on heterotrophic nitrification/aerobic denitrification

Strain A1 was cultured in basal medium with the addition of the following carbon sources: glucose, citrate (trisodium citrate), succinate (sodium succinate), and acetate (sodium acetate). Medium containing glucose was autoclaved for 15 min at 110 °C; all other media were autoclaved for 30 min at 121 °C. The amount of $(NH_4)_2SO_4$ (N source) was fixed and provided 103.64–110.31 mg/L NH_4^+—*N*. The amount of each carbon source was adjusted to give a C/N ratio of 11. The culture conditions were as described above.

2.6. Influence of the amount of carbon on heterotrophic nitrification/aerobic denitrification

The effects of different amounts of a carbon source, as sodium acetate in basal medium, on ammonium removal by strain A1 were measured. The C/N ratio was adjusted to 2, 6, 12, and 26 by fixing the amount of $(NH_4)_2SO_4$ (N source) at 104.27–110.31 mg/L (NH_4^+—*N*) and adding sodium acetate (organic C source) at 239.52 ± 0.15, 669.11 ± 15.48, 1250.02 ± 132.89, and 2986.51 ± 160.35 mg COD/L, respectively. The culture conditions were as described above.

2.7. Analytical methods

During incubation, the cultures were sampled periodically for cell density determinations, chemical analyses, and measurements of DO and pH. The optical density of the culture broth was measured at 600 nm (OD_{600}) using a spectrophotometer (UV-754; Shimadzu, Japan). Ammonium nitrogen was determined by photometric determination with Nessler's reagent (APHA et al., 1998); NO_2^-—*N* was measured by the N-(1-naphthalene)-diaminoethane photometry method; NO_3^-—*N* was analyzed by the phenol disulfonic acid method. TN was determined by UV spectrophotometry, and intracellular nitrogen content was calculated by subtracting the TN of inoculated medium following centrifugation (4 °C, 15 min, 3600*g*) from the TN of non-centrifuged medium. COD was determined using a COD analyzer (AQ4001; Thermo, USA). DO, temperature, and pH were routinely measured with an oxygen electrode (3-star 310D-01, Thermo) and a pH electrode (3-star 310p-02, Thermo), respectively.

2.8. Sequencing batch reactors

The experiment was carried out in two 2-L sequencing batch reactors (SBRs, 1.5-L working volume). The reactors were equipped with a controlled air supply system and distributor with a flow rate of 2.0 L/min. The sludge concentration was maintained at approximately 4.0 g MLSS/L. The SBRs were operated at a cycle of 6 h, with four cycles/d. The cycle consisted of four phases: feeding (0.375 L of influent during 3 min), aerobic growth (325 min), settling (28 min), and decanting (0.375 L of clarified supernatant during 4 min). After the settling period, 1.5 L of supernatant were