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Biological characteristics of a denitrifying phosphorus-accumulating bacterium

Ling Sun [a,b], Xinxin Zhao [b], Huifang Zhang [a,b], Yanqiu Zhang [a,*]

[a] China University of Mining and Technology, Xuzhou 221116, China
[b] Xuzhou Institute of Technology, Xuzhou 221008, China

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ABSTRACT

To isolate highly efficient denitrifying phosphorus-accumulating bacteria (DPAB) for synchronous nitrogen and phosphorus removal, it is necessary to understand their biological characteristics. Using denitrification screening, polyphosphate (poly-P) and poly-β-hydroxybutyrate (PHB) granule staining, nitrate reduction, nitrate reduction producing N_2, and phosphorus uptake tests, a strain of efficient DPAB was isolated. This strain was identified as *Thauera* by 16S rDNA sequencing and physiological–biochemical analyses, and named N11. The growth of the strain N11 was obviously affected by nitrogen source, carbon source, temperature, and pH ($p < 0.05$ or $p < 0.01$). LB was considered the suited nutrient removal medium and the optimum pH and temperature were 8 and 35 °C, respectively. Under the optimal culture conditions for 32 h, the bacteria could consistently keep active, OD_{600} maintained at approximately 1.8, and the count was 1.826×10^{13}–3.218×10^{13} cfu/mL. With anaerobic–aerobic training in synthetic wastewater, the polyhydroxyalkanoate (PHA) content in cells increased from 22.8 mg/L to 150.6 mg/L (under anaerobic conditions, 12 h), and then gradually decreased to 20.4 mg/L (under aerobic conditions, 24 h). The phosphate (PO_4^{3-}–P) concentration in culture supernatants increased from 4 mg/L to 20.8 mg/L (under anaerobic conditions, 12 h), and then gradually decreased to 3.08 mg/L (under aerobic conditions, 24 h), and the phosphorus removal rate was 80.38%. The nitrate–nitrogen (NO_3^-–N) concentration decreased from 75.45 mg/L to 7.12 mg/L. Nitrite–nitrogen (NO_2^-–N) was at a low concentration (≤1.29 mg/L). The denitrification rate was 89.96%. This study describes a novel strain of DPAB and the optimal conditions for nitrogen and phosphorus removal by this strain.

1. Introduction

Nitrogen and phosphorus pollutants are major causes of eutrophication in inland lakes, reservoirs, and rivers. Concurrent removal of these elements is the main objective of biological wastewater treatment. Conventional biochemical processes can effectively reduce wastewater biological oxygen demand (BOD) and the content of suspended solids (SS), but can only remove 10–30% of nitrogen and phosphorus. In the past 30 years, many sewage treatment plants have begun to use microorganisms to remove nitrogen and phosphorus (Kuba et al., 1996; Xia and Liu, 2004). However, denitrifying bacteria compete with phosphorus-accumulating bacteria (PAB) for limited carbon sources, and the relative abundance of nitrifying bacteria and PAB varies with respect to sludge age, which presents a challenge in optimizing sewage treatment systems to achieve simultaneous denitrification and phosphorus removal (Meinhold et al., 1999).

Denitrifying phosphorus-accumulating bacteria (DPAB) are facultative anaerobic bacteria that can utilize O_2, NO_3^-–N, or NO_2^-–N as final electron acceptors to take up phosphorus under aerobic conditions or to release phosphorus under anaerobic conditions. However, these two processes are not balanced in these organisms, with a clear bias toward phosphorus uptake (Li and Huang, 2013). The discovery of these organisms introduces the possibility of solving the contradiction between carbon utilization and sludge age such that nitrogen and phosphorus removal can be achieved simultaneously (Bortone et al., 1999; Lee et al., 2001; Guo et al., 2014). Recently, progress has been made in the isolation of DPAB, with the isolation of DPAB from the genera *Acinetobacter* (Tsuneda et al., 2006), *Alcaligenes* (Li et al., 2006), *Aeromonas* (Wang et al., 2008), *Comamonas* (Lu et al., 2009), *Pseudomonas* (Cai et al., 2010), *Bacillus* (Ma et al., 2011), *Paracoccus* (Zhang and Wu, 2012; Liu et al., 2013), *Planctomycetes* (Liu et al., 2013). In this study, a highly efficient synchronous nitrogen and phosphorus-

* Corresponding author. Tel.: +86 15062190077.
E-mail address: zhangyanqiuls@163.com (Y. Zhang).

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removing DPAB, *Thauera*, was isolated through screening for phosphorus-removal capability, nitrate-reducing N_2, and metachromatic granule staining. However, until now, little information has been available regarding the factors that influence the growth and degradation characteristics of *Thauera*. Determining such factors will enable the optimization of their function and improve the efficiency of wastewater biological nutrient removal processes through biological denitrifying phosphorus removal. Thus, the present study has theoretical value and practical significance.

2. Materials and method

2.1. Media

Lysogeny broth (LB) medium: 5 g of yeast extract, 1 g of peptone, 5 g of NaCl, 1 L of ddH_2O, pH 7.0–7.2 (added 0.025 kg of agar if it is solid).

LB phosphorus-rich medium: LB medium supplemented with 2.5 g K_2HPO_4 and 0.25 g KH_2PO_4, pH 7.0–7.2.

Enrichment medium: 5 g of $CH_3COONa{\cdot}3H_2O$/5 mL of propionic acid, 2 g of KNO_3, 0.2 g of $MgSO_4{\cdot}7H_2O$, 2 g of K_2HPO_4, 2 mL of trace elements, 1 L of ddH_2O, pH 7.2.

1% bromothymol blue (BTB)-denitrification medium: 5.0 g of $CH_3COONa{\cdot}3H_2O$, 2.0 g of KNO_3, 0.2 g of $MgSO_4{\cdot}7H_2O$, 20 g of agar, 4 mL of 1% BTB, 1 L of ddH_2O, pH 7.6.

Denitrification phosphorus-missing medium: 2 g/L of KNO_3, 5 g/L of $CH_3COONa{\cdot}3H_2O$, 0.05 g/L of K_2HPO_4, 0.2 g/L of $MgSO_4{\cdot}7H_2O$, 0.5 g/L of $CaCl_2$, 2 mL/L of trace element solution.

Denitrifying phosphorus-rich medium: 2 g/L KNO_3, 5 g/L $CH_3COONa{\cdot}3H_2O$, 0.05 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L $CaCl_2$, 2 mL/L trace element solution.

Mother liquor of selective medium: $CH_3COONa{\cdot}3H_2O$ 5 g/L; K_2HPO_4 0.05 g/L; KH_2PO_4 0.2 g/L; $MgSO_4{\cdot}7H_2O$ 0.2 g/L; $CaCl_2$ 0.5 g/L; trace element solution 2 mL/L, pH 7.0–7.6.

Trace element solution: 1.5 g/L of $FeCl_3{\cdot}6H_2O$, 0.15 g/L of H_3BO_3, 0.03 g/L of $CuSO_4{\cdot}5H_2O$, 0.03 g/L of KI, 0.06 g/L of $Na_2MoO_4{\cdot}2H_2O$, 0.12 g/L of $MnCl_2{\cdot}4H_2O$, 0.12 g/L of $ZnSO_4{\cdot}7H_2O$, 0.12 g/L of $CoCl_2{\cdot}2H_2O$.

All biochemical reagents except LB were of analytical grade. The pH was adjusted using 0.1 mol/L HCl and 0.1 mol/L NaOH.

2.2. DPAB enrichment culture and screening

2.2.1. Enrichment culture

Ten grams of active sludge was added to the enrichment medium (200 mL). The cultures were incubated at 26 °C and shaken at 140 rpm in anaerobic/hypoxic (12 h/12 h) batch culture. Per 4 days was a cycle, and the mixture was cultured for 10 cycles. Two days before each cycle, sodium acetate was added to provide a carbon source. After 2 days, propionic acid was added to provide a carbon source, and half of the culture solution was replaced with fresh medium. After incubation, 0.5 mL of the culture suspension was collected for serial dilutions of 10^{-1}–10^{-9}. Solid LB agar was inoculated with 0.1 mL of each dilution in duplicate and cultured in a 26 °C incubator for 2–3 days. Single colonies were sub-cultured by picking and streaking 6 times to isolate pure colonies.

2.2.2. Screening of strains

Screening for denitrifiers: single colonies were isolated, streaked on 1% BTB-denitrification medium, and incubated for 2 days at 26 °C. The denitrification medium contained KNO_3 as a substrate and BTB as a pH indicator. Blue colonies were selected as denitrifiers.

Poly-P and PHB staining: metachromatic granules and PHB granules of the selected strains mentioned above were stained. Positive strains were designated as DPAB. All strains were preserved in LB medium slant cultures at 4 °C.

Seed cultures: the above strains were cultured in 100 mL of LB medium at 26 °C with shaking at 140 rpm for 24 h. Cultures were centrifuged at 4000 rpm for 30 min. The optical density (OD_{600}) was adjusted to 1.0 by dilution of the bacterial pellets with sterile saline.

Denitrification and phosphorus removal rates determination: the seed culture was diluted in 100 mL of denitrification phosphorus-missing medium to an OD_{600} of 0.1 and cultured under argon for 12 h at 26 °C with shaking at 140 rpm. Cultures were centrifuged at 4000 rpm for 30 min, washed twice with sterile saline, and resuspended in 200 mL of denitrifying phosphorus-rich medium to an OD_{600} of 0.1. This culture suspension was further incubated under aerobic conditions for 24 h at 26 °C with shaking at 140 rpm. The absorbance of PO_4^{3-}–P, NO_3^-–N, and NO_2^-–N in the culture supernatant was measured by spectrometry as described below. The denitrification and phosphorus removal rates were calculated from the change in absorbance.

The following equations were used to determine phosphorus removal and denitrification rates:

$$\text{Denitrificationrate}: \eta_1 = \frac{(A - A_t - B_t)}{A} \times 100\% \quad (1)$$

$$\text{Phosphorousremoval}: \eta_2 = \frac{(C - C_t)}{C} \times 100\% \quad (2)$$

where η_1, trans-digestibility, %; A, NO_3^-–N initial absorbance, abs; A_t, NO_3^-–N absorbance of the solution at time t, abs; B_t, NO_2^-–N absorbance of the solution at time t, abs; η_2, phosphorus ratio, %; C, PO_4^{3-}–P initial absorbance, abs; C_t, absorbance of the PO_4^{3-}–P in the solution at time, abs.

Strains with denitrification and phosphorus removal rates higher than 70% and 50%, respectively, were chosen as experimental strains.

2.3. Morphological, physiological–biochemical properties of strains

Strain characteristics were determined according to previously described methods (Dong and Cai, 2001).

2.4. 16S rDNA PCR amplification

Bacterial genomic DNA was used as a template to amplify 16S rDNA with a pair of universal primers: upstream primer (7F), 5′-CAGAGTTTGATCCTGGCT-3′; downstream primer (1540R), 5′-AGGAGGTGATCCAGCCGCA-3′. PCR system: template, 0.5 μL; 5× buffer (with Mg^{2+}), 2.5 μL; dNTPs, 1 μL; upstream and downstream primers, 0.5 μL ddH_2O was added to bring the volume to 25 μL. PCR procedures were as follows: denaturation at 98 °C for 3 min; 30 cycles of denaturation at 98 °C for 25 s, annealing at 55 °C for 25 s, and extension at 72 °C for 1 min; a final extension at 72 °C for 10 min; and termination at 4 °C. PCR products were analyzed by agarose gel electrophoresis and sent to China Shanghai Biological Technology Co. for sequencing.

2.5. Effect of different culture conditions on bacterial growth characteristics

Prepared requisite medium (150 mL): cultures with an OD_{600} of 0.1 were injected with oxygen and cultured on a 140 rpm shaker for 24 h, after which the absorbance was measured at 600 nm.

2.5.1. Nitrogen sources

Peptone, yeast extract, $NaNO_2$, and KNO_3 were added into the mother liquor of the selective medium; sodium nitrite and KNO_3