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Application potential of a newly isolated indigenous aerobic denitrifier for nitrate and ammonium removal of eutrophic lake water

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HIGHLIGHTS

- We investigated the bioremediation potential of an aerobic denitrifier.
- The strain was indigenous in the eutrophic lake without climate problem.
- It could remove nitrate and ammonium simultaneously in the lake water samples.
- Via adding this strain, the water qualities ameliorated from Grade V to Grade II.

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ABSTRACT

The aim of this work was to evaluate the utilization potential of a newly isolated indigenous aerobic denitrifier, *Pseudomonas stutzeri* strain T1, for nitrogen removal from the eutrophic Lake Taihu in China. The strain was capable of conducting heterotrophic nitrification–aerobic denitrification and had both excellent nitrate and ammonium removal without nitrite build-up. The characteristics of *P. stutzeri* strain T1 were studied under different cultural conditions. Furthermore, under the optimized cultivation conditions, strain T1 was added into the water samples from Lake Taihu, the ammonium and nitrate removal rates of the strain reached to 60% and 75%, respectively. Via adding this strain, the water qualities of the sample ameliorated from Grade V to Grade II. Thus, the strain T1 should be an useful biological tool to remediate eutrophic lakes and do not meet acclimation problems.

1. Introduction

Over the last few years, tremendously amount of artificial nitrogen fertilizer has been used for high crop yields to meet the rapidly growing human population (Galloway et al., 2008; Lunau et al., 2012; Tilman et al., 2002). Accordingly, substantial amount of nitrate and ammonium entered into water bodies such as lakes, reservoirs and seas (Zhou et al., 2007) via tributary rivers, and hence, an increasing number of aquatic ecosystems in China occurred hyper eutrophication and serious algal blooms in recent years. A large (area 2338 km^2) shallow (mean depth 1.9 m) freshwater lake, Lake Taihu, which is the third largest one in China, has suffered greatly from anthropogenic eutrophication since 1980s (Qin et al., 2007), especially in the northern areas. The proliferation of toxic algae in summer is seriously impacting the public health, local economy and ecosystem in Lake Taihu regions. Moreover, the increased presence of aqueous nitrate and ammonium is a serious environmental problem because ammonium is toxic to fish and other aquatic life (Siljeg et al., 2010) and nitrate damages their immune system (Grguric et al., 2000). Thus nitrogen removal efficiently in eutrophic water bodies is a stringent research.

Conventional nitrogen removal means from wastewater and natural lakes include physical, chemical and biological approaches. Biological aerobic nitrification and anaerobic denitrification have been found to be reliable and should be encouraged due to their easy implementation and efficiency (Borges et al., 2003). Nevertheless, in order to remove ammonium and nitrate in the natural environment, a creation of aerobic and anoxic conditions alternately is needed but technological and economical constrained (van Rijn et al., 2006). The finding of organism *Thiosphaera pantotropha* in 1988 (Robertson et al.) realized the simultaneous nitrification and denitrification and could tolerate oxygen. Nitrogen removal becomes easy in open water body because this process can happen in aerobic environment instead of anoxic environment. Furthermore, alkalinity generated during denitrification can partly

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compensate for the acidification caused by nitrification. Therefore, it is procedural simplicity and can reduce the cost.

In recent decades, more and more heterotrophic nitrification–aerobic denitrification microorganisms were screened and characterized, such as *Alcaligenes faecalis* (Anderson et al., 1993), *Microvirgula aerodenitrificans* (Patureau et al., 1998), *Pseudomonas* sp. (Zhang et al., 2011), *Rhodococcus* sp. (Chen et al., 2012a), *Bacillus methylotrophicus* (Zhang et al., 2012), *Agrobacterium* sp. (Chen and Ni, 2011) and so on. These microorganisms were mostly isolated from activated sludge or wastewater treatment plants. However, these strains may meet acclimation problems in eutrophic freshwater lakes because of the low carbon source level which could limit the denitrification process (Obaja et al., 2005) and the relatively low nitrate and ammonium concentrations. To overcome this problem, an autochthonous strain which can perform heterotrophic nitrification–aerobic denitrification in eutrophic aquatic niche might be helpful.

The aim of this research was to isolate a novel autochthonous heterotrophic nitrification–aerobic denitrification strain from the eutrophic Lake Taihu in order to overcome the acclimation problems in utilization, and evaluate its application potential in both nitrate and ammonium removal from aquatic eutrophication.

2. Methods

2.1. Media

The media used in aerobic denitrifiers isolation were screening medium (SM), solid and semisolid bromothymol blue medium (BTB). The SM (pH 7.2) included the following reagents per liter: sodium succinate, 2.84 g; $NaNO_3$, 10 mM; KH_2PO_4, 1.36 g; $(NH_4)_2SO_4$, 0.27 g; yeast extract, 1 g; $MgSO_4{\cdot}7H_2O$, 0.19 g; TE (trace element) solution, 1 ml. The ingredients of solid BTB (pH 7.0–7.3) were as follows per liter: agar, 20 g; KNO_3, 1 g; KH_2PO_4, 1 g; $FeCl_2{\cdot}6H_2O$, 0.5 g; $CaCl_2{\cdot}7H_2O$, 0.2 g; $MgSO_4{\cdot}7H_2O$, 1 g; sodium succinate, 8.5 g; BTB regent (1% in alcohol), 1 ml. The ingredients of semisolid BTB were identical with solid BTB except that the addition of agar was 10 g L^{-1}. The components of TE solution were described as Ozeki et al. (2001). LB (Luria–Bertani medium) containing 10 g L^{-1} of Peptone, 5 g L^{-1} of yeast extraction and 10 g L^{-1} of NaCl was used for culture preservation.

2.2. Isolation of aerobic denitrifiers

The sources of aerobic denitrifier isolation were surface water samples from Meiliang Bay of Lake Taihu, where obvious denitrification phenomena were detected by Chen et al. (2012b). Water samples (at a depth of 0.5 m) were collected with 1 L sterile bottles on October, 2010. The samples were immediately transported into the laboratory. 100 ml samples and 100 ml SM were transferred to sterilized 500 ml Erlenmeyer flasks with cotton plugs and rotary shaken at 150 rpm at 30 °C for 3 days to enrich denitrifying microflora. The Erlenmeyer flasks were simply sealed with a rubber stopper without bubbling with argon, implying the initial conditions were aerobic. These procedures were repeated three times. The enrichment samples were cultured on BTB plates at 30 °C for 2 days by the gradient dilution and casting methods. Triplicate enrichments were set up under the same culture conditions to increase the population of denitrifiers. Blue cloudy colonies were isolated and then purified by repeated streaking on fresh agar LB plates at 30 °C. Two days later, purified strains were second screened by semisolid agar-stab culture. Each isolate was inoculated into semisolid BTB medium in test tube incubated at 30 °C. Strains with blue upper medium and bubble formation or agar fracture were namely denitrifiers. All enrichment steps were carried out under sterilized conditions. After these procedures, bacteria proceeding denitrification under aerobic conditions were obtained. The most promising one, named strain T1, was Gram-stained and observed under an optical microscope. After purified, it was stored in tubes at −80 °C.

2.3. Bacterial identification and denitrification gene amplification

The 16S rRNA sequence of strain T1 was determined for bacterial identification. Bacterial common primers 27F/1492R were used for 16S rRNA amplification by a thermocycler (My Cycler™ thermal cycler, Bio-Rad, USA), under the following conditions: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 53 °C,1.5 min at 72 °C and a final step of 10 min at 72 °C. PCR products were run and visualized on a 1% agarose gel electrophoresis and ethidium bromide staining. The AxyPrep kit (Axygen, USA) was used to purify the PCR product following the instruction manuals of the manufacture. After that, the 16S rRNA gene fragments were cloned using a TA cloning kit (Invitrogen, USA) and subjected to sequence using an ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) by Shanghai Sangon Biological Technology Co. Ltd. Finally, the 16S rRNA sequence of the isolate was compared with that of other microorganisms by the way of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/bast.cgi).

The *napA* gene encoding periplasmic nitrate reductase which reduces nitrate to nitrite under aerobic conditions and *nirK* or *nirS* gene encoding nitrite reductase which reduces nitrite to nitric oxide were amplified for confirming the aerobic denitrification. Primers NAP1/NAP2 were used for *napA* amplified using the conditions described as Kong et al. (2006); Primers nirK1F/nirK5R and nirS1F/nirS6R were used for *nirK* and *nirS* amplification respectively, conducted as described (Braker et al., 2000). All PCR products were detected and sequenced as the 16S rRNA amplicon.

2.4. Consumption of nitrogen sources by the isolated strain

After incubation 30 h, the strain T1 was observed to remove 68.9% ammonium-N and 35% nitrate-N in SM (Fig. 2), suggesting heterotrophic nitrification–aerobic denitrification ability. In order to evaluate the nitrogen removal ability of strain T1, 100 mg L^{-1} nitrate-N or ammonium-N was used as sole nitrogen source in the medium separately. Other components per liter of these two media were identical: sodium citrate, 6.77 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; KH_2PO_4, 1 g; TE solution, 2 ml; pH 7.0–7.5. Pellets of strain T1 were collected as follows: the bacteria were cultivated in LB medium at 30 °C with a shaking speed of 170 rpm. 500 ml Erlenmeyer flasks containing 100 ml LB were used. Centrifugation was conducted (4000*g* 10 min) after 16 h of cultivation and then the pellets were washed with sterilized double-distilled water three times to purify the bacterial suspension. To start experiments, 100 ml media were placed into 500 ml Erlenmeyer flasks respectively, and 0.5 g wet pellets were inoculated. During experiments, the cultures were sampled periodically to determine cell growth and nitrogen residual.

2.5. Nitrate and ammonium removal performance in eutrophic lake water

In order to get the highest potential nitrogen removal capability of the organism in application in eutrophic Lake Niche, factors affecting heterotrophic nitrification and aerobic denitrification activities of strain T1 in eutrophic lake water were investigated. Lake water was sampled from Meiliang Bay of Lake Taihu. The concentrations of nitrate-N, ammonium-N, total nitrogen (TN) and total carbon were determined. Strain T1 suspension at the late exponential phase of growth was washed and centrifuged as