Bioresource Technology 148 (2013) 144–148

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Biological removal of nitrate and ammonium under aerobic atmosphere by *Paracoccus versutus* LYM

Zhuang Shi, Yu Zhang *, Jiti Zhou, Mingxiang Chen, Xiaojun Wang

Key Laboratory of Industrial Ecology and Environmental Engineering (Ministry of Education), School of Environmental Science and Technology, Dalian University of Technology, Dalian 116024, China

HIGHLIGHTS

- *Paracoccus versutus* LYM could perform aerobic denitrification and HNAD.
- N_2 was sole denitrification product of HNAD by GC.
- Possible pathway of nitrogen was $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$, then NO_3^- was denitrified to N_2.
- Strain LYM could simultaneously remove ammonium and additional nitrate.

ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 4 August 2013
Accepted 8 August 2013
Available online 14 August 2013

Keywords:
Nitrogen removal
Aerobic denitrification
Heterotrophic nitrification and aerobic denitrification
Paracoccus versutus

ABSTRACT

The bacterium isolated from sea sludge *Paracoccus versutus* LYM was characterized with the ability of aerobic denitrification. Strain LYM performs perfect activity in aerobically converting over 95% NO_3^-–N (approximate 400 mg L^{-1}) to gaseous products via nitrite with maximum reduction rate 33 mg NO_3^-–N L^{-1} h^{-1}. Besides characteristic of aerobic denitrification, strain LYM was confirmed in terms of the ability to be heterotrophic nitrification and aerobic denitrification (HNAD) with few accumulations of intermediates. After the nitrogen balance and enzyme assays, the putative nitrogen pathway of HNAD could be $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$, then NO_3^- was denitrified to gaseous products via nitrite. N_2 was sole denitrification product without any detection of N_2O by gas chromatography. Strain LYM could also simultaneously remove ammonium and additional nitrate. Meanwhile, the accumulated nitrite had inhibitory effect on ammonium reduction rate.

1. Introduction

Ammonium along with its oxidation products (i.e. nitrate), widely distributed in the industrial and domestic waste water, can readily cause eutrophication and thus affect ecosystem and human health (Joo et al., 2005). Biological removal of nitrogen has been recognized as the principal method in terms of cost, removal efficiencies and implementation. Generally speaking, this process consists of two steps, i.e. aerobic nitrification and anaerobic denitrification. It is noted that these two biochemical processes are usually separately conducted for the sake of their different requirements for the dissolved oxygen, carbon sources and retention time (Patureau et al., 2000a,b; Third et al., 2005). Firstly found by Robertson and kuenen (1984) in the 1980s, aerobic denitrification provides a potential way to combine them into an integrated one, apparently more cost-effective and manageable than the separated.

So far, there are a host of literatures dealing with the isolation and characterization of aerobic denitrification bacteria, such as *Pseudomonas stutzei* (Wan et al., 2011), *Acinetobacter calcoaceticus* (Zhao et al., 2010), *Bacillus subtilis* (Yang et al., 2011), *Agrobacterium* (Chen and Ni, 2012), *Marinbacter* (Zheng et al., 2012), *Pseudomonas mendocina* (Zhu et al., 2012). Some of them can not only reduce nitrate under the aerobic atmosphere but also convert ammonium to nitrogen gas via hydroxylamine, nitrite, nitrate and nitrous oxide in the order which they occur. These microorganisms are of outstanding interest with regard to the latter capability, termed as heterotrophic nitrification and aerobic denitrification (HNAD), for it is thus more plausible that the biological nitrogen removal could be set in one reaction tank under the same circumstances, even by only one type of microorganism (Joo et al., 2006; Peng and Zhu, 2006; Takaya et al., 2003).

Nevertheless, some recent reports (Joo et al., 2005; Zhao et al., 2010) revealed that a few bacteria capable of transforming ammonium to nitrogen gas heterotrophically could not directly degrade nitrate or nitrite under aerobic conditions. This indicated that another nitrogen removal pathway immediately via hydroxylamine without any accumulation of nitrate and nitrite should be respon-

* Corresponding author. Tel.: +86 411 8470 6240; fax: +86 411 8470 6252.
E-mail address: zhangyu@dlut.edu.cn (Y. Zhang).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.08.052

sible for this phenomenon. The discrepancy hinted that it would be with undue haste to judge whether a new isolate is an HNAD bacterium just according to the ability of heterotrophic nitrogen removal. Necessarily, more details should be done concerned about the profile of nitrate/nitrite reduction as well as the possible denitrification products and corresponding enzymes.

As a candidate for aerobic denitrification and HNAD, strain LYM isolated by our lab, was identified as *Paracoccus versutus*. To our best knowledge, no relative study has been performed to focus on these characteristics of it. In this paper, its performances of aerobic denitrification and HNAD were tested as a first aim. In order to fully elucidate the mechanism of HNAD by strain LYM, work was done as concerns nitrogen balance and enzyme analysis of the crude cell extracts. The influences of nitrate or nitrite were also investigated with respect to the HNAD process.

2. Methods

2.1. Chemicals

Potassium nitrate (99.0%), ammonium chloride (99.0%) and sodium lactate (99%) were purchased from Tianjin Kermel Chemical Reagent Co., Ltd, China. O_2–He (70:30, V:V) gas was obtained from Dalian Guangming Gas Co., China. All other chemicals were analytical grade reagents, commercial available and used without further purification.

2.2. Bacterial strain and cultivation media

Strain LYM, identified as *P. versutus* by 16S rRNA amplification and sequencing, was isolated from seabed sludge by our research group (Dong et al., 2013). This strain (GenBank accession No.JQ328185) was deposited in Guangdong Culture Collection Center, and the collection number of this strain was GIMCC 1.487.

LB broth medium was prepared for enrichment of the isolated bacteria, which contained 10 g peptone, 10 g NaCl and 5 g yeast extract per liter. To investigate the aerobic denitrification ability and HNAD ability of the strain, medium A and medium B were prepared. The ingredients of medium A in 1000 ml distilled water (pH 7.0) were as follows: 0.1 g $MgSO_4{\cdot}7H_2O$, 7.9 g $Na_2HPO_4{\cdot}12H_2O$, 1.5 g KH_2PO_4 and 3 g KNO_3. The composition of medium B in this experiment was 0.1 g $MgSO_4{\cdot}7H_2O$, 6.7 g $Na_2HPO_4{\cdot}12H_2O$, 1 g KH_2PO_4 and 3 g NH_4Cl per liter. Both medium A and medium B used sodium lactate as sole carbon source whose amount depended on the change of external TN with C/N ratio fixed as 10, and were supplied with 1% (v/v) trace elements solution (pH 6.0), which consisted of 50.00 g EDTA, 22.00 g $ZnSO_4$, 5.54 g $CaCl_2$, 5.06 g $MnCl_2$, 4.99 g $FeSO_4$, 1.10 g ammonium molybdate, 1.57 g $CuSO_4$ and 1.61 g $CoCl_2$ (per liter). Each medium was autoclaved for 20 min at 121 °C.

2.3. Shaking culture experiments

Strain LYM was incubated in LB broth medium at 30 °C with a shaking speed of 150 rpm until the OD_{660} reached approximately 3.0. The bacterium was harvested by centrifugation (10,000 rpm, 10 min) and the pellets were washed with phosphate buffer solution (20 mM, pH 7.0). Centrifugation and washing were repeated twice to purify the bacteria suspension.

To start the shaking culture experiments, different inorganic medium (medium A or medium B, 196 mL each) was adjusted to pH 7.0–7.2 with sodium hydroxide solution, and placed into conical flask (500 mL) with 4 mL of bacterial suspension, followed by cultivation at 30 °C with the speed of 150 rpm (dissolved oxygen 5.75 mg/L) for 48 h. During the incubation, the cultures were sampled periodically to determine cell optical density (OD_{660}), pH and then centrifuged (10,000 rpm, 10 min) to acquire supernatants for the assays of ammonium, hydroxylamine, nitrite, nitrate and chemical oxygen demand (COD). Through the time course nitrogen compounds detection of the whole process on strain LYM, nitrogen balance was tested to better explain the pathway of HNAD process.

2.4. Analysis of gas products detection

Analysis of gas products detection of strain LYM was carried out to further demonstrate the performance of HNAD by strain LYM with ammonium as sole nitrogen source. Medium B (98 mL) and strain LYM suspension (2 mL) were injected into the bottle (250 mL) with rubber cap-seal, which was evacuated and fully aerated with O_2–He. Then the system was cultivated at 30 °C with the speed of 150 rpm for 48 h. The gas samples (1000 μL) were periodically sampled by gas tight syringe to detect N_2O, O_2 and N_2 by gas chromatography.

2.5. Enzyme assays

The bacteria prepared for the crude enzyme extract were harvested by centrifugation (10,000 rpm, 10 min). Then the bacteria pellet was suspended with potassium phosphate buffer solution (20 mM, pH 7.0) and centrifuged (4 °C, 20 min) after ultrasonication with lysis for 30 min. The supernatants fraction was concentrated and suspended to obtain the crude enzyme. Nitrite reductase (NIR) activity using reduced methyl viologen as an electron donor was determined by monitoring the reduction of the nitrite (Hira et al., 2012). The activity of nitrate reductase (NR) was measured by the decrease of the nitrate in presence of cell-free extract in a reaction mixture as previously described (Siddiqui et al., 1993). Hydroxylamine oxidase (HAO) activity was assayed spectrophotometrically using cytochrome c as electron acceptors (Josa Marie et al., 1993). Protein concentration in the cell-free extract was measured by the Bradford assay with boving serum albumin as the standard. The specific enzyme activity was defined as the amount of enzyme which catalyzed the transformation of 1 μmol of the substrate per minute by the amount of protein in mg (Barak and Van Rijn, 2000).

2.6. Analytical methods

Cell optical density was assayed at 660 nm using the spectrophotometer (V-560, Jasco International Co. Ltd, Japan). The ammonium (NH_4^+-N), nitrate (NO_3^--N), nitrite (NO_2^--N) were determined according to the standard methods including Nessler reagent photometry, phenol disulfonic acid method and N-(1-naphthalene) – diaminoethane photometry method. Hydroxylamine was analyzed through indirect spectrophotometry (Frear and Burrell, 1955). The intracellular nitrogen in the bacteria was calculated from the relation between OD_{660} and nitrogen content in the dry cell mass, while the dry cell mass was obtained by drying the bacteria at 105 °C after centrifugation (10,000 rpm, 10 min). The nitrogen in the dry biomass was measured by the element analyzer (vario EL III, elementar, Germany). The pH values were measured by means of pH meter (EL20, Shanghai Mettler Toledo Instrument Co. Ltd, China). Gas samples were determined by a gas chromatography (GC-17A, Shimadzu, Japan). All the data showed in the paper were mean numbers from triplicate experiments.

3. Results and discussions

3.1. Aerobic denitrification performance of strain LYM

Data in Fig. 1 presented the profile of nitrate reduction by strain LYM under aerobic conditions. In the initial period of 18 h, no