Chemical Engineering Journal 257 (2014) 90–97

Contents lists available at ScienceDirect

Chemical Engineering Journal

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

Effects of redox mediators on nitrogen removal performance by denitrifying biomass and the activity of Nar and Nir

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HIGHLIGHTS

- Denitrification rate could be enhanced about 2.10 folds with lawsone addition (75 μM).
- Addition of lawsone markedly mitigated the accumulation of nitrite at each dosing concentration.
- 2-Methyl-1,4-naphthoquinone addition (75 μM) could increase the Nar activity about 1.97 folds compared to the control.
- Anthraquinone-2,6-disulphonate (100 μM) addition could enhance the Nir activity about 2.08 folds compared to the control.

ARTICLE INFO

Article history:
Received 18 April 2014
Received in revised form 2 July 2014
Accepted 8 July 2014
Available online 24 July 2014

Keywords:
Nitrogen removal rate
Denitrification
Redox mediators
Nitrite accumulation
Nar activity
Nir activity

ABSTRACT

In this study, effects of four kinds of redox mediators on denitrification performance, nitrate and nitrite reductase activities were investigated, including anthraquinone (AQ), 2-methyl-1,4-naphthoquinone (ME), lawsone (LAW) and anthraquinone-2,6-disulphonate (AQDS). Experimental results demonstrated that the optimum dosing concentrations of AQ, ME and LAW were 75, 25 and 75 μM. The maximum total nitrogen removal rates increased to 26.02, 20.16 and 33.50 mg-N/g-VSS/h at each optimum dosing concentration, which were approximately 1.60, 1.25 and 2.08 times higher compared to that without redox mediator addition. Unlike AQ and ME, addition of LAW appeared to have suppressed the accumulation of nitrite with peak vales about 21.60 mg/L compared to that of 121.9 mg/L with AQ and 180 mg/L with ME addition. Among four kinds of redox mediators, ME increased the nitrate reductase activity about 1.97 folds, and AQDS enhanced the nitrite reductase activity about 2.08 folds compared to the controls. The unbalance of affected nitrate reductase and nitrite reductase activities by redox mediator was considered as the main reason for nitrite accumulation in this study.

1. Introduction

Denitrification is a widely applied biological process in wastewater treatment for ultimately converting nitrate or nitrite to dinitrogen gas (N_2) through a series of intermediate gaseous nitrogen oxide products. Although this process is mainly carried out by facultative anaerobic heterotrophs (such as Paracoccus denitrificans and various pseudomonas) Carlson et al. [6] using carbonaceous compounds as electron donors, some kinds of autotrophic denitrifiers have also been identified (e.g., Thiobacillus denitrificans) Baalsrud et al., [19]. In terms of general nitrogen cycle, denitrification process was ever considered as the only pathway to complete the nitrogen cycle by returning N_2 to the atmosphere until the discovery of anaerobic ammonium oxidation (anammox) process. Although observations from field and laboratory investigations have indicated anammox is an important process for the global nitrogen cycle [23], denitrification process is still considered as the key process for converting fixed nitrogen to the atmosphere. For instance, Ward et al. [3] argued that denitrification process was responsible for 87–99% of total N_2 production in the Arabian Sea.

Recently, quinones were found to play an important role in the anaerobic transformation of organic and inorganic contaminants Field et al. [14]. As the functional active groups in humic substances, quinones and their analogues were proved to catalyze bioconversion of azo dyes [11]; Rau et al., [18], polychlorinated compounds [8], nitroaromatics Kwon et al. [21] and some kinds of heavy metals [16,27]. Recently, there were also a few studies focused on the role of redox mediators on denitrification process. Aranda-Tamaura et al. [7] investigated the impacts of different quinoid redox mediators on the simultaneous conversion of

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http://dx.doi.org/10.1016/j.cej.2014.07.029
1385-8947/

sulphide and nitrate by denitrifying biomass, including anthraquinone-2,6-disulphonate (AQDS), 2-hydroxy-1,4-naphthoquinone and 1,2-naphthoquinone-4-sulphonate (NQS). They demonstrated that NQS had the highest nitrate reduction rate using sulphide as electron donor [7]. Guo et al. [15] explored the possibility of redox mediator catalyzing denitrification process with antrhraquinone (AQ) immobilized by calcium alginate. They also found that addition of 500 antrhraquinone immobilization beads would accelerate the denitrifying rate about 2 times. Liu et al. [12] demonstrated that anthraquinone-2-sulfonate (0.04 mmol/L) immobilized into the functional electropolymerization biocarriers could increase the denitrification rate about 1.5 folds.

It is well known that denitrification process involves several enzymes for completely converting NO_3^- to N_2. Among them, nitrate reductase (Nar) and nitrite reductase (Nir) are responsible for converting nitrate to nitrite, and nitrite to nitrous oxide, respectively Ferguson et al., [25]. Nar usually locates on the cell membrane or outside the cell, and Nir locates in the cell membrane periplasma. Thus, it is likely that RMs directly contact with Nar or Nir and affect their activity. Furthermore, the effects on different enzymes of denitrifying microbes may lead to the different substrate or intermediate converting rate and the accumulation of intermediates, such as nitrite accumulation. Kelson et al., [4] found the competition between Nar and Nir would result in the accumulation of nitrite during denitrification process. Oh et al. [17] also found that nitrite accumulation occurred with a relative lower C/N ratio (acetate as the only carbon source), and Nar could compete against Nir for carbon source at the lower C/N ratio condition. Hunter [26] considered that the difference between Nar and Nir was the main reason for nitrite accumulation during denitrification process.

In this study, we undertook these experiments with the main aim to explore the detailed relationship between denitrification reaction rate and dose concentrations of different redox mediators. Another key purpose of this study is to evaluate the effects of RMs on the activity of Nar and Nir. Four representative redox mediators, including anthraquinone (AQ), 2-methyl-1,4-naphthoquinone (ME), and lawsone (LAW) and AQDS, were used.

2. Materials and methods

2.1. Inoculum and substrate medium

Anaerobic sludge was obtained from a full scale municipal wastewater treatment plant (Lingshui Wastewater Treatment Plant, Dalian, China). The sludge was cultivated in a lab-scale up-flow anaerobic sludge blanket (UASB) reactor (2.5 L) for nearly 6 months. The influent nitrate concentration ranged between 300 and 350 mg/L at hydraulic retention time (HRT) between 8 and 12 h, resulting in nitrogen loading rate between 0.6 and 1.05 kg NO_3–N/m^3/d. Glucose was utilized as the only electron donor with a C/N ratio of about 4:1. The lab-scale UASB reactor demonstrated high denitrification efficiency (>95%) at steady state during which the nitrate was almost consumed up. And the residual nitrogen was mainly in the form of nitrite at a concentration of about 20 mg/L. During the cultivation period, NO_3^-–N was supplied in the form of KNO_3. The substrate medium contained (mg/L): KH_2PO4 (25), $KHCO_3$ (1250), $CaCl_2{\cdot}H_2O$ (300), $MgSO_4{\cdot}7H_2O$ (200), $FeSO_4$ (62.5), ethylene diamine tetraacetic acid (EDTA) (62.5) and 1 ml/L of trace elements solution as described by Cervantes et al. [10].

2.2. Batch experiments

Anoxic batch incubations were carried out in 120 mL serum bottles containing 100 mL substrate medium. The harvested biomass sample taken from the UASB reactor was washed three times with mineral medium to remove any residual nitrogen. 1.0 g (wet weight) of this fresh biomass (MLVSS about 2100 mg/L) was then transferred to the serum bottles. The initial pH was adjusted at 7.5 while the temperature was maintained at 35 ± 1 °C. The contents of the serum bottles were purged with nitrogen gas to remove O_2 and achieve anoxic conditions. Initial nitrate concentration in each serum bottle was set at about 300 mg-N/L and the reaction time lasted only 4 h. The substrate medium used in batch experiments was same as that of the lab-scale UASB reactor. Samples were taken every 1 h and immediately stored in a refrigerator at 4 °C for analysis. All the batch experiments were carried out in triplicate.

2.3. Analytical methods

For the determination of NO_3–N and NO_2–N concentrations, samples were first filtered through 0.22 μm pore size filters. The filtrate was measured for NO_3–N and NO_2–N using an ion-exchange chromatograph (ICS-1100, DIONEX, USA) with an IonPac AS18 anion column. Mixed liquor suspended solid (MLSS) and mixed liquor volatile suspended solid (MLVSS) concentrations were measured according to the Standard Methods [2]. Besides, the denitrifying biomass was determined by16S rRNA analysis. 16S rRNA gene fragments (~1500 bp) were PCR amplified using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TTGGYTACCTTGTTACGACT-3′), The PCR amplification performed under the condition of 5 min at 95 °C followed by 35 cycles of 45 s at 95 °C, 45 s at 95 °C, and 90 s at 72 °C, followed by 10 min of final extension at 72 °C. Replicate PCR products were pooled and purified by gel electrophoresis using a Qiaquick PCR Gel Extraction Kit (QIAGEN, Stanford, CA, USA), Then, the purified PCR products were cloned using the pMDTM18-T Vector System (TaKaRa, Dalian, China) with TOP10 competent Escherichia coli cells (Tiangen, Beijing, China) and plated on LB (Luria–Bertani) plates supplied with ampicillin (Sigma). Colonies were randomly picked, cultured overnight in LB broth supplemented with ampicillin, and then sequenced on both strands using the vector primers M13F-47 and M13R-48 in Invitrogen Inc. (Beijing, China). Obtained sequences were compared with available sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST).

2.4. Measurement of Nar and Nir activity

5 g (wet weight) denitrification biomass was taken from a lab-scale reactor for determining the crude enzyme activity. The biomass samples were centrifuged at 8000 rpm at 4 °C for 10 min followed by washing three times with sodium phosphate buffer solution (20 mM, pH 7.0). The washed pellets were then resuspended in 20 ml of the same buffer and lysed by freezing and thawing followed by sonication (225 W, at 4 °C for 60 min, Ultrasonic processor CPX 750, USA). Cell mass was separated at 4 °C for 40 min by centrifugation (22,000 rpm). The supernatant was stored at −20 °C and used as cell extract in the determination of protein, nitrate reductase (Nar) and nitrite reductase (Nir), respectively. Protein concentration was measured according to the Bradford procedure [22], using BSA as a standard. The Nar and Nir activities were determined according to the methods described by Zhao et al. [5] and Kataoka et al. [20]. The reduced nitrate and nitrite was taken as a measure of Nar and Nir activities. A unit of enzyme activity is defined as the amount of Nar and Nir enzyme activities that is required to reduce 1 μmol of nitrate and nitrite per minute.

3. Results

Batch experiments were carried out to determine the effects of AQ, ME, LAW and AQDS on nitrogen removal by denitrification