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Potential roles of acyl homoserine lactone based quorum sensing in sequencing batch nitrifying biofilm reactors with or without the addition of organic carbon

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ABSTRACT

Two lab-scale nitrifying sequencing batch biofilm reactors, with (SBBR_CN) or without the addition of organics (SBBR_N), were operated to investigate potential roles of acyl homoserine lactone (AHL) based quorum sensing. AHLs of N-[(RS)-3-Hydroxybutyryl]-L-homoserine lactone, N-hexanoyl-L-homoserine lactone (C_6-HSL) and N-octanoyl-L-homoserine lactone (C_8-HSL) were detected in both reactors. C_6-HSL and C_8-HSL were also detected in batch experiments, especially with stimulated nitrite oxidizing bacteria activities. Quorum sensing affected biofilm formation mainly through the regulation of extracellular protein production. By the metagenomics analysis, many identified genera and species could participate in quorum sensing, quorum quenching and extracellular polymeric substances (EPS) production. A high quorum quenching activity was obtained in SBBR_CN, whereas a high quorum sensing activity in SBBR_N. *Nitrosomonas*-like ammonia oxidizing bacteria, *Nitrospira*-like nitrite oxidizing bacteria and Comammox harbored genes for AHL synthesis and EPS production. Possible relationships among AHLs synthesis, biofilm formation and nitrifiers activity were proposed.

1. Introduction

Nitrification is a prerequisite process for removing nitrogen from wastewater. In this process, ammonium nitrogen (NH_4-N) is converted to nitrite nitrogen (NO_2-N) by ammonia oxidizing bacteria (AOB) or archaea, and then to nitrate nitrogen (NO_3-N) by nitrite oxidizing bacteria (NOB). AOB including *Nitrosomonas* and *Nitrosospira*, and NOB including *Nitrospira* and *Nitrobacter* have been found to survive in diverse circumstances (Siripong and Rittmann, 2007). Recently, complete ammonia oxidizer (Comammox) was found to be able to oxidize NH_4-N to NO_3-N in a biofilm system (Kessel et al., 2015). To date, three Comammox species have been identified, including *Candidatus Nitrospira nitrosa*, *Candidatus Nitrospira nitrificans* and *Candidatus Nitrospira inopinata* (Daims et al., 2015). This shows diverse microbial communities and functions existing in nitrogen conversion systems during wastewater treatment. Furthermore, these newly discovered NH_4-N oxidizers raises questions about how these organisms cooperate or compete with each other when they coexist within one system.

Quorum sensing (QS) is a mechanism regulating interactions among inter- and intra-species. Acyl-homoserine lactones (AHLs) are QS signal substances, which are mainly detected in Gram-negative bacteria and their functions are encoded by the LuxI homologue and recognized by the LuxR homologue (March and Bentley, 2004). AHLs, constituted by acyl chain and S-Adenosy-L-methionine (SAM), vary with forms from C_4 to C_{14}, depending on the length of the acyl chain. So far, AHLs mediated extracellular polymeric substances (EPS) production, biofilm/granule formation, nitrification and denitrification have been reported (Huang et al., 2016). In particular, the QS system has been confirmed in both pure and mixed cultures of nitrifying systems (Mellbye et al., 2016, 2017; Hu et al., 2016; Burton et al., 2005). Pure-cultures of *Nitrobacter winogradskyi*, *Nitrosomonas europaea*, *Nitrosospira multiformis*, *Nitrosospira briensis*, *Nitrobacter vulgaris* and *Nitrospira moscoviensis* could produce AHLs (Mellbye et al., 2016, 2017; Burton et al., 2005). AHLs were also detected in nitrifying biofilms, membrane bioreactor (MBR) and autotrophic nitrification/denitrification biofilms (Hu et al., 2016; Song et al., 2014; Sun et al., 2017).

AHLs can be degraded or disrupted by quorum quenching (QQ) enzymes, including lactonase, acylase and oxidoreductase produced by *Bacillus*, *Aeromonas* and *Pseudomonas* etc. (Lade et al., 2014). The coexistence of QS and QQ activities in activated sludge was confirmed in MBRs and sequencing batch granular sludge systems (Song et al., 2014; Tan et al., 2015; Yu et al., 2016). The modulation of AHL-based QS and

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QQ could improve biological wastewater treatment efficiency (Yu et al., 2016). AHLs-mediated QS or QQ processes might regulate nitrifying activities, particularly in biofilm systems. However, how these consortia involving in the AHLs-mediated QS system and in turn how AHLs affecting microbial activity are not yet well evaluated, especially for the specific functions of AHLs in nitrogen removal. AHLs were correlated with the NH_4-N oxidation by AOB (Burton et al., 2005; Batchelor et al., 1997). For examples, AHLs might directly activate cell growth of *N. europaea* in biofilms (Batchelor et al., 1997). Hence, the possible roles of AHLs in nitrifying biofilms need to be further investigated.

With advances in molecular techniques such as high-throughput sequencing, metagenomics and metatranscriptomics, AHL-mediated QS systems could be evaluated by these techniques. AHLs-producing QS bacteria and AHLs-degrading QQ bacteria were both found in activated sludge systems with metagenomics (Tan et al., 2015). Metagenomics was also used to detect LuxI/LuxR homologues from uncultured bacteria (Nasuno et al., 2012). QS function during transition from flocs to granules was detailed by using metagenomics (Barr et al., 2016). In addition, genes involving in nitrification, denitrification, ammonification and nitrogen fixation processes and corresponding functional microbial communities in activated sludge samples were identified by applying combined metagenomic and metatranscriptomic approaches (Ma et al., 2016).

In this study, the possible roles of AHLs based QS in two nitrifying sequencing batch biofilm reactors (SBBRs) with or without the addition of organic carbon were examined. The aims were to (1) investigate system performance and microbial community, (2) examine possible correlation between QS and nitrifying activity, (3) evaluate QS function in biofilm growth, (4) link QS with EPS contents and its producing microorganism, (5) identify genes related to nitrogen conversion and AHLs synthesis pathways using the metagenomic method and (6) finally analyze relationships among AHLs, nitrifying activity and biofilm formation.

2. Materials and methods

2.1. SBBR systems

Two 2 L lab-scale nitrifying SBBRs (20 cm in height and 10 cm in internal diameter) (SBBR_CN with the addition of organics and SBBR_N without) containing 70% of suspended carriers with the specific surface area of 500 m^2/m^3, were operated for 130 days. The experiment was carried out for four phases with varied hydraulic retention times (HRTs): Phase I of 1–29 d/HRT = 24 h, Phase II of 30–51 d/HRT = 12 h, Phase III of 52–89 d/HRT = 6 h and Phase IV of 90–130 d/HRT = 3 h. The SBBR cycle consisted of an intermittent aeration (aeration on/off of 30 min/30 min, including an initial 5-min filling), a 10-min decanting, and a 20-min idle phase. The temperature was in the range of 18–25 °C and the pH was 8.0–8.5. The aeration rate was 300 mL/min. The reactors were seeded with activated sludge taken from a lab-scale intermittent aeration sequencing batch reactor.

The SBBR_CN was fed with synthetic wastewater with the addition of chemical oxygen demand (COD) of 50 mg/L (50% of starch and 50% of tryptone). Other influent components included 382 mg/L NH_4Cl, 14 mg/L $CaCl_2{\cdot}2H_2O$, 90 mg/L $MgSO_4{\cdot}7H_2O$, 23 mg/L Na_2HPO_4, 10 mg/L yeast extract, 500 mg/L $NaHCO_3$, 500 mg/L $KHCO_3$ and 0.4 mL/L trace elements. The composition of trace elements was according to Smolders et al. (1994). The SBBR_N had the same components of SBBR_CN, but without the addition of organics. The synthetic wastewater contained around 50 mg/L of NH_4-N.

2.2. Nitrifying activity and batch experiments

Nitrifying activities were measured during the acclimation period. 30 carriers were taken from different reactor depths. The carriers were gently washed and then re-suspended with 500 mL synthetic wastewater without the addition of NH_4-N and COD. The experiments were started with sufficient aeration (300 mL/min). During experiments, 20 mg/L of NH_4-N or NO_2-N was dosed to assess activities of AOB and NOB, respectively.

Nitrifying batch experiments were carried out under conditions with or without the addition of organic carbon. 20 carriers were taken from different reactor depths. The carriers were gently washed and then re-suspended with 500 mL synthetic wastewater without the addition of NH_4-N and organic carbon in the erlenmeyer flask. The experiments were started with sufficient aeration (300 mL/min) and 20 mg/L of NH_4-N or NO_2-N. For the flasks with the addition of COD, starch and tryptone (about 100 mg COD/L) were also added.

Each experiment was carried out with duplication. During all experiments, liquid samples were taken at intervals of 10 min to analyze concentrations of NH_4-N, NO_2-N and NO_3-N. Biomass in the forms of volatile solids (VS) was measured after each experiment. Dissolved oxygen (DO) and pH were measured on-line. AHLs in the aqueous phase was measured after each experiment by concentrating 450 mL of supernatant.

2.3. Analytical methods

COD, NH_4-N, NO_3-N, NO_2-N and VS were measured according to standard methods (APHA, 1995). DO and pH were measured by a portable DO meter (Flexi, HACH, USA) and a pH meter (pH 30d, HACH, USA), respectively.

Biomass concentration on the carrier was determined as VS. The biofilm biomass was represented as mg VS per carriers or unit volume. Biofilm thickness and density were determined according to Alves et al. (2002).

The oxidization rate of NH_4-N, the production or reduction rate of NO_2-N, and the generation rate of NO_3-N were calculated based on the following equations:

$$r_{NH_4\text{-}N} = \frac{\Delta c_{NH_4\text{-}N}}{VS{\cdot}\Delta t} \quad (1)$$

$$r_{NO_2-N} = \frac{\Delta c_{NO_2-N}}{VS{\cdot}\Delta t} \quad (2)$$

$$r_{NO_3-N} = \frac{\Delta c_{NO_3-N}}{VS{\cdot}\Delta t} \quad (3)$$

where, $r_{NH_4\text{-}N}$, $r_{NO_2\text{-}N}$ and $r_{NO_3\text{-}N}$ are the oxidization rate of NH_4-N, the net rate of NO_2-N, and the production rate of NO_3-N (mg/g VS h).

EPS was extracted using a heating method with some modifications (Li et al., 2014). Briefly, biofilm biomass was gently scraped from 3 carriers and suspended in 50 mL of phosphate buffered saline (pH of 7.4). The mixture was homogeneously mixed and then centrifuged at 6000 r/min for 5 min. The supernatant after filtration with the 0.45 μm glass fiber membrane filter (GF/B 47 mm, Whatman, UK), was recorded as the loose EPS. The residual biomass was re-suspended in 50 mL of phosphate buffer and heated in a thermostat water bath for 30 min at 80 °C. After heating, the mixture was centrifuged at 11,000 r/min for 15 min, and the supernatant, after filtration through the 0.45 μm glass fiber membrane filter (GF/B 47 mm, Whatman, UK), was recorded as the tight EPS. Polysaccharide in EPS was measured by the phenol-sulfuric acid method (Dubois et al., 1956) with glucose as the standard. Protein and humic acid contents in EPS were measured by the modified Lowry method (Hartree, 1972) with bovine serum albumin and humic acid as the standard. DNA was quantified by the diphenylamine method (Hatcher and Goldstein, 1969).

AHLs in water, EPS and biomass phases were quantified based on Sun et al. (2017). Specifically, for the extraction of AHLs in the biomass phase, biofilm biomass without EPS was used. The biomass was dispersed in 5 mL of acetonitrile and was then completely disrupted using ultrasonication at 200 W for 15 min. After ultrasonication, the mixture was centrifuged at 10,000 r/min for 10 min and the supernatant was