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Disintegration of aerobic granules: Role of second messenger cyclic di-GMP

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HIGHLIGHTS

- Adding manganese ions would cause disintegration of aerobic granules.
- Contents of c-di-GMP were reduced in the presence of manganese ions.
- Contents of extracellular polysaccharides and proteins decreased with manganese ions.
- Polysaccharide producers were lost with added manganese ions.
- c-di-GMP is key chemical factor epistatic to quotum sensing to granular stability.

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ABSTRACT

Loss of structural stability of aerobic granular process is the challenge for its field applications to treat wastewaters. The second messenger, cyclic diguanylate (c-di-GMP), is widely used by bacteria to regulate the synthesis of exopolysaccharide. This study for the first time confirmed the correlation between concentration of intracellular c-di-GMP and the granular stability under sequencing batch reactor (MBR) mode. In the presence of manganese ions (Mn^{2+}), the concentrations of intracellular c-di-GMP and of extracellular polysaccharides and proteins in granules were declined. Clone library study revealed that the polysaccharide producers. *Acinetobacter* sp., *Thauera* sp., *Bdellovibrio* sp. and *Paracoccus* sp. were lost after Mn^{2+} addition. The findings reported herein confirmed that the c-di-GMP is a key chemical factor epistatic to quorum sensing to determine granular stability. Stimulation of synthesis of intracellular c-di-GMP presents a potential way to enhance long-term stability of aerobic granules.

1. Introduction

Aerobic granular sludge can be cultivated and applied in sequencing batch reactors (SBR) for treating high-strength industrial wastewaters (Adav et al., 2008). The aerobic granules have compact interior and large hydraulic diameter so exhibiting sufficient settle ability and high resistance to toxic substances in industrial wastewaters. The challenge to the success of aerobic granular sludge is to maintain the structural stability of granules in long-term operations (Lee et al., 2010). The mechanisms corresponding to stability loss of aerobic granules were proposed as follows: (1) outgrowth of filamentous organisms, (2) hydrolysis of the anaerobic core, (3) function loss of functional strains, (4) shifts in compositions of extracellular polymeric substances (EPS) and (5) other mechanisms including changes in environmental stresses and toxicity of heavy metal ions (Lee et al., 2010). These mechanisms lead to decrease in density or in hydraulic diameter of granules so washout of biomass can easily occur in SBR.

Cell–cell interaction is the first step on formation of aerobic granules. Dosing chemicals can enhance or weaken the granule strength under hydraulic shear (Ren et al., 2013; Cai et al., 2013). Microbial community can communicate amongst cells via quorum sensing (QS) to express or repress the QS-controlled genes (Miller and Bassler, 2001). The bacteria in a biofilm were shown to be able to release signal molecules to induce attached growth of suspended cells (Zhang et al., 2011). Cyclic diguanylate (c-di-GMP) molecules were for the first time reported to regulate cellulose synthesis in *Gluconacetobacter xylinus* (Ross et al., 1987). Used by bacteria as second messenger, c-di-GMP regulates the transition from a motile lifestyle to a sessile state of cells (Tamayo et al., 2007) and were proposed as a key intracellular regulator for

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biofilm stability (Thormann et al., 2006). The concentration of c-di-GMP was demonstrated to correlate with biofilm formation from bacteria (Karaolis et al., 2005; Kuchma et al., 2007; Merritt et al., 2007). Waters et al. (2008) noted that that c-di-GMP is epistatic to QS in the control of biofilm formation in *V. cholerae*. The production of exopolysaccharide and cellulose production was noted to be stimulated by high levels of c-di-GMP (Simm et al., 2004).

Whether c-di-GMP presents a key messenger on the stability of aerobic granules remains unclear. The concentration of intracellular c-di-GMP concentration is determined by activities of diguanylate cyclases (DGCs) that synthesize c-di-GMP and those of phosphodiesterases (PDEs) that degrade c-di-GMP (Bobrov et al., 2005). Loo et al. (2003) noted that addition of Mn^{2+} ions could lead to disintegration of biofilms. This paper quantified the concentrations of intracellular c-di-GMP for aerobic granules under Mn^{2+} stress. The clone libraries were established to compare the yielded bacterial communities with and without Mn^{2+} stress. The role of intracellular c-di-GMP on granular stability was discussed.

2. Methods

2.1. Cultivation of aerobic granules

The aerobic granules were cultivated in a column SBR reactor (6 cm × 180 cm) with 2.3 L of working volume. At the beginning of each cycle, 1.6 L of synthetic wastewater was fed at the following compositions (per liter): NH_4Cl 0.2 g, KH_2PO_4 0.66 g, $CaCl_2$ 0.03 g, $MgSO_4$ $7H_2O$ 0.025 g, $FeSO_4$ $5H_2O$ 0.02 g, $NaHCO_3$ 0.013 g, peptone 0.4 g, yeast extract 0.25 g, pH 7.2 ± 0.1, and chemical oxygen demand (COD) at acetate:propionate = 3:1. The seed sludge was collected from sludge recycling tank with mixed liquor suspended solids (MLSS) of 6000 mg L^{-1}. The aeration rate was 5 L min^{-1}. The SBR was operated at 4 h cycles, each comprising filling (3 min), aeration–settling (227 min), decanting (5 min) and idle (5 min). The reactor operation consisted of three phases: Phase I (1–11 d) at COD = 1500 mg L^{-1}, Phase II (12–15) at COD = 3000 mg l^{-1}, and Phase III (after 16 d) at COD = 1500 mg l^{-1}. The settling time was decreased according to the performances, then the aeration time was correspondingly increased. Mature granules were cultivated at the end of Phase III.

2.2. Batch tests with dosed manganese

The mature granules cultivated in Section 2.1 at MLSS of 450 ± 20 mg L^{-1} were fed into four identical SBRs, namely PA, PB, PC and PD, each with a working volume of 1 l with the inner diameter of 4 cm and height of 115 cm. The volumetric exchange ratio was 80%. The compositions of synthetic medium and SBR operations were the same as Section 2.1 but at a total COD of 200 mg L^{-1}. The concentrations of $MnCl_2$ dosed into PA-PD were 0, 10, 30 and 80 mg L^{-1}, respectively.

2.3. Extraction and measurement of c-di-GMP

The extraction of c-di-GMP was revised from procedures proposed in Simm et al. (2004). The aerobic granule was firstly freeze dried at −60 °C and 0.2 g of the dried sludge and 15 ml of Milli-Q water were loaded in 50 ml tubes. Lysozyme (with buffer) was then added with terminal concentration of 1 mg ml^{-1}, and some glass beads (0.1 mm) were also added and vortex for 15 min, then incubated at 37 °C for 1 h. The mixture was centrifuged at 9000 rpm for 15 min at 4 °C. The supernatant was transferred to a new 50 ml tube with ethanol added at ethanol: supernatant = 2:1 and vortex for 10 s. The tube was incubated at 4 °C for 1 h and shaken every 5–10 min, then centrifuged at 9000 rpm for 15 min at 4 °C. The precipitate was kept and incubated at 37 °C for 3 h, and 3 ml of Milli-Q water was added and vortex for 10 s. The mixture was transferred to a 5 ml tube and centrifuged at 12,000 rpm for 10 min. Finally, 1 ml supernatant was loaded into chromatogram vial for high-performance liquid chromatography (HPLC) analysis to measure concentration of c-di-GMP (Hyodo et al., 2005). In brief, the HPLC (Aglient 1260, Aglient Co. Ltd., USA) was performed with a C18 column at 40 °C, detection at 254 nm by diode array detector (DAD). Runs were performed in mixed solvent (95% of solvent A as 0.9% NaCl and 5% solvent B as 100% acetonitrile) at 1 ml min^{-1}.

2.4. Construction of 16S rRNA gene-based clone libraries

The microbial communities of granules were analyzed using 16S rRNA gene-based clone libraries (Zhao et al., 2010). The genomic DNA (100 μl) was extracted according to manual of PowerSoil DNA isolation kit (Mobic Inc, USA). The PCR primers were composed by Takara Co., Ltd. (Dalian, China), and the sequences were as follow: 8F: AGAGTTTGATCCTGGCTCAG; 518R: ATTACCGC GGCTGCTGG.

The PCR mixture (50 μl) comprised 5 μl of 10 × LA Taq buffer, 8 μl of 2.5 mmol dNTP mixture, 1.5 μl of each primer, 2.5 U LA Taq DNA polymerase, 1 ng of DNA, and sterile ddH_2O to a final volume of 50 μl. The PCR conditions were as follow: 94 °C for 10 min; 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 90 s; a final step of 10 min at 72 °C. The genomic DNA and PCR products were analyzed by electrophoresis in 1% agarose gels.

The non-labeled PCR products were purified by agarose gel. The purified amplicons were ligated into pMD-18T and transformed into *E. coli* DH5α cells. The positive clones were randomly picked for sequencing by Sangon Co., Ltd (Shanghai, China). The 16S rRNA gene sequences were assembled in Sequencher 4.2 (Gene Codes Corp., MI, USA) with minimum match percentage of 97%. Thus, the sequences with similarity values >97% were considered to belong to the same operational taxonomic unit (OTU). The coverage of the clone library was estimated according to Good (1953), and the selected OTUs were consequently blast on NCBI.

2.5. Strength of aerobic granules

The strength of aerobic granules was evaluated by ultrasonic method. Three randomly selected aerobic granules were placed into a 25 ml conical flask loading with 15 ml deionized water, and then the tube was placed in an ultrasonic bath at 20–25 kHz, 65 W. The ultrasonic was intermittently applied at 2.5 s (on)–3 s (off) cycles. The treated samples were collected and measured spectrophotometrically at 600 nm.

2.6. Analytical methods

The concentrations of COD, NH_4^+−N, NO_2^-−N, NO_3^-−N and MLSS were measured according to APHA (1998). The extracellular polymeric substances (EPS) of aerobic granules were extracted using formaldehydr–NaOH method according to Liu and Fang, 2002. The extracted proteins and polysaccharides were quantified by Folin–phenol method (Lowry et al., 1951) and phenol–sulfuric acid method (Herbert et al., 1971), respectively. All the experiments in the study were performed in triplicate with mean data being reported.

3. Results and discussion

3.1. Reactor performances

Removal efficiencies of COD in reactor PA−PD were improved with testing time and all were exceeding 90% (data not shown