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# Calcium precipitate induced aerobic granulation

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## HIGHLIGHTS

- Aerobic granulation mechanisms are refined with role of calcium precipitate.
- Under alkaline pH inorganic cores were formed, onto which bacteria would attach.
- Functional strains were identified.
- c-di-GMP production and expression by Psl and Alg genes were promoted.

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## ABSTRACT

Aerobic granulation is a novel biotechnology for wastewater treatment. This study refined existing aerobic granulation mechanisms as a sequencing process including formation of calcium precipitate under alkaline pH to form inorganic cores, followed by bacterial attachment and growth on these cores to form the exopolysaccharide matrix. Mature granules comprised an inner core and a matrix layer and a rim layer with enriched microbial strains. The inorganic core was a mix of different crystals of calcium and phosphates. Functional strains including *Sphingomonas* sp., *Paracoccus* sp. *Sinorhizobium americanum* strain and *Flavobacterium* sp. attached onto the cores. These functional strains promote c-di-GMP production and the expression by Psl and Alg genes for exopolysaccharide production to enhance formation of mature granules.



## 1. Introduction

Aerobic granular sludge is a promising biotechnology for biological wastewater treatment (Lee et al., 2010; Show et al., 2012). With self-immobilized cells in a three-dimensional extracellular polymeric substance (EPS) matrix, aerobic granules have excellent settleability and resistance to toxicity of pollutants in wastewaters (Adav et al., 2008). A few granulation theories were proposed, including internal core theory (Heijnen et al., 1993), hydrophobic surface theory (Wilén et al., 2008), extracellular polymeric substances theory (Di Iaconi et al., 2006), and divalent cation bridging theory (Sobeck and Higgins, 2002), none of which can be considered a universal explanation. Liu and Tay (2002) implied that aerobic granulation is achieved by four stages, including cell-to-cell contact, initial attachment to form aggregates, enhancement by EPS and hydrodynamic packing. Zhou et al. (2013) noted the occurrence of attachment and detachment of bioflocs from the granule biomass, so proposed that random aggregation-disintegration mechanism controls aerobic granulation. Lv et al. (2014) confirmed that the random aggregation-disintegration mechanism determines the cell-to-cell contact and the initial attachment steps of Liu and Tay's proposal; while for granules cultivated from seed sludge that has a network structure, the EPS enhancement and the hydrodynamic packing are essential to shape the granules.

Layered structures with different bacterial strains residing at different depths of granule interior were documented (Tay et al., 2002). Microbial microorganisms can communicate with each other by quorum sensing. Wan et al. (2013a,b) experimentally verified the correlation between the content of a second messenger, cyclic-di-GMP and granular stability. These authors suggested that enhanced synthesis of intracellular c-di-GMP improved granule stability over long-term operations. Restated, the microbial activity significantly affects the granules' performance.

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Inorganic precipitate is presented in aerobic granules in excess quantity. Ren et al. (2008) suggested accumulation of calcium carbonate in aerobic granules which enhance granule strength but reduce bioactivity. Zhou et al. (2013) characterized the calcium compounds in their aerobic granules and tested by energy dispersive X-ray and X-ray diffraction (XRD) the accumulation of calcium carbonate in the granules and certain magnesium compounds as $Ca_7Mg_2P_6O_{24}$ in the granules. Mañas et al. (2011) discovered calcium deposit in the inner part of aerobic granules as hydroxyl-apatite ($Ca_5(PO_4)_3(OH)$). Juang et al. (2010) cultivated stable granules from continuous-flow reactors and noted that large amounts of phosphates and hydroxides of calcium and iron were formed in the granules to enhance the granule stability. The role of the inorganic core inside the mature granules was not satisfactorily explored.

This study refined the granulation theory combining the role of inorganic core and the microbial activities for granulation. Degradation of organic substances increases suspension pH, which leads to calcium precipitate with phosphate. The so yielded precipitate serves as the cores for bacterial strains to attach and to grow with secretion of excess exopolysaccharides. Experimental observations supporting this theory were presented.

## 2. Methods

### 2.1. Cultivation of aerobic granules

The aerobic granules were cultivated in column-type sequencing batch reactors (SBR) (6 cm × 180 cm) of 2.3 L working volume. For each SBR cycle, 1.6 L of synthetic wastewater was pumped in at the compositions of (per liter): $NH_4Cl$ 0.2 g, $KH_2PO_4$ 0.66 g, $CaCl_2$ 0.03 g, $MgSO_4{\cdot}7H_2O$ 0.025 g, $FeSO_4{\cdot}5H_2O$ 0.02 g, $NaHCO_3$ 0.013 g, peptone 0.4 g, yeast extract 0.25 g, and chemical oxygen demand (COD) at acetate: propionate = 2:1 (ranging 1000–2500 mg $l^{-1}$). The wastewater had pH 7.2 ± 0.2.

The operational parameters of the reactor are listed in Table 1. The seed sludge at suspended solid (SS) of 6000 mg $l^{-1}$ was collected from the recycled sludge stream in a municipal wastewater treatment plant in Shanghai, China. Fine air bubbles for aeration were supplied through a fine-bubble diffuser at the reactor bottom with an aeration rate of 5 l $min^{-1}$. The reactor was operated sequentially in 4 h cycles, with 3 min of substrate filling, 227 min of aeration and settling, 10 min effluent withdraw and idle phase.

### 2.2. Analysis of appearance and central core

The appearances of aerobic granules were observed with a digital camera, and inner microstructure was observed using SEM (Hitachi S-4800, Japan). The pretreatment of aerobic granules for SEM tests was referred to Wu et al. (2010). The internal microstructure of granule was viewed through two directions. Firstly, the granular sample was directly bisected from the middle, and then the sectional views were observed by SEM observation. On the other hand, 10–20 granules at day 50 were randomly selected to disintegrate using ultrasonic method (Wan et al., 2013a). The ultrasonic bath (20–25 kHz, 65 W) was intermittently applied at 2.5 s (on)-3 s (off) cycles. The treated mixture were settled for 30 s, and 3/4 (v/v) supernatant was discharged. Then, equivalent pure water was fulfilled and the same screening procedures were repeated for three times, and white tiny particles were left for further SEM observation.

**Table 1**
Detailed operational parameters.

| Time (d) | COD (mg $l^{-1}$) | Volumetric exchange ratio (%) | Settling time (min) |
|---|---|---|---|
| 1–4 | 1000 | 60 | 20 |
| 5–7 | 2500 | 60 | 10 |
| 8–10 | 1000 | 60 | 10 |
| 11–12 | 5000 | 60 | 10 |
| 13–15 | 1000 | 60 | 5 |
| 16–17 | 2500 | 60 | 5 |
| 18–21 | 2500 | 70 | 5 |
| 22–26 | 1000 | 70 | 5 |
| 27–30 | 1000 | 80 | 2 |
| 31–38 | 2500 | 80 | 2 |

### 2.3. Extraction and measurement of intracellular second messenger

The extraction of c-di-GMP was revised from procedures proposed in previous studies (Wan et al., 2013b). The aerobic granule was firstly freeze dried at 60 °C and 0.2 g of the dried sludge and 15 ml of pure water were loaded in 50 ml tube. Lysozyme (with buffer) was 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. The supernatant was transferred to a new 50 ml tube with double volume of ethanol 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 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. 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. Quantitative real-time PCR (RT-PCR)

Particular gene expression was detected by real-time PCR, as a representation of designated exopolysaccharides (Thomas et al., 2011). In different cultivation phases, the Psl or alginate exopolysaccharides were respectively synthesized catalyzing by parts of polysaccharide synthesis locus gene cluster (Ryder et al., 2007). And, particular gene expression was detected by real-time PCR, as a representation of designated exopolysaccharides (Thomas et al., 2011).

(1) RNA extraction: total RNA was extracted using PowerSoil RNA Isolation kit (Mobio Inc., USA) according to the manufacturer's instructions, and two typical bands of 23S RNA and 16S RNA were confirmed by electrophoresis through 1.5% agarose gel.
(2) Reverse transcription: complementary DNA (cDNA) was synthesized by MV First Strand cDNA Synthesis Kit (Life technology, USA) and kept at −20 °C.
(3) Real-time PCR: the RT-PCR was performed using a 20 μl mixture containing 10 μl ABI SybrGreen PCR Master Mix (2X), 1 μl each primer (10 μM), 2 μl of cDNA template, 6 μl of $ddH_2O$. Amplification was carried out on an ABI Stepone plus real-time PCR system (Life technology, USA). The temperature program consisted of 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 40 s, and 72 °C for 40 s. The melt curve was plotted to evaluate the primer specificity. The 16S rRNA was set as housekeeping gene, and forward and backward primer is respectively as follow: 5′-CCTACGGGAGGCAGCAG-3′ (17 bp, $T_m$ = 58 °C), 5′-ATTACCGCGGCTGCTGG-3′ (17 bp, $T_m$ = 56 °C). The genomic sequences encoding Psl and alginate polysaccharide were referred to Ma et al. (2012). The forward primer for PslB is as follow: