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# Formation of bacterial aerobic granules: Role of propionate

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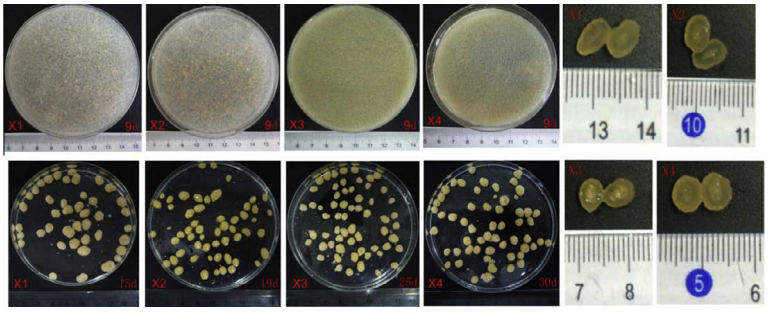
## HIGHLIGHTS

- Propionate is used as main carbon source for aerobic granulation.
- Propionate granules have strong structure but with delayed granulation.
- Propionate shifted microbial community and increased cell hydrophobicity.
- Propionate increased secretion of c-di-GMP to increase EPS production.
- Feed with propionate is promising to cultivate strong aerobic granules.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Propionate presents as one of the major volatile fatty acids in municipal wastewaters, which are not readily degraded as acetate by microorganisms. This study cultivated aerobic granules from column reactors with acetate, acetate/propionate mix (3:1 and 1:3) and propionate as carbon sources and noted that propionate-rich feed would delay granulation, but could generate granules with high structural strength. Propionate feed enriched strains fractionated into the hydrophobic phase, *Sphaerotilus* sp., *Sphingomonadaceae* and *Thauera* sp., in granules and altered hydrophobicity of *Thauera* sp. and *Zoogloea* sp. The enriched strains could secret high quantities of cyclic-di-diguanylate to increase production of extracellular polymeric substances (EPS). The hydrophobic cell surface and increased EPS quantity led to integrated propionate-fed granules. Feed with high propionate concentration is proposed as promising way to cultivate strong aerobic granules for practical use.



## 1. Introduction

Aerobic granulation is an emergent biotechnology for wastewater treatment (Adav et al., 2008; Lee et al., 2010). The dense interior of aerobic granules make the valuable development on the basis of conventional activated sludge, indicating outstanding performances on solid–liquid separation, good toxicity tolerance, and high biomass retention (Liu and Tay, 2004; Nadell et al., 2009; Chen and Lee, 2015). Feeding different carbon sources can yield aerobic granules of distinct characteristics. For example, glucose feed often led to filamentous granules while acetate feed led to bacterial granules (Tay et al., 2002; Du et al., 2011; Wan et al., 2014a). Feeding phenol or pyridine could yield strong granules, although the cultivation time may be long (Adav et al., 2007; Adav and Lee, 2011). Inorganic carbon feed could produce nitrifying bacteria granules (Shi et al., 2010; Wei et al., 2014). Granules cultivated from different carbon sources can have diverse microbial communities, granule morphology and internal structures (Li et al., 2008; Liu and Tay, 2004; Lv et al., 2013).

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With the microorganisms in seed sludge and the feed with specific chemical compositions, the mechanisms for granulation is proposed as a sequential process, including cell–cell contact, self-aggregation as microflocs, entrapment of constituent cells with excess extracellular polymeric substance (EPS), and hydrodynamic compacting of the EPS matrix (Liu and Tay, 2002; Lv et al., 2014). Surface hydrophobicity affects the affinity between microbial cells; at increased hydrophobicity cell–cell interactions are enhanced, also is the granulation rate (Kos et al., 2003; Liu et al., 2004). Microbial microorganisms can communicate amongst cells via certain chemicals, such as cyclic-di-diguanylate (c-di-GMP) to enhance or weaken the cell–cell interactions, hence affecting granule stability (Wan et al., 2013a,b). The contents of intracellular c-di-GMP can be an indicator for tendency of constituent cells to secret EPS in granules in granules.

Acetate and propionate account for 49–70% and 24–33%, respectively, of the volatile fatty acids (VFAs) in municipal wastewater, (Zhang et al., 2008). Microorganisms are generally more readily uptake acetate than propionate since the latter could inhibit activities of microorganisms when at high concentrations (Garrity et al., 2007). Most studies for aerobic granules using acetate as model volatile fatty acids in feed (Lee et al., 2010). Some recent works adopted propionate as one of the main carbon sources for aerobic granulation and noted that, although the granulation may needs extended time, the presence of propionate led to granules with strong interior that can be used without structural deterioration in long-term operation (Lee et al., 2010; Wan et al., 2014b). The role of propionate on aerobic granulation is of practical relevance since it is presented as one of two major VFAs in municipal wastewater needs biological degradation before safe disposal. Additionally, the observations that propionate can yield strong aerobic granules are of great academic interests for exploring the effects of feed substrate on the granulation mechanisms in detail.

This study cultivated aerobic granules from our identical column reactors fed respectively with acetate, acetate/propionate mix (3:1 and 1:3), and propionate. The so yielded granules were characterized on their morphology, strength, content of c-di-GMP, content of EPS, and the hydrophobic and hydrophilic constituent strains in the granules. The role of propionate on granulation process was for the first time detailed.

## 2. Methods

### 2.1. Experimental

Four identical column-type sequencing batch reactors (SBR) (6 cm × 180 cm) of 2.3 L working volume were installed and named as X1–X4. The seed sludge of suspended solid (SS) of 4000 ± 200 mg/L was collected from the recycled sludge stream in a municipal wastewater treatment plant in Shanghai, China. The synthetic wastewater had the following compositions (g/L): $NH_4Cl$ (0.2), $KH_2PO_4$ (0.2), $CaCl_2$ (0.03), $MgSO_4{\cdot}7H_2O$ (0.025), $FeSO_4{\cdot}5H_2O$ (0.02), $NaHCO_3$ (0.013), peptone (0.04), and pH 7.0 ± 0.2. The four reactors were fed with wastewaters containing different carbon sources at the same COD loading: acetate for X1, 3:1 mol/mol mix of acetate and propionate for X2, 3:1 mol/mol mix of acetate and propionate for X3, and propionate for X4. The acetate/propionate ratio of X2 feed was close to those for real municipal wastewater (Zhang et al., 2008). The alternating COD feed proposed by Yang et al. (2014) was adopted herein for granulation. Three different COD concentrations, 1250, 1500 and 5000 mg/L, were adopted in this study. The SBRs were operated with 4 h cycles as air was pumped to the diffuser at the columns' bottom at a volumetric flow rate of 5 L/min. Each cycle comprised 3 min of feeding, 227 min of aeration and settling, 5 min of effluent decanting, and 5 min of idling, with the specific aeration and settling times being set as 30 min and 197 min initially and then regulated to 3 min and 224 min, respectively.

### 2.2. Granule characterization

#### 2.2.1. Microbial community characterization

The total DNA (100 μL) of aerobic granule samples were extracted using Mo-Bio Powersoil DNA Isolation Kit (MoBio Laboratories, Inc., USA) according to the manufacture's protocol. The genomic DNA was evaluated by electrophoresis in 1% agarose gels. The Qubit 2.0 kit was used for DNA quantification. The PCR primers were Bar-PCR IonV3F and IonV4R (CCTCTCTATGGGCAGTCGGTGAT GACTACHVGGGTATCTAATCC). The PCR mixture (50 μL) was mixed according to Wan et al. (2014b) as follows: 5 μL of 10× PCR buffer, 0.5 μL of dNTP (10 mM each), 10 ng of genomic DNA, 1 μL of Bar-PCR primer F(50 μM), 1 μL of primer R(50 μM), 0.5 μL of platinum Taq (5U $\mu L^{-1}$), and sterile $ddH_2O$ to a final volume of 50 μL. The PCR conditions were as follows: 94 °C for 10 min; 5 cycles consisting of 94 °C for 20 s, 45 °C for 20 s, 65 °C for 60 s; 20 cycles consisting of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s; a final step of 10 min at 72 °C. The PCR products were further purified using SanPrep Column DNA Gel Extraction Kit (Sangon Biotech Co. Ltd., Shanghai, China). The extracted PCR products were quantified using Qubit 2.0 kit, and then the PCR products were diluted by sterile $ddH_2O$ with a final concentration of 1:1. Finally, the genomic samples were taken for high-throughput sequenced by iontorrent PGM (life Technology, American).

The sludge mixture was centrifuged at 10,000×*g* for 5 min at 4 °C. The microbial pellet was suspended in 6 mL $ddH_2O$. The 3 mL of microbial mixture and 1 mL n-hexadecane were vibrated for 90 s, and incubated for 10 min at 4 °C (Wan et al., 2014a). The microbial solution in aqueous phase was extracted, regarding as the hydrophilic microorganisms in granules. The microbial communities in initial and hydrophilic solution were analyzed according to Wan et al. (2013a). Briefly, the bacterial community was analyzed by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) technology. Genomic DNA was extracted based on the protocol of PowerSoil DNA isolation kit (Mobio Inc., USA) using PCR primers 8F and 518R (containing GC clamp). The PCR was performed at initial denaturing step at 94 °C for 10 min; then 30 cycles of denaturing at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 30 s; final extension at 72 °C for 10 min. The PCR products were run on 8% polyacrylamide gels with a linear gradient of 30–60% denaturant. Electrophoresis was performed at 140 V for 10 h at 60 °C. Gels were stained with 0.1% $AgNO_3$ and 2.5% $Na_2CO_3$ and were scanned. The nucleotide sequences were compared with those available from the GenBank to identify the closest genes via BLAST searches of the National Center for Biotechnology Information (NCBI) database.

### 2.3. Other analyses

The exterior structures of aerobic granules were observed using cross-sectioning and microscopic photography (Chen et al., 1997). The contents of suspended solids (SS) and mixed liquor suspended solids (MLSS) were measured according to the Standard Methods (APHA, 1998). The contents of extracellular polymeric substances (EPS) of granule samples were extracted using methanol-NaOH method (Liu and Fang, 2002). The protein (PN) and polysaccharides (PS) were measured using by Lowry method and phenol–sulfuric acid method (Chen et al., 2006; He et al., 2006). The strength of aerobic granules was evaluated by ultrasonic method (Wan et al., 2013a). 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