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Short Communication

Species and distribution of inorganic and organic phosphorus in enhanced phosphorus removal aerobic granular sludge

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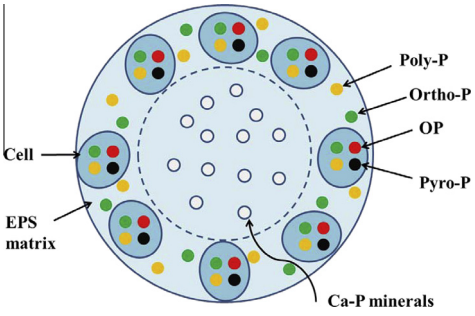
HIGHLIGHTS

- Organic P (monoester-P, diester-P) and poly-P are distributed at the outer layer.
- Poly-P is the primary P specie in granules, microbial cells and EPS.
- Cells and EPS respectively store about 73.7% and 17.6% of TP.
- Mineral precipitates contribute to 5.3–6.4% of TP in EBPR–AGS.
- Hydroxyapatite and calcium phosphate are distributed in the core of granules.

GRAPHICAL ABSTRACT



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ABSTRACT

The species and distribution of phosphorus (P) in an enhanced biological phosphorus removal (EBPR)–aerobic granular sludge (AGS) were fractionated and further analyzed. Results showed that microbial cells, extracellular polymeric substances (EPS) and mineral precipitates contributed about 73.7%, 17.6% and 5.3–6.4% to the total P (TP) of EBPR–AGS, respectively. Inorganic P (IP) species were orthophosphate, pyrophosphate and polyphosphate among which polyphosphate was the major P species in the AGS, cells and EPS. Monoester and diester phosphates were identified as the organic P (OP) species in the AGS and cells. Hydroxyapatite ($Ca_5(PO_4)_3OH$) and calcium phosphate ($Ca_2(PO_4)_3$) were the dominant P minerals accumulated in the core of the granules. Cells along with polyphosphate were mainly in the outer layer of AGS while EPS were distributed in the whole granules. Based on the above results, the distribution of IP and OP species in AGS has been conceived.

1. Introduction

Aerobic granular sludge (AGS) is a promising biotechnology which possesses many incomparable advantages like small footprint, excellent settleability, and high capacity to withstand toxicity and loading rate compared to conventional activated sludge processes (Adav et al., 2008). In an enhanced biological phosphorous removal (EBPR) process, phosphorus accumulating organisms (PAOs) in AGS can accumulate polyphosphate (poly-P) through the release of P to bulk liquor in anaerobic phase and then "luxury uptake" of P in aerobic phase (Zhang et al., 2013a), and P removal is realized by discharging the excess AGS. In general, PAOs are mainly regarded to be responsible for P removal in the EBPR process. However, there are some reports that extracellular polymeric substances (EPS) could accumulate plenty of P contributing to P

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removal as a P reservoir in activated sludge (Cloete and Oosthuizen, 2001; Zhang et al., 2013a,b). Moreover, biologically induced precipitation as the form of hydroxyapatite is estimated to be about 45% of the total phosphorus (TP) removal in AGS process (Angela et al., 2011). Still, the contribution of cell clusters, EPS, and mineral precipitation to P removal in AGS have not been clearly understood.

In addition, identification of P species and their distribution in AGS is helpful to address the characteristics and functions of P in AGS and thus the mechanisms of P removal through this new technology. So far, research works on P species and distribution in aerobic granules are mainly focused on mineral P forms (Angela et al., 2011; Li et al., 2014). Nevertheless, other P species and the distribution of inorganic P (IP, like ortho-P, pyro-P and poly-P) and organic P (OP, like ortho-P monoester and ortho-P diester) in AGS, microbial cells and EPS matrix which are closely related with P removal, have not been documented yet.

This study aimed to investigate the species and distribution of P in the EBPR–AGS. Confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM) were employed to visualize the architectures of the cells, EPS and P distribution in the EBPR–AGS. IP and OP species in AGS, cells and EPS were further analyzed by using ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Based on the obtained results, P distribution in the granules was conceived to better understand the nature of P in aerobic granules.

2. Methods

2.1. Experimental set-up and operation

Aerobic granules were cultivated in two identical sequencing batch reactors (SBRs) and detailed information about the reactors and their operation conditions is shown in Table S1. After aerobic granules appeared, the mixed liquor was withdrawn daily from the reactors in order to keep the solids retention time (SRT) averagely around 10 days (varied between 8 and 12 days). Synthetic wastewater was used in this study. The influent phosphate and ammonium were 20 mg PO_4-P/L and 50 mg NH_4-N/L, respectively. The other influent nutrients were same with a previous work (Huang et al., 2015).

2.2. Extraction and species analysis of P in AGS, microbial cells and EPS

In this study, the cold perchloric acid (PCA) and NaOH extraction procedure was applied to fractionate and characterize P in the AGS and microbial cells according to the schematic diagram shown in Fig. S1a. EDTA – ultrasound extraction method was used for EPS and P extraction from the sludge (Fig. S1b). The extracts were freeze-dried at −50 °C for 48 h, and then the dried extracts were uniformly mixed and stored at −20 °C till ^{31}P NMR analysis.

2.3. Confocal laser scanning microscope (CLSM) observation of AGS

The florescence labeling and CLSM imaging techniques were used to investigate the microbial cells, EPS and poly-P distribution in the EBPR–AGS. The excitation and emission wavelengths for dyes and associated targets are presented in Table S2. This study stained the microbial cells and EPS by following the procedure proposed by Chen et al. (2007). The fluorescent probe 4, 6-diamidino-2-phenylindole (DAPI) was utilized for poly-P observation in AGS (Aschar-Sobbi et al., 2008). The stained granules were sliced into 100 μm sections after frozen at −20 °C, and the sections were then mounted onto a microscopic slide for CLSM (Olympus, FV1000-D) observation.

2.4. ^{31}P NMR analysis

To obtain the ^{31}P NMR spectrum, 200 mg of freeze-dried AGS extracts, EPS powder, or microbial cell extracts were re-dissolved in 0.8 ml of 1 M NaOH and 0.2 ml D_2O followed by 0.2 ml of 100 mM EDTA solution. In order to ensure consistent chemical shifts and optimal spectral resolution during the NMR measurement, EDTA and NaOH solutions were dosed to minimize the interference of divalent/trivalent cations and to adjust the sample pH above 12.0, respectively.

The ^{31}P NMR spectrum was obtained by using a Bruker Avance-600 MHz NMR Spectrometer at 242.94 MHz. 90 °C of pulse width, 25 °C of regulated temperature, and acquisition time of 0.67 s were applied in the experiments. To obtain accurate phosphorus forms, the spectra were collected immediately after preparation and the whole process was completed within 2 h to minimize the transformation of P species. The peaks were assigned to P species according to the reports in literature with peak areas calculated by integration (Turner et al., 2003).

2.5. Other analytical methods

Mixed liquor (volatile) suspended solid (ML(V)SS), chemical oxygen demand (COD), ammonia nitrogen (NH_4-N) and phosphorus (PO_4-P) were measured in accordance with standard methods (APHA, 1998). Total P (TP) in the liquid and sludge was determined with molybdenum blue method after digestion by potassium persulfate at 120 °C. Extracellular proteins (PN) in the extracted EPS were determined with Bradford method (Bradford, 1976). Extracellular polysaccharides (PS) were measured by using phenol–sulfuric acid method (Dubois et al., 1956). The content of Ca-P precipitation in AGS was measured according to the Standards, Measurements and Testing (SMT) Programme extraction protocol (Ruban et al., 1999). The mean granular size was measured by a stereo microscope (STZ-40TBa, SHIMADZU, Japan) with a program Motic Images Plus 2.3S (Version 2.3.0). The morphology of the AGS along with the spatial distribution of elements within the AGS was examined using a scanning electron microscope (SEM, JSM6330F, Japan) combined with an energy dispersive X-ray detector (EDX, EMAX300, Horiba, Japan). The samples used for XRD analysis (Multiflex diffractometer, Rigaku, Japan) were previously dried and calcined in an oven at 500 °C for 2 h in order to remove the organic fraction.

3. Results and discussion

3.1. Aerobic granular cultivation and nutrients removal

Matured aerobic granules were achieved in the SBRs after operation for 90 days with average diameter stabilized at 2.2–2.5 mm. Owing to the stable granules, average COD, NH_4-N and PO_4-P removal efficiencies were 95%, 99% and 98%, respectively in the reactors from day 20 to the end of experiments.

3.2. Distribution of microbial cells, EPS and mineral particles

Fig. S2a–d present a cross-section image of fresh granules on day 100 and their SEM images. The outer layer and core of the granules were yellowish and transparent, respectively, signaling different compositions in these zones of granules. The SEM observations clearly show that most of the bacteria were distributed in the outer layer while very less bacteria in the core of the granules. On the contrary, lots of particle-like substances were observed in the core of the granules with very few found in the outer layer of the granules. Their compositions were further explored in the following experiments.

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