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Distribution of extracellular polymeric substances in anammox granules and their important roles during anammox granulation

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ABSTRACT

Extracellular matrix plays a significant role in formation of matrix structure, biogranulation process and improvement of stability of anammox granules. Distributions of cells and extracellular polymeric substances (EPS) in anammox granules cultured from activated sludge and inactive methanogenic granules were probed. Anammox bacteria secreted more EPS than anaerobic/aerobic granules. The layer of EPS surrounding anammox cells was thicker than other types of granules. In high-enriched granules, the proteins and β-D-glucopyranose polysaccharides were principally distributed at the core, whereas the cells and α-D-glucopyranose polysaccharide accumulated in both the interior and outer layers of granules. In low-enriched ones, cells and α-D-glucopyranose polysaccharides were mainly distributed at the outer rim and the β-D-glucopyranose polysaccharides were located in both the core and the outer layer, whilst the proteins were distributed throughout the whole structure of granules. EPS distribution indicated that low-enriched granules possessed higher stability than high-enriched ones, in consistent with granule strength test.

1. Introduction

The recent discovery of anammox process was first publicly described in the 5th European congress on biotechnology [1]. This unique process is a novel, autotrophic and cost-effective alternative to the traditional biological nitrogen removal process, which possesses plenty of advantages over the nitrification/denitrification techniques, such as low biomass yield, no need for aeration and no addition of external carbon sources, irrespective of the slow growth rate of anammox bacteria [2–4]. Reactors containing granules were considered to be suitable for slowly growing anammox culture. Meanwhile, it was found that the formation of anammox granules could enhance the settleability of biomass and favor physiological conditions for anammox bacteria [3].

In general, bacterial extracellular polymeric substances (EPS) are sticky materials secreted by microorganisms, acting as cementing substances in biofilms and flocs [5]. EPS, which exhibit a rich matrix of polymers, consist of polysaccharides, proteins, nucleic acids, and lipids [6,7]. There is strong evidence that EPS play a significant role in adhesion phenomena, formation of matrix structure, biogranulation process and improvement of long-term stability of granules [8,9]. Liu et al. [10] found that EPS contributed to the matrix structure and stability of anaerobic granules. The contribution of loosely bound EPS and tightly bound EPS to the aggregation of both aerobic and anaerobic sludge was explored using the extended Derjaguin, Landau, Verwey and Overbeek theory [11]. Some studies also found that the metabolic blocking of EPS synthesis and EPS deficiency could result in the failure of microbial aggregation and weakness of granular structure [12,13]. EPS exist in any form of microbial aggregate, such as bioflocs, biofilms, and anaerobic and aerobic granules. Distribution of EPS in aerobic granules and its relationship with structural stability were also investigated recently [6,14]. In comparison with aerobic and anaerobic granules, little is currently known about the role of EPS during anammox granulation, especially the EPS and cells distribution in anammox granules. Our previous study indicated that anammox biomass was tended to form biofilm or granules under high nitrogen substrate environment [3]. It is essential to understand the role of EPS during anammox granulation and the structural details of the extracellular matrix, which is a step toward comprehensive modeling of complex transport/reaction processes in the granules [14].

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Table 1
The stains and collection information used in the proposed staining procedure.

Stain	Emission (nm)	Excitation (nm)
SYTO 63	650–700	633
FITC	505–540	495
Concanavalin A	565–620	555
Calcofluor white	410–479	405

Anammox cells could secrete more EPS contributing to fast anammox granulation [3], while more proof should be supplied to prove this hypothesis, such as the content of EPS and the comparison of different types of granules. This study was to invest the importance of EPS in anammox granules and to probe the internal structural distribution of EPS and cells in anammox granules in combination with the essential role of EPS in granular stability. Two different types of anammox granules cultured from activated sludge and inactive methanogenic granules with different anammox bacterial purities were stained and observed in a CLSM. The staining scheme developed by Chen et al. [14] was adopted to demonstrate qualitatively the distributions of cells (nucleic acids) and EPS (proteins, α- and β-D-glucopyranose polysaccharides) in anammox granules.

2. Materials and methods

2.1. Granules cultivation and sampling

Two types of anammox granules were cultivated in two separate UASB reactors. The running conditions of UASB I were described in Ni et al. [15]. Briefly, a pilot-scale UASB reactor was seeded with mature anammox granules from a landfill-leachate treatment plant at a temperature of 37 °C. Feeding with synthetic wastewater, the reactor was run in a continuously-fed sequence for approximately one year at a hydraulic retention time (HRT) of less than one day. As illustrated in Ni et al. [3], UASB II was a lab-scale glass vessel and inoculated with inactive methanogenic granules and ~1% (volatile suspended solids, VSS) anammox sludge. UASB II was run at a HRT of around 1.5 days at a temperature of 35 °C.

The pH in both reactors was controlled at 7.5 by purging with CO_2 and anoxic conditions were created via argon gas. The synthetic wastewater was prepared by adding ammonium and nitrite to a mineral medium in the required amounts in the form of $(NH_4)_2SO_4$ and $NaNO_2$. The composition of the mineral medium was (g/L): $KHCO_3$ 0.5, KH_2PO_4 0.0272, $MgSO_4{\cdot}7H_2O$ 0.3, $CaCl_2{\cdot}2H_2O$ 0.18 and 1 mL trace elements solutions I and II [4]. The synthetic wastewater was deoxygenated by flushing with argon gas before feeding to the reactor. After the continuous running of one year, granular sludge samples, GI and GII standing for high-enriched anammox granule and low-enriched anammox granule, were taken from UASB I and II for current study, respectively.

2.2. Granule staining

Calcofluor white was purchased from Sigma (St. Louis, MO, USA). The fluorescein isothiocyanate (FITC), Concanavalin A conjugated with tetramethyl rhodamine (Con A), SYTO 63 were ordered from Molecular Probes (Carlsbad, CA, USA). Four dyes and their excitation and emission wavelengths used in this study were listed in Table 1. The collected granules from UASB I and II were kept fully hydrated during the whole staining process. Calcofluor white was utilized to stain the β-D-glucopyranose polysaccharides. The amine reactive dye, FITC was used to stain amine-reactive compounds such as proteins and amino-sugars. Con A was used to bind to α-mannopyranosyl and α-glucopyranosyl sugar residues. SYTO 63, a cell-permeative nucleic acid stain, was utilized to distinguish EPS from cells.

During the sequent staining process, red anammox granule samples were put in 2 mL tubes. The SYTO 63 stain of 100 μL (20 μM) was first added and the sample was placed on a shaker table for 30 min at 150 rpm. After washing with phosphate-buffered saline (PBS, pH 7.2) twice, 0.1 M sodium bicarbonate buffer was added to the sample. 100 μL FITC solutions (10 g/L) were then injected to the sample which was shaken for 1 h at room temperature. Subsequently, the same amount of Con A solution (0.2 g/L) and calcofluor white (300 mg/L) were added and incubated for 30 min, respectively. During the process, after each staining, samples were washed twice by PBS to remove surplus stain. The stained samples were embedded in agarose (with melting point of 65 °C and gelling point of 26–30 °C) for observation. For microscopy observation, stained granules were frozen at -20 °C and sectioned into 50 μm sections before mounting onto a microscopic slide for observation. The staining procedure was described by Chen et al. [14]. A CLSM (Leica TCS SP5Confocal Spectral Microscope Imaging System, Gmbh, Germany) was used to probe the internal structure of granules. The images were analyzed using Leica confocal software.

2.3. EPS extraction and analysis

The EPS in the granules were extracted using cation exchange resin (CER). In general, sludge samples were harvested by centrifugation at 3000 rpm for 15 min at 4 °C and then the sludge pellets were re-suspended in phosphate buffer solution (pH 7.0). The solution was transferred to an extraction bottles, followed by the CER addition with a dosage of 75 g/g suspended solids. These suspensions were stirred at 600 rpm at 4 °C for 2 h. After removing the settled CER, the solutions were centrifuged at 8000 rpm for 30 min to remove remaining sludge components. The supernatants were then filtered through 0.45 μm cellulose membranes and used as the EPS fraction for protein and carbohydrate analyses. The protein content in the EPS was determined according to the Bradford protein assay with bovine serum albumin as the standard [16]. The carbohydrate content in the EPS was measured using the Anthrone method [17] with glucose as the standard. The total EPS content was measured as the sum of these two substances.

2.4. DNA extraction and quantitative real-time polymerase chain reaction (PCR)

Total genomic DNA was extracted by the modified 2% cetyl trimethyl ammonium bromide-based protocol [18]. Genomic DNA preparation was determined with an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and purified DNA samples were stored in sterile deionized water at −20 °C until used. PCR reactions were conducted in 25 μL volumes comprising 300 nM of each primer, DNA samples and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Reactions were performeded in optical-grade 96-well PCR plates in an ABI Prism 7900 Sequence Detection System (ABI, Foster City, CA, USA). The thermal profile was 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Amplification data were analyzed with the Sequence Detection System (SDS) software v. 2.3 (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was then processed based on the description of literature [15].

2.5. Analysis

The granular sludge strength was measured according to the procedure described before [19,20]. The strength of granules is presented in the form of integrity coefficient (IC). The fraction of detached material was used for evaluation of granule strength. To