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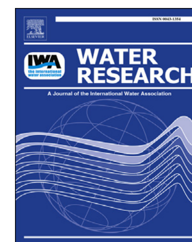

Role of extracellular polymeric substance in determining the high aggregation ability of anammox sludge

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ABSTRACT

The high aggregation ability of anammox sludge has been extensively observed, but the cause for their aggregation is challenging. Here the structure and composition of extracellular polymeric substance (EPS) excreted from anammox sludge were systematically investigated to interpret the high aggregation ability. We combine results of contact angle, zeta potential and surface thermodynamics analysis as well as the following DLVO theory to address this issue. The results show that hydrophobic interaction is the main force determining the aggregation of anammox sludge. To go insight into inherent mechanism, Fourier transform infrared (FTIR) and x-ray photoelectron (XPS) spectroscopy were conducted and demonstrated there were comparatively few hydrophilic functional groups in the EPS of anammox sludge, compared to that of activated sludge, nitrifying and denitrifying sludge. Further, amino acid composition and secondary structure analyses of protein indicated that large amounts of hydrophobic amino acids and high level of protein loose structure for exposing inner hydrophobic groups of protein in EPS significantly contributed to the hydrophobic interaction and further to the high aggregation ability of anammox sludge, which is the critical finding of this work. This investigation is useful for understanding anammox bacteria and then for accelerating the application of the anammox process in wastewater treatment.

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1. Introduction

Anaerobic ammonium oxidation (anammox) is a biological process that uses nitrite as the electron acceptor to convert ammonium to nitrogen gas under anoxic conditions (Jetten et al., 1998). Anammox process for wastewater treatment has several advantages, high efficiency, no need of additional carbon source, decreased oxygen demand and low sludge output etc. (Jetten et al., 2001). Anammox process also can be applied combining with many other biological processes to achieve efficient water treatment (De Graaff et al., 2011; Shi et al., 2013; Jenni et al., 2014; Herbert et al., 2014). As deep-branching monophyletic group of bacteria within the phylum *Planctomycetes*, anammox bacteria exhibit lots of unique features, one of which has been verified that a single

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anammox cell cannot perform anammox reaction and anammox activity can only be detected when the cell density achieves more than 10^{10}/ml (Strous et al., 1998). It is extensively found that anammox sludge is easily to aggregate to display the broccoli shape. This typical shape is characterized as physiognomic feature of anammox bacteria (Egli et al., 2001). During anammox reactor operation, the anammox sludge is frequently found to form biofilm or attach to the container wall (Innerebner et al., 2007). In the suspended activated sludge reactor, high activity anammox sludge is easy to aggregate as red granules settling at the bottom of the reactor (Chen et al., 2013). These existing experiment results undoubtedly verified the high aggregation inclination of anammox sludge. However, a satisfying microscopic description and the origin of the anammox sludge aggregation are still lacking.

It has been well known that extracellular polymeric substance (EPS) released by bacteria play a decisive role in the process of sludge aggregation. Their physicochemical properties and special location make them an important part for maintaining microbial aggregate's structure and function (Liu et al., 2010a, b; Herzberg et al., 2009). For example, it has been found that the electronegative groups of EPS such as carboxyl, hydroxyl and azyl dominate bacterial aggregation due to the bridging ability (Adav et al., 2008; Yuan et al., 2010; Kumar et al., 2004). Proteins and polysaccharides in EPS greatly affect bacteria surface charge, hydrophobicity and aggregates space structure (McSwain et al., 2005; Liu and Tay, 2002). Although the fact that anammox sludge produces more EPS compared to activated sludge, nitrifying sludge and denitrifying sludge (Chen et al., 2013; Cirpus et al., 2006), the key forces determining anammox sludge aggregation and the corresponding structure and composition of EPS for anammox sludge have been remained to be revealed.

The aggregation behavior of anammox sludge is highly significant for the nitrogen removal process. As we known, the slow growth rate of anammox bacteria is the key limitation for the application of the anammox process. Researches demonstrated that the activity of anammox bacteria is obviously dependent on the bacteria density, indicating that the high cell density could lead to the high activity and growth rate (Lu et al., 2012; Ni et al., 2010). In fact, aggregation does not simply mean an increase in cell number. It was recently reported that the information exchange and cooperative action among the bacteria could be strengthened under conditions of high cell density, directly leading to the increased bacteria metabolism and activity (Mostafa, 2012; Strous et al., 1999; Kartal et al., 2011). It is also demonstrated that macromolecular crowding can increase the robustness of gene expression (Tan et al., 2013), indicating that bacterial aggregation could increase the tolerance of bacteria to environmental inhibition. This is very helpful for overcoming another limitation factor -susceptibility to be inhibited for the application of anammox bacteria for wastewater treatment.

To provide insight into the key characteristics of anammox sludge EPS, with the final aim to clarify the aggregation mechanism of anammox sludge, we used activated sludge sludge, nitrifying sludge and denitrifying sludge for comparison in this study. We identified the advantage of anammox sludge in aggregation and the involved main function forces by DLVO theory analysis. Further, we investigated the chemical structure and composition of EPS, proving that protein plays significant roles in anammox sludge aggregation. Finally, we explored the amino acid composition and protein secondary structure analyses to identify the features of proteins from anammox sludge EPS.

2. Material and methods

2.1. *Microorganism*

We primarily focus on anammox sludge below, and other three types of sludge were selected for comparison: nitrifying sludge, denitrifying sludge and activated sludge. Anammox sludge was taken from sequencing batch reactors operated in our laboratory, 80% of which was composed of *Candidatus Brocadia fulgida* (Liu et al., 2013).

Nitrifying sludge was collected from a lab-scale upflow aerobic sludge blanket reactor at Beijing University of Technology, China. The influent NH_4^+-N concentration was around 200 mg/L at hydraulic retention time (HRT) of 8 h. The nitrifying activity was measured as 240 mg NH_4^+-N/gVSS day.

Denitrifying sludge was also collected from a lab-scale upflow anaerobic sludge blanket reactor at Beijing University of Technology, China. The NH_3^--N concentration in influent ranged between 320 and 400 mg/L with COD concentration of 1500 mg/L at HRT of 8 h. The denitrifying sludge activity was measured as 160 mg NH_3^--N/gVSS day.

Activated sludge was collected from the aeration tank at the wastewater treatment plant dealing with domestic wastewater in Beijing, China. At the sampling time, HRT, Sludge Retention Time (SRT), Dissolved Oxygen (DO) and Mixed Liquor Suspended Solids (MLSS) of the aeration tank were maintained at 5 h, 6 d, 2.5 mg/L and 3 g/L respectively.

2.2. *EPS extraction and chemical analysis*

EPS was extracted using the cation exchange resin (CER) method (Frolund et al., 1996). The sludge was harvested using centrifugation at 4000 g for 10 min, and pellets were then washed three times with 0.1 M NaCl solution. The bacterial pellet was then re-suspended to a predetermined volume, and CER was added at a dosage of 70 g/g VSS. The suspensions were stirred for 3 h at 200 rpm and 4 °C. Afterwards, the suspensions were settled for 3 min to remove the CER. EPS were harvested after centrifugation at 9000 G and 4 °C for 20 min. The supernatants were filtered through 0.45 μm acetate cellulose membranes (Advantec Co., Japan) to obtain a crude EPS solution. The crude EPS were then dialyzed (molecular weight cut-off: 8000–14,000 Da, Spectrum Laboratories Co., USA) against deionized water at 4 °C, followed by lyophilization.

For chemical analysis of EPS, polysaccharides were determined using the anthrone method with glucose as the standard (Loewus, 1952). Proteins were determined using the Lowry method with egg albumin as the standard (Sheng and Yu, 2006). Both proteins and polysaccharides results were the average value of three parallel samples.

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