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Quantitative and qualitative characterization of extracellular polymeric substances from Anammox enrichment

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ABSTRACT

Extracellular polymeric substances (EPS) from different Anammox biomass were extracted and characterized by quantitative and qualitative analysis to investigate the link between their characteristics and the enrichment process in lab scale bioreactors. Quantitatively, a decrease of protein to polysaccharide ratio and an increase in total EPS extraction yield were observed during the enrichment process. In the three dimensional excitation emission matrixes, the spectra showed similar location of the fluorescence peaks for all of the samples. Whereas, samples extracted from sludge containing enriched Anammox bacteria possessed two distinct peaks in the low excitation wavelength range (220–230 nm). Multi-excitation peaks might occur as evidenced by the identical fluorescence chromatograms after size exclusion chromatography (SEC) separation at excitation/emission wavelength of 221/350 nm and 280/330 nm. With the process of Anammox enrichment, UV chromatogram at 210 nm after SEC, which is an indicator of polysaccharides, showed increase in both intensity and number of peaks. However, all fluorescence chromatograms, which reflect proteins and soluble microbial by-products, showed similar peak patterns with increased intensity. An increase of EPS hydrophobicity was observed during the enrichment process for both reactors.

1. Introduction

Anaerobic Ammonium Oxidation (Anammox) was firstly observed by Mulder et al. [1] in a denitrifying reactor treating municipal wastewater. It is a process where ammonium is anaerobically oxidized into nitrogen gas with nitrite as electron acceptor mediated by autotrophic bacteria which belong to the group of *Planctomycetes*. Coupled with partial nitrification, the Anammox process provides a cost effective option of autotrophic ammonia removal from wastewater in comparison to the conventional nitrification/denitrification process.

Anammox bacteria tend to grow as aggregates, such as granules and attached growth biofilm, probably as a survival strategy due to their slow growth rate. The production of EPS is essential in the formation of microbial floc, aggregates and biofilm [2]. EPS are sticky highly hydrated matrices which result from microbial cells excretion under stress conditions, cell lysis as well as sorption of molecules from the bulk liquid. The composition of EPS mainly includes polysaccharide, protein, humic-like substances, lipids, nucleic acids, uronic acids and inorganic components [3]. Besides the pure form of proteins and polysaccharides, those molecules also present in composite forms through covalent bonds such lectin-like protein [4], glycoprotein and proteoglycan-like compounds [5]. Different studies on EPS in granular biofilm formation have been conducted for the granulation process of aerobic and anaerobic sludge in which the protein to polysaccharide ratio (PN/PS) ratio was used as an indicator for process performances [6]. The roles of EPS have been believed to include but not limited to: (i) facilitating aggregates formation; (ii) increase of the substrate diffusivity; (iii) influence aggregate morphology by EPS hydrophobicity and (iv) enhanced extracellular enzymatic activity [7,8]. The production of EPS is believed to be triggered by environmental stress, for example, the result of survival strategy under unfavorable condition [9].

A number of EPS characterization methods have been proposed to study the composition and their role in microbiological systems. Quantitative methods provide information about

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global concentration of proteins, humic-like substances, polysaccharides, uronic acids, nucleic acids, etc. To obtain more detailed characteristics of EPS molecules, various qualitative methods have been developed including gas chromatography, Fourier transform infrared spectroscopy (FT-IR), size exclusion chromatography (SEC), 3-dimensionalexcitation-emission matrix (3D-EEM) fluorescence spectroscopy, UV detection, confocal laser scanning microscopy (CLSM), etc. [3]. Among these, 3D-EEM appeared to be a simple and accurate method to identify organic compounds in water and wastewater matrices [10]. Chen et al. [11] empirically defined excitation and emission wavelength boundaries into five regions based on literature survey, which provided the bases of identification of organic matter for other studies [12,13]. As similar to those authors, the specific excitation/emission (ex/em) wavelength couples identified in 3D-EEM matrix with the highest intensity are used for subsequent SEC followed by fluorescence and UV detection in this study. SEC provides qualitative information about the distribution of EPS apparent molecular weight (aMW) from sludge samples [14–16]. UV detections at wavelengths of 210 nm, 254 nm and 280 nm, which assumed to correspond to different fractions of EPS molecules, are frequently used in literature [13,17,18]. Hydrophobicity is also an important parameter in EPS characterization. Hydrophobic interaction plays a main role of sludge performance in settling, flocculation, and/or granulation. Determination of hydrophobicity could be achieved by measurement of contact angle or by fractionation through amberlite XAD resin [19–21].

Studies on EPS extracted from biological wastewater treatment system have been intensively conducted attempting to establish connection between reactor performance and EPS physicochemical characteristics. Those performances include (i) sludge settling and flocculation ability [22,23], (ii) biofilm and granular formation [9] and (iii) membrane fouling [24]. Researches on EPS extracted from Anammox system have been focused on the general composition change in response to salinity stress [25,26]. Chen et al. [27] studied influence of EPS composition and distribution on biomass activity using FT-IR spectra of sludge from a one-reactor partial nitritation/Anammox system without reaching any definitive conclusions on the relationship. *In situ* characterizations after selective staining have been conducted by various authors to reveal the spatial distribution of EPS and cells in Anammox sludge. Both evenly distributed structures [28,29] and layered structures [8,30,31] were found in Anammox and partial nitritation/Anammox granules. Furthermore, Hou et al.[32] found that hydrophobic interaction was the main driving force in the aggregation and that the high hydrophobicity of Anammox sludge was contributed by the presence of highly hydrophobic amino acids and by a loose protein secondary structure facilitating a full exposure of the inner hydrophobic groups [32]. Enrichment processes of Anammox biomass from different seeding sludge have been investigated by various authors [33–35]. However, none of these studies covered the evolution of EPS composition and characteristics. Therefore, the aim of this work is to provide in-depth information regarding EPS characteristics in relation to different stage of Anammox enrichment process and different performance level of enriched Anammox biomass.

2. Materials and methods

2.1. Reactor operation

Seeding sludges were collected from the aeration tank and denitrification tank of a wastewater treatment plant (WWTP) treating municipal wastewater located in Nola, southern Italy. Aerobic sludge (ASR) and denitrification sludge (DSR) were seeded in two identical 5 L glass graduated cylinders operated as sequencing batch reactor (SBR) with working volume of 4 L. Synthetic wastewater was prepared according to deGraaf et al. [36]. Temperature was maintained at 34 ± 1 °C by thermostats. Feeding and discharging of influent and effluent was achieved by peristaltic pumps (Watson Malow, mod. 520Du and Velp Scientific, mod. SP311). Details of the operational conditions of the reactors could be found in a previous study [37]. In brief, ASR was operated under condition with average dissolved oxygen (DO) level of 1 mg/L and a hydraulic retention time (HRT) of 2 days. A total nitrogen removal reached a maximum of 60% after 240 days. DSR was operated with strict oxygen control with DO level of 0.2–0.3 mg/L and a HRT of 4 days. The total nitrogen removal of DSR reached a maximum of 80% after 150 days.

2.2. Extracellular polymeric substances

2.2.1. EPS extraction

Sludge was collected on day 0, 120 and 240 for ASR and day 0 and day 150 for DSR of the whole enrichment period for the investigation of EPS composition and patterns. The number sampling of sludge for EPS characterization was limited by the low amount of biomass due to the slow growth of Anammox bacteria. The interval of sampling was determined according to the shift of nitrogen removal process as well as microbial community [37]. Sampling was conducted when the reactor was fully mixed so that the biomass was representative of the prevailing condition of the reactor. Sludge volatile suspended solid (VSS) was determined according to APHA standard method. The extraction was performed according to the slightly modified CER method provided by Frolund et al. [38]. In brief, the procedure is as follows: sludge was collected and centrifugated at 2000 g for 15 min to separate the solid and liquid phase. Supernatant was discarded and the sludge was resuspended to its original volume by EPS buffer with pH 7.0 ± 0.1 (2 mM Na_3PO_4, 4 mM KH_2PO_4 and 10 mM NaCl). Washed sludge and CER were mixed by a ratio of 70 gCER/gVSS and fully contacted by agitating with magnetic stirrer under 4 °C for 2 h. EPS was separated from the mixture by centrifugation of the mixture at 16,000 g for 15 min at 4 °C.Extracted EPS samples were stored at −20 °C until further analysis.

2.2.2. EPS biochemical analysis

Global concentrations of protein and polysaccharide were measured by colorimetric method. Protein was measured by Lowry method [38]. Humic-like substance was not detected when modified Lowry method [39] was applied probably due to the use of synthetic wastewater [40]. Furthermore, fluorescence emission of extracted EPS solution was never significantly observed in the specific region for humic-like substance in the excitation emission matrix proposed by Chen et al. [11]. Thus humic-like substance was not considered in this study due to their very low concentration in extracted EPS solution. Polysaccharide was measured by phenol-H_2SO_4 method [41]. Total EPS was expressed as total organic carbon (TOC), which was measured by a TOC meter (Shimazu). All the measured results were normalized by TOC of EPS to facilitate comparison.

2.2.3. Excitation emission matrix (EEM) fluorescence spectroscopy

EEM spectra were determined through a spectrofluorometer (Shimadzu RF-5301 PC) to predefine the coupled excitation and emission wavelengths with the highest intensity. EPS samples were filtered by 0.45 μm filter (Sartorius) and diluted by 50–100 times by 50 mM phosphate buffer (pH 7.0 ± 0.1) to achieve a proper concentration for the spectrofluorometer. Temperature was strictly controlled at 20 ± 1 °C in a thermostatic room. Emission intensity was recorded from 225 to 550 nm at each excitation increment of 10 nm ranging from 220 to 400 nm. The 3D EEM graphs were obtained by the software Panorama Fluorescence 3.1 (LabCognition,

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