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Evaluation of the damage of cell wall and cell membrane for various extracellular polymeric substance extractions of activated sludge

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ABSTRACT

Extracellular polymeric substances (EPS) are susceptible to contamination by intracellular substances released during the extraction of EPS owing to the damage caused to microbial cell structures. The damage to cell walls and cell membranes in nine EPS extraction processes of activated sludge was evaluated in this study. The extraction of EPS (including proteins, carbohydrates and DNA) was the highest using the NaOH extraction method and the lowest using formaldehyde extraction. All nine EPS extraction methods in this study resulted in cell wall and membrane damage. The damage to cell walls, evaluated by 2-keto-3-deoxyoctonate (KDO) and *N*-acetylglucosamine content changes in extracted EPS, was the most significant in the NaOH extraction process. Formaldehyde extraction showed a similar extent of damage to cell walls to those detected in the control method (centrifugation), while those in the formaldehyde-NaOH and cation exchange resin extractions were slightly higher than those detected in the control. *N*-acetylglucosamine was more suitable than KDO for the evaluation of cell wall damage in the EPS extraction of activated sludge. The damage to cell membranes was characterized by two fluorochromes (propidium iodide and FITC Annexin V) with flow cytometry (FCM) measurement. The highest proportion of membrane-damaged cells was detected in NaOH extraction (26.54% of total cells) while membrane-damaged cells comprised 8.19% of total cells in the control.

1. Introduction

Extracellular polymeric substances (EPS) are high molecular weight compounds secreted by microorganisms into their environment (Sheng et al., 2010) and include proteins, polysaccharides, humic substances, deoxyribonucleic acids (DNA), lipids, and uronic acid (Frølund et al., 1996; Liu and Fang, 2002; Sheng et al., 2010; d'Abzac et al., 2010). EPS, an important composition of activated sludge (Sheng and Yu, 2006; Nielsen et al., 1996), plays an important role in biological wastewater treatment (Wilen et al., 2003; Neyens et al., 2004; Wang et al., 2005; Long et al., 2009). Because there are other components in activated sludge, EPS must first be extracted before being studied. A number of physical and chemical methods have been applied to extract EPS from activated sludge (Sheng et al., 2010). Common physical methods include centrifugation, ultrasonication and heating (Liu and Fang, 2002; Comte et al., 2006; Sheng et al., 2010) while chemical methods include extractions with ethylenediamine tetraacetic acid (EDTA), formaldehyde, NaOH, NaOH-formamide, H_2SO_4, and NH_3 (Liu and Fang, 2002; Comte et al., 2006; Adav and Lee, 2008; Sheng et al., 2010). However, the quantities and compositions of EPS extracted by different methods varied in previous studies. For example, Liu and Fang (2002) compared the efficacies of extracting EPS from activated sludge and found that EPS extraction by five different methods gave a wide range of efficacies. Extracted EPS, measured as volatile solids (VS), was highest with formaldehyde-NaOH extraction (164.9 ± 3.9 mg/g VS) and lowest with formaldehyde extraction (49.7 ± 1.2 mg/g VS). The constituents of extracted EPS by different methods also varied.

EPS are susceptible to contamination by cellular matter during the extraction process. Because they are the extracellular compounds outside of microbial cells, the released organic matters caused by any damage or lysis of bacterial cells would contaminate the extracted EPS. The EPS must therefore be extracted as much as possible without damaging the integrity of or lysing bacterial cells and it is important to detect any damage to microbial cell structures during the extraction of EPS from activated sludge in order to evaluate the extracted EPS. Monitoring the integrity of bacterial cells and damage to microbial cell structures can be used to evaluate the reliability of EPS extraction methods.

Microbial cell structures are susceptible to be damaged during the EPS extraction include cell walls, membranes and nuclei.

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Table 1
Characteristics of activated sludge used in EPS extraction.

pH	TCOD (mg/L)	SCOD (mg/L)	TSS (mg/L)	VSS (mg/L)
7.03 ± 0.11	2108 ± 35	35 ± 2	2.53 ± 0.06	1.81 ± 0.07

Previous studies had reported the damage of microbial cell structures. Sampathkumar et al. (2003) found that high pH levels during trisodium phosphate treatment caused membrane damage in *Salmonella enterica* serovar enteritidis and resulted in the loss of cell viability; Shen et al. (2009) assessed freeze-thaw and high-pressure low-temperature-induced damage to *Bacillus subtilis* cells with flow cytometry and Pulgarin et al. (2012) studied the damage of cell walls in *Escherichia coli* by TiO_2 suspensions. In some studies of EPS extraction from activated sludge, the damage to microbial cell structures was evaluated by the detection of cellular materials or measuring the composition of cellular structures. Some researchers used the DNA content in extracted EPS to evaluate the damage to nuclei whereby high DNA content indicated contamination with nucleic materials (Frølund et al., 1996; Liu and Fang, 2002; Sheng et al., 2010). Some used the activity of the intracellular enzyme-glucose-6-phosphate dehydrogenase (G6PD) to evaluate the lysis of cell membranes (Wingender et al., 2001; Ras et al., 2008) while others used 2-keto-3-deoxyoctonate (KDO), a characteristic component of lipopolysaccharides in the cell wall of Gram-negative (G^-) bacteria, to evaluate the damage to cell walls (Kumada et al., 1993; Yu et al., 2011; Adav and Lee, 2008). However, the abovementioned tests for cellular materials have their limits: the content of DNA in EPS is uncertain and so the level of DNA contamination resulting from cellular damage is also uncertain; environmental conditions, such as high temperatures and acidic or alkaline pH, can deactivate G6PD (Wang et al., 2002), and only Gram-negative (G^-) bacteria contain KDO (Kumada et al., 1993). It is therefore important to find new, suitable methods for evaluating the damage to cell structures for all microorganisms in EPS extraction.

Additionally, few studies have discussed and evaluated the damage of microbial cell structures in the EPS extraction of activated sludge. It is not clear which microbial cell structures are damaged and to what extent for different EPS extraction methods. Once cell walls and membranes are damaged, the cellular matter would be released because they comprise the outermost structures of cells (Pollard et al., 2007). Thus, the object of this study is to investigate the damage of two important microbial cell structures (the cell wall and cell membrane) in various EPS extractions of activated sludge. Different methods for evaluating the damage of microbial cell structures are also compared.

2. Materials and methods

2.1. Activated sludge

The activated sludge used in the tests was obtained from the aeration tank of a municipal wastewater treatment plant in Beijing, China, which uses an activated sludge process and handles 400,000 t of wastewater daily. The collected sludge samples were first filtered using a 40-mesh sieve to remove the larger particles and then stored at 4 °C before use. Some characteristics of the sludge are summarized in Table 1.

2.2. Extraction of EPS

Nine EPS extraction methods (four physical and five chemical extractions) were compared with centrifugal extraction as the control (Con): heating extraction (He), ultrasound extraction (Ul), cation exchange resin extraction (CER), NaOH extraction (Na), H_2SO_4 extraction (HS), formaldehyde extraction (Fo), formaldehyde-NaOH extraction (FN), formaldehyde-ultrasound extraction (FU) and ethylenediamine tetraacetic acid (EDTA) extraction (ED). Fig. 1 illustrates the detailed procedures of each extraction process, which were carried out according to methods developed by previous studies (Liu and Fang, 2002; d'Abzac et al., 2010; Adav and Lee, 2008). To investigate the changes of sludge characteristics resulting from EPS extraction, the sludge samples (10 mL) were obtained and analyzed before centrifugation at 20,000 × *g* during the extraction process.

2.3. Bacterial cell fluorescent staining and flow cytometry measurement

To study the damage to sludge bacterial cell membranes in the extraction process, approximately 5 mL of sludge obtained before centrifugation was fluorescently stained and measured by flow cytometry (FCM). The sludge cells were stained by two fluorescent dyes: propidium iodide (PI) and FITC Annexin V with an FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen, Heidelberg, Germany). The staining process was conducted according to the manufacturer's instructions. FCM analysis was performed using a flow cytometer (FACSCalibur 4CLR, BD, USA). The operating process of the flow cytometer (include setting gate) were according the manual providing by BD Co. The analyses were finished within 1 h after staining. Approximately 10,000 events were acquired for each sample in the flow-cytometric measurement.

2.4. Chemical analysis

Volatile suspended solids (VSS) and total suspended solids (TSS) of sludge were analyzed according to standard methods (APHA, 1998). Total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) of activated sludge were determined by a COD detector (DR2800, HACH, USA) and SCOD was measured after the sludge was filtered using a 0.45-μm membrane. The pH of sludge was measured by a pH meter (PB-10, Sartorius, Germany). Size distributions and average sizes of sludge samples were determined by a laser particle size analyzer (Mastersizer 2000, Malvern, UK).

The total carbohydrate (Carb) content of extracted EPS was determined by the phenol–sulfuric acid method with glucose as a standard (Dubois et al., 1956) and the total protein (Pro) content by the Lowry et al. (1951) method with bovine serum albumin as a standard. The DNA content of extracted EPS was measured by the diphenylamine colorimetric method using fish sperm DNA and sodium salt as the standard (Sun et al., 1999). KDO content was determined according to Karkhanis et al. (1978), the activity of G6PD according to the method of Lessie and Vander Wyk (1972) and *N*-acetylglucosamine content by the Morgan–Elson colorimetric method with glucosamine as a standard (Morgan and Elson, 1934).