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.

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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 (Wilén 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, H₂SO₄, and NH₃ (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 TiO₂ 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 (UI), cation exchange resin extraction (CER), NaOH extraction (Na), H₂SO₄ 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 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).

Fig. 1. Procedures for EPS extractions.

Fig. 2. EPS extracted by various methods.

3. Results and discussion

3.1. EPS extraction efficiency and changes to sludge characteristics

In this study, the EPS content was determined according to the contents of proteins, carbohydrates and DNA since they are the main components of EPS. Measured quantities were used to evaluate the extraction efficiency of various methods and the results are summarized in Fig. 2.

Extraction of the three organic matters varied with the extraction method. However, the extraction of proteins was the highest for all methods followed by the extraction of carbohydrates. With the exception of the control (centrifugation), proteins extraction was the highest with NaOH (152.72 mg/g VS), followed by extraction upon heating (66.18 mg/g VS) and the lowest with formaldehyde (18.46 mg/g VS). The extraction of carbohydrates was the highest with heating (50.55 mg/g VS) and the lowest with formaldehyde (10.09 mg/g VS). The extraction of DNA was also the highest with NaOH (0.62 mg/g VS), followed by formaldehyde-NaOH extraction (0.36 mg/g VS) and the lowest with formaldehyde (0.06 mg/g VS).

After EPS extraction, the characteristics of sludge were changed (Table 2). It was found that the pH of sludge before and after extraction were different. For extractions involving the addition of an alkali, the pH of sludge after extraction was 12.72 (NaOH extraction) and 12.73 (formaldehyde-NaOH extraction). When acids were added, the pH was 0.98 (H_2SO_4 extraction) and 3.62 (EDTA extraction) after extraction. For other extraction methods, the pH of sludge was slightly increased owing to the release of EPS. Because pH change would result in the denaturation of some organics, such as proteins (Wang et al., 2002), extraction methods resulting in significant changes to pH are not suitable. This is similar to the effects of high temperature and formaldehyde (Wang et al., 2002).

Because of the release of organic matters from microbial cell, the VSS and TSS of sludge decreased. Variations in the VSS and TSS were also introduced by differences in released matters with

Table 2
Characteristics of sludge after EPS extraction.

Extraction method	pH	VSS (mg/L)	TSS (mg/L)	Average size (μm)
Centrifugation (Con)	7.03 $\pm$ 0.11	1.81 $\pm$ 0.07	2.53 $\pm$ 0.06	159.62 $\pm$ 5.23
Heating (He)	7.80 $\pm$ 0.06	1.61 $\pm$ 0.10	2.32 $\pm$ 0.08	149.18 $\pm$ 4.17
Ultrasound (UI)	7.66 $\pm$ 0.08	1.78 $\pm$ 0.06	2.49 $\pm$ 0.05	34.05 $\pm$ 0.92
NaOH (Na)	12.72 $\pm$ 0.10	1.42 $\pm$ 0.06	2.12 $\pm$ 0.06	107.93 $\pm$ 3.45
H_2SO_4 (HS)	0.98 $\pm$ 0.04	1.70 $\pm$ 0.05	2.41 $\pm$ 0.08	97.18 $\pm$ 2.61
Formaldehyde (Fo)	7.41 $\pm$ 0.06	1.79 $\pm$ 0.10	2.51 $\pm$ 0.09	179.33 $\pm$ 7.38
Formaldehyde-NaOH (FN)	12.73 $\pm$ 0.13	1.73 $\pm$ 0.04	2.43 $\pm$ 0.04	137.50 $\pm$ 4.05
Formaldehyde-ultrasound (FU)	7.33 $\pm$ 0.07	1.79 $\pm$ 0.06	2.51 $\pm$ 0.06	30.65 $\pm$ 1.06
EDTA (ED)	7.36 $\pm$ 0.04	1.75 $\pm$ 0.08	2.47 $\pm$ 0.09	128.00 $\pm$ 4.78
Cation exchange resin (CER)	7.24 $\pm$ 0.03	1.76 $\pm$ 0.07	2.48 $\pm$ 0.06	122.68 $\pm$ 2.93

Fig. 3. Size distribution of sludge after EPS extraction.

different extraction methods (Table 2). In contrast to the amount of extracted EPS, the VSS and TSS of sludge was the lowest after NaOH extraction (1.42 and 2.12 mg/L, respectively), followed by heating extraction (1.61 and 2.32 mg/L, respectively) and the highest after formaldehyde extraction (7.41 and 1.79 mg/L, respectively). The changes in VSS and TSS for the same sludge were not consistent because the release of organic and inorganic matters varied for each EPS extraction process.

Because EPS play an important role in maintaining the structure and strength of sludge floc, the extraction of EPS would also affect the structure of the sludge floc (Sheng et al., 2006). All methods except formaldehyde extraction decreased the average size of the sludge floc (Table 2). A decrease in the average size was highest after EPS extraction by formaldehyde-ultrasound followed by extraction with ultrasound. The size distributions of the 10 sludge samples are summarized in Fig. 3. The maximum sizes for sludge samples were decreased after all extractions except formaldehyde extraction and the size distribution curves were clearly shifted to the left (i.e. smaller sizes). From Fig. 3, it can be observed that the largest shift of the distribution curve towards smaller sizes occurred for ultrasound extraction followed by formaldehyde-ultrasound extraction. The maximal volume size for sludge samples after EPS extraction was also changed with some increasing and others decreasing.

3.2. Damage of cell walls in EPS extraction

The cell wall is the outermost layer of cell and provides structural support and protection. In the EPS extraction process, damage to cell walls should be avoided because it eventuates in the release of intracellular matters. Two parameters were used to evaluate the damage of cell walls in EPS extractions: KDO and *N*-acetylglucosamine analyses. The changes to KDO and *N*-acetylglucosamine content in the extracted EPS are summarized in Fig. 4.

Fig. 4. Measured values of three parameters in extracted EPS.

KDO was detected in the control (0.011 ± 0.003 mg/g VSS) because some dead microorganisms (including G^- bacteria) in sludge had released KDO to water (Karkhanis et al., 1978; Kumada et al., 1993). The KDO in the water of sludge after formaldehyde extraction, formaldehyde-NaOH extraction and cation exchange resin extraction were 0.010 ± 0.004 , 0.013 ± 0.006 and 0.012 ± 0.007 mg/g VSS, respectively (Fig. 4). These values are similar to the control and suggested that the three extraction methods resulted in minimal damage to the cell walls of G^- bacteria in sludge (Platt et al., 1985). However, in other extractions, the KDO contents in the supernatants of sludge were much higher than the control with the highest amounts detected after NaOH extraction (0.436 ± 0.012 mg/g VSS) followed by heating extraction (0.268 ± 0.006 mg/g VS). The results suggested that the cell walls of G^- bacteria in sludge were damaged in these EPS extractions. Varying KDO contents for sludge samples also indicated that the ability to damage the cell walls of G^- bacteria differed in these EPS extraction methods.

As a hydrolyzate of peptidoglycan, *N*-acetylglucosamine in the sludge supernatant was also changed after EPS extraction (Bitton, 2005; Nagata, 2003) (Fig. 4). When the cell walls of bacteria (G^+ and G^-) are damaged, the peptidoglycan, a polymer constituent of cell walls, is hydrolyzed and releases *N*-acetylglucosamine (Bitton, 2005; Nagata, 2003). Thus, detection of *N*-acetylglucosamine could be used to detect damage to the cell walls of all bacteria (G^+ and G^-). The *N*-acetylglucosamine content was slightly higher for the formaldehyde-extracted EPS (Fig. 4) (0.011 ± 0.004 mg/g VS) compared with the extracted EPS of the control (0.010 ± 0.003 mg/g VS) (Fig. 4). The formaldehyde-NaOH and cation exchange resin-extracted EPS contained 0.013 ± 0.005 and 0.015 ± 0.007 mg/g VS of *N*-acetylglucosamine, respectively, which were slightly higher than those of the control and formaldehyde-extracted samples. The *N*-acetylglucosamine content of NaOH-extracted EPS was the highest (0.597 ± 0.014 mg/g VS) followed by heating extracted supernatant (0.313 ± 0.011 mg/g VS). Varying contents of *N*-acetylglucosamine in the extracted EPS suggested that the damage to cell walls differed according to the extraction processes whereby higher content corresponded to more damage.

In comparing the contents of *N*-acetylglucosamine and KDO for different extraction methods, similar trends suggested that the damage to cell walls indicated by the two parameters was also similar and that both tests are suitable for detecting cell wall damage during EPS extraction. However, the similar changes to KDO and *N*-acetylglucosamine may be a result of microorganisms in activated sludge being mostly G^- bacteria (Seviour et al., 2000). *N*-acetylglucosamine is released in the damage of cell walls of both G^- and G^+ bacteria (Bitton, 2005) whereas KDO is only released in the damage of G^- bacteria (Platt et al., 1985). *N*-acetylglucosamine analysis is thus more suitable for evaluating the damage to bacterial cell walls in the EPS extraction of activated sludge.

3.3. Damage of cell membranes in EPS extraction

Cell membranes are components of cells beneath the cell wall that protect the cell from its surroundings. During EPS extraction, damage to cell membranes would cause the interior components of cells to be released and increase the content of EPS. Researchers have shown that some treatments (e.g. alkaline treatment, heating, chemical treatment and ultrasound) can damage microbial cell membranes (Goodford, 1971; Virto et al., 2005; Tachibana et al., 1999; Sampathkumar et al., 2003). For example, Sampathkumar et al. (2003) found that high pH (>10.0) during trisodium phosphate treatment causes membrane damage of *S. enterica*. The damage of cell membranes in the EPS extraction should thus be detected. Two analytical methods were used to detect the

Fig. 5. Example of flow-cytometric dot plots with the indication of the four regions to distinguish between intact cells, damaged cells and damaging cells.

damage of cell membranes in the extraction process: G6PD activity measurements and FCM. The results are summarized in Figs. 4–6.

The G6PD activity was highest in the ultrasound-extracted EPS followed by EDTA-extracted EPS; both activities were higher than those found in the control (Fig. 4). Meanwhile, the G6PD activity in the cation exchange resin-extracted EPS was only slightly higher than that in the control. The results suggested that the abovementioned methods damaged cell membranes during the extraction process with CER causing the least damage. In contrast, no G6PD activity was detected in the extracted EPS from other methods owing to the inactivation of G6PD by heating or chemicals (i.e. acid, alkali, EDTA and formaldehyde) (Wang et al., 2002). The results therefore suggest that the activity of G6PD cannot be used to detect the damage of cell membranes in all EPS extractions.

Following the EPS extraction process, cellular membranes may exist in three possible states: intact, damaging and damaged states (Pollard et al., 2007). When the membrane is intact, it can exclude fluorochromes such as propidium iodide. When it is damaged, it becomes permeable and allows the leakage of PI into the cell and the consequent staining of nucleic acids inside the cell. When it is damaging or undergoing apoptosis, phosphatidylserine is no longer restricted to the cytosolic part of the membrane, and instead becomes exposed on the surface of the cell and susceptible to staining by FITC Annexin V (Davey and Hexley, 2011; da Silveira et al., 2002; Zhang et al., 1997). After the cells were stained by the joint use of FITC Annexin V and PI, FCM was used to distinguish the state of the cellular membranes (Vermes et al., 1995). The use of the two dyes (PI and FITC Annexin V) and the setting of thresholds on PI and FITC Annexin V generated four regions in the FCM cytograms, as shown in the example in Fig. 5. Depending on the intensity of the green and red fluorescence emissions, the following regions are distinguished: intact cells ($PI^{-}FITC^{-}$), membrane-damaged cells

Fig. 6. The ratios of different cells in sludge after EPS extraction.

($PI^{+}FITC^{-}$ and $PI^{+}FITC^{+}$) and damaging membrane cells ($PI^{-}FITC^{+}$). The ratios of the three cell types in the sludge samples after EPS extraction are summarized in Fig. 6. The intact cell count was highest in the sludge following formaldehyde extraction (86.55%) and was slightly lower than those in the control (87.01%); the membrane-damaged cell count was highest in the sludge after NaOH extraction (26.54%); cells with damaging membranes were highest in the sludge after EDTA extraction (9.64%). The results suggested that the extent of damage to cell membranes differed for the various extraction processes; NaOH extraction caused the most damage to cell membranes while formaldehyde extraction caused the least. The proportions of damaged and damaging cell membranes by formaldehyde–NaOH extraction and cation exchange resin extraction were 15.55 and 15.48%, respectively, which were higher than the control (12.99%). The results suggested that FCM measurement with propidium iodide (PI) and FITC Annexin V staining is suitable for evaluating the extent of damage to bacterial cell membranes in the EPS extraction of activated sludge.

4. Conclusions

The damage to two important microbial cell structures (the cell wall and cell membrane) in nine EPS extraction methods for activated sludge was evaluated in this study. The experimental results showed that NaOH extraction obtained the most EPS and formaldehyde extraction obtained the least EPS in the nine methods. The cell walls and membranes of sludge microorganisms were all damaged in the nine EPS extraction processes to varying degrees. Although EPS extraction was the highest for the NaOH extraction process, the damages of cell walls and cell membranes were also the highest for this method. In contrast, extraction with formaldehyde resulted in the least EPS but also the least damage to cell walls and membranes. *N*-acetylglucosamine content changes and flow cytometry (FCM) measurement could be used to evaluate the damage of cell wall and cell membrane in EPS extraction of activated sludge, respectively. Up to now, cation exchange resin extraction and formaldehyde–NaOH extraction are the most suitable methods for EPS extraction because they exhibited slightly more damage to cell walls and membranes than the control and their extracted EPS were considerable.

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References

- Adav, S.S., Lee, D.J., 2008. Extraction of extracellular polymeric substances from aerobic granule with compact interior structure. *J. Hazard. Mater.* 154, 1120–1126.
- APHA, 1998. *Standard Methods for the Examination of Water and Wastewater*, 20th ed. APHA, Washington, DC, USA (2-54-2-56, 2-80-2-81).
- Bitton, G., 2005. *Wastewater Microbiology*, third ed. A John Wiley & Sons, Inc., Publication, Hoboken, New Jersey, USA, pp. 7–9.
- Comte, S., Guibaud, G., Baudu, M., 2006. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties: Part I. Comparison of the efficiency of eight EPS extraction methods. *Enzyme Microb. Technol.* 38, 237–245.
- da Silveira, M.G., San Romão, M.V., Loureiro-Dias, M.C., Rombouts, F.M., Abee, T., 2002. Flow cytometric assessment of membrane integrity of ethanol-stressed *oenococcus oeni* cells. *Appl. Environ. Microbiol.* 68, 6087–6093.
- d'Abzac, P., Bordas, F., Joussein, E., van Hullebusch, E., Lens, P.N.L., Guibaud, G., 2010. Characterization of the mineral fraction associated to extracellular polymeric substances (EPS) in anaerobic granular sludge. *Environ. Sci. Technol.* 44, 412–418.

- Davey, H.M., Hexley, P., 2011. Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. *Environ. Microbiol.* 13 (1), 163–171.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.
- Frølund, B., Plamgren, R., Keiding, K., Nielsen, P.H., 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Res.* 30, 1749–1758.
- Goodford, P.J., 1971. The effect of temperature changes on the cell membrane. *Ann. R. Coll. Surg. Engl.* 48 (2), 70–71.
- Karkhanis, Y.D., Zeltner, J.Y., Jackson, J.J., Carlo, D.J., 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. *Anal. Biochem.* 85, 595–601.
- Kumada, H., Haishima, Y., Kondo, S., Umemoto, T., Hisatsune, K., 1993. Occurrence of 2-keto-3-deoxyoctonate (KDO) and KDO phosphate in lipopolysaccharides of *Bacteroides* species. *Curr. Microbiol.* 26, 239–244.
- Lessie, T.J., Vander Wyk, J.C., 1972. Multiple forms of *Pseudomonas multivorans* glucose-6-phosphate and 7-phosphogluconate dehydrogenases: differences in size, pyridine specificity and susceptibility to inhibition by adenosine 5'-triphosphate. *J. Bacteriol.* 110, 1107–1117.
- Liu, H., Fang, H.H.P., 2002. Extraction of extracellular polymeric substances (EPS) of sludges. *J. Biotechnol.* 95, 249–256.
- Long, G.Y., Zhu, P.T., Shen, Y., Tong, M.P., 2009. Influence of extracellular polymeric substances (EPS) on deposition kinetics of bacteria. *Environ. Sci. Technol.* 43, 2308–2314.
- Lowry, N.J., Rosebrough, A.L., Farr Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Morgan, W.T.J., Elson, L.A., 1934. A colorimetric method for the determination of *N*-acetylglucosamine and *N*-acetylchondrosamine. *Biochem. J.* 28, 988–995.
- Nagata, T., 2003. Microbial degradation of peptidoglycan in seawater. *Limnol. Oceanogr.* 48 (2), 745–754.
- Neyens, E., Baeyens, J., Dewil, R., de Heyder, B., 2004. Advanced sludge treatment affects extracellular polymeric substances to improve activated sludge dewatering. *J. Hazard. Mater.* 106, 83–92.
- Nielsen, P.H., Frølund, B., Keiding, K., 1996. Changes in the composition of extracellular polymeric substances in activated sludge during anaerobic storage. *Appl. Microbiol. Biotechnol.* 44 (6), 823–830.
- Platt, R.M., Geesey, G.G., Davis, J.D., White, D.C., 1985. Isolation and partial chemical analysis of firmly bound exopolysaccharide from adherent cells of a freshwater sediment bacterium. *Can. J. Microbiol.* 31, 675–680.
- Pollard, T.D., Earnshaw, W.C., Earnshaw, J., Lippincott-Schwartz, J., 2007. *Cell Biology*, second ed. W.B. Saunders Company, Philadelphia, PN.
- Pulgarin, C., Kiwi, J., Nadtochenko, V., 2012. Mechanism of photocatalytic bacterial inactivation on TiO₂ films involving cell-wall damage and lysis. *Appl. Catal., B: Environ.* 128, 179–183.
- Ras, M., Girbal-Neuhauser, E., Paul, E.M., Sperandio, M., Lefebvre, D., 2008. Protein extraction from activated sludge: an analytical approach. *Water Res.* 42, 1867–1878.
- Sampathkumar, B., Khachatourians, G.G., Korber, D.R., 2003. High pH during trisodium phosphate treatment causes membrane damage and destruction of *Salmonella enterica* serovar enteritidis. *Appl. Environ. Microbiol.* 69, 122–129.
- Seviour, R.J., Maszenan, A.M., Soddell, J.A., Tandoi, V., Patel, B.K., Kong, Y., Schumann, P., 2000. Microbiology of the 'G-bacteria' in activated sludge. *Environ. Microbiol.* 2 (6), 581–593.
- Shen, T., Bos, A.D., Brul, S., 2009. Assessing freeze–thaw and high pressure low temperature induced damage to *Bacillus subtilis* cells with flow cytometry. *Innov. Food Sci. Emerg. Technol.* 10, 9–15.
- Sheng, G.P., Yu, H.Q., Li, X.Y., 2006. Stability of sludge flocs under shear conditions: roles of extracellular polymeric substances. *Biotechnol. Bioeng.* 96, 1095–1102.
- Sheng, G.P., Yu, H.Q., Li, X.Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. *Biotechnol. Adv.* 28, 882–894.
- Sheng, G.P., Yu, H.Q., 2006. Characterization of extracellular polymeric substances of aerobic and anaerobic sludge using 3-dimensional excitation and emission matrix fluorescence spectroscopy. *Water Res.* 40, 1233–1239.
- Sun, Y., Clinkenbeard, K.D., Clarke, C., Cudd, L., Highlander, S.K., Dabo, S.M., 1999. Pasteurella haemolytica leukotoxin induced apoptosis of bovine lymphocytes involves DNA fragmentation. *Vet. Microbiol.* 65, 153–166.
- Tachibana, K., Uchida, T., Ogawa, K., Yamashita, N., Tamura, K., 1999. Induction of cell-membrane porosity by ultrasound. *Lancet* 353 (9162), 1409.
- Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods* 184 (1), 39–51.
- Virto, R., Mañas, P., Álvarez, I., Condon, S., Raso, J., 2005. Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. *Appl. Environ. Microbiol.* 71, 5022–5028.
- Wang, J., Zhu, S., Xu, C., 2002. *Biochemistry*. Higher Education Press, Beijing, pp. 368–380 (In Chinese).
- Wang, Z.W., Liu, Y., Tay, J.H., 2005. Distribution of EPS and cell surface hydrophobicity in aerobic granules. *Appl. Microbiol. Biotechnol.* 69, 469–473.
- Wilen, B.M., Jin, B., Lant, P., 2003. The influence of key chemical constituents in activated sludge on surface and flocculation properties. *Water Res.* 37, 2127–2139.
- Wingender, J., Strathmann, M., Rode, A., Leis, A., Flemming, H.-C., 2001. Isolation and biochemical characterization of extracellular polymeric substances from *Pseudomonas aeruginosa*. *Methods Enzymol.* 336, 302–314.
- Yu, R., Ou, Y., Tan, J., Wu, F., Sun, J., Miao, L., Zhong, D., 2011. Effect of EPS on adhesion of *Acidithiobacillus ferrooxidans* on chalcocopyrite and pyrite mineral surfaces. *Trans. Nonferrous Met. Soc. China* 21, 407–412.
- Zhang, G., Gurtu, V., Kain, S.R., Yan, G., 1997. Early detection of apoptosis using a fluorescent conjugate of annexin V. *Biotechniques* 23, 525–531.