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Extraction of extracellular polymeric substances from aerobic granule with compact interior structure

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Abstract

Extracellular polymeric substances (EPS) were extracted from aerobic granules of compact interior structure using seven extraction methods. Ultrasound followed by the chemical reagents formamide and NaOH outperformed other methods in extracting EPS from aerobic granules of compact interior. The collected EPS revealed no contamination by intracellular substances and consisted mainly of proteins, polysaccharides, humic substances and lipids. The quantity of extracted proteins exhibited a weak correlation with quantity of extracted carbohydrates but no correlation with quantity of extracted humic substances. The total polysaccharides/total proteins (PN/PS) ratios for sludge flocs were approximately 0.9 regardless of extraction method. Protein content was significantly enriched in the granules, producing a PN/PS ratio of 3.4–6.2. This experimental result correlated with observations using excitation–emission matrix (EEM) and confocal laser scanning microscope technique. However, detailed study disproved the use of EEM results as a quantitative index of extracted EPS from sludge flocs or from granules.

Keywords: Extracellular polymeric substances; Granules; Extraction; Ultrasound

1. Introduction

Extracellular polymeric substances (EPS) are secreted largely by bacteria in harsh environments [1]. The content of EPS significantly affects flocculation efficiency [2,3] and dewatering [4] of activated sludge. Also, EPS play a crucial role in building and maintaining structural integrity of aerobic granules through cohesion and adhesion of microbial cells [5–8]. Extracellular polymeric substances are a matrix rich in polymers, including polysaccharides, glycoproteins, proteins, nucleic acids and phospholipids [2,9–16]. Organic compounds such as humic substances and uronic acids have also been detected in EPS [17]. Evans et al. [18] noted that the EPS produced in biofilm was high at low cell growth rate, and was low at high cell growth period. Jahn and Nielsen [19] noted that the protein contents in biofilm from sewers were much higher than polysaccharides or humins. Zhang et al. [20] reported proteins/polysaccharides ratio of 0.5–0.7 for EPS in biofilms on a rotating angular reactor. Bura et al. [21] compared the composition of EPS in activated sludge and noted that the proteins presented the major part of the EPS in all activated sludge samples. Lawrence et al. [22], Neu and Lawrence [23] and Staudt et al. [24] utilized a lectin-binding analysis and the use of confocal laser scanning microscope (CLSM) to detect glycoconjugates in biofilm systems.

Physical means of extracting EPS from sludge include centrifugation, ultrasonication and heating as well as chemical means such as NaOH, ethylenediamine tetraacetic acid (EDTA) and cation exchange resins [25]. When heating a bio-sample in NaOH solution at 80 °C to extract EPS, McSwain et al. [16] noted DNA contamination in the extracted EPS due to cell lysis. Liu and Fang [17] compared different physical and chemical EPS extraction methods in aerobic, acidogenic and methanogenic sludges and concluded that chemical reagents formaldehye plus NaOH were most effective for extracting EPS from their tested sludges. Formaldehyde fixed the cell walls to prevent lysis, and NaOH increased local pH to dissociate acidic groups in EPS. However, the authors of that study also noted that staining-confocal laser scanning microscope (CLSM) tests indicated the proposed formaldehyde + NaOH scheme could extract only a limited portion of EPS in sludge (for example, 165–179 mg g^{-1} out of 435–536 mg g^{-1} sludge).

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The content and composition of EPS require elucidation to clarify the role of EPS in various sludge processes. Comte et al. [26,27] noted that applied chemical reagents could contaminate collected EPS. Further study by Comte et al. [28] revealed that applied chemical reactants could affect the high-pressure size exclusion chromatography (HPSEC) fingerprint of EPS whereas physical extraction methods only affect corresponding molecular weight distributions. The authors additionally noted that physical means (such as centrifugation) were either inefficient for extraction or could induce significant cell lysis (e.g., heating) and contaminate the EPS.

Aerobic granules are compact bioaggregates [29–31]. Wang et al. [8] determined that non-soluble β-polysaccharide forms the outer shell of aerobic granules, providing granular strength. Further, Chen et al. [32] described the distribution of EPS (proteins, α- and β-polysaccharides and lipids) and cells (total and dead) in aerobic granules using a novel six-fold staining scheme and CLSM technique. Extracting EPS from aerobic granules is more difficult than from sludge flocs due to the low surface-to-volume ratio of chemical exposure [17]. Tay et al. [41], McSwain et al. [16] and Sheng and Yu [42] extracted EPS from aerobic granules by heating the samples at 80 °C (NaOH) or by mixing them with cation exchange resins. Particularly, McSwain et al. [16] noted due to the large size of the granules satisfactory EPS extraction requires mechanical breakup of aggregates before chemical extraction. As noted by Liu and Fang [17] the chemical reagents formaldehyde + NaOH are effective for extracting EPS from sludge flocs. However, no systematic studies have examined how formaldehyde + NaOH extract EPS from aerobic granules, with or without mechanical breakup stages.

This study examined the efficiency of EPS extraction from aerobic granules using formaldehyde + NaOH as a chemical reagent. Phenol-fed granules of compact interior were herein used. Extractions were also applied on activated sludge as a comparison. Ultrasonication was applied before or after extraction. Formaldehyde was used to fix the cell walls from lysis during NaOH extraction. In clinical studies, formamide is widely used to detach tubules by shrinking without breaking the cells [33]. In this study, extraction of EPS was tested by replacing formaldehyde with formamide. Restated, eight extraction schemes (including controls) were analyzed to compare EPS extraction efficiency from aerobic granules.

2. Material and methods

2.1. Samples

Aerobic activated sludge was collected from a local municipal wastewater treatment plant in Taipei, Taiwan. The sludge sample was inoculated in three geometrically identical acrylic columns 120 cm high and 6 cm in diameter and supplied with 2.0 l min^{-1} air from the reactor base. The columns were fed with synthetic wastewater containing phenol (400 mg l^{-1}) as a sole carbon source with the following composition: 1000 mg l^{-1} $(NH_4)_2SO_4$; 200 mg l^{-1} $MgCl_2$; 100 mg l^{-1} NaCl; 20 mg l^{-1} $FeCl_3$; 10 mg l^{-1} $CaCl_2$; phosphate buffer (1350 mg l^{-1} KH_2PO_4, 1650 mg l^{-1} K_2HPO_4); pH 6.8 and, micronutrients (g l^{-1}): H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2$, 0.03; $MnSO_4{\cdot}H_2O$, 0.05; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2{\cdot}6H_2O$, 0.05; and NiCl, 0.05 [34]. The medium was sterilized via autoclaving for 15 min at 121 °C. The phenol solution was filter sterilized and added to the autoclaved medium. The reactor was operated in 12 h cycles. Aeration was provided through the reactor bottom. The granules formed and matured in three weeks.

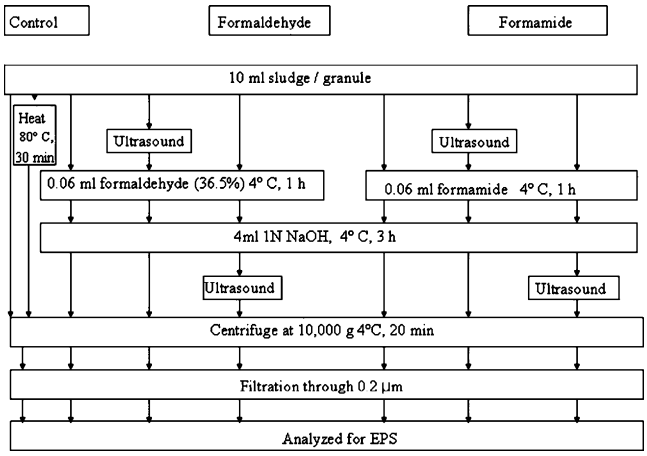

Fig. 1. Flow chart of EPS extraction methods.

At steady-state, the phenol removal rates of the original sludge and the present mature granule systems were 82.1 and 96.3%, respectively. The granules appeared in near round shape and with compact structure. Both the original sludges and the mature granules were used as testing samples. The granules were washed with Milli Q water before test. The sludge was settled for 1.5 h at 4 °C with the supernatant decanted. Then the thickened sludge was centrifuged at 2000 × *g* for 10 min at 4 °C and the pellets were resuspended in Milli Q water.

2.2. Extraction of EPS

Fig. 1 illustrates the details of the EPS extraction processes. As a control, the EPS was physically extracted with high speed centrifugation (10,000 × *g*) for 20 min without adding chemicals. Some sludge or granule samples were first extracted by NaOH with or without pre-pretreatment using formaldehyde or formamide. The extracts were then filtered and analyzed. In some tests, low frequency ultrasound at 120 W for 5 min, in ice bath before or after the chemical extraction were applied. Hence, seven extraction procedures were tested and compared: heat, formaldehyde + NaOH, ultrasound + formaldehyde + NaOH, formaldehyde + NaOH + ultrasound, formamide + NaOH, ultrasound + formamide + NaOH, and formamide + NaOH + ultrasound. Following extraction, all samples were centrifuged and filtered through 0.2 μm filters to collect soluble fractions. The extracted samples were stored at −20 °C in aliquots until analyses were performed.

The extents of EPS contamination by cell lysis during extraction of alkaline or heat treated samples are hard to measure since high pH or heat are known to denaturize enzymes and proteins, such as glucose-6-phosphate dehydrogenase [16]. The extents

Table 1
EPS components of sludge by various extraction methods

Extraction method	Proteins (mg g VSS^{-1})	Carbohydrates (mg g VSS^{-1})	Humic substances (mg g VSS^{-1})	Lipids (mg g VSS^{-1})	DNA (mg g VSS^{-1})	KDO (mg g VSS^{-1})	PN/PS[a]
Sludge							
Formaldehyde–NaOH	35.9 ± 4.5	44.1 ± 5.4	52.4 ± 10.7	12.5 ± 7.4	0.34 ± 0.03	0.10 ± 0.02	0.82
Formaldehyde–NaOH–ultrasound	40.5 ± 7.8**	50.2 ± 7.6***	71.6 ± 15.3***	15.3 ± 8.2	0.37 ± 0.04	0.12 ± 0.04	0.81
Ultrasound–formaldehyde–NaOH	49.7 ± 17.1**	59.1 ± 8.1***	77.5 ± 18.1***	13.5 ± 3.5	0.27 ± 0.03	0.16 ± 0.03	0.85
Formamide–NaOH	49.0 ± 9.4	49.9 ± 19.4	45.8 ± 6.6	12.9 ± 5.9	0.29 ± 0.05	0.12 ± 0.02	0.99
Formamide–NaOH–ultrasound	48.1 ± 12.9	49.6 ± 19.2	71.6 ± 21.1$^{\$\$\$}$	15.7 ± 3.6	0.21 ± 0.03	0.17 ± 0.07	0.97
Ultrasound–formamide–NaOH	53.5 ± 18.5$^{\$\$}$	54.9 ± 12.1$^{\$}$	77.5 ± 15.2$^{\$\$\$}$	18.3 ± 4.7	0.38 ± 0.01	0.19 ± 0.05	0.98
Heat	62.2 ± 28.8$^{**\$\$}$	50.2 ± 7.9	58.7 ± 12.3	10.7 ± 8.2	0.20 ± 0.7	0.10 ± 0.03	1.04
Control	5.8 ± 1.1	7.1 ± 0.7	7.4 ± 0.9	14.6 ± 5.2	0.08 ± 0.01	0.01 ± 0.02	0.81
Granules							
Formaldehyde–NaOH	309.5 ± 11.2	70.0 ± 21.7	52.4 ± 5.2	54.1 ± 5.8	0.29 ± 0.01	0.14 ± 0.02	4.4
Formaldehyde–NaOH–ultrasound	384.6 ± 28.1***	85.1 ± 13.54**	91.7 ± 15.4***	59.2 ± 9.8	0.48 ± 0.03	0.18 ± 0.04	4.5
Ultrasound–formaldehyde–NaOH	455.2 ± 19.6***	85.1 ± 25.3**	85.2 ± 27.3***	68.4 ± 14.2	0.43 ± 0.01	0.21 ± 0.08	5.3
Formamide–NaOH	374.9 ± 24.5	61.0 ± 14.9	49.3 ± 13.8	58.3 ± 15.1	0.37 ± 0.06	0.19 ± 0.06	6.2
Formamide–NaOH–ultrasound	377.8 ± 68.7	106.1 ± 16.7$^{\$\$\$}$	71.7 ± 12.5$^{\$\$\$}$	59.1 ± 17.2	0.54 ± 0.02	0.21 ± 0.09	3.6
Ultrasound–formamide–NaOH	537.2 ± 36.3$^{\$\$\$}$	109.3 ± 15.3$^{\$\$\$}$	85.2 ± 20.5$^{\$\$\$}$	49.2 ± 15.4	0.57 ± 0.07	0.22 ± 0.08	4.9
Heat	238.2 ± 28.8$^{***\$\$\$}$	57.2 ± 20.7$^{*\$}$	59.6 ± 14.2	38.2 ± 17.2	0.38 ± 0.08	0.08 ± 0.06	3.6
Control	15.1 ± 0.2	6.2 ± 0.1	5.5 ± 0.4	45.1 ± 14.4	0.09 ± 0.01	0.01 ± 0.04	2.4

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. formaldehyde–NaOH alone and $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ vs. formamide–NaOH alone test (Tukey–Kramer multiple comparisons test).

[a] PN/PS, proteins/polysaccharides ratio.

of cell lysis during EPS extraction was estimated by analyzing the quantity of 2-keto-3-deoxyoctonate (KDO) in samples, using KDO (0.1–2 μg, Sigma Chemicals Co.) as a standard [35]. KDO is part of the cell membrane of bacteria and therefore can be used as a marker for membrane compound contamination [36]. With low contents of the DNA and KDO indicated that the EPS extracted was not contaminated by a significant amount of intracellular materials.

2.3. Chemical analysis

The dry weight of granules and volatile suspended solids (VSS) in the separate samples before extraction of EPS were measured according to Standard Methods [37]. The carbohydrate content in EPS was measured by the Anthrone method with glucose as the standard. The protein and humic content in EPS was measured by the modified Lowry method using bovine serum albumin and humic acid (Fluka, USA) as the respective standards. The DNA content was measured by the diphenylamine colorimetric method [38] using fish DNA as the standard.

In each test, blanks with respective extracting reagents but with no sample were performed and treated as control. The total lipid content, polar and neutral lipid, were extracted separately from sludge and granules by the treatment described above and then adding methanol/chloroform (1:2, v/v). After 5 min centrifugation, the supernatant was treated with sodium chloride (0.9%, w/v). The mixture was centrifuged, and the organic phase (oily phase) was recovered as a lipid extract. Total lipid content was obtained by evaporating the organic solvents and drying in the oven at 45 °C for 15 min and accurately weighing.

2.4. EEM fluorescence spectroscopy

The EEM spectra of extracted EPS for sludge and aerobic granule samples were recorded by luminescence spectrometry (Cary Eclipse, Varian Inc., USA). The EEM spectra were obtained by scanning the sample over excitation wavelengths from 200 to 550 nm and emission wavelengths from 200 to 550 nm. The blank EEM spectrum obtained for double distilled water was used to ensure the quality of the scanned samples.

2.5. Staining and CLSM imaging

Proteins in the samples were stained by fluoresceinisothiocyanate (FITC). Concanavalin tetramethylrhodamine conjugate (ConA) was used to bind to α-mannopyranosyl and α-glucopyranosyl sugar residues. The whole cells were probed with the wall-permeable nucleic acid stain SYTO 63. The dead cells were stained by cell wall-impermeable stain SYTOX blue. Nile red was used to stain lipids. Calcofluor white was used to stain β-polysaccharides. All probes were purchased from Molecular Probes (Carlsbad, CA, USA).

Confocal laser scanning microscopy (CLSM) (Leica TCS SP2 Confocal Spectral Microscope Imaging System, Germany) was used to visualize cell or EPS distributions in bio-samples. The fluorescence of SYTO 63 was detected via excitation at 633 nm and emission at 650–700 nm. The fluorescent intensity of SYTOX blue was analyzed via excitation at 458 nm and emission at 460–500 nm. The fluorescence of Nile red, FITC and Calcofluor white were detected via excitation at 514, 448 and 400 nm and emission at 625–700, 500–550 and 410–480 nm, respectively. Staining details are available in Chen et al. [32].

3. Results

3.1. Characterization of EPS in sludge samples

Table 1 summarizes the quantities of EPS extracted from sludge by the eight mentioned processes (including control). The quantities of DNA in collected EPS were all less than 0.4 mg g^{-1} VSS for sludge flocs. The KDO contents in the extracted EPS were in the range of 0.1–0.22 mg g^{-1} VSS. The low contents of DNA and KDO in the extracted EPS indicated negligible contamination of intracellular substances in the collected EPS.

The results for sludge flocs indicated that the collected EPS was primarily composed of proteins, carbohydrates, lipids and humic substances. The amount of extracted EPS significantly varied according to the extraction sequences and chemicals used. The quantities of EPS extracted with chemical reagents were 6–10 times that of controls. Quantity of EPS extracted from sludge samples using formamide was comparable to that using formaldehyde and heat extraction. More specifically, each gram of VSS in original sludge contained 35.9–53.5 mg proteins, 44.1–54.9 mg carbohydrates, 52.4–77.5 mg humic substances and 12.5–18.3 mg of lipids. The proteins, carbohydrates and humic substances in the EPS extracted by formaldehyde–NaOH with pre- or post-ultrasound were higher ($p < 0.01$, one way ANOVA) than those extracted with formaldehyde–NaOH alone, indicating the enhancement yielded by ultrasound. The protein and carbohydrate contents in the ultrasound + formaldehyde–NaOH extraction was higher ($p < 0.01$) than those with post ultrasound, suggesting that ultrasound should be applied prior to chemical extraction to enhance the EPS extraction yield. Notably, the total polysaccharides/total proteins (PN/PS) ratios were all approximately 0.9 regardless of extraction scheme.

3.2. Characterization of EPS from aerobic granules

The quantities of DNA and KDO in collected EPS were all less than 0.6 and 0.25 mg g^{-1} VSS for aerobic granules, also indicating negligible contamination of intracellular substances in the collected EPS.

The protein extracted from aerobic granules was greater than that from polysaccharides (Table 1). Extraction using formamide yielded a greater quantity of EPS than extraction with formaldehyde. Specifically, the extracted EPS quantities were 255–309 mg g^{-1} of proteins, 55.5–109 mg g^{-1} of carbohydrates and 49–93 mg g^{-1} of humic substances. Ultrasound significantly enhanced EPS extraction from aerobic granules, and extraction by ultrasound + formamide–NaOH was up to 52 times that in controls. McSwain et al. [16] also noted a high extraction yield for granular samples using homogenization treatment.

The efficiencies of EPS recovery were as follows: ultrasound–formamide–NaOH > ultrasound–formaldehyde–NaOH > formamide–NaOH–ultrasound > formaldehyde–NaOH–ultrasound > formamide–NaOH > formaldehyde–NaOH > heat > control. Pre-ultrasound produced higher yields of proteins, polysaccharides and humic substances than post-ultrasound. Moreover, the content of proteins and carbohydrates in formamide-extracted EPS was significantly higher than in formaldehyde extracted EPS. Therefore, pre-ultrasound plays an important role in breaking down the structural integrity of granules for easier extraction by chemical reagents. The cell shrinkage action by formamide also largely enhanced efficiency of EPS extraction.

3.3. Characterizing EPS by EEM

Three major peaks in the EEM spectra were identified in the EPS collected from all samples (peaks A–C in Fig. 2). Peak A was at excitation/emission wavelengths (Ex/Em) of 220–230 nm/340–350 nm, peak B at Ex/Em of 270–280 nm/340–350 nm and peak C at Ex/Em 330–340 nm/420–430 nm. Based on the classification scheme developed by Chen et al., peaks A, B and C were located in regions II (aromatic proteins), IV (soluble microbial by-product-like) and V (humic acid-like), respectively. The EEM results revealed the presence of proteins and humic substances, corresponding to the extraction results with redundant proteins and humic substances.

Table 2 lists maximum fluorescence peak intensities for peaks A–C. The greater the quantity of proteins and humic substances

Table 2
Fluorescence spectral analysis of the EPS extracted by using formamide or formaldehyde

Sample	Method	Peak A		Peak B		Peak C		B/C
		Ex/Em	Intensity	Ex/Em	Intensity	Ex/Em	Intensity	
Sludge	Formaldehyde–NaOH	230/350	168	280/360	227	330/420	79.0	2.87
	Ultrasound–formaldehyde–NaOH	220/365	207	280/355	258	330/420	72.2	3.56
	Formaldehyde–NaOH–ultrasound	220/355	248	270/355	242	330/420	103	2.35
	Formamide–NaOH	220/360	23.9	280/350	112	330/425	21.3	5.27
	Ultrasound–formamide–NaOH	230/340	32.4	280/340	134	330/420	21.3	6.29
	Formamide–NaOH–ultrasound	230/340	19.2	270/350	123	330/420	26.6	4.61
Granules	Formaldehyde–NaOH	220/340	61.4	280/345	173	330/420	78.6	2.19
	Ultrasound–formaldehyde–NaOH	220/350	108	270/355	242	330/420	103	2.36
	Formaldehyde–NaOH–ultrasound	220/350	119	270/355	270	330/420	105	2.58
	Formamide–NaOH	220/340	43.5	270/325	976	330/420	34.4	28.4
	Ultrasound–formamide–NaOH	220/310	108	270/330	917	330/420	39.0	23.5
	Formamide–NaOH–ultrasound	220/230	69.2	270/330	983	330/420	47.6	20.7

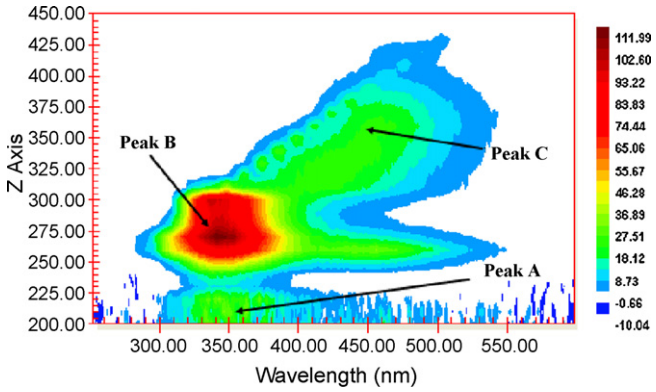

Fig. 2. The EEM fluorescence spectra of sludge flocs with designated peak locations.

extracted using the tested scheme (Table 1), the stronger the peak intensities noted in the EEM spectra. However, regression analysis revealed that corresponding peak intensities correlated weakly with quantities of extracted proteins (peaks A or B) or extracted humic substance (peak C). Restated, the EEM spectra alone cannot quantify extracted EPS from sludge flocs or aerobic granules.

4. Discussion

4.1. Extraction efficiency

Formaldehyde–NaOH effectively extracted EPS from sludge flocs. The use of pre- or post-ultrasound or the replacement of formaldehyde with formamide incrementally improved extraction efficiency. This result partly supported the proposal by Liu and Fang [17] to use formaldehyde–NaOH for EPS extraction from sludge flocs. The use of ultrasound or formamide is not justified in sludge flocs, probably due to the correspondingly loose attachment between EPS and cells.

Conversely, for aerobic granules, the efficiencies of EPS recovery were as follows: ultrasound–formamide–NaOH > ultrasound–formaldehyde–NaOH > formamide–NaOH–ultrasound > formaldehyde–NaOH–ultrasound > formamide–NaOH > formaldehyde–NaOH ≫ control. The (pre- or post-) ultrasound and/or the use of formamide are needed to significantly enhance efficiency of EPS extraction from aerobic granules without noticeable contamination by intracellular substances. The floc breakup action of ultrasound helps expose the inclusive EPS to NaOH while the cell shrinking action of formamide helps loosen the EPS from cell walls.

Fig. 3 shows the results of cryosection staining of aerobic granules. Correlating with the extraction results revealed substantial proteins (FITC-stained) in the granule interior, with cells (SYTO 63 and SYTOX blue), lipids (Nile red) and α-polysaccharides (Con A) accumulating at the outer rim regime and β-polysaccharides (calcofluor white) over the granule.

Regression analysis revealed that the quantities of extracted proteins only weakly correlated with those of carbohydrates ($r^2 = 0.37$). The contents of extracted humic substances did not correlate with the contents of extracted proteins or carbohydrates.

Compared with the other six chemical or physical treatment schemes, centrifugation at 10000 rpm for 20 min (control) sufficiently extracted most lipids from both sludge flocs and aerobic granules. The improved extraction may be attributable to accumulation of lipids at the granule surface (Fig. 3). Additionally, extraction of humic substances by ultrasound was more effective than extraction by formamide (Table 1). Hence, humic

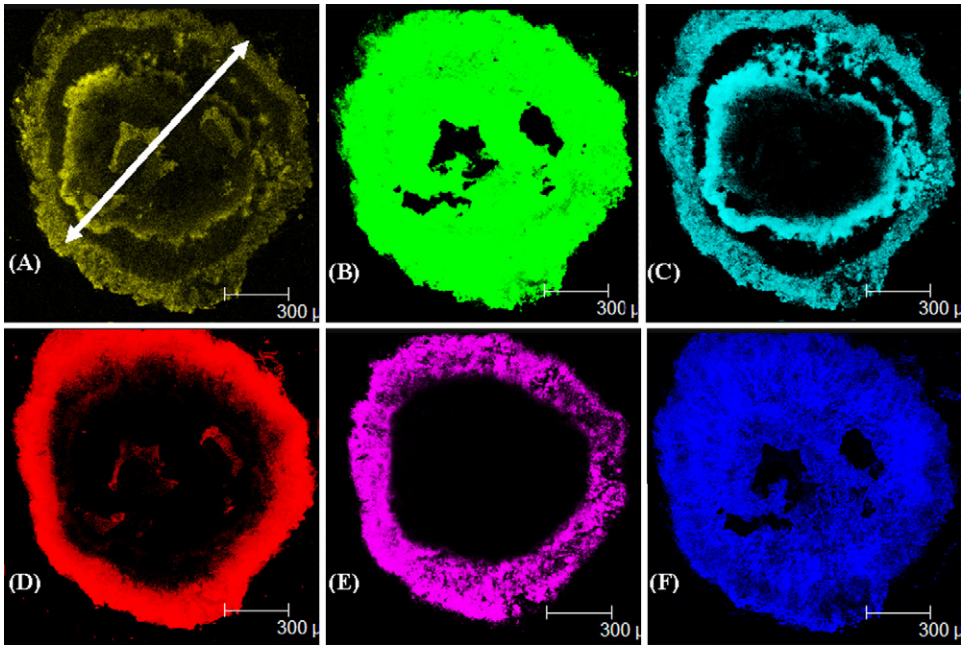

Fig. 3. The CLSM images of granules of extracellular polymeric substances. (A) lipids (yellow): Nile red; (B) proteins (green): FITC; (C) α-polysaccharide (light blue): Con A rhodamine; (D) living cells (red): SYTO 63; (E) dead cells (violet): Sytox blue; (F) β-polysaccharide (blue): calcofluor white. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

substances should locate inside the granule interior (to be released by ultrasound) but not bind with the cell walls (no role of formamide).

In all granule tests, pre-ultrasound always yielded more EPS than post-ultrasound. Therefore, deterioration of the compact structure of the granule is apparently a prerequisite for effective EPS extraction from the granules.

4.2. PN/PS ratio

Polysaccharides are the major constituent of EPS in bacterial cultures [39,40]. Recent analysis of wastewater treatment reveals protein to be an important constituent in EPS [41]. The current study suggests that the constituents of EPS are proteins, polysaccharides, humic acid and lipids.

The PN/PS ratio was approximately 0.9 with formaldehyde or with formamide for sludge flocs. Hence, proteins and polysaccharides may present relatively homogeneously over the entire flocs and bind at common sites during extraction. The protein content was significantly high in aerobic granules, and PN/PS ratios were 3.4–6.2. McSwain et al. [16] also reported high protein content in their analysis of peptone and glucose fed aerobic granules. Tay et al. [41] reported that hydrodynamic shear increases polysaccharide production, thus yielding aerobic granules. However, the present study noted that proteins, rather than polysaccharides, were enriched in sheared granule systems, which correlated with the results of McSwain et al. [16]. Chen et al. [32] noted that proteins and dead cells were mainly distributed at the core regime of granules while the live cells and α-polysaccharides were located at the outer rim regime of the granules. Meanwhile, the β-polysaccharides were distributed in the core and at the outer rim regimes of the phenol-fed granule. The protein core noted in Fig. 3 should have a stabilizing effect on granule integrity, but the possible role of β-polysaccharides on granule stability cannot be ruled out since it presents a network over the entire granule.

4.3. EPS and EEM

Sheng and Yu [42] analyzed shifts in position or changed intensity of EEM peaks as evidence of chemical changes in extracted EPS. The present study also revealed the following shifts/changes: pre-ultrasound and post-ultrasound sludge showed 17 and 5 nm shifts in peak A, 7 and 5 nm shifts in peak B and no shift in peak C; EPS extracted from sludge by using formamide showed a 9 nm shift in peak A; intensities of peak B of EPS extracted from granules were significantly higher than flocs when formamide was used in extraction. However, the peaks can easily shift up to 15 nm using different interpolation schemes on the same EEM scan. For instance, data interpolation with or without pre-smoothing could yield such a difference in positioning. Hence, shifts in EEM peak position cannot confirm a change in chemical nature of collected EPS.

Moreover, quantities of extracted proteins and humic substances should correlate with intensities of EEM peaks for protein-like or humic-like fluorophores when EEM is used for quantitative analysis as proposed by Sheng and Yu [42]. No correlation is apparent between these parameters (data not shown for brevity). More specifically, distinct correlations between protein peak intensity are noted in EPS from flocs or from granules, which may have different chemical natures. However, the use of different extraction schemes, even from the same samples, yields different peak intensities. These observations also indicate the ineffectiveness of EEM peak intensities for quantitatively comparing EPS content from different samples.

5. Conclusions

Aerobic granules are compact bioaggregates. The efficiency of EPS extraction from phenol-fed, aerobic granules was studied using formaldehyde + NaOH as a chemical reagent. Ultrasonication was applied to enhance EPS extraction before or after extraction. A total of eight extraction methods were tested, all effectively extract EPS from sludge flocs of loose interior. Conversely, the efficiencies of EPS recovery were as follows: ultrasound–formamide–NaOH > ultrasound–formaldehyde–NaOH > formamide–NaOH–ultrasound > formaldehyde–NaOH –ultrasound > formamide–NaOH > formaldehyde–NaOH > heat > control. Specially, extraction using pre-ultrasound + formamide–NaOH yielded quantity of EPS from aerobic granules up to 52 times that in controls. The EEM results revealed the presence of proteins and humic substances in the extracts. However, EEM spectra alone cannot quantify the extracted EPS. The PN/PS ratio was approximately 0.9 for sludge flocs, and was 3.4–6.2 for aerobic granules.

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