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Article *in* Algal Research · October 2016

DOI: 10.1016/j.algal.2016.10.024

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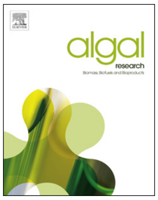

Using flow cytometry to evaluate thermal extraction of EPS from *Synechocystis* sp. PCC 6803

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ARTICLE INFO

Article history:
Received 25 July 2016
Received in revised form 19 September 2016
Accepted 30 October 2016
Available online xxxx

Keywords:
Extracellular polymeric substances (EPS)
Flow cytometry (FC)
Thermal treatment
STTOX Green (SG) dye
Synechocystis sp. PCC 6803

ABSTRACT

Soluble microbial products (SMP) and extracellular polymeric substances (EPS) produced by photoautotrophic cyanobacteria become substrates for heterotrophic bacteria in photobioreactors (PBRs). Understanding the roles of SMP and EPS depends on reliable extraction and measurement methods. While SMP can be separated from biomass using filtration, EPS extraction is more challenging. Flow cytometry (FC) with the nucleic-acid (NA) stain SYTOX Green (SG) was used to evaluate EPS solubilization and cell lysis during thermal extraction of EPS from biomass of *Synechocystis* sp. PCC 6803. Fluorescence intensity (FI) was used to assay the binding of SG with NA, and FC made it possible to distinguish extracellular NA from intracellular NA. Thermal treatment affected the yield and accuracy of the measurement in systematic ways. For a 20-min extraction, solubilization of EPS increased and the emission FI of SG binding with extracellular NA decreased with temperature from 30 °C to 60 °C. Cell lysis and EPS denaturation occurred for temperature higher than 70 °C. High EPS-extraction efficiency without cell lysis and EPS denaturalization was achieved with thermal extraction at 60 °C for 20 min for *Synechocystis* PCC 6803. This work lays the foundation for using the FC + SG methodology to evaluate the effectiveness of any EPS-extraction method.

1. Introduction

Extracellular polymeric substances (EPS) are organic macromolecules that are located outside the cell and are part of the solid-phase biomass [1,2]. Carbohydrate and protein usually are the major components of EPS, and humic substance may also be an important component of EPS from activated sludge in biological wastewater treatment reactors [3]. In addition, the EPS matrix always contains small amounts of nucleic acid, lipid, uronic acid, and other polymeric compounds [1]. The composition of EPS depends on the active microorganisms in the aggregates, their history, and also the extraction method and analytical tool used to assay for EPS [2,4]. Van der Waal forces, electrostatic interactions, hydrogen bonds, and hydrophobic interactions are involved in binding of the EPS matrix [5].

In a microalgae-based system, such as a photobioreactor (PBR), EPS and soluble microbial products (SMP) produced by the microalgae can become sources of carbon and electrons for heterotrophic bacteria [6,7]. Because SMP are soluble and biodegradable, they are the direct electron-donor substrates for heterotrophic bacteria [8]. As the primary source of SMP, EPS also become indirect substrates for heterotrophs. Decho et al. [9] found that cyanobacterial EPS was rapidly transformed post-secretion through heterotrophic degradation, especially by sulfate-reducing bacteria, which led to accumulation of refractory remnant polymers. A mass balance of ^{14}C–EPS showed 30–50% mineralization of its C (as $^{14}CO_2$) over 48 h, with the remaining ^{14}C in the refractory polymer. As the ultimate goal of a cyanobacteria-based PBR is to produce cyanobacteria biomass or products generated by the cyanobacteria, heterotrophs usually are not desired.

If the goal is to minimize the growth of heterotrophs, the first step is to quantify EPS, the dominant source of SMP. Since the best extraction method for EPS may depend on the type of interactions that keep the EPS components together in the matrix [1,10], no universally accepted extraction method exists for the quantitative extraction of EPS from microorganisms [11]. The various methods developed for the EPS extraction can be classified as physical, chemical, and a combination of physical and chemical [12–15].

Among the physical methods for the EPS extraction, thermal treatment decreases van der Waal forces and hydrogen bonding [1] and thus solubilizes the EPS. However, thermal treatment also might cause cell lysis that releases soluble non-EPS components. Previous studies [10,16] used the nucleic acid (NA) content in extracted materials as an indicator of cell lysis during extraction. Because a small amount of nucleic acid normally is found in the EPS matrix [1], simply detecting NA is not a foolproof way to gauge lysis.

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http://dx.doi.org/10.1016/j.algal.2016.10.024
2211-9264/

In order to evaluate the reliability of EPS extraction, we combined flow cytometry (FC) with a fluorescent-dye, a combination previously employed to evaluate aspects of ecology, morphology, physiology, and biochemistry for microalgae [17]. FC counts and sorts cells based on characteristics detectable by multi-dimensional and quantitative measurement of light scattering and fluorescence emission [17]. SYTOX Green (SG) is an unsymmetrical cyanine dye that binds strongly with NA, but is completely excluded by the intact membrane of live eukaryotic and prokaryotic cells [18]. SG stain is excited by light with a wavelength of 488 nm, which is emitted from argon ion laser; its green fluorescence emission (530 ± 20 nm) occurs when it binds with NA.

Although NA are mainly present in the cell interior, a small amount of NA also can be found in EPS [8,19]. Because of its large molecular size, SG dye cannot penetrate an intact cell membrane [19,20], and the fluorescence intensity (FI) emitted by SG is due only to NA in the EPS. However, thermal treatment to solubilize EPS may be accompanied by cell lysis that releases intracellular NA [20]. Since internal NA is much greater than NA naturally in EPS, the FI emitted by SG that binds with intracellular NA will be much larger than extracellular NA with the compromised cells [10,19,21]. Thus, FC with SG can sensitively differentiate extracellular NA from intracellular NA, and this is a means to judge if cell lysis is occurring during EPS extraction.

Our ultimate aim is to develop a reliable method to quantify EPS from microalgae. Here, we use FC with SG to ascertain if thermal-treated *Synechocystis* were lysed. From that, we can determine the optimal conditions for thermal extraction without cell lysis. We are aware of no previous work applying FC to evaluate the EPS solubilization and cell lysis during EPS extraction. The work presented here lays the foundation for evaluating the effectiveness of any EPS-extraction method applied to any type of microalgae.

2. Materials and methods

2.1. Cultivation of Synechocystis

Wild-type *Synechocystis* sp. PCC 6803 was provided by the laboratory of Dr. Willem F. J. Vermaas (School of Life Sciences, Arizona State University). We grew *Synechocystis* biomass in a 500-mL (working volume) Erlenmeyer flasks with standard BG-11 medium (30.5 mg/L K_2HPO_4, 1469 mg/L $NaNO_3$, 6 mg/L ferric ammonium citrate, 20 mg/L Na_2CO_3, 75 mg/L $MgSO_4 \cdot 7H_2O$, 36 mg/L $CaCl_2 \cdot 2H_2O$, 6 mg/L citric acid, 0.91 mg/L Na_2EDTA, and trace minerals [22]) bubbled with air filtered through a 1.0-μm air filter (Pall, Port Washington, NY, USA). We maintained the culture temperature at 30 °C by automated air cooling, and the incident light intensity was 300 $\mu E/m^2$ s provided from T5 fluorescent plant grow lamps (Envirogro Hydrofarm, USA). We maintained the pH of the culture at 8.5 using a pH-Stat that automatically sparged CO_2 when the pH rose above 8.51 [23]. Prior to the inoculation, the flasks and BG-11 medium were sterilized by autoclaving, and the pH probe was sterilized using 75% ethanol.

2.2. Thermal treatment of Synechocystis

After 10 days of incubation, the optical density (OD) of the culture increased to ~3.7, giving a biomass dry weight of ~1 g/L (shown in Fig. S1 of Supplementary Information). We then diluted the culture to an OD of 0.5 using a buffer solution consisting of 2 mM Na_3PO_4, 4 mM Na_2HPO_4, 9 mM NaCl, and 1 mM KCl [24].

To evaluate EPS extraction and cell lysis by thermal treatment, we used a water bath with a hot plate (model 528F, VWR, USA) and applied temperatures of 30, 40, 50, 60, 70, 80, and 90 °C. Each temperature experiment utilized three 15-mL polypropylene centrifuge tubes (BD Falcon, VWR, USA) that held 12 mL of 0.5-OD culture. For a given temperature, the three tubes were heated in the water bath to the target temperature, and one tube was removed after 10, 20, or 30 min at the target temperature.

We rapidly cooled each tube to room temperature (23.8 °C) using a 4 °C water bath and then filtered the culture through a 0.2-μm cellulose acetate membrane filter (Whatman, Germany) to remove the cells and other particles. Filtration also was performed directly on culture (no thermal treatment) as a control. We prewashed the membrane filters using 5 mL deionized water to ensure that the filters did not "bleed" any soluble organic matter [25]. We stored the filtrates at 4 °C in a freezer (UGL3020A, Thermo Scientific, USA) prior to COD, carbohydrate and protein analyses.

2.3. Analytical methods

2.3.1. Cell density and biomass dry weight

We measured the Optical Density (OD) of the culture using a UV–vis BioSpec-mini spectrometer at 730 nm (Shimadzu Corp., Japan). We auto-zeroed the spectrometer with deionized ion (DI) water before

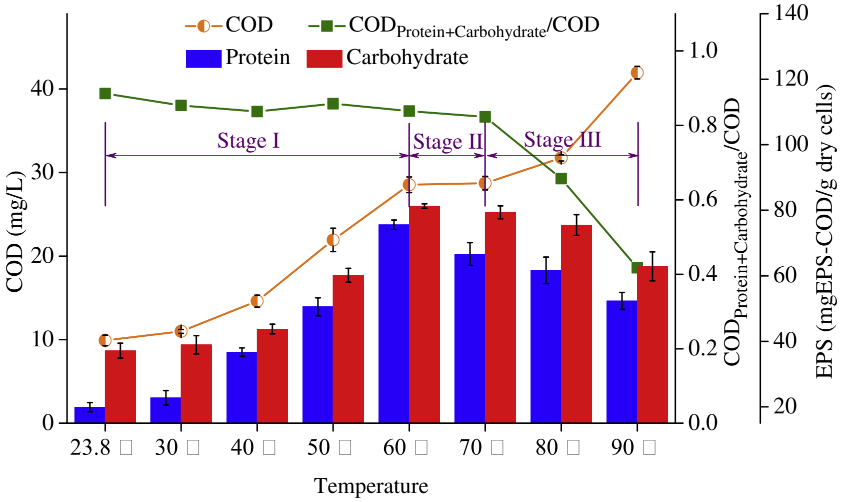


Fig. 1. Soluble COD, the concentrations of protein and carbohydrate, the fraction of the soluble COD that is protein + carbohydrate, and the EPS yield (mg EPS-COD/g dry cells) for *Synechocystis* extracted after thermal treatment for 20 min at the noted temperatures. Values represent mean ± standard deviation ($n = 3$). The soluble COD of the BG-11 medium was ~6.2 mg/L, and this was subtracted from the measured value to compute the EPS yield.

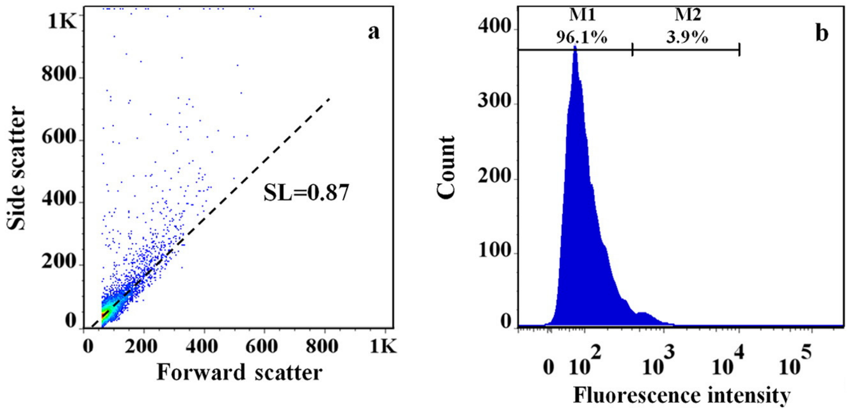


Fig. 2. FC results for (a) light scattering alone and (b) FI of NA-complexed SG in *Synechocystis* without thermal treatment. SL, the slope of the linear relationship between FS and SS, is inversely related to cell size. M1 designates SG emissions lower than 400 FIU, and M2 shows SG emissions higher than 400 FIU.

each measurement, and samples with high OD were diluted with DI water to obtain an OD < 0.8 [23]. We converted the OD_{730} value to biomass dry weight (DW) using calibration curve for *Synechocystis*. For the calibration, we determined the DW using total suspended solids, assayed by Method 2540D in *Standard Methods* [26].

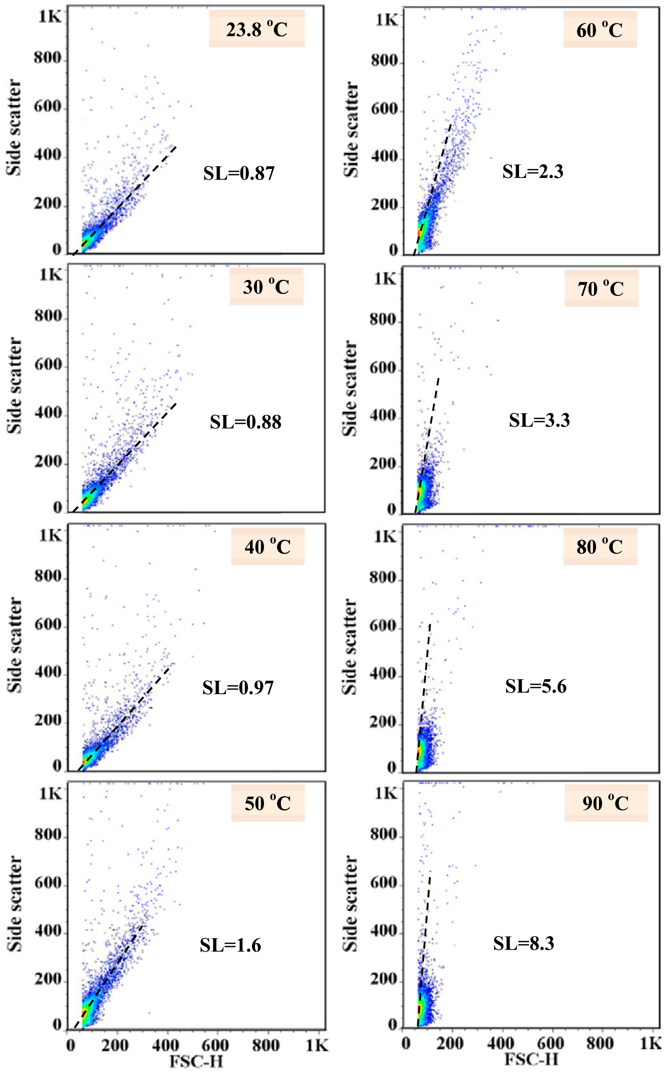


Fig. 3. FC of *Synechocystis* after thermal treatment for 20 min at the noted temperatures.

2.3.2. Organic composition

The chemical oxygen demand (COD) of the filtrate determined the total concentration of EPS that had been solubilized from EPS, plus any soluble organic matter that was released from inside the cell during thermal treatment. We measured the COD using HACH TNT822 kits (0–60 mg/L, Loveland, CO, USA) and a HACH DR2800 spectrophotometer. We measured the protein fraction of the filtrate with a QuantiPro BCA Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin (BSA) as the standard; BSA equivalents were converted to COD using a conversion factor of 1.4 mg COD/mg BSA [27]. We measured the carbohydrate fraction of the filtrate with the phenol-sulfuric acid method using glucose as the standard [28] and converted glucose equivalents to COD using a conversion factor of 1.07 mg COD/mg glucose [27].

2.3.3. SYTOX Green staining and flow cytometry

We applied the fluorescent dye SG according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA). After thermal treatment, we did not filter the sample, but directly mixed 2 mL of cooled sample with 1 μL SG and then reacted them in a rocker mixer (Lab-Line, TX, USA) for 15 min in the dark [21]. We used *Synechocystis* biomass without thermal treatment and SG stain to adjust the FI to zero.

After staining, we performed FC using a FACSAria flow cytometry (BD Biosciences, CA, USA) having an air-cooled 20-mW argon ion laser with an excitation wavelength of 488 nm. We used an FITC filter with a wavelength band of 510–550 nm to detect the SG emission. We diluted the samples stained with SG to a concentration suitable for the instrument's counting speed of 300 to 400 events/s, and we counted 10,000 events for each sample. We performed the data analysis and graphical outputs with FlowJo 7.6.1 software (Treestar, Inc., San Carlos, CA, USA).

2.4. Statistical analyses

For thermal-treatment experiments, we used three tubes for each temperature, and the sample in each tube was assayed one time for COD, protein, and carbohydrate. The results are expressed as the mean and standard deviation of the three measured samples (mean ± SD). When presenting the results of light scattering and the spectra from FC, we show one typical result for each sample.

3. Results and discussion

3.1. Effects of temperature on the release of soluble organic material

Temperature had the greatest effect on soluble organic material. Fig. 1 shows the concentration of total soluble organics as COD after thermal treatment for 20 min at all treatment temperatures; results

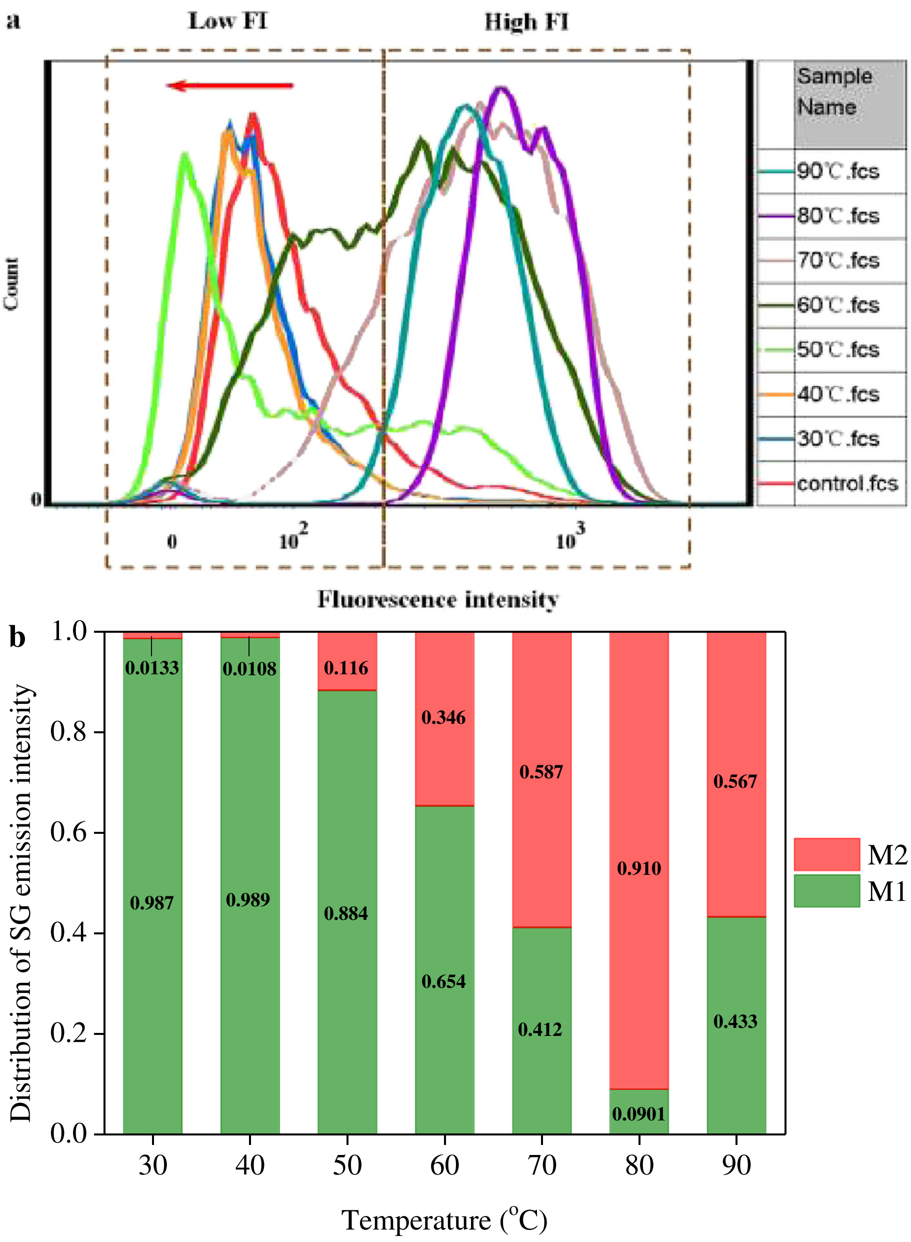


Fig. 4. (a) Fluorescence spectra and (b) the distribution of SG emission intensity lower than 400 FIU (M1) and higher than 400 FIU (M2) in *Synechocystis* after thermal treatment for 20 min at the noted temperatures.

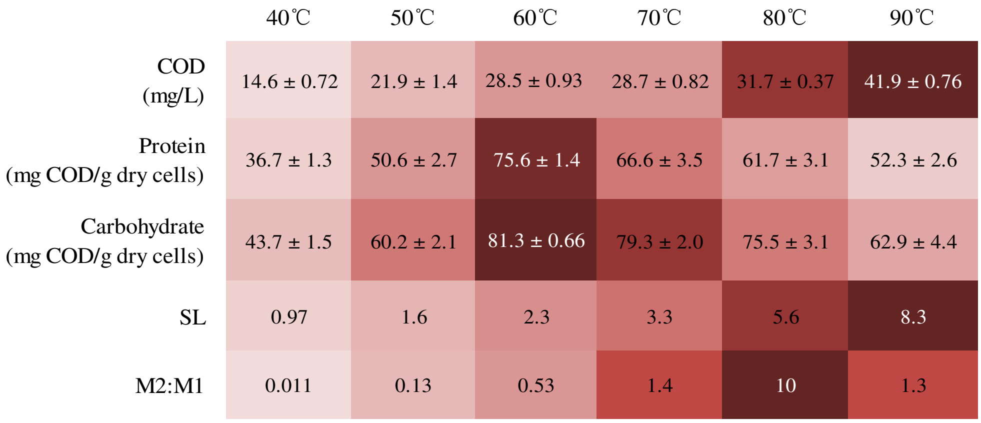
for all times and temperatures are provided in Fig. S2. The soluble COD changed systematically with temperature and can be grouped into three stages. In stage I, 30 °C to 60 °C, soluble COD increased steadily from 10 to 28.5 mg COD/L with increasing temperature. This increase represents increasing solubilization of EPS [12], the goal of heat treatment. In stage II (from 60 °C to 70 °C), COD remained stable, which implies that all EPS was removed from the cell surface, but leakage of intracellular organics was minimal. In stage II, the EPS from protein + carbohydrate was ~150 mg EPS-COD/g dry cells. Soluble COD increased dramatically in stage III (70 °C to 90 °C), a sign of cell lysis and release of intracellular soluble organics [29].

Fig. 1 also shows that the fraction of COD coming from protein + carbohydrate was stable at about 90% in Stages I and II. The proportion of protein + carbohydrate declined at temperatures higher than 70 °C, since other organic materials were being released from lysed cells. In parallel, the COD fractions of protein + carbohydrate in filtrates declined at temperatures higher than 70 °C, especially for protein, which began to decrease as the treatment time longer than 20 min at 60 °C (Fig. S2). The loss of protein most likely was caused by denaturation and precipitation [29–32], which led to solids removal during the filtration process [31,33,34]. Thus, protein and carbohydrate were the dominant fractions (90%) in the EPS of *Synechocystis* as long as the soluble COD was not influenced by soluble organics released due to cell lysis.

3.2. Flow cytometry analysis

3.2.1. Untreated Synechocystis

In FC, cell size is correlated with forward scatter (FS), and side scatter (SS) reflects the conformations of interior structures [17]. The slope (SL) between the two values in the region with the highest density of points is inversely related to cell size [35]. Fig. 2a shows that the size for most of the *Synechocystis* cells before thermal treatment was lower than 300 Cell Size Units (CSU), and the SL was 0.87. Fig. 2b presents the corresponding SG-emission FI versus cell count ordered by intensity for non-treated *Synechocystis*. 96% of cells had SG emission <400 Fluorescence Intensity Units (FIU), which is denoted as the M1 region; this



	40℃	50℃	60℃	70℃	80℃	90℃
COD (mg/L)	14.6 ± 0.72	21.9 ± 1.4	28.5 ± 0.93	28.7 ± 0.82	31.7 ± 0.37	41.9 ± 0.76
Protein (mg COD/g dry cells)	36.7 ± 1.3	50.6 ± 2.7	75.6 ± 1.4	66.6 ± 3.5	61.7 ± 3.1	52.3 ± 2.6
Carbohydrate (mg COD/g dry cells)	43.7 ± 1.5	60.2 ± 2.1	81.3 ± 0.66	79.3 ± 2.0	75.5 ± 3.1	62.9 ± 4.4
SL	0.97	1.6	2.3	3.3	5.6	8.3
M2:M1	0.011	0.13	0.53	1.4	10	1.3

Fig. 5. Synthesized results for Synechocystis extracted after thermal treatment for 20 min at the noted temperatures. The color darkness represents the value of the metric. A greater difference between adjacent temperatures indicated a faster change of the metric with increasing temperature. SL relates to cell size, and a greater SL means a smaller of the cell size. M2:M1 is related to SG binding with intracellular NA, and a greater M2:M1 means more SG could bind with intracellular NA.

low fluorescence was from the binding of SG with NA naturally in EPS [19]. <4% of the cells had SG emission >400 FIU (the M2 region), which probably was from SG binding with NA in the small fraction of dead cells naturally present [36].

3.2.2. Thermal-treated Synechocystis

Good thermal treatment should decrease cell size by solubilizing EPS, but without disrupting the cell membrane [10,20]. Fig. 3 presents FS versus SS for *Synechocystis* thermally treated for 20 min at the noted temperatures; results of FS versus SS for *Synechocystis* thermally treated at all temperatures and times are shown in Fig. S3. In Fig. 3, SL values clearly were >0.87 for temperature ≥ 40 °C. Longer treatment time (Fig. S3) also increased SL, but not as dramatically as for the increase in temperature. The increase in slope up to 70 °C means that the cells became smaller from thermal treatment [17], a trend consistent with EPS solubilization (Fig. 1). The increase in SL became dramatic for temperatures higher than 70 °C (up to 11 for 90 °C and 30-min treatment time, Fig. S3), and this is another sign of cell lysis [20,29,36].

3.2.3. Fluorescence intensities of thermal-treated Synechocystis

Fig. 4 shows the fluorescence spectra and the distribution of SG-emission intensity between the M1 and M2 regions after thermal treatment for 20 min. Figs. S3 and S4 give the full spectra and whole SG emission intensity distributions, respectively. Temperatures of 30 °C and 40 °C had only one peak with low FI in each histogram, and the distribution of the M1 and M2 regions was the same as for the untreated control (Fig. 1B), ~96% M1. This confirms that the cells were not compromised at all up to 40 °C, as SG dye could not penetrate into the cell. The low-FI signal decreased as the temperature rose from 40 to 60 °C and disappeared for temperature higher than 70 °C. The loss of the low-FI signal confirms increasing loss of EPS by its solubilization. All EPS was lost from the surface for temperature higher than 70 °C.

Temperatures of 50 °C or more shifted the distribution more towards M2, a signal of increasing cell-membrane permeability [35,37] that allowed SG to pass through the membrane and bind with intracellular NA, resulting in an increase of FI. The M2 ratio continued to increase up to 80 °C, where it was 91%, but the M2 ratio decreased to 33% at 90 °C, when large-scale cell lysis occurred and resulted in the loss of NA inside the cells [20,36].

3.2.4. Synthesizing the results

Fig. 5 is a heat map that synthesizes all results for *Synechocystis* extracted after thermal treatment for 20 min at the noted temperatures. To achieve a high yield of extracted EPS, but with minimal cell lysis, the values of SCOD, SL, and M2:M1 should be high, but without the dramatic increases associated with cell lysis. Temperatures ≤60 °C had all of the characteristics that signal complete EPS solubilization: consistent increases of SCOD, protein, and carbohydrate concentrations that signal, as well as a modest increase of SL. Temperatures higher than 70 °C clearly incurred the problems associated with cell lysis and EPS denaturalization: the dramatic increases of SCOD and SL, and the losses of protein and carbohydrate concentrations.

Synthesizing all of the results in Fig. 5 indicates that thermal treatment at 60 °C and for 20 min provided a reliable extracting condition of EPS from *Synechocystis* sp. PCC 6803.

4. Conclusions

FC combined with SG staining is a novel and sensitive method for evaluating EPS solubilization and cell lysis during EPS extraction. We applied them to find the optimal conditions for thermal extraction of EPS from the cyanobacterium *Synechocystis*. FC with SG could distinguish solubilization of EPS (the desired outcome) from cell lysis (the undesired outcome) based on parallel and consistent changes in soluble COD, cell size, and emission FI of SG bound with extracellular NA versus intracellular NA. Thermal treatment at 60 °C for 20 min provided a reliable extraction of EPS from *Synechocystis*, because it achieved the high extraction efficiency, but without evidence cell lysis and EPS denaturalization. While these optimum conditions apply only to thermal extraction from *Synechocystis* PCC 6803, FC with SG should be applicable to any strain of microalgae and any method of EPS extraction. An optimal extraction method achieves a high yield of extracted EPS, but with minimal cell lysis and EPS denaturalization.

Acknowledgements

This work was supported by the China Scholarship Council (CSC NO. 201506260022), and the National Science and Technology Pillar Program (2013BAD21B03). We thank Dr. Dong Fu at the Center of Infectious Diseases and Vaccinology, Biodesign Institute at Arizona State University, for her expertise in flow cytometry for sample quantification; and Levi Straka and Megha Patel for valuable contributions to the experimental design, setup and data analysis; and Dr. Willem Vermaas and his laboratory in the School of Life Sciences at Arizona State University for providing *Synechocystis* sp. PCC6803 wild type.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.10.024.

References

[1] G.P. Sheng, H.Q. Yu, X.Y. Li, Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review, Biotechnol. Adv. 28 (2010) 882–894.

[2] J. Wingender, T.R. Neu, H.C. Flemming, What are bacterial extracellular polymeric substances? Microbial Extracellular Polymeric Substances, Springer 1999, pp. 1–19.

[3] B. Frølund, R. Palmgren, K. Keiding, P.H. Nielsen, Extraction of extracellular polymers from activated sludge using a cation exchange resin, Water Res. 30 (1996) 1749–1758.

[4] P.H. Nielsen, A. Jahn, Extraction of EPS, in: J. Wingender, T.R. Neu, H.C. Flemming (Eds.), Microbial Extracellular Polymeric Substances: Characterization, Structure and Function, Springer-Verlag, Berlin Heidelberg 1999, pp. 49–72 (Chapter 3).

[5] B.E. Christensen, W.G. Characklis, Physical and chemical properties of biofilms, Biofilms 93 (1990) 130.

[6] B.T. Nguyen, B.E. Rittmann, Electron partitioning in soluble organic products by wild-type and modified Synechocystis sp. PCC 6803, Biotechnol. Bioeng. 90 (2016) 237–242.

[7] A.S. Zevin, T. Nam, B. Rittmann, R. Krajmalnik-Brown, Effects of phosphate limitation on soluble microbial products and microbial community structure in semi-continuous Synechocystis-based photobioreactors, Biotechnol. Bioeng. 112 (2015) 1761–1769.

[8] C.S. Laspidou, B.E. Rittmann, A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass, Water Res. 36 (2002) 2711–2720.

[9] A.W. Decho, P.T. Visscher, R.P. Reid, Production and cycling of natural microbial exopolymers (EPS) within a marine stromatolite, Palaeogeogr. Palaeocl. 219 (2005) 71–86.

[10] H.Q. Chu, H. Yu, X.B. Tan, Y.L. Zhang, X.F. Zhou, L.B. Yang, D.Y. Li, Extraction procedure optimization and the characteristics of dissolved extracellular organic matter (dEOM) and bound extracellular organic matter (bEOM) from *Chlorella pyrenoidosa*, Colloids Surf. B 125 (2015) 238–246.

[11] J. Wingender, T.R. Neu, H.-C. Flemming, Microbial Extracellular Polymeric Substances: Characterization, Structure and Function, Springer Science & Business Media, 2012.

[12] X.Y. Li, S.F. Yang, Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge, Water Res. 41 (2007) 1022–1030.

[13] Z.Q. Zhang, Y. Zhou, J. Zhang, S.Q. Xia, Copper (II) adsorption by the extracellular polymeric substance extracted from waste activated sludge after short-time aerobic digestion, Environ. Sci. Pollut. R. 21 (2014) 2132–2140.

[14] G.P. Sheng, H.Q. Yu, Z. Yu, Extraction of extracellular polymeric substances from the photosynthetic bacterium *Rhodopseudomonas acidophila*, Appl. Microbiol. Biotechnol. 67 (2005) 125–130.

[15] M.W. Breedveld, L. Zevenhuizen, A.J.B. Zehnder, Osmotically induced oligo-and polysaccharide synthesis by *Rhizobium meliloti* SU-47, Microbiology 136 (1990) 2511–2519.

[16] M.J. Brown, J.N. Lester, Comparison of bacterial extracellular polymer extraction methods, Appl. Environ. Microbiol. 40 (1980) 179–185.

[17] P. Hyka, S. Lickova, P. Přibyl, K. Melzoch, K. Kovar, Flow cytometry for the development of biotechnological processes with microalgae, Biotechnol. Adv. 31 (2013) 2–16.

[18] P. Lebaron, P. Catala, N. Parthuisot, Effectiveness of SYTOX Green stain for bacterial viability assessment, Appl. Environ. Microbiol. 64 (1998) 2697–2700.

[19] B.L. Roth, M. Poot, S.T. Yue, P.J. Millard, Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain, Appl. Environ. Microbiol. 63 (1997) 2421–2431.

[20] H. Zipper, H. Brunner, J. Bernhagen, F. Vitzthum, Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications, Nucleic Acids Res. 32 (2004) e103,1–e10310.

[21] J. Sheng, R. Vannela, B.E. Rittmann, Evaluation of cell-disruption effects of pulsed-electric-field treatment of Synechocystis PCC 6803, Environ. Sci. Technol. 45 (2011) 3795–3802.

[22] R. Rippka, J. Deruelles, J.B. Waterbury, M. Herdman, R.Y. Stanier, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, Microbiology 111 (1979) 1–61.

[23] B.T. Nguyen, B.E. Rittmann, Predicting dissolved inorganic carbon in photoautotrophic microalgae culture via the nitrogen source, Environ. Sci. Technol. 49 (2015) 9826–9831.

[24] G.H. Yu, P.J. He, L.M. Shao, P.-P. He, Stratification structure of sludge flocs with implications to dewaterability, Environ. Sci. Technol. 42 (2008) 7944–7949.

[25] B.T. Mulling, A.M. Soeter, H.G. van der Geest, W. Admiraal, Changes in the planktonic microbial community during residence in a surface flow constructed wetland used for tertiary wastewater treatment, Sci. Total Environ. 466-467 (2014) 881–887.

[26] A. Apha, WEF (American Public Health Association, American Water Works Association, and Water Environment Federation), Standard Methods for the Examination of Water and Wastewater, 1998 19.

[27] B.E. Rittmann, P.L. McCarty, Environmental Biotechnology: Principles and Applications, New York: McGrawHill 400, 2001.

[28] K. Chojnacka, A. Noworyta, Evaluation of Spirulina sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures, Enzym. Microb. Technol. 34 (2004) 461–465.

[29] E. Neyens, J. Baeyens, A review of thermal sludge pre-treatment processes to improve dewaterability, J. Hazard. Mater. 98 (2003) 51–67.

[30] K. Nishinari, H. Zhang, S. Ikeda, Hydrocolloid gels of polysaccharides and proteins, Curr. Opin. Colloid Interface Sci. 5 (2000) 195–201.

[31] D.M. Webster, Protein Structure Prediction: Methods and Protocols, Springer Science & Business Media, 2000.

[32] A. Rothen, The present status of molecular weights of proteins, Ann. N. Y. Acad. Sci. 43 (1942) 229–241.

[33] M.L. Anson, Protein denaturation and the properties of protein groups, Adv. Protein Chem. 2 (1945) 361–386.

[34] C. Tanford, Protein denaturation, Adv. Protein Chem. 23 (1968) 121–282.

[35] J.L. Collier, Flow cytometry and the single cell in phycology, J. Phycol. 36 (2000) 628–644.

[36] G. Karp, Cell Biology, Wiley Online Library, 1979.

[37] P. Foladori, S. Tamburini, L. Bruni, Bacteria permeabilisation and disruption caused by sludge reduction technologies evaluated by flow cytometry, Water Res. 44 (2010) 4888–4899.