Comparison of EPS Extraction Efficiences from *Spirogyra fluviatilis* by Chemical and Physical Extraction Methods

Shui-Ping Chang, Hwey-Lin Sheu, Yi-Chao Lee*
Department of Environmental Engineering, Kun Shan University, Tainan City, Taiwan.

* Corresponding author. Tel.: +886-6-2055011; email: lyc007@mail.ksu.edu.tw
Manuscript submitted November 23, 2018; accepted June 4, 2019.
doi: 10.17706/ijbbb.2019.9.4.202-209

Abstract: The algal extracellular polymeric sustances (EPS) have been studied as natural resources for a number of applications. For a macroalgae Spirogyra fluviatilis, the aboundant extracellular matrix on the surface of algae cell. EPS has a gel-like structure that could fractioned into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). The contents and extraction rate of EPS were compared two chemical (formaldehyde-NaOH and NH_4OH) and two physical (ultrasonication and heating) extraction methods. The analysis demonstrated that extraction rates of EPS followed heating (7.94 mg g^{-1} fresh algae) > ultrasonication (5.02 mg g^{-1} fresh algae) > formaldehyde-NaOH (3.12 mg g^{-1} fresh algae) > NH_4OH (2.01 mg g^{-1} fresh algae). The protein to polysaccharides ratio (P/S) in EPS is between 0.23 to 0.35 by chemical methods and between 0.27 to 0.37 by physical methods. The P/S ratio is LB-EPS is higher than TB-EPS. The results indicated that the EPS could be efficiently extracted by the heating method, the influence of P/S ratios were obvious different.

Key words: *Spirogyra fluviatilis*, algae, extracellular polymeric substances (EPS), protein, polysaccharide.

1. Introduction

EPS are the products of cellular lysis and hydrolysis of macromolecules with a high molecular weight, and they usually produced by microoganisms [1]-[3]. Based on binding force with cell, the EPS are usually divided into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) [4]-[6].

The application of microbial EPS in the industry has been valued highly. In the food industry, EPS can be used as a thickener and gel to improve food quality and texture [7]. In medicine, EPS is used as a hydrophilic matrix to control the release of medicines, or can be used in the development of bacterial vaccines and increase non-specific immunity [8]. In addition, EPS also improves the process of heavy metal and radio nuclides contaminated water by enhancing the water retention capacity of the soil [9], [10]. The demands for natural polymers in various industrial applications have increased lately, and therefore, novel algae have been used to synthesize high value products, such as products of high aggregated value such as pigments, osmoprotectant, metabolites, fatty acids and proteins, may also be exploited for EPS as biosurfactants and/or bio- emulsifiers [11], [12].

The extraction and analysis of EPS is a most challenging endeavor. The isolation method for algal EPS can in turn influence the compositions, contents, structure, properties and functions of EPS [13], [14]. Different extraction methods can change the properties of EPS [15]. There is no universal extraction and analysis procedures which have to be adapted to the specific EPS. Extraction protocols were compared to investigate

how different extractant affect the chemical characteristics of collected EPS [14], [16]-[20].

EPS can be found in bacteria, Cyanobacteria, marine microorganisms and fungi. *Spirogyra* is a genus of large abundant filamentous green algae, commonly found in freshwater areas all over the world. It is easy for harvested. The cell surface of *Spirogyra* spp. can excrete large amounts of EPS and then form a unique mucilage layer. However, the extraction methods of *Spirogyra* spp were not explored.

This study compared the EPS extraction yield of the large freshwater algae *Spirogyra fluviatilis*. and the levels of its primary major components of polysaccharides and proteins with two different physical extraction methods of ultrasonication and heating, also two different chemical extraction methods of formaldehyde-NaOH and NH_4OH. We expect to establish an EPS extraction-based biotechnology from *Spirogyra* spp. and explore its potential in industrial development.

2. Materials and Methods

2.1. Algae

The algae that were used in this study was the large filamentous green algae *Spirogyra fluviatilis*, which grows naturally in freshwater areas. The algae samples were collected from the campus of Kun-Shan university southern Taiwan (N22.998415,E120.254504). The algae samples were washed twice with tap water, placed in a strainer at room temperature for 2h to naturally drain the water, and then used for EPS extraction.

2.2. EPS Extraction

The harvested algae samples were suspended in a 0.05% NaCl solution and centrifuged at 5000g, 4℃ for 20 min. The liquid was collected carefully for measurement of LB-EPS [21], [22]. The remaining algae samples were resuspended with NaCl solution for following TB-EPS extraction.

Four widely used EPS extraction protocols using formaldehyde-NaOH, NH_4OH, ultrasonication and heating were adopted. Two protocols (Fig. 1) were on the use.

2.2.1. Chemical extraction methods

The remaining algae samples were resuspended with 0.05% NaCl solution for TB-EPS extraction using formaldehyde-NaOH and NH_4OH chemical method.

1) NH_4OH: remaining algae samples +0.05% (w/w) NaCl solution to 100ml+50ml 0.1M NH_4OH at 4°C for 1h.
2) Formaldehyde-NaOH: remaining algae samples +0.05% (w/w) NaCl solution to 100ml+50ml 36.5% Formaldehyde-NaOH at 4°C for 1h.

The treated suspension was centrifuged (15000g) for 20 min with the suspension being collected.

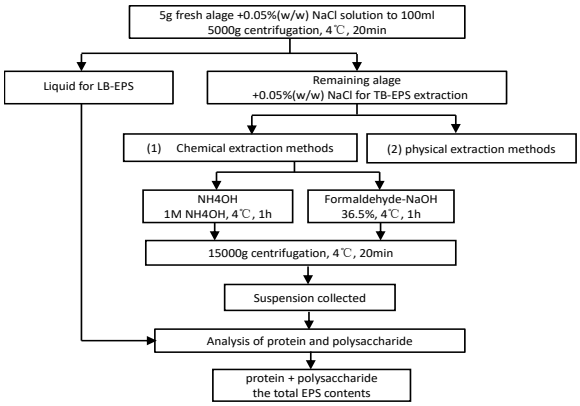

Fig. 1. Procedure for the two EPS extraction process of *S. fluviatilis* EPS.

2.2.2. Physical extraction methods

In physical extraction methods, cationic exchange resin (CER) is not suitable for EPS extraction from large filamentous algae [23], [24]. Therefore we used two physical methods, heating, and ultrasonication, to extract EPS.

1) Ultrasonication: remaining algae samples + 0.05% (w/w) NaCl solution to 100ml, was sonicated at 120W for 10 min.
2) Heating: remaining algae samples + 0.05%(w/w) NaCl solution to 100ml, was heated at 80℃ for 10 min.

The treated suspension was centrifuged (10,000 × g) for 10 min with the suspension being collected. All supernatants were diluted by Mili-Q water to at the same level and then filtrate using 0.45μm cellulose nitrate membrane filter with filtrate collected.

The flowchart of extraction procedures is shown in Fig. 2.

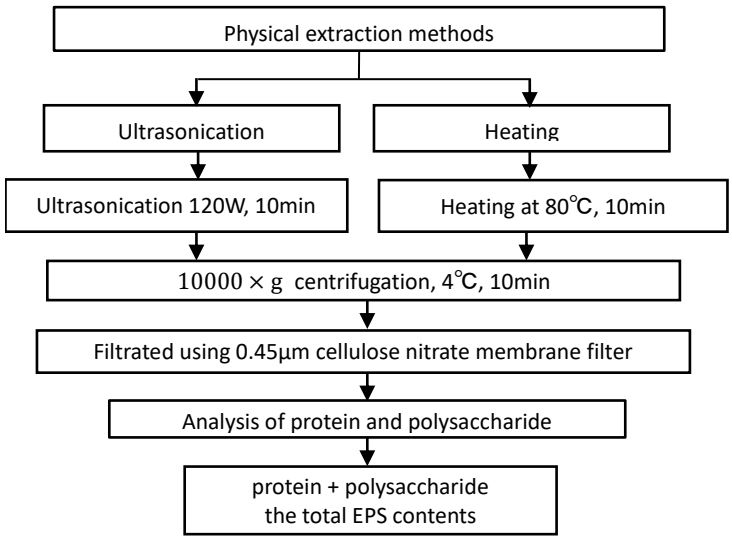

Fig. 2. Procedure for physical extraction process of *S. fluviatilis* EPS.

2.3. Analysis Method

All chemicals used in this work were of analytical grade. Polysaccharide content was determined by the phenol-sulfuric acid using glucose as a standard [25]. Protein content was determined according to Bradford (1976) with bovine serum albumin BSA, Sigma A2153 100mg/ml [26]. The total EPS content was measured as the sum of these two substances.

3. Results and Discussion

3.1. Microscopy and Comparison of Polysaccharide and Protein in LB-EPS

Microscopic examination by India ink reverse staining showed appearance of extracellular matrix on the surface of algae cell. The EPS had a gel-like structure that could fractionated into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) (Fig. 3).

For more detailed information, EPS matrix in this study were specifically divided into LB-EPS and TB-EPS fraction. As shown in Table 1 and Fig. 4, the total EPS in extracted LB-EPS matrix was 3.12±0.52 mg g^{-1} fresh alage, while 2.24±0.36 mg g^{-1} fresh alage of protein and 0.88±0.17 mg g^{-1} fresh alage of polysaccharides. These result demonstrated that uneven distribution pattern of protein/ polysaccharide

ratios in the EPS matrix may facilitate the stabilization of cell structure under complex ecological environments [27].

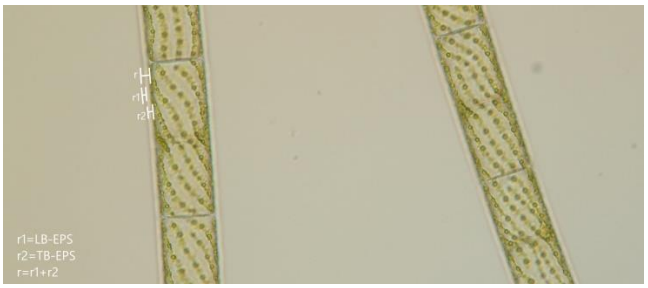

Fig. 3. Mucilage layer stucture of *S. fluviatilis.*

Table 1. Comparison of Contents and Extraction Yields of LB-EPS

EPS	Organic matter contents (mg g^{-1} fresh algae)			
LB-EPS	protein	polysaccharide	EPS(protein+ polysaccharide	Potein /polysaccharide
	2.13	0.81	2.94	2.62
	1.93	0.66	2.59	2.91
	2.49	1.08	3.57	2.31
	2.62	0.97	3.59	2.72
	1.75	0.72	2.48	2.42
	2.53	1.03	3.56	2.46

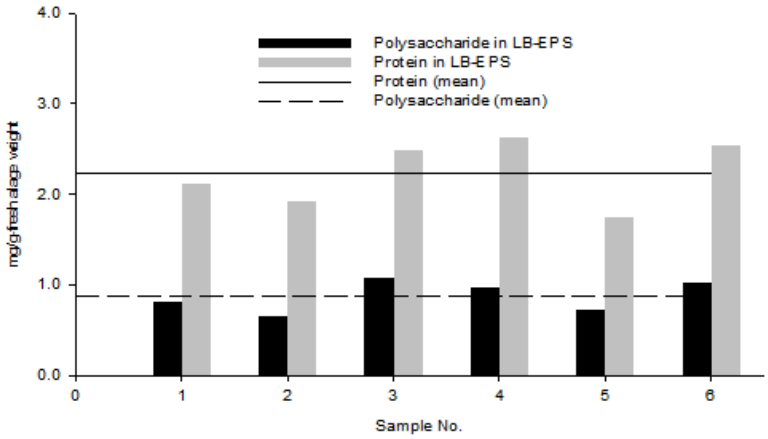

Fig. 4. Comparison of protein and polysaccharide in LB-EPS.

3.2. Comparison of Polysaccharide and Protein in TB-EPS by Physical and Chemical Extraction Methods

The TB-EPS contents and compositions resulting from the applied 2 physical extraction methods are shown in Fig. 5. Further comparison showed that the heating method exhibited higher efficiency, with the protein and polysaccharide contents of 1.82±0.24 mg g^{-1} fresh algae and 6.12±0.85 mg g^{-1} fresh algae in the TB-EPS fraction, respectively. The results were similar with the results of Xu *et al*. [28].

Two chemical extraction methods (formaldehyde-NaOH and NH_4OH) were applied to extract the TB-EPS. The TB-EPS and contents resulting are showed in Table 2, Fig. 6. The formaldehyde-NaOH method exhibited the higher efficiency, with the polysaccharide and protein contents of 0.64±0.13 mg g^{-1} and 2.48±0.19 mg g^{-1} in the TB-EPS, respectively. Which compare with the 0.48±0.05 mg g^{-1} fresh alage and 1.53±0.15 mg g^{-1} fresh alage for protein and polysaccharide by NH_4OH extraction method.

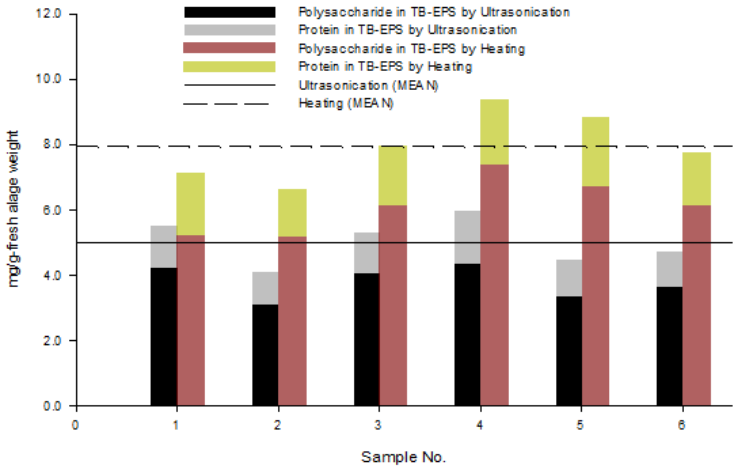

Fig. 5. Comparison of protein and polysaccharide in TB-EPS from physical extraction method.

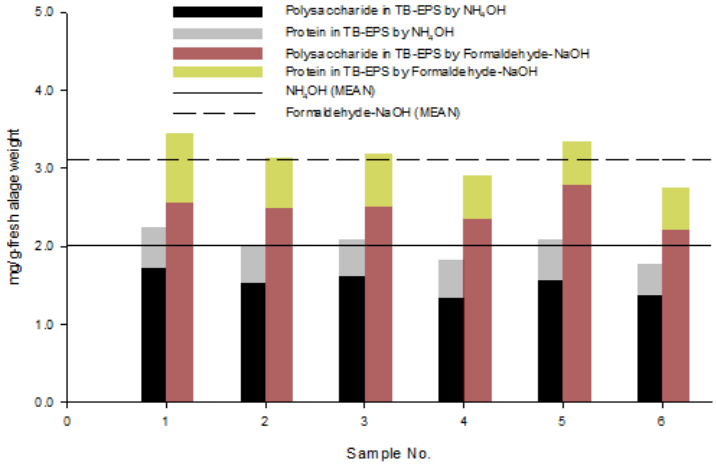

Fig. 6. Comparison of protein and polysaccharide in TB-EPS from chemical extraction method.

3.3. Comparison of Contents and Composition at Different Extraction Methods

The results are shown in Table 2 and Fig. 7. The data reflect the average results from 6 repeated measurements, for two chemical (NH_4OH and formaldehyde-NaOH) and two physical (heating and ultrasonication) extraction methods, which showed in LB-EPS fractions, proteins rather than polysaccharides were the predominant composition. From the TB-EPS fraction procedure which could give a deeper insight into the EPS structure and composition.

Table 2. Comparison of Contents and Extraction Yields of EPS by Different Extraction Methods

EPS extractions	Extracted method	Organic matter contents (mg g^{-1} fresh algae)			
		protein	polysaccharide	EPS(protein+ polysaccharide	Potein /polysaccharide
LB-EPS		2.24±0.36	0.88±0.17	3.12±0.52	2.57±0.22
TB-EPS	Heating	1.82±0.24	6.12±0.85	7.94±1.02	0.30±0.04
	Ultrasonication	1.22±0.21	3.80±0.50	5.02±0.70	0.32±0.03
	Formaldehyde-NaOH	0.64±0.13	2.48±0.19	3.12±0.26	0.26±0.05
	NH_4OH	0.48±0.05	1.53±0.15	2.01±0.18	0.32±0.03

The extracted total EPS in TB-EPS from the four extraction methods followed heating (7.94±1.02 mg g^{-1}

fresh algae) > ultrasonication (5.02±0.70 mg g^{-1} fresh algae) > formaldehyde-NaOH (3.12±0.26 mg g^{-1} fresh algae) > NH_4OH (2.01±0.18 mg g^{-1} fresh algae). This result together with the different trend in P/S ratios are shown in Fig. 7, indicating the two chemical alkaline extractants could dissociate aggregated carbohydrate (polysaccharide) in EPS [28], whereas the proteins in EPS could be more effectively extracted by heating.

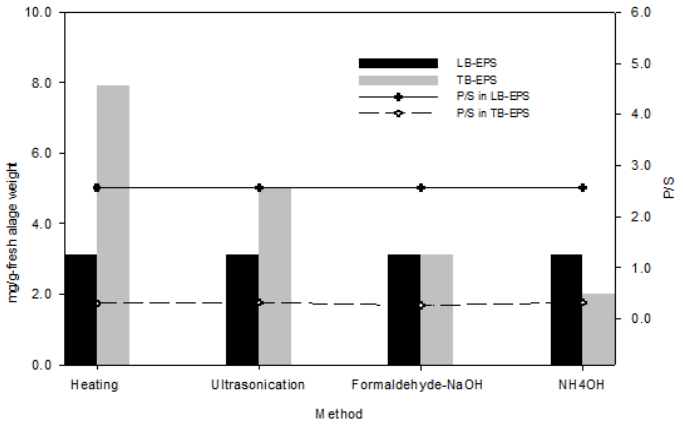

Fig. 7. Comparison of EPS and protein to polysaccharide ratio in from different extraction methods.

This study further compared the primary contents of EPS with different extraction methods. The results showed that using different extraction methods can lead to different extraction yields of EPS, the ratios of protein to polysaccharide in EPS also significant different [29].

4. Summary

In this study of macroalgal EPS, the extraction and analysis of EPS is a most challeging work. To compare the extraction yields of EPS from *S. fluviatilis* mucilage layer (LB-EPS and TB-EPS) and the changes in its primary contents of polysaccharides and proteins with two different chemical (formaldehyde-NaOH and NH_4OH) and physical (ultrasonication and heating) extraction methods, the study results show that: (1) EPS (Both contents of polysaccharide and protein) could be efficiently extracted by heating method. (2) The ratios of protein to polysaccharide in TB-EPS are obvious different， but not for LB-EPS. (3) The extraction method for macroalgal EPS can in turn influence the extraction rate, contents and properties.

S. fluviatilis is a widely distributed dominant fresh water macro-green algae. In its natural habitat, *S. fluviatilis* has great potential in EPS extraction. This preliminary study results help us understand the extraction methods for the influence on EPS isolation.

References

[1] Chi, Z., Su, C. D., & Lu, W. D. (2007). A new exopolysaccharide produced by marine *Cyanothece* sp. 113. *Bioresource Technol., 98(6)*, 1329-1332.

[2] McSwain, B. S., Irvine, R. L., Hausner, M., & Wilderer, P. A. (2005). Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. *Applied and Environmental Microbiology, 71(2)*, 1051-1057.

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

[4] Wu, Z., *et al.* (2013). Effect of ultrasonic power density on extracting loosely bound and tightly bound extracellular polymeric substances. *Desalinat., 329(15)*, 35-40.

[5] Wang, L. P., Shen, Q. R., Yu, G. H., Ran, W., & Xu, Y. C. (2012). Fate of biopolymers during rapeseed meal and wheat bran composting as studied by two-dimensional correlation spectroscopy in combination with multiple fluorescence labeling techniques. *Bioresour. Technol., 105,* 88-94.

[6] Sheng, G. P., Yu, H. Q., & Li, X. Y. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatmentsystems: A review. *Biotechnology Advances*, *28(6)*, 882-894.

[7] Cheng, Y. S., Zheng, Y., Labavitch, J. M., & VanderGheynst, J. S. (2011). The impact of cell wall carbohydrate composition on the chitosan flocculation of *Chlorella*. *Process Biochem., 46(10)*, 1927-1933.

[8] Cheng, Y. S., Zheng, Y., Labavitch, J. M., & VanderGheynst, J. S. (2013). Virus infection of Chlorella variabilis and enzymatic saccharification of algal biomass for bioethanol production. *Bioresour. Technol., 137*, 326-331.

[9] Zheng, Y., Roberts, M., Kelly, J., Zhang, N., & Walker, T. (2015). Harvesting microalgae using the temperature-activated phase transition of thermoresponsive polymers. *Algal Res*., *11*, 90-94.

[10] Zheng, Y., Xiao, R., & Roberts, M. (2016). Polymer-enhanced enzymaticmicroalgal cell disruption for lipid and sugar recovery. *Algal Res., 14*, 100-108.

[11] Zheng, Y., & Xiao, R. (2016). Overview of microalgal extracellular polymeric substances (EPS) and their applications. *Biotechnology Advances, 34(7)*, 1225-1244.

[12] Shen, L., Li, Z. F., Wang, J. J., Liu, A., Li, Z. H., Yu, R. L., Wu, X. L., Liu, Y. D., Li, J. K., & Zeng, W. M. (2018). Characterization of extracellular polysaccharide/protein contents during the adsorption of Cd(II) by Synechocystis sp. PCC6803. *Environmental Science and Pollution Research, 25(21)*, 20713-20722.

[13] Takahashi, E., Ledauphin, J., Goux, D., & Orvain, F. (2009). Optimising extraction of extracellular polymeric substances (EPS) frombenthic diatoms: Comparison of the efficiency of six EPS extraction methods. *Mar. Freshw. Res., 60(12)*, 1201-1210.

[14] Matsumoto, T., Yamamura, H., Hayakawa, J., Watanabe, Y., & Harayama, S. (2014). Influence of extracellular polysaccharides (EPS) produced by two different green unicellular algae on membrane filtration in an algae-based biofuel production process. *Water Sci. Technol., 69(9)*, 1919-1925.

[15] Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nat. Rev. Microbiol., 8(9)*, 623-633.

[16] Wingender, J., Neu, T. R., & Flemming, H. C. (1999). What are bacterial extracellular polymeric substances? *Microbial Extracellular Polymeric Substances: Characterization, Structures and Function*, 1-19. Berlin: Springer Verlag.

[17] Liu, H., & Fang, H. H. P. (2002). Extraction of extracellular polymeric substances (EPS) of sludges. *Journal of Biotechnology, 95(3),* 249-256.

[18] Eskicioglu, C., Kennedy, K. J., & Droste, R. L. (2006). Characterization of soluble organic matter of waste activated sludge before and after thermal pretreatment. *Water Research, 40(20)*, 3725-3736.

[19] Domínguez, L., Rodríguez, M., & Prats, D. (2010). Effect of different extraction methods on bound EPS from MBR sludges. Part I: Influence of extraction methods over three-dimensional EEM fluorescence spectroscopy fingerprint. *Desalination, 261(1-2)*, 19-26.

[20] Jiang, J. Q., Zhao, Q. L., Wei, L. L., & Wang, K. (2010). Extracellular biological organic matters in microbial fuel cell using sewage sludge as fuel. *Water Research, 44(7)*, 2163-2170.

[21] Huacheng, X., Guanghui, Y., & Jiang, J. H. (2013). Investigation on extracellular polymeric substances from mucilaginous cyanobacterial blooms in eutrophic freshwater lakes. *Chemosphere, 93(1)*, 75-81.

[22] Lixiao, N., Danye, L., Shiyi, R., Lili, S., Wei, Z., Peifang, W., Chao, W., Shiyin, L., & Kumud, A. (2017). Characterization of extracellular polymeric substance (EPS) fractions produced by Microcystis aeruginosa under the stress of linoleic acid sustained-release microspheres. *Environmental Science and*

Pollution Research, 24(26), 21091-21102.

[23] Sun, M., Li, W. W., Yu, H. Q., & Harada, H. (2012). A novel integrated approach to quantitatively evaluate the efficiency of extracellular polymeric substances (EPS) extraction process. *Appl. Microbiol. Biotechnol., 96(6)*, 1577-1585.

[24] Wang, Z. M., Cheung, Y. C., Leung, P. H., & Wu, J. Y. (2010). Ultrasonic treatment or improved solution properties of a high-molecular weight exopolysaccharide produced by a medicinal fungus. *Bioresource Technol., 101(14)*, 5517-5522.

[25] 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(3)*, 350-356.

[26] Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem., 72(1-2)*, 248-254.

[27] Xu, H. C., Cai, H. Y., Yu, G. H., & Jiang, H. L. (2013). Insights into extracellular polymeric substances of cyanobacterium Microcystis aeruginosa using fractionation procedure and parallel factor analysis. *Water Res., 47(6)*, 2005-2014.

[28] Park, C., Novak, J. T., Helm, R. F., Ahn, Y. O., & Esen, A. (2008). Evaluation of the extracellular proteins in full-scale activated sludges. *Water Research, 42(14)*, 3879-3889.

[29] Mahendran, B., Jared, F., & Steven, N. L. (2015). Protein and polysaccharide content of tightly and loosely bound extracellular polymeric substances and the development of a granular activated sludge floc. *Water Research, 82*, 104-117.

Shui-Ping Chang was born in 1954. From 1973-1977, he got the B.S. in botany from National Chung Hsing University (Taichung, Taiwan). From 1982 to 1985, the M.S. in environmental science, SUNY at Syrcause, New York, U.S.A.; From 1992 to 1997, the Ph.D. of environmental engineering, National Cheng Kung University. His research interests are algae, bioremediation, water treatment.

From 1985-1992, he is the senior engineer and section chief at EPA in Taiwan. Form 1997, he is an associate professor, Department of Environment Engineering, Kun Shan University in Taiwan.

Hwey-Lin Sheu is an associate professor at Department of Environment Engineering, Kun Shan University in Taiwan. She was original born in Taiwan, Nantow, on January 3, 1969. She received her Ph.D. degree at National Cheng Kung University in Taiwan. Her main research areas focus on the protection of environment.

Yi-Chao Lee is an assistant professor at the Department of Environment Engineering, Kun Shan University in Taiwan, Tainan. She was born in Taiwan, Chiayi on November 14, 1966. She obtained a master's degree in civil engineering from National Chiao Tung University in Taiwan. Her main research areas focus on the environmental engineering.