Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 204 (2018) 287–294

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Assessment of physiological responses and growth phases of different microalgae under environmental changes by Raman spectroscopy with chemometrics

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ARTICLE INFO

Article history:
Received 16 March 2018
Received in revised form 21 May 2018
Accepted 16 June 2018
Available online 20 June 2018

Keywords:
Water eutrophication
Raman mapping spectroscopy
Cell physiology and growth phases of microalgae
Principal component analysis
Support vector machine

ABSTRACT

The assessment for cell physiology and growth phases of microalgae plays important roles in ecological and environmental fields since it can be used to forecast water eutrophication level worldwidely. Herein, growth phases and environmental conditions of microalgae were assessed by combining resonance Raman mapping spectroscopy with multivariate analysis methods. And, primary Raman characteristic peaks of microalgae were mined with two-dimensional synchronous spectra. Thereafter, algal growth phases and environmental conditions of microalgae were preliminary classified with different tendencies of characteristic Raman peaks by unsupervised principal component analysis (PCA) and support vector machine (SVM) methods. Our results demonstrated that resonance Raman mapping spectroscopy with PCA and SVM classification models can be used to assess algal growth phases and preliminary predict environmental conditions with characteristic Raman spectra of microalgae in water bodies.

1. Introduction

Eutrophication, the enrichment of nutrients within a waterbody, is one of the most serious water pollution problems worldwidely. Recently, it is aggravated by human activities. Large amounts of nitrogen, phosphorus, and other inorganic salt nutrients, which are from sewage and industrial wastewater, rainfall and surface runoff, have promoted the growth and rapidly reproduction of planktonic microalgae in water bodies [1]. What's worse, the eutrophication can result in water blooms frequently [2]. Usually, physiological responses and growth phases of microalgae are used to predict eutrophication even water blooms during the past decades [3]. Therefore, the assessment for physiological responses and growth phases of microalgae plays important roles in ecological and environmental fields [4]. However, it is challenging to find a sensitive, non-destructive, time saving and automated method for the study of algal physiological responses and identification of growth phases so as to predict the water eutrophication level.

Traditionally, physiological status of microalgal communities and water eutrophication levels are assessed with the concentrations of total chlorophylls and cell densities of microalgae in water bodies. Among them, concentrations of total chlorophylls are estimated from remote sensing images of analyzed waters [5]. And, cell densities of microalgae are done with the help of microscopy by manually or intelligent methods by experienced scientists [6]. Other established analytical methods like high performance liquid chromatography and mass spectrometry already provide detailed biochemical information about cellular components, metabolites and physiological responses of microalgae under environmental changes [7, 8]. However, these techniques are restricted to bulk volumes or require further sample preparation steps such as extraction and chemical derivatization.

For investigating physiological responses and growth phases of microalgae, vibrational spectroscopy demonstrates a label-free, nondestructive and widely applicable method that probes the cellular

Abbreviations: 2D correlation Raman analysis, two-dimensional correlation Raman analysis; PCA, principal component analysis; SVM, support vector machine; FACHB, Freshwater Algae Culture Collection at the Institute of Hydrobiology; AsLS, asymmetric least squares; N-limited, nitrogen-limited; HL-Flux, high light flux; LL-Flux, low light flux; PC, principal components.

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https://doi.org/10.1016/j.saa.2018.06.060
1386-1425/

components and physiological responses of individual cells in different environmental water bodies [9, 10]. At the same time, most applications of monitoring microalgae necessitate fast deployable and sensitive methods to assess cellular physiology, preferably at unicellular scale [11]. Additionally, extraordinary vibrational Raman spectroscopy can provide characteristics fingerprint information of intracellular proteins, lipids, and pigments in microalgae. Therefore, it is used to evaluate composition or distribution changes of these organic compounds under different environmental conditions [12, 13]. Cellular physiology and nutrient status are also predicted with multivariate classification methods by using Raman microspectroscopy at unicellular scale [14]. Physiological responses and morphological changes of microalgae are qualitatively or semi-quantitatively assessed by FT-Raman and normal Raman spectroscopy [15–17]. Under high/low-light flux and nitrogen-limited conditions, conformation changes of carotenoids and lipids, accumulations of lipids and pigments are visualized at unicellular scale by resonance Raman spectroscopy with a non-destructive manner. Meanwhile, non-invasive model-based chemometrics technique-ordinary least square is used to evaluate the fat composition of living marine diatoms which are cultured under six stress conditions by using Raman spectroscopy [18–22]. However, different algal growth phases under environmental changes have not been in vivo assessed with physiological responses by using resonance Raman mapping spectroscopy at unicellular scales.

In order to assess eutrophication level even predict water blooms preliminary, combining resonance Raman spectroscopy with chemometrics methods, physiological responses and growth phases of different microalgae will be analyzed with their Raman characteristic information under different environmental conditions. Primary characteristic Raman peaks and tendencies of characteristics Raman spectra under changing environments are mined with two-dimensional (2D) correlation Raman analysis firstly [23]. Thereafter, growth phases and different culture conditions of different microalgae will be tentatively classified with an unsupervised exploratory principal component analysis (PCA) [24, 25] and support vector machine (SVM) [26] classification method respectively. The heuristic approaches would provide a possibility non-destructive assessment of water eutrophication level worldwidely, even predict environmental changes in water bodies.

2. Materials and Methods

2.1. Organisms and Cell Cultures

Microcystis flos-aquae (*M. flos-aquae*, FACHB-1341), and *Chlorella vulgaris* (*C. vulgaris*, FACHB-32) were purchased from Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-collection), Chinese Academy of Sciences. Algal cells were firstly enriched in BG11 medium with an aeration rate of 70 ml/min under 25 ± 2 °C in a 1 L Erlenmeyer flask. And, a 12:12 h light/dark cycle was set with a light intensity of 22 μmol/m^{-2} s^{-1} which was illuminated by cool-white fluorescent lamps. Thereafter, algal suspensions were washed three times with sterile 1 M $NaHCO_3$ solution and then re-inoculated into five aliquots for further cultivation in 500 ml Erlenmeyer flasks with cell densities of 5.8 × 10^6 cells/ml and 5.08 × 10^6 cells/ml for *M. flos-aquae* and *C. vulgaris* respectively. They were all cultured under 25 ± 2 °C with a 12:12 h light/dark cycle. In order to simulate changing environments, nitrogen-limited (N-limited, concentration of nitrogen: 0.5 mg/L), aeration (aeration rate: 0.6 vvm), high light flux (HL-Flux, light intensity: 55 μmol/m^{-2} s^{-1}), and low light flux (LL-Flux, light intensity: 10 μmol/m^{-2} s^{-1}) were applied to algal suspensions in comparison with control group (Control, light intensity was 22 μmol/m^{-2} s^{-1}, concentration of nitrogen was 250 mg/L, no aeration) respectively. Culture flasks were manually shaken 2–3 times daily to ensure thorough mixing of algal suspensions. The total algal cultivations ran 18 days without adding other nutrients. The algal harvests and Raman measurements were performed 8 times in the algal growing process.

2.2. Raman Spectroscopy and Measurements

Renishaw inVia micro-Raman spectroscopy system (Wotton-under-Edge, Gloucestershire, UK) was used to collect back-scattered Raman signals of microalgae with a Peltier cooled CCD detector (−70 °C) and a 532 nm DPSS laser (the maximum laser power: 50 mW). The spectral resolution was 1 cm^{-1} upon 2400 lines mm^{-1} grating. The diameter of laser spot on specimen was approximately 0.88 μm while 100 × objectives was chose for laser excitation within Leica microscope system. Prior to measurement of algal cells, Raman spectrometer was calibrated daily with Raman shift and intensity of silicon at 520 cm^{-1} firstly.

For each culture condition, 1 ml algal suspensions were taken from Erlenmeyer flask 2 h after onset of light cycle to avoid daily variabilities in pigment composition and cellular metabolism prior to Raman measurements. 1 μl algal suspensions were dropped on glass slide overcoated with poly-L-lysine (Sigma-Aldrich). Algal cells were adhered to glass slide and maintained about 1 min. Then, Raman mapping spectra of 10 single algal cells were acquired with the aid of microscope for each culture condition. Scanning step of Raman mapping was 1 μm. At least 36 points in a rectangle filled area were chose on the surface of each algal cell. For each single spectrum in mapping spectra, acquisition time of each spectrum was set as 1 s and this was repeated only 1 time. The range of wavenumber was from 596 cm^{-1} to 1760 cm^{-1}. 0.05% of laser power was chosen and then approximately 0.01 mW laser power irradiated on algal cells. It was low enough to prevent photo damage of algal cells during Raman mapping measurements. As shown in Fig. S1, the images of *M. flos-aquae* cells, before and after exposure with 0.05% of laser power, both showed little differences in morphology of single and double cells. Their mapping spectra at 1514 cm^{-1} were also basically consistent with cell morphologies.

2.3. Spectral Preprocessing

Four strongest spectra in a mapping dataset were chose as characteristic spectra of each algal cell upon Raman intensities. Therefore, for each experimental day, total 400 spectra were used to model and classify growth phases or culture conditions with multivariate analysis methods. Firstly, 2D correlation Raman analysis of *M. flos-aquae* and *C. vulgaris* under different culture conditions were performed with *2Dshige* (Shigeaki Morita, Kwansei - Gakuin University, 2004–2005) respectively [26]. Then, characteristic Raman intensities, which were fitted with *Lorenz* function (WiRE3.4), were to gather statistics information to assess physiological responses and growth phases of microalgae in each culture condition. Before multivariate analysis methods were done to spectra datasets, mismatching of Raman shifts for each day was calibrated by cubic smoothing spline fitting method [24]. What's more, asymmetric least squares (AsLS) baseline correction method was used to correct background noises which came from samples themselves [27, 28].

2.4. Principal Component Analysis (PCA) and Support Vector Machine (SVM)

Chemometrics methods PCA and SVM were used to assess growth phases and culture conditions of microalgae. Few principal components (PCs) can express main characteristics in Raman spectral datasets of algal cells under environmental induction and their growth phases for each culture condition preliminary. Then, Raman spectra of algal cell induced with different environmental conditions were classified with their PCA scores. In addition, PCA was also used for spectral dimensionality reduction before multivariate analysis. And, SVM was a machine learning method based on structural risk minimization principle and empirical risk minimization. Owing to the advantages of generalization error minimum, it was widely used in the small samples statistics analyses. Therefore, based on PCA scores, SVM was applied to identify the