Algal Research 19 (2016) 246–252

Contents lists available at ScienceDirect

Algal Research

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

Assessment of growth phases of the diatom *Ditylum brightwellii* by FT-IR and Raman spectroscopy

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ARTICLE INFO

Article history:
Received 13 May 2016
Received in revised form 9 August 2016
Accepted 5 September 2016
Available online xxxx

Keywords:
Raman microscopy
FTIR microscopy
Cell physiology
Biomolecules
Chemometrics

ABSTRACT

Most applications of monitoring microalgae necessitate fast deployable and sensitive methods to assess cellular physiology, preferably at the scale of single cells. Vibrational spectroscopies, namely Fourier transform infrared (FT-IR) and Raman spectroscopy, have been proven to be valuable tools since they are label-free, nondestructive, and fast applicable. Infrared spectra contain spectral contributions of macromolecules, such as proteins, nucleic acids, carbohydrates, biosilica and lipids. A major concern of this method is the requirement to dry the sample, which might severely affect cellular physiology. Raman spectroscopy, on the other hand, can be applied in aqueous environments, and provides complementary information to FT-IR spectroscopy, because Raman spectra of microalgae are dominated by the photosynthetic pigments, e.g. chlorophyll *a* and carotenoids. In our current study we applied both spectroscopy methods to study chemical variations within individual cells of the diatom *Ditylum brightwellii* during the course of growth phases. Culture growth was monitored via cell counting and related spectral changes were examined employing partial least square (PLS) regression and linear discriminant analysis (LDA) classification. FT-IR models revealed a pronounced decrease of protein and carbohydrate content, a concurrent increase in cellular lipid level and constant biosilica content during culture growth. Interestingly, the highest nucleic acid content appeared in the stationary phase. Raman based models could easily identify cells in the exponential phase indicating highest chlorophyll *a* amount in that particular stage. Further cell proliferation was accompanied with distinct variances in the carotenoid pool. Comparison of model performances showed that Raman based models were more stable and more accurate than respective FT-IR based models. This leads us to conclude that Raman spectroscopy is a promising method for examining microalgal cell physiology.

1. Introduction

During the past decades microalgae have been intensely debated as a renewable and commercially competitive feedstock of diverse chemicals [1]. Since then they have been subjected to a systematic research, which includes fields from bio-fuel to pharmaceutical industries and environmental sciences [2,3]. Resulting discoveries led to the development of microalgae-based biorefineries where both fuels and value-added co-products are extracted from algal biomass [4]. Still, many key aspects remain elusive, e.g. selecting optimal growth conditions and choosing productive algal strains. The characterization of the metabolic response function is a critical point in order to control the cultivation environment, and to maximize yields of highly unsaturated triglycerides in the bio-fuels production or in photosynthetic pigments as antioxidant agents in the pharmaceutical and cosmetic industry. In a broader sense monitoring physiological status of microalgal assemblages in sea and fresh water samples can serve as a sensitive tool for water quality assessment [5]. Established analytical methods like gas and high-pressure liquid chromatography and mass spectrometry already provide detailed biochemical information about cellular metabolites and are suitable for online monitoring in bioreactors. However, these techniques are restricted to bulk volumes and require further sample preparation steps such as extraction, chemical derivatization as well as costly instrumentation. For investigation of single-cell physiology vibrational spectroscopy displays a label-free, nondestructive and fast applicable method that probes the biomolecular content of individual cells. FT-IR spectroscopy has already been applied to microalgae in several studies [6–9]. IR spectra consist of rather broad absorption bands that can be assigned to functional groups of specific macromolecules and can be used to unravel relative changes in the macromolecular composition of cells, induced by external stress. Based on FT-IR spectroscopy in combination with chemometric classification models it is also possible to taxonomically differentiate microalgae in the same water body [10,11]. While this can be accurately done for unrelated

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http://dx.doi.org/10.1016/j.algal.2016.09.007
2211-9264/

species, the differentiation of closely related species is more challenging. Driver et al. recently showed that identification of different species by FT-IR spectroscopy is considerably hampered due to their phenotypic plasticity [12]. Another possible limitation to chemometric classification models of conventional IR spectra is the restriction to measure dried microalgae, since water is a strong IR absorber and prohibits a straightforward interpretation of the results. So far it has not been evaluated how the drying procedure alters the macromolecular structures, but considering even marginal perturbations on the molecular structure it certainly induces conformational changes in proteins and nucleic acids, which causes spectral shifts in the peptide and phosphate backbone band regions, respectively [13,14]. Approaches to overcome this limitation involve measurements of microalgae in thin layers of water using IR synchrotron radiation, which paves the way to combine high spatial resolution FT-IR imaging and microfluidics [15]. Raman spectroscopy offers another convenient modality for non-destructive and label-free in vivo investigations of phytoplanktonic organisms in an aqueous environment [16,17]. It probes different modes of molecular vibrations with higher spatial resolution compared to FT-IR spectroscopy. Raman spectra of microalgal cells are dominated by signals originating from photosynthetic pigments, such as chlorophyll *a* and carotenoids, but also compounds with long hydrocarbon chains, such as esterified fatty acids and other molecules of the lipid class. The majority of Raman studies on algae focuses on lipid body formation and quantification in nutritionally stressed cells to select potential bio-fuel producers [18–20]. Recently, Moudříková et al. used confocal Raman spectroscopy to resolve starch compartments and polyphosphate bodies inside a *Desmodesmus quadricauda* coenobium after photobleaching [21]. Extracted cell walls of the diatom *Stephanopyxis turris* were investigated by FT-IR and Raman microspectroscopic imaging and allowed to resolve the spatially heterogenous distribution of embedded organic and inorganic material [22]. Heraud et al. correlated microalgal nutrient status with pigment composition [23]. Their results already indicated that relevant physiological information can be derived from Raman spectra [21].

The aim of the current study was to compare the suitability of FT-IR and Raman spectroscopy to investigate cell physiology of microalgae in defined growth stage phases using the diatom *Ditylum brightwellii* as a model system. Diatoms constitute one major group of the algae community that exerts an enormous impact on the planetary carbon cycle. Besides their ecological relevance diatoms are also able to build intricately structured species-specific cell walls made of biosilica. Based on single-cell spectra two classification models for the assessment of growth phases were developed and validated using partial least squares linear discriminant analysis (PLS-LDA). Both models were examined with respect to interpretability and prediction performance. We found that Raman based models performed considerably well in identification of cells in the exponential and stationary phases due to compositional changes in the photosynthetic apparatus.

2. Materials and methods

2.1. Cell culture and sample preparation

D. brightwellii was isolated from the North Sea and cultivated in sterile filtered artificial seawater medium (ASW) which was prepared according to the protocol in [24]. Cultures were grown in a homebuilt cultivation chamber at a temperature of 18 °C, 14/10 h day/night cycle and an illuminance of approximately 1000 lx with fluorescent tubes (Osram T5 36 W 640). Three batches of *D. brightwellii* cultures were prepared as follows: 70 mL of ASW in a 250 mL culture flask (Greiner) was inoculated with 1 mL of a stock culture resulting in a final cell density of 2.5×10^3 cells per mL. Culture flasks were shaken twice daily to ensure thorough mixing of algal suspension. Cell density was estimated via daily counting cells using a Fuchs-Rosenthal hemocytometer. For spectroscopic measurements a total sample volume of 1.0 mL was taken from the suspension two hours after onset of the day cycle in the cultivation chamber to avoid daily variability in pigment composition and cellular metabolism. Half of this volume was placed in a centrifugal filter unit (Ultrafree-CL, Millipore) and centrifuged for 2 min at 20,000 rpm in order to maintain cell integrity. Cells on the filter were resuspended in 1 mL deionized water and centrifuged as mentioned before. This procedure was repeated four times in order to wash out ingredients of the medium that show strong IR absorbance. Subsequent spectroscopic measurements were performed within a time frame of three hours after sampling.

2.2. FT-IR measurements

Before measurements the cell pellet on the centrifugal filter unit was resuspended in 100 μL deionized water and transferred onto a CaF_2 slide. The slide was placed in a FT-IR microscope chamber (Varian 620-IR, Agilent, USA) which was enclosed in a homebuilt box and purged by dried air to dry the sample as well as to reduce spectral contributions from water vapor. The microscope was coupled to a FT-IR spectrometer (Varian 670-IR, Agilent, USA) and equipped with a Cassegrain 15×/NA 0.4 objective and a liquid nitrogen cooled MCT detector. The microscope aperture was adjusted to match single cell size and before each measurement a background spectrum from a vicinal spot on the slide was acquired. Every day 30 individual cells of one batch were studied and for each cell 64 scans in transmission mode were co-added at a spectral resolution of 4 cm^{-1} in the interval 900 to 4000 cm^{-1}.

2.3. Raman measurements

Raman images of 30 single diatoms in ASW were collected per day on an upright Raman microscope system (Holoprobe, Kaiser Optical System, USA), consisting of a multi-mode diode laser at 785 nm emission (Invictus), an f/1.8 spectrograph with a holographic transmissive grating (Kaiser Optical System, USA) and a Peltier-cooled back illuminated deep depletion CCD detector (iDus420, Andor, Ireland). The microscope was coupled to the Raman system by multi-mode fiber with a core diameter of 105 μm (Thorlabs, Germany). Raman images were recorded at a mean step size of 8 μm with 2 s exposure time per spectrum, using a 60×, 1.0 NA water immersion objective lens (Nikon). Cosmic spike removal was performed automatically by the acquisition software Holograms (Kaiser Optical System, USA) by subsequently recording two spectra of the same spot in the sample. For intensity and wavelength calibration of the spectrometer a calibration lamp (Kaiser Optical System, USA) was used daily before starting the measurements. The laser power was adjusted to 30 mW measured at the sample plane with a digital power meter (Thorlabs, USA). The power was low enough to prevent photo-damage of the cell during the measurement, as verified by visual inspection. In order to avoid laser trapping of diatoms the CaF_2 slide surface was modified with poly-L-lysine. Thus, the CaF_2 slide was incubated with a solution of poly-L-lysine in deionized water (0.1 mg/mL, Sigma-Aldrich) for 10 min. Afterwards the supernatant solution was removed by suction and the slide was left to air-dry.

2.4. Spectral preprocessing

Preprocessing of raw spectra was performed in R using the hyperSpec package [25]. Spectral smoothing and interpolation was done by locally weighted polynomial regression (LOESS). All IR spectra exhibited strong Mie type scattering signature and were corrected using an algorithm (Matlab) provided by Bassan et al. [26]. One IR spectrum with minimal scattering background was chosen as the reference spectrum and using 10 iterations per spectrum with eight principal components a sufficient correction for Mie scattering could be achieved. Corrected IR spectra were area-normalized relative to the spectral range from 1000 to 1800 cm^{-1} and 2800–3000 cm^{-1}, respectively.