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Dedicated to Professor Dr. Cozar Onuc on His 70th Anniversary

CYANOBACTERIA DETECTION AND RAMAN SPECTROSCOPY CHARACTERIZATION WITH A HIGHLY SENSITIVE, HIGH RESOLUTION FIBER OPTIC PORTABLE RAMAN SYSTEM

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ABSTRACT. Raman spectroscopy of cultivated Synechocystis sp. AICB 51 strain cyanobacteria is reported both at single cell level and in bulk solution. Two types of equipment, a lab-based Raman system and a portable, high resolution fiber optic Raman system respectively, were employed, to characterize the bacteria and to assess the capability of the portable instrument to reproduce the lab results for environmental application purpose. Additional cyanobacteria isolated from the Adriatic Sea, probably Synechococcus (not yet genetically identified) was employed for Raman spectroscopy characterization. Both cyanobacteria strains showed characteristic bands at 1516, 1156 and 1006 cm^{-1}, consistent with the three main Raman modes of beta-carotene in protein-carotenoid-complex from cyanobacteria, slightly different from those of pure beta-carotene. The portable system showed additional performance in recording higher signal to noise ratio in a faster and flexible way. A photoprotective energy dissipating mechanism involved upon laser exposure could be responsible for the similar Raman output of the two strains.

Keywords: *cyanobacteria, Raman spectroscopy, portable Raman system, carotenoids.*

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INTRODUCTION

Cyanobacteria are among the widest spread microorganisms worldwide and their capability to adapt, survive in extreme conditions and fabricate the building blocks of the organic world within the photosynthetic process, still attach large interest for the nanotechnology-based sensing of environmental conditions as well as on their response to the environmental changes. Detection of cyanobacteria is of crucial importance in understanding microbial world and cyanobacteria research evolved in a broad area of biomedical, environmental or terrestrial and extraterrestrial applications [1-3]. Here we describe the Raman spectroscopy detection of cyanobacteria using a portable, high resolution fiber optic Raman system. Further, we demonstrate the unique capability of the Raman spectroscopy with portable instruments to promptly assess the carotenoid species in the cultivated batches of microorganisms when the 532 nm line is pre-resonantly used.

Anticipating that portable Raman mini-spectrometers could be part of a suite of analytical instrumentation in the forthcoming environmental monitoring programs or submersible and extraterrestrial missions, it is critically important to examine the spectral information that could be acquired with portable instruments from attempts to determine key molecular signatures of microorganisms under various (hostile) conditions or in real world environmental samples. Furthermore, the exploration of the Raman signature of various cyanobacteria strains poses a double aim: to understand their behavior when microorganisms are exposed specific conditions and to achieve the enhanced capability of detection in real world samples. Although abundant data are available on microbial pigments [3], it is surprisingly to note that limited information or applications are available to date on these aspects raised above.

EXPERIMENTAL

Cyanobacteria. A batch sample of cyanobacteria (Synechocystis sp. AICB 51 strain) was kindly provided by Professor N. Dragos from Biology Department of Babes-Bolyai University. The genus is a mesophilic cyanobacterium able to use the inorganic carbon added to the growth medium (Zarrouk) [4] as $NaHCO_3$. The sample was taken before the exponential grow phase and the cells density was estimated as about 0.5 $x10^5$ cells/ml from the optical density measurements. Cyanobacteria growing conditions as well as the chemical composition are described in details elsewhere [5, 6]. The genus was found strongly dependent on the temperature,

with optimal growing condition at 30°C, in fluorescent light with 630 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance. A specific growth rate of 0.8 $days^{-1}$ and a doubling time of 1.2 days was concluded at optimal temperature. An additional genus of cyanobacteria (most likely Synechococcus sp. in a mixed culture, strain not yet genetically identified) was isolated from open waters of South Adriatic Sea. A small batch sample of about 20 ml was available courtesy of Eng. biologist Dr. Stijepo Ljubimir, from Institute for Marine and Coastal Research, University of Dubrovnik, Croatia.

Equipment. A Renishaw InVia confocal Raman system equipped with a video camera was used for high resolution (0.5 cm^{-1}) Raman spectra acquisition and micrographs collection. A diode pumped solid state (DPSS) air cooled laser operating at 532 nm line was used for pre-resonant excitation of the cyanobacteria, with 15 s integration time, 6 acquisitions and 5% output power (10 mW).

A portable, i-Raman Plus spectrometer from B&W TEK [7] was employed for the fast assessment of the real world samples of salty waters as well as the cultivated batches. The instruments provide both the 532 and 785 nm line for excitation and the adaptability to a microscope and a fiber optic probe. The *BAC102 Raman Trigger Probe* offers a trigger function which is designed for versatility in reaching samples with difficult accessibility.

RESULTS AND DISCUSSIONS

Typical Raman signal recorded with the lab-based Raman system of the bulk cyanobacteria is shown in the Fig. 1a) in comparison with the FT-Raman signal of powder beta-carotene [8]. The beta-carotene spectrum characterization and vibrational analysis was provided elsewhere [8]. A detail of the ν_1 mode of the polyene chain centered at 1512 cm^{-1} and assigned to the conjugated C=C bond is showed in the Fig. 1b), where the corresponding band of cyanobacteria appeared shifted to 1516 cm^{-1} and considerably broadened. Tschirner et al [9, 10] shown that the prominent peak at ca. 1525 cm^{-1} of beta-carotene in solutions is composed of two closely spaced modes, both of which dominated by C=C stretching coordinates of the polyene chain, and this is an intrinsic characteristic of the molecule. Therefore, the observation of the main carotenoid band at 1522 cm^{-1} at single cell level, does not necessarily reveal a different carotenoid compound. Micrograph (20 x objective) of the Synechocystis collected with the Raman microscope from the drop coated depositions of cyanobacteria culture on a hydrophobic slide is showed in the Fig. 1c and their optical microscopy image obtained with an inversed microscope (100x objective) is showed in the Fig. 1 d.

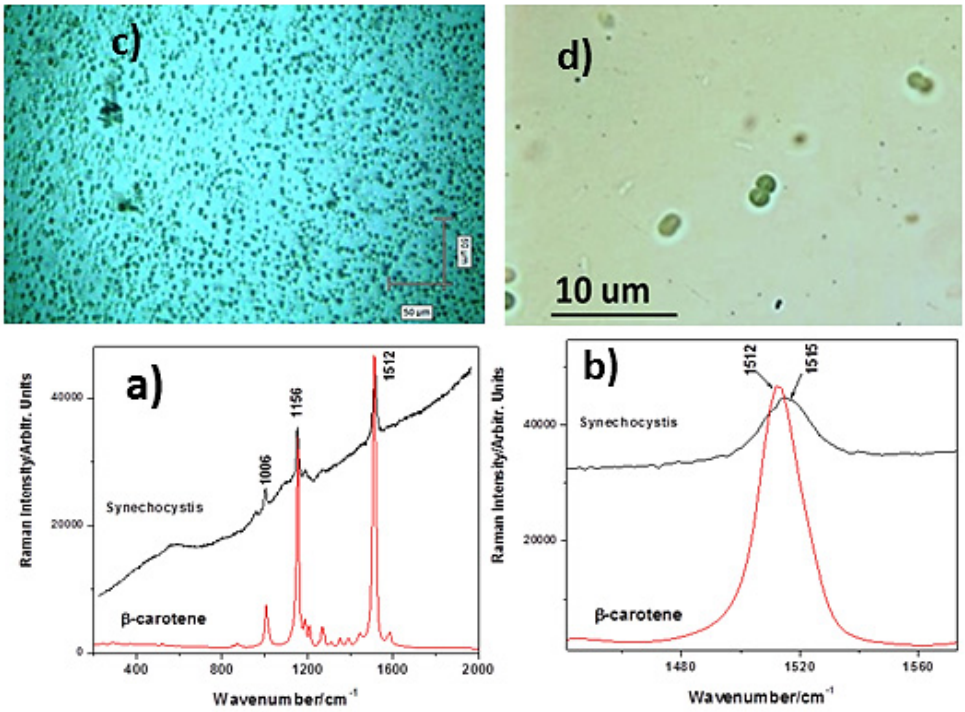

Fig. 1. Raman signal collected with a lab-based instrument (InVia, Renishaw) with 15 sec integration time from Synechocystis cyanobacteria (bulk) compared to the FT-Raman spectrum of beta-carotene powder a); detail of the v_1 Raman mode b); micrograph of the cyanobacteria seen through the Raman microscope (20x objective) c) and through an inversed optical microscope (100x objective), d).

Live cells from both cyanobacteria cultures were further characterized by Raman micro-spectroscopy with a lab-based high resolution instrument. Video-camera of the system allowed the visualization of their motility before complete aqueous medium evaporation. Wetting again the completely dried strain resulted after drop coating deposition on the hydrophobic slide with distilled water, resulted in restarting cell gliding and/or rolling motility, confirming their live status. The measured cyanobacteria were optically inspected before and after careful laser exposure and showed intact image. Raw micro-Raman spectra and their corresponding background subtracted signals are showed in the Fig. 2.

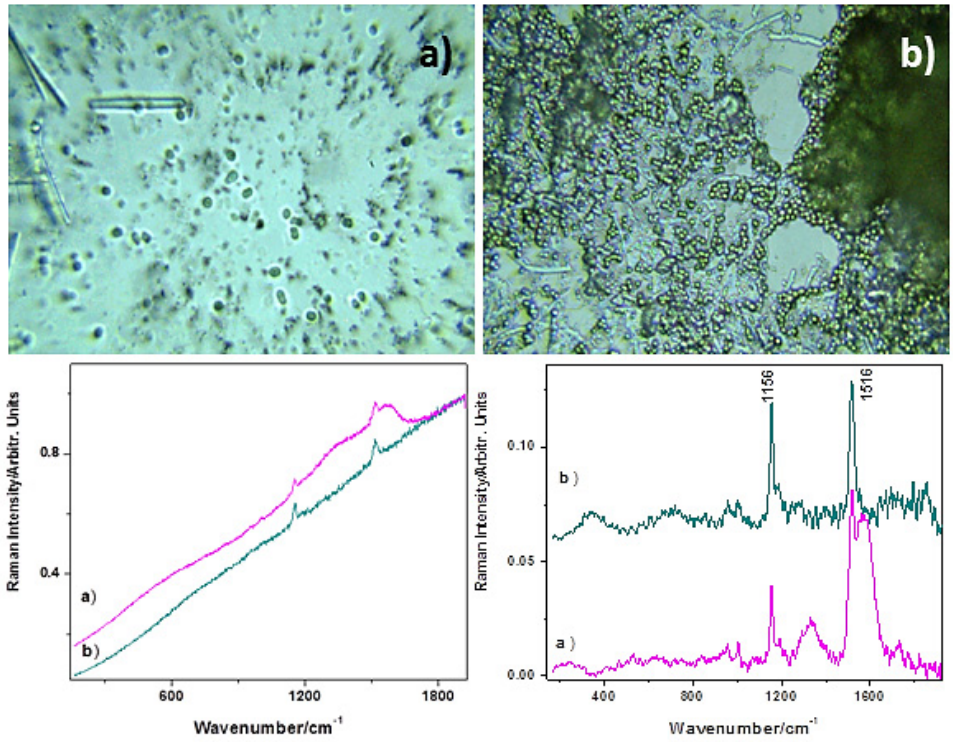

Fig. 2. Normal Raman spectra collected from the *in vivo* cyanobacteria single cells deposited on a hydrophobic slide: a) Synechocystis, b) Synnechococcus. The spectra were normalized by total integration time. Excitation: 532 nm. Laser power 1 mW. Left: raw signal; right: background subtracted. Y offset was applied for clarity. Excitation: 532 nm, 1mW. The corresponding optical microscopy images (50x objective) of the bacteria are showed in the top.

Raman spectroscopy of single cyanobacteria cell with a portable Raman system coupled with the Raman Video Microsampling System (BAC151B) allowed assessing cyanobacteria specimens from the optical field. Although their motility is reduced during water evaporation on the hydrophobic plate, during laser exposure a motility drift was discernable. However, their spectra revealed the typical bands observed at 1522 and 1158 cm^{-1} upon substantial fluorescence background.

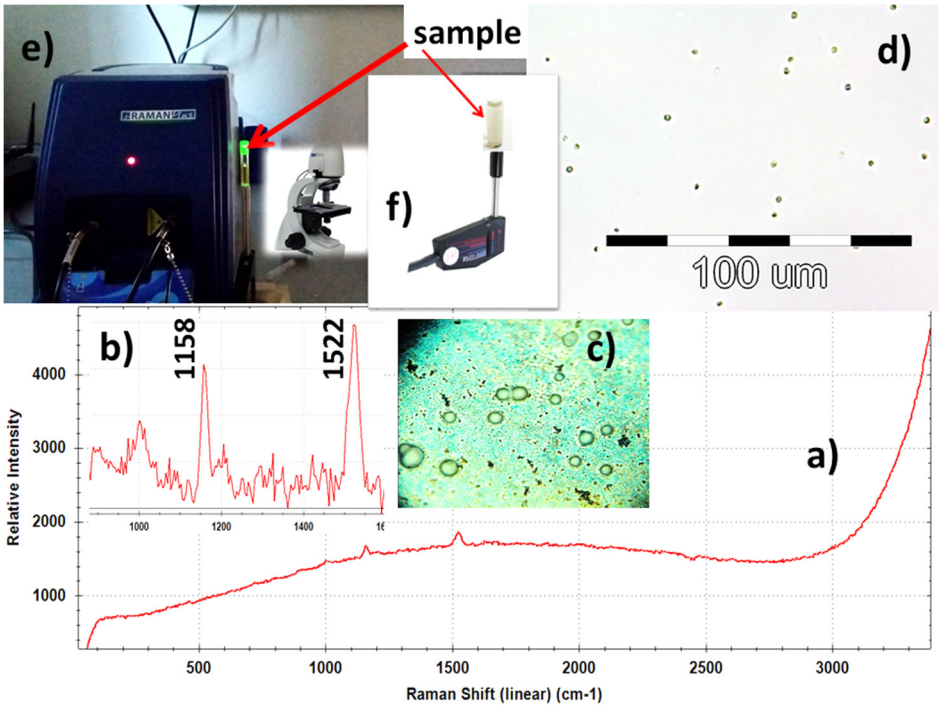

Fig. 3. Raman spectrum of single cyanobacteria (Synechocystis sp. AICB 51 strain a) collected through the Raman Video Micro-sampling System (BAC151B) with the portable instrument. On passing from bulk measurement to the single cells, the trigger was coupled to the microscope equipped with a video camera. Excitation: 532 nm, 5 s acquisition. The background subtracted signal from single cell is shown in insertion (b), highlighting the two major bands characteristic to carotenoids. Micrograph of the cyanobacteria (c) deposited on a hydrophobic slide was collected with the Raman instrument video-camera and the light microscopy image obtained with an inversed optical microscope (Leica, 100x objective) is shown in d). A picture of the instrument highlighting the fiber optic probe with a detail of the trigger and a bulk sample is shown in f) insertion.

Bulk cyanobacteria Raman spectra

Typical Raman signal of bulk cyanobacteria in 1ml glass vial collected with the portable system is shown in the Fig. 4, in comparison with the Raman spectrum of the same sample collected with the lab-based system under lateral laser focus. The spectra were normalized to the total integration time in the BWSpec acquisition software.

An increased background of the bulk cyanobacteria was observed in the case of lateral illumination through the vial glass wall, in detriment of the spectral information. In the case of portable system, the trigger facility allows to set the sample vial on its top (as shown in the Fig. 3e), resulting a longer optical path through the same sample and avoiding the signal loss due to the bacteria deposition on the bottom of the vial which results in decreasing cells concentration during measurement. Regarding the possible "defense" mechanism of live cyanobacteria to avoid too strong light energy during laser exposure, the complex photochemical processes [11] could certainly influence their Raman spectroscopy as well as their fluorescence output. These aspects will be addressed elsewhere. Technically assessing, taking the same cyanobacteria sample from a lab-based Raman characterization to a portable system, an improved signal-to-noise ratio and a substantially decreased background was observed as showed in the Fig. 4.

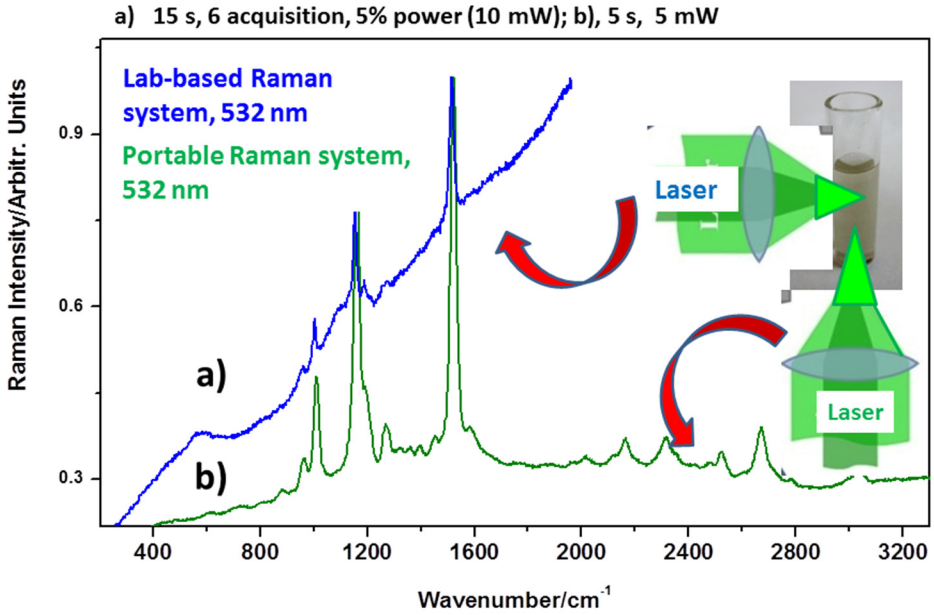

Fig. 4. Raman spectra of bulk cyanobacteria (Synechocystis sp. AICB 51 strain recorded with the lab-based Raman system, a), compared to the corresponding signal provided by the portable system b) through the bottom of a glass vial of 1 ml capacity. Excitation: 532 nm, 15 s, 6 acquisition, 10 mW (a) and 5 s, 5 mW (b).

Thus, in the high wavenumber range the overtones [8] of the carotenoids bands could be easily observed. Additional weak bands arising from other components of the bulk cells were discernable. The culture medium revealed notable band arising from the sulfate group stretching mode at 978 cm^{-1} (spectrum not shown here).

The main advantage of the portable instrument arises from the unique combination of wide spectral interval and high resolution, in the range from 65 cm^{-1} to 4000 cm^{-1}, enabling one step fast measurement both in the low and high spectral range. The detection is achieved with a high quantum efficiency CCD array. Passing from micro-sampling accessory to trigger and back is fast and user friendly, requiring several seconds trivial operation. Unlike portable system, the lab-based one requires longer time for setting the experimental acquisition, impose longer acquisition time when all the spectral range (100-3600 cm^{-1}) is envisaged, making difficult Raman measurement of light sensitive samples over the whole spectral range in one acquisition. Additionally, on passing from micro-Raman to bulk liquid measurement accessory in a lab-based system is supplementary time consuming, in detriment of the experiment flexibility when dealing with live cells or large number of samples.

The flexible, lightweight design of the portable instrument and low power consumption provide substantially improved research grade Raman spectroscopy capabilities over the existing lab-based systems. Additionally, the fiber optic probe can be used with a cuvette holder, a video microscope, an XYZ positioning stage probe holder and a BWIQ multivariate analysis software and BWID identification software. The software output provides all the spectral information like peak pixel, relative intensity, Raman shift, FWHM, in just one click, thus adding enormous help for fast analysis of large number of samples. Thus, the portable instrument capability predicts a wide range of biomedical or environmental applications that certainly are under way.

CONCLUSIONS

The results clearly suggest that cyanobacteria could be instantly Raman spectroscopy characterized *in vivo* and under the 532 nm excitation, the Raman signal is consistent with the characteristic pre-resonance modes of carotenoids. Both cyanobacteria strains exhibited similar Raman modes associated to their carotenoids content, which are known to be pre-resonantly excited when the 532 nm laser line is employed. Regarding the capability of the portable instrument to promptly achieve Raman characterization of cyanobacteria, it demonstrated better performances in obtaining high quality Raman signal than the lab-based instrument, due to its optimized

technical characteristics, prompt response, complex information providing, user friendly software for data acquisition and processing and high flexibility for sampling. These capabilities combined with Raman spectroscopy user expertise, suggest further possibility to monitor cyanobacteria cultivation and growing in specific bioreactors, in a non-destructive manner, where the growing conditions are determinant for their chemical composition and carotenoids accumulation.

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