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Utilization of Laser-Ablation Based Analytical Methods for Obtaining Complete Chemical Information of Algae

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***Abstract*—**Themain goal of this article is to find efficient methods for elemental and molecular analysis of living microorganisms (algae) under defined environmental conditions and cultivation processes. The overall knowledge of chemical composition is obtained utilizing laser-based techniques, Laser-Induced Breakdown Spectroscopy (LIBS) for acquiring information about elemental composition and Raman Spectroscopy for gaining molecular information, respectively. Algal cells were suspended in liquid media and characterized using their spectra. Results obtained employing LIBS and Raman Spectroscopy techniques will help to elucidate algae biology (nutrition dynamics depending on cultivation conditions) and to identify algal strains, which have the potential for applications in metal-ion absorption (bioremediation) and biofuel industry. Moreover, bioremediation can be readily combined with production of 3rd generation biofuels. In order to use algae for efficient fuel production, the optimal cultivation parameters have to be determinedleading to high production of oil in selected cellswithout significant inhibition of the photosynthetic activity and the culture growth rate, e.g. it is necessary to distinguish conditions for algal strain containing high amount of higher unsaturated fatty acids. Measurements employing LIBS and Raman Spectroscopy were utilized in order to give information about alga *Trachydiscusminutus* with emphasis on the amount of the lipid content inside the algal cell and the ability of algae to withdraw nutrients from its environment and bioremediation (elemental composition), respectively. This article can serve as the reference for further efforts in describing complete chemical composition of algal samples employing laser-ablation techniques.

***Keywords*—**Laser-Induced Breakdown Spectroscopy, Raman Spectroscopy, Algae, Algal strains, Bioremediation, Biofuels.

I. Introduction

WITH expanding economies and increasing global population the society looks for viable alternatives to fossil fuels. Algae may provide a solution, yet there are many aspects to be dealt concerning algal industrial production, before it reaches the point of viable competitiveness on the fuel market, e.g. high amount of oil production in algal cells, daily crop harvest, effective algal biomass to 3rd generation biofuel conversion, improvement of the economics of the entire system. Algae are the most perspective among other alternatives to fossil fuels with no requests for arable land [1]. Algae convert the solar energy to lipids, carbohydrates and proteins via photosynthesis. Typical storage form of lipids in algae is triacylglycerol: tri-esters of glycerol with saturated or unsaturated fatty acids. Algae have 200 times higher yield of oil per acre per year than the best-performing plant/vegetable oils [2]. Algae can be grown in open pond systems [3, 4] such as in bioreactors [4] with possibility of daily harvest because some algal strains are capable to double their mass several times per day [5]. Every algal strain has to be grown under optimized conditions to obtain high amount of crop harvest per day, e.g. sufficient sunlight, nutrients, protection against natural predators. Each algal strain has different properties and reacts differently on the conditions in which they grow. That is the reason why the algal strains are bioengineered. Algae bioengineering leads to improvement of specific algal strains, e.g. mainly the enhancement of the ability of algae to produce more lipids stored in the cell, theirresistance against natural predators and the ability to be invariant to different cultivation processes [1, 6].

The entire process of algal biofuel production is time-consuming and very expensive compared to fossil fuel production. There are many possibilities of optimizing the algal biofuel production in order to increase competitivenessin respect to fossil fuel production, e.g. optimizing the growth conditions, improving bioreactors or open ponds respectively, bioengineering of algal strains, selling of co-products. Algae require nutrients, light, water and CO_2 for efficient growth. The major nutrients required by most algae include phosphorus and nitrogen being the major components of agricultural fertilizers, and furthermore iron and sulfur. In order to reduce growing expenses and especially the nutrient control cost, it is possible to use not only agricultural wastewaters to grow algae [1]. The ability of algae to withdraw nutrients from its environment is called bioremediation [7, 8, 9].

The properties of algal strains differ,whichimpliesvarious utilization purposes, i.e. some algal strains can be used for biofuel production as stated above, others in food industry or nutrition. The proper understanding of algal properties can only be reached by exploiting thecomplex information of both molecular and elemental composition simultaneously. For obtaining overall chemical information two laser-based

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techniques were employed, Laser-Induced Breakdown Spectroscopy (LIBS) and Raman Spectroscopy [10, 11]. LIBS setup for measurements for algal suspensions employing liquid jet excels among others atomic emission spectroscopy (AES) techniques with no need for sample preparation, fast and simple data obtaining and processing, etc. Algal strains are measured *in-situ*, *in-vivo*, *on-line* and in real time utilizing LIBS, this technique can be used directly in the bioreactor or at the open ponds, respectively. LIBS measurements were carried out to analyze the elemental composition of individual algal strainsproviding the quantitative information concerning minor or trace elements. Elemental analysis is essential while selecting different algal strains on the basis of the best abilities for bioremediation. Calibration curves from selected elements may provide information about lipid content inside the algal cell; in other words LIBS can partially substitute Raman measurements. LIBS measurements can lead to acceleration of the quality control process during algal growth [12].

Raman spectroscopy technique is powerful tool for observing complex biological systems due to highly specific vibrational spectra, i.e. it identifies different molecules using their unique vibrational spectra [12]. As mentioned above, algae have higher lipid content and therefore can be used more effectively as the source for biofuel production. The content of the fatty acids refers to the amount of lipids within the algal cell. Raman Spectroscopy is applied for quantitative measurements of the amount of the higher unsaturated fatty acids via observing the vibrational spectra for C=C stretching mode and CH2 scissoring mode peaks respectively. Intensities of the stretching mode peak versus scissoring mode peak correspond with the amount of higher saturated, unsaturated respectively, fatty acids [14, 15].

II. Experimental Setups

Newly constructed Laser-Induced Breakdown Spectroscopy setup at Brno University of Technology for measurements of the liquid samples is employed for elemental analysis. The measurements of algal suspension were performed in custom-built glass vessel Fig. 1. We have used LIBS setup with a water jet to reduce splashing and signal quenching in suspension.

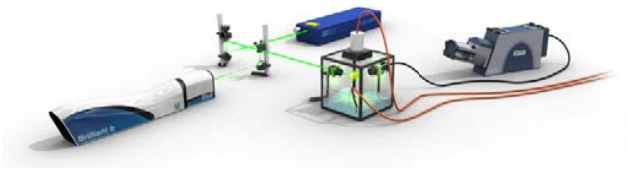

Fig. 1 LIBS system arrangement employing water jet

Continuous and relatively steady thin flow of liquid sample (algal suspension) has to be achieved for liquid LIBS measurements. We used peristaltic pump PCD 81 (Kouřil, CZ) working at 100ml/min. Liquid sample was introduced to the thin jet via silicone tubes – 0.6mm thin jet was mounted to the XY movement (ThorLabs, US) for positioning of the liquid flow.

Plasma plume was generated employing the Nd:YAG laser LQ529A (Solar, BY) operated at 532 nm with 6 ns pulse duration and 10 Hz repetition rate. Second laser pulse provided by Nd:YAG laser Brilliant B (Quantel, FR) operated at 1064 nm with 10 ns pulse duration and 10 Hz repetition rate. The energy of primary/ablation laser pulse was 12 mJ per pulse and the energy of secondary/reexciting laser pulse was 110 mJ per pulse. Laser pulses are led through the optical system (ThorLabs, US/Newport, UK) in collinear geometry, where harmonic separator (Eksma Optics, LT) with reflectance for 1064nm and transmittance for 532 nm is used. Lens with 75mm focal length focus the laser beams into the thin flow of liquid and luminous micro-plasma is created.

The LIBS plasma radiation was collected with UV-NIR achromatic collimating mirror system CC52 (Andor, UK) and transported by a fiber optic system (25 μm in diameter) onto the entrance of spectrometer ME5000 in echelle configuration (Andor, UK). As a detector an ICCD camera iStar 734i (Andor, UK) was employed.

The time-resolved studies were performed by controlling the gate width t_W (time during which the spectra are integrated), the gate delay time t_d (time at which the spectra are acquired by the detector) and the delay between the two pulses Δt. Both lasers and ICCD camera were triggered by delay generator DG535 (Stanford Research System, US) supplemented by special developed electronic switch and controlled via computer equipped with laboratory-made software. Times were optimized to obtain the best signal to noise ratio and adjusted at t_W = 16 μs, t_d = 1.5 μs and Δt = 1.5 μs.

The LIBS analysis was performed in the air at standard atmospheric pressure. Each spectrum obtained from algal suspension was accumulated from 30 shots and gain level on an ICCD camera was set on 100.

Raman measurements were observed at Institute of Scientific Instruments of the ASCR v.v.i., Academy of Sciences of the Czech Republic, employing custom-built experimental system Fig. 2.

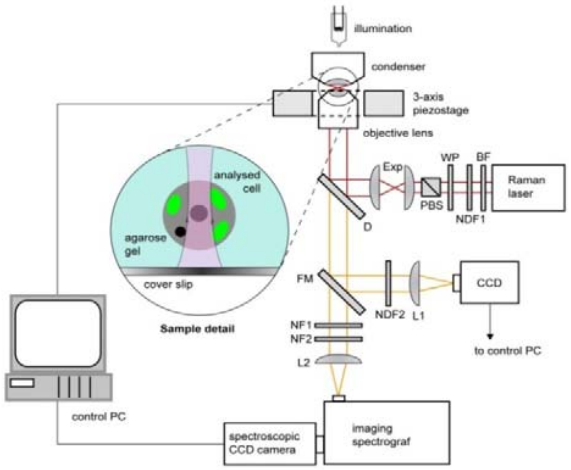

Fig. 2 Schematic diagram of the experimental set-up for Raman spectroscopy

The Raman laser beam (Ti:Sapphire, λ = 785nm, beam diameter 0.6mm; 899-01 Coherent, US) was delivered to the setup by the optical fiber. After exiting from the fiber, laser beam passed through bandpass filter (transmission bandwidth 3nm centered on 785nm; MaxLine LL01-785, Semrock, US) in order to clean up the excitation laser line. Beam diameter was further enlarged by 2x beam expander before coupling to the objective lens via dichroic mirror (LPD01-785RS, Semrock, US). Maximal laser power available for excitation was estimated to be approximately 60mW at the specimen.

The Raman excitation beam was focused on the specimen with an IR-optimized water-immersion objective lens (Olympus UPLSAPO 60x, NA 1.20). The lens was mounted on a custom-made aluminum frame that also provided a stable support for condenser and illumination light source and for 3-axis piezo-driven stage (P-517.3CL, PhysikInstrumente, D) which served for nanometer-precise positioning of the sample relative to the objective lens. In our experiments, the cells were immobilized in agarose gel placed between standard microscope coverslips. This mounting procedure allowed us to select a target cell within the specimen and focus the Raman beam on a well-defined intracellular location.

Raman scattering spectra from the target cellular compartment were collected by the objective lens and subsequently focused by lens into the entrance slit of an imaging spectrograph (focal length 300mm, f/3.9; SpectraPro 2300i, PI Acton, US). The Raman scattered light was dispersed with a 600 gr/mm diffraction grating, imaged on the chip of a high-sensitivity liquid-nitrogen-cooled spectroscopic CCD camera (Spec-10:100BR/LN, Princeton Instruments, US).

Trachydiscusminutus (Bourrelly) Ettl [16], CCALA, were obtained from the Culture Collection of Autotrophic Organisms, CCALA (Insitute of Botany, Academy of Sciences of the Czech Republic). *T. minutus* was cultivated in 50% Šetlík-Simmer medium in 100ml air-bubbled batch cultures. The irradiance during the cultivation was 400μmol(photons).$m^{-2}.s^{-1}$ and temperature 28°C. The cells were harvested in early stationary phase. For LIBS measurements, *Trachydiscusminutus* were doped with high amount of copper (50mg/L).

The technique of vital Nile Red staining was used in our study in order to visualize lipid bodies within the algal cells. Consequently, the lipid bodies were targeted by the focused laser beam and as a result one observes Raman scattering. Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) was prepared according to [17].

III. Results and Discussion

Elemental composition of the algal strain Trachydiscusminutus was obtained employing LIBS setup, the spectra on Fig. 3. For elemental analysis were selected macro elements (calcium, magnesium and potassium) and copper as a toxic heavy metal. Corresponding peaks and wavelengths are shown in the Fig. 3. From the LIBS spectra is obvious that the alga *Trachydiscusminutus* is able to readily withdraw species of heavy metals from its environment. Trace amount of copper was found in the spectra, approximately 50ppm (parts per million).

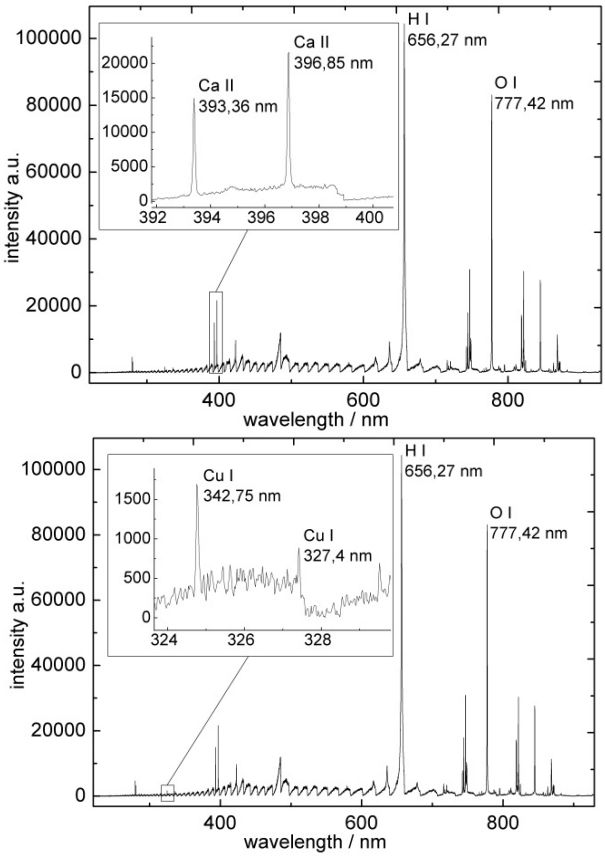

Fig. 3 LIBS spectra of selected elements (calcium, copper)

In experiments employing Raman spectroscopy setup, we focus on the ratio of unsaturated-to-saturated carbon-carbon bonds in algal lipid molecules. We employ two specific spectral peaks, shown in the Tab. 1. We found these peaks free

of any significant interference or overlaps with Raman signal of other cellular components. The average ratio of double-to-single bonds NC=C/NCH$_2$ in the specimen – specimen mass unsaturation - can be estimated. It is possible to directly convert the measured values of NC=C/NCH$_2$ to the iodine value for a given sample [18]. Iodine value refers to the determination of the amount of unsaturation contained in fatty acids. This unsaturation is in the form of double bonds which react with iodine compounds. The higher the iodine number, the more unsaturated fatty acid bonds are present in fat.

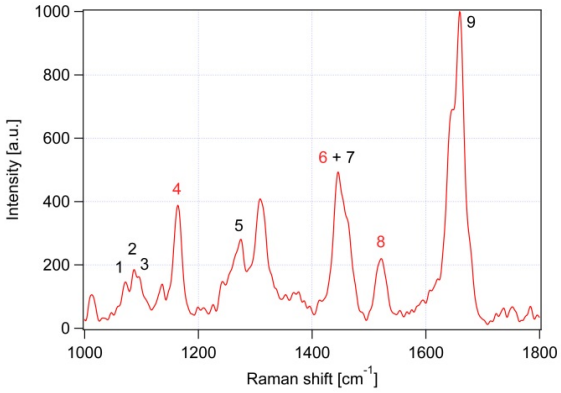

Fig. 4 Raman scattering spectrum

TABLE I
SELECTED PEAK FROM RAMAN SCATTERING SPECTRUM

Peak no.	Raman feature [cm^{-1}]	Suggested assignment
1	1060	C-C skeletal stretching vibration, out/of/plane
2	1085	C-C skeletal stretching vibration, gauche chain conformer
3	1125	C-C skeletal stretching vibration, trans chain conformer, in-plane
4	1157	β-caroten
5	1267	*cis* double bond =C-H, in-plane
6	1442	β-caroten
7	1445	CH2 bend, scissoring deformation; saturated fat indicator
8	1525	β-caroten
9	1656	*cis* C=C stretchingvibration; unsaturatedfatindicator

It is clearly visible that the *Trachydiscusminutus* strain has a significantly higher content of the unsaturated fatty acids. Algal strain with larger iodine values might not be the species of choice for the biofuel production. This Alga might excel in the food industry applications, where high amount of unsaturated fatty acids is required.

IV. Conclusion

LIBS technique showed its ability to measure low concentrations of different elements (trace amounts). Systematic measurements of various concentrations of heavy metal elements (Pb, Cu, Sr, Cd…) can be used for plotting calibration curves. Employing these calibration results the unknown amount of heavy metal should be readily stated. LIBS technique excels with its simplicity and fast measurement evaluations.

Raman spectroscopy needs further study to be established as a spectroscopic method for a rapid and massive investigation and analysis of various potentially inhomogeneous algal strains. Raman spectroscopy is suitable tool for evaluation of the amount of unsaturated fatty acids in the algal lipid body.

Laser based techniques, Laser-Induced Breakdown Spectroscopy and Raman Spectroscopy, have great potential among others to be employed in selecting different algal strains for bioremediation and biofuel production respectively.

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