Prospects of fluorescence methods application for monitoring of cyanobacterial cultures in biotechnology

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Abstract. Cyanobacteria are photosynthetic microorganisms that possess a high potential for innovative applications in agriculture, food production, cosmeticals, wastewater remediation, biofuels, antioxidative enzymes production, etc. During the industrial cultivation of cyanobacteria several parameters as growth rate, physiological state and algological purity of the culture should be controled permanently. One of the methods that can provide on-line monitoring of cyanobacterial cultures and further process automatization is a fluorescence spectroscopy. In this work several fluorescence techniques are analysed and their possible adaptation for biotechnological applications is suggested.

1 Introduction

Biotechnology is a technology that utilizes biological systems, living organisms or parts of them to produce different products. In the last few decades cyanbacteria became a valuable object of biotechnology. Cyanobacterial cultures have various applications in the modern biotechnology from food production to the treatment of wastewater, from production of various secondary metabolites like toxins, exopolysaccharides, enzymes, vitamins, and nutraceuticals to biofuel production. In agriculture cyanbacteria and their metabolites are used as fertilizers, cattle and fish feed, ect. [1-2]. Cyanobacteria have tremendous bioindustrial potential in production of UV-absorbing amalgams, solar cells, bioplastics and coating materials. [3].

In some South American and Southeast Asian countries mass cultivation of cyanobacteria already became an important part of agriculture and food industry [4-7]. During the industrial cultivation cyanobacterial cultures should be permanently checked to control their algological purity and the absence of additional toxic species. Moreover, monitoring of physiological state and the developmental stage of cultivated cyanobacteria is very desirable in such industrial fields as food and drugs production, to gain the optimal value of proteins, antioxidative enzymes, toxins or exopolysaccharides. The most convenient, cheap, fast, non-

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invasive, in vivo and ready for automation monitoring technique is a self-fluorescence analysis.

Usually, to control growth rate and species composition of cyanobacterial cultures absorbtion spectra and so called optical density (OD) are used [8-12]. Unfortunately, absorption spectrum includes the information only about the chemical structure of photosynthetic cells, thus it contain no information about developmental stage and viability of the culture. Moreover, only a rough discrimination of big classes of phytoplankton is available in this case. Opposite to the absorption spectra, the in-vivo fluorescence spectra are much more informative. Although self-fluorescence spectra contain the information only about photosynthetic apparatus of cyanobacteria, it reflects the species composition of the mixed cultures, as well as the developmental stage and viability of monocultures.

Fluorescence methods for monitoring physiological state and biological diversity of cyanobacterial cultures are well-known and widely used [13-15]. In this work we try to emphasize methods of fluorescence spectroscopy most perspective for industrial and biotechnological applications. Several examples are given to demonstrate the advantages of fluorescence methods application for monitoring of growth, physiological state and algological purity of cyanobacterial cultures during the industrial cultivation.

2 Material and methods

Cyanobacterial cultures. For this work cyanobacterial strains were obtained from CALU collection of the Core Facility Center “Centre for Culture Collection of Microorganisms” of the Research Park of St. Petersburg State University [16]. Cyanobacteria used in this investigation were grown on liquid medium № 11. Experimenal culture was prepared from a preliminarily cultivated stock culture by one-week incubating in 30 mL of medium at room temperature and under continuous illumination from fluorescent lamps (2000 lux).

Fluorescent spectroscopy. An intrinsic self-fluorescence spectra of cyanobacteria were recorded with Cary Eclipse (Varian Cary) scanning fluorimeter. The measurements were carried out at room temperature. The living-cell suspension was placed in a standard 10-mm-long quartz cuvette. Fluorescence spectra were excited at 440, 488, and 625 nm, which corresponds to maximum absorbtion of the main photosynthetic pigments (440 and 625 nm) and to one intermediate excitation wavelength. Spectra were recorded from 580 to 800 nm. The bandwidth of the excitation and emission monochromators was 5 nm. During preprocessing anti-aliasing filter was used to obtain the final curves.

Confocal Laser Scanning Microscopy (CLSM). For spectroscopic studies of individual cyanobacterial cells Leica TCS-SP5 confocal laser-scanning microscope, equipped with three lasers: diode laser (405 nm), argon laser (458, 476, 488, 496, and 514 nm), He-Ne laser (543, 633 nm) was used. Fluorescence spectra in the range of 520-785 nm were recorded using a standard procedure of Lambda-scanning presented in ‘Leica Confocal Software’. A series of spatial distributions of fluorescence intensity, with medium quality 512×512 pixels, were recorded at a bandwidth of 6 nm and with step 6 nm. An immersion lens with an aperture 1.3 (HCX PL APO 63.0×1.30 GLYC 37°C UV lens) and with glycerol immersion (glycerol 80% H_2O) was used. The size of a single pixel of the image corresponds to 53.5 × 53.5 nm. During the fluorescence spectra processing, graphical averaging was used for the so-called “region of interest” (ROI), covering a single cell [17-18].

3 Results and discussion

Monitoring of developmental stage and physiological state of cyanobacterial cultures during incubation is a primary task in biotechnological applications, which deal with biomass ptoduction of cyanobacteria. Usually this process is reduced to an optical density measurenments and determining the total volume of chlorophyll in dry weight. With such approach, actual physiological state and viability of cyanobacterial culture can not be detected, because measured parameters include both living and dead cells. In this case only fluorescence spectromety can give adequate result. The intensity and spectral composition of fluorescence, emitted by cyanobacterial cells in vivo, depends on the operational effectiveness of photosynthetic apparatus, reflecting the in-time physiological state of the culture.

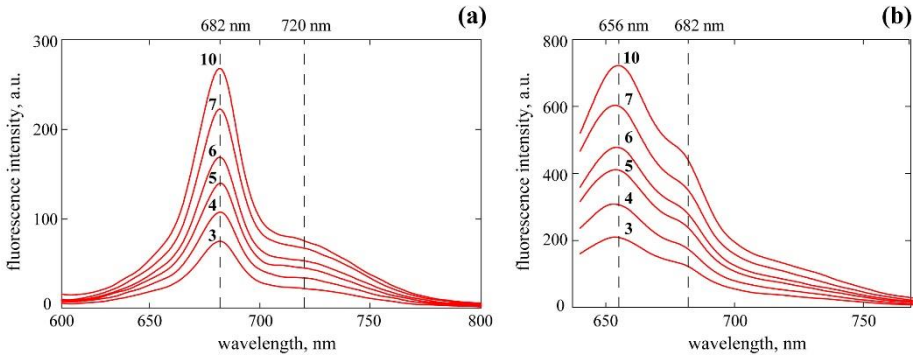

Fig. 1. Growth rate and main photosynthetic pigments monitoring via self-fluorescence spectra for *Microcystis aeruginosa Kuetz. CALU 972*. (a) – chlorophyll a fluorescence, excitation wavelength 440 nm, (b) – phycocyanin fluorescence, excitation wavelength 625 nm. Figures near curves denote the number of incubation days. Vertical lines indicate wavelengths for fluorescence maximum of corresponding photopigment.

To monitor the groth rate and developmental stage of incubated cyanobacterial culture standard fluorimeter can be used. However, fluorescence spectra should be recorded at several excitation wavelengths corresponding to the main photosynthetic pigments inherent by particular cyanobacterial species. For example, presented in Fig.1 several fluorescence spectra were recorded during growth of cyanobacterial culture *Microcystis aeruginosa Kuetz. CALU 972* at 3, 4, 5, 6, 7, 10 day. The photosynthetic apparatus of this cyanobacterial species contain only two main photopigments: phycocyanin (and allophycocyanin, the excitation and emission wavelengths of which cannot be clearly separated from phycocyanin ones at room temperature) and chlorophyll a. Thus for monitoring growth rate and developmental stage of such culture only two excitation wavelengths are enough (625 and 440 nm, respectively). Moreover, for the purpose of in-line monitoring only several ranges in emission spectra are interesting. For chlorophyll a fluorescence (Fig.1 (a)) the ranges of interest are near 682 and 715 nm. These are the wavelength of maximal fluorescence of chlorophyll a in photosystem II (PSII) and photosystem I (PSI), correspondingly. And for phycocyanin emission spectra (Fig.1 (b)) only one wavelength range near 656 nm is of interest. These three values give the information about growth and developmental stage of cyanobacterial culture under consideration. For the first one, maximal fluorescence of chlorophyll a PSII at 682 nm should be detected. And for the last one the ratio of main photosynthetic pigments phycocyanin to chlorophyll a PSII and chlorophyll a PSII to chlorophyll a PSI should be recorded.

To control the variations in physiological state of cultivated cyanobacteria, the changes in the ratio of main photosynthetic pigments, namely phycocyanin to chlorophyll a PSII

(PC/Chl a), and the rate of fluorescence intensity should be monitored. In Fig. 2 two examples of changes in self-fluorescence spectra for cyanobacterial culture *Synechocystis aquatilis CALU 1336* under weak external actions are given. The first example (Fig 2 (a)) corresponds to the culture irradiated by ultrasound with frequency of 60 kHz and power of 5.85 mWt/mL (within the experimental volume) during three days. This physical treatment causes the changes in both fluorescence intensity of main photopigments and PC/Chl a ratio. These changes are well seen in the main panel of Fig. 2(a). In the insert the difference in self-fluorescence spectra of controlled and treated culture at 488 nm is presented. Such commonly used intermediate wavelength reflects the total effect of the external action on the photosynthetic system of cyanobacteria. More detailed description of this effect see in [14-15].

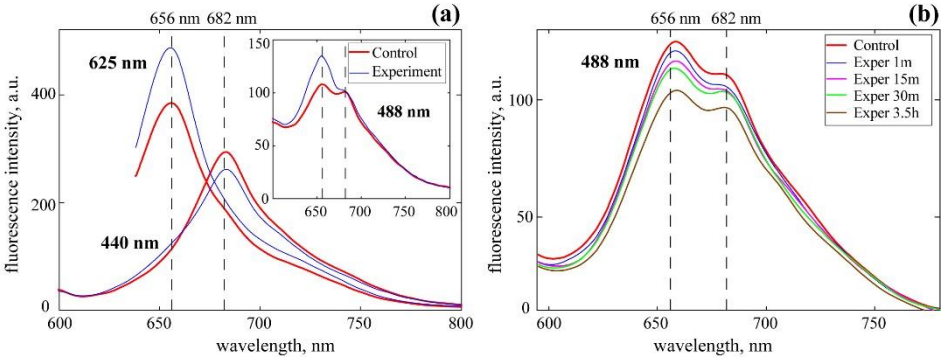

Fig. 2. Changes in fluorescence intensity and spectral composition at physical (a) and chemical (b) treatment of *Synechocystis aquatilis CALU 1336*. (a) – three-days ultrasound treatment of cyanobacterial culture. Excitation wavelengths are shown next to the curves. Inset shows the effect at excitation wavelength 488 nm. (b) – fast dynamics of fluorescence changes during 2 mg/l copper sulfate pentahydrate treatment (CuSO4 x 5H2O). Excitation wavelength 488 nm. Spectra were recorded at 1, 15, 30 and 210 min (3.5 hours). Vertical lines indicate wavelengths for fluorescence maximum of phycocyanin (656 nm) and chlorophyll a (682 nm).

In Fig. 2(b) the results of chemical treatment of *Synechocystis aquatilis CALU 1336* are presented. In this case the culture was affected by 2 mg/l copper sulfate pentahydrate ($CuSO_4$ x $5H_2O$). The presented in Fig. 2(b) results corresponds to the control culture and to the culture after 1, 15, 30 and 210 min (3.5 hours) of the treatment. Spectra were recorded at the excitation wavelength 488 nm. Fast inhibition of self-fluorescence of both photopigments is caused by the direct arrest of the electron transport in phycobilisome and reaction center of PSII by copper ions [19-20]. Again we see that only several regions of the fluorescence spectra are informative, i.e. wavelength ranges near 656 and 682 nm. Thus for biotechnological applications the on-line monitoring of only these spectral ranges is really needed.

Monitoring of algological purity of the industrially cultivated cyanobacterial cultures also can be automated by means of fluorescence spectroscopy. It is well-known that different phytoplankton species possess a wide variety of photosynthetic pigment composition, and thus possess different fluorescence properties. Comparative analysis of the series of single-cell fluorescence spectra recorded via confocal laser scanning microscope (CLSM) for cyanobacterial species reveals visible variations in their shape (Fig.3). In the investigation, presented in [13], it was shown, that if the single-cell fluorescence spectra were taken from alive cyanobacterial cells in normal physiological state, which are cultured in the same growth conditions, then the interspecies variations in pigment/chl a ratios are more pronounced than variations within the individual species. Moreover, in some works it was shown, that even nutrient and light limitations (i.e. chromatic adaptation) do not significantly

change the initial fluorescence spectra of cyanobacteria and cannot impede the species discriminaton [21].

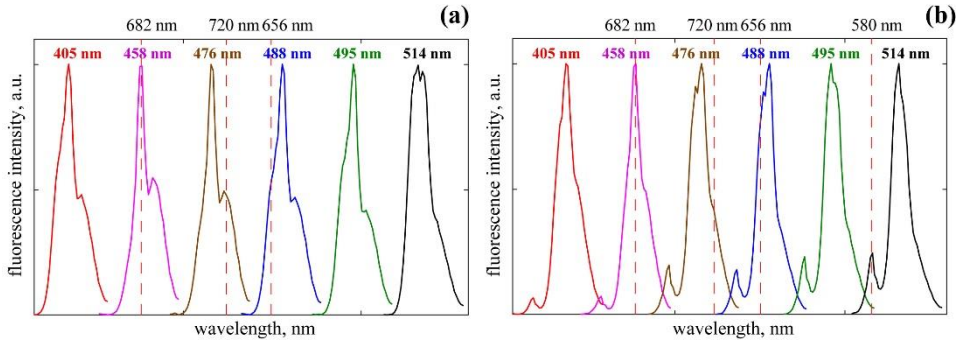

Fig. 3. Two characteristic sets of single-cell fluorescence spectra for *Spirulina platensis CALU 550* and *Merismopedia punctata CALU 666*. The excitation wavelengths (405, 458, 476, 488, 496, 514 nm) are given over the curves. All spectra are normalized to the maximum intensity and shifted along x-axis for convenience of observation. The dashed lines indicate fluorescence maxima of the individual pigments (PE – 580 nm; PC – 656 nm; Chl a – 682, 715 nm).

The possibility of complete (up to 97%) species discriminaton based on single-cell self-fluorescence spectra was demonstrated in [13]. During monitoring of algological purity much more simple problem should be solved. Only the deviations from the fluorescence spectra shape should be detected. For this purpose, it is enough to select only a few characteristic excitation wavelengths and periodically compare the fluorescence spectra of controlled culture with the reference spectra of an algologically pure culture and register any changes in the fluorescence intensity of main photosynthetic pigments.

4 Conclusion

In this work a novel technique for monitoring of industrially cultivated cyanobacteria, based on a strict relation of the intensity and shape of intrinsic self-fluorescence spectra with the physiological state and genera affiliation of the culture, is presented. This technique allows to register weak variations in physiological state and species composition of cyanobacterial cultures and can become very promising for monitoring of biomass cultivation process in biotechnological applications.

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