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Isolation of a heterocyts - forming Cyanobacterium and quantification of its biotechnological potential with respect to redox properties at single cell level

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Abstract

This paper describes the isolation of an oxygenic heterocyst- forming cyanobacterium (IS-H) from meso-thermal samples (Obanul Mare - Mangalia), and quantitative results concerning its biotechnological potential, namely the ability of individual cell within the filament to reduce extracellular added artificial electron acceptor. Special emphasis is focused on quantitative determinations by automated image analysis of the ability of each individual cell to reduce MTT, an artificial electron acceptor. Our results show a strong decrease (almost 4 times in 24 hours) in the blue signal during MTT reductions by each individual analyzed cell, as a consequence of orange light absorption by reduced MTT. Up to our best knowledge this is the first report on the use of automated image analysis for the measurement of reduction of artificial electron acceptors at single cell level in cyanobacteria. This paper argues for the importance of mathematical methods of signal and image processing, a methodology capable of precise, detailed, tireless, and objective analysis of color intensity measurement of each individual cell.

Keywords: filamentous cyanobacterium, artificial redox acceptors, automated digital image analysis, bio-fuel cells.

Introduction

Cyanobacteria are the most largest and most diversified, ecologically most successful and evolutionary most important group of prokaryotes (Peschek & al. [23]), clearly defined by the ability to carry out both oxygenic photosynthesis within the thylakoid membranes and respiration within plasma (cell) membranes (CM) and thylakoid membranes (ICM) as well. The ability of (cyano)bacteria to reduce artificial electron acceptors is important for their use as convertors of light energy in electricity or as biosensors (Ardelean & al. [5] [4]; Ardelean and Zarnea [6]; Margineanu & al. [18]; Ardelean [3]; Lovley [17]; Nishio & al. [20]; Ardelean and Peschek [1]) and still an important tool in studying photosynthetic and respiratory electron transport in these photosynthetic prokaryotes (Nicholls [19]). Microbiology opens the view to understand more and more microbial processes as they occur at the level of each individual cell (Brehm - Stecher and Johnson [9]; Gross & al. [11]). One way to study bacteria at single cell level is automated image analysis of classical images of individual bacterial cells as they are obtained using different types of microscopes. Automated image analysis is very useful in microbiology to quantify important parameters such as cell numbers, cell volumes, frequencies of dividing cells, *in situ* classification of bacteria, enumerate actively respiring bacteria, characterization of bacterial growth on solid medium, viability and physiological activity in biofilms (e.g. Ploem & al. [25]; Pettipher and Rodrigues [24]; Bloem & al. [8], Pernthaler & al. [22], Van Wambeke [32]; Shopov & al. [31]; Ogawa & al. [21]; Belyaev & al. [7]; Heydorn & al. [15]; Yang & al. [34]; Lehmussola & al. [16]; Chavez de Paz [10]; Edelstein & al. [12]). The advent of digital imaging and automated microscope systems has transformed the image based analyses from tedious manual work into high-throughput science, automated by the algorithms of digital image analysis. Traditionally,

images have been taken by an analogue camera, by opening a shutter and letting photons on film, where chemical compounds react to the incoming light and change intensity, forming an image. Today, the film has been replaced with a digital photosensitive sensor, resulting in nearly all scientific imaging to be performed digitally. The images obtained from microscope offer any help in scientific studies, but it is the *image analysis*, the methods producing qualitative of quantitative data from the images, how useful information is obtained (Selinummi & al. [30]). Digital image processing is very useful in microbiological studies and it is difficult to see any other methodology capable of detailed, tireless, and objective analysis of large image databases showing up in many fields of science. The aim of this paper is to investigate the ability of a cyanobacterial strain, our isolate IS-H, to reduce MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)], an artificial electron acceptor, with special emphasis on quantitative determinations at single cell level using automated image analysis for precise color measurement of cells within the filaments of this strain. Up to our best knowledge this is the first report on the use of automated image analysis for the measurement of reduction of artificial redox carriers at single cell level in cyanobacteria.

Materials and methods

Strain. Samples were collected in sterile bottles from sulfurous mesothermal spring Obanul Mare, placed in north of Mangalia City (43˚49'53.6''N; 28˚34'05.3''E) (Romania) (Ardelean & al. [2]; Sarchizian and Ardelean [28]), in order to isolate and purify unicellular and filamentous cyanobacteria by inoculation into either BG_{11} medium or nitrate - free BG_{11} medium (BG_0), either solid of liquid (Rippka & al. [26]). Samples were incubated in culture room at 25 ± 1ºC and illuminated with fluorescent tubes having the photon rate of 50 μmol m^{-2} s^{-1} at surface of the culture vessels. From these samples several strains have been isolated and purified, one being the filamentous cyanobacterium able to produce heterocysts when cultivated in the absence of nitrate, on BG_0 medium, preliminary named IS-H, which is used in the studies reported here. Cultivation was done in the same conditions as those used for isolation.

For microscopic assays, an MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide)] stock solution (5 mg of MTT/mL of distilled water) was filter and kept for no more than 2 weeks at 4°C. To start the reaction, stock solution was added to growing cultures (final concentration, 0.5 mg/mL). The mixture was incubated for 24 h in light at 28°C, without agitation.

Digital image analysis. As manual image analysis suffers from numerous drawbacks, being always subjective (Webb, 2003), in our study we used automated image analysis for obtaining quantitative data from changing colors of cyanobacterial cell during MTT. The Image J software used in this study allowed us to obtain color histogram and the correct values of mean of pixel in RGB images with cyanobacterial filaments taken with a 10 Megapixel Nikon Coolpix S220 10MP Digital Camera with 3x Optical Zoom and 2.5 inch LCD. Image J package is constantly improved by the very active user community, ranging from small user interface improvements all the way to fully featured tools for controlling automated microscopy equipment (Edelstein et al., 2010).

Results and discussion

As one can see in figure 1, MTT is reduced by suspensions of culture of cyanobacteria IS-H when incubated in light.

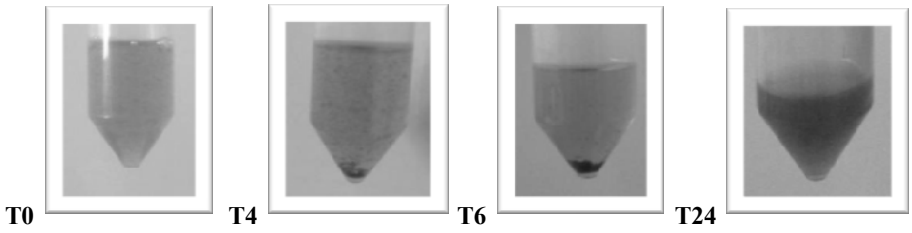


Fig.1. Macroscopic view of MTT reduction by suspensions of IS-H incubated in light for different periods of time (T0- initial time; T4- 4 hours of incubation; T6- hours of incubation and T24- 24 hours of incubation).

The increase in MTT reduction was followed by bright filed microscopy. In figure 2 one can see the microscopic images showing the color change of cyanobacterial culture due to MTT reduction, as they look in these black/white images.

The whole process from image analysis to result validation and data storage requires careful planning, and especially knowledge on both the biological samples as well as computer science to select the right tools. Successful automated image analysis starts by describing the analysis task precisely; image analysis software only does what it is programmed to do. It does not make any predictions, nor does it have any knowledge of the context of the study. Therefore, all types of samples and errors have to be taken care of beforehand in order to get reliable results: in automated image analysis unpredictable events often lead to unpredictable results. If properties of the sample change during the analysis, for example by unintentionally changing the microscope lighting settings, the results of automated analysis will most likely fail, although some automated error detection logic might detect the anomaly and discard the results. These limitations must be thoroughly understood before utilizing automation, requiring biologists to have basic understanding of image analysis methodology. Vice versa, computer scientists implementing the image analysis methods need experience in cell biology in order to develop software packages that truly benefits biologists, not just relying on novel technological advances without practical use. Shortly, successful automation requires engineers and biologists to work closely together, starting from interdisciplinary study programs (Selinummi personal communication).

Microphotographs obtained in bright field microscopy were used to determine the color of cyanobacterial cells from filaments. All images used in this study is JPG digital images and we automatically obtained with ImageJ software the mean of pixel of each 10 cells from cyanobacterial filaments in T0, T2, T6, T24 after MTT addition and light cultivation on 30ºC (T0 is the moment of MTT addition in BG_0 cyanobacterial culture; T2 – after 2 hours of cultivation on light with MTT; T6 – after 6 hours; T24 – after 24 hours).

The automated image analysis procedure is divided in three parts (Gonzale and Woods [14]), namely preprocessing, segmentation, and measurements. Preprocessing aims at improving and normalizing the image quality to enable accurate analysis in the subsequent steps. Using *digital filtering*, preprocessing step suppresses noise, we optimized contrast of images, and filters out different artifacts such as staining residue. One common defect in images is uneven background, especially in image corners. One approach in reducing the effect is to mathematically fit a 2-D surface to the image pixels, and subtract it from the original image (Russ [27]). All digital images used for automated analysis was done in the same condition without uneven background or other residue.

ImageJ allowed us to calculate and displays histograms of the distribution of gray values in the active image or in ROI's (in this study, region of interests represents the analyzed cells from cyanobacterial filament). According to user guide of Image J software, the X-axis represents the possible gray values and the Y-axis shows the number of pixels

found for each gray value. The horizontal LUT bar below the X-axis is scaled to reflect the display range of the image. The total pixel *Count* is also calculated and displayed, as well as the *Mean*, standard deviation (*StdDev*), minimum (*Min*), maximum (*Max*) and modal (*Mode*) gray value. Using the *List* or *Copy* buttons of the ImageJ software we save the histogram data, and clicking on *Log* we can display a log-scaled version of the histogram (overlaid in gray). In this report we used only the mean of pixel to compare in time the color of cyanobacterial cells treated with MTT.

Value / *Count* pairs (i.e., grayscale value corresponding to the X-axis cursor position / the number of pixels that have that intensity) are displayed on the bottom right while mousing over the histogram window. With RGB (color) images, the histogram is calculated by converting each pixel to grayscale using the formula gray = (red + green + blue)/3 or gray = 0.299 × red + 0.587 × green + 0.114 × blue if *Weighted RGB Conversions* is checked in Edit, Options, Conversion (user guide Image J software).

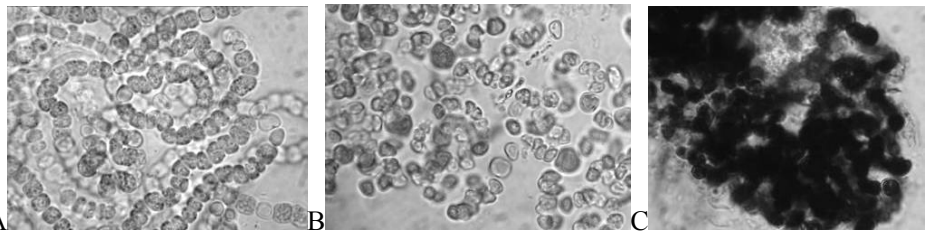
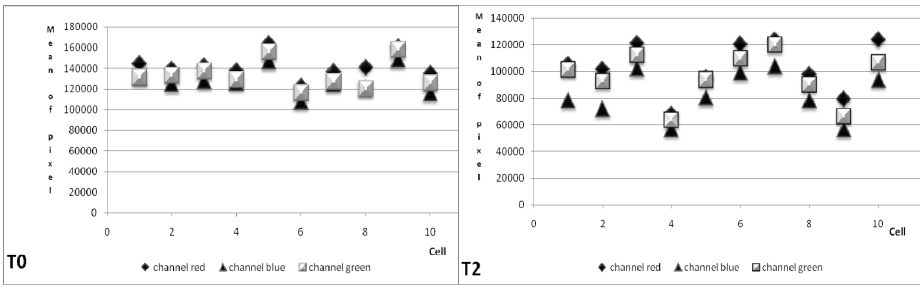


Fig 2. A- Microphotographs of filamentous cyanobacterium IS-H isolated on BG_0 medium; A) IS- H, cyanobacterial culture without any added MTT; B – 1 hour of incubation; C – 24 hours of incubation.

These types of images were further used to measure reduction of MTT occurring at single cell level by automated color image analysis taking into account the change in color due to formation of purple formazan (reduced, insoluble MTT).

The bright field channel, although readily available in all microscopes, is often neglected in cell population studies. Firstly, the cells are often nearly transparent, making the contrast very poor. Even by manual visual cell analysis it is often impossible to reliably detect the locations of cell borders, especially if the cells are clumped together. Recently, however, a number of studies have been published showing the usefulness of the bright field channel in cell detection and automated image analysis of cell populations (Selinummi & al. [30]). We observed that all images taken in bright field microscopy can be analyzed with Image J software and can detect any region of interests (ROI's) and after that measure the mean of pixel for each cells from cyanobacterial filament. This steps used in our study allowed to determine the mean of pixel in channel red, green and blue (RBG) for analyzes cells and also to study the different aspect of MTT reduction in cyanobacterial filaments in time.

In figure 3 there are presented the results of automated color image analysis in time, for 10 cells belonging to a cyanobacterial filament during reduction of MTT, and automated results of mean of pixel in RGB channels.



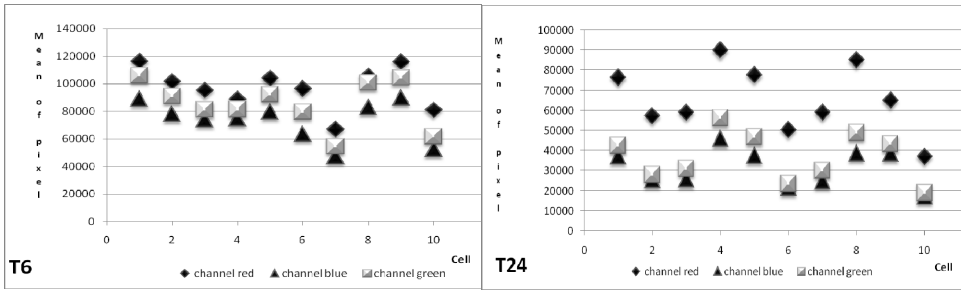


Fig 3. Automated color image analysis in time (T0, T2, T6, T24) for 10 cells belonging to a cyanobacterial filament treated with MTT, and automated results of mean of pixel in RGB channels.

As one can see in the figure 3, there is a decrease in time with respect to the overall intensity of light passing through each individual cell (the scale are decreasing from 20,000 pixels at time zero to 10,00 pixels after 24 hours of incubation with MTT), suggesting that each analyzed cell absorbs and/or reflect more incident light, thus less light is available to pass through each cell. As reduced MTT is colored (purple) and insoluble both processes of (increased) light absorption by the colored compound and (increased) light diffusion by the crystals of reduced MTT should be taken into account for the decreased light passing through each individual cyanobacterial cell. In this paper we only focus on the decreased light passing through each individual cell as the results of MTT reduction, and the possibility to measure this light by RGB automated image analysis. It is also evident that there are differences between each analyzed cells with respect to Red, Blue and Green light passing through cells, differences which appears to increase from the beginning of incubation toward its end (24 hours). To better understand the evolution of each analyzed color (red, blue and green) during the incubation time, in the figure 4 there are presented the results with respect to the arithmetic mean of each 10 analyzed cells.

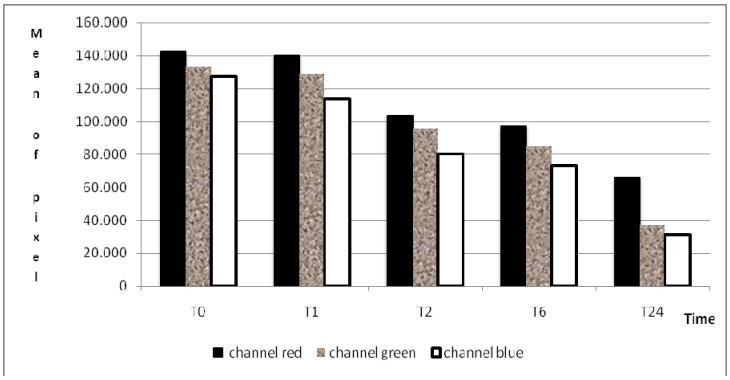


Fig.4. The evolution of pixel's arithmetic mean in RGB channels during the incubation time of cyanobacterial suspension in the presence of MTT (0.5mg/mL)

The dramatic decrease in blue channel could logically be attributed to the absorption of the complementary color, orange, by the reduced, purple, MTT; the same for the decrease in red channel as a consequence of absorption of green light by the reduced MTT. When it comes to the decrease in the green channel, its signification is under investigation being probably related to the occurrence of multiple light processes (absorption, reflection, transmission) whose interaction with different (colored) cell components, and processes, is not yet understood.

Conclusions

This study shows the importance of mathematical methods of signal and image processing for research at single cell level. Our results show a strong decrease in the blue signal during MTT reductions by each individual analyzed cell, as a consequence of orange light absorption by reduced MTT. Up to our best knowledge this is the first report on the use of automated image analysis for the measurement of reduction of artificial electron carriers at single cell level in cyanobacteria.

These results are important for fundamental research within the increasing field of single cell microbiology and for biotechnological research, linking the redox properties of cyanobacteria with their use as convertors of light energy in electricity, as biosensors or as cell factories for nanoparticles synthesis (Focşan & al. [13]). This article is only a beginning for image analysis at single cell level in filamentous cyanobacteria. One further task is to compare the quantitative results at single cell level obtained by automated image analysis with those obtained by (classically) measuring dehydrogenase activity with a spectrophotometer at populations level (bulk activity). Preliminary experiments sustain that quantification (using a spectrophotometer) of MTT reduction or 2, 6 dichlorphenol indophenol reduction (as it is, or in the presence of lipophilic electron carriers, phenazine methosulphat or 2, 6 dichloro benzoquinone) parallels the reduction of MTT at single cell level, measured as numerical decrease in the blue channel (results not shown). Further experiments are needed to measure dehydrogenase activity at population and at single cell level, and to compare the results obtained by different methodologies, using different artificial electron acceptors, both in light and in darkness.

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