

ADDRESSING ENVIRONMENTAL CHALLENGES THROUGH MICROBIAL FUEL CELLS

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In the pursuit of sustainable energy solutions, the development of alternative energy sources that minimize environmental impact remains a major challenge in mitigating climate change. Achieving electricity generation with zero emissions is regarded as an essential strategy for lowering greenhouse gas outputs and promoting environmental sustainability [5]. However, since practical obstacles persist, the implementation of innovative technologies is crucial to counteracting the growing effects of lowering greenhouse gas emissions and advancing toward a safer, more sustainable environment. In this regard, microbial fuel cells as a type of bioelectrochemical systems appear as the best option for solving these urgent issues.

A microbial fuel cell (MFC) is an innovative bioelectrochemical device that combines the principles of an electrochemical system and a biological reactor. Generally, it comprises two electrodes separated by an ion-exchange membrane. The electrodes function as the electron-releasing anode and the electron-accepting cathode, divided by a separator such as a membrane or salt bridge [2]. Each chamber has an electrode that not only mediates electron transport but also supports microbial growth and activity. The anode compartment is supplied with organic compounds, which act as the primary electron donors in the system. Microorganisms colonize the anode surface, forming a dense biofilm. The release of electrons from microbial cells is primarily driven by the redox potential gradient between the anode and cathode compartments [6]. Through metabolic oxidation of the organic matter, bacteria generate carbon dioxide, protons, and electrons [3]. Unlike conventional fuel-based energy systems, MFCs operate under mild conditions and produce only environmentally benign by-products such as carbon dioxide, water, and recoverable heat, making them a sustainable and eco-compatible alternative for energy generation [1].

The ultimate goal of this study was the investigation of electrogenic properties of certain *Pseudomonas* strains and possibility to create the MFC system on their basis.

In this work, the following strains of microorganisms were used: *P. aeruginosa* ATCC 27853, ATCC 15692, *P. chlororaphis* ONU 111 and *P. fluorescens* ONU 110, which are the part of the collection of microorganisms of the Department of Microbiology, Virology and Biotechnology of Odesa I. I. Mechnikov National University. Cultivation of microorganisms during the main experiment was carried out for 48 hrs in sterile polystyrene plates in King's medium, without the agar-agar supplementation. Samples were taken after 4, 24 and 48 hrs from the cultivation beginning for the relevant studies.

DREAM assay (dye reduction-based electron-transfer activity monitoring) using methylene blue was applied to determine the redox potential of microorganisms [11]. At the appropriate times, samples of the supernatant were taken, to which methylene blue was added. Recovery of methylene blue was measured at a wavelength of 660 nm

using a μ Quant spectrophotometer (BioTek Instruments, USA). The control consisted of a sample of the nutrient medium, based on the absence of the studied microorganisms in it, which were subjected to analysis. The redox potential of the studied microorganisms was quantified using the DREAM coefficient, calculated as the difference between the control and experimental absorbance values at 660 nm at the time of the analysis. The coefficient of the DREAM analysis indicates the level of microbial electron transfer activity in the sample. Student's t-test was used in the comparative analysis of measurement results. The difference was considered significant at $p \leq 0.05$ [7].

The method of biofilm detection was used in the experiment based on the crystal violet dye application [8]. Derived from the obtained optical density values, the coefficient of biofilm formation was calculated, which was equal to the ratio of the optical density of the sample to the optical density of the control. Nutrient medium in the absence of the studied microorganisms was used as a control.

Microscopy of the studied biofilms was carried out using a Zeiss Primo Star light microscope (Germany), with a magnification of x1000. Biofilm images were collected at seven randomly selected locations on the glass. Photographs were taken using a Canon EOS 750D camera (Japan) and Zen 2 software (ZEISS Efficient Navigation), which is a modular software for collecting, processing and analyzing images for digital microscopy.

Selected microorganisms were tested for the possibility of producing electrons, which in turn actively interact with organic substances, in particular methylene blue [9]. The obtained DREAM coefficient values indicate that microorganisms can generate electrons, thus reducing methylene blue to the colorless leuco-form.

The obtained data indicate that the studied microorganisms are capable of producing compounds that participate in redox reactions (Table 1).

Table 1 – Redox ability of the studied microorganisms

Strain	DREAM coefficient (hrs)		
	4	24	48
<i>P. aeruginosa</i> ATCC 27853	0,310	1,607	1,791
<i>P. aeruginosa</i> ATCC 15692	0,378	1,561	1,741
<i>P. aeruginosa</i> ATCC 10145	0,300	0,856	0,954
<i>P. chlororaphis</i> ONU 111	0,065	0,506	0,565
<i>P. fluorescens</i> ONU 110	0,082	0,310	0,346

P. aeruginosa strains ATCC 27853, ATCC 15692 and ATCC 10145 were characterized by an almost 4.6-5.8 times higher DREAM coefficient compared to *P. chlororaphis* ONU 111 and *P. fluorescens* ONU 110. Over time, both 24 and 48 hrs, the level of redox activity of microorganisms increased, but the significant difference between the two groups of pseudomonads was also preserved during these periods.

For successful bioelectrochemical system creation a culture of microorganism with attachment ability to the surface, in particular anode, colonization is needed.

During this process such bacteria could create biofilms on the solid anode surface and at the same time produce redox-active compounds (electrogenic shuttles) [6].

At the same time, behind the electrogenic properties, the intensity of biofilm formation was distinguished (Table 2).

Table 2 – Characteristics of biofilm formation by the studied microorganisms

Strain	Biofilm formation coefficient (hrs)		
	4	24	48
<i>P. aeruginosa</i> ATCC 27853	1,15	3,57	4,21
<i>P. aeruginosa</i> ATCC 15692	1,00	5,16	5,87
<i>P. aeruginosa</i> ATCC 10145	1,21	4,54	5,22
<i>P. chlororaphis</i> ONU 111	1,09	1,68	2,40
<i>P. fluorescens</i> ONU 110	1,14	1,98	2,61

In general, according to Stepanovic et al. the studied microorganisms were characterized by either a moderate intensity of this process or the formation of a high-density biofilm [10]. As a result, *P. aeruginosa* strains were characterized by more intensive formation of associations on the dense surface of the wells of the polystyrene plate. This is proved by higher biofilm formation coefficient values, especially starting from 24 hrs after the start of cultivation. Thus, *P. aeruginosa* ATCC 27853 and *P. aeruginosa* ATCC 10145 had biofilm formation coefficient in the range from 4,5 to 5,2. Other representatives of pseudomonads also formed biofilms, but at a lower rate. Hence, the increase in biofilm formation coefficient over the studied period of time was from 30% to 80%, while it was smaller within 24-48 hrs.

Not only the intensity, but also the structure of biofilm plays an important role in such systems. Continuous layer of mature biofilm is characterized by stable gradients of electrogenic compounds [4]. In this study the structure changes of biofilm formation *in vitro* of *Pseudomonas* strains were detected (Fig.1, only result for *P. aeruginosa* ATCC 27853 is demonstrated).

During initial stage of biofilm formation to solid surface, adhesion of individual cells is detected. Such characteristics like microbial surface charge and flagella presence contribute to the following process. Thus, microorganisms' selection becomes a crucial stage in the creation of MFC's system [4].

In the current work *P. aeruginosa* ATCC 27853 started the adhesion in 4 hrs after cultivation beginning. Separate individual cells which attached to the solid wells' surface could be seen on the received photos. At this period no microcolonies are formed. Obviously, this depends on the period of microbial adaptation to the nutrient medium.

On the contrary, on 24 hrs *P. aeruginosa* ATCC 27853 cells replicated on the solid plate surface, eventually forming microcolonies: intensive violet color area on Fig.1 Zen 2 software processed photos. The growth of separate microcolonies indicates the immature continuous layer: white color site between cells association.

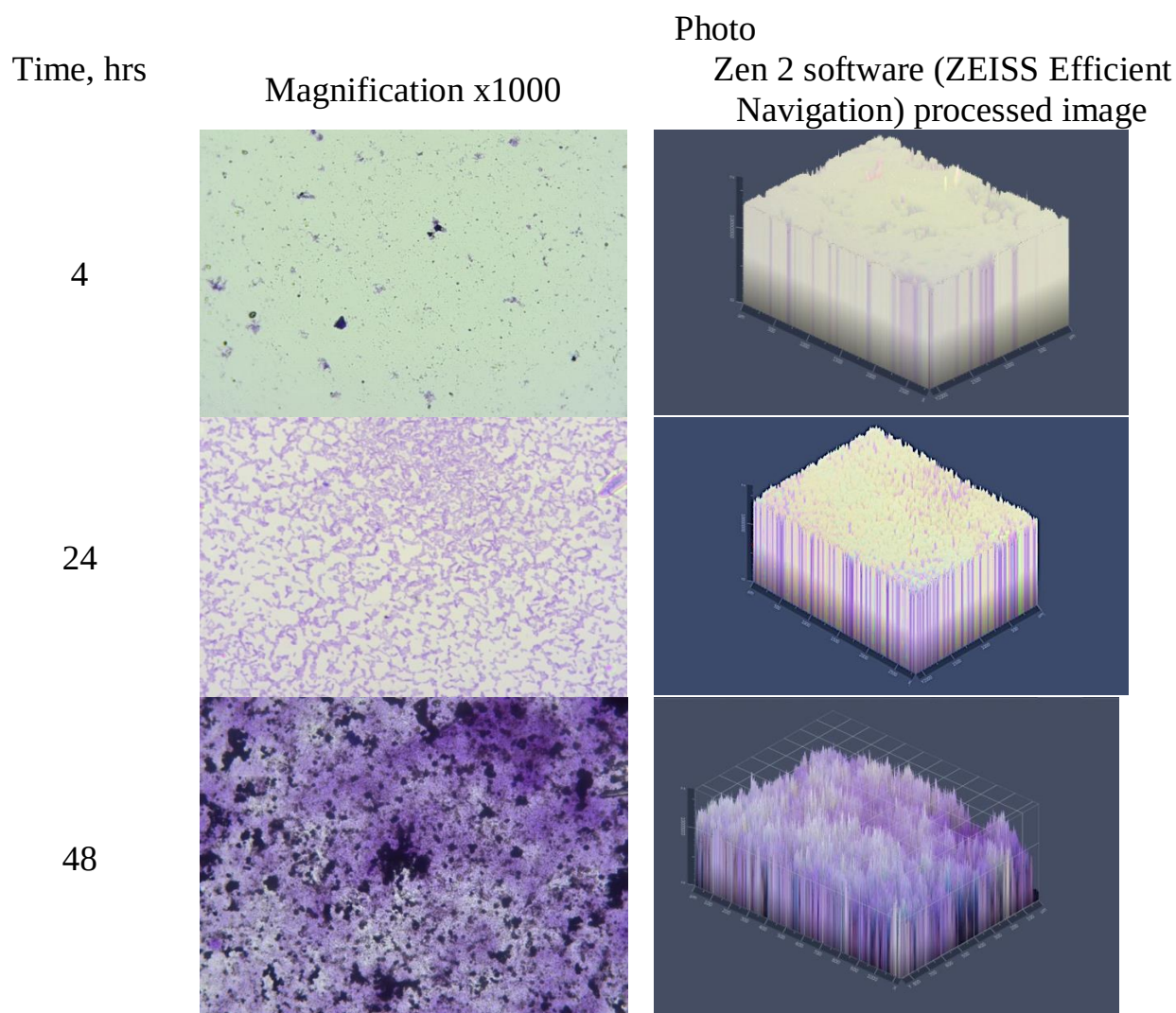


Fig. 1 – 3D-shadow representations of the biofilm structures developed *in vitro* by *P. aeruginosa* ATCC 27853. Biofilms were stained with the Crystal violet dye and observed by visible microscopy (Zeiss Primo Star light microscope (Germany), with a magnification of x1000).

During next maturation of *P. aeruginosa* ATCC 27853 biofilm, on 48-hour cultivation, creation of continuous layer was observed. Analyzing the corresponding photos there was noted fusion of separate microcolonies in the general association. Multi-layer thick biofilms produce higher currents than thin or mono-layer biofilm. And it was detected that the thickness of *P. aeruginosa* ATCC 27853 biofilm also increased comparing with the previous stage. However thickness can become a limiting factor to current production [6].

To sum up, all studied representatives of *Pseudomonas* genus were characterized by the ability to form a biofilm: this process occurred most intensively in the case of strains of *Pseudomonas aeruginosa* ATCC 15692, ATCC 27853 and ATCC 10145. According to the level of redox activity, the studied cultures of pseudomonads were divided into two groups: for representatives of the first *Pseudomonas aeruginosa* group, the corresponding indicator exceeded the value for the second *non-aeruginosa* group by 4.6-5.8 times. Moreover, anode-respiring bacteria, in particular *P. aeruginosa*

ATCC 27853, is characterized by the ability to biofilm formation on the artificial surface *in vitro* and resulted in the 3D-architected structure creation. Thus, these findings indicate that the studied strains possess key electroactive biofilm properties, forming a promising foundation for the development of microbial fuel cells capable of addressing current environmental challenges.

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