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Original article

HIGH EXOELECTROGENIC BIOFILMS FORMATION IN MICROBIAL FUEL CELLS

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Background. Microbial fuel cells are devices in which electricity is generated by microorganisms called exoelectrogens. During the process of anaerobic respiration exoelectrogens emit electrons outside the cell. These electrons can be transferred to the anode of biofuel cell via several different mechanisms. Electricity generation in microbial fuel cells depends primarily on the electrochemical activity of the exoelectrogens present in the anode space. Nowadays, the usage of microorganisms, immobilized as biofilms on the anode, is constantly increasing. Natural sources for exoelectrogens selection such as activated sludge, biofilter biofilms, sediments of seas and rivers have a very diverse microbial composition. Therefore, it is important to immobilize relatively deficient in natural sources exoelectrogens on the anode during the biofilm formation process. The main research areas are the development of a technique for obtaining of electroactive biofilms enriched with exoelectrogens along with reduction of the period of biofilm formation process.
Objective. We set a goal to study the process of high exoelectrogenic biofilm formation basing on the combination of different methods of exoelectrogens isolation and immobilization at the anode of a microbial fuel cell.
Methods. A three-stage technique was used to obtain a highly exoelectrogenic biofilm which, due to the combination of typical isolation and immobilization techniques of exoelectrogens, allows obtaining the biofilm in which the vast majority of microorganisms are exoelectrogens. In the first stage, a biofilter biofilm was used as a source of exoelectrogens. The biofilm formed in the first stage was used as an inoculum for the second stage of biofilm formation. During the second stage an additional selective factor (applied additional potential in the electrical circuit of the microbial fuel cell) was used. The third stage of biofilm formation was the isolation of exoelectrogens capable of reducing ferum (III) compounds from secondary biofilm with subsequent application of these cells as inoculum.
Results. The usage of the proposed method allows obtaining of a biofilm enriched with exoelectrogenic bacteria. The maximum current density generated by the biofilm, obtained during the first stage, reaches 140 μA/cm^2, during the second – 400 μA/cm^2, during the third – 615 μA/cm^2. The duration of biofilm formation at each stage was 110 h, 40 h, and 60 h, respectively.
Conclusions. It has been proven that the duration of biofilm formation is reduced almost twice as a result of a combination of typical methods of isolation and immobilization of exoelectrogens; obtained biofilm has high electrochemical activity and properties similar to biofilm, formed by pure cultures of exoelectrogens.
Keywords: microbial fuel cells; biofilm; exoelectrogens; electrochemical activity.

Introduction

The rapid growth of interest in the usage of biofuels (primarily biodiesel) is damaging to farmland. It leads to soil depletion and deterioration. It is also accompanied by a sharp reduction in the area for growing crops, intended for food. Development of new technologies for renewable energy sources generation with the possibility of simultaneous wastes utilization is a very urgent problem. Algae are currently being promoted as an ideal third generation biofuel feedstock [1, 2]. But another promising technology for renewable energy production and waste water treatment is bioelectrochemical method of electricity generation in microbial fuel cells. An important advantage of this method is the possibility of using wastewater with biodegradable organic compounds. The systems in which such a process occurs are called microbial fuel cells (MFCs) [3].

MFCs are bioelectrochemical systems that generate electrical current using bacteria. This process is based on the catabolism of organic substances such as glucose, acetate, butyrate or other organic compounds contained in wastewaters. Bacteria oxidize the organic compounds and release electrons to the anode. Electrons are delivered to the cathode through an external electric circuit. MFCs could be defined as devices able to transform chemical energy of organic compounds into electricity via electrochemical reactions involving biochemical pathways [3].

The basis of the MFCs is the ability of certain microorganisms to extracellular electrons transfer to the anode during the process of organic substances consumption. In such systems, the anode is a terminal electron acceptor, while electron donors are organic compounds of the nutrient medium. Microorganisms of such systems are called exoelectrogens [3, 4]. Exoelectrogens are presented in the form of biofilms in MFCs rather than free-flowing microorganisms in recent studies.

To release electron to the electric circuit the exoelectrogenic cell should contact with the MFC anode. The interaction between the cells and the anode may take place in a form of direct or indicrect contact – through soluble mediators. That is why the presence of mediators is required almost in all cases of the free-flowing biomass usage. It has been revealed that widespread artificial mediators are metabolites, generated by the degradation of the certain dyes [5], such as methylene blue, neutral red [6], resazurin, humic acid, safranine O [7], malachite green [5], potassium permanganate, potassium ferricyanide, bromocresol green [8]. Artificial mediators are expensive [9] and can often cause the additional pollution of the treating wastewater because of their toxicity for bacteria and animals [8, 9]. It is also known that intensive mixing or bubble aeration is necessary to maintain biomass in a suspended state [10]. Another way is to use different types of flow reactors [11]. But all these methods add expenditures to the technology. The latest studies confirm that MFCs with biofilm have higher overall productivity than others with free-flowing biomass.

According to modern concepts, a biofilm is a continuous multilayer formation of microorganism cells attached to the phase separation surface and one to another, and immersed in a biopolymer matrix. The biofilm is characterized by attachment to a solid surface, structural heterogenity, significant genetic diversity, complex interactions within the grouping and extracellular matrix of polymeric substances.

When it comes to actual application, the usage of MFCs with mixed anode consortia is more profitable than pure culture of exoelectrogens. It has been proven that the electricity generation capacity and the ability to adapt to the complex substrates are lower for MFC systems, operated with pure cultures of exoelectrogens than for the systems, operated with mixed consortia [12, 13]. Thus, in order to create the biofilm, enriched with different types of microorganisms, natural sources of exoelectrogens are preferred. The big challenge is to grow the biofilm, enriched with exoelectrogens while activated sludge or sediments are being used as the source of inoculum. Activated sludge and other natural sources of exoelecrogens are rich in different types of organisms, which can colonize the anode and prevent electron transfer. That's why the biofilm growing process aims not only on immobilization of bacterial cells on the anode surface, but also on some kind of selection.

There is a certain number of techniques for formation of biofilm with exoelectrogenic activity. They are based on supporting of conditions that can promote the development of exoelectrogens and suppress other bacteria [13–17].

Vogl [17] determines the most common approaches to improvement of anode biofilm formation process: providing conditions that simulate the natural environment of the inoculant bacteria, poising the anode potentials, chemical correction of wastewater used as a substrate, variation of MFCs configuration, temperature, pH and other parameters variation, chemical or physical pretreatment of the anode surface, management of the substrate effects on the startup, MFC inoculation with the preacclimated cultures from another MFC, using effluent of another MFC as the source of inoculum, scraping off the biofilm of a populated anode and applying it to a fresh anode. All these methods are designed with the purpose of increasing the number of exoelectrogens' cells in the biofilm and reduction of the duration of biofilm formation process.

The objective of this work is to study the process of anode biofilm formation with high exoelectrogenic activity, basing on combining and comparing different methods of exoelectrogens selection and immobilization.

Materials and Methods

The process of biofilm selection and formation was carried out in anaerobic conditions at a temperature of 37 ± 2 °C. Biofilter biofilm from the anaerobic reactor was used as a source of exoelectrogens. A laboratory self-made cylindrical two chamber MFCs were made of plexiglass. MFCs were constructed as previously described [18]. A total volume of anode compartment was 0.5 dm^3; a total liquid volume of cathode compartment was 0.4 dm^3. A carbon felt (16 cm^2) fixed on a stainless steel wire was used as the anode. The cathode (9 cm^2) was made according to the method described in [19] and had 4-polytetrafluoroethylene layers and 0.5 mg/cm^2 of Pt. The electrode compartments were separated by a proton-selective membrane (10 cm^2, Nafion 112, Dupont). The anode chamber was filled with a medium, containing 15 мМ of sodium acetate in 50 mM

phosphate buffer solution (pH 7), metal salt and vitamin solutions as described in study [20]. The cathode chamber contained phosphate buffer solution (pH 7).

The biofilm formation on the first stage was carried out according to the methods described in researches [3, 21]. But this time biofilter biofilm was used as inoculum. The biofilm (10 g) was immersed into the flasks, containing 500 cm^3 15 мМ of sodium acetate in 50 mM of phosphate buffer solution (pH 7.0) and glass beads. The flasks were shaken (30 minutes) to suspend cells from the biofilm. The anode chamber was inoculated with the suspend cells (20 cm^3).

The second stage of the biofilm formation was carried out in the same conditions, but previously formed anode biofilm was used as a source of exoelectrogens. During the second stage of the biofilm forming the anode was polarized at a positive potential (e.g., at 0.25 V vs. Ag/AgCl) [22]. The current density was measured with the view to monitor the process of exoelectrogens immobilization on the anode. Cultivation was carried out under semi-batch conditions with the replacement of a regular nutrient medium, but without adding the inoculum.

The third stage of exoelectrogens selection was carried out in flasks, filled with phosphate buffer, metal salts and vitamin solutions, as well as with the addition of Fe (III) salt (100 mM) and sodium acetate (10 mM) as electron acceptor and donor, respectively. The secondary biofilm from the anode of the MFC was used as the source of exoelectrogens for third stage of the biofilm formation. Part of the anode carbon material (1 cm^2) from the MFC was immersed into the flasks, containing 30 ml of the phosphate buffer solution (pH 7.0) and glass beads. The flasks were shaken (5 minutes) to suspend cells from the biofilm. Then they were diluted to 10^{-6} with the buffer into bottles, containing Fe (III)-acetate medium (50 ml). The bottles were incubated at 37 ± 2 °C. After exoelectrogens selection, they were immobilized at the anode of MFC [21]. The stages of biofilm formation are illustrated in Fig. 1.

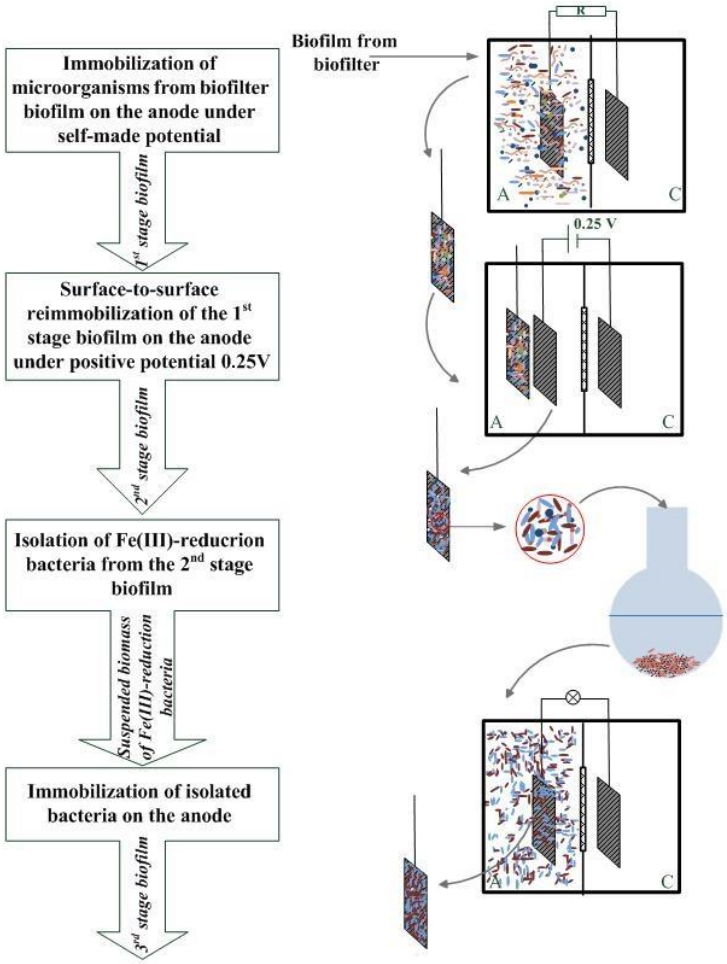

Figure 1: Stages of biofilm formation

The consumption of organic compounds from the nutrient medium was determined by measuring COD value before and after cultivation. Upon the exhaustion of organic compounds in the nutrient medium the complete replacement of the solution in anode chamber was carried out, but without adding an inoculum.

Results

During the first stage of biofilm formation the current density increased to 140 ± 5 μA/cm^2 after 110 hours. Then current density decreased after 48 hours. After replacing of the nutrient medium in the anode chamber the current density increased to 140 ± 5 μA/cm^2. After adding of nutrients and new portion of the inoculum to anodic chamber, the exoelectrogenic activity of the biofilm increased and during the subsequent 3 cycles it has reached the value of 140 ± 5 μA/cm^2. Since the repeated adding of nutrients and inoculum did not provoke the increase of current density, the process of biofilm forming could be considered complete (Fig. 2).

At the second stage of the biofilm formation a pure anode material was immersed into the anode chamber of MFC with a formed biofilm on the anode. Such process of re-forming the biofilm of exoelectrogens was studied under batch conditions of cultivation, and the biofilm that was formed in such a way was called secondary. Upon immersion of a sterile (without microorganisms) carbon material in an anode chamber with a pre-formed anode biofilm the formation of a new biofilm with an exoelectrogenic property was observed at a much shorter period of time. The maximum value of the current density of such biofilm was achieved after 40 hours already (Fig. 3). During three repeated cycles the value of current density has reached approximately the same value of 400 ± 10 μA/cm^2. Further adding of pure carbon materials did not provide receiving a biofilm with a higher current density.

Higher values of the current density of the biofilm were achieved upon application of the procedure of exoelectrogens selection from natural associations; the procedure was described in the following study [21]. In this rapid selection strategy, described in this study, the growth of the primary biofilm on the anode of MFC takes place along with its subsequent transfer to medium with crystalline iron oxide (III), which is an electron acceptor for exoelectrogens.

After natural consortium of microorganisms was cultivated in such environment, a large number of microorganisms with the ability to reduce Fe (III) has developed. Therefore, such an enriched consortium was transferred to an anode chamber of the MFC, in which they were able to use anode as the terminal electron acceptor, but not the crystalline Fe(III)-oxide.

After the cultivation of resuspended cells for 10 days at a temperature of 37 ± 2 °C the black particles were observed in the solution. According to the results, described in one of recent studies, [23], these black particles could be Fe_3O_4 and $FeCO_3$ compounds, which were formed as a result of Fe (III) reduction into Fe (II) with the electrons, emitted during the metabolism by exoelectrons. The obtained microorganisms suspension was used to form a biofilm in MFC. The received maximum value of the biofilm current density was 615 ± 10 μA/cm^2 (Fig. 4). For this sample, the maximum value of current density was obtained in the following period (during 60 hours).

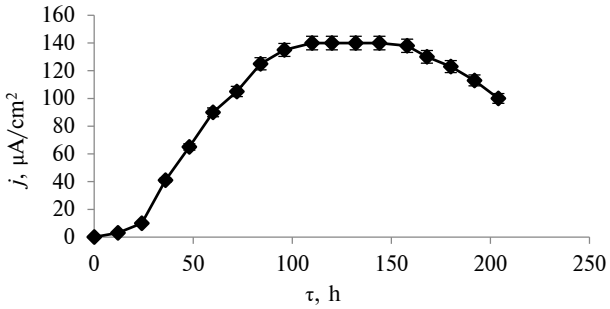

Figure 2: Current density of a microbial biofilm during the first stage of biofilm formation

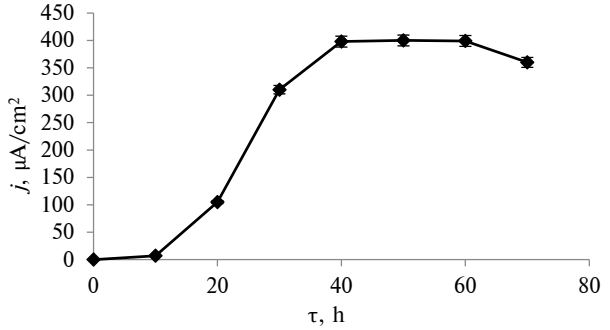

Figure 3: Current density of a microbial biofilm during the second stage of biofilm formation

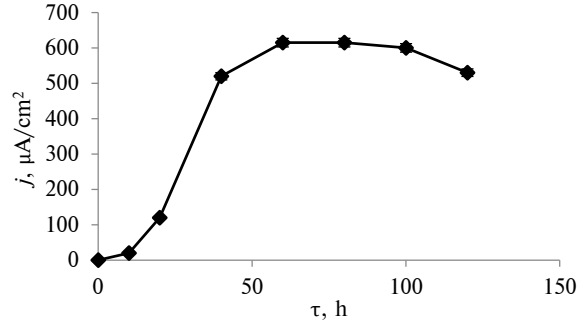

Figure 4: Current density of a microbial biofilm during the third stage of biofilm formation

Discussion

Comparing the results of biofilm formation on the first stage with results, described in [23, 24], it may be concluded that the value of the current density of biofilm, obtained by this procedure, is almost twice as lower (140 μA/cm^2 comparatively to 230 μA/cm^2). These results can be explained via the fact that the formed anode biofilm contained microorganisms that were could use other electron acceptors. Exoelectrogens' metabolites may be such terminal electron acceptors in anaerobic conditions. The difference in the values of the current density can also be explained by the fact that during the anode biofilm formation not only the nutrient medium was replaced, but also new portions of inoculum were added. The steady current values were reached for almost thrice less time (5 days vs. 17 days). Such a reduction in the biofilm formation duration can be explained via higher temperature of cultivation (37 ± 2 °C in this study and 22 °C in [24]). Comparing the results with results, described in [25], we can notice that the duration of biofilm formation is reduced by 40 hours (110 hours compared to 150 hours), and stable current density values are obtained in 3 cycles, not in 4 as in [25]. These results can be explained by higher temperature of cultivation and higher concentration of microorganisms in the inoculum.

The biofilm, formed on the second stage, had similar properties (400 μA/cm^2 vs 480 μA/cm^2) as described in [24], although the current density of the primary biofilm was different. This is proven by the fact that such a procedure allows the selection of exoelectrogens and the biofilm formation from an inoculum with a different concentration of exoelectrogenic microorganisms. Comparing the results, described in [25], we can conclude that secondary biofilm, formed in this way, had similar current density, but this value was reached 20 hours earlier (40 hours vs 60 hours).

Studying of the three-stage procedure for the biofilm formation showed that the duration of the process of formation was reduced twice via the combination of different procedures for its formation, and the exoelectrogenic properties of such a biofilm are similar to the biofilm, formed of the pure culture of *Geobacter sulfurreducens* [23, 26]. Comparing the results with study [25], we can conclude that increase of the temperature of cultivation allowed to reduce the period of cultivation by 20 hours (60 hours vs 80 hours) and increase the current density by 15 μA/cm^2 (615 μA/cm^2 vs 600 μA/cm^2).

Conclusions

The results of research of formation of exoelectrogenic anode biofilm for microbial fuel cells are presented. The suggested method of mixed culture biofilm formation includes integration of previously used methods such as using of additional voltage, growing the secondary biofilm on the basis of the biofilm, formed from natural source of inoculum (biofilter biofilm) with following isolation of exoelecrogens with common electron acceptor. It is shown that the combination of known procedures for biofilm forming provides the possibility to reduce the duration of its formation almost twice. The second and third stages of biofilm formation lasted almost twice as less than the first one (40 and 60 hours compared to 110 hours). At the same time, the value of the current density increased from 140 ± 5 μA/cm^2 to 400 ± 10 μA/cm^2 (in the second stage) and 615 ± 10 μA/cm^2 (in the third stage).

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ФОРМУВАННЯ ВИСОКОЕКЗОЕЛЕКТРОГЕННИХ БІОПЛІВОК У МІКРОБНИХ ПАЛИВНИХ ЕЛЕМЕНТАХ

Проблематика. Мікробні паливні елементи – це пристрої, в яких відбувається генерування електричної енергії мікроорганізмами – екзоелектрогенами. В процесі анаеробного дихання екзоелектрогени здатні виділяти електрони назовні клітини, звідки останні за рахунок різних механізмів передаються на анод біопаливного елемента. Генерування електричної енергії в мікробних паливних елементах залежить насамперед від електрохімічної активності мікроорганізмів, присутніх в анодному просторі. Нині все частіше використовують мікроорганізми, що іммобілізовані у вигляді біоплівки на аноді. Природні джерела виділення екзоелектрогенних мікроорганізмів, такі як активний мул, біоплівки біофільтрів, донні осади морів та річок, мають дуже різноманітний мікробний склад. Тому важливо, щоб у процесі формування біоплівки саме екзоелектрогени, які чисельно становлять досить малу частку від загальної кількості мікроорганізмів із природних джерел, іммобізувалися на аноді. Основними напрямами досліджень є розробка методики отримання електроактивних біоплівок, збагачених екзоелектрогенами, та скорочення тривалості процесу формування біоплівки.

Мета. Дослідження процесу формування високоекзоелектрогенної біоплівки, заснованого на поєднанні різних методик виділення та іммобілізації екзоелектрогенів на аноді мікробного паливного елемента.
Методика реалізації. Для одержання високоекзоелектрогенної біоплівки було використано тристадійну методику, яка за рахунок поєднання типових методик ізолювання та іммобілізації екзоелектрогенів дає можливість отримати біоплівку, в якій переважну більшість мікроорганізмів становлять екзоелектрогени. На першому етапі як джерело екзоелектрогенів використовували біоплівку біофільтра. Біоплівку, що утворилася на першому етапі, використовували як інокулюм для другої стадії формування біоплівки. На другій стадії також використовували додатковий селективний фактор – прикладали додатковий потенціал в електричне коло мікробного паливного елемента. Третя стадія формування біоплівки полягала у виділенні з біоплівки, сформованої на другій стадії, клітин екзоелектрогенів, які здатні відновлювати сполуки заліза (III), з подальшим використанням цих клітин як інокулюму.
Результати. Використання запропонованої методики дає змогу отримати біоплівку, збагачену екзоелектрогенними бактеріями. Максимальна густина струму, яку генерувала біоплівка, отримана на першій стадії, досягає 140 мкА/см^2, на другій – 400 мкА/см^2, на третій – 615 мкА/см^2. Тривалість формування біоплівки на кожній зі стадій становила 110, 40 та 60 год відповідно.
Висновки. Показано, що в результаті поєднання типових методик ізолювання та іммобілізації екзоелектрогенів тривалість формування біоплівки скорочується майже вдвічі, а отримана біоплівка має високу електрохімічну активність і властивості, аналогічні біоплівкам, сформованим чистими культурами екзоелектрогенів.

Ключові слова: мікробні паливні елементи; біоплівка; екзоелектрогени; електрохімічна активність.

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ФОРМИРОВАНИЕ ВЫСОКОЭКЗОЭЛЕКТРОГЕННЫХ БИОПЛЕНОК В МИКРОБНЫХ ТОПЛИВНЫХ ЭЛЕМЕНТАХ

Проблематика. Микробные топливные элементы – это устройства, в которых происходит генерирование электрической энергии микроорганизмами-экзоэлектрогенами. В процессе анаэробного дыхания экзоэлектрогены способны выделять электроны наружу клетки, откуда последние за счет различных механизмов передаются на анод биотопливного элемента. Генерирование электрической энергии в микробных топливных элементах в первую очередь зависит от электрохимической активности микроорганизмов, присутствующих в анодном пространстве. Сейчас все чаще используют микроорганизмы, иммобилизованные в виде биопленки на аноде. Природные источники выделения экзоэлектрогенных микроорганизмов, такие как активный ил, биопленки биофильтров, донные осадки морей и рек, имеют очень широкий микробный состав. Поэтому важно, чтобы в процессе формирования биопленки именно экзоэлектрогены, которые численно составляют весьма малую долю от общего количества микроорганизмов активного ила, иммобилизировались на аноде. Основными направлениями исследования являются разработка методики получения электроактивных биопленок, обогащенных экзоэлектрогенами, и сокращение продолжительности процесса формирования биопленки.
Цель. Исследование процесса формирования высокоэлектрогенной биопленки, основанного на сочетании различных методик выделения и иммобилизации экзоэлектрогенов на аноде микробного топливного элемента.
Методика реализации. Для получения высокоэлектрогенной биопленки была использована тристадийная методика, которая за счет сочетания типовых методик изолирования и иммобилизации экзоэлектрогенов позволяет получить биопленку, в которой подавляющее большинство микроорганизмов составляют именно экзоэлектрогены. На первом этапе в качестве источника экзоэлектрогенов использовали биопленку, выделенную из биофильтра. Образовавшуюся биопленку на первой стадии использовали как инокулюм для второго этапа формирования. На второй стадии также использовали дополнительный селективный фактор – прикладывали дополнительный потенциал в электрическую цепь микробного топливного элемента. Третья стадия выделения экзоэлектрогенных микроорганизмов заключалась в выделении из биопленки, сформированной на второй стадии, клеток экзоэлектрогенов, которые способны восстанавливать соединения железа (III), с последующим использованием этих клеток в качестве инокулюма.
Результаты. Использование предложенной методики позволяет получить биопленку, обогащенную экзоэлектрогеннымы бактериями. Максимальная плотность тока, которую генерировала биопленка, полученная на первой стадии, достигает 140 мкА/см^2, на второй – 400 мкА/см^2, на третьей – 615 мкА/см^2. Длительность формирования биопленки составляла 110, 40 та 60 ч соответственно.
Выводы. Показано, что в результате сочетания типовых методик изолирования и иммобилизации экзоэлектрогенов продолжительность формирования биопленки сокращается почти вдвое, а полученная биопленка имеет высокую электрохимическую активность и свойства, аналогичные биопленкам чистых культур экзоэлектрогенов.

Ключевые слова: микробные топливные элементы; биопленка; экзоэлектрогены; электрохимическая активность.