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EXPERIMENTAL ARTICLES

Intensification of Bioelectricity Generation in Microbial Fuel Cells Using *Shewanella oneidensis* MR-1 Mutants with Increased Reducing Activity

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Abstract—Electrogenicity of *Shewanella oneidensis* MR-1 mutants FRS1 and FRB1 with reducing activity 30–40% higher than in the original strain was studied in various microbial fuel cells (MFC) developed in the course of the work. The voltage and current density developed by the mutants were 1.7 times higher than in the case of *S. oneidensis* MR-1. A correlation was found between reducing activity of the cells and the voltage and current density developed in MFC. The possibility for enhanced bioelectricity production in MFC by genetic modification of *S. oneidensis* MR-1 was demonstrated.

Keywords: microbial fuel cells, electrogenic bacteria, reducing activity

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Search for alternative ways of energy generation from renewable organic sources resulted in development of microbial fuel cells (MFCs), where electric current is generated in the course of anaerobic oxidation of organic compounds by electrogenic microorganisms [1]. These bacteria transport electrons to the outer surface of the cell membrane, where they may be accepted by exogenous and endogenous mediators of the electron transfer (dyes, quinones, riboflavin, etc.) or by the anode of an MFC [2]. These processes result in the generation of electric current. The possibility to generate energy in the course of microbial transformation of organic wastes of food industry, agriculture, and waste plants require further optimization of MFCs [3]. MFC-generated electricity may be used to power the equipment located in remote, hard-to-reach areas [4], as well as in closed systems, such as submarines, orbital space stations and, in the future, spaceships [5]. These technologies are environmentally friendly, combine waste utilization with electricity generation, and are capable of long-time operation.

Strains of *Shewanella, Geobacter, Aeromonas*, and other bacteria are used for investigation of electricity generation by microorganisms. *Shewanella oneidensis* MR-1, a gram-negative gammaproteobacterial facultative anaerobe, which has been retrieved from soil and marine and freshwater bottom sediments. *S. oneidensis* MR-1 has been studied by molecular genetic techniques, its chromosomal DNA was completely sequenced [6], and techniques for exchange of genetic material were developed [7]. MFCs were developed using various organic substrates.

The power generated by MFCs is presently low, and improvements are required for their industrial application. MFC optimization may be achieved by improvement of their design or by genetic modification of the microorganisms. Modification of the technical parameters resulted in an increase of current density from 0.1 mA/m^2 to 4.3 A/m^2, so that MFCs could be used for low-power appliances [8, 9]. There are, however, almost no publications reporting on power generation in MFCs increased by genetic modification of microorganisms. Modification of the oxidative processes of *S. oneidensis* MR-1 resulting in increased rates of electron generation is one of the possible approaches to enhancing the MFC efficiency. We have reported obtaining the mutants of *S. oneidensis* MR-1 with increased reducing activity and intensified lactate consumption [10]. The mutants FRB1 and FRS1 were selected for resistance to the antibiotic fosfomycin, a toxic analogue of phosphoenolpyruvate. Since phosphoenolpyruvate plays a central role in the carbon metabolism, we expected the variants with enhanced activity of the enzyme systems responsible for carbohydrate transport and oxidation, as well as for production of reduced NAD(P)H equivalents and ATP bio-

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synthesis, among fosfomycin-resistant mutants. Lactate consumption by strains FRS1 and FRB1 was 10 and 30% higher, respectively, compared to the original strain. Reducing activity of the mutants determined by the rate of methylene blue discoloration by the cell suspensions of equal optical density was ~30–40% higher than that of the original strain.

The goal of the present work was comparative analysis of the voltage and current density developed in MFCs by the original strain *S. oneidensis* MR-1 and the mutants FRB1 and FRS1 with enhanced reducing activity. To achieve this goal, the conditions for aerobic and anaerobic cultivation were optimized, the titers of the cell suspensions for incubation in MFCs were determined, laboratory models of two types of MFCs were constructed, and their working parameters were determined.

MATERIALS AND METHODS

Strains. The *S. oneidensis* MR-1 mutants FRS1 (Fosfomycin Resistant Small) and FRB1 (Fosfomycin Resistant Big) with enhanced reducing activity were obtained previously [10]. The strain *S. oneidensis* MR-1 was obtained by the All-Russian Collection of Industrial Microorganisms, State Research Institute of Genetics and Selection of Industrial Microorganisms, from the microbial collection of Pasteur Institute (no. CIP106686, France).

Media and biomass collection for MFCs. The strains were grown on TSB (Tryptic Soy Broth, Sigma) medium (40 g TSB and 17 g agar per 1 L distilled water) for 24 h at 30°C. The cells were then washed off with the physiological saline, and this suspension was used to inoculate liquid TSB medium (1.0 mL per 750-mL flask with 100 mL of the medium). The flasks were incubated aerobically for 18 h at 30°C on a rotary shaker (220 rpm). The cultures were then centrifuged at 6000 *g* for 20 min, the pellet was washed with saline and centrifuged under the same conditions. The biomass with 1 mL of sterile distilled water was transferred into MM synthetic medium [8] with lactate (4 g/L). The suspensions were adjusted to equal OD prior to their introduction into the anode chamber of an MFC.

MFCs. Two devices, MFC1 and MFC2, which have different design factors, were used. The voltages created by the strains in MFC1 and MFC2 were measured by different methods.

MFC1 was a two-chamber electrochemical cell with the MK-40 cation exchange membrane (Shchekinoazot, Russia). The volume of each chamber (cathode and anode) was 295 cm^3. The anode (64 cm^2) and cathode (15.5 cm^2) were made of carbon material. The anode was connected to the cathode via an electric circuit with resistive load of 150 kΩ. Electrical parameters of MFC1 were monitored throughout the experiment with 10-min intervals using a E-270 encoder (LCard, Russia) and the LabVIEW software package (National Instruments, United States).

MFC2 was a multi-chamber electrochemical cell with a Nafione cation exchange membrane (DuPont, United States), a common cathode chamber, and several isolated anode chambers. The volume of a single anode chamber was 13 cm^3. The anode was a combined construction of a graphite rod (0.5 cm in diameter, 9.0 cm long, 14.1 cm^2) and a disk of graphitized textile (1.6 cm in diameter, 2.0 cm^2). The cathode (150 cm^2) was of graphitized textile. Electric parameters of MFC2 (current and voltage) were determined once every 24 h by an ammeter and a voltmeter.

RESULTS AND DISCUSSION

Consumption of organic substrates by the mutants FRS1 and FRB1. Since *S. oneidensis* MR-1 is unable to utilize exogenous glucose, the usual carbon sources in experimental MFCs using *Shewanella* strains are lactate, formate, or acetate [11]. Electrogenic strains use lactate as a source of new cell material and as an electron donor for their metabolism. Formate may be used as an electron donor oxidized in the periplasm or at the cell surface. Experiments with washed living *Shewanella* cells revealed that methylene blue (MB) discoloration became much more rapid in the presence of 0.1% sodium formate. For example, MB discoloration time by *S. oneidensis* MR-1 suspensions in water with and without formate was 225 and 115 s, respectively. MB discoloration time by suspensions of the FRS1 mutant in water with and without formate was 135 and 40 s, respectively. Thus, MB discoloration time in the presence of formate decreased about twofold for the parent strain and more than threefold for FRS1. Comparable results were obtained for strain FRB1. Thus, the rates of oxidation-reduction processes were higher in the mutants than in the original strain. These results suggested the application of these mutants in microbial fuel cells.

Electricity generation in MFCs by *Shewanella* strains. Numerous modifications of MFCs exist, differing in the chamber volume, anode and cathode materials, distance between the electrodes, etc. An MFC consists of two chambers (anode and cathode ones) separated by an ion-selective membrane (Fig. 1). Organic matter and bacteria are located in the anode chamber under anaerobic conditions. The cathode is under aerobic conditions. The membrane permits proton transport from the anaerobic anode chamber into the aerobic cathode one and prevents oxygen diffusion into the anode chamber. The anode is connected with the cathode via an electric circuit with a certain resistive load. The electrons arrive to the terminal acceptor (a proton in the cathode chamber) via the anode and the electric circuit, thus creating the current [8].

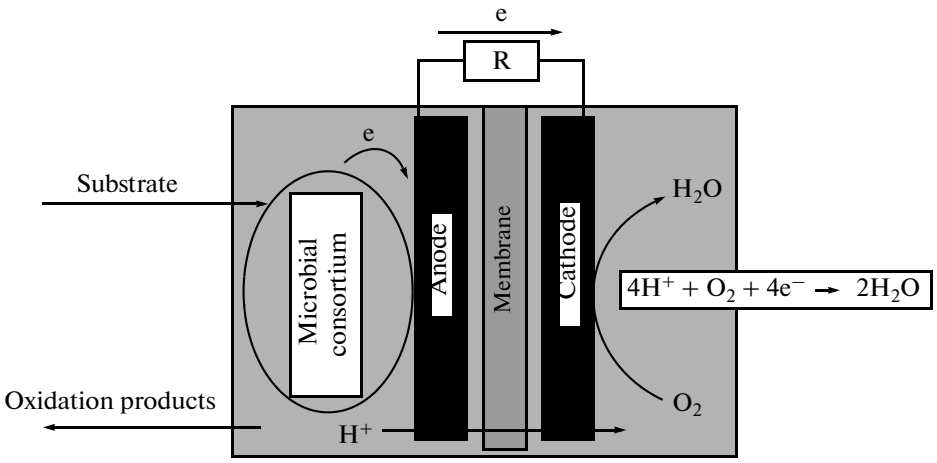

Fig. 1. Diagram of a microbial fuel cell (MFC).

Comparative analysis of the electrogenic activity of the original strain *S. oneidensis* MR-1 and of the FRS1 and FRB1 mutants was carried out in two laboratory models, MFC1 and MFC2. In each experiment, three cells of each MFC model were used simultaneously for strain MR-1 and for the mutants FRS1 and FRB1. The anode chambers were aseptically filled with cell suspensions of equal cell density in MM medium with lactate and incubated under anaerobic conditions. The cathode chamber contained distilled water and was open to air. The fuel cells were incubated at room temperature (MFC1) or at 30°C (MFC2). Identity of the parameters of all the cells and of the results obtained in different cells with the same strain is important for the MFC experiments. For different strains, the cells giving stable reproducible results under the same experimental conditions were used [9].

In order to compare electrogenic activity of the mutants FRS1, FRB1, and the original strain in MFCs, three experiments were carried out. Electrogenicity of the cultures depends significantly on their physiological state, cultivation conditions, preliminary treatment of the strains, and variations of the temperature regime of an MFC. While the voltage and current density developed by a strain may vary from experiment to experiment (in our case the voltage variations reached 50–70 mV), the relative differences between the mutants and the original strain were observed in each individual experiment.

The growth phase of the cells transferred into MFCs, specifically the duration of aerobic cultivation for biomass accumulation, have a significant effect on the electrogenicity of the cultures and their viability in MFCs. The optimal cultivation time was shown to be 18 h at 30°C, which corresponds to the mid-exponential growth phase. The cells of this growth phase continue division under anaerobic conditions in MFCs, so that the cell titer and electrogenic activity are maintained. Although longer aerobic cultivation (for 24 and 30 h) may result in higher cell titers, the reducing activity of the cultures (determined as the rate of MB discoloration) and the voltage in MFCs decreased. Initial cell density in the anode chamber of an MFC is important for electricity generation. The rate of methylene blue discoloration (a measure of reductive activity of the cells) was shown to increase at higher cell titers in suspensions [10]. The cell titer in an MFC should be at least 1.0×10^7 cells/mL. The suspensions used in our experiments had the initial titer of 5×10^7–1×10^8 cells/mL. Analysis of cell survival after 30 days of incubation in MFCs revealed cell titers of 1.0×10^5–1.0×10^6 cells/mL.

Results of one of the experiments on voltage measurement for the mutants FRS1 and FRB1, and the original strain *S. oneidensis* MR-1 strain in MFC1 are presented on Fig. 2. The data are presented in the manner compatible with the similar publications [12, 13]. It can be seen that throughout 30 days of the experiment the mutants generated higher voltage. The maximal difference in voltage between the original strain and the mutants at days 10–25 of the experiment was 200 mV. The maximal voltage for the mutants and the original strain was 620 and 600 mV, respectively. Importantly, the original strain maintained the maximal voltage for 4 days, with a subsequent drastic decrease. Unlike the original strain, the mutant FRS1 maintained 620 to 570 mV until day 25 of the experiment. After 10 days of incubation, the voltage generated by strain MR-1 decreased to 370 mV, while in the case of the mutants it was 570 mV. The diagrams presented on Fig. 3 show the current density calculated for days 13 and 20 of the experiment. It can be seen that the current density generated by the mutants on day 13 was ~1.7 times higher compared to the original strain. On day 20, the current density generated by FRS1 retained at the same high level, while in the case of FRB1 it decreased, remaining, however, 1.2 times higher than the current density

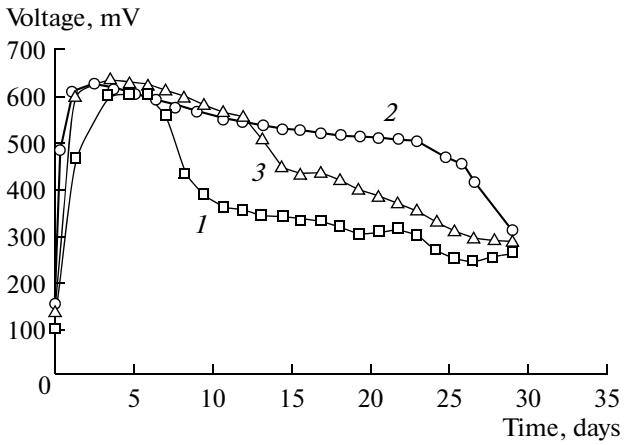

Fig. 2. Voltage generated in MFC1 by *Shewanella oneidensis* MR-1 (*1*), FRS1 (*2*), and FRB1 (*3*).

generated by the original strain. Thus, in MFC1 both the voltage and the current density were more stable in the case of FRS1 than for FRB1. The major characteristics of the mutant strains FRS1 and FRB1 were higher intensity, stability, and duration of electricity generation. Measurement of MFC2 revealed lower voltage for all three strains, probably due to the design philosophy of the MFC2 cells. For example, the maximal voltage for the original strain was 350 mV. For FRS1 and FRB1, this parameter was 35 and 20% higher, respectively.

Thus, the mutants with increased reductive activity exhibited higher levels of electric current generation in MFCs of both types. A correlation between reductive activity (determined as the rate of MB discoloration) and the level of electricity generation (measured as MFC voltage) was found. Our results demonstrate the possibility of increased electricity generation by MFCs resulting from enhanced redox processes in genetically modified electrogenic bacteria.

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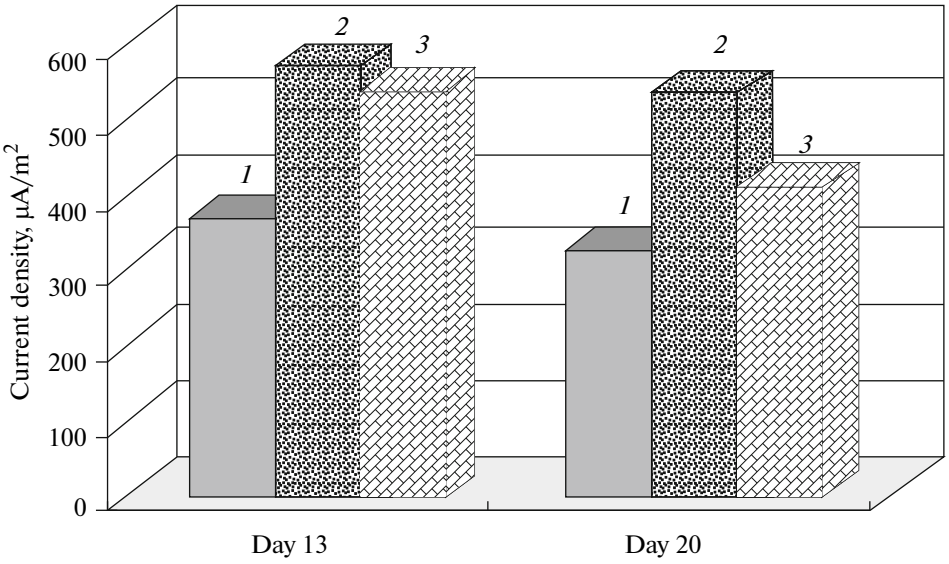

Fig. 3. Current density generated by *Shewanella oneidensis* MR-1 (*1*), FRS1 (*2*), and FRB1 (*3*) on days 13 and 20 of cultivation in MFC1.

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