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Isolation of Fe(III)-reducing bacterium, *Citrobacter* sp. LAR-1, for startup of microbial fuel cell

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ABSTRACT

The LAR-1 strain was isolated from sediment samples collected at the– intersection of A-Shi River and Songhwa River, China with high iron-reducing capability. Biochemical tests (API 20A, API-20NE and API-ZYM), fatty acids analysis, and polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) tests revealed that LAR-1 is closely related to *Citrobacter* sp. The LAR-1 was used as anode chamber seed to start up microbial fuel cell (MFC). The tested MFC had an open-circuit voltage of 610 mV and a maximum power density of 610 mW/m^2 at 360 mV. The analytical results confirmed that the iron-reducing bacterium can be used as anodic respiratory bacterium for MFC.

Introduction

Microbial fuel cells (MFCs) convert electron donors on an anode to their oxidized form to produce electrons. An external circuit then transfers the electrons to a] cathode [1–4]. For instance, sulfide can be converted on anode to elementary sulfur with excess electrons being received by oxygen on cathode [5,6]. The anodic biofilm in a microbial fuel cell (MFC) oxidizes substrates to generate electricity. The anode-respiring bacteria (ARB) can conduct extracellular electron transfer *via* direct electron transfer (DET) and mediated electron transfer (MET) [7], whose rate determines the performances of specific MFCs. The anodes are sparsely soluble solids that can accept electrons from bacteria. Iron-reducing bacteria (FRB) such as *Shewanella* and *Geobacter* sp. grow upon reduction of Fe(III) [8], a process similar to ARB on anodes in MFC applications [9].

The numerous ARB strains identified in mixed cultured anodic biofilms of MFC include *Acidiphilium*, *Aeromonas*, *Arcobacterbutzleri*, *Bacillus*, *Bacteroides*, *Citrobacter*, *Citrobacter*, *Comamonas*, *Desulfuromonas*, *Enterobacter*, *Escherichia*, *Geobacter*,

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Geopsychrobacter, *Geothrix*, *Klebsiella*, *Ochrobactrum*, *Pseudomonas*, *Rhodoferax*, *Rhodopseudomonas*, *Shewanella*, *Thermincola* [10–29]. Only a few studies started up MFC with pure culture FRB [30]. Huang et al. [31] isolated an exoelectrogenic bacterium *Citrobacter freundii* from the anodic biofilm of an MFC Z7. The MFC was then used to cultivate an anodic biofilm with citrate as the electron donor. However, these studies did not describe the Fe(III)-reducing capability of the tested Z7. Hence, the hypothesis that FRB can be directly adopted as an ARB for MFC has not been tested.

In this study an isolate with high-iron reducing capacity was used as the inoculum for the anodic compartment to start up a MFC. The experimental results confirmed that the pure culture FRB can be used as ARB for MFC.

Materials and methods

Strain screening, isolation and identification

Sludge samples were collected from sediment at the intersection of A-Shi River and Songhwa River, China. The samples were cultivated in several tubes containing iron-reducing medium (Na_2HCO_3, 2.5 g/L; KH_2PO_4, 0.6 g/L; NH_4Cl, 1.5 g/L; yeast extract 0.5 g/L; sodium acetate, 1.36 g/L; iron citrate, 50 mM; Wolfe's vitamin solution, 5 mL/L; Wolfe's trace metal solution, 10 mL/L; pH 6.9) at 30 °C, 130 rpm shaking. Samples with white, black or gray precipitates were collected and centrifugated to remove supernatant. The sediment was then recultivated in iron-reducing medium to produce another batch. This procedure was repeated more than ten times to enrich the consortium.

The obtained consortium samples were diluted and spread onto the solidified agar plates containing iron-reducing medium. The colonies formed on the agar were picked up, diluted, and spread on new agar plates. This process was repeated at least 10 times to obtain pure isolates. The cell morphology of obtained isolates was observed by a phase-contrast microscope.

Biochemical tests of the isolated strains were performed with API 20A, API-20NE and API-ZYM. The fatty acids were extracted, saponified and methylated according to the Sherlock Microbial Identification System (MIDI) protocol. The genomic DNA of collected cells was extracted with a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer instructions. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using primers:968 (5′-AACGCGAAGAACCTTAC-3′) and 1401R (5′-CGGTGTGTACAAGACCC-3′). The PCR solutions contained 10 × buffer 5 μL, ExTaq(R) 0.25 μL (TAKARA), 1 μL of each primer, 2.0 μg DNA template, and sterile deionized water to obtain a total volume of 42.25 μl. The PCR amplification was performed by TMin an iCycleriQ (Bio-Rad Laboratories, Hercules, CA, USA) with an initial denaturation of DNA for 5 min at 94 °C followed by 30 cycles of the following program: 45 s at 94 °C, 45 s at 58 °C, 1 min at 72 °C, and a final extension for 7 min at 72 °C. The PCR products were purified with UNIQ-10 gel (1%) (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China). Denaturing gradient gel electrophoresis (DGGE) tests were performed with the Bio-Rad universal mutation detection system with 10% (w/v) polyacrylamide gels. The range of denaturants (100% denaturant corresponds to 7M urea and 40% (v/v) deionized formamide) was 35–65%.The DGGE was performed at 60 °C for 12 h at 30 V. Gels were stained with ethidium bromide and photographed with a UV transilluminator. Purified PCR products were ligated to vector pMD19 and cloned into *Escherichia coli* DH5α competent cells. The randomly selected 50 clones were sequenced using the ABI Prism model 3730XL (Applied Biosystems, CA, USA). The 16S rRNA gene sequences were analyzed using BLASTN and EzTaxon.

MFC and tests

Double-chambered MFCs were prepared for testing. Square chambers 7 × 7 cm^2 and length 10.5 cm were connected to a cation exchange membrane (CEM) (Ultrex CMI-7000, Membrane International Inc., Glen Rock, NJ, USA). Both electrodes were made of carbon cloth of area 14.0 cm^2 (W0S1002, CeTech. Co., Taichung, Taiwan). The cathode contained 0.5 mg/cm^2 Pt catalyst.

The reactor was 10.5 cm long, 7 cm wide, and 7 cm high. Both the anode and cathode compartments had volumes of 70 ml. The anode compartment was flushed with nitrogen before the experiment. Between compartments, a Ultrex proton-exchange membrane (Membranes International, Inc, Glen Rock, NJ, USA; 80 cm^2) was set up. The membrane was incubated in 2% NaCl at 45 °C for 18 h before use. The anode and cathode were made of carbon cloth, each with area of 14 cm^2. Both electrodes were connected through a 1000Ω resistor. Reference Ag/AgCl electrode (type 217, XianRen Industries Co., Shanghai, China) was installed in the anodic chamber for electrochemical measurements.

The enriched Fe(III)-reducing consortium was fed into the anode chamber of the MFC in medium (per L): Na_2HPO_4 4.57 g, NaH_2PO_4 2.45 g, NH_4Cl 0.31 g, Wolfe's vitamin solution 5 ml, and Wolfe's mineral solution 12.5 ml, at pH 6.9. The carbon source for all MFC tests was NaAc (20 mM). The cathodic medium was a mixture of 50 mM potassium ferricyanide and 100 mM PBS.

The voltage drop over the external load was recorded using a data acquisition system (Advantech Co., Taipei, Taiwan). Linear sweep voltammetry (LSV) tests of the MFC were performed with an electrochemical workstation (model CHI611, CH Instruments, Inc. Austin, TX, USA). The working electrode of a potentialstat (LPS 505N, Motech Industries, Inc., Taipei, Taiwan) was connected to the anode to control its local potential, using Ag/AgCl reference electrode. Potential sweep experiments were performed at a scan rate of 1 mV/s from the open-circuit cell voltage (OCV) to the short-circuit cell voltage. The so obtained V–I was then used to obtain the polarization curve and power density curve of the tested MFC.

The electrochemical impedance spectroscopy (EIS) experiments were performed by Zahner™ IM6ex potentiostat-AC frequency analyzer equipment at the end of tests. The frequency of the AC signal was varied from 100 kHz to 10 mHz with an amplitude of 5 mV. Impedance experiments were performed under galvanostatic closed circuit conditions at 400 mA for the tested biofilms. The initial electrical potential for anode tests was −0.5 V while that for cathode tests was

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