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Exoelectrogenic Bacterium Phylogenetically Related to *Citrobacter freundii*, Isolated from Anodic Biofilm of a Microbial Fuel Cell

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Abstract An electrogenic bacterium, named *Citrobacter freundii* Z7, was isolated from the anodic biofilm of microbial fuel cell (MFC) inoculated with aerobic sewage sludge. Cyclic voltammetry (CV) analysis exhibited that the strain Z7 had relatively high electrochemical activity. When the strain Z7 was inoculated into MFC, the maximum power density can reach 204.5 mW/m^2 using citrate as electron donor. Series of substrates including glucose, glycerol, lactose, sucrose, and rhammose could be utilized to generate power. CV tests and the addition of anode solution as well as AQDS experiments indicated that the strain Z7 might transfer electrons indirectly via secreted mediators.

Keywords *Citrobacter freundii* · Exoelectrogens · Microbial fuel cell · Biofilm · Bioelectricity

Introduction

Exoelectrogens, the microorganisms which are capable to transfer electrons extracellularly [1], are a key element for power generation. In the anode compartment of MFC, the thick biofilm is formed on anode due to the accumulation of electrogenic bacteria that release electrons produced during degradation of substrate, while in the cathode compartment the electrons are then transferred to electrodes through kinds of pathways. Recent investigations have shown that different kinds of exo-electrogens undergo various processes of metabolism, which results in diverse electrogenic activities on same conditions, such as the discrepancies of power density and the characteristics of carbon sources utilization [2–5]. Moreover, power generation from different kinds of substrates for an identical microorganism varies significantly [6, 7].

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Thus, it is important to investigate and understand the exoelectrogens and their electron transfer manners.

Nowadays, over 20 species of electricigens have been reported previously in the field of MFC, including *Shewanella* [4], *Pseudomonas* [8], *Escherichia* [9], *Enterobacter* [10], *Klebsiella* [11, 12], *Citrobacter* [6], *Comamonas denitrificans* [13], *Desulfuromonas* [3], *Geobacter* [2], *Aeromonas* [14], *Geopsychrobacter* [15], *Rhodoferax* [16], *Rhodopseudomonas* [17], *Ochrobactrum* [7], *Acidiphilium* [18], *Arcobacter butzleri* [19], *Geothrix* [20], *Clostridium* [21], *Thermincola* [22], *Bacillus* [23] and *Bacteroides* [5]. Additionally, it has been found that the amount of electrochemically active bacteria reported was pretty limited. Therefore, it is necessary to isolate novel electrogenic pure cultures.

Electrons can be transferred from different kinds of electricigens via various mechanisms. Since the strain of *Shewanella putrefaciens* which was able to transfer electrons without the addition of exo-mediators was reported [4], mediator-less MFCs have gained more concerns in this area. The electron transfer mechanisms of mediator-less MFCs could be divided into three classes, directly contact by outer membrane cytochromes [24] and by conductive nanowires [25, 26] as well as indirectly by electrochemical mediators [8, 11, 27]. Moreover, for a specific electrogenic pure culture, two or more mechanisms described above maybe exist together in a MFC [1, 28]. Thus understandings the electron transfer mechanisms of electricigens attribute to enhance the process of electricity generation.

A latest study showed that *Citrobacter* sp. SX-1 could produce electricity in a MFC with current density around 205 mA/m^2 [6]. In this study we isolated and characterized a strain Z7 related to *Citrobacter freundii* from an air-cathode single-chamber MFC. The results of cyclic voltammetry (CV) test and electricity generation using seven kinds of substrates manifested that strain Z7 had strong electrochemical activity and can generate maximum power density as high as 204.5 $mW/m^2$when using citrate as electron donor. In order to examine the electron transfer mechanisms of the isolated strain Z7 in MFC, CV tests were carried out to measure the anode solutions of the MFC sampled at different time. In addition, exogenous mediator such as anthraquinone-2,6-disulfonic acid disodium salt (AQDS) was added into anode chamber so as to observe whether AQDS could contribute to electrons transfer or not.

Materials and Methods

Isolation and Characterization of *C. freundii* Z7

The bacterium Z7 was isolated from an air-cathode MFC utilizing 1 g/L glucose and aerobic sewage sludge sampled from a local wastewater treatment plant as inoculation. The anodic biofilm samples were gathered and inoculated into sterilized Luria-Bertani (LB) liquid medium containing 20 mmol/L ferric citrate as electron acceptor. After incubating in sealed serum bottle for 2 days at 30 °C, the cultures were diluted into a serial of concentrations on agar plates. Two days later, single colonies appeared on the plates. Then each single colony was selected and purified on a fresh agar plate. The isolate, designated as Z7 was obtained after six times repeated purification. All the operations of isolation were conducted under anaerobic conditions (Glove box anaerobic chamber, Xinmiao, Shanghai, China) at 30 °C. The isolate (authorized by the Chinese Patent as no. ZL201310119749.3) has been stored in the China Center for Type Culture Collection under depository number CCTCC M2012447.

The morphological and biochemical characteristics of the isolate were examined. LB medium containing 20 mmol/L ferric citrate was used to observe the colonies characteristic. The size of the cells and its motility was observed using scanning electron microscope

(S-3000N, Hitachi, Japan) at an accelerating voltage of 15 kV and transmission electron microscope (HITACHIH-7650, Japan) at an acceleration voltage of 80 kV. Growth curve was plotted using the standard method. Carbon sources utilization and biochemical tests were conducted according to the methods described previously [29].

Near-full length 16S rDNA of the isolate was sequenced and used for phylogenetic analysis. Genomic DNA of the isolated strain was extracted and purified using a TIANamp Bacteria DNA Kit (Tiangen Biotech., China) according to the manufacturer's instructions. The 16S rDNA was amplified using the universal primer pair 27F (5 -AGA GTT TGA TCC TGG CTC AG-3) and 1492R (5 -GGT TAC CTT GTT ACG ACT T-3). The PCR reaction system was 50 μL final volume consisted of 20 ng of template, 4 μL of dNTPs (each 2.5 mmol/L), 0.5 μL each primer (10 μmol/L), 5 μL of 10× PCR buffer, 1.25 U of DNA polymerase, and deonized water. The PCR was done as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and finally 72 °C extension for 10 min. Then, the PCR products were subjected to sequencing (BGI, Shenzhen, China). Sequence identification was initially estimated by BLAST facility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/). All available subsets of 16S rDNA sequences were selected and aligned using CLUSTALX 1.8. The phylogenetic tree was constructed based on available 16S rDNA sequences by MEGA 4.1. The 16S rDNA sequence of the isolate in this research has been deposited in the GenBank under accession number JX185134.

MFC Construction and Operation

An air-cathode single-chamber MFC was constructed to evaluate power generation characteristics of the isolated strain under 30 °C at pH 7 (the optimum condition for the growth of bacteria). MFC consisted of a cylindrical chamber containing the electrodes which were placed on two sides of reactor with the distance of 2 cm between two electrodes. The anode was made of carbon fiber felt (BET 1,200 m^2/g, 2 mm thickness, Liaoyang Jingu Carbon Fiber Sci-Tech Co., Ltd., China) which was treated with nitric acid prior to being used [30]. Carbon paper used as cathode was coated with 0.5 mg/cm^2 platinum catalyst and four layers of PTFE on the facing solution and air sides respectively. Both electrodes provided projected geometrical area of 3.14 cm^2. A cation-exchange-membrane (CEM, Zhejiang Chiaki sewage treatment Co., Ltd, China) was placed directly onto the air-cathode side. Titanium wires were used to connect the electrodes and external circuit. The volume of the reactor was 6.28 cm^3.

The assembled MFC was sterilized under the UV light. Bacteria in mid-log phase was centrifuged and resuspended in 50 mmol/L phosphate buffer. The reactor was inoculated with bacterial suspension and medium at ratio of 1:5 (*v*/*v*) in order to form the biofilm more available which led to the successful startup. The external circuit resistance was 1,000 Ω. The medium which consisted of carbon sources, phosphate buffer solution, vitamins, and minerals [31] was replaced when the output voltage decreased below 50 mV. The anode compartment was only refilled with fresh medium after the potential reached 100 mV. Citrate, glucose, glycerol, lactose, sucrose, rhammose, and acetate were used as carbon sources at a concentration of 30 mmol/L. To determine whether the anode solution could enhance power generation or not, anode solution of a complete cycle was filtered with 0.22-μm membrane and fed back to the cell at ratio of 1:1 (*v*/*v*) with fresh medium. AQDS was also added to the citrate medium as an exogenous electron transfer mediator at a final concentration of 50 μmol/L to investigate its effect on the power generation.

Electrochemical Analysis

The electricity-generation ability of the strain Z7 was evaluated in MFCs. Seven kinds of substrates were tested and the corresponding power generation characteristics were determined. The voltage (U) was measured using a data acquisition system (Keithly 2700). To obtain polarization curve, a variable external load of 1,300, 1,200, 1,100, 1,000, 900, and 800 was used in a full batch cycle. Anodic and cathodic potentials were measured at the same time against the Ag/AgCl (197 mV vs NHE) reference electrode.

CV tests of the bacterial suspension, the anode solution without bacteria and the anodic biofilm at different power-generating stages were carried out. Bacterial cells cultivated in LB medium (containing 20 mmol/L ferric citrate or not) were harvested at the mid-log phase, washed, and suspended in phosphate buffer (50 mmol/L, pH 7.0). The CV tests were obtained using a conventional three-electrode electrolysis cell of 15-ml capacity in which a glassy carbon working electrode, a platinum counter electrode, and an Ag/AgCl reference electrode were used. Measurements were performed at 30 °C at the scanning rate of 100 mV/s over the range from −700 mV to 700 mV. Moreover, For CV tests of anode solutions, the solutions were filtered with 0.22-μm membrane to remove bacteria and the filtrates were subjected to CV tests as previously described. Additionally, in situ CV tests of anode biofims were conducted using a working electrode (anode), a counter electrode (cathode), and an Ag/AgCl reference electrode at the scanning rate of 1 mV/s.

Results

Isolation and Identification of Z7

A pure culture, named as strain Z7, was isolated from the anodic biofilm of a stable running MFC inoculated with aerobic sludge. Solid medium containing ferric citrate as electron acceptor was chosen for screening. Colonies grown on ferric citrate medium were round, flat, reddish brown in center, light gray edge, and 2 mm in diameter after 2 days cultivation, similar to that observed in the *Citrobacter* genus [32, 33]. The cells of strain Z7 are gram-negative and rod-shaped, 1–5 μm length, and 0.4–0.7 μm width. It could be motile by means of clear flagella and lateral as well as branched filaments. A near-full length 16S rDNA sequence (1,409 bp) of strain Z7 was obtained for phylogenetic analysis. Phylogenetic tree of strain Z7 indicated that the 16S rDNA sequence similarity was almost close to the strain of *C. freundii* M59291.1 (99.0 %). The results exhibited strain Z7 was capable of utilizing citrate, acetate, dulcitol, glycerol, myo-inositol, lactulose, D-lyxose, maltitol, D-melibiose, palatinose, D-raffinose, L-sorbose, sucrose, glucose, rhamnose, and lactose as energy sources. However, the strain did not use malonate, D-tartrate as nutrients (Table 1). It was indicated that the isolate was the closest related to *C. freundii* species in term of the carbon sources utilization. Above all, all characteristics suggested that strain Z7 could be identified as a member of *C. freundii*.

Electricity Generation by Strain Z7

After inoculating strain Z7 into the single-chamber air-cathode MFC, the MFC went through 15-h lag phase. Afterwards, the voltage was increased (Fig. 1a). After eight batches, the voltage output became stable and reached 0.255 V when the cell was operated under the conditions of 30 °C, 1,000 Ω external resistance, and 30 mmol/L sodium citrate. It could be found that the electricity-generation time was around 30 h. When the output voltage became

Table 1 Physiological and morphological characteristics of strain Z7 and the most closely phylogenetically related species of the genus *Citrobacter*

Characteristic	Strain Z7	*C. freundii*	*C. braakii*	*C. werkmanii*
Cell length (μm)	1–5	1–5	NT	NT
Cell shape	Rod	Rod	Rod	Rod
Motility	+	+	+	+
Citrate	+	+	+	+
Use of carbon sources and electron donors				
Malonate	−	−	−	+
Acetate	d	d	d(+)	d(+)
D-Tartrate	−	−	−	+
Dulcitol	+	−	d	−
Glycerol	+	+	+	+
Myo-Inositol	+	+	−	−
Lactulose	+	+	d	−
D-Lyxose	+	d(+)	d	+
Maltitol	d	d	d	−
D-Melibiose	+	+	+	−
Palatinose	d	d	d	−
D-Raffinose	+	d(+)	−	−
L-Sorbose	+	+	−	+
Sucrose	+	+	−	−
Glucose	+	+	NT	NT
Rhamnose	+	NT	NT	NT
Lactose	+	NT	NT	NT
Sodium malonate	−	NT	NT	NT

Data for *C. freundii*, *C. brakii*, and *C. werkmanii* were obtained from reported references [29, 33]
"+"positive reactions for 83 %;
"–"negative reactions for 83 %;
"d" positive for 18~83 % in 2 days, positive for 83 % in 3~4 days; *NT*,not tested

stable (0.255 V), polarization curve was obtained by varying external resistance from 400 to 10,000 Ω (Fig. 1b). It was indicated that the 204.5-mW/m^2 maximum power density produced by strain Z7 was achieved when the external resistance was set at 700 Ω. To determine the role the anode biofilm played, the electrodes potentials against a reference electrode (Ag/AgCl) were monitored (Fig. 1c). It was demonstrated that the anode potential increased with the rise of current density, while the cathode potential decreased. The results implied that the anodic biofilm was enriched on the anode and the electricigens existed in the biofilm would attributes more negative for the anode potential which resulted in the more significant difference of potential for MFC.

Anodes with and without biofilms of the MFCs were examined by SEM technique (Fig. 2). After 1-month operation, the anode of the MFC was observed using SEM. It showed that a thick biofilm was developed on the surface of the anode. The biofilm consisted of rod-shaped bacterial cells, which were in consistent with the morphology of strain Z7 (Fig. 2b, c). In addition, cells seemed to connect with each other and pilus-like appendages and extracellular polymeric substance (EPS) existed between cells and electrodes (Fig. 2d).

In order to further investigate the ability of *C. freundii* Z7 to generate electricity from other electron donors, another six kinds of substrates including glucose, glycerol, lactose, sucrose, rhammose, and acetate were selected at the initial concentration of 30 mmol/L. The maximum power density of 120.4 mW/m^2 was achieved from glycerol, 81.3 mW/m^2 from sucrose,

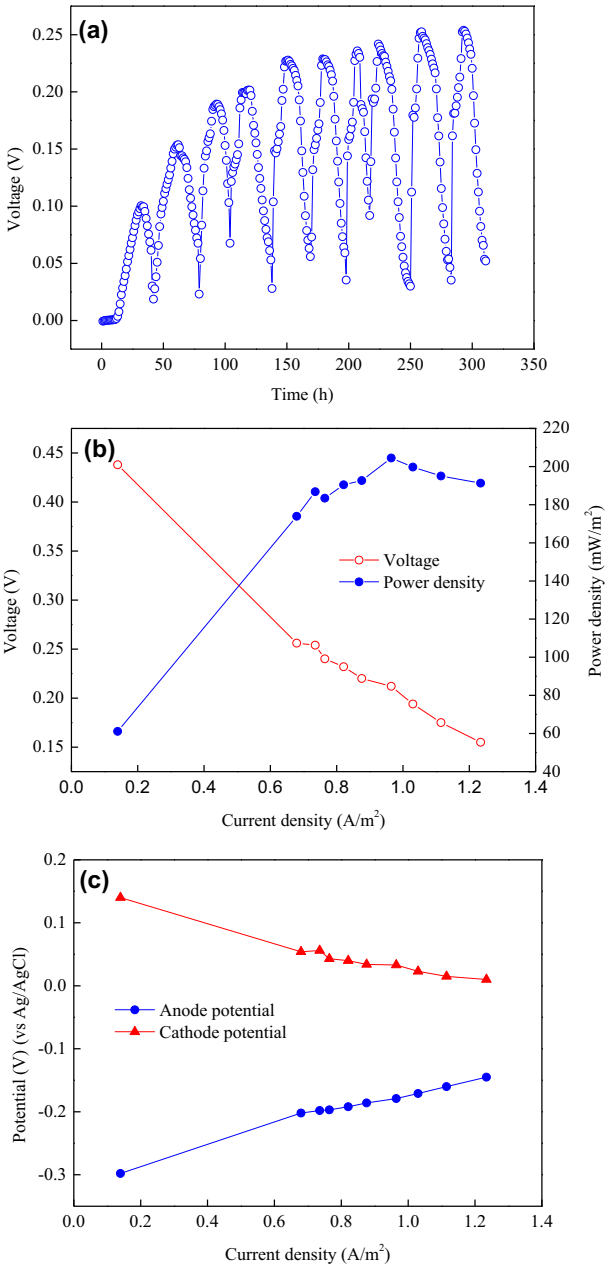

Fig. 1 Electricity generation (**a**), polarization curve (**b**), and electrodes potentials (**c**) of the MFCinoculated with *C. freundii* Z7

68.8 mW/m^2 from rhamnose, 63.7 mW/m^2 from lactose, and 41 mWm^{-2} from glucose; however, no power was produced from acetate (Fig. 3). Under the same conditions of 30 °C, 1,000 Ω external resistance, and 30 mmol/L substrate, the higher output voltage, the greater the maximum power density.

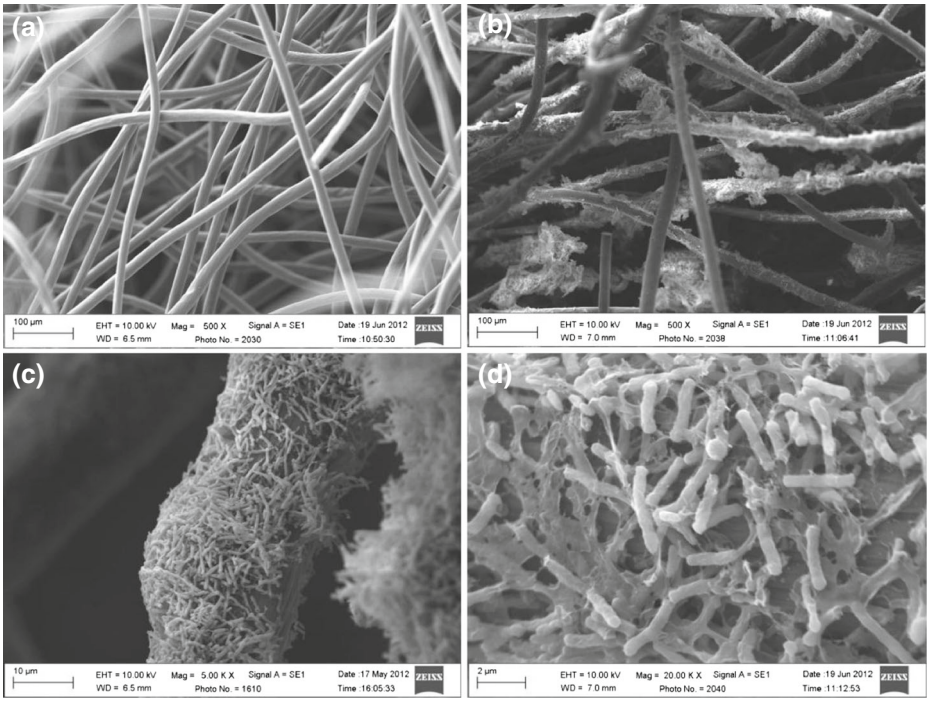

Fig. 2 SEM images of new anode (**a**) and anodic biofilm (**b**, **c**, and **d**)

CE was also calculated based on the COD removal and the total Coulombs in a complete cycle. CE of the MFC utilizing sodium citrate as energy source was the highest 29.8 %, glycerol was followed by 19.35 %, glucose, lactose, rhamnose was 3.59, 2.47, 2.37 %, respectively (Fig. 3). A possible reason for this phenomenon might be attributed to the

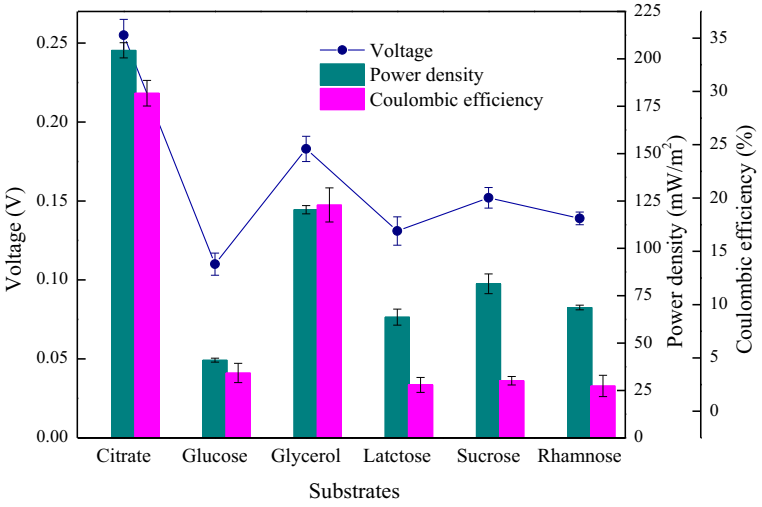

Fig. 3 The generated voltage, maximum power density, and coulombic efficiency when various substrates

different substrate degradation pathways of the strain Z7, fermentation and/or methanogenesis was therein to reduce the number of electrons flowing to the electrode [31, 34].

CV Analysis

The electrochemical activity of *C. freundii* Z7 was firstly determined by means of CV after growing under anaerobic conditions. CV of the cell suspension of strain Z7 using ferric citrate which not only provided the nutrients but also played an important role as the reducing substrate for the strain showed a pair of oxidation and reduction peaks (Fig. 4a). The reduction peak and the oxidation peak were observed at −155 mV (−130~−180 mV) and 170 mV (140~200 mV) vs the Ag/AgCl reference electrode, respectively. The asymmetric peaks indicated that quasi-reversible redox reaction may occur in the cell suspension. However, no apparent redox peaks were observed when strain Z7 suspension utilizing ferric citrate as electron donor was employed for CV test.

The color of anode solution in the MFC inoculated with strain Z7 became yellow along with the electricity production from sodium citrate. CV tests of the anode solutions in complete cycles were performed to investigate whether the cells transfer electrons to anode via secreting intermediates or not. The results showed that pairs of oxidation/reduction peaks were appeared, which implied that quasi-reversible redox reactions might occurred (Fig. 4b). Conversely, the control group of the fresh medium did not show any peaks in CV curve.

In order to evaluate biocatalytic properties of the developed anodic biofilms, in situ CV tests of MFC at different electricity-generating stages were executed. A pair of oxidation/reduction peaks appeared at −120 mV (−80~−60 mV) and −60 mV (−40~−100 mV), respectively (Fig. 4c). The redox peaks of the voltage-decreasing stage were more obvious than that of the increasing stage. The redox peaks observed in both samples almost appeared at the identical locations. It was implied that the strain Z7 might secrete certain electrons transfer mediators which tended to be accumulated during the electricity-generating process.

Effects of Filtered Anode Solutions and Quinone Addition on MFC Performances

To further investigate the electron transfer mechanism, filtered anode solutions of complete cycles were fed back to the MFC with the fresh medium (1:1, *v*/v*)*. In a complete electricity-generation stage, the output voltage was a little bit higher than that of feeding only fresh medium (Fig. 5a). It might be explained for the mediator-like substance in the anode solution secreted by strain Z7.

Moreover, after addition of 50 μmol/L AQDS, the output voltage was increased rapidly up to 0.41 V, with 0.16 V higher than that of no addition of AQDS (Fig. 5b). When the addition of AQDS was stopped, the output voltage of the MFC returned back to the normal level immediately, which indicated that AQDS, well-known as electron transfer mediator [2, 11], could promote the electricity production from sodium citrate biocatalyzed by strain Z7.

Discussion

Citrobacter sp., a genus of gram-negative *coliform* bacteria in the γ-*proteobacteria* class, *Enterobacteriales* order and *Enterobacteriaceae* family, is the facultative aerobic and widely survive in environment, such as soil, water, and sewage. *C. freundii* plays an important role in

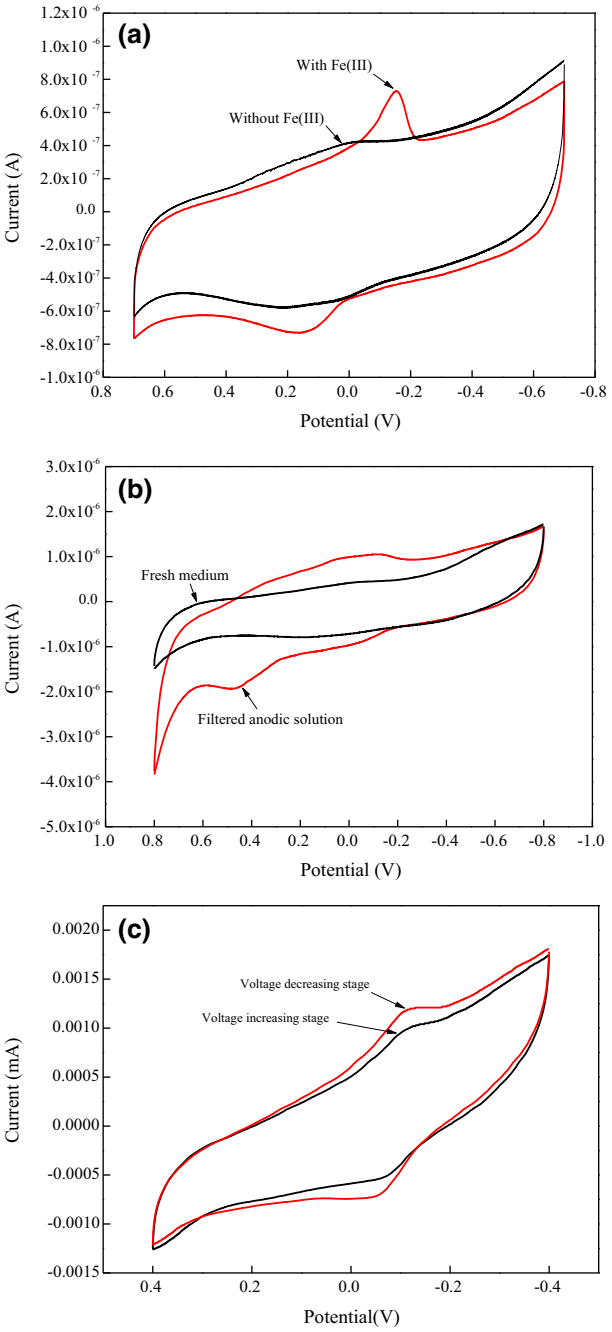

Fig. 4 CV analysis of *C. freundii* Z7 cells suspension (**a**), filtered anode solutions (**b**), and anodic biofilms (**c**)

environment [35]. Few *Citrobacter* species have been reported in the field of microbial fuel cells. Recently, *Citrobacter* sp. has been detected in the anodic biofilms of MFCs inoculated

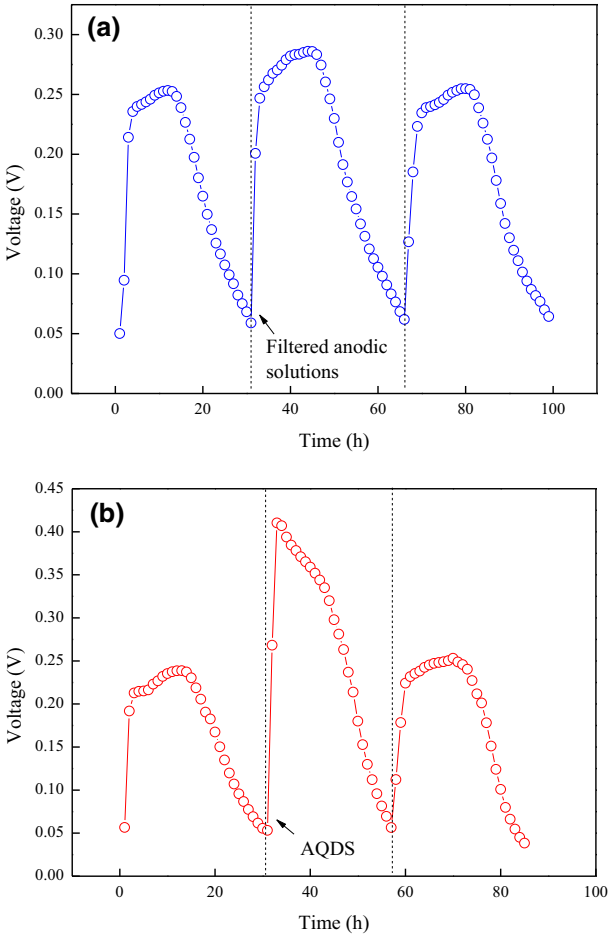

Fig. 5 Voltages of MFC before and after adding filtered anodic solutions (**a**) and exogenous AQDS (**b**)

with natural environmental samples by DGGE analysis [36, 37]. Xu and Liu isolated *Citrobacter* sp. strain SX-1 from a single-chamber air-cathode MFC [6]. Up to date, there is no record on isolation of *C. freundii* which is capable of electricity generation from various substrates from anodic biofilm.

In terms of the electricity-generating characteristics of *Citrobacter* sp., there are only few reports on this topic. The recent reported *Citrobacter* sp. SX-1 could generate the highest current density of 205 mA/m^2 from sodium citrate in a single-chamber air-cathode MFC [6]. In this study, compared to other electricigens reported previously, the strain we isolated show superior advantages over others. Firstly, the strain *C. freundii* Z7 isolated from anodic biofilm obtained the maximum output voltage and the maximum power density of 0.26 V and 204.5 mW/m^2 from sodium citrate in a single-chamber air-cathode MFC, respectively (Fig. 1). Secondly, some electrogens, such as *Shewanella* and *Geobacter*, are only able to oxidize few kinds of

substrates, however, the isolated strain Z7 could utilize kinds of substrates to generate electricity, including glucose, glycerol, lactose, sucrose, and rhammose (Fig. 3). In brief, the isolated strain Z7 can use a wide range of substrates to generate electricity and possesses perfect electrochemical activity. Moreover, the exocellular electron transfer mechanisms of *C. freundii* Z7 were proposed in this study. CV analysis of the filtered anode solutions indicated that two kinds of redox substances might exist in the solutions. It was implied that the strain Z7 may secret mediators to promote extracellular electron transfer. To further investigate the possible electron transfer mechanism, filtered anode solutions of complete cycles were fed back to the MFC along with the fresh medium. The increase of the resulting voltage after adding filtered anode solutions provided a deeper evidence that mediators may be secreted to promote the electricity generation by the strain Z7 (Fig. 5a). It has been proven that some kinds of exoelectrogens are capable of secreting mediators to enhance electron transfer. *Pseudomonas aeruginosa* strain KRP1 secreted pyocyanin and phenazine-1-carboxamide; *Shewanella* secreted flavins that mediated extracellular electron transfer; *Lactococcus lactis* could improve electron transfer by secreting 2-amino-3-dicarboxy-1,4-naphthoquinone (ACNQ); *Klebsiella* sp. ME17 secreted quinones during electricity production [11, 27]. Xu and Liu claimed that *Citrobacter* sp. SX-1 might transfer electrons via self-secreted redox-active compounds during the electricity production [6].

Quinones, especially AQDS, are widely used in MFCs to improve power production as an electron mediator [2, 11]. In this study, effects of exogenous AQDS on power generation from sodium citrate biocatalyzed by the strain Z7 were investigated. The increase of generated voltage indicated that AQDS could promote the electricity production (Fig. 5b). However, further investigations could be focused on identification of the secreted mediators by the strain Z7, which can be realized by using qualitative identification techniques, such as high-performance liquid chromatography (HPLC) or gas chromatography–mass spectrometry (GC-MS). The other concern is to understand the electron production and transfer with the preferential substrate in the process of metabolisms.

Besides secreting mediators, electricigens might transfer electrons via cytochromes on the cytomembrane and/or pili. It was known that *Shewanella* and *Geobacteraceae* could transfer electrons through the cytochromes on the cytomembrane [24]. Previous study also indicated that pilus-like appendages and EPS played an important role in the formation of anode biofilms [38]. Moreover, it had been confirmed that *Shewanella oneidensis* and *Geobacter sulfurreducens* was able to transfer electricity over long distances through secreted pilin filaments [25, 28, 38]. In this study, SEM investigations showed that the thick biofilm was developed and the anode surface was covered by strain Z7; pilus-like appendages and EPS was also observed (Fig. 2 c, d). However, further work is required to determine whether these structures especially the pilus and filaments are conductive or not [25, 28].

An electricigen of strain Z7 was isolated from anodic biofilm of an air-cathode single-chambered MFC inoculated with aerobic sewage sludge. Strain Z7 was identified as a member of *C. freundii* based on 16S rDNA gene sequencing and morphological and biochemical characterization. C. *freundii* Z7 had relatively high electrochemical activity and could produce electricity from a wide range of substrates including sodium citrate, glucose, glycerol, lactose, sucrose, and rhammose. C. *freundii* Z7 could generate maximum power density of 204.5 mW/m^2 from citrate. The strain Z7 might transfer electrons indirectly via secreted mediators.

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