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# Isolation of a facultative anaerobic exoelectrogenic strain LZ-1 and probing electron transfer mechanism *in situ* by linking UV/Vis spectroscopy and electrochemistry

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ABSTRACT

A new facultative anaerobic exoelectrogenic strain LZ-1, belonging to *Citrobacter freundii*, has been isolated. This strain can produce current densities of 843.9 and 865.6 μA $cm^{-2}$ using citrate or acetate as carbon source in a three-electrode configuration. The electricity generation performance was also analyzed in a dual-chamber MFC system, reaching a maximum power density of 1233 mW $m^{-2}$. In addition to acetate and citrate, other carbon sources such as pyruvate, formate, acetate, citrate and fumarate could also be utilized to produce current by strain LZ-1. Data supports the presence of electroactive *c*-type cytochromes in *C. freundii* sp. when grown on ITO electrodes, by linking spectroscopy and electrochemistry *in situ*. Since facultative strains possess many desirable properties compared to anaerobic strains, strain LZ-1 represents a promising exoelectrogenic species in engineering of biological catalysts for microbial electrochemistry.

## 1. Introduction

Microbial fuel cells (MFCs) show promising potential applications in wastewater treatments, due to the ability of MFCs to directly couple organic matter degradation to electricity production using the activity of exoelectrogens (Call et al., 2011; Pandey et al., 2016; Zhou et al., 2015). Exoelectrogenic species such as *Geobacter sulfurreducens, Shewanella putrefaciens, Pseudomonas aeruginosa, Rhodopseudomonas palustris, Gluconobacter oxydans and Rhodoferax ferrireducens* have been reported as possible biocatalysts in MFCs (Bond et al., 2002; Chaudhuri et al., 2003; Kalathil et al., 2016; Kim et al., 1999; Navanietha Krishnaraj et al., 2014; Rabaey et al., 2004; Xing et al., 2008). To date, a subset of exoelectrogenic strains are reported to be facultative, including *Bacillus subtilis* (Nimje et al., 2009), *Acidiphilium cryptum* (Borole et al., 2008), *Rhodoferax ferrireducens* (Chaudhuri et al., 2003), *Shewanella putrefaciens* (Kim et al., 2002) *Pseudomonas aeruginosa* (Rabaey et al., 2004) and *Klebisella pneumonia* (Deng et al., 2010). In general, representatives of the genus *Shewanella* are more convenient to study and easier to genetically manipulate than anaerobic strains such as *Geobacter, and Shewanella oneidensis* MR-1 is one of the most widely studied species for potential applications in bioremediation, heavy metal leaching and carbon cycling of freshwater sediments (Millo et al., 2011; Nielsen et al., 2010).

However, the electricity producing capability of *Shewanella* is much lower than that of strictly anaerobic *Geobacter* strains under the same conditions (Jung, 2012). Therefore, there is a need for novel facultative exoelectrogens with higher ability for electricity generation. Members of the facultative *Citrobacter* genus are usually studied for their clinical applications (Campos et al., 2015; Kataria et al., 2015). Recent work reported isolation and electricity generation by *Citrobacter* in MFCs, suggesting that *Citrobacter* could be a good biocatalysts candidate for MFC-like applications (Huang et al., 2015; Liu et al., 2015a; Nguyen et al., 2014; Xu et al., 2011). To the best of our knowledge, there is no report about the electron transfer mechanism between *Citrobacter* and an electrode. *In situ* spectroscopy linked with electrochemistry is a non-destructive technique that has been successfully applied to study the electron transfer mechanisms of *Geobacter* or *Shewanella* species (Collinson et al., 1992; Liu et al., 2012, 2011; Nakamura et al., 2009; Busalmen et al., 2008). In this work, the strain *Citrobacter* strain LZ-1 was isolated from wastewater and its electricity generation ability and electron transfer mechanism were investigated. Along with high current densities, evidence for functioning *c*-type cytochromes in living *Citrobacter freundii* sp. biofilms on ITO electrode surfaces is provided by linking spectroscopy and electrochemistry techniques.

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## 2. Materials and methods

### 2.1. Growth medium, inoculation and cultivation

Domestic sewage from a wastewater treatment plant in Yangling of Shaanxi (China) was used as inoculum (with chemical oxygen demand (COD) of 315 mg $L^{-1}$). The growth medium (per liter) containing 0.6 g KCl, 1.5 g $NH_4Cl$, 0.3 g $KH_2PO_4$, 0.1 g $MgCl_2$, 0.1 g $CaCl_2$ and 10 mL of trace element solution as well as 10 mL of vitamin solution was prepared as the previously described (Lovley et al., 1988). 20 mM acetate sodium and 40 mM fumarate were used as electron donor and acceptor, respectively. All media (pH 7.2) were sterilized and cell growth was conducted at 30 ± 1 ℃ with air or under high purity nitrogen saturated environment. All experiments were performed in triplicate. *G. sulfurreducens* PCA, purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, was cultured in the growth medium.

### 2.2. Chemicals and electrode materials

The whole chemicals used here were of analytical or biochemical grade. Polished polycrystalline carbon sheets (geometric surface area of 2.60 $cm^2$) connected by a copper wire were used as working or counter electrodes, respectively. Indium tin oxide (ITO) coated glass (square resistance of 12–15 Ω $cm^{-2}$) with a geometric surface area of 3.5 $cm^2$ was used as the working electrode for spectral analysis. All solutions and electrode materials were sterilized under UV light or autoclaved.

### 2.3. Half-cell experiments

Electrochemical experiments were performed by using a three-electrode system, consisting working electrode, counter electrode and saturated calomel reference electrode (SCE, $Hg/Hg_2Cl_2$ saturated KCl, +0.244 V *vs*. hydrogen standard electrode (SHE)) at 30 ± 1 ℃. All potentials are versus SCE unless otherwise stated. In addition, all electrochemically active biofim (EAB) growth steps were carried out by a 16-channel potentiostat (BioLogic Science Instruments, France) at a constant potential of 0.30 V using chronoamperometry. The anaerobic bioreactor was purged with high purity nitrogen for at least 20 min before running.

The polished graphite with geometric surface area 2.60 $cm^2$ was used as working electrode in three-electrode system. The total biomass of both *G.sulfurreducens* PCA and strain LZ-1 were tested by the dying method with coomassie brilliant blue when mature biofilms formed on graphite electrode with the highest current densities (Sedmak et al., 1977). All experiment was repeated for three times and the mean value was evaluated to eradicate any discrepancies.

### 2.4. Isolation and identification of the exoelectrogenic strain

To select facultative anaerobes, 5 mL wastewater and 40 mL medium were stirred with air for one week to reduce the presence of anaerobic bacteria and then were transferred into the growth medium with 5% inoculation under anaerobic condition for 4 days at 30 ± 1 ℃. Finally, as-obtained facultative cells were used as inoculation for further screening of electrochemically active cells using chronoamperometry in the three-electrode system.

Once the EAB was enriched on the working electrode, the EAB was detached by strongly shaking and then incubated in the growth medium. Then growth medium was used for ten-fold serial dilutions up to $10^8$ and each diluted solution was plated on a petri dish with solid media (1.5% agar) for isolation. The single purified cells were used as template for PCR analysis with the universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) and the 16S rDNA gene sequences were compared as the previous report (Deng et al., 2015).

### 2.5. MFCs experiments

For the dual-chamber MFCs, each chamber had a volume of 150 mL and a magnetic stirring bar was at the bottom. In the anode chamber, a carbon rod with geometric surface area of 11.1 $cm^2$ was used as anode. Anolyte consisted of 100 mL growth medium containing 20 mM citrate and 20% inoculum using the isolated strain LZ-1 or *G. sulfurreducens* PCA, respectively. Meanwhile, a house-built carbon brush was used in the cathodic chamber, which contained 50 mM potassium ferricyanide and 0.1 M phosphate buffer solution to simplify the process and avoid oxygen effects. The output voltages of MFCs were recorded using Keithley instrument (model 2400) with 1000 Ω resistance connected in the circuit. All measurements were run at 30 ± 1 ℃. All MFCs experimental data were from at least in triplicate, using individual MFC design.

### 2.6. Morphological analysis

The morphological analysis of EAB on carbon electrode, grown for 2 days with 400 μA $cm^{-2}$ was studied on a Hitachi S-3400N SEM (Hitachi, Tokyo, Japan) using previous methods (Liu et al., 2014). A Nikon A1R confocal laser scanning microscope (CLSM, Nikon, Japan) was used to image the biofilm-covered ITO electrodes. The biofilm was stained and measured as the previously reported (Liu et al., 2010).

### 2.7. UV/Vis spectroscopic analysis

A house-built bioreactor using a quartz cuvette with 50 mL volume was used to grow the biofilms on the ITO electrodes. Absorption spectra of the strain LZ-1 biofilm covering ITO electrodes were recorded between 200 and 800 nm wavelength on an Agilent Cary 60 UV–vis spectrometer under step-wise potentials of 50 mV from 0.3 to −0.6 V (Liu et al., 2012) using 16-channel potentiostat (BioLogic Science Instruments, France).

## 3. Results and discussion

### 3.1. Enrichment of facultative exoelectrogenic bacteria, isolation and identification of strain LZ-1

Once EAB were enriched from wastewater, the EAB were used to isolate the facultative exoelectrogens by streaking using agar medium and re-isolating colonies at least 5 times (Figs. S1–S3). The single strain named LZ-1 was analyzed and identified using PCR through 16S rDNA sequencing. Comparison with sequences in GenBank suggested that the facultative strain LZ-1 belongs to the genus *Citrobacter* and is related to *Citrobacter freundii* ATCC $8090^T$ (with similarity of 99.78%) (Fig. S4).

Chronoamperometrometry demonstrated that the maximum current density (880.0 μA $cm^{-2}$) was higher than that of *G. sulfurreducens* PCA under similar conditions (605.7 μA $cm^{-2}$) (Fig. S5). The strain LZ-1 could produce current from more different carbon sources than standard *G. sulfurreducens* strain (Fig. S6 and Table S1). In addition, the maximum current density of strain LZ-1 oxidizing citrate was only 3% higher than that using acetate. Meanwhile, various carbon sources such as formate, pyruvate and fumarate as electron donor were also studied, and results are shown in Fig. 1A. Strain LZ-1 could produce a current density between 865 and 925 μA $cm^{-2}$ from acetate, formate, pyruvate and citrate.

The electricity generation performance of strain LZ-1 was further investigated *via* MFCs system using strain LZ-1 or *G. sulfurreducens* PCA as inoculum in the anodic chamber, respectively (Fig. 1B). Fed batch MFCs showed output voltages as high as 0.69 V, and with enough acetate a voltage above 0.5 V could be obtained for at least 600 h. With an external resistance of 1 kΩ, the maximum current density reached 600 mA $m^{-2}$. Previous studies reported an operational voltage of