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Short Communication

The parallel electron transfer pathways of biofilm and self-secreted electron shuttles in gram-positive strain *Rhodococcus pyridinivorans* HR-1 inoculated microbial fuel cell

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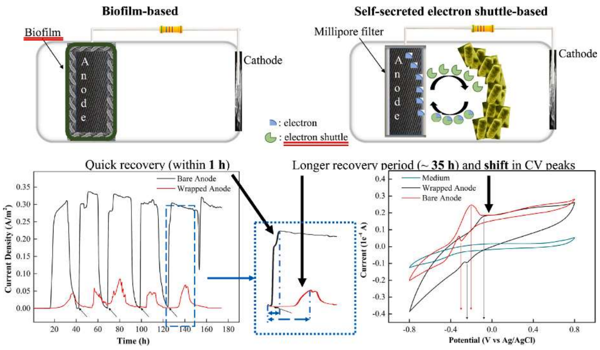
HIGHLIGHTS

- Two electron transfer pathways in *R. pyridinivorans* HR-1 MFC were identified.
- As a pure strain MFC, HR-1 showed quick recovery of current after substrate exchange.
- *R. pyridinivorans* strain HR-1 amplified the member of gram-positive exoelectrogen.
- Co-existing biofilm and self-secreted electron shuttle were practical in real application.

GRAPHICAL ABSTRACT



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ABSTRACT

Microbial fuel cell (MFC) exhibits huge potentials in disposing wastewater and extra energy consumption. Exploring useful microorganisms for MFC is the crucial section. Herein, the electrochemical mechanism of extracellular anaerobic respiration in MFC inoculated with gram-positive *Rhodococcus pyridinivorans* HR-1, was first revealed. The MFC exhibited rapid recovery of currents on anode, and could recover to maximum output within one hour, with redox peaks near −0.38 and −0.18 V through electron transfer between the biofilm and anode. When the biofilm-based pathway was blocked by wrapping the anode with Millipore filter membrane, HR-1 inoculated MFC could still generate electricity within a longer recovery period (~35 h) during analyte

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exchange. This was proposed as a self-secreted electron shuttle pathway for electron transfer in *R. pyridinivorans* HR-1. Cyclic voltammetry analysis revealed that the biofilm-based and self-secreted electron shuttle-based pathways co-existed in *R. pyridinivorans* HR-1 inoculated MFC, which could play synergistic roles in electricity generation.

1. Introduction

Developing clean wastewater disposal method is crucial for urban sustainable development. As one of section, microbial fuel cell (MFC) is a bioelectrochemical device that can transfer chemical energy into electricity with bacteria as anode catalysts (Santoro et al., 2017). Compared to chemical fuel cells, MFCs using biocatalysts are tunable and capable for various substrates (Hatzell et al., 2014). Exoelectrogens, which play the key role in MFCs, are environmental bacteria with electrochemically activity that can transfer electrons from substrates to solid electrodes. Until recently, many species have been explored, most of which belong to *Proteobacteria*, *Firmicutes*, *Acidobacteria*, and *Actinobacteria* (Liu and Li, 2020). Research on electron transfer in MFCs by harnessing these screened exoelectrogens, are mainly focused on gram-negative bacteria, such as *Geobacter sulfurreducens* and *Shewanella putrefacien* (Liu and Li, 2020). Gram-positive bacteria have been recently reported to exhibit electrochemically activity in communities of *Thermincola* sp. and *Corynebacterium sp.* (Lusk *et al.*, 2018). However, there are few studies on MFCs engineered with gram-positive bacteria, and the mechanism of extracellular electron transfer remains confusing.

Understanding how electrons were transferred to the electrodes is essential to enhancing the device efficiency and sustainability in MFCs. When inoculated into the MFC chamber, exoelectrogens (e.g. *P. Shewanella*) can adhere on the anode surface to form biofilm (Logan et al., 2006). These bacteria extend the respiration chain into the outer membrane, with the electron-transport proteins accumulated on the cell envelope surface, which can mediate electron transfer to the electrode (Sun et al., 2016). However, due to the contact inhibition of microbes, the limited surface areas prevent some exoelectrogens from establishing direct contact with the anode. Electron shuttles are small organic molecules that can mediate electrons between the anode and the microbe surface (Luo et al., 2020). Recent studies reported that *Pseudomonas* sp. and *Desulfitobacterium* sp. inoculated MFCs could operate steadily with addition of soluble electron shuttles (Wan et al., 2019). However, these electron shuttles, such as phenazine, was highly toxic and non-recyclable during cation exchange (Qiao et al., 2017). Gram-positive bacteria have characteristic peptidoglycans and teichoic-acids in cell wall, which are considered to be unsuitable for electron shuttle during extracellular electron transfer. How to evaluate the existence of electron shuttles in the presence of biofilm is of great value in practical application. Biofilm adhered firmly on the anode surface to initiate a stable operation while electron shuttles could mediate distant electron transfer, which allowed MFC to exceed the limitation of anode surface area to achieve higher output. *Rhodococcus pyridinivorans* HR-1 provided an alternative that could transfer electrons in MFCs without exogenous electron shuttles (Cheng et al., 2018). This thermophilic exoelectrogen could operate at elevated temperatures in extreme conditions, thus producing higher levels of current and Coulombic efficiency due to its high-intensity metabolism and inactivation capability of contaminating bacteria (Yin et al., 2019). Besides, gram-positive strains also showed unique potentials in environmental remediation such as metal detoxification (Zhang et al., 2021), and antibiotic resistance genes reduction (Chen et al., 2021).

Despite a high performance achieved in *R. pyridinivorans* HR-1 inoculated MFC, for engineering the electrochemical reactors and electrode materials which requires fundamental information, the electrochemical process from the exoelectrogen biomembrane to extracellular anode remains nebulous. In this study, *R. pyridinivorans* HR-1 was inoculated routinely to demonstrate the formation of biofilm. Meanwhile, in the experiment group, the anode was tailored with Millipore filter to inhibit the formation of biofilm so as to detect the existence of self-secreted electron shuttles. The electrochemistry of two devices was comprehensively characterized by polarization curve and cyclic voltammetry analysis. Two parallel electron transfer pathways on HR-1 inoculated anodes under different conditions were proposed.

2. Materials and methods

2.1. Culture preparation

R. pyridinivorans HR-1 was isolated from a leachate inoculated MFC and then cultivated in lab. The purified strain was inoculated into the MFC to verify its power-generation capacity. The anolyte medium was consisted of a phosphate buffer solution (PBS) and 1.0 g/L of substrate, and the PBS contained the following compounds (per liter): 2.93 g KH_2PO_4, 5.87 g K_2HPO_4, 0.3 g $FeSO_4$, 2 g NaCl, 5 g $(NH_4)_2SO_4$, 10 mL of trace mineral solution. To eliminate the interfere of O_2, the medium was dispensed into culture bottles under inflation of N_2 for 20 min.

2.2. MFC construction

A single-chamber air cathode microbial fuel cell was constructed as described by Milliken with an external resistor of 1000 Ω. The available volume was 28 mL and the anode was unilaminar carbon cloth with an effective area of 6.7 cm^2. The carbon was connected to a titanium wire using conductive silver epoxy. The chamber was assembled with a cation exchange membrane (Nafion 117) clamped to a carbon-platinum (10 %) cloth cathode. Since *R. pyridinivorans* HR-1 was not an obligate anaerobe, the MFC compartment was UV-disinfected for at least 24 h before inoculation. The MFC was inoculated with a 5 % (v/v) culture transfer from *R. pyridinivorans* grown on sodium acetate and insoluble iron-hydroxides with 100 % culture transfer at 30 °C, and the medium exchange for anode exoelectrogens occurred when the output voltage dropped below 20 mV. The voltage was measured for every 15 min.

2.3. Electrochemical analysis

The voltage output was monitored using a Keithley multichannel data-acquisition system (Keithley 2750, USA), and the electric current was calculated by Ohm's law. The current and power density were normalized according to the working area of the electrode. To obtain a polarization curve, the external resistance was varied using a slide rheostat, and the voltage at each resistance was recorded after the output data was stable for 2 min. To reduce deviation, the experiments were repeated three times. To further explore the existence of the electron-shuttle mechanism, the anode was wrapped with a microporous membrane (MF-MFC) before inoculation to prevent direct contact between the exoelectrogen and electrode. The microporous membrane (Aladdin, Shanghai) allowed the solution to move freely, which could only result in a trivial change to the MFC's internal resistance. Meanwhile, the pore size (0.22 μm) was smaller than that of the bacteria, which would impede the formation of a biofilm.

The CV result was obtained using a potentiostat (CHI1010, Shanghai) at a scan rate of 2 mV/s within the range from −0.8 V to 0.8 V. To prevent the external interference, the chamber was purged with pure filtered N_2 for 15 min to remove O_2 before test. During the experiment, the anode was set as the working electrode, the cathode as the counter electrode, with an Ag/AgCl (assumed + 197 mV versus standard hydrogen electrode) electrode (MF-2052, BAS) as the reference electrode.

2.4. Scanning electron microscopy (SEM)

Biofilm and anode morphology were detected by SEM (JEM-5410LV, Japan). The pre-treatment process for SEM observation was described as following. The anode carbon cloth was fixed with 2.5 % glutaraldehyde for 4 h, and then washed with 0.1 mol/L PBS for five times. It was dehydrated using different concentrations of alcohol from 30 % to 100 % to replace the dehydrated anode carbon cloth with *tert*-butyl alcohols. Samples were then sputter coated with a SC7640 (Polaron) sputter coater and visualized under SEM.

3. Result and discussion

3.1. Morphology of anode surface

Before inoculation of *R. pyridinivorans* HR-1, the anode was wrapped with 0.22-μm microporous membrane to prevent the formation of biofilm. This could further block the electron transfer through direct contact. SEM was conducted on the anode surface to observe the HR-1 biofilm on anode before and after a continuous operation of MFC (**see supplementary material**). Before the operation, a large crowd of bacteria were observed to adhere on the anode surface, indicating the compatibility of HR-1 on the carbon cloth anode. A HR-1 bacterium in metaphase was further observed on anode, indicating that the strain could proliferate on anode to form a structural biofilm. Since *R. pyridinivorans* HR-1 was a rod-shape bacterium without flagellum, the biofilm of HR-1 should facilitate a direct contact between anode and bacteria that was responsible for electricity generation. In contrast, on the surface of wrapped anode, there was no bacteria found to adhere, indicating that the microporous membrane successfully prevented the biofilm formation on anode. In this case, the HR-1 strain was labile in medium condition near the anode.

3.2. Performance of electricity generation

As shown in Fig. 1a, the maximum current density on wrapped anode was significantly smaller than those on bare anode. Besides, wrapped anode required a much longer period (30 ± 5 h) to recover. These indicated that the pathway based on HR-1 biofilm was more efficient in electron transfer. Interestingly, even without an exogenous electron shuttle in HR-1, the biofilm-blocked MFC could still generate currents with a maximum power density of 0.086 W/m^2. Considering the present mechanism for electron transfer in exoelectrogens, the self-secreted electron shuttles in *R. pyridinivorans* would probably contribute to the electricity generation. Fig. 1b illustrates the polarization curve of the HR-1 inoculated bare anode. The maximum power density was 0.667 W/m^2 under a voltage of 3 V, which was significantly higher than that of wrapped anode. The bare anode was also found to be more stable on maximum voltage and during the whole cycle (Fig. 1a). All of these demonstrated that HR-1 biofilm facilitated a sustainable and powerful electrification process, while self-secreted electron shuttles only assisted this process during the initial stage, or under specific conditions unfavorable to biofilm formation. However, whether the two pathways could appear simultaneously, is to be further confirmed by separating the biofilm-based process from the self-secreted electron shuttle process.

Biofilm is noteworthy during MFC design. It has been reported that the thick bio-membrane rendered gram-positive exoelectrogens internal resistance. Extreme conditions such as high alkaline concentrations or high temperatures could enhance several exoelectrogens to generate electricity (Lusk et al., 2016). Gram-positive exoelectrogens withstood the severe tests and additionally. Since that, explaining the electron transfer in significant in development of MFC inoculated with gram-positive exoelectrogens.

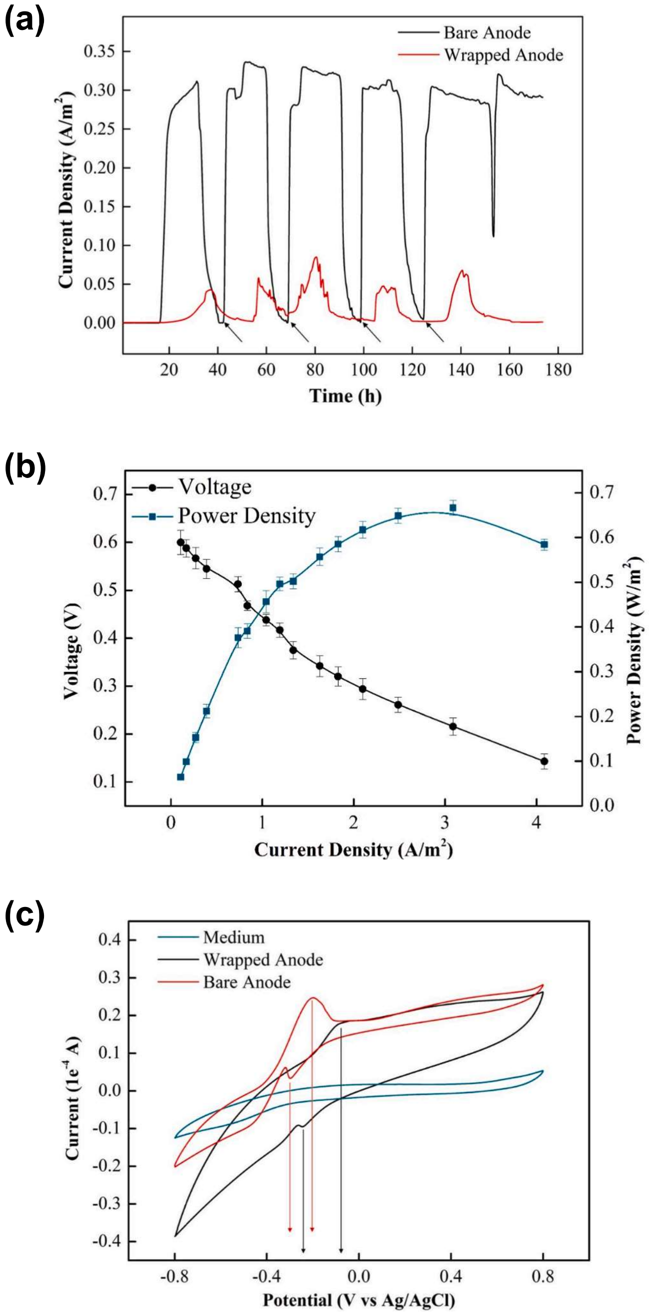

Fig. 1. (a) Sustainable current generation by *R. pyridinivorans* in bare or wrapped anode MFC; (b) Polarization and power density curve. The voltage was measured with sliding rheostat (10^1–10^5 Ω) after 2 min equilibration; (c) Cyclic voltammogram of *R. pyridinivorans* inoculated MFC immediately after anolyte exchange.

3.3. Cyclic voltammetry evaluation of R. pyridinivorans

In the existence of HR-1 biofilm, the electrochemical activity of the self-secreted electron shuttles was hard to separately evaluate. To solve this, CV analysis was conducted on the initial medium, and on bare anode and wrapped-anode after stabilization (Fig. 1c). No redox signals were observed in the medium where the MFC was initially inoculated with the cell suspension of HR-1. After several operation cycles, redox peaks were found at approximately −0.4 and −0.3 V (versus Ag/AgCl). This result was in accordance with previous studies, which explained the electron transfer by the exoelectrogen self-secretion of electrochemically active substances such as riboflavin and pyocyanine (Jin et al., 2019; Yong et al., 2017). The redox peaks of the wrapped MFC were significantly distinguished from those on bare anode, which illustrated that the electrons were transported via a pathway different from biofilm mechanism. Furthermore, even without exogenous electron shuttles in HR-1, the redox signals of two anodes were much stronger as compared to the medium, demonstrating that both the two pathways in *R. pyridinivorans* were ascribed to long-distance electron transfer from extracellular environment to anode. In general, the CV analysis reinforced the co-existence of electron transfer pathways in *R. pyridinivorans* HR-1 inoculated MFC.

Electron shuttles were small molecules as mediators of electron transfer between the electrode and exoelectrogens when they were not physically contacted (Zhou et al., 2022). Understanding the self-secreted electron shuttle in *R. pyridinivorans* HR-1 could provide fundamental to microbial electron mediation, and further apply this strain to other fields such as sewage disposal. External addition of exogenous electron shuttles like Cu (II) and neutral red were high-cost and environment-toxic (Deng et al., 2022). In HR-1 inoculated MFC, biofilm and self-secreted electron shuttles were demonstrated to co-exist, which contributed to the stability during electricity generation. Due to the characteristic of HR-1, when the bacteria suspension was inoculated into the MFC chamber, the non-motile HR-1 would adhere to the anode to form biofilm, which was tightly fixed to facilitate an exchangeable anolyte (Miskan et al., 2016). Meanwhile, the upper-layer labile bacteria were speculated to self-secret electron shuttles to mediate electron transfer. The soluble small molecules improved the electron transportation and resulted in enhancement of power output. Besides, the industrial MFCs were usually designed with large volume, which further require an ample contact between microorganism with stable and sustainable power-generation capacity (Hiegemann et al., 2019). Self-secreted electron shuttles could avoid high-cost and pollutant exogenous addition, and improve the whole electrical performance. Further study is required to characterize the electron shuttles with spectrum analysis of the supernatant, and probe the molecular mechanism behind the self-secreted electron shuttle-based electron transfer.

4. Conclusion

Paralleled electron transfer pathways in a gram-positive exoelectrogen *R. pyridinivorans* HR-1 were demonstrated by wrapping the anode to block the formation of biofilm. Electron transfer of biofilm was based on the direct contact with the anode, which exhibited a quick recovery with anolyte exchange. However, when the formation of biofilm was blocked, currents were also formed after a long period, which revealed that HR-1 could self-secret electron shuttles to mediate electron transfer. Further explorations on the electron transfer pathway with diverse physiologies and phenotypes, are urgent to discover the common features of exoelectrogens that can be employed in real wastewater treatment.

CRediT authorship contribution statement

Peng Cheng: Data curation, Methodology, Formal analysis, Investigation, Writing – original draft. **Yingchuan Zhang:** Data curation, Writing – review & editing. **Nianfang Ma:** Data curation, Formal analysis. **Lining Wang:** Data curation, Writing – review & editing. **Liqun Jiang:** Data curation, Formal analysis. **Zhen Fang:** Supervision, Writing – review & editing. **Yitong Wang:** Data curation, Formal analysis. **Xiangping Tan:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.128514.

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