Short communication

Improving the power generation performances of Gram-positive electricigens by regulating the peptidoglycan layer with lysozyme

Wenrui Shen[a,b,1], Xiaoran Zhao[c,1], Xiaoliang Wang[a], Siqi Yang[a], Xindi Jia[a], Xiaodi Yu[a], Jing Yang[d], Qinzheng Yang[a,b,*], Huazhang Zhao[e,**]

[a] State Key Laboratory of Biobased Material and Green Papermaking, Qilu University of Technology, Shandong Academy of Sciences, Jinan, 250353, Shandong, PR China
[b] Department of Bioengineering, Qilu University of Technology (Shandong Academy of Sciences), Jinan, 250353, Shandong, PR China
[c] State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing, 100191, PR China
[d] School of Electronic and Information Engineering (Department of Physics), Qilu University of Technology (Shandong Academy of Sciences), Jinan, 250353, Shandong, PR China
[e] State Key Lab Plateau Ecology and Agriculture, Qinghai University, Xining, 810016, PR China

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ABSTRACT

The power generation performance of a microbial fuel cell (MFC) greatly depends on the relative amount of electricigens in the anodic microbial community. Running the MFC multiple times can practically enrich the electricigens, and thus improve its power generation efficiency. However, Gram-positive electricigens cannot be enriched well because of their thick non-conductive peptidoglycan layer. Herein, we report a new Gram-positive electricigen enrichment method by regulating the peptidoglycan layer of the bacteria using lysozyme. Lysozyme can partially hydrolyze the peptidoglycans layer of Gram-positive *Firmicutes* to improve the permeability of cell wall, and thus enhance its electricity generation activity. The stimulation of Gram-positive electricigen endows MFCs a high power generation community structure, which results in the power density 42% higher than that of the control sample. Our work has provided a new and simple method for optimizing the anode community structure by regulating weak electricigens in the community with lysozyme.

1. Introduction

Microbial fuel cells (MFCs) are the electrochemical devices that directly convert the chemical energy of organic compounds into electrical energy via the catalytic action of electrochemically active bacteria (EAB) (Lovley, 2008; Luo et al., 2015), and thus have attracted considerable attentions in both renewable energy production and wastewater treatment (Logan, 2009; Zhou et al., 2011). The current generation performance of MFCs is greatly affected by the power generation capacity of anode respiring bacteria (ARB) which is either a pure culture or mixed community (Armato et al., 2019; Singh et al., 2018). Compare to pure cultures, the mixed microbial communities display more stable and robust power generation abilities, and thus are more conducive to wastewater treatments (Dessì et al., 2018; Nevin et al., 2008).

The power generation performance of mixed microbial MFCs is closely related to the relative content of electricigens that transfer electrons directly via cell-surface proteins or indirectly through excreted mediator molecules (Bond and Lovley, 2003; White et al., 2016). Conventionally, the electricigen dominated microbial community can be obtained by running the bioelectrochemical system multiple times, where the Gram-negative electricigens are enriched. However, the power generation performance of this microbial community cannot be further improved by electrochemical enrichment method (Kokko et al., 2018; B. E. Logan and Regan, 2006). In the enriched microbial community, there are a certain amounts of Gram-positive electricigens, in addition to the large amounts of Gram-negative electricigens (Finch et al., 2011; Modestra et al., 2020). They display relatively weak power generation capabilities because of their thick (30–80 nm) and non-conductive peptidoglycan layer (Vollmer and Seligman, 2010). The low permeability of peptidoglycan layer severely limits the shuttle of electron mediator, which affects not only the electricity generation ability,
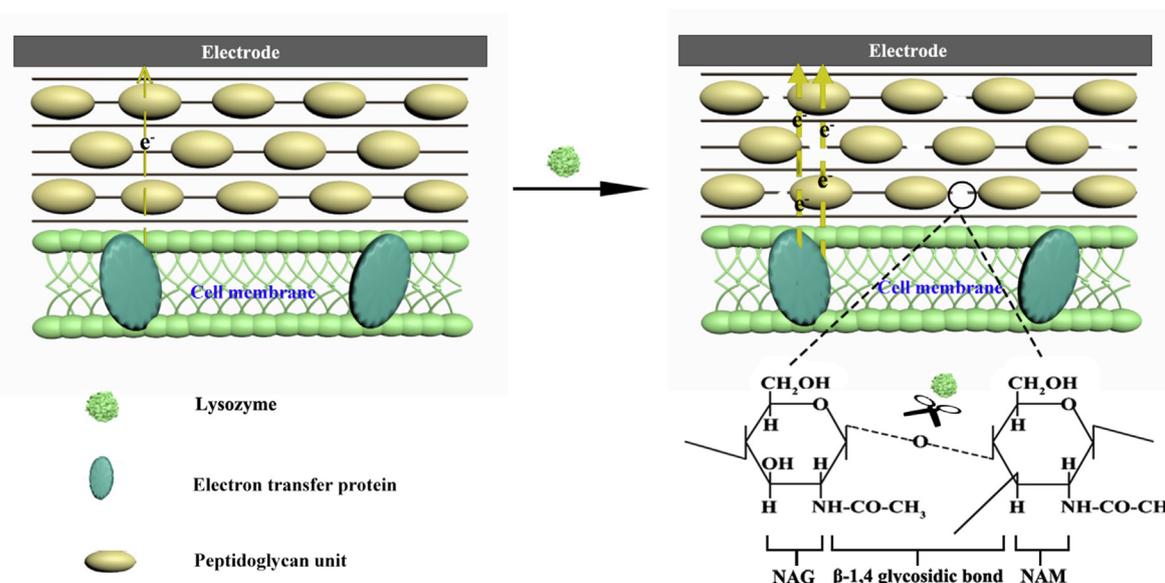
Fig. 1. Schematic preparation process of lysozyme partially hydrolyzes the peptidoglycan layer of Gram-positive bacteria in biofilms, enhancing the efficiency of electron transfer from bacteria to electrodes.

but also the enrichment of these bacteria during the electrochemical enrichment.

The electricigens in a microbial community can be effectively enriched by regulating their activity (Chen et al., 2018; Gavrilov et al., 2012). Various chemical reagents, such as rhamnolipid and tween 80, have been used to improve the permeability of peptidoglycan layer in the cell wall of Gram-positive bacteria, resulting in 2–8 times higher electron transfer efficiencies (Wen et al., 2010, 2011). However, these non-specific chemical reagents also act on the cell wall of Gram-negative bacteria, thus affecting the structure and the electricity production efficiency of the whole microbial community (Yang et al., 2019). Hence, green, mild, and non-secondary contaminating enzyme reagents with specific actions on the polysaccharide layer of Gram-positive bacteria are highly desired to obtain high-performance electricity-generation microbial communities for improving the output power of mixed microbial MFCs (Luo et al., 2015).

In the present work, lysozyme was explored as a such specific enzyme. The effects of lysozyme on the power generation of weak electricigens (Fig. 1) and the structure of mixed microbial communities were investigated. The microbial community was treated with different concentrations of lysozyme, and the residual peptidoglycan contents were determined. The corresponding microbial community structures were imaged by scanning electron microscope (SEM), and the electrochemical performances of MFCs with the microbial communities were evaluated.

2. Material and methods

2.1. Configuration and operation of MFC

Four groups of 12 dual-chamber MFCs, with three parallel MFCs in each group, were fabricated using the double-layer carbon cloth anodes (2×2 cm). Both volumes of the cathode and anode chambers are 26.88 mL. The 5% waterproof carbon paper (2×2 cm) with one side covered with 108 Pt catalyst (0.5 mg/cm^2 with 20% Pt, E-TEK division, USA) was used as the cathodes of the MFCs. Two compartments were separated with a proton exchange membrane (PEM, Nafion 117, Alfa Aesar). M9 salt solution containing sodium acetate (1 g/L NH$_4$Cl, 0.5 g/L NaCl, 17.8 g/L Na$_2$HPO$_4$·12H$_2$O, 0.11 g/L CaCl$_2$, 0.247 g/L MgSO$_4$, 3 g/L KH$_2$PO$_4$ and 2.24 g L^{-1} C$_3$H$_5$O$_3$Na) and aqueous solution containing potassium ferricyanide and potassium chloride (3.756 g/L KCl,

8.426 g/L K$_3$[Fe(CN)$_6$]) were used as the anolyte (Lamberg and Bren, 2016) and catholyte, respectively. The MFCs were fed with a source of mixed bacteria (obtained from the Jinan Wastewater Treatment Plant) and operated with an external resistance of 1000 Ω at 30 $\pm$ 1 °C to generate a stable voltage (about 450 mV). After the voltage of the stable MFCs dropped to 50 mV, the lysozyme (Hen egg white lysozymes w95%, w50,000 units/mg protein, Beijing BioDeeBio technology Co. Ltd.,P.R. China) anolyte was activated by shaking at 35 °C for 30 min. The anolyte in the original MFC was replaced with anolytes containing different concentrations of lysozyme (0 g/L, 1 g/L, 2 g/L, and 4 g/L), respectively, and the mixed bacterial anode was immersed in the lysozyme-containing anolyte for 2 h. The treatment time was set to be 2 h according to the optimization experiment (Fig. S1). Then the lysozyme-containing anolyte was discarded. One carbon cloth was taken out and tested for peptidoglycan content. The anolyte was replaced with the M9 salt solution containing sodium acetate. The data card was connected and the operating voltages of the 4 groups batteries were recorded. The polarization and power density curves were recorded at the steady state of the MFC as the external resistance varied from 10 to 0.1 kΩ.

2.2. Peptidoglycan content of biofilm on MFC anode

A small piece (~0.25 cm^2) was cut from the carbon cloth for peptidoglycan content analysis and shred in the phosphate buffered saline (PBS, 0.27 g/L KH$_2$PO$_4$, 1.42 g/L NaHPO$_4$, 8 g/L NaCl, 0.2 g/L KCl, pH = 7.2). Then anodic mixed bacteria was disrupted by ultrasonication, cooled to room temperature, and centrifuged at 3000 rpm for 20 min. The supernatant was collected for the peptidoglycan content analysis with a PG ELISA Kit (Shanghai Jianglai industrial Limited By Share Ltd., Shanghai, China).

2.3. Microbial community investigation

The biomass on the anode after lysozyme treatment and enrichment was collected, centrifuged at 5000 rpm and 4 °C for 5 min, and extracted with the FastDNA® Spin Kit for Soil (MP Biomedicals, LLC, Illkirch, France) according to the manufacturer's instruction. The extracted total genomic DNA was subject to PCR amplification targeting the 16S rRNA hypervariable regions V3–V4 with the universal bacterial primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGA-CTACHVGGGTWTCTAAT-3′). The bacterial community was analyzed

with an Illumina MiSeq high-throughput sequencing system (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China).

2.4. Morphological analysis

The morphology of the biofilm after the MFCs were run for 65 h was imaged by scanning electron microscopy (SEM) using a HITACHI Regulus 8220 microscope. Firstly, small pieces of carbon cloth (~0.25 cm²) were cut from the MFC anode, fixed in glutaraldehyde solution at 4 °C overnight, washed three times with phosphate buffer brine (PBS, 0.27 g/L KH_2PO_4、1.42 g/L $NaHPO_4$、8 g/L NaCl, 0.2 g/L KCl, pH = 7.2), dehydrated stepwise in a series of ethanol-water solutions with 50%, 70%, 80%, 90%, and 100% v/v concentration, dried in a desiccator, and then imaged by SEM at room temperature (Du et al., 2017).

2.5. Electrochemical measurements

Electrochemical analyses were conducted on a CHI 660E electrochemical working station (Shanghai Chenhua Co. Ltd., China) composed of a three-electrode system. An Ag/AgCl electrode (relative to SHE + 197 mV, saturated KCl), the cathode and anode treated with different concentrations of lysozyme were used as the reference electrode, counter electrode and working electrode, respectively. Electrochemical impedance spectroscopy (EIS) analysis was performed on the anode in the frequency range of 0.01 Hz–100 kHz. The initial voltage was set according to the open circuit voltage (OCV) of each MFC. The cyclic voltammetry was measured at the scan rate of 5 mV s^{-1} in the range from 0 V to −0.80 V (VS. SCE) (Burkitt et al., 2016).

3. Results and discussion

3.1. Hydrolyzation of the peptidoglycan layer of Gram-positive electricigens

To evaluate the hydrolysis effect of lysozyme on the peptidoglycan layer in anodic microbiome, microbial communities were respectively treated with different concentrations of lysozyme and their peptidoglycan contents were measured using a PG ELISA Kit. As shown in Fig. 2, the lysozyme treatment significantly reduces the peptidoglycan
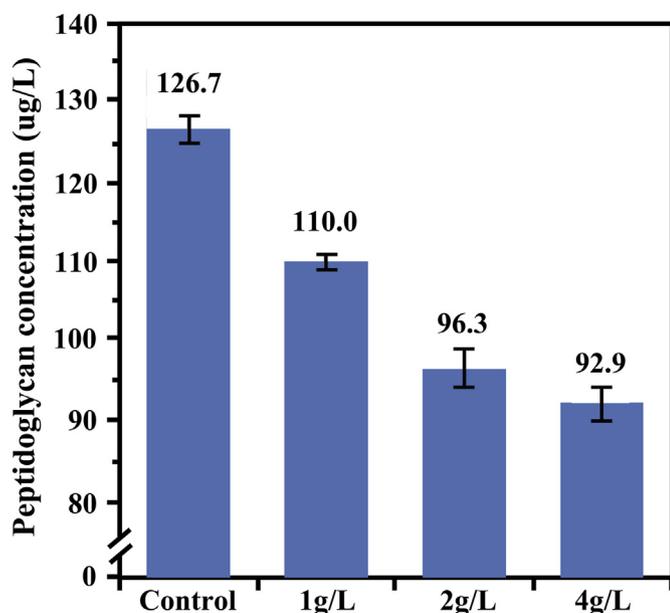
Fig. 2. Peptidoglycan contents of the microbial communities treated with different concentrations of lysozyme.

content of the microbes. The peptidoglycan content of the untreated microbial community is 126.67 μg/L, and is reduced to 110 μg/L, 96.3 μg/L and 92.9 μg/L as the microbes are treated with 1 g/L, 2 g/L and 4 g/L lysozyme, respectively. These results suggest that lysozyme can degrade the peptidoglycan layer of Gram-positive bacteria into the anolyte. The treatments with 1 g/L and 2 g/L lysozyme reduce the peptidoglycan content by 13.1% and 24.0% respectively, which are approximately proportional to the lysozyme concentration. Therefore, the peptidoglycan layers of Gram-positive bacteria are partially degraded by lysozyme and the degradation degree increases with the increase of lysozyme concentration at low levels. The significantly decreased peptidoglycan content by the treatment with 2 g/L lysozyme can greatly improve the cell permeability. However, the degradation degree of peptidoglycan only be increased by 2.73% as the lysozyme concentration increased from 2 g/L to 4 g/L, suggesting that further increasing the lysozyme does not affects the degree of degradation significantly in the experimental period. In all, the peptidoglycan in the cell wall of Gram-positive weak electricigens can be effectively degraded with 2 g/L lysozyme, which may be able to alter the permeability of the cell wall and thus improve their electricity-generating efficiency.

3.2. Effects of lysozyme treatment on anodic microbial population

The mixed microbial community samples on anodes were respectively enriched by running the corresponding dual-chamber MFCs until the voltage became stable to reduce non-electricigens, treated with different concentrations of lysozyme, and then re-enriched by running the MFCs for 65 h. Their microbial populations were determined by the 16S rRNA gene sequencing method (Fig. 3). At the phylum level, the bacterial species in all anode biofilms are dominated by Gram-negative Bacteroidetes and Proteobacteria. Their relative percentages in the anode biofilms of the MFCs treated with 0 g/L, 1 g/L and 2 g/L are 77.49%, 77.78% and 75.60%, respectively, suggesting that these two negative bacteria are the main electricigens for the MFC power generation and the lysozyme treatment does not affect their population. Gram-negative Porphyromonadaceae, Lentimicrobium and Geobacter are also found in the biofilms. The first two genera belong to Bacteroidetesin phylum and the last one is the major genus of Proteobacteria phylum (Fig. S2) (Gustave et al., 2019; Kim et al., 2006; Saratale et al., 2017). The total abundance of these three genera is not affected by the lysozyme treatment significantly, with the values of 42.11%, 42.63% and 45.25% in the biofilms of the MFCs treated with 0 g/L, 1 g/L and 2 g/Llysozyme. These results are in accordance with the previous report that the Proteobacteria and Bacteroidetesin in the mixed bacterial MFC can be enriched by pre-running the MFCs.

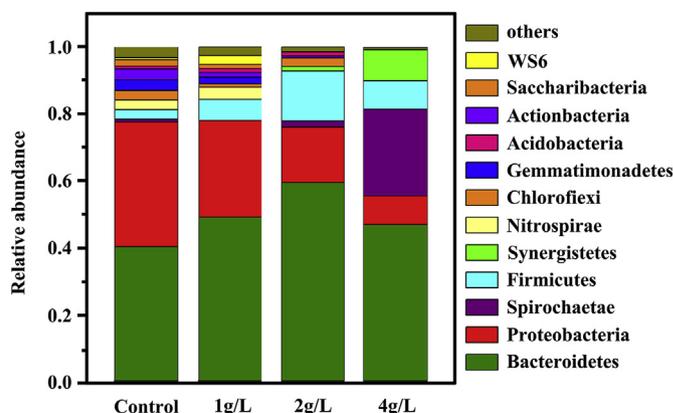The relative abundance of Fermicutes, a Gram-positive weak

Fig. 3. Relative abundances at the phylum level of each microbe found in the anode biofilms treated with different concentrations of lysozyme.

electricigen, is determined to be 2.84% in the control biofilm, and dramatically increases to 6.35% and 14.87% as the biofilm treated with 1 g/L and 2 g/L lysozyme, respectively. The total percentage of the electricigens in the biofilm of MFC treated with 2 g/L lysozyme is 90.47%, significantly higher than that of the untreated MFC (80.33%). These results indicate that the hydrolysis of the peptidoglycan in *Firmicutes* with lysozyme results in local bond rupture and increases the permeability of bacterial cell wall. In addition, at the genus level, *Fimicutes* is mainly composed of *Clostridium* and *Thermincola* (Li et al., 2018; Wrighton et al., 2011; Park et al., 2001). The abundances of *Clostridium* and *Thermincola* increase with the increase of the concentration of lysozyme and reach to 7.57% and 6.35% at the lysozyme concentration of 2 g/L (Fig. S2). These results suggest that the lysozyme treatment stimulates the electricigenic activities of the Gram-positive *Clostridium* and *Thermincola*. Therefore, pre-running a MFC can enrich the microbial population in its anodic biofilm. Our observations are in good agreement with the results reported by Wen et al. that the treatments with Tween 80 and rhamnolipid can improve cell permeability and electrochemical activity of electricigens (Wen et al., 2010, 2011). Further increasing the lysozyme concentration to 4 g/L decreases the abundance of *Fermicutes* to 8.48%. It can be explained that the high concentration of lysozyme hydrolyzes the peptidoglycan excessively, and thus affects the electricity generation activity and the competitive advantage of *Fermicutes*. The Gram-positive non-electricigen, *Actinobacteria*, is found in the anode biofilm of untreated MFC. Its relative abundance decreases from 1.81% to 1.12% and 0.2%, and eventually undetectable with the increase of lysozyme concentration from 1 g/L to 4 g/L. The abundance of the *Arthrobacter* genus that belong to the *Actinobacteria* phylum decreases with the increase of lysozyme concentration (Fig. S2) (Li et al., 2018). These results suggest that the hydrolysis of peptidoglycan layer with lysozyme can effectively inhibit and remove anode non-electricigenic Gram-positive bacteria. Low concentrations of lysozyme can partially hydrolyze the peptidoglycan in the cell wall of the Gram-positive electricigens, which increases the permeability of cell wall without affecting the Gram-negative electricigens. The electricigens in the anode biofilm can be enriched by treating the anode with an appropriate amount of lysozyme and running it in an MFC for a certain period of time.

3.3. Morphologies of anode biofilms treated with different concentrations of lysozyme

The anodes of the MFCs treated with and without lysozyme were imaged by SEM after the MFCs were run for 65 h. As shown in Fig. 4, the anodes treated with 1 g/L and 2 g/L lysozyme are covered with much denser biofilms than that of the control MFC. In addition, the lysozyme treatment also enhances the bacterial adhesion, especially at the lysozyme concentration of 2 g/L. It can be explained that the partial hydrolysis of peptidoglycan in the cell wall of Gram-positive bacteria improve the permeability of cell wall, and thus the bacteria are easily enriched during the operation of MFC. The high amounts of bacteria form the denser and crosslinked biofilms on the anodes (Fig. 4b and c), which are conducive to electron transfer. It is worth noting that the treatment with 4 g/L lysozyme results in a slimy biofilm on the anode surface and less total biomass. These results further suggest that high concentrations of lysozyme excessively hydrolyze the peptidoglycan layer and thus affect the activity of the Gram-positive bacteria with weak electricity-generation abilities. In all, an appropriate amount of lysozyme can significantly promote the formation and attachment of electricigen biofilm on anode.

3.4. Performances of the MFCs with the lysozyme-treated anode biofilms

To evaluate the effects of lysozyme treatment on the performance of MFC, cyclic voltammetry (CV) was conducted on the anodes treated with different concentrations of lysozyme in M9 buffer under a nitrogen

atmosphere in the potential range from 0.0 V to −0.8 V. A cyclic voltammogram usually contains a reverse and a forward scan, corresponding to the reduction and oxidation reactions, respectively. As shown in Fig. 5a, the lysozyme-treated anodes exhibit much higher double layer currents than the control anode. The current generated on the anode treated with 2 g/L lysozyme is the highest among those on the four anodes at the same potential with the peak reduction current of 0.963 mA and peak oxidation current of 0.487 mA, indicating the high redox catalytic activity and excellent electron transfer properties of the anode. Our results is consistent with the results reported by Luo et al. that lysozyme can promote the redox activity of Gram-positive bacteria (Luo et al., 2015).

To further understand the high redox catalytic activity of the anode biofilm treated with 2 g/L lysozyme, electrochemical impedance spectroscopy (EIS) analysis was conducted. In general, the diameter of the semicircle in an electrochemical impedance plot reflects the interfacial charge transfer resistance (Rct). Based on the electrochemical impedance plots (Fig. 5b), the Rct of the anode treated with 2 g/L lysozyme is calculated to be 41.24 Ω, much lower than that of the control anode (~63.79 Ω). The Rct of the anode treated with 4 g/L lysozyme is the highest with the value of ~82.55 Ω. A lower Rct indicates faster charge transfer. Therefore, the lysozyme treatment can optimize the structure of anode microbial community, and thus promote the charge transfer between electrode and electrolyte.

The power generation performances of the dual-chamber MFCs with untreated and lysozyme-treated bioanodes are compared for a 65 h-operation. The untreated MFC shows the exponentially increasing of output for 10 h with the highest output voltage of 520.8 mV. Compared with those of the untreated one, the exponential growth rate and the maximum voltage of the lysozyme-treated anode are higher (Fig. 5c). The MFCs with the anodes treated with 1 g/L and 2 g/L lysozyme output the highest voltages of 562.5 mV and 646.7 mV, respectively. Further increasing the lysozyme concentration results in dramatically drop in both exponential growth rate and maximum voltage. These results can be explained that an appropriate amount of lysozyme partially degrades peptidoglycan and improves the activity of Gram-positive electricigens. The electricigens are enriched during the operation of MFCs, resulting in the excellent power generation performance. These results are in good agreement to the report that rhamnolipid can improve cell permeability and promote electricigenic activity of Gram-positive electricigens (Wen et al., 2010).

The effect of lysozyme treatment on the power generation performance of MFC was further evaluated with the polarization curve and power density curve obtained using a variable external load resistance (Fig. 5d). The highest open circuit voltages (OCV) on the anodes treated with 0 g/L, 1 g/L, 2 g/L and 4 g/L reach up to 653 mV, 728 mV, 740 mV and 640 mV, respectively. The maximum power density of the corresponding MFCs are determined to be 1472.3 mW m^{-2}, 1650 mW m^{-2}, 2083.3 mW m^{-2} and 1300.6 mW m^{-2} (Fig. 5d). These observations most probably rely on the fact that lysozyme hydrolyzes the peptidoglycan layer and increases the permeability of microbial membranes and the diffusion of electron in cell membrane is promoted. It is clear that the treatments with low concentrations of lysozyme (1 g/L and 2 g/L) significantly increase the open circuit voltage and power density of MFC. The slope of polarization curve reflects the internal resistance of MFC. The internal resistances of the MFCs with the anodes treated with 0 g/L, 1 g/L, 2 g/L and 4 g/L lysozyme are calculated with their polarization curves to be 88 Ω, 67 Ω, 55 Ω, and 116 Ω, respectively. These results indicate that the treatment with appropriate amounts of lysozyme can lower the internal resistance of MFC and improve its power generation performance possibly due to the good conductivity of the optimized anode microbial community structure.

4. Conclusions

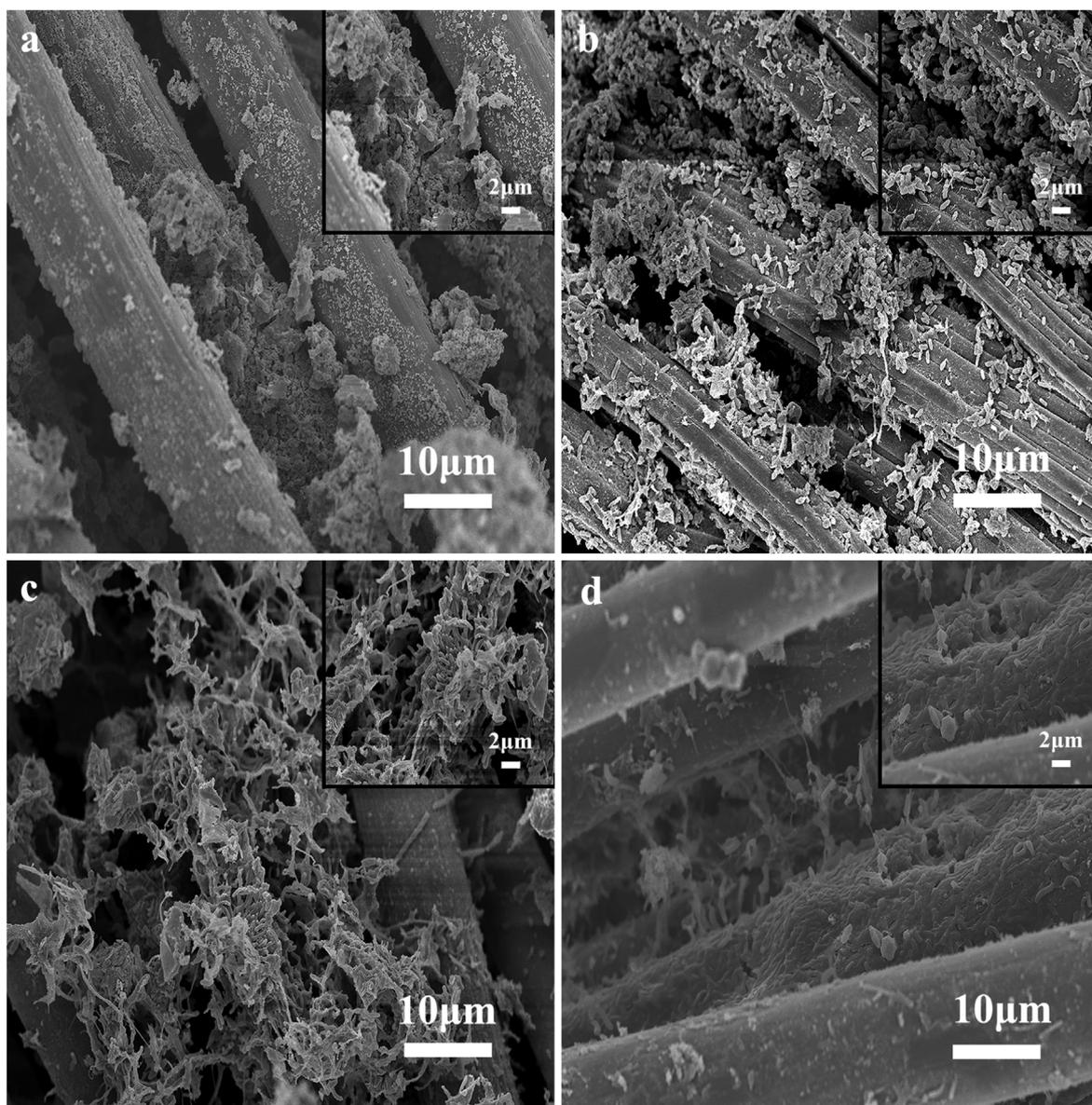In summary, a new approach has been developed to enrich the

Fig. 4. SEM images of the anode biofilms treated with 0 g/L (a), 1 g/L (b), 2 g/L (c) and 4 g/L (d) lysozyme and run in MFC for 65 h.

anode electricigens of mixed MFC by specifically hydrolyzing the peptidoglycan layer in the cell wall of Gram-positive bacteria in the microbial communities. The lysozyme treatment decreases of the peptidoglycan content of microbial community 26.69%, which results in improved activity of Gram-positive *Firmicutes*. Therefore, the abundance of anodic electricigens was increased 14.26% during the following 65 h-operation of the corresponding MFC. The MFC exhibits the power generation capacity 42% higher than that of the control. In all, our study has provided a whole novel strategy to acclimate electricigens as an effective anodic catalyst and improve the application potentials of Gram-positive electricigens in MFCs.

CRediT authorship contribution statement

Wenrui Shen: Software, Resources, Data curation, Writing - original draft. **Xiaoran Zhao:** Investigation, Visualization, Writing - original draft. **Xiaoliang Wang:** Validation, Formal analysis. **Siqi Yang:** Data curation, Software. **Xindi Jia:** Visualization. **Xiaodi Yu:** Investigation. **Jing Yang:** Methodology. **Qinzheng Yang:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition. **Huazhang Zhao:** Methodology, Supervision, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109463.
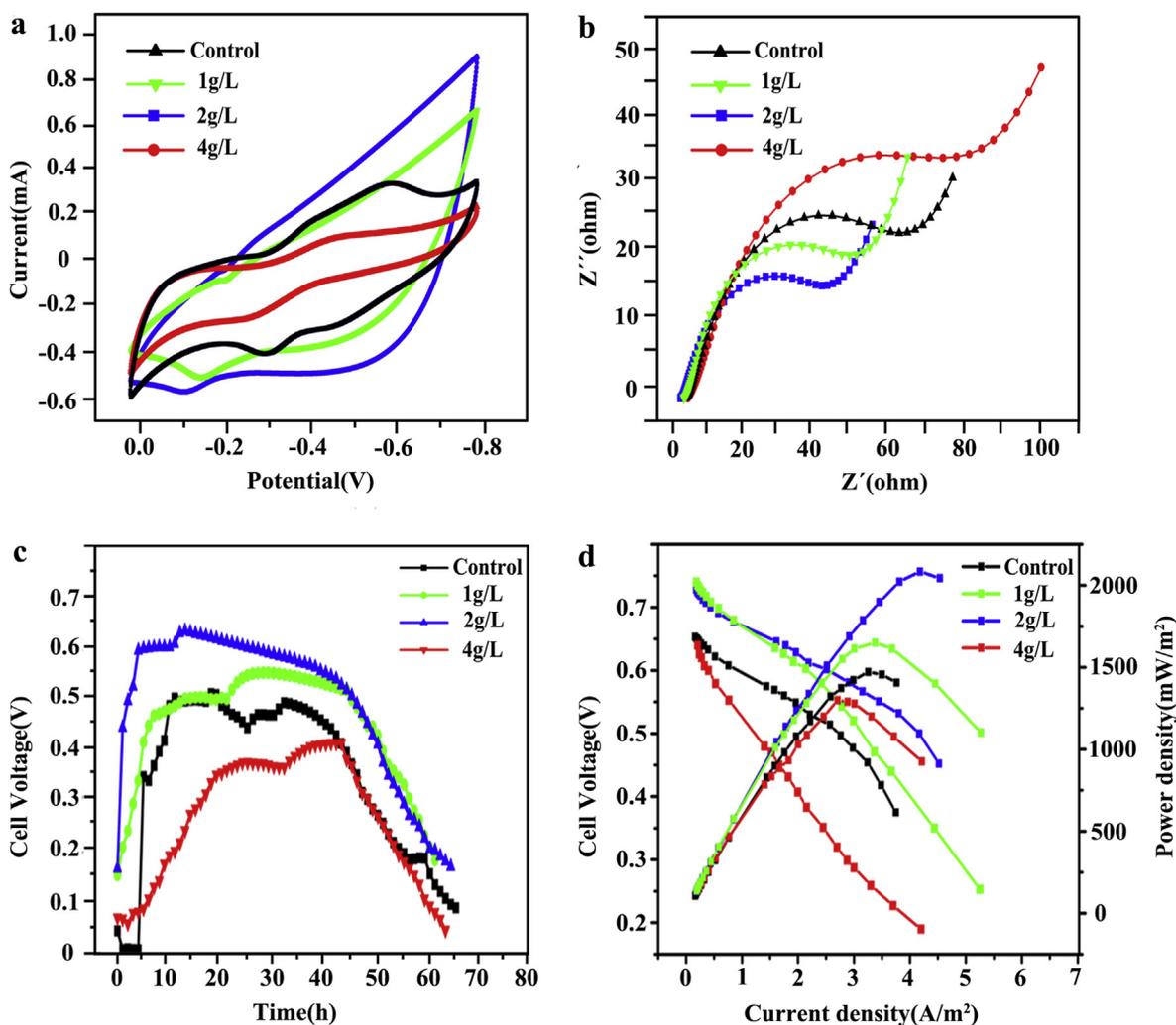
Fig. 5. Cyclic voltammograms (a), electrochemical impedance plots (b), voltage-time curves (c) and polarization curves (d) of the MFCs with the anodes treated with 0 g/L (control), 1 g/L, 2 g/L and 4 g/L lysozyme.

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