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Metabolic Engineering Modification and Electricity Production Condition Optimization of Salt-Tolerant Electricigen *Shewanella Algae*

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Abstract: Electricigens are a type of microorganism with extracellular electron transfer ability, which can convert stored chemical energy in organic matter into electrical energy through their own metabolism. Previous studies have shown that electricigens can be used as catalysts to construct microbial fuel cells (MFCs). However, low electricity production remains the most urgent issue for electricigens. This study is based on a salt-tolerant electricigen, *Shewanella algae*, and uses CRISPRa technology to regulate the expression of *pflb* and *mdh* genes in the extracellular electron metabolism pathway, thereby enhancing the ability to produce electricity. Simultaneously, we aim to optimize power generation conditions of *Shewanella algae* by adding surfactants or electron mediators in the bipolar chamber of fuel cells to enhance electron transfer. The results show that overexpression of *pflb* and *mdh* genes increases the current density by 3.51% and 14.17%, respectively. The use of surfactants such as Tween-80 increases the current density by approximately 20%–40%. These results indicate that an increase in extracellular electron transfer or electron metabolism can increase power generation, providing a basis for further optimization and transformation of salt-tolerant electricigen *Shewanella algae*.

Keywords: *Shewanella algae*; metabolic engineering; microbial fuel cell (MFC); surfactant; electron mediator
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0 Introduction

The microbial fuel cell (MFC) is a bioelectrochemical system (BES) that utilizes live microorganisms to convert organic matter into electrical energy[1]. MFCs have been developed for biomass waste power generation[2] and wastewater treatment[3]. MFC technology treats wastewater in the anode chamber and reduces heavy metals in the cathode chamber. However, the low extracellular electron transfer (EET) rate remains a limiting factor for the practical application of MFCs[4]. Research has shown that MFC systems can enhance the EET rate and enable the construction of conductive biological anodes by screening strains with excellent EET capability. The electricity generated from anode wastewater treatment can be used to drive the reduction of heavy metals in the cathode, enabling self-sustaining energy supply and effectively solving the problem of heavy metal pollution[4]. Electricigens are organisms capable of transferring electrons outside of cells[5], and in other studies, they are referred to as electrochemically active bacteria, anodic respiratory bacteria, and electropositive bacteria[6]. Research has found that bacteria capable of iron reduction can generate energy in MFCs without external media, indicating that energy production may be a rare characteristic limited to certain microorganisms. Dissimilatory metal-reducing bacteria can transfer electrons directly through contact with outer membrane cytochromes, or by using excretory mediators[3,5,7], or by synthesizing appendages capable of transmitting electrical currents, namely nanowires[5].

Electricity production has been detected in four subdivisions within Proteobacteria, as well as in Firmicutes and Acidobacteria. *Shewanella algae* is one of the Proteobacteria and is a multifunctional electricigen that has attracted much attention due to its unique salt tolerance and electricity production ability. *Shewanella* strains undergo incomplete oxidation of organic matter under anaerobic conditions (including power generation conditions) to produce acetate as the final product. However, studies have shown that *Shewanella algae* can generate electricity from acetic acid. Therefore, the catabolic pathway of *Shewanella algae* is different from that of the typical *Shewanella* strains[8], and studying the catabolic pathway will be an interesting research direction. A recent study has shown that a new *Shewanella algae* isolate exhibits the ability to oxidize acetate salts using manganese (III) as the sole electron acceptor[9]. Therefore, acetic acid oxidation under anaerobic conditions may be a common feature that distinguishes *Shewanella algae* from other *Shewanella* strains. There are over 70 species of strains with multiple respiratory abilities in the genus *Shewanella*, all of which

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have the potential to serve as electrogenic microorganisms. Most studies have used *Shewanella oneidensis* MR-1 as a model strain[10-11], but *Shewanella algae* selected in this study has multiple advantages. Genome sequencing reveals the presence of highly reliable CRISPR/Cas9 system elements, which can be used for gene editing and metabolic engineering. Its genome also includes eight types of antibiotic resistance genes, indicating strong environmental adaptability and evolutionary potential[12]. With the wide distribution in marine water quality, it has the advantages of low cultivation requirements and good modification potential, making it suitable as the chassis cell strain for this study.

The EET process driven by external power sources involves the flow of intracellular electrons generated by carbon source oxidation metabolism to extracellular electron acceptors (e. g., minerals and inert electrodes) via a pathway mediated by c-type cytochromes and soluble electron shuttles[13-15]. However, the low EET rate remains a critical issue that severely restricts applications such as MFCs. Many researchers are committed to improving the EET rate, expanding the operating parameters and scaling up MFCs by optimizing electrode materials[16-19]. At the same time, another feasible strategy to enhance bioenergy production is to improve the electrogenic capacity of bacteria[16,20-21]. This includes genetic modification of bacteria to boost the electron output and improve the efficiency of electron transfer to the anode[3,16,21]. The reduced nicotinamide adenine dinucleotide (NADH) serves as the primary intracellular reducing equivalent and is the main electron carrier and source of EET that can be released in cells[22]. Therefore, cofactor engineering has been developed through metabolic engineering methods to manipulate intracellular NAD (H/$^+$) levels, which has been shown to effectively control intracellular NAD (H/$^+$) availability. Cofactor engineering mainly includes introducing NAD biogenesis to control total NAD (H/$^+$) levels[23-24] and manipulating NADH consumption and regeneration to regulate the NAD (H/$^+$) ratio[25], thereby improving the power output in MFCs[22]. For example, researchers conducted expression analysis on 16 genes related to NADH regeneration in the four modules of glycolysis, C1 metabolism, pyruvate fermentation, and tricarboxylic acid (TCA) cycle in the central carbon metabolism pathway of *Shewanella oneidensis*, aiming to explore their effects on EET. The results showed that *pflb* and *mdh* are key genes that enhance NADH regeneration, and overexpression of these genes significantly increases the intracellular NADH/NAD^+ ratio and the NADH level, thereby significantly enhancing their electrical output in MFCs[22].

Surfactants, as a means of increasing extracellular electron transfer, alter the structure of cell membranes by forming micropores or other structures, thereby increasing the permeability of electrogenic bacteria and reducing their internal resistance[26]. Surfactants can be classified into non-ionic, anionic, cationic, and zwitterionic types based on their charge properties. Tween-80 is a non-ionic surfactant with the advantages of strong emulsifying and solubilization ability, but the disadvantage is that it may affect cell metabolism at high dosages. Triton X-100 is also a non-ionic surfactant, which has the advantage of regulating cell membrane permeability and facilitating electron transfer in cells. However, its disadvantage is that it may cause damage and degradation of cell membrane proteins. CTAB is a cationic surfactant with good ability to adsorb and encapsulate lipid substances. Cations can assist in electron transfer, but they have certain cytotoxicity. Similarly, electron mediators, as substances that enhance extracellular electron transfer, can also accelerate the process of electron transfer and increase electricity production. Electron mediators can be divided into two categories: naturally formed and artificially synthesized. Naturally formed electron mediators are mostly generated by some electricity-producing microorganisms, such as pyocyanins or riboflavin. In order not to change the electricity production mechanism of the electricigens, they were not selected in this article. Among artificially synthesized electron mediators, neutral red is a common and widely used substance, which is an oxidation-reduction type electron mediator that can effectively promote extracellular electron transfer and increase output power by directly capturing electrons through the cell membrane. Potassium ferrocyanide is an artificially synthesized redox electron mediator that can accelerate electron transfer and reduce internal resistance, but its disadvantage is that it is sensitive to pH.

In this study, we used CRISPRa technology to construct plasmids overexpressing *pflb* and *mdh*, and transformed them into *Shewanella algae* by electroporation. Afterward, we constructed a bipolar chamber type MFC to measure the power generation. In addition, various surfactants were used as electron transfer promoters in the cathode chamber, and neutral red was used as an electron mediator in the anode chamber. The effects of these optimized conditions on the power generation were tested to propose a better MFC using *Shewanella algae* as an electricigen.

1 Materials and Methods

1.1 Strain source and growth conditions

The chassis strain *Shewanella algae* was purchased from the China Center of Industrial Culture Collection. 2216E medium was used as the amplification medium (with a pH of 7.6±0.2), SOC medium was used as the electroporation buffer medium, and MSM buffer was used as the anode chamber electroporation buffer. The ingredients and mass concentrations of the media or buffers are shown in Tables 1–3.

Table 1 2216E medium ingredients and mass concentrations

Ingredient	Mass concentration/(g/L)
Peptone	5.000 00
Yeast extract	1.000 00
Ferric citrate	0.100 00
Sodium chloride	19.450 00
Magnesium chloride	5.900 00
Magnesium sulfate	3.240 00
Calcium chloride	1.800 00
Potassium chloride	0.550 00
Sodium bicarbonate	0.160 00
Potassium bromide	0.080 00
Strontium chloride	0.003 40
Boric acid	0.002 20
Sodium silicate	0.000 40
Sodium fluoride	0.000 24
Ammonium salt	0.000 16
Disodium hydrogen phosphate	0.000 80

Table 2 SOC medium ingredients and mass concentrations

Ingredient	Mass concentration/(g/L)
Peptone	20.000
Yeast extract	5.000
Sodium chloride	0.500
Potassium chloride	0.186
Magnesium chloride	0.952
Glucose	0.360

Table 3 MSM buffer ingredients and mass concentrations

Ingredient	Mass concentration/(g/L)
Sodium chloride	44.000 00
Magnesium chloride	1.650 00
Calcium chloride	1.375 00
Potassium dihydrogen phosphate	0.070 00
Dipotassium hydrogen phosphate	0.105 00
Disodium hydrogen phosphate	0.280 00
Ferric sulfate	0.050 00
Manganese sulfate	0.025 00
Ammonium chloride	0.015 50
Calcium chloride	0.010 00
Cobalt chloride	0.005 00
Copper sulfate anhydrate	0.000 50
Boric acid	0.005 00
Sodium molybdate	0.001 25
Nickel sulfate	0.001 20
Ethylenediamine tetraacetic acid	0.005 00

1.2 Electroporation *of Shewanella algae*

Shewanella algae was mixed with 1 μL plasmid (100 ng/μL), and electroporation was conducted via the Epulse device. Immediately after electroporation, 1000 μL SOC medium was added to the electroporation cuvette, and the cells were resuspended by pipetting and mixing. The resuspended bacterial solution was incubated with shaking on a horizontal shaker at 30 ℃ and 250 r/min for 1 h to allow cell recovery and resistance gene expression. After incubation, 20 μL of the transformed product was mixed with 180 μL of medium for dilution. A 20 μL aliquot of the diluted mixture was then streaked onto 2216E culture medium containing the corresponding antibiotics using the plate streaking method and incubated at 30 ℃ for 12–16 h. Monoclonal colonies were then selected as the transformed strains.

1.3 Construction of MFCs

A bipolar chamber type MFC made of organic glass material was used as the battery body. The inner diameter of the anode and cathode chambers was 75 mm, with a volume of 500 mL. Graphite felt was employed as the electrode material in both chambers. *Shewanella algae* transformed with the plasmid was cultured to the logarithmic growth phase. The bacterial culture was then mixed with MSM medium at a ratio of 1∶4 and added to the anode chamber, after which the chamber was sealed. An equal volume of ultrapure water was added to the cathode chamber as the catholyte. All components were connected with copper wires, and the MFC was placed in a water bath maintained at 37 ℃. The current between the two electrodes was measured using a multimeter. Current values were recorded every 5 min, and the current variation over a 150 min period was plotted.

1.4 Optimization of power generation conditions

To evaluate the effect of a single factor, the power generation was measured according to the method described in Subsection 1.3 while sequentially changing one operational parameter, such as a surfactant or electron mediator, and keeping all other conditions unchanged.

2 Results and Discussion

2.1 Design and prediction of sgRNA for *pflb* and *mdh* genes

The National Center for Biotechnology Information (NCBI) was used to search for the transcription start sites of *pflb* and *mdh* genes, as well as their positions and transcription directions in *Shewanella algae*. The 200 base pairs (bp) upstream sequence of the transcription start site was input into the CRISPOR website for sgRNA design and prediction. *Shewanella algae* JCM 21037 was used as a reference genome; sequences containing the NGG protospacer adjacent motif (PAM), which is recognized by SpCas9, were identified for sgRNA design. Three models were used to predict the candidate results, and the higher the score,

the better the feasibility and efficiency of sgRNA. Fusi-score was more suitable for predicting the efficiency of sgRNA used for in vivo transcription (U6 promoter). CRISPRScan is more suitable for predicting the efficiency of in vitro transcription (T7 promoter). RS3-score provides a more comprehensive and general prediction of sgRNA efficiency. As shown in Fig. 1(a), seq6 has the highest comprehensive score among the six candidate sgRNAs for *pflb*, and the predicted results indicate that there is no mismatch or off-target. Therefore, seq6 was chosen as the sgRNA for *pflb*. As shown in Fig. 1(b), seq5 has the highest comprehensive score among the six candidate sgRNAs for *mdh*, and the predicted results also indicate that there is no mismatch or off-target. Therefore, seq5 was chosen as the sgRNA for *mdh*.

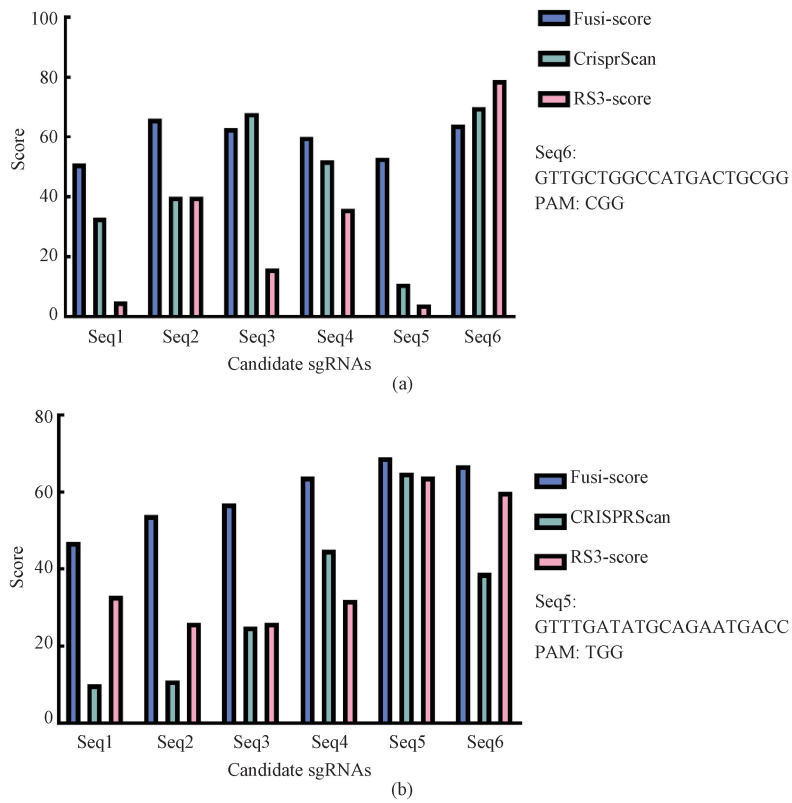


Fig. 1 Design and prediction of sgRNA: (a) prediction score of candidate sgRNAs for *pflb*; (b) prediction score of candidate sgRNAs for *mdh*

2.2 Construction of sgRNA and pdCas9 plasmids

After obtaining the sgRNA sequence, the sgRNA was inserted into the gRNA scaffold region of the pgRNA vector via homologous recombination. Simultaneously, a VP64 sequence was inserted into the pdCas9 bacteria plasmid through homologous recombination to enhance the expression of dCas9 protein. The effect of plasmid construction was verified by agarose gel electrophoresis. Figure 2(a) shows the plasmid map after the final construction. As shown in Fig. 2(b), the electrophoresis result indicates a single and clean band without obvious heterobands. In Fig. 2(b), M represents the standard molecular marker; lane 1 represents the *pflb* pgRNA-bacteria plasmid, with a size of approximately 2 584 bp; lane 2 represents the pdCas9-bacteria-VP64 plasmid, with a size of approximately 7 080 bp; lane 3 represents the *mdh* pgRNA-bacteria plasmid with a size of approximately 2 584 bp; lane 4 represents the pdCas9 bacteria plasmid, with a size of approximately 6 705 bp. All of them meet the expectations, indicating successful plasmid construction, and can be used for metabolic engineering modification of *Shewanella algae*.

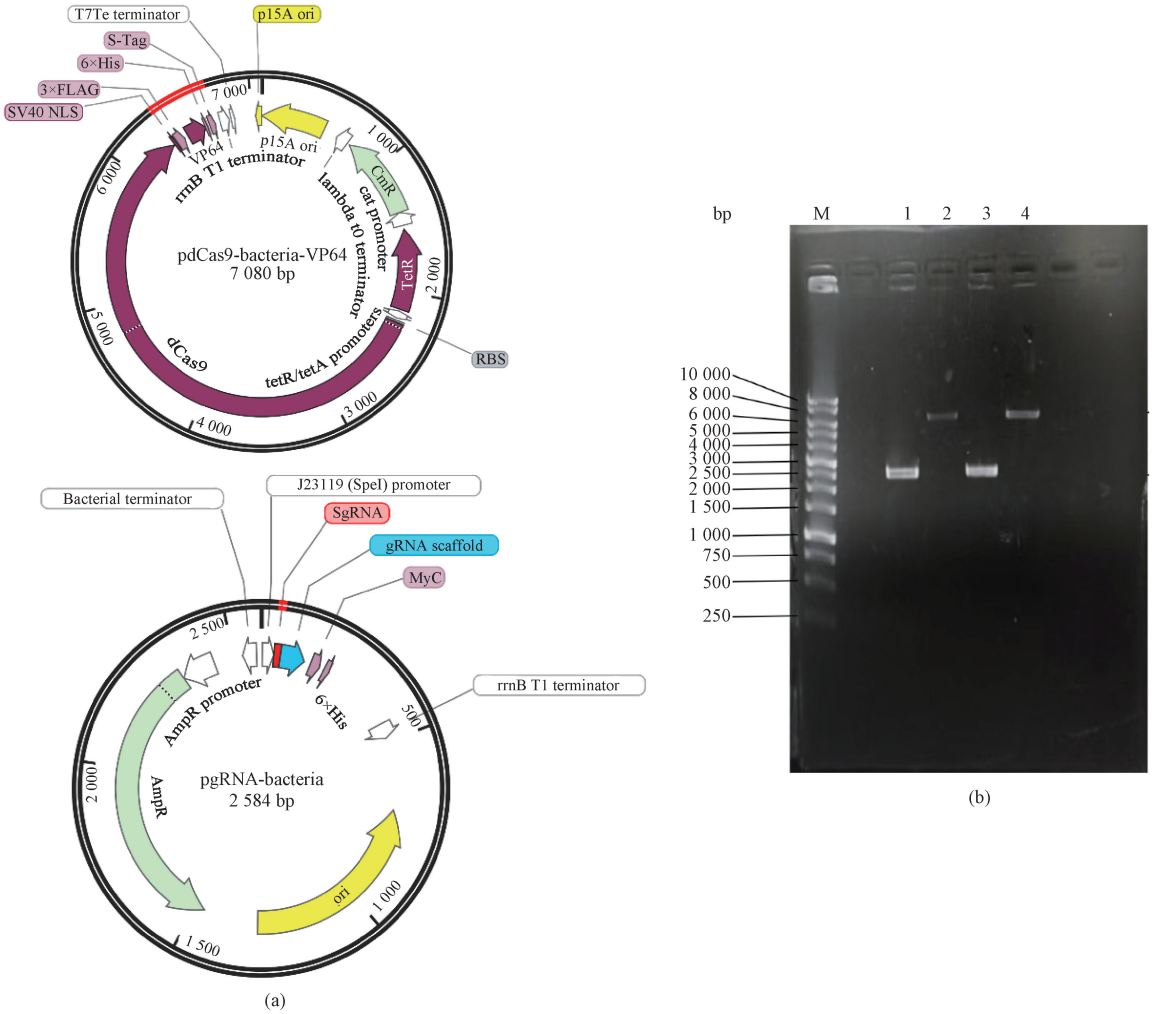


Fig. 2 Construction and validation of pdCas9 and sgRNA plasmids: (a) plasmid map of pdCas9-bacteria-VP64 and pgRNA-bacteria; (b) agarose gel electrophoresis results

2.3 Metabolic engineering modification of *Shewanella algae* to increase electricity production

The growth curve of wild-type *Shewanella algae* was plotted by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer. As shown in Fig. 3(a), the logarithmic growth period of wild-type *Shewanella algae* is 4–6 h, where its OD_{600} is between 0.4 and 0.8. The sgRNA plasmid and pdCas9 plasmid were transformed into *Shewanella algae* by electroporation and further cultured to the logarithmic growth stage to construct an MFC for electricity production detection. Wild-type *Shewanella algae* without metabolic engineering modification was used as a control, as shown in Figs. 3(b)–3(d). All strains exhibit a rapid increase in current density, with a noticeable rise already observed within the first 10 min, reaching a peak within 20–30 min, followed by minor fluctuations and a gradual stabilization over the remaining measurement period up to 150 min. The current densities from 10 to 150 min are taken as the power generation results, and the average value is calculated. The average current density of the wild-type *Shewanella algae* during the measurement time from 10 to 150 min is 136.25 μA/cm^2. The strain that transformed *pflb* sgRNA and pdCas9-bacteria-VP64, denoted as *Shewanella algae pflb*$^+$, has an average current density of 141.03 μA/cm^2, an increase of 3.51% compared to the wild type, but the change is not significant. The strain that transformed *mdh* sgRNA and pdCas9-bacteria-VP64, denoted as *Shewanella algae mdh*$^+$, has an average current density of 155.56 μA/cm^2, an increase of 14.17% compared to the wild-type *Shewanella algae*. This improvement is greater than that observed in *Shewanella algae pflb*$^+$.

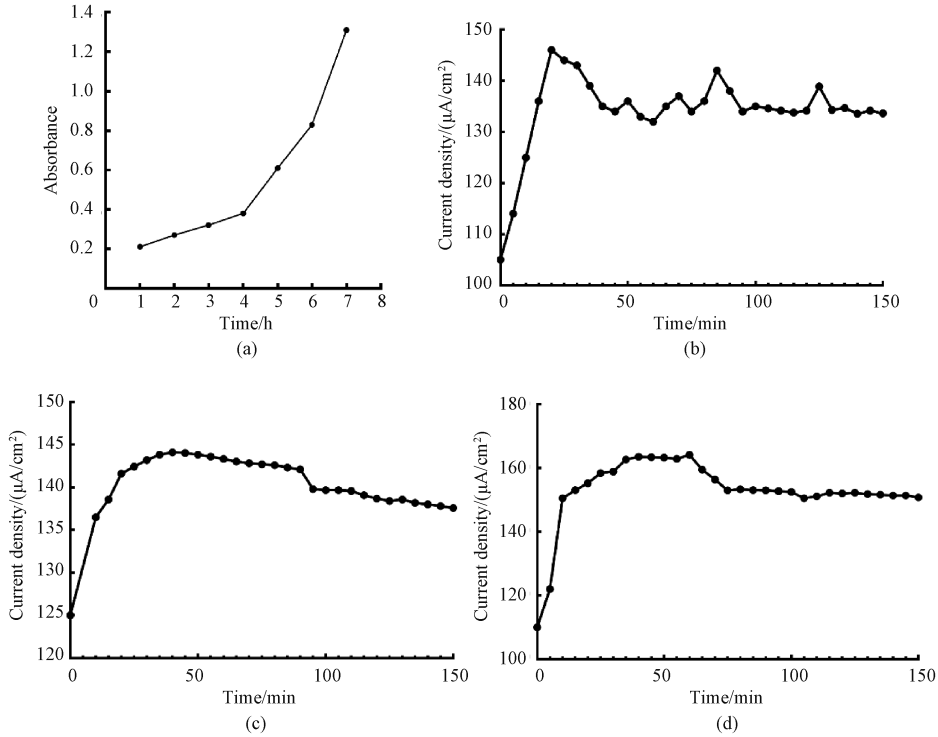


Fig. 3 Current density of *Shewanella algae* after metabolic engineering modification: (a) growth curve of wild-type *Shewanella algae*; (b) current density of wild-type *Shewanella algae*; (c) current density of *Shewanella algae pflb*$^+$; (d) current density of *Shewanella algae mdh*$^+$

2.4 Optimize *Shewanella algae* power generation conditions to increase electricity production

In addition to metabolic engineering modifications, we also attempted to enhance electricity production efficiency by adjusting and optimizing the composition of the cathode or anode chamber. This was mainly achieved by adding surfactant to the anode chamber, including Tween-80, Triton X-100, and cetyltrimethylammonium bromide (CTAB). We also added an electron mediator, neutral red, to the cathode chamber. After adding surfactants, the current density of *Shewanella algae* also rapidly increases in the first 10 min, as shown in Fig. 4(a). After adding 0.8 g/L Triton X-100 to the cathode chamber, the average current density from 10 to 150 min is 187.54 μA/cm^2, an increase of 37.64% compared to that before the modification. Similarly, as shown in Fig. 4(c), adding 5 g/L Tween-80 to the cathode chamber results in an average current density of 191.81 μA/cm^2 for 10–15 min, an increase of 40.78% compared to that before the modification, representing a more significant change. As shown in Fig. 4(b), after adding 0.8 g/L CTAB to the cathode chamber, the average current density from 10 to 150 min is only 164.35 μA/cm^2, an increase of 20.62% compared to that before the modification. The change is not significant, but its trend indicates that it can make *Shewanella algae* reach the power generation state faster. Compared with other results, its current density reaches 194.2 μA/cm^2 at 5 min, and maintains 193.9 μA/cm^2 and 186.9 μA/cm^2 at 10 and 15 min, respectively. The results are quite unique.

Similar to this, as shown in Fig. 5(a), after adding the electron mediator neutral red to the anode chamber, it can be observed that the current density no longer increases rapidly, but exhibits a slow growth process. It maintains an increase in current density from 0 to 150 min. However, its average current density is relatively low, even lower than that without any modification, only 95.15 μA/cm^2, a decrease of about 30.17%. Afterward, we attempted to use potassium ferrocyanide as an electron mediator. The results are shown in Fig. 5(b). The addition of 50 mmol potassium ferrocyanide significantly increases the current density, which slowly increases within 150 min like neutral red. The average current density can reach 5.85 mA/cm^2, which is about 43 times higher than that before the addition.

Overall, the metabolic engineering modification of key genes in the respiratory chain is not significant enough, but there is a certain degree of increase. The overexpression modification effect of *mdh* is more obvious. The optimization effect on the electricity production environment is more obvious and unique, with the addition of surfactant causing a greater change in electricity production compared to gene editing. Although the addition of some electron mediators does not increase electricity production, they cause a unique change in the

trend of electricity production, and potassium ferrocyanide shows an exponential increase.

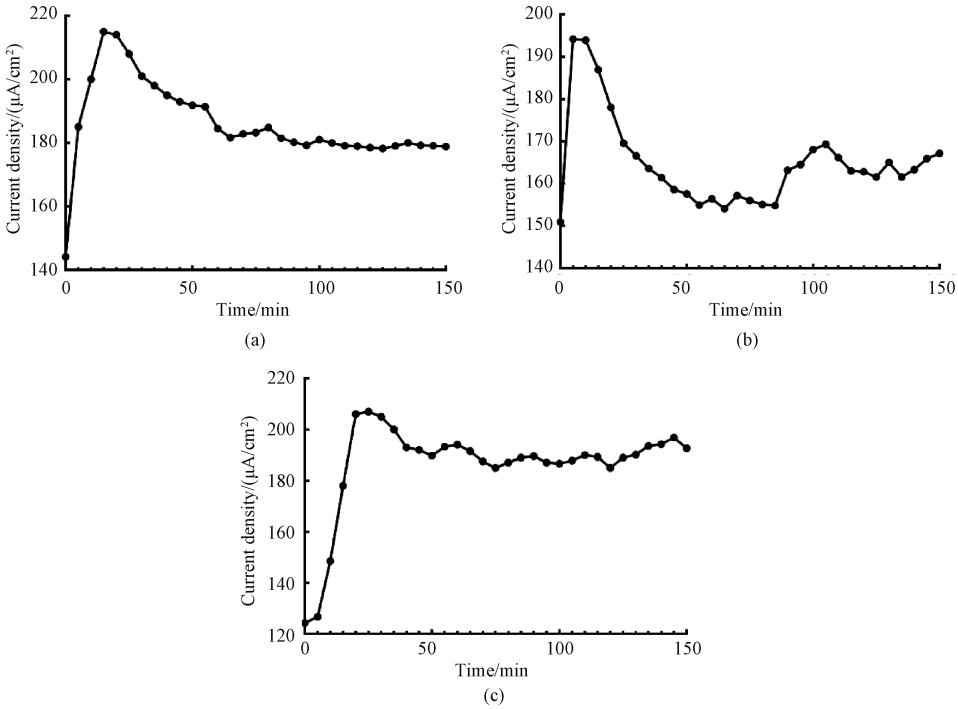


Fig. 4 Current density of *Shewanella algae* after addition of various surfactants: (a) Triton X-100; (b) CTAB; (c) Tween-80

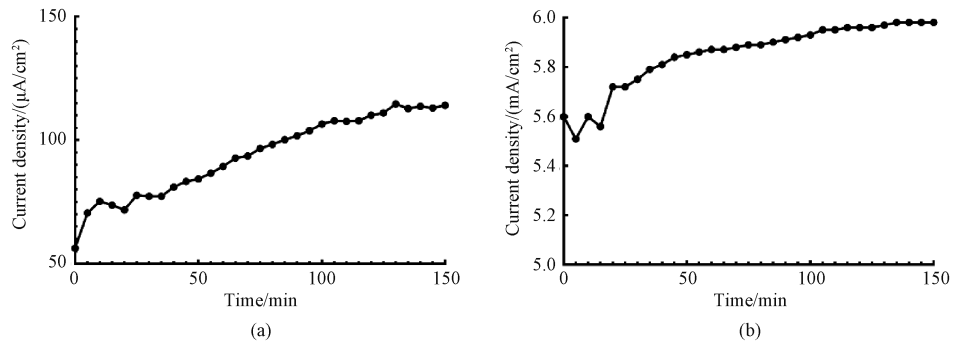


Fig. 5 Current density of *Shewanella algae* after addition of various electron mediators: (a) neutral red; (b) potassium ferrocyanide

We believe that the ineffective results of metabolic engineering modification are due to incomplete gene editing and incomplete selection of modification targets. Although electroporation was used for gene editing and resistance screening, there was no confirmation of gene editing results after transformation, which might result in poor editing results. At the same time, both genes played an important role in NADH production, but NADH production was not only influenced by these two genes; *pflb* also seemed to have bidirectional regulation of NADH. Its function of optimizing electron allocation direction seemed to be improved as its expression level decreased, and these two genes seemed to have a synergistic effect, which appeared to require joint editing to achieve significant results.

During the experiment, we also considered detecting electricity production when the bacterial count changes slowly during the stationary period. The reason for choosing the logarithmic growth period is to consider the overall activity of the strain. Since the strain used in this study generates electricity via EET during its respiratory process, cells harvested at the stationary phase are not ideal. Although stationary-phase cultures exhibit slow growth, they typically show low metabolic activity, reduced respiratory chain EET rates, and consequently poorer electricity production compared to cells in the logarithmic growth phase.

3 Conclusions

In this study, we attempted to enhance the electricity production of wild-type *Shewanella algae* by using gene editing and modifying power generation conditions, including the addition of surfactants or electron

mediators. For gene editing, both *pflb* knock-in and *mdh* knock-in led to a slight increase in power generation. However, modifying power generation conditions led to a more significant improvement. We chose Triton X-100, CTAB, and Tween-80 as surfactants, and neutral red and potassium ferrocyanide as electron mediators. All surfactants increased the current density by approximately 20% – 40%, while both electron mediators altered the trend of power generation.

Although the metabolic engineering modifications in this study are less effective than surfactant modification, there is still potential for further research. As mentioned earlier, surfactants require specific optimal concentrations or types, and many are slowly consumed by electricigens as carbon sources. However, a successful metabolic engineering modification is permanent, allowing the entire fuel cell to function stably for a long period without maintenance or replacement.

In summary, we used metabolic engineering and other methods to optimize power generation capacity, which had potential applications in military fields such as submarines. Additionally, we modified the buffer system in the dual-chamber fuel cell. Adding surfactants to the cathode chamber or electron mediators to the anode chamber each offered distinct advantages, significantly increasing power generation and providing a basis for further optimization of fuel cells using *Shewanella algae* as an electricigen.

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耐盐产电菌 *Shewanella algae* 的代谢工程改造及产电条件优化

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摘　要：产电菌是一类具有胞外电子转移能力的微生物，能够通过自身代谢将有机物中储存的化学能转化为电能。已有研究表明可将产电菌作为催化剂构造微生物燃料电池。然而，微生物燃料电池产电性能不佳仍是当前面临的关键瓶颈。本研究基于一种耐盐产电菌 *Shewanella algae*，通过 CRISPRa 技术调控产电菌胞外电子代谢途径中 *pflb* 和 *mdh* 基因的表达，增加产电菌产电强度。同时优化产电环境，在燃料电池的双极室中添加表面活性剂或电子中介体，从而增强电子传递。结果表明，*pflb* 和 *mdh* 基因的过表达分别使电流密度增加了 3.51%和 14.17%。而使用 Tween-80 等表面活性剂使电流密度增加约 20%～40%。这些结果表明电子转移的增加或者电子代谢的增加都可增加产电强度，为进一步优化改造耐盐产电菌 *Shewanella algae* 提供了依据。

关键词：*Shewanella algae*；代谢工程；微生物燃料电池；表面活性剂；电子中介体