Genetically engineered microbial electrocatalysts for high performance biofuel cells

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**GENETICALLY ENGINEERED MICROBIAL
ELECTROCATALYSTS FOR HIGH PERFORMANCE
BIOFUEL CELLS**

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**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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**GENETICALLY ENGINEERED MICROBIAL
ELECTROCATALYSTS FOR HIGH PERFORMANCE BIOFUEL
CELLS**

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School of Chemical and Biomedical Engineering

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Abstract

Nowadays, the sustainable treatment and exploitation of wastewater is obtaining extensive studies because of the increasing deficiency in water resources, shortage of fossil fuel, and pollution in water. So far, most conventional wastewater remedy procedures need energy and also bring about the issues of pollutants. The microbial fuel cell (MFC) is one type of microbe-catalyzed fuel cell that is able to transfer the energy in chemical form directly from an inorganic or organic materials into electricity by means of a series of bio-chemical reactions. Electricigens are the crucial factor that controls the entire microbial fuel cell performance by means of their metabolic activities and extracellular electron transport (EET).

Electron export from the microbial metabolism of the electricigen itself to the anode is carried out by two major ways, that is, direct electron transport as well as mediated electron transport, dependent upon whether the electron shuttles are used in these systems. The extracellular electron transport efficiency is regulated by the voltage difference between the electron donor and the anode acceptor. Both the cell inner and outer membrane and the cell respiratory chain protein complex provide the reacting place for MFC to extract energy from microbes. These electrons are then exported to the anode either through the direct electron transport by c-cytochromes at the inner and outer membrane of the microbes as well as by conducting pili, or through the mediated electron transport induced by electron shuttles.

The electron transport is considered as the main constraint condition that restrains the output performance of the microbial fuel cell. The electron shuttle induced electron transport is one of the most widely used electron transport routine for a lot of electricigens. In this work, we recombine the *Escherichia coli* BL21 (DE3), a strain commonly utilized to express proteins, in order to upregulate the secretion of electron shuttles so that after immobilizing it as the bio-cocatalyst beads, the current and power output of MFC could be raised by more than 9-fold. Then, we overexpress the type two NADH dehydrogenase in the inner-membrane of the electricigens to accelerate electron trans-inner-membrane motion to bridge the gap between substrate oxidation and electron transport of microbial electrocatalyst. The power density of mutant strain increases by 3.3-fold. Thirdly, we coexpress the MtrCAB electron transport protein conduits from wild-type *Shewanella oneidensis* MR-1 strain as well as the ribAB genes which encodes the first two step of RF biosynthesis in the *E.coli* BL21(DE) strain so as to improve the power density of *E.coli*-catalyzed MFCs by 26-fold.

Keywords: microbial fuel cell, MFC, bioelectrocatalyst, electricigen, electrochemical, electrode, electricity

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List of Abbreviations

AQDS: anthraquinone-2,6-disulfonate

AES: artificial electron shuttles

c-Cyts: c-type cytochromes

CC: carbon cloth

CF: carbon felt

CFU: colony forming units

CO: carbon monoxide

COEs: conjugated oligoelectrolytes

CV: cyclic voltammetry

DI: deionized

DET: direct electron transport

2D: two-dimensional

EES: exogenous electron shuttles

EET: extracellular electron transport

EIS: electrochemical impedance spectroscopy

FAD: flavin adenine dinucleotide

FMN: flavin mononucleotide

HPLC: high performance liquid chromatography

LB: Luria-Bertani

IM: inner membrane

IPTG: isopropyl-beta-D-thiogalactopyranoside

MCS: multiple cloning sites

MET: mediated electron transport

MFC: microbial fuel cell

NDH I: type I NADH dehydrogenase

NDH II: type II NADH dehydrogenase

NQR: Na⁺-translocating NADH dehydrogenase

NR: neutral red

OCP: open circuit potential

OD: optical density

OM: outer membrane

PCR: polymerase chain reaction

RF: riboflavin

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List of Publications

L. Tao, M. Xie, L. Pu, G. G. Y. Chiew, W. N. Chen and X. Wang, Enhancing the performance of *Escherichia coli*-catalyzed MFC based on RF secreting, prepared.

L. Tao, M. Xie, G. G. Y. Chiew, Z. Wang, W. N. Chen and X. Wang, Improving electron *trans*-inner membrane movements in microbial electrocatalysts, Chemical communications, 2016, 52, 6292.

L. Tao, H. Wang, M. Xie, L. Thia, W. N. Chen and X. Wang, Chemical communications, 2015, 51, 12170. (selected as front cover paper)

Chapter 1. Introduction and thesis scope

1.1. Background

Over the last few decades, environmental problem and energy issues have become the central problem which can affect the survival and development of human beings. For nearly more than one hundred years, the pernicious effects of energy crisis and environmental pollution appears in front of the world during the process of great-leap-forward development of human society promoted by the science and technology which is almost based on the fossil power. Even if the global energy saving projects could be implemented rapidly, the demand for energy in the worldwide will increase at a high speed, the grow amplitude of which is expected to rise by more than fifty percent in 2030.

The Seventies oil crisis of the twentieth century gives a great push forward to those research on the sustainable energy sources. To cut down CO₂ emissions and meet the increasing demand for energy, it is wise to use sustainable energy resources instead of fossil resources. The expectation of exhaustible deposits of fossil fuels as well as the environmental concerns for green gases emission will push people into developing and implementing the production of sustainable energy. Compared to other alternatives such as exploiting solar energy, wind energy as well as geothermal energy, it seems to be much easier to exploit biological energy (biomass). In fact, biomass comprises more than seventieth percent of total renewable energy source

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(Figure 1.1) in Europe now.^{204,205,206}

Figure 1.1. The share market of renewable energy in Europe.

Nowadays, biomass is gradually considered as one kind of green energy sources, which is in fact the solar energy fixed by photosynthesis in the form of trees, herbs as well as other photosynthetic organisms. Through photosynthesis, the atmospheric carbon dioxide is converted into biomass in photosynthetic organisms and when taking this "green" energy from biomass, e.g. via carbohydrate oxidation, the carbon dioxide is released once again. Since the biomass follows the natural "short-chained" carbon cycle path and could be generated and utilized in a sustainable way, it belongs to renewable energy sources. In contrast, fossil fuels is made for millions of years by converting the primitive organisms (biomass) into the gas or liquid fossil states, that is,

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natural gas and crude oil. Thus, using these fossil fuels will lead to the release of deposited carbon in the form of carbon dioxide, which results in the accumulation of green house gases today.

Currently, the continuous growth of energy demands around the world and the carbon neutrality requirement of carbon dioxide emission vigorously promote the production of biological energy from biomass. For example, the anaerobic digestion, which is considered as one type of low-cost waste and wastewater treatment system, can produce biological energy as well. Besides, the production of biomass is abundant and found across the globe, which is in great contrast with the traditional fossil fuels, given the fact that the number of regions in the world which is rich in fossil fuel deposits could be "counted on the fingers of one hand" and, not to mention the fact that some of these regions are always experiencing the geopolitical unrest.

In fact, the wastewater, which used to be thought to have low recovery utilization value, is the optimal substrate for the generation of biological energy. The improvement in the conversion process will increase the capacity of the continuous supply of biological fuels as well as reduce its environmental influence and increase its efficiency. By far, the fossil fuels suppresses the large-scale application of biological alternatives still be means of their relative low prices. However, after falling sharply recently, the traditional energy prices are about to rise. Moreover, it is expected that the overall prices of fossil fuels will continuously increase in the coming decades. It is difficult to make sure whether people are ready to pay such a high price for energy in the future. Thus, it is more feasible to extract biological

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energy from the biomass. For the utilization of biomass, it is better for people to extract various ingredients from both food crops and energy crops in order to produce a series of goods like energy and chemicals, each of which can create investment returns. That might be of more benefit and can also build up a biological refining market in the future.

In the last few years, as an effective new technology that can recover energy as well as resources, e.g. electricity, hydrogen gas and methane, bioelectrochemical system (BES) exhibits a great and broad development potential and has drawn universal concerns all over the world. The concept of BES is proposed in recent years and both bioelectricity and bioelectronics are its underlying conceptual basis at the early stage. The microbial fuel cell (MFC) is the first as well as the most familiar direction in these research areas. Then, based on this, the microbial electrolysis cell and the microbial desalination cell as well as other new functions and technologies develop.

The MFC could be defined as a system which can utilize microorganisms as the catalyst to convert chemical energy into electricity. The MFC is one type of biological fuel cells, which take advantage of biocatalysts more widely, as compared to the MFC, from the cell to enzymes to complete those redox reactions. Although there are other ways to use biomass to produce energy, for example, through the anaerobic digestion of biomass, the biohydrogen, bioethanol as well as biodiesel can be produced, the MFC can convert the chemical energy contained in biomass directly into electricity, which is regarded as one type of the most clean, safe and efficient energy source with

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high quality. In contrast, the other types of utilizing biomass either needs noble catalysts to further transform chemical energy into electricity or converts the chemical energy into mechanical work via thermal engine, all of which are relatively uneconomical from the standpoint of cost and energy conversion efficiency. In this decade, the research on MFC is not only directly related to generating electricity and treating wastewater, but also dependent on the breakthrough progress in microbiological and engineering aspects.

1.2. Overall Scope

Our understanding of microbial reactions in the MFCs and BES is still in the initial stage. In the process of microbial treatment, the application of biotechnologies typically originate from the discovery, investigation and recognition of the natural microbial reactions. Molecular biological tools can not only help us better understand the protein reactions, but also let us influence the characteristics of these protein reactions. To achieve the practical application effectively, we are able to utilize the modern biotechnologies to regulate these microbial reactions in order to optimize their functions that we need. The main path of electron transferring to the anode of MFC is the direct electron transport through cell membrane-anchored redox proteins. Although the true path of electron transport as well as the importance of specific proteins are still under study, both of them are involved in cell respiration on the

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electrode. Molecular biological technologies can help us interpret the basic reaction steps of electron transferring. Genetic engineering tools can be applied in studying and improving out-cellular respiratory reactions and especially in designing the biocatalysts which could be used in MFCs. Therefore, the aim of this study will be classified as the resulting three respects:

1.2.1. To improve the mediated electron transfer using a decoupled biococatalyst

After immobilizing the recombinant riboflavin-secreting *E.coli* cells as a type of biococatalyst, we propose a new method of taking advantage of the biococatalyst decoupled from anodic biocatalyst to greatly boost the performance of MFC. The proposed strategy has the following advantages: The bio-cocatalyst metabolizes the substrates which cannot be utilized by the anodic electricigen to secrete electron shuttles; the usage amount of these biococatalysts can be regulated depending on such factors as the flavinogenic activity, reactor volume as well as hydraulic retention time, to achieve the desired mediator concentration in the anodic compartment; the biococatalysts have good reusability and recyclability, which means it can be applied in the constant stream reactor to treat wastewater in future; since it is decoupled from the anodic biocatalyst (electricigen), the biococatalyst beads do not cut the effectual anode surface area used to accumulate electricigens on the anode and this strategy avoids direct contact and accordingly inexpectant interactions between the nonelectricigen and electricigen at the electrode surface, which means it has the possibility to be exploited combined with other bioelectrocatalysts besides *S.*

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oneidensis MR-1. Thus, our work provides a new perspective on the design of anodic bioelectrocatalysis of MFC.

1.2.2. To Bridge substrates oxidation and electron transport in microbial electrocatalysis

The type two NADH dehydrogenase is overexpressed in the innermembrane of the microbial cell to accelerate electron trans- innermembrane motion and at the same time, bridge the gap between substrate oxidation and electron transport of microbial electrocatalyst. Moreover, as the electron trans- innermembrane motion is a necessity for exporting from electron transport route to the extracellular anode, it means that the improved bioelectrocatalyst can be used together with exogenous electron mediators, conjugated oligoelectrolytes as well as nanostructured electrodes to raise the upper-limits of their current maximum outputs.

1.2.3. Enhancing the performance of Escherichia coli-catalyzed MFC based on RF secreting

Nowadays, Engineering the well-known electron transport protein conduits into the most widely used biochemical engineering bacteria, *Escherichia coli*, by means of the complementary method become a necessity. Thus, the MtrCAB electron transport protein conduits from wild-type *Shewanella oneidensis* MR-1 strain as well as the ribAB genes which encodes the first two step of RF biosynthesis are co-expressed in the the *E.coli* BL21(DE) strain. When the MtrCABribAB mutant strain is loaded in the MFC reactor, it can significantly improve the performance of *E.coli*-catalyzed

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MFCs, probably due to the fact that the presence of MtrCAB electron transport protein conduit enables the electron export from the interior of the cell to the exterior of the cell as well as that the self-secretion of RF will enhance the electron transport from the bacteria to the electrode.

1.3. The structure of thesis

This thesis consists of six parts, which contains the introduction (Chapter One), the literature review (Chapter Two), the experiments and results of studies (from Chapter Three to Chapter Five), as well as the last portion-the conclusion and future prospect (Chapter Six). All of them are described briefly as follows:

The Chapter One is a concise introduction about the research background and thesis scope of this work. In the Chapter Two, a comprehensive literature review on both fundamental mechanisms and the practical applications of the MFC system reported recently are summarized as the research premise. From the Chapter Three to Five, the experiments and results of this work is presented. In the Chapter Three, the engineered *E.coli* cells is used as biococatalyst to secrete electron shuttles decoupled from anodic biocatalyst to greatly promote the output performance of MFC. In the Chapter Four, the type II NADH dehydrogenase is overexpressed in the innermembrane of the electricigens to accelerate the electron trans-innermembrane motion. In the Chapter Five, the MtrCAB electron transport protein conduits from

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wild-type *Shewanella oneidensis* MR-1 strain as well as the ribAB genes which encodes the first two step of RF biosynthesis are co-expressed in the *E.coli* BL21(DE) strain. After the MtrCABribAB mutant strain is inoculated in the MFC reactor, the loaded MFC can greatly improve the performance of *E.coli*-catalyzed MFCs. In the Chapter Six, the conclusions generalized from all the studies mentioned-above are outlined. And on the basis of that, the research perspective and feasible studying directions is suggested so that the work of this thesis could be further developed.

Chapter 2. Literature Review

2.1. Microbial fuel cell

The microbial fuel cell (MFC) is one type of microbe-catalyzed fuel cell that is able to transfer chemical energy directly contained in the organic or inorganic substrate into electricity by means of a series of bio-chemical reactions.^{1,2,3,4,5} The microbial metabolism is connected through electron donors and acceptors such as the electrode, which creates the potential difference that functions as the driving power for the production of electricity.^{6,7,8,9} MFC is capable of using a wide scope of soluble simple or complex substrates from wastewater so that it provides the advantage in producing renewable energy as well as simultaneous pollutant remediation, which enables the operation environmental friendly.^{10,11}

2.1.1 Wastewater treatment

Nowadays, the sustainable treatment and exploitation of wastewater is obtaining extensive studies because of the increasing deficiency in water resources, shortage of fossil fuel, and pollution in water. So far, most conventional wastewater remedy procedures need energy and also bring about the issues of pollutants.¹² For example, during treatment of wastewater great amounts of such greenhouse gases as NO_x, CO as well as other volatile substances are released into the environment. In addition, a large quantity of surplus sludge will be generated during the remedy process, whose

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removal consumes energy and is expensive too.¹³ However, wastewater is known to contain a large amount of energy. It is reported that municipal wastewater has about nine times more energy than what is consumed during its treatment in the wastewater treating factory.¹⁴ Thus, how to extract the energy potential in wastewater efficiently is very important to reducing wastewater treating cost. For this purpose, diverse energy-recovering methods and devices such as the anaerobic decomposing and dark fermenting reactors and protocols have been developed.^{15,16} Since MFC is able to recover electricity from organic pollutants during the wastewater treating process, it gains more and more attention at present.^{17,18,19}

2.1.2 Biological catalysts

MFC utilize microbes as the bio-catalysts for transforming the chemical energy available in the wastewater directly into electricity. Only those microbes, which are able to transport electrons from the cell interior to the cell exterior and finally to the insoluble electron acceptors such as metal oxides as well as solid electrodes, named as electricigens, contribute to current production in MFC.¹⁷ The most widely reported electricigens belong to the α -, β -, γ -, and δ -proteobacteria, including *Geobacter sulfurreducens*, *Geobacter metallireducens*, *Shewanella oneidensis*, *Escherichia coli*, *Rhodopseudomonas palustris* and so on.^{20,21,22,23,24} At the same time such non-proteobacteria as *Geothrix fermentans* and yeasts are reported to have the ability of extracellular electron transport too.^{25,26} The difference between electricigens and nonelectricigens lies in whether they are able to export electrons from the interior of

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the cell to the exterior electrode. There are some bacteria like *Shewanella oneidensis* and *Geobacter sulfurreducens* which are much well studied electricigens because they can synthesize electrically active proteins localized in the electrically nonconductive membrane that is comprised mainly of lipid and peptidoglycan. On the basis of this capability, these bacteria turn into the electrical active bacteria which are effective in creating electricity.

2.1.3 Components of MFC setup

There are two major types of MFCs. That is, one chamber MFC and two chamber MFC. A standard one chamber MFC setup is mainly made up of one anodic compartment, one air-cathode and one gas diffusion layer. The anodic compartment and the air-cathode are segregated by the gas diffusion layer, which has to enable the passive oxygen diffusion to the cathode and eventually into the anodic compartment to complete the electric circuit.

A standard two chamber MFC setup is mainly made up of one anodic compartment, one cathodic compartment and one proton-exchanging membrane (Fig. 2.1). In the anodic chamber, electricigens oxidize substrates as electron donors, producing electrons and H^+ . The electrons are transported to the anode and then travel through an external load to the cathode. In the meantime, H^+ diffuses from the anodic chamber to the cathodic chamber through the proton exchange membrane and then reaches electroneutrality. At the cathode, such terminal electron acceptors as O_2 , NO_3^- , or SO_4^{2-} , receive the electrons together with H^+ to generate new chemical products.

Figure 2.1. Schematic of the working principle of MFC. Reproduced from Ref. 181 with permission from the PCCP Owner Societies.

MFC is capable of producing electricity from almost all kinds of degradable organic sources in the wastewater, which comprises simple molecules such as acetate, glycerol or lactate, as well as polymers like proteins.^{5,11}

2.2. Electricigens

In general, electricigens are the crucial factor that controls the entire MFC performance by means of their metabolic activities and extra-cellular electron transport (EET). Electron delivering can come about by outer-membrane cytochromes, namely direct electron transport or electron shuttles, which is known as mediated electron transport.^{27,28} Different kinds of single and mixed culture of electricigens from diverse sources are studied for their performances of electricity generation in the MFC systems. Nourishing the electro-active microorganisms at the electrode surface is reported to contribute to higher power densities.²⁹

2.2.1 Single electricigen at the anode

A lot of microbes have been studied to evaluate their performances of electricity generation during the MFC operation. Among all reported microbes, *G. sulfurreducens*,^{20,30} *R. ferrireducens*,³¹ *A. hydrophila*,³² *P. aeruginosa/otitidis*,^{33,34} *G. electrodiphilus*,³⁵ *D. propionicus*, *E. coli*, *R. palustris* DX-24 *S. oneidensis*, *S. halotitis*³⁶ are electro-active and facultatively anaerobic microbe, which are able to reduce metals. Oxygen diffusion through the cathodic compartment into the anodic compartment could regulate the microbial metabolic actions, and thus the unobligated anaerobic microbes raised well in the anodic compartment.³⁷ Metal-reducing microbes have a specific capability of delivering electrons to the anode by means of direct contact. Outer-membrane protein cytochrome c (c-cyts) could transport the electrons from the interior of the microbe to the exterior of the cell. These microbes also synthesize bionanowires to enhance the microbe-electrode physical contact and consequently enhance their power density.³⁸ *Geobacter* and *Shewanella* strains are the most studied pure strains in this area. Until now MFC inoculated with single microbe have been mainly used at laboratory-scale for mechanism purpose rather than for industrial applications. In addition to exploiting bacteria kingdom, MFC can be run by taking the yeast as the bioelectrocatalyst.³³

2.2.2 Microbial community at the anode

Mixed cultured microbes gained from different sources such as anaerobic or aerobic reactors, sludge, municipal waste and so on, are also studied during MFC

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operation as biocatalyst. In contrast to the pure culture at anodic chamber, MFC loaded with mixed cultures is a naturally open system and according is more close to the practical applications and is responsible for its economic feasibility particularly when utilizing the wastewater as the electrolyte.³⁹ But, the bacterial community of a hybrid cultured MFC changes according to the source of microbe, fuels diversity, reaction container types and other running requirements.^{6,7,40,41,42} The altering of microbial consortia affects electricity production efficiency and consequently the MFC performance.⁴³ It is reported that mixed cultured MFC generated higher power outputs but lower faradic efficiencies because of the likely various microbial metabolic reactions as well as the increased mass transport losses. Other factors such as the quorum sensing is also described to regulate the electrogenic ability of microbes when cultivating them in hybrid.^{44,45} Thus, mixed cultured MFC will be a potential bioanode for electricity production as well as for the waste remediation.

2.2.3 Biofilm at the anode

Apart from planktonic microbes growing in the anodic chamber, the anode materials of MFC containing pure bacteria or mixed cultured bacteria provides the substrates for the growth of biofilm. Both the planktonic cells and the biofilm residing on the electrode are known to be able to take part in the extracellular electron transport. The importance of biofilm in the performance of MFC is well known. Most of the microbes in MFCs rely on exogenous or endogenous^{46,47,48,49} small electron shuttles for electron transporting and a few of them are capable of transferring the

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electrons directly to the electrode.^{50,41,51,52} Planktonic microbes are able to carry out the electron transport only by soluble electron shuttles, while microbes in the biofilm at the anodic surface are able to deliver electrons straightly from the interior of the cell to the electrode outside the cell or via the bionanowires. Some researchers reported that direct electron transport has higher kinetics than mediated electron transport.⁵³ Thus, it is reasonable that the ability of the electricigens to attach to the anode surface as biofilm will play an important part in enhancing the output power density of MFC.

It is reported that the living of electricigens as planktons or as the biofilm entirely relies on the metabolic level of the electricigens themselves and running environments of MFC. The capability of the biofilm formation at the anode surface is known to be modulated by such factors as biological, chemical as well as physical conditions. The electrochemical-active biofilm residing at the electrode surface by the electricigens has a few potential practical applications, for example the production of energy substances and generation of valuable bio-chemicals.⁴⁶ Thus, our understanding of the biofilm formation as well as its function in microbial-catalyzed MFC will contribute to the improvement of the electricity generating ability. Direct transport of electrons is attainable from inside of the biofilm to outside the electrode surface.⁵⁴ A number of studies are performed focused on the impact of biofilm forming on electricity production^{9,55} and accordingly the activity of biofilm forming at the electrode surface also affected the output results of MFC.⁹ Although the biofilm forming is important to electron export and MFC performance, the thicker biofilm

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may obstruct the electron delivering. Therefore, the best biofilm thickness at the MFC anode surface will be studied to achieve the maximum power outputs. In addition, our understandings of the electroactive biofilms can be stretched to other biofilm-induced progresses such as biosensing as well as biocorroding.

2.3. Mechanisms of electron transport

The chief role of MFC is on the basis of extracting the available electrons by the external electrode as the terminal electron acceptor. The fuel oxidizing is catalyzed by microbes, which happens inside the microbial cells residing at the bio-anode (Equ. 1). This process then generates reducing power (electrons). At the same time reduction occurs at the cathode (Equ. 2). H^+ travels through the proton-changing membrane and arrives at the cathode, during which a relative positive cathodic voltage is created, while a relative negative anodic potential is also created at the anode (Fig. 2.2). The entire reaction consists of the oxidation of substrate to CO_2 and H_2O together with the accompanying electricity generation (Equ. 3). The divergence between the cathodic and anodic voltages is regarded as cell potential that propels the electrons to travel from the anode to the cathode.

2.3.1 Extracellular electron transport (EET)

The so-called electrochemically active microbes are those who have excellent current producing abilities, which are very important to the MFC operating.^{1,4,56,57,58} Electron export from the microbial metabolism of the electricigen itself to the anode is carried out by two major ways, that is, the direct electron transport (DET) and mediated electron transport (MET), dependent upon whether the electron shuttles are used (Fig. 2.3). The EET efficiency is regulated by the voltage divergence between the electron donor and the anode acceptor, in spite of the underlying pathways.^{59,60} Both the cell inner and outer membrane and the cell respiratory chain protein complex provide the reacting place for MFC to extract energy from microbes.⁶⁰ Electricigens uptake and digest various types of substrates by differing anaerobic metabolic pathways inside the cell residing at the anodic compartment of MFC to create cytoplasmic electrons. These electrons are then exported to the anode either through DET by c-cyts localized at the inner and outer membrane of the microbes as well as by the conducting pili, or through MET induced by small soluble redox molecules.^{61,17,62,63,64} EET is a main constraint condition that restrains the output performance of MFC. The electron mediator induced EET is one of the most widely used electron transport routine for a lot of electricigens, such as *Shewanella sp.*, *Pseudomonas sp.*,⁴⁹ and *Escherichia coli*.⁶⁵ But, the outermembrane of these microbe usually has weak permeability so that it is an obstacle for transporting both the electrons and other small molecules over their membranes (contains one or two), which makes the effectiveness of EET unfavorably and may account for the weak

output power density of MFC.

Figure 2.2. Schematic mechanisms of electrons and protons generation and transport within cell outer-membrane and the electrodes. Reproduced with permission from ref. 203. Copyright 2014 Elsevier.

One strategy of enhancing the EET induced by the electron mediators is to make the bacterial membranes more permeable.⁶⁶ This method of increasing membrane penetrability is comparatively simple in the Gram-negative bacteria (G-) compared with the Gram-positive bacteria (G⁺) because of their difference in membrane structure.⁴⁴ After subjected to constant external pressure for the discharging of current, *E. coli* is reported to form large holes on the outer membrane structure and thus the secreting of endogenous electron carriers across cell membrane could be accelerated so that it achieve more effective EET.²³ Membrane permeabilizing agents such as chitosan as well as polyethyleneimine are also utilized to make pores on the microbial

outer membrane. These chemicals are capable of increasing membrane permeability and thus enhancing the secretion of small electron carriers, resulting in an accelerated EET. Membrane permeability is regarded to be very important to the efficiency of EET.⁵⁶ A porin protein OprF from *P. aeruginosa* PAOI is recombinantly expressed in *E. coli*, which could increase membrane penetrability and show the superior electric current than the control bacteria. Other ways such as quorum sensing and synergetic interactions within the microbial consortia also take an important part in helping electron export for increased power output. The small signal chemicals like acyl-homo-serine lactones, peptides and scattering signal molecules could accelerate the bacterial electron export too.⁴⁴

Figure 2.3. Different types of electron transport mechanisms from the electricigen to the anode. Reproduced with permission from ref. 202. Copyright 2012 Elsevier.

2.3.2 Direct electron transport

The DET occurs by means of membrane bound c-cyts or conductive cell

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appendages without soluble electroactive small molecules being used in the extracellular electron transport from the cellular interior to the solid anode outside the cell.^{56,1,63,38} DET is a type of the physical contact between the microbial cell and the anode without involving any electron shuttles. The electricigen ought to have membrane-bound electron transferring proteins that are capable of aiding in the electron transport from the outermembrane of the electricigen to the outside electron acceptor like the anode. It is reported that many electro-active bacteria have demonstrated effective DET capability, for example, *Geobacter*, *Rhodoferrax* and *Shewanella*.^{62,67,31,68} These electricigens have membrane-bound electron transferring proteins which are capable of exporting the electrons from inside of electricigen cells to their outermembranes and eventually to an exterior hard electron acceptor (e.g. the anode). The cytochrome c family and multi-heme proteins are regarded as likely components for DET. One constraint of this mechanism is that the electricigen have to attach to the anode to form the so-called biofilm in order to export the electrons more efficiently. Many of the Gram⁺ ones of the electricigens are proposed to develop the DET mechanisms by means of biofilm forming on the anode surface, which provides a straight contact between the proteins localized at the bacterial membrane and the anode. However only the electricigens localized at the first layer within the biofilms next to the anode are related to the c-cyts mediated DET. The other type of DET is by means of the conducting pili (bionanowire) grown on the electricigen surface associated with the cytochromes which is able to transport electrons from the interior of the biofilm to the outside anode.^{38,69} Electricigens such as *Geobacter* and

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Shewanella are able to create electrically conductive appendages (nanowires).⁵⁰ These appendages might help for forming thick electro-active biofilm, which could result in enhanced MFC performances.⁴¹ DET by electricigen appendages is also possible travelling through membrane-bound c-cyts, because their standard potential are similar. The outer-membrane protein functions involved in DET are reported.^{70,32} Although a large number of research work has been performed about DET principles and the proteins involved, a completely understanding of this phenomena still needs further investigation.

2.3.3 Mediated electron transport

The MET occurs by means of the electroactive electron shuttles which are able to help the electron transferring from the microbial cytosolic area to the electrode. The outermembranes of lots of microbes consist of lipid membrane, peptido glycans and lipopoly saccharides, which are non-conductive so that will obstruct the electron transport to the outside electrode. Electron shuttles are capable of propelling the electron moving rates. These electron shuttles in oxidized form could be reduced in the existence of electrons provided by the cytochrome protein on the cell membrane. The electron shuttles then travel over the cell membrane and be delivered to the anode and be oxidized by the anode. This reversible progress thus enhances the electron transport rate as well as accordingly strengths the electricity generation of MFC. MET happens either by adding man-made small molecules into the reactor or by soluble primary and secondary metabolic products secreted by electricigens themselves

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coming from their own metabolism pathway. An good electron shuttle ought to be capable of travelling over the cell membrane freely and gaining electrons from the terminal cytochromes of the electron export chains easily. In general, they should have a high interfacial reaction rate and good dissolvent ability in the solution. In addition, they ought to be non-biodegradable, non-toxic to the microorganisms as well as inexpensive. An electron shuttle with a upper standard redox voltage is thought to be able to deliver a higher total power output than that with the lower redox potential.⁷¹

Experiments shown that MET is an effective way to connect the energy from the inside microbial metabolism with the outside anode. However, the performance differs according to the redox properties of each electron shuttle. A large variety of chemicals including the inorganic potassium ferricyanide and the organic benzoquinone group, are able to accelerate electron transport efficiency. Other synthetic exogenous mediators contains such as neutral red, methylene blue, thionine, 2-hydroxy-1,4-naphthoquinone as well as Fe^{3+} EDTA.⁷² However, the toxicity and cost of these electron shuttles constraint their practical utilizations in MFCs. Anaerobic bacterial fermentation is capable of producing primary and secondary metabolic products that can act as electron mediators. It is reported that phenazines, phenoxazines, quinines and so on, are the electron shuttles that be secreted by electricigens themselves.⁷³ Electricigens cultivated under soluble electron shuttles removed environments and separated from the outside solid anode are reported to secrete small molecular-weight electron mediating chemicals, such as pyocyanin and

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2-amino-3-carboxy-1,4-naphthoquinone, through their cytosolic metabolisms.^{74,33,75}

Pyocyanin secreted by *P.aeruginosa* is deeply reported because it is effective in electron transport,³⁴ which may also facilitate electron delivering from other microbes to the anode in mixed culture. Since the biosynthesis of these self-secreted chemicals is able to be independent of electron acceptors, the mechanisms of their secreting needs to be further investigated. Generally speaking, these electron shuttles should be reversible in redox status and thus they will be reoxidized during discharging electricity at the anode and then be ready for accepting electrons once again.⁷⁴ However, because their amount in the anolytes are too low, their isolation and determination is very difficult. Till now only pyocyanin chemicals from *P.aeruginosa*³⁴ as well as flavin shuttles from *S. oneidensis*⁷⁶ are reported. In addition, it is reported that the electron shuttles secreted by one microbe could be utilized by the other microbe. This cooperative functions will undoubtedly lead to the enhancement of the mixed cultured MFC in electric energy production.

2.4. Biosynthesis of riboflavin

All the animals as well as a lot of microbes are unable to synthesize riboflavin (vitamin B2, RF) *de novo*.^{77,78,79} However, all the plants plus fungi and most microorganisms are able to generate RF and thus are the origin of vitamin B2 for humans as well as other animals.^{80,81,82} In the meantime, all of the creatures have enzymes that are capable of transforming RF to flavin mononucleotide (FMN) and

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flavin adenine dinucleotide (FAD).^{83,84,85}

The RF biosynthetic pathway in bacteria (Fig. 2.4) begins from two precursors, one of which is GTP and the other one is ribulose-5-phosphate.^{86,87,88} The first step of RF bioproduction is catalyzed by GTP cyclohydrolase II (encoded by *ribA* gene), which is capable of deleting the C-8 from the GTP molecule to generate formate;^{89,90} at the same time, pyrophosphate is removed by the same enzyme too.^{91,92,93} This enzyme is identified and purified from *Escherichia coli* for the first time.^{94,95} It is reported that the GTP cyclohydrolase II of *E. coli* is a homo-dimer which includes Zn^{2+} ions as its cofactor.^{96,97,98} It can be stimulated by Mg^{2+} ions. Thus, this step begins by removing its pyrophosphate and this is also the rate-limiting step of the RF biosynthesis pathway, after which imidazole ring opens, accompanied by formate releasing.⁹⁹

During the following two steps, the amino group at position-2 is de-aminated and the ribosyl side-chain is reduced to ribityl by the bi-functional de-aminases-reductase, which is the product of the *E. coli* gene *ribD*. 5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate, which is created after the first three steps of the RF synthesizing pathway, is further de-phosphorylated to generate 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione, which will be the reactant in the lumazine synthase reaction, the following reaction in the bacterial RF biosynthesizing pathway.¹⁰⁰ After that, the pyrimidine progenitor of flavin molecules, 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione, is transformed into the pteridine molecule, 6,7-dimethyl-8-ribityllumazine.¹⁰¹ The transformation from the

Figure 2.4. Biosynthetic pathway of riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Reproduced with permission from ref. 124. Copyright 2011 American Society for Microbiology.

pyrimidine molecule (containing only one ring in the structure) to the pteridines (containing two combined rings) is catalyzed by the 3,4-dihydroxy-2-butanone

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4-phosphate synthase.^{102,103,104,105,106}

The lumazine synthase in the following step will catalyze condensing the molecule of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and the molecule of 3,4-dihydroxy-2-butanone 4-phosphate. However, this lumazine synthase catalyzed step is able to carry on without the aid of any enzyme in diluted nearly neutral-pH solutions at room temperature, which indicates the fact that the reaction rates enhanced by this enzyme is very little.^{107,108}

The last reaction in the RF synthesizing pathway is catalyzed by the RF synthase, which includes dismutating of two of the 6,7-dimethyl-8-ribityllumazine molecule, in which an interchange of a four-carbon part happens, during which one molecule of them is converted to the RF molecule.^{109,110}

After that, FMN is created by phosphorylating the RF molecule at the ribityl chain catalyzed by RF kinase. Then FAD synthetase catalyzes moving adenyl elements from the ATP molecule to FMN to produce FAD.¹¹¹

2.5. Genetically engineering electricigens

Shewanella oneidensis MR-1 is a Gram-negative facultative anaerobic microorganism which is famous for its respiratory plasticity. In last decades, *Shewanella oneidensis* MR-1 is widely used as one type of model microorganism to explore its ability of bioremediation in the environment. Then, it is found that it can be utilized as a good bioelectrocatalyst for MFC application. Thus, new genetic vector tools are developed, by means of which in principle any metabolic pathway

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within this bacterium can be modified using the genetic engineering method. This offers researchers a good method to improve this bioelectrocatalyst to enhance the performance of MFCs.

Besides traditional electricigens like *Shewanella oneidensis* and *Geobacter sulfurreducens*, recently some scientists are trying to engineer the electron transport protein complex from the electricigens into the *E.coli* strain by the complementary method. Because *E.coli* is the most extensively studied and used bacteria in the biochemical engineering industry.^{197,198} For instance, it is reported that coexpressing MtrA and CymA proteins in the *E.coli* strains will make them become able to reduce the extracellular soluble Fe^{3+} ions except for the solid Fe_2O_3 .^{199,200} Further, coexpressing the MtrCAB electron transport protein complex enables the *E.coli* strain to reduce the extracellular solid Fe_2O_3 .¹⁹⁷

Chapter 3. Improving mediated electron transfer by a bio-cocatalyst

3.1. Introduction

Shewanella oneidensis MR-1, a facultative anaerobe, has been widely used as a model anode biocatalyst in microbial fuel cells (MFCs) due to its easiness of cultivation, adaptability to aerobic and anaerobic environment and both respiratory and electron transfer versatility. Proposed mechanisms for *S. oneidensis* EET include: (i) MET through electron shuttles; (ii) DET through physical contact between outer-membrane c-type cytochromes (c-Cyts) or bacterial appendages and the electrode surface. Although *S. oneidensis* possesses versatile EET mechanisms, their efficiencies are relatively low and could be further improved considering the fact that it exhibits an excess of substrate oxidation ability.¹²⁹ *S. oneidensis* MR-1 is straight rod shaped bacterium¹³⁶ and consequently its cell surface is incapable of achieving complete contact with any two-dimensional (2D) electrode within MFC anodic chamber, which is partly responsible for the slow kinetics of EET to anode. A reasonable solution to this problem is to add exogenous artificial electron shuttles (AES) into the anolyte, which accept electrons from terminal c-Cyts of bacterial EET chain that cannot touch the anode directly, move to anode surface and release electrons to the anode. Exogenous AES such as neutral red,¹³⁷ anthraquinone-2,6-disulfonate (AQDS),²² humic acids,¹³⁸ methyl viologen,¹³⁹

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porphyrins,¹⁴⁰ phenazine¹⁴¹ or Fe-NTA²⁰⁸ are usually utilized. However, they are generally costly and toxic to anodic electricigens, for example, Fe-NTA belongs to the renal and hepatic carcinogen,²⁰⁷ causing difficulties to practical applications of MET-type MFCs. *S. oneidensis* MR-1 is capable of utilizing self-secreted flavins like riboflavin (RF) as endogenous electron shuttles to accelerate EET more efficiently than exogenous AES.^{28,129} Moreover, RF is vitamin B2 and accordingly anodic electricigens have a better tolerance to RF than other toxic AES. Thus, increasing RF concentration in the anolyte is proposed to improve MFC performance.

There are two rational strategies for achieving that goal: (i) *Shewanella* strains could be genetically engineered to enhance their ability to secrete RF. However, only a narrow range of carbon sources such as formate, lactate, pyruvate and amino acids^{141,142} can be used as electron donors by *Shewanella* strains under strictly anaerobic conditions. Metabolizing the limited categories of fuels by engineered *Shewanella* strains to not only generate electricity but also increase the yield of self-secreted RF will undoubtedly lower the coulombic efficiency and consequently reduce energy harvesting from biomass by MFCs. Recently, *S. oneidensis* MR-1 has been engineered to gain the ability to utilize glucose, which is a platform chemical in biorefinery and cannot be metabolized by *Shewanella* strains under strictly anaerobic conditions,^{143,144} as carbon source to generate electricity in MFCs.¹⁴⁵ Thus, there exists a theoretical possibility that this recombinant glucose-utilizing *S. oneidensis* MR-1 strain could be further engineered to increase its RF secretion. However, the fact that this recombinant *S. oneidensis* MR-1 strain cannot survive without external

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electron acceptors like fumarate or electrode under anaerobic condition¹⁴⁵ indicates that anode surface area will limit the amount of the recombinant riboflavin-secreting strain and eventually limit the concentration of RF in the anolyte; (ii) *S. oneidensis* MR-1 could be co-cultivated in combination with riboflavin-producing microbes in MFCs. Recent studies have shown that the interactions between microbes in mixed culture systems are complicated and consequently their electrochemical performances are quite unpredictable.^{146,147} a synergistic effect with enhanced electricity generation;¹⁴⁸ a food-web relationship with similar power output;¹⁴⁹ and a negative effect with reduced electricity production^{150,151}, compared to a pure culture of anodic electricigen. Ideally, to achieve the maximum electrical output, the anode surface ought to be completely occupied by electricigens. However, the presence of nonelectricigen within the anode microbial community in MFCs will inevitably reduce the effective anode area available for electricigen accumulation on the electrode, which might partly account for differing MFC performances in mixed culture systems.

Escherichia coli, an easily grown facultative anaerobe capable of utilizing both nonfermentable and fermentable carbon sources, can be cultivated under strictly anaerobic condition without external electron acceptors. Herein, we engineer *E. coli* BL21 (DE3), a widely used strain for protein expression, to enhance its anaerobic RF secretion. The recombinant *E. coli* cells are then immobilized in alginate beads, placed at the bottom of MFC reactor without any occupation of anode surface and metabolize glucose to secrete RF, as a bio-cocatalyst, to improve the electricity

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generation by *S. oneidensis* MR-1, as a biocatalyst, at the anode. In principle, these bio-cocatalyst beads can be loaded into the continuous flow reactor for wastewater treatment, saving the cost of adding exogenous AES and in particular, the amount of bio-cocatalyst beads used can be adjusted according to the flavinogenic activity, hydraulic retention time (HRT), reactor volume, etc. Recently, it has been reported that flavins promote EET of *Enterococcus faecalis*, three *methanotrophic bacteria*, *alkaliphilic Bacteria consortium*, and even *Geobacter sulfurreducens* as either electron shuttles or redox cofactors. It suggests that our riboflavin-secreting bio-cocatalyst beads, given that the immobilized bio-cocatalyst avoids direct contact with anodic electricigen and consequently unpredictable interactions between nonelectricigen and electricigen at the anode, can be applied in combination with diverse biocatalysts besides *S. oneidensis* MR-1 in MFCs in the future.

3.2. Materials and methods

3.2.1. Bacteria strains and culture conditions

All *E.coli* strains are conventionally grown in Luria-Bertani (LB) broth at 37 °C with shaking at 220 rpm. When needed, ampicillin (100 µg/ml) is used to select positive clones and maintain plasmids. When the optical density of bacterial culture at 600 nm (OD₆₀₀) reached 0.5, 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) is added to induce overexpression of recombinant proteins for 24 h.

1 ml *S. oneidensis* MR-1 culture is inoculated in 100 ml LB broth and incubated at

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30°C with shaking at 220 rpm until OD₆₀₀ value reached about 1.0. The bacteria culture is then harvested by centrifugation (4000 rpm×10 min). The bacteria pellets are washed three times with M9 minimal medium (6.78 g/L Na₂HPO₄; 3g/L KH₂PO₄; 1g/L NH₄Cl; 0.5g/L NaCl; 0.011 g/L CaCl₂; 0.12 g/L MgSO₄) and suspended in 100 ml anolyte (5% LB broth plus 95% M9 minimal medium containing 26mM lactate and 15 g/L glucose, PH 7.0). The bacteria suspension (10⁹ cells / mL) is then transferred into the microbial fuel cell (MFC) anodic chamber and purged over nitrogen gas via a 0.2µm filter for 35 min to remove oxygen.

3.2.2. DNA manipulation and recombinant construction

To initiate protein synthesis accurately from the start codon of each rib gene itself, the upstream start codon (ATG) next to multiple cloning sites (MCS) of pQLinkN vector is replaced with AAG, by using KOD Plus Mutagenesis Kit (Toyobo, Japan) and the primers pQlinkN-Mut-F/R (Table 3.1). The resulting vector is named as pQLinkNMut. Genomic DNA of *E.coli* K-12 strain is purified by using GeneJET Genomic DNA Purification Kit (Thermo scientific, USA). The five genes of riboflavin (RF) biosynthetic pathway, *ribA* (Gene ID: 945763, NCBI), *ribB* (Gene ID: 947526, NCBI), *ribD* (Gene ID:945620, NCBI), *ribC* (Gene ID:945848, NCBI), and *ribE* (Gene ID:946453, NCBI), are amplified by polymerase chain reaction (PCR) using gene specific primers (Table 3.1), respectively. The PCR products are digested with BamHI and NotI, and the restriction fragments are ligated into expression vector pQLinkNMut at the downstream of Ptac promoter (Figure 3.1). The recombinant

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vectors are electroporated into *E. coli* BL21 (DE3) electrocompetent cells. The transformants are screened by PCR and then verified by sequencing (Aitbiotech, Singapore). The plasmids of positive clones are purified and named as pQLinkNMut-RibA, pQLinkNMut-RibB, pQLinkNMut-RibD, pQLinkNMut-RibC and pQLinkNMut-RibE, respectively.

For constructing the coexpression plasmid of pQLinkNMut-RibAB, 0.4 pmol plasmid of pQLinkNMut-RibA is digested for 20 hour at 25 °C with 5 U *Swa*I in 10µl and 0.4 pmol plasmid of pQLinkNMut-RibB is digested for 20 hour at 37°C with 5 U *Pac*I (New England Biolabs, USA) in 10µl. Enzymes are inactivated by incubating at 65°C for 20 min and 0.2 pmol DNA is treated with 1 U LIC-qualified T4 DNA Polymerase (Merck, USA) in total volume of 20µl (T4 DNA Polymerase Buffer, 10mM DTT, Nuclease-free Water, 2.5mM dGTP for *Swa*I digest and 2.5mM dCTP for *Pac*I digest). Upon incubation at 22 °C for 30 min and sequentially at 75 °C for 20 min, 2µl of the above T4 DNA Polymerase treated solution (*Pac*I digest, containing RibB insert) is mixed thoroughly with 1°C of that solution (*Swa*I digest, containing linearized pQLinkNMut-RibA) and heated to 65°C for 10s and then incubated at 22 °C for 5 min. The mixture is supplemented with 1µl 25mM EDTA, followed by incubating at 22 °C for 25 min. The resulting product is electroporated into *E. coli* BL21 (DE3) electrocompetent cells and screened by PCR and verified by sequencing. Following procedures mentioned above, the coexpression plasmid of pQLinkNMut-RibCE is produced from pQLinkNMut-RibC and pQLinkNMut-RibE. The coexpression plasmid of pQLinkNMut-RibABD is produced from

pQLinkN-Mut-RibAB and pQLinkN-Mut-RibD. A ~9.6k coexpression plasmid of pQLinkN-Mut-RibABDCE is eventually constructed from pQLinkN-Mut-RibABD and pQLinkN-Mut-RibCE (Figure 3.1). The recombinant plasmid is electroporated into *E. coli* BL21 (DE3) electrocompetent cells. The positive clones are screened by PCR and sequenced using specific designed primers V-pQLinkN-F/R (Table 3.1) unless otherwise specified.

Tabel 3.1 Primers used in chapter 3

Primer name	Primer sequences
pQlinkN-Mut-F	5' -GAGAAATTA ~~ACTAAGGGATCCAGTCTTCG~~ -3'
pQlinkN-Mut-R	5' -CTCTTTAATGAATTCTGTGTGAAATTGTTATC-3'
V-pQLinkN-F	5' -CGACCGAGTTGCTCTTGC-3'
V-pQLinkN-R	5' -ATCAGGCGGGCAAGAATG-3'
ribA-F	5' -CGGGATCCATGCAGCTTAAACG-3'
ribA-R	5' -ATAGTTTAGCGGCCGCTTATTTGTTC-3'
ribB-F	5' -CGGGATCCATGAATCAGACGCTACTT-3'
ribB-R	5' -ATAGTTTAGCGGCCGCTCAGCTGG-3'
ribC-F	5' -CGGGATCCATGTTTACGGGGATTGTAC-3'
ribC-R	5' -ATAGTTTAGCGGCCGCTCAGGCTTCT-3'
ribD-F	5' -CGGGATCCATGCAGGACGAGTATTACA-3'
ribD-R	5' -ATAGTTTAGCGGCCGCTCATGCACC-3'
ribE-F	5' -CGGGATCCATGAACATTATTGAAGCTAAC-3'
ribE-R	5' -ATAGTTTAGCGGCCGCTCAGGCCT-3'

* Restriction enzyme sites for *Bam*HI or *Not*I are shown in *italics*

3.2.3. Anode preparation, MFC setup and operation

Dual-chamber glass MFCs (136 ml each chamber) separated by Nafion 117 membrane (DuPont, USA) are constructed. The cathode is made from a carbon cloth (GasHub, Singapore) with geometric area of 6 cm². The anode is made from a carbon

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cloth or carbon felt (6 cm² for both). Before used, both carbon cloth and carbon felt are treated with acetone and 1 M HCl. When required, the anode is treated with oxygen plasma atmosphere using plasma cleaner system (Harrick, USA) to enable it to become more hydrophilic for better electricigen adhesion. The sidewall of carbon felt is sealed with silicone rubber (Dow Corning, USA). The cathodic chamber contains 50 mM K₃Fe(CN)₆ and 50 mM KCl. The catholyte level is adjusted to the anolyte level. A 2k Ω load resistor is connected into external MFC circuits, unless noted otherwise. The voltage across the load resistor is recorded by using a digital multimeter (ESCORT 3146A). MFCs are incubated at 30 °C.

3.2.4. Preparation of bio-cocatalyst beads, operation and reactivation

Recombinant *E.coli* cells cultivated in 3L LB broth at 37 °C (supplemented with 100 μ g/ml ampicillin and 1 mM IPTG) are harvested by centrifugation at 4000 rpm for 15 min at 4 °C, rinsed three times with anolyte and the cell pellets (approximately 9 g/ wet weight) is suspended and mixed thoroughly with 30 ml 3% sterile alginate (completely dissolved in anolyte). The mixture is then extruded via a syringe into a sterilized 0.1 M CaCl₂ solution to form calcium alginate beads with the average diameter of 2 mm. The beads are cured in the same solution at 4 °C for 24 h. Before used, the bio-cocatalyst beads (approximately 39 g/ wet weight) are rinsed three times with anolyte to remove excess CaCl₂ and free cells. To test flavinogenic activity of immobilized bio-cocatalyst, the beads (39 g/wet weight) are transferred into an anaerobic culture bottle containing 100 ml anolyte, which is purged over nitrogen gas

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(filtered by 0.2 μ m membrane) for 35 min to remove dissolved oxygen and incubated anaerobically at 30 °C. After completion of one batch (168 h), the beads are filtered, rinsed three times and transferred into a new bottle containing fresh anolyte for the next batch under the same experimental conditions. After completion of the eighth batch, the bio-cocatalyst beads are reactivated by replacing anolyte with 100 ml fresh LB broth (supplemented with 100 μ g/ml ampicillin, 1 mM IPTG and 0.1 M CaCl₂) and incubated at 37 °C for 24 h. The beads are then filtered, rinsed three times and transferred again into a new bottle containing fresh anolyte for the ninth batch. Samples (100 μ l) are taken at 12 h intervals for HPLC analysis.

After a stable output of MFC appeared (about 500 h after inoculation, electrolytes are replenished during operation when required), the bio-cocatalyst beads (39 g/wet weight) are loaded into MFC anodic chamber containing 100 ml fresh anolyte and purged over nitrogen gas for 35 min to remove oxygen.

3.2.5. Chemical and biological analysis

Electrochemical characterization

All MFC performance measurements are performed after a stable output appeared (about 500 h after inoculation, electrolytes are replenished during operation when required). The polarization and power output curves are obtained by varying external load resistors. Both current density and power density are normalized to the geometric area of anode. All electrochemical experiments are conducted by using VersaSTAT-3F workstation (Princeton Applied Research) with a Ag/AgCl (saturated KCl) reference

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electrode. For the cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements, the reference electrode is inserted into anodic compartment. The EIS measurement is carried out over a frequency range of 0.3 Hz to 300 kHz at open circuit potential (OCP) with ac amplitude of 10 mV. For the Tafel measurement, the reference electrode is inserted into cathodic compartment to relieve limitations to current density exerted by the cathode.¹¹⁷ Tafel plots are recorded for the anode being swept at 0.2 mV/s from $\eta=0$ to 0.25 V, where $\eta=0$ is the OCP of the anode versus the reference electrode. All electrochemical tests are performed at 30°C.

High-performance liquid chromatography (HPLC) analysis

The HPLC analysis is performed at an Agilent 1100 HPLC system (Agilent Technologies, USA) equipped with a UV detector. The column is a Hibar 250-4 Lichrospher 100 RP-18 (Merck, USA). According to the procedures of Vasilaki et al¹¹⁸ and Yong et al,¹¹⁹ flavins are eluted with a mobile phase of acetonitrile and 50 mM Sodium dihydrogen phosphate (15:85, PH 3.0) at the flow rate of 0.4 ml/min and monitored at 270 nm. According to the procedure of Baere et al,¹²⁰ lactate is eluted with a mobile phase of acetonitrile and 25 mM Sodium dihydrogen phosphate (5:95, PH 2.2) at the flow rate of 0.4 ml/min and detected at 210 nm.

Colony forming units determination

According to the procedure of Merritt et al,¹²¹ immediately after CV measurements, the anodic carbon cloth is aseptically transferred into a 15 ml tube containing 10 ml sterile Phosphate buffered saline (Life technologies, USA) and vortexed thoroughly to detach all bacteria from the carbon cloth. Then the vortexed

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sample is 10-fold serially diluted and 100µl of each diluted solution is spread onto a separate LB agar plate. The plates are incubated overnight at 30°C and the number of colony forming units (CFU) per anodic carbon cloth are determined in triplicate by using cell counter and the results are recorded only when between 50 and 400 colonies are counted each plate.

Coulombic efficiency

To determine coulombic efficiency, MFC is connected using a 1kΩ load resistor.

Coulombic efficiency is calculated as:^{122,123}

$$\varepsilon_c = \frac{M \int_0^t I dt}{F b_e V_{an} \Delta C} \times 100\%$$

where M is the molecular weight of the substrate lactate (M=90.08), I is the current over a period of time t, F is Faraday's constant (96,485 C/mole of electrons), b_e is the theoretical number of mol of electron produced per mol of substrate during full oxidation of lactate (b_e=12), V_{an} is the liquid volume of in the anodic chamber, ΔC is the substrate concentration change over the batch cycle.

3.3. Result and discussion

3.3.1. Recombinant *E.coli* construction and beads preparation

The five-gene biosynthetic pathway of RF is constructed by cloning ribA, ribB, ribD, ribC and ribE¹²⁴ genes from *E.coli* K-12 genome into pQLinkNMut vector, a derivative of pQLinkN vector for protein coexpression,¹²⁵ under the control of Ptac

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promoter, resulting in a ~9.6k plasmid of pQLinkNMut-RibABDCE, which is then electroporated into *E. coli* BL21 (DE3) electrocompetent cells (Fig. 3.1 and Table 3.1). To examine the anaerobic flavinogenic activity of bio-cocatalyst beads, 39 g of beads (9 g recombinant *E. coli* cell pellets, wet weight) are placed into an anaerobic culture bottle containing 100 ml anolyte and incubated anaerobically at 30 °C. Samples are collected at 12 h intervals and analyzed by HPLC. As shown in Fig. 3.2, the RF concentration in anolyte can reach up to $82.04 \pm 0.83 \mu\text{M}$ ($n=3$) in the first batch (168 h). Although RF is known as a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD),¹²⁴ both of which could also be used as electron shuttles to enhance EET,^{126,127} neither of them is detected within the anolyte by HPLC analysis (data not shown). Thus, the contribution of bio-cocatalyst to MFC performance is attributed solely to the increase of RF concentration. To evaluate the reusability of these bio-cocatalyst beads, experiments of repeated batch operation are performed for eight cycles.

Figure 3.1. Flowchart of DNA manipulation and construction of co-expression vectors. A: pQLinkNMut-RibA, B: pQLinkNMut-RibB, C: pQLinkNMut-RibC, D: pQLinkNMut-RibD, E: pQLinkNMut-RibE, AB: pQLinkNMut-RibAB, CE: pQLinkNMut-RibCE, ABD: pQLinkNMut-RibABD

Figure 3.2. Anaerobic flavinogenic activity measurements. 39 g of beads (9 g recombinant *E. coli* cell pellets, wet weight) are placed into an anaerobic culture bottle containing 100 ml anolyte (5% LB broth plus 95% M9 minimal medium containing 26 mM lactate and 15 g/L glucose, PH 7.0) and incubated anaerobically at 30 °C. After completion of 8 batches, the bio-cocatalyst beads are reactivated by replacing anolyte with 100 ml fresh LB broth (supplemented with 100 µg/ml ampicillin, 1 mM IPTG and 0.1 M CaCl₂) and incubated at 37 °C for 24 h. Samples (100 µl) are taken at 12 h intervals for HPLC analysis. All measurements are carried out in triplicates.

RF secretion by bio-cocatalyst beads gradually decreased from the first cycle to the eighth cycle. After eight reuse cycles, approximately 42% of flavinogenic activity of bio-cocatalyst beads still remained (Fig. 3.2). To test the ability to recover their flavinogenic activity, these beads are reactivated by 100 ml fresh LB broth supplemented with 100 µg/ml ampicillin, 1 mM IPTG and 0.1 M CaCl₂. After

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incubated at 37 °C, the restored activity of bio-cocatalyst beads is approximately 60% of original flavinogenic activity (Fig. 3.2).

3.3.2. MFC performances with or without bio-cocatalyst beads

Dual chamber reactors with the *S. oneidensis* MR-1 bio-anode, Potassium ferricyanide cathode and 2 k Ω load resistor are used to evaluate MFC performances, except as noted. The anode is made from carbon cloth, which could be made more hydrophilic for better electricigen adhesion by oxygen plasma treatment.¹²⁸ After a stable output of MFC appeared (about 500h after inoculation), the number of living microbes at the O₂ plasma treated anodic carbon cloth determined is $15.89 \pm 0.35 \times 10^6$ colony forming units per square centimeter (CFU/cm²), compared to $2.61 \pm 0.08 \times 10^6$ CFU/cm² at the anodic carbon cloth without O₂ plasma treatment. Then, 39 g of bio-cocatalyst beads are loaded into this MFC anodic chamber containing 100 ml fresh anolyte. After another 160 h, the stable output of MFC occurred again. At this point, 100 μ l of sample is taken for HPLC assay and the MFC is used for electrochemical measurements.

Figure 3.3. (a) Power output and polarization curves of MFCs with oxygen plasma treated carbon cloth anodes. RF concentration in MFC anodic chamber containing bio-cocatalyst beads accumulated to 81 μ M after loading these beads for about 160 h. (b) Power output and polarization curves of MFCs using carbon cloth anodes without oxygen plasma treatment. RF concentration in MFC anodic chamber containing bio-cocatalyst beads accumulated to 46 μ M after loading these beads for about 66h. (c) Power output and polarization curves of MFCs with oxygen plasma treated carbon felt anodes.

Given that flavins are capable of increasing the amount of microbes colonizing the anode,¹²⁹ the *S. oneidensis* MR-1 bio-anode incubated with bio-cocatalyst beads under the same condition mentioned above is transferred into MFC reactor without bio-cocatalyst beads as the control. The polarization and power output curves (Fig. 3.3a) show that although the OCP of the MFC with O₂ plasma treated anode containing bio-cocatalyst beads (799.6mV) is slightly higher than that of the control MFC (785.8mV), the former MFC delivers a maximum power density of 1079.3 mW/m², corresponding to a current density of 2721.5 mA/m² at a cell potential of 396.6 mV, which is 9.6-fold as much as that of the control MFC (112.2 mW/m²), obtained at a current density of 210.7 mA/m² with a cell potential of 532.4 mV. The maximum current output of the former MFC determined by the polarization curve is 3804.3 mA/m², which is almost 8-fold higher than that of the control MFC (477.5 mA/m²). The role of bio-cocatalyst beads in promoting performance of the MFC using anode without O₂ plasma treatment is also examined. The polarization and power output curves reveal (Fig. 3.3b) that the maximum power density of the MFC without plasma treatment containing bio-cocatalyst is 434.1 mW/m², which is

Figure 3.4. Power output and polarization curves of MFCs (a) without fuel using oxygen plasma treated carbon cloth anodes. 82 μ M RF is added into the MFC anodic chamber containing anolyte without lactate. (b) without fuel and oxygen plasma treatment. 47 μ M RF is added into the MFC anodic chamber containing anolyte without lactate.

9.9-fold as much as that of the control MFC (43.7 mW/m^2). Thus, MFCs loaded with

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bio-cocatalyst beads have much lower polarization for better energy conversion efficiency, regardless of the amount of electricigen at the anode.

The polarization and power output curves of the MFC using O₂ plasma treated anode in the anolyte without lactate containing 82 μ M RF is compared with that containing no additional RF. The results (Fig. 3.4a) showed that the former MFC delivered a maximum power density of 36.1 ± 0.5 mW/m², corresponding to a current density of 68.2 ± 1.0 mA/m² at a cell potential of 529.7 ± 0.4 mV, which is 4.6-fold as much as that of the MFC containing no additional RF (7.8 ± 0.3 mW/m²), obtained at a current density of 14.5 ± 0.5 mA/m² with a cell potential of 539.5 ± 0.5 mV. The maximum current output of the former MFC is 110.9 ± 0.7 mA/m², which is almost 3-fold higher than that of the MFC containing no additional RF (37.9 ± 0.3 mA/m²). According to the measurements, the maximum power density of the MFC with no fuel containing 82 μ M RF (36.1 ± 0.5 mW/m²) accounted for 3.3% of that of the MFC with bio-cocatalyst beads containing fuel (1079.6 ± 5.1 mW/m², Fig. 3.3a). The maximum current output of the MFC with no fuel containing 82 μ M RF (110.9 ± 0.7 mA/m²) accounted for 2.9% of that of the MFC with bio-cocatalyst beads containing fuel (3804.3 ± 9.4 mA/m², Fig. 3.3a).

The polarization and power output curves of the MFC using untreated anode in the anolyte with no fuel containing 47 μ M RF are also examined (Fig. 3.4b). It delivered a maximum power density of 7.8 ± 0.2 mW/m², which accounted for 1.8% of that of the MFC with bio-cocatalyst beads containing fuel. Its maximum current output (42.6 ± 1.0 mA/m²) accounted for 2.3% of that of the MFC with bio-cocatalyst

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beads containing fuel (Fig. 3.3b).

Electrochemical impedance spectroscopy (EIS) measurements are performed and the Nyquist plots (Fig. 3.5a) represent semicircles over the high frequency range, followed by straight lines. A Randle equivalent circuit is used to fit the impedance data, in which the interfacial charge transfer resistance (R_{ct}) is equal to the diameter of semicircle.¹³⁰ For *S. oneidensis* MR-1 bio-anode in the MFC containing bio-cocatalyst beads, R_{ct} greatly declines compared to that in the MFC without bio-cocatalyst. To determine the coulombic efficiency, the load resistor of MFC is replaced with 1 k Ω . The Coulombic efficiency of MFC containing bio-cocatalyst beads is 10.3 %, which is higher than that (7.6 %) of the control MFC. The low charge transfer resistance may reduce energy loss during electron transport and could increase the coulombic efficiency of MFC containing bio-cocatalyst. The Tafel plots (Fig. 3.5b) show that the exchange current density of MFC loaded with bio-cocatalyst beads, determined by extrapolation of Tafel slopes back to $\eta=0$ (corresponds to the OCP of the bio-anode *versus* the reference electrode),¹³¹ is 4-fold as much as that of the control MFC. It is speculated that RFs are reduced by out-membrane *c*-Cyts, move *via* diffusion to the anode and oxidized by the electrode.¹³² The bio-cocatalyst beads are capable of raising RF concentration in the anolyte and thus increase RF flux between *c*-Cyts in the biofilm and the anode. Accordingly, the concentration of reduced RFs close to the anode surface is increased, which leads to the enhanced current density (j).

Figure 3.5. (a) Electrochemical impedance spectra of *S. oneidensis* MR-1 bio-anode in anodic chamber. (b) Tafel plots recorded at the scan rate of 0.2 mV/s from $\eta = 0$ to 0.25 V versus OCP.

3.3.3. Comparison between mediated and direct electron transport

To further explore the underlying mechanism of *S. oneidensis* EET kinetics enhanced by RF-secreting bio-cocatalyst, the electrocatalytical response of *S. oneidensis* MR-1 bio-anode to the presence of varying RF concentrations is analyzed. After anolyte in MFC is replaced with the fresh one, RF is added into the anodic chamber and cyclic voltammetry of *S. oneidensis* MR-1 biofilm residing on the O₂ plasma treated carbon cloth anode in the presence of increasing levels of additional RF is recorded (Fig. 3.6a). The magnitude of catalytic current is measured at -0.2 V to minimize the interference of nonspecific current and maximize the contribution to the catalytic current from RF.^{129,126} The catalytic current demonstrated saturating behavior with the increase of RF concentration. The data are fit to the Michaelis-Menten equation to yield an apparent Michaelis constant (K_m) of $30.4 \pm 1.5 \mu\text{M}$ which represents an average of *c*-Cyts in the electroactive *S. oneidensis* MR-1 biofilm. The cyclic voltammetry of *S. oneidensis* MR-1 biofilm at the anodic carbon cloth without O₂ plasma treatment in the presence of increasing levels of additional RF is also examined (Fig. 3.6b). After fitting, these data yielded an apparent K_m of $30.5 \pm 2.1 \mu\text{M}$. K_m is known as a physical constant characteristic of enzyme-catalyzed reactions and for the *c*-Cyts catalyzed reduction of RF (eqn(1)), K_m is defined as (eqn(2)).

$$K_m = (K_1 + \dot{K}_2)/K_{-1} \quad (2)$$

Although the reaction rate j captured by electrochemical workstation corresponds to the concentration of reduced RF at the surface of electrode rather than that in the vicinity of *c*-Cyts in the biofilm, according to the model developed by Torres *et al.*¹³², transfer of RF to the electrode is accomplished by diffusion obeying Fick's law and consequently j is proportional to the concentration of reduced RF in the vicinity of

Figure 3.6. (a) Typical cyclic voltammograms (1 mV/s) from a *S. oneidensis* MR-1 biofilm residing on the O₂ plasma treated carbon cloth anode in 0, 1.2, 2.26, 3.22, 5.44, 11.8, 17.1, 22.4, 33, 43.6, 54.2, 70.1, 86 and 101.9 μ M RF. The electrochemical potential (-0.2 V) where catalytic current is analyzed is illustrated by a dash line. Insert: Variation of the magnitude of the catalytic current, measured at -0.2 V, with RF concentrations (background value at 0 μ M RF is subtracted). The line shows the catalytic current arising from a Michaelis-Menten type of kinetics with a K_m value of 30.4 μ M. (b) Typical cyclic voltammograms (1 mV/s) from a *S. oneidensis* MR-1 biofilm

residing on anodic carbon cloth without O₂ plasma treatment in 0, 1.1, 2.16, 3.22, 5.34, 11.8, 17, 22.3, 32.9 and 43.5 μ M RF. The electrochemical potential (-0.2 V) where catalytic current is analyzed is illustrated by a dash line. Insert: Variation of the magnitude of the catalytic current, measured at -0.2 V, with RF concentrations (background value at 0 μ M RF is subtracted). The line shows the catalytic current arising from a Michaelis-Menten type of kinetics with a K_m value of 30.5 μ M.

outer-membrane *c*-Cyts saturated with RF. Accordingly, K_m obtained by fitting data on variation of current with added RF concentrations is identical to that obtained by variation of reduced RF concentration in the vicinity of *c*-Cyts with added RF concentration and this method has the superiority that the reactivity of outer-membrane *c*-Cyts in the electroactive biofilm with RF can be easily determined in real time by workstation. Thus, K_m could be used to estimate the turnover number (K_{cat}) of *c*-Cyts in the biofilm. As K_{cat} / K_m values with RF for purified MtrC and OmcA are 1.2×10^5 and $5.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ respectively,¹³³ the estimated K_{cat} ranged

Figure 3.7. Peak potential separation versus log(scan rate) for *S. oneidensis* MR-1 biofilm and RF on the carbon cloth. The former experiment is performed in the fresh

anolyte, whose data are fit to a k_{s1} of 2 s^{-1} and the latter one is performed in the fresh anolyte containing $1 \mu\text{M}$ RF, whose data are fit to a k_{s2} of 7 s^{-1} , according to the Laviron theory.

from 3.7 to 17.9 s^{-1} . Although this K_{cat} window is at least one order of magnitude slower than that (from 617 to 1045 s^{-1}) estimated from data obtained by measuring relative fluorescence unit changes of cells cultivated in flasks,¹³⁴ the electron transfer rate constant between *c*-Cyts in the biofilm and RF (from 7.4 to 35.8 s^{-1}) is still higher than that ($k_{s1}=2$) between *c*-Cyts in the biofilm and the carbon cloth anode (Fig. 3.7), given that each RF accepted 2 electrons from *c*-Cyts at a time. *In vitro* studies have observed that *c*-Cyts redox kinetics are at least two orders of magnitude faster

Figure 3.8. Schematic of the mechanism of MFC anodic electrocatalyzed process

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enhanced by the bio-cocatalyst beads.

than that *in vivo*.^{129,133,135} As an explanation for this inconformity, transporting electrons from the cell interior to the external electrode directly through *c*-Cyts resulted in cation accumulation within the cell, which produced the liquid junction potential to retard the electron export.¹³⁵ RF is assumed to have the capability of coupling H^+ export with electron transport, which could alleviate this retardation and accelerate electron transfer. Furthermore, the interfacial electron transfer rate constant (K_{s2}) between RF and carbon cloth electrode determined is $7s^{-1}$ (Fig. 3.6b), which is faster than that ($k_{s1}=2$) between *c*-Cyts in the biofilm and the electrode. Therefore, we propose that electron export to electrode *via* RF shuttles has a faster kinetics than that *via* direct contact (Fig.3.8).

3.4. Conclusion

In conclusion, by immobilizing engineered RF-secreting *E.coli* cells as bio-cocatalyst beads, we present a novel strategy of using bio-cocatalyst beads to significantly enhance the performance of MFCs without any noble metal catalyst, which has the following potential advantages: The maximum RF concentration is unlimited; The anode surface area is unoccupied; They are reusable. Owing to lack of direct contact with anodic electricigen, they also have the potential to be applied together with diverse biocatalysts besides *S. oneidensis* MR-1.

3.5. Declaration

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L. Tao, H. Wang, M. Xie, L. Thia, W. N. Chen and X. Wang, Chemical communications, 2015, 51, 12170.

Chapter 4. Bridging fuel oxidation and electron transport in microbial electrocatalysis

4.1. Introduction

The microbial electrocatalysts can utilize the intact living microbes to oxidize fuels to generate electricity. The microbial electrocatalytic processes are thus divided into metabolism of substrates as well as the electron transport pathway, which is in turn composed of the quinone pool of the inner membrane, the inner membrane-anchored periplasmic CymA, the periplasmic MtrA, the integral outer membrane (OM) porin MtrB and the OM-anchored MtrC/OmcA (the latter five proteins, a.k.a. electron conduit),¹⁵² both of which are separated spatially and accordingly functionally by the inner membrane (IM) of the cell. Some studies are centered on microbial metabolism, in which overexpression of glycerol dehydrogenase¹⁵³ or manipulation of cofactor¹⁵⁴ can enhance the electricity production while many more investigations are focused on the improvement of electron transport from the electron transport protein conduit to the extracellular electrode: exogenous electron shuttles (EES) such as neutral red,¹⁵⁵ methyl viologen¹⁵⁶ and riboflavin^{157,158} are usually used to accelerate electron transport between electricigens and electrode; conjugated oligoelectrolytes (COEs) are added to assist the electron transport via the protein conduit and self-secreted flavins;^{159,160,161} and the electrodes are modified with nanostructure materials such as nickel oxide

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nanoflaky arrays,¹⁶² graphene/Au composites¹⁶³ and iron sulfide nanoparticles to make better contact with OM-cytochromes of the microbial electrocatalysts and hence result in fast interfacial electrochemistry. However, the improved maximum output power densities reported from these studies range from about 200 mW/m² to about 1300 mW/m², which are still at least three orders of magnitude lower than that of chemical fuel cell.^{164,165,166} These maximum performances are restrained partly because of the fact that these improvements can only facilitate electron transfer from electron transport pathway to electrode but cannot influence the amount of electrons that will enter the transport pathway, which is spatially impeded by the IM of cell. Although such physicochemical methods as addition of DSSN+,¹⁶⁷ treatment with polyethyleneimine¹⁶⁸ or electrochemical tension¹⁶⁹ are utilized recently to perforate the cell membrane to enhance performance, physical permeation and accordingly disruption of cell membrane, on one hand, will cause leakage of cell components inducing bacterial apoptosis-like cell death¹⁷⁰ and on the other, cannot directly connect electron transport with metabolism of substrates, the latter of which is the ultimate source of electron and energy. Therefore, there is an urgent need to develop nondestructive methods, wherein the amount of appropriate enzyme in the IM of cell is increased, to enhance trans-IM electron flow and meanwhile strengthen the link with substrate metabolism.

The corresponding selection criteria for the enzyme are as follows: the enzyme ought to be (i) localized to the IM; (ii) facing the cytoplasm; (iii) and capable of oxidizing a certain substrate to reduce quinone pool in the IM. There are several

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enzymes that meet these criteria such as glycerol-3-phosphate dehydrogenase,¹⁷¹ pyruvate oxidase,¹⁷² lactate dehydrogenases,¹⁷³ D-amino acid dehydrogenase¹⁷⁴ and NADH dehydrogenase¹⁷⁵ which can oxidize glycerol-3-phosphate, pyruvate, lactate, D-amino acid and NADH, respectively, to eventually pass electrons on to quinone pool. The substrate catabolism of lactate by *Shewanella* species under anaerobic or microaerobic conditions, both of which are quite common in bioanodic chambers, will eventually lead to production of acetate or CO₂ (Fig. 4.1), respectively, during which besides stored in the form of ATP/GTP, most of the metabolic energy is transferred to NADH,^{176,177} which is also acting as a cellular energy currency.¹⁷⁸ Thus, compared to those enzymes oxidizing only a single substrate, NADH dehydrogenase is selected to oxidize the primary electron donor NADH to reduce quinone pool, which means it can indirectly expand the substrate scope and namely, the energy contained in any substrate capable of being released in the form of NADH can be utilized.

In bacteria three families of NADH dehydrogenases are identified: Na⁺-translocating NADH dehydrogenase (NQR) that couples NADH oxidation to pumping Na⁺ across the membrane is composed of six subunits with a molecular mass of about 210 kDa;¹⁷⁹ H⁺-pumping type I NADH dehydrogenase (NDH I) that oxidizes NADH to generate protonmotive force is composed of 14 subunits with a mass of about 550 kDa;¹⁸⁰ and non-proton-pumping type II NADH dehydrogenase (NDH II) consists of a single polypeptide chain with a mass of 50~60 kDa.¹⁸¹ NDH II

Figure 4.1. Schematic of the microbial electrocatalytic processes. The electron transport pathway is in turn composed of the quinone pool in IM, the IM-anchored periplasmic CymA, the periplasmic MtrA, the integral OM-porin MtrB and the OM-anchored MtrC/OmcA. The OM is omitted for simplicity. *Shewanella* anaerobic and microaerobic catabolism are in yellow and violet background, respectively.

is superior to its counterparts not only because it does not pump H^+ during NADH oxidation, which indicates it won't be hampered by a high ions gradient across the membrane and consequently the oxidation rate of substrate won't be slowed down due to feedback inhibition as is the case with NDH I,¹⁸² but also because of its small molecular weight, which means its gene manipulation is much easier than others. Herein, we overexpress NDH II in the IM of cell to promote electron nondestructive

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trans-IM movement and meanwhile, bridge the gap between substrate oxidation and electron transport of microbial electrocatalyst. Further, since electron trans-IM movement is the prerequisite for its transfer from electron transport pathway to electrode, it suggests that our improved microbial electrocatalyst can be applied in conjunction with EES, COEs or modified nanostructure electrodes to raise the upper limits of their current maximum performances in the future.

4.2. Materials and methods

4.2.1. Gene manipulation of NDH II strain

The gene expression sequence consists of the IPTG responding element (PlacIq-lacI-Ptac) and *ndh II* (Gene ID: 4921489, NCBI) which encodes the type II NADH dehydrogenase. The PlacIq-lacI-Ptac sequence is designed according to the pMAL-c plasmid (New England Biolabs, USA). The DNA sequence is in vitro synthesized (Aitbiotech, Singapore) and amplified by PCR using gene specific primers rNDH II-F / rNDH II-R (Table 4.1). The underline is the restriction enzyme sites for EcoRI and XhoI, respectively. In the thermo-cycling reactions the high-fidelity hot start enzyme (Kapa, USA) is used and the conditions are as follows: Initial denaturing at 95°C for 5 min; denaturing at 98 °C for 20 sec,

Figure 4.2. The schematic of type II NADH dehydrogenase expression vector. The *PlacIq-lacI-Ptac-ndh II* sequence is inserted into the pHG101 and named as the pHG-NDH II vector.

annealing at 55 °C for 15 sec, extending at 72 °C for 2 min 6 sec, repeating 2 cycles; denaturing at 98 °C for 20 sec, annealing at 69 °C for 15 sec, extending at 72 °C for 2 min 6 sec, repeating 30 cycles; final extending at 72 °C for 5 min; cooling at 4 °C. The reaction products are purified by PureLink Quick Gel Extraction Kit (Lifetechnologies, USA). Then these products as well as pHG101 empty vector1 are double digested by *EcoRI* and *XhoI* (NEB, USA) at 37 °C for 12 h, respectively. Both products are mixed by 2μ l Pellet Paint (Merck, USA) followed by 10% (v/v) 3 M NaOAc. After 2 volume of ethanol added and vortexed, they are incubated at -80 °C for 2 h. Then the samples are centrifuged at 13000 rpm for 5 min and the supernatant are removed. The resultant pellets are rinsed with 70% and 100% ethanol respectively

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and resuspended in sterile deionized (DI) H₂O. 8μ l of DNA fragment dissolved in DI H₂O together with 2μ l of vector in DI H₂O are mixed thoroughly with 10μ l of DNA ligation enzyme solution I (Takara, Japan) and incubated at 16 °C for 16 h. The recombinant products are mixed by 2μ l Pellet Paint followed by 10% (v/v) 3 M NaOAc. After 2 volume of ethanol added and vortexed, they are incubated at -80 °C for 2 h. Then the samples are centrifuged at 13000 rpm for 5 min and the supernatant are removed. The resulting pellets are rinsed with 70% and 100% ethanol respectively, resuspended in 10μ l sterile DI H₂O and then is electroporated into *S. oneidensis* MR-1 electrocompetent cells (700V, 5ms). The cells are immediately transferred into 0.6 ml SOC recovery broth (20% tryptone, 5% yeast extract, 0.5% NaCl, 5% MgSO₄·7H₂O and 20 mM dextrose) and recovered at 30 °C for 1.5 h with shaking at 100 rpm. After selecting with a LB Agar

Tabel 4.1 Primers used in this chapter

Primer name	Primer sequences
rNDH II-F	5'-CCGGAATTCCCGACACCATCGAATGG-3'
rNDH II-R	5'-CCGCTCGAGTTAGTGCAGCTTGAGGTG AG-3'
V-pHG101-F	5'-GCTAGCCCCGGGTGGTAC-3'
V-pHG101-R	5'-CTTATGTCTATTGCTGGTTTACCG-3'

medium containing 50 μ g/ml kanamycin, the transformants are screened by PCR and sequenced using specific designed primers V-pHG101-F / V-pHG101-R (Table 4.1). The recombinant vector is named as pHG-NDH II (Figure 4.2).

4.2.2. Electrochemical Measurements *Bacteria strains and cultivation*

1 ml *S. oneidensis* MR-1 culture is inoculated in 100 ml LB broth (10g/L NaCl; 10 g/L tryptone; 5 g/L yeast extract) and incubated at 30 °C under shaking at 220 rpm until the optical density of bacterial culture at 600 nm (OD₆₀₀) reached about 1.0. 1 ml aliquot of the recombinant *S. oneidensis* MR-1 NDH II strain culture is inoculated in 100 ml LB broth containing 50µ g/ml kanamycin and incubated at 30 °C under shaking at 220 rpm. When the OD₆₀₀ value reached about 0.5, 1 mM IPTG is added to induce overexpression of the type II NADH dehydrogenase for about 6 h. Then both types of bacteria culture are harvested by centrifugation (8000 rpm×8 min). Both bacteria pellets are washed three times with M9 minimal medium (0.5g/L NaCl; 1g/L NH₄Cl; 3g/L KH₂PO₄; 6.78 g/L Na₂HPO₄; 0.12 g/L MgSO₄; 0.011 g/L CaCl₂).

The MR-1 strain is suspended in 100 ml anolyte (95% M9 minimal medium plus 5% LB broth containing 18 mM lactate, PH 7.0) and the recombinant strain is suspended in 100 ml anolyte supplemented with 25µ g/ml kanamycin and 0.01 mM IPTG. Both types of bacteria suspension (~10⁹ cells / mL) are then transferred into the microbial fuel cell (MFC) anodic chamber respectively and purged with nitrogen gas filtered through 0.2µ m membrane for 30 min to remove oxygen.

4.2.3. MFC construction and operation

Dual-chamber glass MFCs (105 ml volume of each chamber) are constructed with separation by a Nafion 117 membrane (DuPont, USA). Both anode and cathode of MFC are made from carbon cloth (GasHub, Singapore). The geometric surface of

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cathode is 6 cm² while that of anode is 4 cm². Before used, carbon cloth is cleaned by acetone and 1 M HCl treatment sequentially and the anode is further treated with oxygen plasma atmosphere by the plasma cleaner system (Harrick, USA) to make it more hydrophilic for better bacteria adhesion. The catholyte is 50 mM K₃Fe(CN)₆ plus 50 mM KCl. An external resistor of 2k Ω is used to connect MFC circuits and the voltage across the external resistor is measured by a digital multimeter (ESCORT 3146A).

4.2.4. Colony forming units measurement

After the output of MFC turned up stable, the the anodic carbon cloth is aseptically transferred to a 15-ml tube containing 10 ml 4oC pre-cold sterile PBS buffer (8g/L NaCl; 0.2g/L KCl; 1.42g/L Na₂HPO₄; 0.27g/L KH₂PO₄; 1 mM CaCl₂; 0.5 mM MgCl₂). Then, samples are vortexed thoroughly to make all bacteria detached from the carbon cloth. After the vortexed samples are 10-fold serially diluted, each dilution is plated on a separate LB Agar plate. These plates are incubated at 30 °C for about 18 h and the number of CFU per unit geometric area of anodic carbon cloth are measured three times independently for both wild-type and recombinant strains employing the cell counter and the results are recorded only when between 50 and 400 colonies per plate are counted.

4.2.5. NAD^+ / $NADH$ assay

To detect the intracellular concentrations of $NADH$ and NAD^+ of the bioelectrocatalyst on the anode surface, the NAD^+ / $NADH$ quantification colorimetric kit is used (BioVision, USA). Briefly, after the stable output of MFC appeared, the anodic carbon cloth is aseptically transferred into a sterile tube containing pre-cold PBS buffer, which is then vortexed thoroughly to detach all bacteria from the carbon cloth. The samples are centrifuged at 13000 rpm for 6 min and the cell pellets are washed twice with pre-cold PBS and extracted with 400 μ l of NAD^+ / $NADH$ extraction buffer by subjecting the bacteria cells to three cycles of freeze/thaw followed by homogenization for 5 min and then centrifuged at 13000 rpm for 5 min. The extracted NAD^+ / $NADH$ supernatant is filtered through the 10kDa molecular weight cut-off membrane (BioVision, USA). To determine the total concentration of NAD^+ / $NADH$ (tNAD), 50 μ l of this filtrate from each extracted sample is transferred into a 96-well micro-plate (Thermo scientific, USA) in triplicates. After the NAD cycling mix is added into each well, the plate is incubated at room temperature for 5 min to allow the enzymatic reactions to proceed completely. Subsequently 10 μ l of $NADH$ developer is added into each well and incubated at room temperature for 1 h 31 min in the dark. To determine the concentration of $NADH$, 200 μ l of extracted solution from each sample is heated at 60°C in a dry bath for 30 min to decompose NAD^+ and then 50 μ l of extracted solution from each sample is transferred into the 96-well plate in triplicates. The rest steps are the same as the procedures of detecting tNAD mentioned above. Eventually, after 1 h 31 min in the dark, the plate is read at

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450 nm on a Victor 3 Model Microplate reader (PerkinElmer, USA). The concentration of NAD^+ is calculated as follows: $\text{NAD}^+ = \text{tNAD} - \text{NADH}$.

4.2.6. Membrane protein extraction and quantification

To verify the successful expression of heterogeneous NDH II protein in the membrane, the membrane protein is extracted by using the Membrane I-ReadyPrep protein extraction kit (Bio-Rad, USA) and then quantified by using the BCA protein assay kit-reducing agent compatible (BioVision, USA). Briefly, the samples of NDH II strain induced by IPTG and MR-1 strain are harvested, respectively and the wet cell pellets are resuspended in 0.5 ml M1 buffer and sonicated on ice with an ultrasonic probe to break open the bacterial cells. Then 0.5 ml M2 buffer are added in the cell extracts and mixed well, after which the samples are incubated on ice for 10 min and at 37 °C for 30 min and centrifuged at $16000 \times g$ for 5 min. The samples then produced an upper aqueous phase, containing the hydrophilic proteins and a lower detergent-rich phase, containing membrane proteins. Repeated these steps mentioned above once again. Thus, the membrane proteins partitioned into the lower detergent-rich phase while the cytoplasmic and periplasmic proteins are in the aqueous phase. Finally, the concentrations of proteins in the lower phase are measured by BCA method. The bovine serum albumin (BSA) is used as the protein standard. Protein samples in the 96-well plate are read at 562 nm on a SpectraMax M5 microplate reader system (Molecular Devices, USA) in triplicates.

4.2.7. Characterization of electrochemical behaviors

All electrochemical analyses of bioanode and measurements of MFC performance are performed after a stable output of MFC appeared (~500 h after inoculation during which electrolytes are replenished when required). To reduce the constraint of a whole-cell system on the evaluation of bioelectrocatalyst, the electrochemical analyses of bioanode are conducted in a three-electrode half-cell system (all three electrodes are in the anodic chamber) with an Ag/AgCl (saturated KCl) and a Pt coil as the reference and counter electrodes. All electrochemical experiments are carried out at room temperature (~30 °C) by using VersaSTAT-3F workstation (Princeton Applied Research). The cyclic voltammograms (CVs) are recorded with a scan rate of 1 mV/s from -0.8 V to 0.2 V (versus Ag/AgCl). The chronoamperometry (CA) experiments are performed at 0 V or +0.2 V and the electrochemical impedance spectroscopy (EIS) measurements are carried out over a frequency range of 0.3 Hz to 300 kHz at $\eta = 0.2$ V with ac amplitude of 10 mV. Tafel plots are recorded for the anode being swept at 1 mV/s from $\eta = 0$ to 0.25 V, where $\eta = 0$ is the OCP of the anode versus the reference electrode.⁵ The measurements of MFC performance are conducted in a whole-cell system. The polarization and power output curves are measured by varying external resistances. Both current density and power density are normalized to the projected surface area of anode. For the discharge experiment, an external load resistor of 2k Ω is connected to MFC and the voltage across the resistor is recorded. All the experiments are repeated three times and representative results are reported.

4.2.8. High-performance liquid chromatography

Riboflavin in the anodic chamber of MFC is quantitated by the HPLC analysis employing an Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) with a UV detector. The column is a LiChrospher 100 RP-18 (5 μ m) Hibar RT 250-4 (Merck, USA). Riboflavin is measured (mobile phase: 15% acetonitrile, 50 mM Sodium dihydrogen phosphate, PH 3.0) with a flow of 0.4 ml/min and monitored at 270 nm.

4.3. Results and Discussion

4.3.1. Expression of NDH II protein in the membrane

Figure 4.3. Membrane protein concentration of MR-1 and NDH II.

To verify the successful expression of heterogeneous NDH II protein in the membrane, the membrane protein is extracted by using the Membrane I-ReadyPrep

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protein extraction kit and then quantified by using the BCA protein assay kit-reducing agent compatible (Figure 4.3). The amount of membrane proteins of NDH II strain is significantly increased compared to that of MR-1 strain. Since the backbone of recombinant vector pHG-NDH II didn't include any other proteins localized to the cell membrane except NDH II protein, this increase in the amount of membrane protein of the mutant strain is solely due to the expression of heterogeneous NDH II protein in the cell membrane.

4.3.2. Measurements of intracellular NADH / NAD⁺ and CFU

For the heterologous expression of type II NADH dehydrogenase in *S. oneidensis* MR-1, *ndh II* (Gene ID: 4921489, NCBI) from *Shewanella loihica* PV-4, which could produce the highest current density among *Shewanella strains*,¹⁸³ is cloned into pHG101 vector,¹⁸⁴ under the control of Ptac promoter, resulting in a 7.455 kb plasmid of pHG-NDH II, which is then electroporated into *S. oneidensis* MR-1 electrocompetent cells (Figure 4.2). Dual-chamber MFCs with microbial anode, potassium ferricyanide cathode and 2k Ω resistor are utilized to allow anodic biofilm formation on the O₂ plasma-treated carbon cloth¹⁸⁵ as well as test the performance of whole-cell system. After a stable output of MFC is achieved, the number of living wild-type MR-1 and NDH II recombinant strains at the carbon cloth are determined at $15.89 \pm 0.37 \times 10^6$ CFU/cm² and $15.98 \pm 0.40 \times 10^6$ CFU/cm², respectively. The intracellular concentrations of NADH and NAD⁺ of microbial electrocatalysts on the

anodic carbon cloth are also detected. Upon the overexpression of *ndh II* in the IM, the intracellular NADH and NAD^+ pool size of NDH II strain are decreased by 0.58 fold and augmented by 0.64 fold, respectively, while the pool size of $\text{NAD(H}^+)$ is approximately identical to that of MR-1 strain (Figure 4.4). These results demonstrated that although the total amount of $\text{NAD(H}^+)$ remained relatively constant, NDH II in the IM accelerated the utilization of the cytoplasmic primary electron donor NADH, which indicated that more electrons and hence energy could be pumped into the quinone pool of the IM. Considering that MR-1 maintained a high concentration of quinones in the IM and

Figure 4.4. Quantitative analysis of intracellular NADH, NAD^+ and $\text{NAD(H}^+)$ of bioanodes for NDH II and MR-1 strains.

consequently fast quinones-CymA reaction kinetics, those electrons and energy

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stored as quinol could move rapidly into the electron transport protein conduit and eventually arrive directly, or accepting outside help, at the electrode.

4.3.3. Electrochemical Measurements and MFC performances

After the output of MFC became stable, electrolytes in the anodic chambers of MR-1 and NDH II strains are replenished, respectively, with fresh anolytes without any antibiotics or IPTG. Then the electrochemical analyses of bioanode are performed in a three-electrode half-cell system (all three electrodes are in the anodic chamber) with an Ag/AgCl (saturated KCl) reference electrode and a Pt counter electrode in order to reduce the constraint of a whole-cell system on the evaluation of microbial electrocatalysts. *S. oneidensis* MR-1 possessed two electron transport processes which are distinguishable by the characteristic potential ranges of -0.4 V to 0 V vs. AgCl for MET and 0 V to 0.2 V vs. AgCl for DET. Thus, to evaluate the capability of DET of MR-1 and NDH II strains, chronoamperometry (CA) experiments are performed at +0.2 V to help to pull electrons from the transport protein conduit to the electrode after the anolyte replenishment. When poised at +0.2 V, the current density of NDH II reached 1116.6 ± 33.4 mA/m², which is 2.5-fold as much as that of MR-1 (447.1 ± 6.5 mA/m²) (Figure 4.5a). To mimic the scenario that the electron transport pathway has been greatly improved, 200 μ M riboflavin (RF), which is at least 100-fold higher than the usual concentration of self-secreted RF in the reactor, is added into the anodic

Figure 4.5. (a) CA of NDH II and MR-1 bioanodes poised at +0.2 V, (b) poised at 0 V with additional 200 μ M RF.

chamber and CA is performed at 0 V to reduce the effects of DET.¹⁸⁶ The current density of NDH II reached 6033.7 ± 76.8 mA/m² when poised at 0 V, which is 2.0-fold as much as that of MR-1 (3016.9 ± 34.0 mA/m²) (Figure 4.5b). This

difference of current density between NDH II and MR-1 in 200 μ M RF is smaller than that of DET (2.5-fold) because RF could only transfer electrons from OM-cytochromes to the electrode, which indicated that the electrons accepted by RF needed to pass through the electron transport protein conduit, whose poor efficiency might limit the maximum value of this difference, regardless of how much difference previously in capability of pumping electrons into the protein conduit between NDH II and MR-1. Since neutral red (NR) is able to not only act as electron shuttle but also come into the IM and accept electrons in place of quinones,¹⁸⁸ NR is utilized to

Figure 4.6. CA of NDH II and MR-1 bioanodes poised at 0 V with additional 100 μ M NR.

probe the electron transfer process from the IM, bypassing the electron transport protein conduit, directly to the electrode. The current density of NDH II in 100 μ M NR reached 1536.3 ± 28.6 mA/m², which is 2.6-fold as much as that of MR-1 (591.3

Figure 4.7. (a) CV of NDH II and MR-1 bioanodes, (b) EIS of the NDH II and MR-1 bioanode in MFCs.

± 6.1 mA/m²) (Figure 4.6). These results demonstrated that although NR, as electron shuttle, is worse than RF probably because the more positive E_0' value of RF (-0.208 V) than NR (-0.325 V) thermodynamically favored the microbial metabolism,¹⁸⁹ the difference of current density between NDH II and MR-1 in NR (2.6-fold) is larger than that in RF (2.0-fold) because the former omitted electron transfer process via the protein conduit. The cyclic voltammetry (CV) measurements of bioanodes are conducted under conditions as follows: when the anolyte replenished (without any detectable RF), 22 h after anolyte replenishment (containing $1.09 \pm 0.08 \mu$ M RF, $n=3$) and 200μ M additional RF added. The CVs revealed that at all concentrations, as the potential swept from -0.45 V to -0.25 V, the amplitudes of flavins-mediated catalytic waves of NDH II are larger than that of MR-1 as well as the initiation point of catalytic waves of NDH II exhibited negative shift compared to that of MR-1 (Figure 4.7a), both of which indicated that much more RF could be reduced by NDH II than MR-1 and similar phenomena occurred in previous studies.¹⁸⁷ In addition, the magnitude of current rise of NDH II in the potential range of 0 V to 0.2 V for DET is slightly larger than that of MR-1 (Figure 4.7a), which also demonstrated that NDH II enzyme in the IM could pump more electrons into electron transport protein conduit for DET. Electrochemical impedance spectroscopy (EIS) (Figure 4.7b) showed the high-frequency semicircles followed by straight lines, whose diameter represented the apparent interfacial charge transfer resistance (R_{ct}) of bioanode.¹⁹⁰ For the NDH II strain bioanode, the R_{ct} significantly decreased compared to that of MR-1 strain bioanode. Recent studies on EIS in anodic biofilms are summarized and the R_{ct}

measured ranged from 2Ω to 156Ω (Table 4.2).

Table 4.2 Summary of the reported resistances of biofuel cells measured by EIS

Cell type	Electrode	Bioelectrocatalyst	Resistance(Ω)	Reference
D	Graphite felt	Anaerobic sludge	11	<i>International Journal of Hydrogen Energy</i> , 2012, 37 , 16935
S with air cathode	Carbon felt	Anaerobic sludge	18.1 ± 2.4	<i>Electrochimica Acta</i> , 2011, 58 , 58
SMFC	Carbon paper	Mixed culture in domestic wastewater	2	<i>Bioresource technology</i> , 2012, 118 , 412
FMFC	Granular graphite	Mixture of anaerobic and aerobic sludge	20	<i>Bioresource technology</i> , 2012, 114 , 308
S with air cathode	Graphite felt	Anaerobic sludge	50	<i>Journal of Microbial & Biochemical Technology</i> , 2013, 01
S	Glassy carbon electrode	Glucose oxidase with OMC; with OMC and PVA**	17.5 for OMC; 5.2 for OMC and PVA	<i>Journal of Power Sources</i> , 2010, 195 , 4090
SIMFC	Carbon cloth	River sediment	105.5 for rotating; 78 for non-rotating cathode	<i>Biosensors & bioelectronics</i> , 2007, 22 , 3252
D	Carbon cloth	Electrical tension evolved <i>E.coli</i> K-12	107.5	<i>Chemical communications</i> , 2008, 1290; <i>Chemical communications</i> , 2009, 6183
D	Carbon cloth	Effluent from a previous MFC	15.1 ± 0.5	<i>International Journal of Hydrogen Energy</i> , 2014, 39 , 19148
D	CHI/VSG**	<i>Pseudomonas aeruginosa</i>	150	<i>Nano letters</i> , 2012, 12 , 4738
D	CNT/PANI composite	<i>E.coli</i> K-12	156	<i>Journal of Power Sources</i> , 2007, 170 , 79
D	Graphite felt	<i>Shewanella oneidensis</i> DSP10 with additional RF	16	<i>Biotechnology and bioengineering</i> , 2009, 104 , 882
D	PANI/TiO ₂ composite	<i>E.coli</i> K-12	~12.5	<i>Acs Nano</i> , 2008, 2 , 113
S	Graphene / nickel foam	<i>Shewanella putrefaciens</i>	~6	<i>RSC Advances</i> , 2014, 4 , 21788
D	Carbon cloth	GldA overexpressed <i>E.coli</i> BL21 (DE3)	125	<i>Electrochemistry Communications</i> , 2009, 11 ,

D	TiO ₂ /rGO hybrid on carbon cloth	<i>Shewanella putrefaciens</i> CN32	28	<i>Journal of Power Sources</i> , 2015, 276 , 208
S	O ₂ plasma treated carbon cloth	<i>Shewanella oneidensis</i> MR-1; NDH II mutant	42.81 ± 0.02; 24.55 ± 0.15	This work

* Dual chamber: D; single chamber: S; submersible MFC: SMFC; floating MFC: FMFC; sediment MFC: SiMFC;

** Ordered mesoporous carbon: OMC; poly(vinyl alcohol): PVA

*** Chitosan and vacuum-stripped graphene: CHI/VSG

Nonturnover CVs are also performed (Figure 4.8a) and the results showed that the NDH II strain bioanode had better electrocatalytic activity than that of MR-1 bioanode because the oxidation and reduction currents at peaks from both RF and OM-cytochromes of NDH II anode are higher than that of MR-1 anode (The capacitive currents of NDH II and MR-1 bioanode are considered to be the same because their amounts of alive cells on the electrodes are nearly identical and NDH II strain had no alteration in its OM-surface structure compared to MR-1 strain). Moreover, the results of nonturnover CVs in 200 μ M RF (Figure 4.8b) demonstrated that the peak currents from OM- cytochromes are higher than what are shown in Figure 4.8a, which is due to the fact that the presence of large amount of RF could accelerate DET of OM-cytochromes to the electrode. Their peak currents from RF looked the same because in 200 μ M RF, the peak currents from RF could be covered by signals caused by large amount of RF molecule itself.

Figure 4.8. (a) Nonturnover CV of NDH II and MR-1 bioanodes. (b) nonturnover CV of NDH II and MR-1 bioanodes in 200 μM RF. The potential scan rate is 1 mV/s.

The Tafel plots (Figure 4.9) illustrated that the exchange current density of NDH II is 2.3-fold as much as that of MR-1 in the fresh anolyte (without RF). Further, the Tafel slope of NDH II at a fixed potential is nearly identical to that of MR-1, suggesting that they underwent the same interfacial reaction mechanism over these potential ranges.^{191,192,193} It is comprehensible since the NDH II enzyme is in the IM and thus engineering the wild-type strain didn't get involved in any alteration in the OM surface structure of the microbial electrocatalyst.

Figure 4.9. Tafel plots recorded at a scan rate of 1 mV/s from $\eta = 0$ to 0.25 V versus OCP.

Then the MFC performance is evaluated using a whole-cell system. The polarization and power output curves (Figure 4.10a) revealed that the NDH II strain inoculated MFC generated a maximum power density of $371.5 \pm 3.7 \text{ mW/m}^2$,

Figure 4.10. (a) Power output and polarization curves of NDH II and MR-1 inoculated MFCs. (b) Discharge performance of NDH II and MR-1 inoculated MFCs. The arrow indicates electrolyte replenishments.

Table 4.3 Summary of the reported performances of MFCs

Cell type*	Electrode	Bioelectrocatalyst	Power density (mW/m ²)	Reference
D	Carbon Cloth	Electrical tension evolved <i>E.coli</i> K-12	1300	<i>Chemical communications</i> , 2008, 1290
D	NiO/carbon cloth	<i>Shewanella putrefaciens</i>	1024 ± 46	<i>Journal of Power Sources</i> , 2014, 266 , 226
D	PANI/TiO ₂ composite	<i>E.coli</i> K-12	1495	<i>Acs Nano</i> , 2008, 2 , 113
D	Graphene/nickel foam	<i>Shewanella putrefaciens</i>	3903	<i>RSC Advances</i> , 2014, 4 , 21788
D	Carbon fiber brush	<i>Ochrobactrum</i> sp. 575	2625**	<i>RSC Advances</i> , 2014, 4 , 39839
D	CHI/VSG***	<i>Pseudomonas aeruginosa</i>	1530	<i>Nano letters</i> , 2012, 12 , 4738
D	Carbon nanocage	GldA overexpressed <i>E.coli</i> BL21 (DE3)	1304	<i>Electrochemistry Communications</i> , 2009, 11 , 1593
D	rGO-5-Ni composite	<i>Shewanella oneidensis</i> MR-1	27000**	<i>Nanoscale</i> , 2013, 5 , 10283
D	TiO ₂ /rGO hybrid on carbon cloth	<i>Shewanella putrefaciens</i> CN32	3169	<i>Journal of Power Sources</i> , 2015, 276 , 208
D	O ₂ plasma treated carbon cloth	<i>Shewanella oneidensis</i> MR-1 with biococatalysts	1079.6 ± 5.1	<i>Chemical communications</i> , 2015, 51 , 12170
S	Carbon fiber brush	<i>Shewanella oneidensis</i> MR-1	332 ± 21	<i>Biotechnology Bioengineering</i> , 2010, 105 , 489–498
D	3D graphene/PA NI	<i>Shewanella oneidensis</i> MR-1	768	<i>ACS Nano</i> 2012, 6 , 2394
D	Carbon paper	<i>Shewanella oneidensis</i> MR-1	104	<i>Biosensors and Bioelectronics</i> 2011, 26 , 3987
S	Graphite felt	SO_3350 mutant MR-1	~110	<i>Bioscience, Biotechnology, and Biochemistry</i> 2011, 75 , 2229
D	Carbon cloth	3D rGO hybrid biofilm	843	<i>Angewandte Chemie International Edition</i>

D	O ₂ plasma treated carbon cloth	NDH II mutant	371.5±3.7; 2277.4±11.1 with 200 uM RF	This work

* Dual chamber: D; single chamber: S;
** Unit: mW/m³;
*** Chitosan and vacuum-stripped graphene: CHI/VSG

corresponding to a current density of 792.3 ± 6.9 mA/m² at a cell potential of 468.8 ± 0.5 mV, which is 3.3-fold as much as that of the MR-1 strain inoculated MFC (112.1 ± 3.1 mW/m²), obtained at a current density of 210.7 ± 5.6 mA/m² with a cell potential of 531.9 ± 0.6 mV. The maximum power densities reported in recent literature are listed in Table 4.3. Interestingly, this difference (3.3-fold) is larger than that of current density between NDH II and MR-1 (2.5-fold) in CA experiments performed at +0.2 V without additional RF, which might be due to the fact that the CA is conducted under the strong anodic polarized condition, however, this condition could not be easily reached during their maximum power density measurements (without additional RF), when the effects of overpotentials of microbial electrocatalysts themselves predominated and consequently the difference of their own overpotentials would enlarge the difference of their performances at this point. The discharge curves (Figure 4.10b) determined by measuring the output current with a 2 kΩ external resistor showed that the NDH II-inoculated MFC delivered a significant higher steady-state current density than that of MR-1-inoculated MFC.

The MFC performances in 200μ M RF are also measured. The polarization and power output curves showed (Figure 4.11) that the maximum power density of the NDH II-inoculated MFC is 2277.4 ± 11.1 mW/m², which is 2-fold as much as that of

the MR-1-inoculated MFC ($1135.3 \pm 6.3 \text{ mW/m}^2$).

Figure 4.11. Power output and polarization curves of NDH II and MR-1 inoculated MFCs with additional 200 μ MRF.

4.4. Conclusions

In summary, by overexpressing the NDH II enzyme in the IM of *S. oneidensis* MR-1 strain, we present a novel nondestructive strategy of enhancing electron trans-IM movement as well as bridging the gap between substrate oxidation and electron transport of microbial electrocatalyst. Moreover, since electron trans-IM movement is the prerequisite for its transfer from electron transport protein conduit to electrode, our engineered microbial electrocatalyst has the potential to be applied in combination with EES, COEs or modified nanostructure electrodes to raise their

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current upper limits of the maximum performances of MFCs in the future.

4.5. Declaration

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Chapter 5. Enhancing the performance of *Escherichia coli*-catalyzed MFC based on RF secreting

5.1. Introduction

The microbial fuel cells (MFCs) is a type of equipment that can transform the energy in organics into electricity by means of biochemical reactions catalyzed by the microbes. Accordingly, in MFCs, the microbes, as bioelectrocatalysts, are essential for exploiting various kinds of organics, such as wastewater, sludge and biomass as substrates to produce electric energy. The electricity-producing ability of the microorganisms is composed of the bioelectrocatalysts metabolizing a variety of organic matter to creating electrons as well as the electron transferring from the organics to the anode and finally to the external circuit as electricity when the circuit is ready for generating the electric current. In the electron transferring process, electrons can't be easily transported from the fuel oxidation sites to the anode outside the cell because of the probably nonconductive attribution of both cell membrane (contains one or two membranes) and the bacterial out-surface structure. For example, some bacteria like *Shewanella oneidensis* and *Geobacter sulfurreducens* synthesize electrically conductive proteins which are located in the electrochemical inactive membrane which is comprised primarily of lipid and peptidoglycan. On the basis of this capability, these bacteria turn into the electrical active bacteria (EAB) which are effective in creating electric current. In general, the bacteria have three types of

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electron transporting from the interior of the cell to the exterior electrode. i) direct electron transport through the electrochemical active proteins (the electron transport protein conduit); ii) mediated electron transport through electrochemical active chemicals (electron shuttles); iii) electron transport through bacterial pili.

As a model bacteria for electricity generation studies, *Shewanella oneidensis* MR-1 has been deeply investigated about its electron transport mechanism on the scale of molecular level. During the long period of natural evolvement, *Shewanella oneidensis* MR-1 has developed its own electron transport pathway which enables itself to live under anaerobic conditions with respiring mainly by either the exterior solid mineral or the electrode. The basic electron transport pathway of *Shewanella oneidensis* MR-1 includes at least four protein complexes: CymA, located in the inner membrane of the cell facing the periplasmic space; MtrA, located in the outer membrane of the cell facing the periplasmic space; MtrC/OmcA, located in the outer membrane of the cell facing the exterior environment; MtrB, located in the outer membrane of the cell as a porin which can help MtrC/OmcA complex become stable. When *Shewanella oneidensis* MR-1 is living on the anode (electrode-respiring in the anaerobic environment), the reducing power from the oxidation of the organic matters is directed to the quinone pool in the inner membrane and then to the CymA, a type of cytochrome c containing multihemes. When the external circuit is closed, the electron from CymA is transferred, either directly or through other intermediate proteins like FccA, to MtrA. After that, CymA is reoxidized, during which the quinone/quinol cycle accomplished and the bacteria itself also can acquire the energy for growth

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through the proton transmembrane transporting. Furthermore, the outer-membrane MtrC or OmcA can be reduced by MtrA with the help of MtrB. Finally, the electron from MtrC or OmcA will be either directly passed to the exterior electrode or indirectly by taking advantage of the electron shuttle-riboflavin (RF) to reduce the anode. It is reported that about seventy to eighty percent of electric current created by *Shewanella oneidensis* MR-1 depends on the RF-mediated routine while the rest part is dependent on the direct electron transport routine.

Since such bacteria as *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* have developed their own electron transport pathways which enable themselves to respire mainly by either the exterior solid mineral or the electrode in the anaerobic environment, they are able to transport the electrons generated from bacterial metabolism to the extracellular space. Apparently, this kind of ability could be widely used in the fields of nanoparticle synthesis, bioremediation, biocomputing as well as biomining. Thus, the practical application of this kind of ability makes precise controlling and systematical modulation of the bacterial electron transport a necessity. However, these naturally occurring electricigens always contain multiple redundant electron transport pathways due to the selective pressure, which makes it difficult for us to regulate their electron transport capability. Moreover, the genetic tools suitable for engineering these naturally occurring electricigens are relatively limited. Therefore, recently many researchers are devoted to engineering the well-known electron transport protein conduits into the *E.coli* by means of the complementary method, the most extensively studied and used bacteria in the biochemical

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engineering industry.^{197,198} For example, it is reported that the coexpression of both MtrA and CymA proteins in the *E.coli* strains can make them become able to reduce the extracellular soluble ferric ions except for the solid ferric oxides.^{199,200} In addition, coexpressing the MtrCAB electron transport protein conduits enables the *E.coli* strain to reduce the extracellular solid ferric oxides.¹⁹⁷ But the reducing rates of this mutant *E.coli* strain is much lower than that of the wild-type *Shewanella oneidensis* MR-1 strain. This might be due to two reasons: i) the electron transfer kinetics of the outer-membrane proteins (MtrC or OmcA) of *Shewanella oneidensis* MR-1 is much slower than that of RF-mediated electron transfer; ii) this mutant *E.coli* strain has a significantly slow growth rate, which results in a small amount of biomass. On the other hand, we have previously reported that increasing the amount of RF in the reactor could not only enhance the electron transfer rates of both direct and mediated transport among the bacteria and the extracellular hard surface (the anode), but also improve the growth of electricigens on the electrode.²⁰¹ Hence, it is reasonable that the research findings mentioned-above can be applied in the area of improving the electron transport between the *E.coli* strain and the electrode.

In this study, we coexpress the MtrCAB electron transport protein conduits from wild-type *Shewanella oneidensis* MR-1 strain as well as the ribAB genes which encodes the first two step of RF biosynthesis in the *E.coli* BL21(DE) strain. After the genetic engineering, the mutant *E.coli* BL21(DE) strain are able to not only enhance its electron transfer rate from the interior the bacteria to the exterior electrode, but also increase the biofilm formation due to the self-secretion of RF molecules in the

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reactor. Thus, this type of bioelectrocatalyst can be loaded in the MFC reactor, which will significantly improve the performance of *E.coli*-catalyzed MFCs.

5.2 Materials and methods

5.2.1. Engineering of recombinant *E.coli* BL21(DE3)

The empty plasmid, pQLinkNMut, is used in the gene cloning. The pSB1ET2-MtrCAB plasmid is used as the target template. The MtrCAB gene expression elements are amplified and subcloned from the pSB1ET2-MtrCAB plasmid by PCR using gene specific primers (Table 5.1). The PCR product is simultaneously digested by both *NotI* and *BamHI* restriction enzymes, and the PCR products is ligated into expression vector pQLinkNMut under the control of Ptac promoter (Figure 5.1). The recombinant plasmid is electroporated into the *E. coli* BL21 (DE3) electrocompetent bacteria. The transformants is screened by PCR, after which is verified by gene sequencing (Integrated DNA Technologies, Singapore). The plasmids of positive clones are purified and called as pQLinkNMut-MtrCAB.

To construct the coexpression vector of pQLinkNMut-MtrCAB-ribAB, 0.4 pmol vector of our previously developed pQLinkNMut-ribAB¹⁵⁸ is digested at 25 °C with 5 U *SwaI* in 10µ l and 0.4 pmol vector of pQLinkNMut-MtrCAB is digested for 20 hour at 37°C with 5 U *PacI* in 10µ l, whose incubation periods are more than twenty hours for both. All the Enzymes are inactivated by 65°C incubation for 25 min and

Figure 5.1. The flowchart of pQLinkNMut-MtrCAB-ribAB plasmid construction.

0.2 pmol DNA is treated with 1 U LIC-qualified T4 DNA Polymerase in total volume of 20 μ l (T4 DNA Polymerase Buffer, 10mM DTT, Nuclease-free Water, 2.5mM dGTP for SwaI digest and 2.5mM dCTP for PacI digest). Upon incubation at 22 $^{\circ}$ C for 30 min and sequentially at 75 $^{\circ}$ C for 20 min, 2 μ l of the above T4 DNA Polymerase treated solution (PacI digest, containing the MtrCAB fragment) is mixed thoroughly with 1 $^{\circ}$ C of that solution (SwaI digest, containing the pQLinkNMut-ribAB) and heated to 65 $^{\circ}$ C for 10s and then incubated at 22 $^{\circ}$ C for 5 min. The mixture is supplemented with 1 μ l 125mM EDTA, followed by incubating at 22 $^{\circ}$ C for 25 min. The resulting product is transformed into *E. coli* BL21 (DE3) bacteria and screened by PCR and verified by sequencing. Finally, the coexpression vector of pQLinkNMut-MtrCAB-ribAB is constructed from pQLinkNMut-MtrCAB

and pQLinkN-Mut-ribAB (Figure 5.1). The recombinant vector is transformed into *E. coli* BL21 (DE3) bacteria again. The positive clones are screened by PCR and sequenced using specific designed primers V-pQLinkN-F/R (Table 5.1).

Table 5.1 Primers used in Chapter 5

Primer name	Primer sequences
ribA-Forward	5' -CGGGATCCATGCAGCTTAAACG-3'
ribA-Reverse	5' -ATAGTTTAGCGGCCGCTTATTTGTTC-3'
ribB-Forward	5' -CGGGATCCATGAATCAGACGCTACTT-3'
ribB-Reverse	5' -ATAGTTTAGCGGCCGCTCAGCTGG-3'
MtrCAB-Forward	5' -CGCGGATCCATGATGAACGCACAAAAATC-3'
MtrCAB-Reverse	5' -ATAGTTTAGCGGCCGCTTAGAGTTTGTAATCATGCTCA-3'
V-pQLinkN-Forward	5' -CGACCGAGTTGCTCTTGC-3'
V-pQLinkN-Reverse	5' -ATCAGGCGGGCAAGAATG-3'

* Relevant restriction enzyme sites are shown in italics (*Bam*HI / *Not*I).

To construct the *E.coli* mutant strain, both the pQLinkN-Mut-MtrCAB-ribAB plasmid and the pEC86 plasmid, the latter of which carries the *ccm* genes that enable the cytochrome *c* to mature in the *E.coli* strains, are co-transformed into the *E. coli* BL21 (DE3) electrocompetent cells. Then positive clones are selected for our research.

5.2.2. Cultivating bacteria strains

All *E.coli* strains are cultivated in 2×YT medium at 37 °C with shaking at 200

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rpm. When needed, ampicillin (100 μ g/ml) and chloramphenicol (34 μ g/ml) is used to select positive clones and maintain plasmids. When the OD₆₀₀ of bacterial culture reached 0.35, 0.7 mM IPTG is added to induce over-expression of recombinant proteins for one day.

Then both mutant and wild-type *E.coli* culture are harvested by centrifugation (8500 rpm $\times$ 6 min). Both bacteria pellets are washed three times with M9 minimal medium. The wild-type strain is resuspended in 50 ml anolyte (95% M9 minimal medium plus 5% 2 $\times$ YT medium containing 10 g/L glucose) while the recombinant strain is suspended in 50 ml anolyte supplemented with 50 μ g/ml ampicillin, 17 μ g/ml chloramphenicol and 0.01 mM IPTG. Both types of bacteria suspension are then moved into the microbial fuel cell (MFC) anodic chamber respectively and purged with nitrogen gas for 15 min to remove O₂.

5.2.3. Constructing and operating MFC

Dual-chamber glass MFCs (50ml volume each chamber) separated by Nafion 117 membrane are constructed. The cathode is made from a carbon cloth with projected area of six cm². The anode is made from a six cm² carbon felt. Before used, both carbon cloth and carbon felt are treated with acetone and 1 M HCl sequentially. The catholyte is 50 mM K₃Fe(CN)₆ and 50 mM KCl. A 600 Ω external resistor is connected into external MFC circuits. The voltage across the resistor is recorded by using a digital multimeter. MFCs run at room temperature (about 25 $^{\circ}$ C).

5.2.4. Electrochemical Measurements

All electrochemical analyses of MFC performance are performed after a stable output of MFC appeared (about 10 days after inoculation). All electrochemical experiments are carried out at room temperature ($\sim 25^{\circ}\text{C}$) by using electrochemical workstation. The cyclic voltammogram (CV) is recorded with sweeping from -0.75 V to 0.05 V (versus Ag/AgCl) (scan rate, 1 mV/sec). The chronoamperometry (CA) experiment is carried out at $+0.2\text{ V}$ and the electrochemical impedance spectroscopy (EIS) measurement is carried out over a frequency range of 0.1 Hz to 1000 kHz with ac amplitude of 5 mV . Tafel plot is recorded by sweeping at 1 mV/s from $\eta = 0$ to 0.25 V , where $\eta = 0$ is the OCP of the anode versus the reference electrode. The polarization and power output curves of MFC are measured by varying external resistances. Both current density and power density are normalized to the geometric surface area of anode. For the discharge experiment, a load resistor of 600Ω is connected to MFC and the voltage across this resistor is recorded.

5.2.5. Colony forming units determination

According to the procedure of^{158,201}, after a stable output of MFC appeared, the anodic carbon felt is aseptically transferred into a 15 ml tube containing 10 ml sterile Phosphate buffered saline (Life technologies, USA) and vortexed thoroughly to

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detach all bacteria from the carbon felt. Then the vortexed sample is 10-fold serially diluted and 100 μ l of each diluted solution is spread onto a separate LB agar plate. The plates are incubated overnight at 37°C and the number of CFU per anodic carbon felt are determined in triplicate by using cell counter and the results are recorded only when between 50 and 400 colonies are counted each plate.

5.2.5. HPLC analysis

The HPLC analysis is carried out at an Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) equipped with a UV detector. A LiChrospher 100 RP-18 column is used. The mobile phase contains 15% acetonitrile and 85% 50 mM Sodium dihydrogen phosphate. The eluted rate is 0.4 ml/min. Riboflavin is monitored at 270 nm (retention time: ca. 16 min) while HNQ is monitored at 263 nm (retention time: ca. 29 min).

5.3 Results and Discussion

5.3.1. Secreting RF by the *MtrCABribAB* mutant *E.coli* strain

The RF biosynthetic pathway in bacteria begins from two precursors, one of which is GTP and the other one is ribulose-5-phosphate. The gene *ribA* and *ribB* encode the GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate

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synthase, respectively. Both of these two enzymes are the first reaction step where the two precursors, the GTP and ribulose-5-phosphate, enter the RF biosynthesis pathway, respectively. Reasonably, they are also the rate-limiting steps in the RF biosynthesis pathway. Hence, the overexpression of these two genes in the *E.coli* BL21 (DE3) strain is capable of increasing the biosynthesis and secretion of the RF molecule of the *E.coli* strain.

The two rate-limiting enzymes in the RF biosynthetic pathway and the MtrCAB electron transport protein conduit are combined together by cloning *ribA* and *ribB* genes from *E.coli* *K-12* genome as well as subcloning the MtrCAB gene expressing elements from the pSB1ET2-MtrCAB plasmid into pQLinkNMut empty plasmid following the *Ptac* promoter, leading to the pQLinkNMut-MtrCABribAB plasmid, which is transformed into *E. coli* BL21 (DE3) cells later (Figure 5.1 and Table 5.1). In order to measure the RF biosynthetic capability of the MtrCABribAB strain in the MFC reactor under the strictly anaerobic condition, After a stable output of MFC turns up (around 10 days after inoculation), the anolyte is replenished with the fresh one. Then samples are collected at one day intervals and tested by HPLC. As shown in Figure 5.2 and Figure 5.4, although the RF peak (turns out at ca. 16 mins, 270 nm detected) looks much smaller than those noise peaks caused by bacterial secondary metabolites, it can be captured and quantified by the machine. The results show that after one day operation of MFC, the RF concentration in the anodic chamber

Figure 5.2. HPLC analysis of the MtrCABribAB inoculated anodic chamber. RF is monitored at 270 nm, HNQ at 263 nm. (a) day zero; (b) day one; (c) day two; (d) day three.

Figure 5.3. HPLC analysis of the wild-type E.coli inoculated anodic chamber. RF is monitored at 270 nm, HNQ at 263 nm. (a) day one; (b) day two; (c) day two; (d) day three.

inoculated with the MtrCABribAB mutant strain accumulates to $1.16 \pm 0.09 \mu M$ (n=3). After the second day, it accumulates to $1.62 \pm 0.16 \mu M$ (n=3). After the third day, it can reach up to $2.25 \pm 0.19 \mu M$ (n=3), which is much higher than that of the

wild-type electricigen, *S. oneidensis* MR-1 (usually 0.5~1 μ M in the reactor). On the contrary, as shown in Figure 5.3, there isn't any detectable RF from day one to day three in the anodic chamber inoculated with the wild-type *E.coli* BL21 (DE3) strain. Thus, the MtrCABribAB mutant strain can greatly increase the RF concentration in the anodic chamber of MFC compared to the wild-type *E.coli* BL21 (DE3) strain.

Figure 5.4. RF concentrations in the MtrCABribAB inoculated MFC from Day zero to Day three

Moreover, it reported that under long-term electro-tension, the wild-type *E.coli* strain might secrete HNQ molecule into the reactor, which can be used as the electron shuttle to accelerate the electron transport from the bacteria to the electrode. Thus, the concentration of HNQ in the anodic chamber of MFC is also detected at 263 nm (retention time ca. 29 min). The results (Figure 5.2 and Figure 5.3) demonstrate that

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neither the MtrCABribAB mutant strain nor the wild-type *E.coli* BL21 (DE3) strain will secrete detectable HNQ molecules into our MFC reactor. Hence, all the changes in the performance of bioelectrocatalytic activity have nothing to do with HNQ.

5.3.2. Electrochemical behaviors of bioelectrocatalyst

The anolyte is replenished with the fresh one, after a stable output of MFC turns up. The cyclic voltammetry of the anodic biofilms are carried out at zero day, one day, two days and three days after the anolyte replenishment, respectively. The turnover CV of the MtrCABribAB mutant strain (Figure 5.5a) demonstrates two peaks, the former of which centers around -0.4 V vs. Ag/AgCl while the latter of which centers around -0.2 V vs. Ag/AgCl. Accordingly, the first peak can be attributed to the electrochemical activity of RF molecules and the other peak can be attributed to the electrochemical activity of MtrC proteins in the outer-membrane of the cell. With the MFC operating, the RF concentration in the anodic chamber is increasing (Figure 5.4) and consequently the bioelectrocatalytic activity of the mutant strain enhances from day zero to day two (Figure 5.5a). However, its bioelectrocatalytic activity after three day operation decreases compared to that of day two, although the RF concentration is still accumulating in the reactor. That might be due to the poor capability of

Figure 5.5. Turnover CV (a) and nonturnover CV (b) of the MtrCABribAB inoculated MFC.

Figure 5.6. Turnover CV (a) and nonturnover CV (b) of the wild-type *E.coli* inoculated MFC.

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transferring electrons from the interior of the cell to the exterior of the cell as well as the biofilm living activity of the *E.coli* strains. Thus, further increasing of RF concentration can rarely extract electrons from the bacteria to the electrode. Then, the turnover CV of the wild-type *E.coli* BL21 (DE3) strain is also performed. The results shows (Figure 5.6a) that there isn't any redox peak can be found from the he wild-type *E.coli* BL21 (DE3) cells.

The nonturnover CV of the MtrCABribAB mutant strain also demonstrates (Figure 5.5b) clearly that there are two redox peaks in the CVs. The first one are the RF peaks (mediated electron transport) and the other one are MtrC proteins (direct electron transport). The results also shows that, as the operation period goes on, the activity of the biofilm on the anode increases and reaches a maximum two days after the replenishment, after which its activity decreases probably due to its own biological characteristics no matter whether the RF concentration is still increasing. The turnover CV of the wild-type *E.coli* BL21 (DE3) strain is also performed (Figure 5.6b). And again, like its turnover CVs, there is no redox peak from the wild-type *E.coli* BL21 (DE3) strain.

In addition, there exists a linear correlation between the peak currents of redox pairs centers around -0.4 V vs. Ag/AgCl and the square root of scan rates extracted from the CVs of the MtrCABribAB mutant strain at various scan rates (Figure 5.7a), indicating that this peak derives from a diffusive electroactive species. In contrast, the linear correlation between the peak currents of redox pairs centers around -0.2 V vs. Ag/AgCl and scan rates extracted from the same series of CVs indicates that a direct

Figure 5.7. (a) Function of peak current vs. $(\text{scan rate})^{1/2}$; (b) Function of peak current vs. scan rate.

electron transport is responsible for this peak (Figure 5.7b). These results further substantiate the different origins of these two redox pairs and the fact that the MtrCABribAB mutant strain has two type of electron transfer mechanisms.

The Tafel plots are performed after two days operation and the results demonstrate that (Figure 5.8) the exchange current density of the MtrCABribAB bioanode, acquired by extrapolating Tafel slopes back to the OCP (open circuit potential) vs the counter electrode, at least fifty six-fold as much as that of the wild-type *E.coli* BL21 (DE3) bioanode. It indicates that the electrocatalytic activity of the mutant strain is much higher than that of the wild-type strain.

Figure 5.8. Tafel plots of the MtrCABribAB and wild-type *E.coli* inoculated MFCs

Figure 5.9. EIS of the MtrCABribAB and wild-type *E.coli* inoculated MFCs

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Electrochemical impedance spectroscopy (EIS) determinations are executed and the Nyquist figures turn out as hemicycles in the superior frequency area. The Randle equivalent-circuit is utilized to fit the impedance parameter and the the interfacial charge transfer resistance (R_{ct}) can be acquired from it. For the R_{ct} of the MtrCABribAB bioanode (Figure 5.9a) decreases nearly 80-fold as much as that the wild-type *E.coli* BL21 (DE3) bioanode (Figure 5.9b). The great increae of the interfacial charge transfer rate of the MtrCABribAB bioanode is probably due to the fact that the presence of RF can enhance the electron transport between the bioelectrocatalyst and the eletrode combined with the fact that the overexpression of MtrCAB protein conduit can help electron transport from the cytoplasm of the cell to the exterior the cell.

5.3.3. MFC performance

The MFC performance when loaded with these bioelectrocatalysts are assessed taking advantage of a full-cell system. The polarization and power output plots (Figure 5.10a) reveal that the OCP of the MFC inoculated with the MtrCABribAB mutant strain (513 mV) is comparable to that of the MFC inoculated with the wild-type *E.coli* BL21 (DE3) strain (512.2 mV). And the MFC inoculated with the MtrCABribAB mutant strain generates a maximum power density of 39.8 ± 0.3 mW/m², aiming to the current density of 147.6 ± 0.5 mA/m² at a cell voltage of 269.9 ± 0.1 mV, which is at least 26-fold as much as that of the MFC

Figure 5.10. (a) The polarization and power output curves a; (b) the discharge curves of the MtrCABribAB and wild-type *E.coli* inoculated MFCs

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inoculated with the wild-type *E.coli* BL21 (DE3) strain (1.5 ± 0.1 mW/m²), gained at the current density of 6.3 ± 0.2 mA/m² with a cell voltage of 243.8 ± 0.2 mV. The maximum current generation of the former MFC achieved by the polarization plot is 522.3 ± 0.6 mA/m², which is more than 33-fold larger than that of the MFC inoculated with the wild-type *E.coli* BL21 (DE3) strain (15.5 ± 0.3 mA/m²).

The discharge plots (Figure 10b) acquired by testing the output voltage with a 600 Ω external resistor during one batch (five days) reveal that the MFC inoculated with the MtrCABribAB mutant strain generates a substantial larger steady-state current density (27.3 ± 0.6 μ A/m²) than that of the MFC inoculated with the wild-type *E.coli* BL21 (DE3) strain (0.8 ± 0.3 μ A/m²).

5.3.4. The underlying possibilities causing these improvements

The results in Figure 5.5b and Figure 5.6b have already reveal that, as the operation period of MFCs goes on, the activities of the biofilm of either the MtrCABribAB mutant strain or the wild-type *E.coli* BL21 (DE3) strain on the anode increase and reach a maximum two days after the replenishment and then after that, their activities decrease. The CFU of the bioanode are also measured. The CFU of the MtrCABribAB mutant strain inoculated MFC (Figure 5.11a) is $5.97 \pm 0.35 \times 10^8$ CFU/cm² at the anode, which is about 1.9-fold as much as that of the wild-type *E.coli* BL21 (DE3) strain (Figure 5.11b) inoculated MFC ($3.13 \pm 0.12 \times 10^8$ CFU/cm²) at the anode. Moreover, as shown in the nonturnover CVs,

Figure 5.11. CFU of (a) the MtrCABribAB and (b) the wild-type *E.coli* inoculated MFCs

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the CFU of both the MtrCABribAB mutant strain and the wild-type *E.coli* BL21 (DE3) strain on the anode increase over the first two days after the replenishment and reach the maximum at day two, followed by a consecutive decline from day three to day five (five days a batch). And the at the day five, both biofilms become very unsteadily probably due to the accumulation of metabolic wastes of bacteria, which are also reflected in their electrochemical performances in the reactors.

To test the response of both the MtrCABribAB mutant strain and the wild-type *E.coli* BL21 (DE3) strain in the anolytes containing RF molecules, the chronoamperometry (CA) experiments are carried out at +0.2 V to aid to extract electrons from inside the bacteria to the outside anode. The results demonstrate that when hung at +0.2 V, the MtrCABribAB mutant strain at the anode (Figure 5.12a) delivers a much higher current density compared to the wild-type *E.coli* BL21 (DE3) strain (Figure 5.12b), the latter of which is negligibly small. In addition, when a additional 2 μ M RF is added into the reactor, the MtrCABribAB mutant strain at the anode shows an increase in the current density while the wild-type *E.coli* BL21 (DE3) strain at the anode shows negligible response to the RF. That might be because the presence of MtrCAB electron transport protein conduits in the mutant strain could transfer cytoplasmic electrons to the cell exterior, which can be further transferred to the electrode with the help of RF. However, the wild-type *E.coli* BL21 (DE3) strain doesn't have this electron transport chain so that it cannot transfer the cytoplasmic electrons to the cell exterior, without which the RF can not help electron transport to the electrode either.

Figure 5.12. CA of (a) the MtrCABribAB and (b) wild-type *E.coli* inoculated MFCs with or with additional 2 uM RF

5.4 Conclusions

The MFC is able to transform the energy in organics into electricity by means of biochemical reactions catalyzed by the microbes. During the long period of natural evolvement, some electricigens have developed their own electron transport pathways, which are able to transport the electrons generated from bacterial metabolism to the extracellular space, which can be utilized in the MFC systems. However, they cannot be precisely regulated and there is a limitation of the genetic tools of engineering them for further usage. Thus, engineering the well-known electron transport protein conduits into the most widely used biochemical engineering bacteria, *E.coli*, by means of the complementary method become a necessity. In this work, we coexpress the

Figure 5.13. Mechanisms of the MtrCABribAB strain

MtrCAB electron transport protein conduits from wild-type *Shewanella oneidensis* MR-1 strain as well as the ribAB genes which encodes the first two step of RF biosynthesis in the *E.coli* BL21(DE) strain (Figure 13). When the MtrCABribAB mutant strain is loaded in the MFC reactor, it can significantly improve the performance of *E.coli*-catalyzed MFCs, probably due to the fact that the presence of MtrCAB electron transport protein conduit enables the electron export from the interior of the cell to the exterior of the cell as well as that the self-secretion of RF will enhance the electron transport from the bacteria to the electrode.

Chapter 6. Conclusions and Outlook for the future work

6.1. Conclusions

MFC is a microbe-catalyzed electrochemical cell that is capable of converting the chemical energy contained in the organic or inorganic substrate into electricity taking advantage of a series of bio-chemical reactions. The MFC is one kind of biological fuel cells, which exploits bioelectrocatalysts more widely, as compared to the MFC, from the cell to enzymes to complete those redox reactions. MFC, with a variety of derivational applications, is regarded as one of the potential candidates to substitute the conventional fossil fuel cell to solve environmental problem and energy problems in the future. Although our understanding of microbial behaviors in the MFCs and BES is still in the primary stage, the molecular biological technologies can help us better understand the microbial reactions in the MFCs as well as let us affect the characteristics of these microbial reactions. By using these tools, we are able to utilize the modern biotechnologies to modify these microbial reactions so as to optimize their functions as what we want to do. Genetic engineering technologies can be applied in studying and improving extracellular respiratory chains of bacteria on the electrode and especially in designing the bioelectrocatalysts which could be loaded in MFCs. Therefore, in this thesis, the main results and findings are summed up in the following three aspects:

In the first project, the engineered *E.coli* cells that can secrete riboflavin (electron

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shuttles) is immobilized as the biococatalyst, after which the beads can be used as the biococatalyst decoupled from anodic biocatalyst to greatly promote the performance of MFC. This strategy has many advantages. The biococatalyst utilizes the fuels that cannot be metabolized by the electricigen to secrete electron mediator, which increase the substrates utilization efficiency of the whole MFC system. The quantity of biococatalyst used can be altered and the beads have good reusability and recyclability, which shows its excellent functional flexibility and reusability. Since the biococatalyst beads do not occupy the electrode area, they do not cut the efficient anode surface area used to accumulate the electricigens on the electrode, which do not cause any interference with the electricigen on the electrode.

In the second study, the type II NADH dehydrogenase is overexpressed in the innermembrane of the *Shewanella oneidensis* MR-1 strain to promote electron nondestructive trans-innermembrane motion, which is capable of bridging the gap between the fuel oxidation and the electron transport of microbial electrocatalyst. In addition, considering the fact that the electron trans-innermembrane motion is a necessity for its export from the cytoplasm of the cell to the extracellular electrode, it indicates that the designed bioelectrocatalyst can be exploited together with various exogenous electron mediators, conjugated oligoelectrolytes or nanostructured electrodes to raise the upper-limits of their current maximum outputs in future.

In the third study, the MtrCAB electron transport protein conduits from wild-type *Shewanella oneidensis* MR-1 strain as well as the ribAB genes which encodes the first two steps of RF biosynthesis are co-expressed in the *E.coli* BL21(DE) strain. After

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genetic modifications, the designed MtrCABribAB mutant strain is able to lead to the synthesis of MtrCAB electron transport protein conduit which enables the electron export from the interior of the cell to the exterior of the cell as well as the self-secretion of RF which will enhance the electron transport from the bacteria to the electrode. Therefore, when the MtrCABribAB mutant strain is inoculated in the MFC reactor, it can significantly improve the performance of *E.coli*-catalyzed MFCs.

6.2. Outlook for the future work

Although a lot of previous work centered on the modification of the microbial cell on the electrode has been completed, the interest in enhancing the bioelectrocatalytic activity of the bioelectrocatalysts of BES to improve the performance of MFC is still increasing. The research focused on developing the methods of the heterogeneous expression of the extracellular respiratory proteins as well as the construction of effective and possible metabolic pathways of a variety of potential MFC/BES application systems. For example, the protein family-cytochrome c can be set as a starting point. For the application of biological technologies in engineering the extracellular respiratory protein chain, the host bacteria should be easy for the researcher to cultivate, maintain and manipulate. With the overexpression of the cytochrome c proteins, it is hoped that the amount of electrons transferred to the electrode can be maximized. Hence, if the more complicated and effective fuels can be converted directly to electricity, the increase in the efficiency of the fuel

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conversion will make the MFC system a better application.

In addition, if the bioelectrocatalysts can be designed according to the flow of the fuel, the energy conversion efficiency of the MFC system will be increased again. It should be pointed out that, although in this thesis, only *Shewanella oneidensis* MR-1 strain and the *Escherichia coli* BL21 (DE3) strain are used as the host bacteria, other host bacteria, especially have good metabolizing ability, can be regarded as the host bacteria for the heterogeneous expression of recombinant proteins too. However, the *Escherichia coli* strain is the best model host in this area only because of the lack of molecular biological tools in other microorganisms. Therefore, developing the necessary biological tools for heterogeneously expressing the recombinant proteins as well as knocking out genes in other host microorganisms will greatly prompt the development of designing and modifying the bioelectrocatalysts in the MFC in future.

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