Enhancing extracellular electron transfer of Shewanella to improve the performance of microbial fuel cells

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**ENHANCING EXTRACELLULAR ELECTRON
TRANSFER OF *SHEWANELLA* TO IMPROVE THE
PERFORMANCE OF MICROBIAL FUEL CELLS**

TING LIU

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	I
TABLE OF CONTENTS	II
LIST OF TABLES.....	V
LIST OF FIGURES	VI
SUMMARY	1
CHAPTER 1 INTRODUCTION	3
1.1 Background.....	3
1.2 Objectives.....	6
CHAPTER 2 LITERATURE REVIEW	9
2.1 The basic principles of MFCs	9
2.1.1 The working principles of MFCs	9
2.1.2 Fundamentals of voltage and power generation in MFCs.....	10
2.2 Exoelectrogens.....	16
2.2.1 Exoelectrogens	16
2.2.2 Well-known exoelectrogens.....	17
2.2.3 Communities analysis.....	19
2.2.4 Mechanisms of extracellular electron transfer	21
2.2.5 <i>Shewanella</i> biofilm in MFCs.....	26
2.3 Materials and architecture.....	30
2.3.1 Electrode materials	30
2.3.2 Membrane.....	32
2.3.3 Architecture	32
2.4 Diverse applications based on bioelectrochemical systems	33
2.4.1 Microbial fuel cells for wastewater treatment.....	33
2.4.2 Microbial electrolysis cells for hydrogen production.....	34
2.4.3 Microbial electrosynthesis for chemical production	35
CHAPTER 3 ENHANCED <i>SHEWANELLA</i> BIOFILM PROMOTES BIOELECTRICITY GENERATION 37	
3.1 Abstract.....	37
3.2 Introduction.....	37

3.3	Materials and Methods	40
3.3.1	Bacterial strains, plasmids, growth conditions and media	40
3.3.2	Construction of engineered strains of <i>S. oneidensis</i>	41
3.3.3	Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)	43
3.3.4	MFCs setup and Operations	44
3.3.5	Electrochemistry characterization	44
3.3.6	Biofilm characterization	45
3.3.7	Quantification of riboflavin	46
3.4	Results and discussion	46
3.4.1	The expression of <i>ydeH</i> gene in <i>Shewanella</i> enhanced biofilm formation	46
3.4.2	Enhanced biofilm improved bioelectricity generation	51
3.4.3	Flavins-mediated and contact-based EET increased by enhanced biofilm	57
3.5	Conclusions	64
 CHAPTER 4 A SYNTHETIC MICROBIAL CONSORTIUM OF SHEWANELLA AND BACILLUS TO BOOST BIOELECTRICITY GENERATION		
4.1	Abstract	66
4.2	Introduction	66
4.3	Materials and methods	68
4.3.1	MFCs setup and operation	68
4.3.2	Electrochemical characterization	69
4.3.3	Quantification of riboflavin	70
4.4	Results and discussion	70
4.4.1	Mixed-culture of <i>Shewanella</i> and <i>Bacillus</i> improved bioelectricity generation	70
4.4.2	Both mediated and direct electron transfer efficiency were enhanced in mixed-culture	73
4.5	Conclusions	78
 CHAPTER 5 THE EFFECT OF EXTERNAL RESISTANCE ON BIOFILM FORMATION AND INTERNAL RESISTANCE IN SHEWANELLA INOCULATED MICROBIAL FUEL CELLS		
5.1	Abstract	79
5.2	Introduction	79
5.3	Materials and methods	81

5.3.1	MFCs setup and operation.....	81
5.3.2	Electrochemical characterization	82
5.3.3	Biofilm characterization	83
5.3.4	Riboflavin quantification.....	83
5.4	Results and discussion	84
5.4.1	Bioelectricity generation and internal resistance were affected by external resistance.	84
5.4.2	The external resistance affected the biofilm formation.....	90
5.5	Conclusions	93
CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS.....		94
6.1	Conclusions	94
6.2	Recommendations	95
REFERENCE.....		97
LIST OF PUBLICATIONS.....		118

LIST OF TABLES

Table 3-1 Strains and plasmids used in this study	41
Table 3-2 Primers used in RT-qPCR	43
Table 5-1 Fit parameters of EIS for MFC at maximum output voltage.....	88
Table 5-2 Mediated electron transfer-based and direct electron transfer-based catalytic current density with different external resistances	89

LIST OF FIGURES

Figure 1-1 Schematic of microbial electrochemical systems. (A) Microbial fuel cell; (B) microbial electrolysis cell; (C) microbial electrosynthesis. Diagram derived from published work (Wang and Ren, 2013).....	4
Figure 1-2 Summary of my work. Strategies using biofilm engineering and synthetic electroactive microbial consortium were used to enhance extracellular electron transfer. The effect of external resistance on the performance of MFCs was also studied.	6
Figure 2-1 Schematic of the basic components of a microbial fuel cell (not to scale). Electrochemical active bacteria grow on the anode and oxidize organic substrates, producing CO ₂ , electrons and protons. Diagram derived from published findings (Logan et al., 2006).	10
Figure 2-2 Standard potentials (adjusted to pH = 7) of different redox species. The potentials here are relative to NHE. Diagram derived from published work (Rabaey and Rozendal, 2010)	12
Figure 2-3 Potential losses in MFC. The cell voltage is usually smaller than the overall cell electromotive force due to various potential losses. Diagram derived from published finding (Rabaey and Verstraete, 2005).	13
Figure 2-4 Three proposed extracellular electron transfer pathways for exoelectrogens: via outer membrane c-type cytochromes, electrically conductive nanowire and electron shuttles. Diagram derived from published work (Logan et al., 2006).	17
Figure 2-5 Proposed syntrophic relationship within the electroactive microbial communities.	20
Figure 2-6 Schematic of extracellular electron transfer pathways in <i>Shewanella oneidensis</i> MR-1. Three extracellular electron transfer pathways have been proposed for MR-1: Mtr respiratory pathway that need direct contact, electrically conductive nanowire and electron shuttles. Mtr respiratory pathway consists of five primary protein components: OmcA, MtrA, MtrB, MtrC and CymA.	23
Figure 2-7 Formation and hydrolysis of c-di-GMP. C-di-GMP can be generated from two molecules of GTP by diguanylate cyclases (DGCs). GGDEG domain is essential for DGC activity. Specific phosphodiesterases (PDEs) hydrolyze c-	

di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG), which then split into two molecules of GMP. PDEs are a class of protein whose enzymatic activity is associated with the EAL or HD-GYP domains. Diagram derived from published findings (Hengge, 2009).	28
Figure 3-1 We overexpressed the <i>ydeH</i> gene (encoding a diguanylate cyclase to synthesize c-di-GMP) in <i>Shewanella oneidensis</i> MR-1, which dramatically enhanced its biofilm formation and electroactivity. The MFCs inoculated with these engineered strains accomplished a maximum power density of 167.6 ± 3.6 mW/m ² , which was ~2.8 times of that achieved by the wild-type MR-1 (61.0 ± 1.9 mW/m ²).	40
Figure 3-2 Genetic construction of the two plasmids: pHG- <i>ydeH</i> _{inducible} (i.e., pHG- <i>ydeH</i> 1) and pHG- <i>ydeH</i> _{constitutive} (i.e., pHG- <i>ydeH</i> 2). Km ^r is the kanamycin resistance gene; rep is replication origin; mob is mobilization gene. In pHG- <i>ydeH</i> 1, <i>LacI</i> ^{q1} -P _{tac} - <i>ydeH</i> represents an inserted sequence, in which <i>ydeH</i> gene is under the control of an IPTG-inducible promoter P _{tac} . In pHG- <i>ydeH</i> 2, <i>ydeH</i> gene is under the control of a constitutive promoter P _{arcA}	42
Figure 3-3 Characterization of the <i>ydeH</i> gene' expression level and the biomass of biofilm formed by engineered <i>Shewanella</i> strain HG1 and HG2. (A) The relative <i>ydeH</i> cDNA abundance of HG1 upon induction by different IPTG concentrations. The control is the <i>ydeH</i> cDNA's basal expression in HG1 (in the absence of IPTG) and The error bars were calculated from triplicate experiments. (B) Relative biofilm biomass of MR-1 and HG1 at 8, 16, 24 and 48 hours upon induction by different IPTG concentrations in the static biofilm analysis under aerobic conditions, respectively. The control is the biofilm biomass of MR-1 (after 8 hours' incubation upon inoculation). The error bars were calculated from four parallel experiments.	48
Figure 3-4 Characterization of MFC's anodic biofilms after 479 hours' operation. (A) SEM image of the biofilm of the wild-type strain MR-1, (B) SEM image of the biofilm of the strain HG1 (with the induction of 1 mM IPTG), (C) SEM image of anode from MFCs with the strain HG2, and (D) the relative biofilm mass of MR-1 and HG1, respectively. To calculate the relative biofilm mass of the HG-1 strain, the MR-1 biofilm biomass formed on the anode of MFC was used as the control (designated as "1"). The error bars were calculated from triplicate experiments.	50

Figure 3-5 SEM images of anode carbon cloth from MFCs with (A) MR-1, (B) HG1 and (C) HG2. (D), (E) and (F) are SEM images of sonicated anode carbon cloth from MFCs with MR-1, HG1 and HG2, respectively.	51
Figure 3-6 Bioelectricity generation of the MFCs inoculated with wild-type strain MR-1 and engineered <i>Shewanella</i> strain HG1, respectively. (A) Output voltage vs. operation time of MFCs with the strain HG1 (with 1 mM IPTG), (B) Output voltage vs. operation time of MFCs with the strain HG2.	52
Figure 3-7 Output voltage vs. operation time of MFCs inoculated with the wild-type strain MR-1 (adding 1mM IPTG) and the strain HG1 (without IPTG), respectively.	53
Figure 3-8 Polarization curves of MFCs with MR-1, HG1 (with 1 mM IPTG) and HG2; solid: output voltage vs. current density (V-j curve), hollow: power density vs. current density (P-j curve).....	54
Figure 3-9 Linear scanning curve of the MFCs' cathode before (blue) and after (red) nutrient depletion in the MFC anodic chamber. Insert arrow indicates the current value and its corresponding cathode voltage at steady MFCs discharge.	55
Figure 3-10 SEM images of carbon cloth aerobically incubated in LB medium inoculated with (A) the strain MR-1, (B) the strain HG1 induced by 1 mM IPTG and (C) the strain HG2. (D) Their relative biofilm mass. The control is the carbon cloth electrode cultured in LB broth inoculated with MR-1, and the error bars are calculated from triplicate experiments.	56
Figure 3-11 Current discharge curves of the bioelectrochemical system under poised-potential conditions, i.e., -0.2V, 0V and 0.2V (vs. SCE), for the three <i>Shewanella</i> strains (MR-1, HG1 and HG2), respectively.....	57
Figure 3-12 Cyclic voltammetry (CV) characterization of the MFCs with inoculations of <i>Shewanella</i> strain MR-1 and HG1 (with 1mM IPTG induction) under non-turnover condition, respectively. The scanning rate of the CV curves was 50 mV/s. Inset is the baseline-corrected anodic curves of the CVs (from -0.45 V to -0.1 V).....	58
Figure 3-13 (A) CV curves at different scanning rates for the MFCs inoculated with the engineered strain HG1 (with 1 mM IPTG); (B) relationship between peak current densities and scanning rates.	59

Figure 3-14 CV curves of the MFCs inoculated with the <i>Shewanella</i> strain MR-1 and HG1 (with 1mM IPTG induction) under turnover condition, respectively, with a scanning rate of 1 mV/s.	61
Figure 3-15 (A) Cyclic voltammetry (CV) curves of MFCs with HG2 at non-turnover condition (50 mV/s). (B) CV curve of MFCs with HG2 at turnover condition (1 mV/s).	62
Figure 3-16 Riboflavin concentration of anolyte with MR-1, HG1 (with 1mM IPTG) and HG2.	63
Figure 4-1 Schematic of a synthetic microbial consortium, in which high concentration of riboflavin produced by <i>Bacillus subtilis</i> RH33 can be utilized by <i>Shewanella oneidensis</i> MR-1 to enhance extracellular electron transfer. The maximum power density of MFCs with this synthetic microbial consortium was 277.4 mW/m ² , which was 4.9 times of that by MR-1 (56.9 mW/m ²) and 40.2 times of that by RH33 (6.9 mW/m ²), respectively.	68
Figure 4-2 Bioelectricity generation of MFCs with MR1 (blue), RH33 (red) and mixed-culture of MR-1 and RH33 (green), respectively. The error bas (mean ± standard error) were derived from triplicate experiments for each group of MFCs.	71
Figure 4-3 Polarization curves of MFCs with different external resistances. (A) Output voltage vs. current density (V-j) curves and (B) Power density vs. current density (P-j) curves.	72
Figure 4-4 Differential pulse voltammetry curves for MR-1, RH33, mixed-culture of MR-1 and RH33.	74
Figure 4-5 Riboflavin concentration of anolytes in the MFCs with MR1, RH33 and mixed-culture, separately.	74
Figure 4-6 Current discharge curves of the bioelectrochemical system under poised potential (0 V vs. SCE). MFCs were first discharged for ten hours and then the anlyote of MR-1 and RH33 were exchanged.	76
Figure 4-7 CV curves of the MFCs inoculated with MR-1, RH33 and mixed-culture under turnover condition, respectively, with a scanning rate of 1 mV/s.	77

Figure 5-1 Bioelectricity generation of MFCs with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω , and 22 k Ω resistors, respectively. The error bas (mean $\pm$ standard error) were derived from triplicate experiments for each group of MFCs.	84
Figure 5-2 Polarization curves of MFCs with different external resistances. (A) Output voltage vs. current density (V-j) curves and (B) Power density vs. current density (P-j) curves.	86
Figure 5-3 (A) EIS (Nyquist plot) for MFCs with different external resistances at respectively maximum output voltages. (B) CV curves of MFCs with different external resistances under turnover conditions at a scanning rate of 1 mV/s.	88
Figure 5-4 Riboflavin concentration of anolytes in the MFCs with different external resistances.	90
Figure 5-5 Characterization of anode biofilm with different external resistances. (A) The relative biofilm mass. To calculate the relative biofilm mass, the biofilm mass of anode with 250 Ω resistance was used as the reference (designated as “1”). (B) Carbohydrate and protein contents of EPS from anode biofilm.	91
Figure 5-6 SEM images of the biofilm on carbon cloth anode with (A) 250 Ω , (B) 500 Ω , (C) 2 k Ω , (D) 6 k Ω , and (E) 22 k Ω external resistances.	92

SUMMARY

Microbial electrochemical technologies, also known as bioelectrochemical systems (BESs), including microbial fuel cells (MFCs), microbial electrolysis cells (MECs), microbial electrosynthesis (MES) *etc.*, serve as a diverse platform that combines waste treatment and energy or chemical production utilizing microbial catalytic reactions. MFCs, as a green and sustainable technology enabling simultaneous wastewater treatment and bioelectricity harvest, have attracted extensive attention in recent decades. Nevertheless, MFCs still face some challenges that restrict their real-world application. One of the main challenges is the relatively low energy output compared to traditional fuel cells. However, with the rapid development in exoelectrogens, materials, reactor architecture and operation optimization, power output has increased hundreds of times in the past decade. So it is reasonable to expect that MFC-originated microbial electrochemical technologies have the potential to become an interdisciplinary and flexible platform. Many studies have been done to understand the extracellular electron mechanisms and improve the performance of MFCs.

In this thesis, different strategies have been implemented to enhance extracellular electron transfer of the well-known electroactive strain, *Shewanella oneidensis* MR-1, to improve the performance of MFCs. Electroactive biofilms play essential roles in determining the power output of MFCs. To engineer the electroactive biofilm formation of *Shewanella oneidensis* MR-1, a c-di-GMP biosynthesis gene *ydeH* was heterologously overexpressed in *S. oneidensis* MR-1, generating an engineered strain in which the expression of *ydeH* is under the control of IPTG-inducible promoter, and a strain in which *ydeH* is under the control of a constitutive promoter. Such engineered *Shewanella* strains had significantly enhanced biofilm formation and bioelectricity generation. The MFCs inoculated with these engineered strains accomplished a maximum power density of $167.6 \pm 3.6 \text{ mW/m}^2$, which was ~ 2.8 times of that achieved by the wild-type MR-1 ($61.0 \pm 1.9 \text{ mW/m}^2$). The engineered strains in the bioelectrochemical system at poised potential of

0.2 V vs. saturated calomel electrode (SCE) generated a stable current density of 1100 mA/m², ~3.4 times of that by wild-type MR-1 (320 mA/m²).

In addition, a synthetic microbial consortium containing exoelectrogen *Shewanella oneidensis* MR-1 and riboflavin producing strain, *Bacillus subtilis* RH33, was rationally designed and successfully constructed, enabling a stable, multiple cycles of MFC operations for more than 500 hours. The maximum power density of MFCs with this synthetic microbial consortium was 277.4 mW/m², which was 4.9 times of that with MR-1 (56.9 mW/m²) and 40.2 times of RH33 (6.9 mW/m²), separately. At the same time, the Coulombic efficiency of the synthetic microbial consortium (5.6%) was higher than MR-1 (4.1%) and RH33 (2.3%). In the synthetic microbial consortium, it was found that both mediated and direct electron transfer efficiency were enhanced in mixed-culture. By exchanging the anolyte of MR-1 and RH33, it was confirmed that the improved MFC performance with the synthetic microbial consortium was because MR-1 could efficiently utilize the high concentration of riboflavin produced by RH33.

Furthermore, external resistance is one of the important factors that affect the performance of MFCs. Bioelectrochemical and biofilm characterization was conducted for *Shewanella oneidensis* MR-1 inoculated MFCs with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω , and 22 k Ω resistors. In overall, smaller external resistance resulted in higher maximum power density and more riboflavin secretion. Maximum power density of 136.8 ± 3.1 mW/m² was achieved when MFCs were operated with 500 Ω resistor, which was 3.7 times of that with 22 k Ω resistor. Electrochemical impedance spectra analysis verified an increased internal resistance along with higher external resistance. Meanwhile more biofilm mass and extracellular polymer substances were confirmed on MFC anode with higher external resistance.

CHAPTER 1 INTRODUCTION

1.1 Background

Renewable bioenergy is thought to have the potential to alleviate the global energy crisis and climate change. Microbial electrochemical technologies, also known as bioelectrochemical systems (BESs), including microbial fuel cells (MFCs), microbial electrolysis cells (MECs), microbial electrosynthesis (MES) *etc.*, serve as a diverse platform that combines waste treatment and energy or chemical production utilizing microbial catalytic reactions (Du et al., 2007; Harnisch and Schroder, 2010; Logan and Rabaey, 2012; Wang and Ren, 2013). MFCs, as a green and sustainable technology enabling simultaneous wastewater treatment and bioelectricity harvest, have attracted extensive attention in recent decades (Logan, 2009; Rabaey and Verstraete, 2005). The MEC is a recent invention. Microbial electrolysis is a process for the production of green hydrogen from organic matter by microorganisms. MEC system is operated in a similar mode with MFC system. But an additional external voltage is invested to the cell to augment the total voltage to be sufficient to produce hydrogen gas by reducing protons (Rabaey and Rozendal, 2010). MES is a promising technology to feed microbes electricity to convert carbon dioxide and water to multicarbon organic chemicals and fuels. The electricity can be produced by solar energy with photovoltaic technology. So microbial electrosynthesis is an attractive strategy to convert solar energy and store it as covalent chemical bonds (Nevin et al., 2010).

Figure 1-1 Schematic of microbial electrochemical systems. (A) Microbial fuel cell; (B) microbial electrolysis cell; (C) microbial electrosynthesis. Diagram derived from published work (Wang and Ren, 2013).

The earliest reported MFC concept is credited to Potter in 1911 (Potter, 1911). But only about 80 years later, did works about MFCs become of more interest due to greatly enhancement in current density and power generation by the addition of chemical mediators (Allen and Bennetto, 1993). The find that electron shuttles were not necessarily needed brought the breakthrough in MFCs (Chang et al., 1999; Kim et al., 1999). The demonstration that MFCs can combine wastewater treatment and electricity harvest drew people's attention in this field (Bond et al., 2002). Since then, studies about MFCs have mushroomed.

The MFC technology is distinguished in several aspects when compared with traditional bioenergy technologies. Firstly, it has broad fuel source. MFCs can utilize different organic matters, such as wastewater, biomass and sludge for electricity generation. Second, MFC is a green way for electricity generation, without second pollution and pollutant production. Furthermore, MFC can be operated in mild condition, such as room temperature. This distinguishes it from anaerobic digestion and other fermentation processes. In addition, instead of aeration, MFCs offer a feasible method to degrade the biomass under anaerobic conditions because of electrode respiration. Finally, despite the fact that MFCs was initially developed for wastewater treatment, MFCs and technologies originated from MFCs have broad applications, such as hydrogen production, bioproduction, bioremediation, toxicity and biochemical oxygen demand (BOD) sensors (Harnisch and Schroder, 2010; Knight et al., 2013; Logan, 2009; Logan and Rabaey, 2012).

Nevertheless, MFC still has some bottlenecks that restrict its real-world application. One of the main challenges is that the energy output is still low compared with traditional fuel cells. However, with the rapid development in exoelectrogens, materials, reactor architecture and operation optimization, power output has increased by orders of magnitude in past decade (Hamelers et al., 2010; Logan, 2010). So it is reasonable to expect that MFC-originated microbial electrochemical technologies have the potential to become an interdisciplinary and flexible platform, integrating microbiology,

electrochemistry, materials science and other related fields, for different applications, such as sustainable energy production, bioproduction and so on.

It is important to understand MFC technology since it is the archetype of BES and shares similar features with other microbial electrochemical technologies. Thus understanding MFC technology is of great help in optimizing all BESs.

1.2 Objectives

This study aimed to enhance extracellular electron transfer of the well-known electroactive strain, *Shewanella oneidensis* MR-1, thus to improve the performance of MFCs. Different strategies were developed to enhance direct and mediated electron transfer pathways, such as biofilm engineering and synthetic electroactive microbial consortium (Fig. 1-2). In CHAPTER 2, the basic principles of MFCs, studies about electroactive bacteria, materials and architecture development for MFCs, and various applications based on microbial electrochemical systems are reviewed.

Figure 1-2 Summary of my work. Strategies using biofilm engineering and

synthetic electroactive microbial consortium were used to enhance extracellular electron transfer. The effect of external resistance on the performance of MFCs was also studied.

CHAPTER 3 describes a strategy to improve bioelectricity via enhancing electroactive biofilm formation of *Shewanella oneidensis* MR-1 was implemented. A c-di-GMP biosynthesis gene *ydeH* was heterologously overexpressed in *S. oneidensis* MR-1, generating an engineered strain in which the expression of *ydeH* is under the control of IPTG-inducible promoter, and a strain in which *ydeH* is under the control of a constitutive promoter. Bioelectrochemical and biofilm characterization was conducted for engineered strains inoculated MFCs. The MFCs inoculated with these engineered strains accomplished a maximum power density of $167.6 \pm 3.6 \text{ mW/m}^2$, which was ~ 2.8 times of that achieved by the wild-type MR-1 ($61.0 \pm 1.9 \text{ mW/m}^2$), caused by higher levels of bacterial cells in anode biofilm, and thus higher levels of cytochromes involved in extracellular electron transfer.

In CHAPTER 4, a synthetic microbial consortium containing exoelectrogen *Shewanella oneidensis* MR-1 and riboflavin producing strain, *Bacillus subtilis* RH33, was rationally designed and successfully constructed to improve bioelectricity generation. The high concentration of riboflavin produced by RH33 could be effectively used by MR-1 as electron mediator. The maximum power density of MFCs with this synthetic microbial consortium was 277.4 mW/m^2 , which was 4.9 times of that by MR-1 (56.9 mW/m^2) and 40.2 times of that by RH33 (6.9 mW/m^2), separately. It was found that both mediated and direct electron transfer efficiency were enhanced in the synthetic microbial consortium.

The effect of external resistance on biofilm formation and internal resistance in *Shewanella* inoculated MFCs was studied in CHAPTER 5. Bioelectrochemical and biofilm characterization and riboflavin quantification were conducted for *Shewanella oneidensis* MR-1 inoculated MFCs with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω , and 22 k Ω resistors. Generally, smaller external resistance resulted in higher maximum power density and more riboflavin secretion.

Maximum power density of 136.8 ± 3.1 mW/m² was achieved when MFCs were operated with 500 Ω resistor, which was 3.7 times of that with 22 k Ω resistor. Electrochemical impedance spectra analysis verified an increased internal resistance along with higher external resistance. Meanwhile more biofilm mass and extracellular polymer substances were confirmed on MFC anode with higher external resistance.

CHAPTER 2 LITERATURE REVIEW

2.1 The basic principles of MFCs

2.1.1 The working principles of MFCs

A schematic of an MFC system is shown in Figure 2-1. Electrochemical active bacteria (i.e., exoelectrogens) grow on the anode and oxidize organic substrates, producing CO_2 , electrons and protons. The anode and the cathode are connected by a wire with a load, which is usually a resistor in laboratory. Exoelectrogens can transfer electrons to the anode instead of usual electron acceptors (O_2 , NO_3^- , etc.), that this process is called as extracellular electron transfer (EET). To be different with normal respiration pathway, extracellular electron transfer utilizes extracellular solid electrode as the final electron acceptor other than intracellular final electron acceptor, such as O_2 , NO_3^- , etc. Electrons can be transferred to the anode through mainly three mechanisms: electron shuttles or mediators, outer-membrane c-cytochromes directly attached to anode surface and conductive nanowires (Hernandez and Newman, 2001). The electrons are then transferred to the cathode via external electric circuit. The protons produced in the anode chamber are transferred to the cathode through a proton exchange membrane that only allows protons to transfer between the electrodes. Reduction reaction happens in the cathode chamber. If air-cathode is used, then the electrons react with the protons and oxygen to produce water. Chemical oxidizers can also be used in cathode, for example Mn (IV) and ferricyanide, etc.

Figure 2-1 Schematic of the basic components of a microbial fuel cell (not to scale). Electrochemical active bacteria grow on the anode and oxidize organic substrates, producing CO₂, electrons and protons. Diagram derived from published findings (Logan et al., 2006).

2.1.2 Fundamentals of voltage and power generation in MFCs

Only if the overall reaction is thermodynamically favorable, can electricity be generated in an MFC. The theoretical amount of energy available for electricity generation can be calculated in terms of Gibbs free energy (Joules),

$$\Delta G_r = \Delta G_r^0 + RT \ln(\Pi) \quad (2-1)$$

where ΔG_r is the change of reaction in Gibbs free energy, ΔG_r^0 is the standard change of reaction in Gibbs free energy, R is the gas constant, T is the absolute temperature (K), and Π (unitless) is the reaction quotient. The reaction quotient is the ratio of the activities of the products divided by the reactants raised to their respective stoichiometric coefficient,

$$\Pi = \frac{[products]^p}{[reactants]^r} \quad (2-2)$$

This is the maximum amount of work that can be gained from a reaction.

The overall reaction in a MFC can also be evaluated from the perspective of overall cell electromotive force, E_{emf} , which is the potential difference between the cathode and anode.

$$E_{emf} = E_{emf}^0 - \frac{RT}{nF} \ln (\Pi) \quad (2-3)$$

where E_{emf}^0 is the cell electromotive force when all reactions are evaluated at standard conditions. This E_{emf} is the theoretical maximum cell voltage, but the actual voltage would be lower than the value because of various potential losses. In biological systems, the reported potentials are usually pre-adjusted to neutral pH. The total potential that can be produced by MFC is the potential difference between anode and cathode potentials,

$$E_{emf}^{0'} = E_{anode}^{0'} - E_{cathode}^{0'} \quad (2-4)$$

where “'” on E is used to indicate the neutral pH conditions. The anode reaction mainly involves the biodegradation of biomass and release the electron to the anode. There are at least two recognized inner metabolic pathways that involve EET and NADH probably work as the electron source for EET (Rabaey and Verstraete, 2005). For bacteria, NADH is usually the electron donor into respiratory chain, not the substrate. NADH/NAD⁺ couple is the redox species directly involved in transferring electrons to electrode. The standard redox potential of NADH/NAD⁺ under neutral pH conditions is $E^{0'} = -0.32$ V (Fig. 2-2). But the redox potential of NADH/NAD⁺ varies upon different NADH/NAD⁺ ratio. However, the theoretical anodic potential at open circuit is determined by the redox species, for example, c-type cytochromes or soluble shuttles, which directly react at the anode surface.

$E'_0(\text{V})$	Redox reaction
+0.84	$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$
+0.771	$\text{Fe}^{3+} + \text{e}^- \rightarrow \text{Fe}^{2+}$
+0.44	$\text{NO}_2^- + 8\text{H}^+ + 6\text{e}^- \rightarrow \text{NH}_4^+ + 2\text{H}_2\text{O}$
+0.436	$[\text{Fe}(\text{CN})_6]^{3-} + \text{e}^- \rightarrow [\text{Fe}(\text{CN})_6]^{4-}$
+0.421	$\text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$
+0.254	$\text{Cytochrome c}(\text{Fe}^{3+}) + \text{e}^- \rightarrow \text{Cytochrome c}(\text{Fe}^{2+})$
+0.1	$\text{Ubiquinone} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{Ubiquinol}$
+0.75	$\text{Cytochrome b}(\text{Fe}^{3+}) + \text{e}^- \rightarrow \text{Cytochrome b}(\text{Fe}^{2+})$
+0.31	$\text{Fumarate}^{2-} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{Succinate}^{2-}$
-0.18	$\text{FAD} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{FADH}_2$
-0.185	$\text{Pyruvate}^{2-} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{Lactate}^{2-}$
-0.22	$\text{SO}_4^{2-} + 10\text{H}^+ + 8\text{e}^- \rightarrow \text{H}_2\text{S} + 4\text{H}_2\text{O}$
-0.274	$\text{S} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{S}$
-0.320	$\text{NAD}^+ + \text{H}^+ + 2\text{e}^- \rightarrow \text{NADH}$
-0.42	$\text{Ferredoxin}(\text{Fe}^{3+}) + \text{e}^- \rightarrow \text{Ferredoxin}(\text{Fe}^{2+})$
-0.42	$2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$

Figure 2-2 Standard potentials (adjusted to pH = 7) of different redox species. The potentials here are relative to NHE. Diagram derived from published work (Rabaey and Rozendal, 2010) .

The theoretical maximum potential for cathode using oxygen is $E^{0'} = +0.84$ V. But the actual cathode potential is much less than the predicted one. The measured maximum potential, i.e. open-circuit potential (OCP), of air cathode is about +0.4 V, while the working potential is about +0.25 V. The most commonly used catholyte other than oxygen is ferricyanide. The standard redox potential of ferricyanide is +0.37 V, and it is +0.436 V at pH = 7. MFCs using ferricyanide as catholyte usually show higher power generation than these using air cathode because the working potential of ferricyanide is close to the standard redox potential.

The cell electrochemical force is the maximum value from the point of thermodynamics. The open circuit voltage (OCV), occurring in the absence of current, is the maximum voltage available. The OCV should approach the value

of E_{emf} , theoretically. Nevertheless, the value of OCV is usually much lower than that of E_{emf} because of various potential losses (Fig. 2-3).

Figure 2-3 Potential losses in MFC. The cell voltage is usually smaller than the overall cell electromotive force due to various potential losses. Diagram derived from published finding (Rabaey and Verstraete, 2005).

The cell voltage decreases significantly when the electrical loop is close. Generally speaking, overvoltage is the difference between the cell electrochemical force and cell voltage. It includes the overpotentials of the electrode (anode and cathode) and the ohmic loss of the system, where overpotential is the potential difference between the theoretical potential based on thermodynamics under and the actual observed potential

$$E_{cell} = E_{emf} - (\sum \eta_a + |\sum \eta_c| + IR_{\Omega}) \quad (2-5)$$

where E_{cell} is cell voltage, $\sum \eta_a$ and $|\sum \eta_c|$ are the overpotentials of the anode and the cathode, and I and R_{Ω} are the current and ohmic resistance of the system. The overpotentials of the electrode usually includes three parts, activation losses, bacterial metabolic losses and concentration losses. IR_{Ω} represents the sum of all ohmic losses (Logan et al., 2006; Rabaey et al., 2005b).

Activation losses happen because oxidation or reduction reactions need activation energy to transfer electrons from bacteria to anode or from cathode to final electron acceptor. Activation losses appear to be the major factor in overpotentials, increasing with the current density (Rabaey et al., 2005b).

Bacterial metabolic losses occur when electrons are transferred from a substrate to the final electron acceptor (anode in MFC), which is at a higher potential than the substrate. The potential difference determines the metabolic energy gained by bacteria. The attainable metabolic energy gained by bacteria increases with the potential difference, while the possible maximum voltage of MFC decreases with it. Thus, lower anode potential is good for maximizing the MFC voltage. However, extracellular electron transport will be inhibited if the anode potential is set too low.

Concentration losses, also known as concentration polarization, happen when current production is restricted due to limited mass transfer of chemical species from or to the electrode (Larminie and Dicks, 2003). At the anode, accumulation of oxidized species or lack of reduced species at the electrode surface may cause concentration polarization, resulting in an increased ratio between the oxidized and the reduced species, leading to an increased anode potential. At the cathode, the contrary is the case, causing a decreased cathode potential.

Ohmic losses include the resistance to electron flow via the interconnections and electrodes, and the resistance of ions through proton exchange membrane and electrolyte. It is usually written in the form of IR_{Ω} . To reduce the ohmic

resistance, it can be achieved by improving conductivity of electrode and electrolyte, minimizing the resistivity of membrane, and reducing the distance that the electrons and protons need to travel (Larminie and Dicks, 2003; Logan et al., 2006).

In an MFC, the cell voltage can also be described in the form of OCV and internal resistance (R_{int})

$$E_{cell} = OCV - IR_{int} \quad (2-6)$$

where I is the generated current. IR_{int} includes all internal losses. From eq. 2-5 and 2-6, it can be seen that OCV includes the overpotentials of electrodes under open circuit conditions, and internal losses include the ohmic losses and overpotentials which depend on current. Despite the fact that internal resistance and ohmic resistance are used by researchers indiscriminatively, it should not be ignored that internal resistance includes more than ohmic resistance.

One of the important factors to evaluate the performance of MFCs is power output

$$P = IE_{cell} \quad (2-7)$$

where I can be calculated according to Ohm's law. Thus the power can be calculated as

$$P = \frac{OCV}{R_{int} + R_{ext}} E_{cell} = \frac{OCV^2 R_{ext}}{(R_{int} + R_{ext})^2} \quad (2-8)$$

where R_{ext} is the external resistance. It can be seen that the power of MFC is maximum when the internal resistance equals to the external resistance. To make the power output of different systems more comparable, power density is often used by normalizing the power to some characteristic of the system, such as projected anode surface area and reactor volume (Liu et al., 2004; Park and Zeikus, 2003; Rabaey et al., 2004).

2.2 Exoelectrogens

2.2.1 Exoelectrogens

Exoelectrogens, also known as electrochemical active bacteria, electricigens or anode respiring bacteria, refer to the microorganisms that have the ability of extracellular electron transfer (EET) (Fig. 2-4). A final electron acceptor is needed for cellular respiration. For instance, respiration utilizes oxygen as the final electron acceptor is termed as aerobic respiration, while respiration utilizes other intracellular soluble chemicals, such as NO_3^- , as the final electron acceptor is called anaerobic respiration. To be different with normal respiration pathway, EET utilizes extracellular strong soluble oxidizing agent or solid compounds, such as electrode, Mn(III/IV) oxides and Fe(III) oxides, as the final electron acceptor other than intracellular final electron acceptor (Kumar et al., 2015; Logan, 2009).

Figure 2-4 Three proposed extracellular electron transfer pathways for exoelectrogens: via outer membrane c-type cytochromes, electrically conductive nanowire and electron shuttles. Diagram derived from published work (Logan et al., 2006).

2.2.2 Well-known exoelectrogens

It is found that various microbes from *Proteobacteria*, *Acidobacteria*, *Firmicutes*, yeast, fungi and microalgae has the ability to generate electricity in MFCs without addition of mediators, with bacteria being the majority (Kim et al., 2005a; Park et al., 2001; Pham et al., 2003; Wang et al., 2012). The most efficient exoelectrogens are from two genera, *Geobactor* and *Shewanella*, being intensively studied to understand its mechanisms of EET and related metabolisms (Coursolle et al., 2010; Flynn et al., 2012; Pinchuk et al., 2010; Shi et al., 2007).

All *Geobacter* species are gram negative, anaerobic, being in the *Geobacteraceae* family, *Bacteria* domain, *Proteobacteria* phylum, *Deltaproteobacteria* class, and *Desulfuromonadales* order. Generally speaking, *Geobacter* species were found to be more abundant than other Fe(III)-reducing microorganisms where reduction of Fe(III) is occurring. *Geobacter* can utilize various final electron acceptors for respiration, such as electrode, elemental sulfur, humic substances, Fe(III), Mn(IV), U(VI), Np(V), and Co(III)-EDTA, etc. (Caccavo et al., 1994; Lovley and Phillips, 1988; Lovley et al., 1987; Lovley et al., 1991; Lovley et al., 1998; Phillips et al., 1993). Among pure culture MFCs, MFCs with *Geobacter* species nearly produce the highest current densities. *Geobacter metallireducens* GS-15 is the first strain being isolated from sand sediment in Potomac River in Washington D.C. by Derek Lovley in 1987, and it is also the first organism found to be capable of EET (Lovley et al., 1987). There are a large number of *Geobacter* pure cultures being isolated, sharing the common features, oxidizing acetate and being capable of EET. *Geobacter sulfurreducens* and *Geobacter metallireducens* are the species that have been studied mostly (Lovley et al., 2011).

Shewanella species are the only genus in *Shewanellaceae* family of the *Proteobacteria* phylum. All *Shewanella* species are gram negative and facultative anaerobic. *Shewanella putrefaciens*, found in 1931, was formerly known as *Achromobacter putrefaciens*. It was later classified to *Pseudomonas*, and finally reclassified to the new genus *Shewanella* that was named after the late Dr. James M. Shewan. *Putrefaciens* is the first isolated species in *Shewanella* (Debby and Hammer, 1931; Lee et al., 1977; MacDonell and Colwell, 1985; Shewan et al., 1960). There are about 40 species in *Shewanella* genus based on 16S rRNA sequences analysis and DNA: DNA hybridization. *Shewanella* species are commonly distributed in communities existing in marine and fresh water environments. *Shewanella* began to draw vast attention since the discovery of *Shewanella oneidensis* MR-1, being capable of metal-respiration. *Shewanella oneidensis* MR1, which was named after Oneida Lake where it was isolated in 1998, is the first species that was found to produce bioelectricity without addition of mediator (Hau and Gralnick, 2007; Kim et al., 1998).

Shewanella species are among the most diverse respiratory microorganisms, being able to respire about 20 inorganic and organic compounds in environments, including some solid metals and toxic elements, such as Fe(III) oxide, Mn(III and IV) oxides, dimethyl sulfoxide, trimethylamine-N-oxide, arsenate, arsenite, fumarate, and succinate, etc. (Hau and Gralnick, 2007).

2.2.3 Communities analysis

Due to the various range of inocula, system architectures, operating conditions, electron donors and acceptors, the bacterial communities developed in MFCs show great diversity. Important information about electrode-colonizing microbial communities are gained through the rapid development of interdisciplinary research combining metagenomics and phylogeny based on 16S rRNA, metatranscriptomics and microbial electrochemistry. The information is of great help for optimizing the MFC systems. Analysis of electricity-producing communities reveals that diverse bacteria other than model species of the electrogens exist in the communities. It was found that *d-Proteobacteria* predominated in sediment MFCs, while communities developed in other systems include *a-, b-, d- or g-Proteobacteria*, yeast, *Firmicutes* and other unknown species. Only in sediment MFCs, was *Geobacteraceae* found to predominate in the communities in anode. It was revealed that 71% of the sequences in 16S rDNA clone library of the anode community in a marine sediment MFC belonged to *d-Proteobacteria*. And 70% of these sequences were *Geobacteraceae* (Bond et al., 2002). In a kindred MFC, 76% of the 16S rDNA sequences were *d-Proteobacteria*, and 59% of these belonged to *Geobacteraceae* (Tender et al., 2002). An analysis of different freshwater, marine and salt marsh sediments showed that the *d-Proteobacteria* predominated in these samples, accounting for 54–76% of 16S rDNA sequences of the anode communities (Holmes et al., 2004). And *Shewanella* species only played as the majority in cysteine-fed system (Logan et al., 2005). Other communities in anode show a broad diversity, including Gram-positive- and Gram-negative- dominated systems and a large number of uncharacterized 16S rDNA sequences (Kim et al., 2004; Lee et al., 2003). The role of different species in the anode community remains unknown due to the complexity of

metabolic crosstalk and short of correlation between functional correlation and phylogenetic identity. However, preliminary evidence from several studies suggested syntrophic relationships within the microbial communities (Fig. 2-5). In a syntrophic relationship, the growth of one species depends on, or improved by the products of another species, such as nutrients, growth factors, substrate and other components. Within the electricity-producing communities, it was found that the syntrophic association can be based on metabolites or electron shuttles (Pham et al., 2008; Venkataraman et al., 2011; Wang et al., 2014). For a better understanding of electricity-producing communities developed in the anode, it is necessary to analyze the effect of MFC architecture, substrate and inoculum. Studying the way microbial ecology develops and evolves in the anode communities is of great help for exploring the complex microbial communities and optimizing the systems for better energy production (Logan and Regan, 2006; Zhi et al., 2014).

Figure 2-5 Proposed syntrophic relationship within the electroactive microbial communities.

2.2.4 Mechanisms of extracellular electron transfer

Microorganism was found to be capable of utilizing versatile final electron acceptors. Under anaerobic conditions, some microorganisms grow using fermentation, in which organic compounds serve as the final electron acceptor. While other microorganisms grow using inorganic compounds other than oxygen, such as sulfate ions (SO_4^{2-}), nitrate ions (NO_3^-), fumarate, Fe (III) oxide or Mn(III and IV) oxides, as the final electron acceptor. To be different with normal respiration pathway, extracellular electron transfer (EET) utilizes extracellular strong soluble oxidizing agent or solid compounds (e.g., electrode, Mn(III/IV) oxides and Fe(III) oxides), as the final electron acceptor other than intracellular final electron acceptor (Kumar et al., 2015; Logan, 2009). These important microbial respiration pathways have drawn vast attention in recent decades, leading to the development of attractive research about bioelectrochemical systems for applications regarding waste treatment and energy or chemical production.

A transport system for extracellular electron transfer is needed to overcome the physical barrier of bacterial cell. There are two main extracellular electron transfer mechanisms: direct electron transfer (DET) and mediated electron transfer (MET). DET pathway mainly involves the electron transfer via membrane-spanning cytochromes and electrically conductive nanowires or pili, requiring direct contact between the microbes and electrodes (Bond and Lovley, 2003; Busalmen et al., 2008b; Gorby et al., 2006; Okamoto et al., 2012; Reguera et al., 2005). MET, alternative EET pathway, is facilitated by exogenous or self-excreted redox mediators (Brutinel and Gralnick, 2012; Feng et al., 2010; Marsili et al., 2008; Park and Zeikus, 2000; Rabaey et al., 2005b; Tang et al., 2010; von Canstein et al., 2008). While these two main mechanisms are distinct, they are not mutually exclusive in several species, all hinging upon the microbes and their strategy to accomplish EET.

The involvement of c-type cytochromes in DET has been reported mainly for *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens* (Bond and Lovley, 2003; Inoue et al., 2011; Inoue et al., 2010; Meitl et al., 2009a;

Okamoto et al., 2012). Mtr respiratory pathway, which consists of five primary protein components: OmcA, MtrA, MtrB, MtrC and CymA, has been established for *S. oneidensis* MR-1 (Fig. 2-6). OmcA and MtrC are outer-membrane (OM) c-type cytochromes, contacting the electrode directly, and MtrA is a periplasmic decahaem c-type cytochrome. MtrB, which is an OM β -barrel nonheme protein, connects the MtrA and OM c-type cytochromes. The electrons from the inner quinone pool are finally transferred to outer membrane-associated OmcA and MtrC via inner membrane-associated quinol oxidase CymA firstly and then through MtrA.

The electrons are transferred from MtrCAB-OmcA complexes to electrodes via direct contact or flavins (Coursolle et al., 2010; Hartshorne et al., 2009; Okamoto et al., 2013; Shi et al., 2009). Another complex MtrFDE, which is a homologous complex of MtrCAB, was also discovered in *S. oneidensis* MR-1 (Coursolle and Gralnick, 2010). OmcA, being a homologue of MtrF and MtrC, can receive electrons from MtrFDE or MtrCAB complexes. However, it was found that MtrC is more important than OmcA in EET (Baron et al., 2009; Carmona-Martínez et al., 2013; Coursolle et al., 2010; Jain et al., 2012; Shi et al., 2006). The DET pathway through c-type cytochromes was also identified in other *Shewanella* strains, such as *Shewanella putrefaciens* and *Shewanella loihica* (Carmona-Martínez et al., 2013; Jain et al., 2012).

Figure 2-6 Schematic of extracellular electron transfer pathways in *Shewanella oneidensis* MR-1. Three extracellular electron transfer pathways have been proposed for MR-1: Mtr respiratory pathway that need direct contact, electrically conductive nanowire and electron shuttles. Mtr respiratory pathway consists of five primary protein components: OmcA, MtrA, MtrB, MtrC and CymA.

There are a lot of studies about *G. sulfurreducens* DET pathway via OM c-type cytochromes. The electrons are transferred from the inner membrane protein MacA to PpcA, a periplasmic protein, which transfers the electrons to the OM c-type cytochromes. Several OM c-type cytochromes, such as OmcB, OmcS and OmcZ, have been found to be involved in extracellular electron transfer of *G. sulfurreducens*. Among these cytochromes, OmcZ plays the predominant role in EET, which has been revealed genetically and electrochemically (Holmes et al., 2006; Marsili et al., 2010; Nevin et al., 2009a; Richter et al., 2009; Srikanth et al., 2008). Some of the OM c-type cytochromes

involved in EET to metal oxides were found not necessarily required for transferring electrons to electrodes (Holmes et al., 2006; Richter et al., 2009).

The nanowires in EET, being electronically conductive, are pili or extracellular appendages similar to pili (Malvankar and Lovley, 2012). The involvement of nanowires in EET has been shown for *G. sulfurreducens* (Malvankar et al., 2011; Nevin et al., 2009b; Reguera et al., 2006). Decrease of nanowire production and thus electricity generation was found due to deletion of the PilA gene (Nevin et al., 2009b). Under transmission electron microscopy, it was showed that OM c-type cytochromes OmcS is aligned along the nanowires (Leang et al., 2010). The pili of *G. sulfurreducens* were found to be crucial for long-distance DET (Malvankar et al., 2011). Notwithstanding, for better understanding the role of *G. sulfurreducens* nanowires in EET across anodic biofilms, more studies are needed. Extracellular electron transfer facilitating by nanowires was also discovered in *S. oneidensis* MR-1, despite the fact that nanowires of are filamentous, which is different from *Geobacter* nanowires (divergent pili). Experiments about gene deletion in *S. oneidensis* MR-1 revealed that OM c-type cytochromes are necessary for the nanowires to perform EET (Gorby et al., 2006). Despite the fact that there is no sound proof to suggest that cytochromes are aligned along the filaments of MR-1, it was found that these filaments are electronically conductive (El-Naggar et al., 2010; Malvankar and Lovley, 2012). However, it is still questionable about the contribution of *Shewanella* nanowires in EET (Bouhenni et al., 2010; Fitzgerald et al., 2012).

There have been interesting debates on relevance of the different EET pathways for *Shewanella* and *Geobacter* species. Tender's group insisted that electron transport along *Geobacter* pili and biofilm proceeds via electron hopping, while Lovley's group thought it is via metallic-like conductivity (Malvankar et al., 2012; Strycharz-Glaven and Tender, 2012; Strycharz-Glaven et al., 2011). And recently people tend to accept the idea that flavin electron shuttles dominate in EET by *Shewanella* and DET dominates in *Geobacter* (Kotloski and Gralnick, 2013).

Mediated electron transfer is facilitated by self-excreted or artificial redox mediators, also known as electron shuttles. Such mediators are capable of gaining electrons from OM cytochromes or intracellular electron carriers. In order to deliver electrons to the electrodes, mediators are usually electrochemically reversible and have a proper redox potential propitious to EET (Rabaey and Verstraete, 2005). There are several discovered endogenous electron shuttles, such as phenazines, quinones and flavins, which have been studied intensively (Brutinel and Gralnick, 2012; Marsili et al., 2008; Park and Zeikus, 2000; Rabaey et al., 2005b; Tang et al., 2010; von Canstein et al., 2008). Exogenous electron shuttles were usually added in the initial period of development of MFCs (Akiba et al., 1987; Kim et al., 2000; Park et al., 1999; Thurston et al., 1985). The artificial electron shuttles, such as neutral red, quinone derivatives and thionin, could facilitate EET (Feng et al., 2010; Park and Zeikus, 2000; Tang et al., 2010; Thurston et al., 1985). These redox mediators are usually used to help microorganisms incapable of extracellular electron transfer. These artificial mediators have several drawbacks for practical application, such as the toxicity presented at higher concentrations, the unstable nature of some exogenous mediators and the need of continuous addition of mediators to the system (Schröder, 2007). Since the discovery that artificial mediators are not necessarily needed for EET, the use of exogenous mediators has been declined. Phenazine-1-carboxylic acid (PCA), pyocyanin (PYO) and phenazine-1-carboxamide (PCN) produced by *Pseudomonas aeruginosa* are the first identified shuttles (Rabaey et al., 2005a). Several electrochemical studies suggested that flavins-based DET was found to be the important EET pathway for *S. oneidensis* MR-1. Riboflavin and riboflavin-5'-phosphate (FMN) were found to be secreted by MR-1 to mediate EET, with riboflavin as the dominant component at biofilm mode (Marsili et al., 2008; von Canstein et al., 2008). The current produced by *Shewanella oneidensis* in MFC decreased after changing the original medium with fresh medium, but recovered immediately by putting the original medium after centrifugation to remove planktonic cells (Marsili et al., 2008). *Geobacter* species that are not thought to use extracellular electron shuttles were not affected by changing with fresh medium. Deletion of *bfe* (bacterial flavin adenine dinucleotide exporter) gene resulted a mutant strain of *Shewanella oneidensis* MR-1, which was unable to secrete flavins. Without

supplement of flavins, this mutant strain produced remarkably less current than the wild type in MFC. However, after addition of same concentration of flavins, the mutant strain generated as much current as the wild type (Kotloski and Gralnick, 2013). It was found that proteins in the Mtr pathway, especially OM c-type cytochromes MtrC and OmcA, were essential for the activity of flavins-based electron shuttling (Coursolle et al., 2010; Okamoto et al., 2013). Several mutants that lack genes in Mtr respiratory pathway were tested for their ability to reduce riboflavin and FMN. These mutants were remarkably diminished in their abilities to reduce both riboflavin and FMN, while complemented strains restored their flavin reduction ability (Coursolle et al., 2010). Removal of flavins resulted in severe defect of EET of MR-1 and vice versa (Baron et al., 2009; Okamoto et al., 2013; Yong et al., 2013b). The relatively low concentration of secreted flavins restricted the EET efficiency of MR-1. It was an effective way to improve the EET efficiency of *Shewanella* by adding riboflavin to anolyte directly (Baron et al., 2009). Recently, the involvement of flavins in EET for *S. loihica* PV-4 has also been reported (Jain et al., 2012). Several bacteria were found capable of secreting endogenous electron shuttles to facilitate EET (Bond and Lovley, 2005; Nimje et al., 2009; Rabaey et al., 2007). It was found that electron shuttles generated by certain kind of microorganisms in anode communities can be used by other microorganisms to conduct EET, thus enhancing the performance of MFCs by this mutualism (Boon et al., 2008; Pham et al., 2008; Venkataraman et al., 2011; Wang et al., 2014; Yang et al., 2015).

2.2.5 *Shewanella* biofilm in MFCs

Shewanella oneidensis MR-1 is known for its versatile metabolic pathways and electron transfer capacities (Flynn et al., 2012; Fredrickson et al., 2008; McLean et al., 2008a; Pinchuk et al., 2011; Pinchuk et al., 2010; Wang et al., 2010). Oxygen is a critical factor among numerous factors that affect the biofilm formation of MR-1. It was found that detachment of cells from established aerobic biofilms of MR-1 in flow cell chambers was induced after the flow was stopped. Induction of detachment was rapid, suggesting the quick detection and response of biofilm to oxygen fluctuations (Thormann et al.,

2005). Genes encoding cell-to-cell and cell-to-surface adhesion factors whose transcription increased upon exposure to increased oxygen concentrations were identified by global transcriptome analysis comparing aerobic aggregated to oxygen-limited unaggregated cells (McLean et al., 2008b). Biofilms formed under aerobic conditions showed a hollow and seeding dispersal structure, while a round and densely-packed structure under anaerobic conditions. It was also found MR-1 biofilms grown in medium equilibrated with air were poised to rapidly reduce a variety of anaerobic electron acceptors (McLean et al., 2008a). This aerobic-to-anaerobic shift might induce MR-1 to utilize anode as the electron acceptor.

Cyclic diguanylate (c-di-GMP) is a ubiquitous second messenger in bacteria. High concentration of intracellular c-di-GMP facilitates biofilm formation and low level of c-di-GMP is favorable for planktonic phenotype. It was found that c-di-GMP influences many processes related to biofilm formation and disperse, including exopolysaccharide biosynthesis, cell cycle progression, flagellar genes, motility and secretion system. c-di-GMP interacts with specific receptors that coordinately regulate a cascade of proteins at the transcriptional, translational, post-transcriptional, and post-translational levels. The process controlled by c-di-GMP is complex and not completely understood. The model species for c-di-GMP signaling is *Pseudomonas*, *Escherichia*, *Vibrio*, and *Salmonella* (Hengge, 2009; Jenal and Malone, 2006; McDougald et al., 2012). There are only a few publications about c-di-GMP signaling in *Shewanella* (Chao et al., 2013; Rakshe et al., 2011; Thormann et al., 2006a).

It is recognized that bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) acts as the central role in the network which controls the switch from biofilm to planktonic phenotypes (Hengge, 2009). c-di-GMP was discovered in 1987, but only almost 20 years after its discovery, was it recognized that c-di-GMP is a ubiquitous second messenger in bacteria (Jenal and Malone, 2006). High level of intracellular c-di-GMP level promotes the expression of adhesive matrix components and result in multicellular behavior and biofilm formation. Low level of c-di-GMP concentration is favorable for planktonic phenotypes

(Hengge, 2009). Thormann et al. proposed that c-di-GMP of *Shewanella oneidensis* MR-1 is a key intracellular regulator for controlling the shift of a biofilm cell between attachment and detachment. Deletion of *mxdA* gene coding for protein that can indirectly modulate intracellular c-di-GMP concentration caused severe defects in biofilm formation and three-dimensional architecture. The impacted phenotype was complemented by VCA0965 that is a known c-di-GMP-forming enzyme (Rakshe et al., 2011; Thormann et al., 2006b).

Figure 2-7 Formation and hydrolysis of c-di-GMP. C-di-GMP can be generated from two molecules of GTP by diguanylate cyclases (DGCs). GGDEG domain is essential for DGC activity. Specific phosphodiesterases (PDEs) hydrolyze c-di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG), which then split into two molecules of GMP. PDEs are a class of protein whose enzymatic activity is associated with the EAL or HD-GYP domains. Diagram derived from published findings (Hengge, 2009).

As shown in Figure 2-3, diguanylate cyclases (DGCs) are responsible for producing c-di-GMP from two molecules of GTP. GGDEG domain, named after the specific amino acid sequence motif, is essential for DGC activity (Hengge, 2009). Ryjenkov et al. found that individual GGDEF domains possessed a low level of DGC activity and the oligomeric states of GGDEF domains and full-length proteins were similar. Despite the fact that the sensory protein domains near GGDEF domains regulate DGC enzymatic activity, GGDEF domains are sufficient to obtain DGC activity and c-di-GMP specific (Ryjenkov et al., 2005). Specific phosphodiesterases (PDEs) hydrolyze c-di-GMP into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG), which then split into two molecules of GMP. PDEs are a class of protein whose enzymatic activity is associated with the EAL or HD-GYP domains (HD-GYP domain proteins are

less common) (Jenal and Malone, 2006). Large-scale genome sequencing found that the number of GGDEF, EAL and HD-GYP domain is highly variable across different species. *Shewanella oneidensis* MR-1 has 51 proteins containing the GGDEF domain, 27 with EAL domain, and 20 that carry HD-GYP domain (Thormann et al., 2006b). The c-di-GMP signaling network regulates responses of bacteria due to environmental stimuli and cell-cell communication (Jenal and Malone, 2006). Generally, there are four basic components in c-di-GMP control module. c-di-GMP is produced and degraded by DGC and PDE which response to certain signals through their amino-terminal sensory domains, and binds and allosterically regulates an effector molecule which can be protein or RNA. The effector subsequently affects the molecular output of a target molecule, which can be enzyme, promoter DNA, or complex cellular structure (exopolysaccharide synthesis and secretion apparatus, flagellar basal body, etc) (Hengge, 2009). The actual regulation network of c-di-GMP is much more complex and diverse due to the variety of all four basic components in a single species. A large number of cellular and environmental signals Most GGDEF, EAL and HD-GYP domains are associated with various terminal sensory domains. Singles, such as oxygen, temperature, redox conditions, light, nutrition, intercellular signaling molecules and various extracellular substances (polyamines, antibiotics, etc.) are integrated into the c-di-GMP signaling network by being perceived by these sensory domains, such as the oxygen-binding haemerythrin domain (French et al., 2008), flavin-associated Per-Arnt-Sim (PAS) domain (Qi et al., 2009), the blue-light sensing proteins that used flavin adenine dinucleotide (BLUF) (Hasegawa et al., 2006), the bacteriophytochromes that sense red and far red light (Tarutina et al., 2006). c-di-GMP binds and allosterically regulates an effector molecule which can be protein or RNA. There are four types of c-di-GMP effector proteins identified, PliZ, FleQ, PelD and I site effector (Hengge, 2009). Proteins of PliZ family, which represent the best-studied class of c-di-GMP effector, were identified in *Pseudomonas aeruginosa*, *Caulobacter crescentus*, *Vibrio cholera*, *Escherichia coli* and various Gram-negative bacteria (Amikam and Galperin, 2006; Christen et al., 2007; Merighi et al., 2007; Pratt et al., 2007; Ryjenkov et al., 2006). Sudarsan *et al.* identified that c-di-GMP is also a ligand to a riboswitch class which has highly conserved RNA domain

GEMM, which means RNA element occurring in genes for the environment, membranes and motility (Sudarsan et al., 2008).

2.3 Materials and architecture

2.3.1 Electrode materials

Various materials, with carbon materials being the widely used electrodes, have been used in MFCs studies. There are three kinds of materials often used as anodes in MFCs: metal-based, carbon and composite materials. Carbon materials, such as carbon cloth, carbon felt, carbon paper, graphite rod, and graphite fiber brush, have several advantages: they are chemically stable and cost-effective, and have good biocompatibility. Carbon cloth was used as the electrode in a single-chamber MFC, in which brewery wastewater was used as the anolyte, achieving a maximum power density of 482 mV/m² (Wang et al., 2008). Graphite rod was often used as the anodes. However, the application of the graphite rod was limited because of its low porosity and surface area for microorganism adsorption. It was found that the performance of MFCs was improved when graphite felt was used instead of graphite rod, suggesting that the performance of MFCs could be enhanced by increasing the surface area of electrode (Chaudhuri and Lovley, 2003). Graphite fiber brush could further improve the surface area of electrode. In a work, the use of graphite fiber brush as anode increased the power density 3 times (Logan et al., 2007). Despite the fact that carbon materials are competitive for lab-scale experiments, the relatively low mechanical strength and electrical conductivity of them restrict their application at practical applications (Liu et al., 2005). Recently, the development of biomass-derived black carbon (biochar) as a sustainable electrode material for MFCs enables a promising environmentally friendly and cost-effective choice of electrode materials. The cost of biochar is about 10 times less than granular activated carbon and graphite granules (Huggins et al., 2014; Yuan et al., 2013).

Metal materials, such as platinum, gold, and platinum, etc., have the advantages of high mechanical strength and good electrical conductivity.

Notwithstanding, they are much more expensive than carbon-based materials. Furthermore, the biocompatibility of metal materials needs further improvement since the smoothness of metal materials restricts the adhesion of microorganisms (Guo et al., 2015; Hernández-Fernández et al., 2015; Zhou et al., 2011).

Composite materials are a combination of different materials, such as conductive polymers, carbon and metal materials, having the advantages of diverse materials. Graphene-based composite materials have excellent conductivity, high surface area and good biocompatibility, being a good choice for MFC electrodes. Using a combination of 3D structures of graphene and conductive polymer polyaniline resulted in a maximum power density of 768 mW/m² (Yong et al., 2012). Another type of new composite material utilized in MFC systems is polymeric-metals, such as a combination of platinum and polyaniline (Niessen et al., 2004; Schröder et al., 2003). Nanomaterials, such as nanotubes, have also been used in composite materials (Higgins et al., 2011; Qiao et al., 2007). However, the biocompatibility of these materials needs to be taken into consideration since nanomaterials might affect the activity of microorganisms.

The cathode materials are also very important. The most commonly used cathode materials includes carbon cloth, carbon paper and graphite (Guo et al., 2015; Hernández-Fernández et al., 2015; Zhou et al., 2011). Chemical catholyte, especially ferricyanide, has been extensively used in lab for anode study because of its small overpotential. But the unsustainability of ferricyanide restricts its practical application. Air-cathode, where oxygen is the final electron acceptor, requires an effective catalyst for oxygen reduction reaction. Platinum is usually used as the catalysis. But the cost of noble metal and pH change in air-cathode recede its application. The recent development of percarbonate as sustainable catholyte enabled comparable MFCs performance with potassium catholyte and air-cathode, which makes it a good choice for catholyte (Forrestal et al., 2014).

2.3.2 Membrane

Ion exchange membrane, being closely related with the cost and performance of MFCs, affecting the internal resistance, substrate loss, oxygen diffusion and biofouling, is an important factor in MFC systems. Proton exchange membranes (PEMs) are widely used in MFC systems. DuPontTM Nafion® membranes, as a widely used commercial materials in MFCs to separate anolyte and catholyte, are perfluorinated ionomer membranes, which has high proton conductivity. Notwithstanding, Nafion® membranes not only allow protons to pass through, but also allow cation species, such as K^+ , Na^+ , Mg^{2+} or NH_4^+ , to go through. Due to consumption of protons in cathode reaction and accumulation of cation species, the pH in cathode chamber increases as a consequence, leading to the drop of cathode potential, thus impairing the performance of MFCs. Furthermore, electrochemical activity of microorganisms is usually undermined by a decrease of anode pH (Rozendal et al., 2006). The thermodynamic potential of MFCs is impaired by pH gradients (Gil et al., 2003). In addition, the relatively high cost of Nafion® membranes restrict its adoption in large-scale applications. Other types of membrane, such as anion-exchange membranes (AEMs), cation-exchange membranes (CEMs), ultracentrifugation membranes (UCMs) and bipolar membranes (BPMs), were also tested in MFCs (Kim et al., 2007; Rozendal et al., 2007; ter Heijne et al., 2006). In a study, researchers compared the performance of acetate-fed air-cathode MFCs using different types of membrane, such as Nafion®-based PEM, CEM, AEM and UCM. The MFCs with AEM produced higher power density than others (Kim et al., 2007).

2.3.3 Architecture

Various range of MFC architectures have been applied in lab-scale works. There are several key parameters of MFC architecture, such as the surface area of electrodes, the separator used and the distance between anode and cathode. Two-chamber configuration is a typical MFC architecture, including an anodic and cathodic chamber separated by a membrane, usually proton exchange

membrane (Bergel et al., 2005; Bond et al., 2002; Min et al., 2005; Oh et al., 2004). The traditional H-configuration usually includes two bottles, which are connected via a tube with a separator inside. H-shape MFCs are usually used for research regarding the basic parameters. However, the power density of these systems usually remains low due to the limited surface area of the membrane and long distance between electrodes. Notwithstanding, the greatest challenge related to two-chamber MFCs is the scaling-up in practical applications.

Single-chamber architecture, only having an anodic chamber, offers an alternative configuration. The cathode of single-chamber MFC keep exposed in air. It was found that using air-cathode instead of aqueous-cathode results in larger power densities. A MFC set-up using an air-cathode and a graphite anode was developed, suggesting that an open-air cathode has the potential for practical implementation (Rabaey et al., 2005c). To avoid liquid leaking to the cathode, a membrane can be used in air-cathode systems. It also helps to prevent oxygen diffusing into the anode chamber (Oh and Logan, 2006). Novel MFC architecture enables these devices to open up novel applications. A single-chamber MFC without membrane was developed to generate energy for implantable medical devices (Liu and Logan, 2004).

2.4 Diverse applications based on bioelectrochemical systems

2.4.1 Microbial fuel cells for wastewater treatment

The demonstration that MFCs can combine wastewater treatment and electricity harvesting drew people's attention in this field (Bond et al., 2002). Since then, studies on using different kinds of waste streams, from brewery, paper recycling, agricultural, food processing, refinery and municipal wastewater, as substrates with various separator and electrode materials and reactor architecture have mushroomed (Pant et al., 2010). The energy stored in wastewater is estimated 2-4 folds of that used for wastewater treatment. So it is

feasible to sustain wastewater treatment system by converting the energy consumption process to energy gaining process by integrating large-scale MFC system into wastewater treatment process, though the efficiency and scale up remain as a major challenge. These are several advantages of combining MFC with wastewater treatment. First of all, using MFCs in wastewater treatment saves energy by elimination of aeration. The aeration in traditional activated sludge system consumes about 45–75 % of total energy consumption. A work compared the effect of raw wastewater treatment by an aeration reactor and a MFC in lab-scale. It was found that both reactors were capable of reducing chemical oxygen demand (COD) from 1100 to 30 mg/L. Notwithstanding, a maximum power density of 168W/m^3 was produced the MFC, while an average 2.1 kWh/m^3 electricity was consumed by the aeration reactor (Huggins et al., 2013). The lab-scale MFCs was reported to produce a maximum power density as high as 2.87 kW/m^3 , being promising for practical application (Fan et al., 2012). In addition, MFC systems can save the cost for sludge treatment since they produce low amount of biomass. In traditional wastewater treatment process, sludge treatment costs up to 60% of the total cost. Utilizing MFC in wastewater treatment can reduce 50-70% of sludge production, which in turn reduce the plant operation cost (Wang and Ren, 2013).

2.4.2 Microbial electrolysis cells for hydrogen production

Microbial electrolysis cells (MECs) generate hydrogen gas in cathode by adding an external voltage on top of the potential produced by MFCs. In traditional MFCs, exoelectrogens grow on the anode and oxidize organic substrates, producing CO_2 , electrons and protons. The electrons are finally transferred to the cathode via first through anode, and the protons are transferred to the cathode through a separator. If oxygen is present in the cathode chamber, the electrons will combine with oxygen and produce water. A small voltage can be added externally in MECs, allowing hydrogen gas production by reducing protons at the cathode (Liu et al., 2005; Logan et al., 2008). The external voltage usually ranges from 0.6 to 0.8V, with lowest

external voltage being 0.2 V confirmed by several studies, which is much less than the voltage used in traditional water electrolysis (1.8 to 2.0 V). In addition, waste and renewable materials can be used as substrates, and the hydrogen gas yield can be as high as 11 mol H₂/mol glucose and production rate can be higher than 1 m³/day/m³ reactor, which is more than 4 folds of that produced in dark fermentation (Liu et al., 2010a; Logan et al., 2008).

Hydrogen gas generation rate was significantly increased by converting double-chamber architecture to single chamber MECs. However, it was found that the produced hydrogen was likely used to produce methane by methanogenesis (Liu et al., 2010; Logan et al., 2008). Various methods, such as controlling the redox potentials and pH, adding methanogen inhibitors, and periodically exposing solution in air, to inhibit methane production, but the contamination of methane still remains as a major challenge of single-chamber MECs (Liu et al., 2010a; Logan et al., 2008). A process called microbial reverse-electrodialysis electrolysis cell was recently established by combining the energy from oxidation of organic matter by exoelectrogens on the anode and the salinity gradient between river water and seawater. (Kim and Logan 2011a).

Other kinds of inorganic chemicals were found to be generated in the cathode chamber via using similar principles of MECs. Phosphate was found to be recovered as struvite in a modified microbial electrolysis cell (Cusick et al., 2012). Hydrogen peroxide and alkaline solutions were also produced by applying an external voltage (Rabaey and Rozendal, 2010).

2.4.3 Microbial electrosynthesis for chemical production

Microbial electrosynthesis (MES) is an emerging microbial electrochemical technology, using cathode derived electrons to reduce CO₂ or other chemicals to produce a wide range of organic compounds, especially some precursors for high value chemicals (Lovley and Nevin, 2011; Rabaey and Rozendal, 2010). There are several advantages of MES. First of all, MES is capable of carbon

sequestration and produce organic compounds at the same time. Furthermore, since MES can be driven by any kind of electricity, it also has the potential to address the problems related to storage, and distribution renewable but intermittent forms of energy, such as wind and solar, by converting them into desirable fuels or chemicals. And the efficiency of converting solar energy into organic chemicals by microbial electrosynthesis is about 100 times of that by plants. Uncomplicated organic reactions, such as one-step reduction or oxidation reactions may utilize chemical catalysts. There are several works reported to produce chemicals by electrochemical synthesis (Lovley and Nevin, 2011). However, microbial cathodes can achieve complex reactions preferably.

The initial idea of MES was brought up with the finding that methane was produced from a biocathode predominated by *Methanobacterium palustre* (Cheng et al., 2009). It was found that biofilms of *Sporomusa ovata* was capable of producing acetate and a small amount of 2-oxobutyrate by reducing carbon dioxide with the electrons derived from cathode (Nevin et al., 2010). Several acetogenic bacteria, such as *Sporomusa sphaeroides*, *Moorella thermoacetica*, *Clostridium aceticum*, and *Clostridium ljungdahlii*, were found to be capable of producing organic acids by using electrical current (Nevin et al., 2011). It was reported that acetate could be converted to ethanol by microbial communities in the cathode (Steinbusch et al., 2009). The microbial communities from brewery wastewater was demonstrated to utilize a posed potential to produce acetate, methane and hydrogen gas with carbon dioxide severing as the solo carbon source (Marshall et al., 2012) (Marshall et al., 2012). Research on isolation of microbial strains and genetically engineered microorganisms may significantly facilitate microbial electrosynthesis. Generally speaking, MES has great potential to bring the breakthrough of microbial electrochemical technologies, however, there are also many challenges related to technological and economic problems need to be addressed to enable real implementation (Lovley and Nevin, 2011; Rabaey and Rozendal, 2010)

CHAPTER 3 ENHANCED *SHEWANELLA* BIOFILM PROMOTES BIOELECTRICITY GENERATION¹

3.1 Abstract

Electroactive biofilms play essential roles in determining the power output of microbial fuel cells (MFCs). To engineer the electroactive biofilm formation of *Shewanella oneidensis* MR-1, a model exoelectrogen, a c-di-GMP biosynthesis gene *ydeH* was herein heterologously overexpressed in *S. oneidensis* MR-1, constructing a engineered strain in which the expression of *ydeH* is under the control of IPTG-inducible promoter, and a strain in which *ydeH* is under the control of a constitutive promoter. Such engineered *Shewanella* strains had significantly enhanced biofilm formation and bioelectricity generation. The MFCs inoculated with these engineered strains accomplished a maximum power density of $167.6 \pm 3.6 \text{ mW/m}^2$, which was ~ 2.8 times of that achieved by the wild-type MR-1 ($61.0 \pm 1.9 \text{ mW/m}^2$). In addition, the engineered strains in the bioelectrochemical system at poised potential of 0.2 V vs. saturated calomel electrode (SCE) generated a stable current density of 1100 mA/m^2 , ~ 3.4 times of that by wild-type MR-1 (320 mA/m^2).

3.2 Introduction

Microbial electrochemical technologies, derived for microbial fuel cells (MFCs), serve as a diverse platform combining waste treatment and energy or chemical production utilizing microbial catalytic reactions (Du et al., 2007; Harnisch and Schroder, 2010; Logan and Rabaey, 2012; Wang and Ren, 2013). MFCs, as a green and sustainable technology enabling simultaneous wastewater treatment and bioelectricity harvest, have attracted extensive attention in recent decades (Logan, 2009; Rabaey and Verstraete, 2005). Exoelectrogens (*i.e.*,

¹ The findings described in CHAPTER 3 have been published. Liu, T., Yu, Y.Y., Deng, X.P., Ng, C.K., Cao, B., Wang, J.Y., Rice, S.A., Kjelleberg, S., Song, H., 2015. Enhanced *Shewanella* biofilm promotes bioelectricity generation. *Biotechnol. Bioeng.* 112, 2051-2059

electroactive bacteria) on anode surfaces are responsible for oxidizing organic compounds and transferring electrons to anodes, which determine the power output of MFCs. However, electricity power output of MFCs remains low, restricting its adoption in practical applications.

Electroactive biofilm is an important factor in determining power density output of MFCs. Understanding the impact of biofilms on electricity generation is of great help in the rational optimization of MFCs and the improvement of the efficiency of biological conversion processes in MFCs (Borole et al., 2011; Leang et al., 2013; Patil et al., 2013). *Shewanella oneidensis* MR-1 is an exoelectrogen being intensively studied to understand its mechanisms of extracellular electron transfer (EET) and related metabolisms (Coursolle et al., 2010; Flynn et al., 2012; Pinchuk et al., 2010; Shi et al., 2007). As a facultative anaerobic bacterium, biofilm formation of *S. oneidensis* MR-1 on MFCs' anodes under anaerobic conditions is usually less dense and thick in comparison to *Geobacter* (Liu and Bond, 2012; Malvankar and Lovley, 2012). Many researches had thus focused on developing a wide variety of anodic nanomaterial to facilitate *Shewanella* biofilm formation and enhance direct-contact based EET via outer-membrane c-type cytochromes. However, few studies were conducted on genetic engineering *Shewanella* to enhance its electroactive biofilm formation (Deng et al., 2010; Huang et al., 2011b; Peng et al., 2010a).

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), as a second messenger, was recently recognized to play central roles in determining bacterial biofilm formation (Chao et al., 2013; Jenal and Malone, 2006; McDougald et al., 2012; Rakshe et al., 2011; Ross et al., 1987; Thormann et al., 2006b). High level of intracellular c-di-GMP promotes the expression of adhesive matrix components, facilitating bacterial biofilm formation, while low-level of c-di-GMP is favorable for planktonic phenotype. Diguanilate cyclases (DGCs) catalyze the biosynthesis of c-di-GMP from two molecules of GTP (Hengge, 2009). Meanwhile, specific phosphodiesterases (PDEs) can hydrolyze c-di-GMP into 5'-phosphoguananylyl-(3'-5')-guanosine (pGpG), which is then split into two molecules of GMP (Hengge, 2009).

YdeH is a DGC originated from *Escherichia coli*, and is able to catalyze the biosynthesis of c-di-GMP (Zähringer et al., 2011). Herein, we constructed a plasmid pHG-ydeH_{inducible} (see its construction procedure in Materials and Methods, and Schematic S1), in which the expression of the *ydeH* gene is under the control of the isopropyl- β -D-1-thiogalactopyranoside (IPTG)-inducible promoter Ptac. This plasmid was then transferred into *S. oneidensis* MR-1, resulting in an engineered *Shewanella* strain HG1. We also constructed a *Shewanella* strain harboring a plasmid in which the expression of *ydeH* is under the control of a constitutive promoter. Thus, these engineered strains allowed us to study the relationship between c-di-GMP controlled electroactive biofilm formation and EET in *Shewanella*. These engineered *Shewanella* strains could significantly enhance biofilm formation and bioelectricity generation. The MFCs inoculated with these engineered strains could accomplish a maximum power density of $167.6 \pm 3.6 \text{ mW/m}^2$, which was ~ 2.8 times of that achieved by the wild-type MR-1 (Fig 3-1). The engineered strains in the bioelectrochemical system at poised potential could generate a stable current density of 1100 mA/m^2 , ~ 3.4 times of that by MR-1.

Figure 3-1 We overexpressed the *ydeH* gene (encoding a diguanylate cyclase to synthesize c-di-GMP) in *Shewanella oneidensis* MR-1, which dramatically enhanced its biofilm formation and electroactivity. The MFCs inoculated with these engineered strains accomplished a maximum power density of 167.6 ± 3.6 mW/m², which was ~ 2.8 times of that achieved by the wild-type MR-1 (61.0 ± 1.9 mW/m²).

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids, growth conditions and media

All bacterial strains and plasmids used in this study were listed in Table 3-1. For genetic manipulation, *Escherichia coli* DH5 α and WM3064 were grown in Luria-Bertani (LB) medium at 37 °C, and *S. oneidensis* strains were grown in LB medium at 30 °C (Learman et al., 2009). Whenever required, the medium was supplemented with 50 μ g/ml kanamycin. 0.3 mM 2,6-Diaminopimelic acid (DPA) was added in *E. coli* WM3064 culture. Gene induction of the *S.*

oneidensis strain HG1 (harboring the pHG-ydeH_{inducible} plasmid) was achieved by the addition of 1 mM IPTG.

Table 3-1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Bacterial strain		
<i>Escherichia coli</i>		
DH5 α	F ⁻ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rK ⁻ , mK ⁺) <i>phoA supE44 λ-thi-1 gyrA96 relA1</i>	Invitrogen
WM3064	Donor strain for conjugation; <i>AdapA</i>	(Learman et al., 2009)
<i>Shewanella oneidensis</i>		
MR-1	Wild-type	(Venkateswaran et al., 1999)
HG1	MR-1 harbouring the plasmid pHG-ydeH _{inducible} ; Km ^r	This study
HG2	MR-1 harbouring the plasmid pHG-ydeH _{constitutive} ; Km ^r	This study
Plasmid		
pHG101	Vector (without promoter) derived from the broad host range vector pBBR1MCS-2; Km ^r	(Wu et al., 2011)
pHG102	pHG101 containing the <i>arcA</i> promoter	(Wu et al., 2011)
pHG-ydeH _{inducible}	<i>ydeH</i> under control of <i>lacI^q</i> -P _{tac} in pHG101; Km ^r	This study
pHG-ydeH _{constitutive}	<i>ydeH</i> under control of <i>arcA</i> promoter in pHG 102; Km ^r	This study

The strains used for MFCs' inoculation were firstly activated overnight in 5 ml LB broth medium after taken out from -80 °C fridge. Then the strains were cultured in 100 ml LB broth medium. After aerobically shaking for 8 hours at 30 °C, the cultures were centrifuged at 5000 rpm for 7 minutes. The cell pellets were washed with PBS, and inoculated into the nitrogen bubbled (5 minutes) MFC electrolyte (95% M9 salts and 5% LB broth, supplemented with 18 mM sodium lactate).

3.3.2 Construction of engineered strains of *S. oneidensis*

All genetic manipulations were carried out according to the standard protocols (Sambrook and Russell, 2001). Kits for purification of plasmids and PCR fragments were purchased from Invitrogen. DNA polymerase was

obtained from Kapa Biosynthesis, and all other enzymes were purchased from New England Biolabs.

The plasmid pHG-ydeH_{inducible} was constructed as shown in Fig 3-2. The P_{lacI^{q1}}-lacI^{q1}-P_{tac}-ydeH fragment sequence was designed according to the National Center for Biotechnology Information, and synthesized by GenScript (Glascok and Weickert, 1998; Zähringer et al., 2011). Two restriction sites, KpnI and BamHI, were introduced at the 5' and 3' ends, respectively. After being digested by the two restriction enzymes (KpnI and BamHI), the fragment and vector pHG101 were ligated to construct the plasmid pHG-ydeH_{inducible} (Wu et al., 2011). The plasmids pHG-ydeH_{inducible} was then amplified in *E. coli* DH5α and confirmed by sequencing.

Similarly, the plasmid pHG-ydeH_{constitutive} was constructed by ligating ydeH fragment and the digested plasmid pHG102, which has P_{arcA} promoter (Schematic S1B). The plasmids pHG-ydeH_{constitutive} were then amplified in *E. coli* DH5α and confirmed by sequencing.

Figure 3-2 Genetic construction of the two plasmids: pHG-ydeH_{inducible} (i.e., pHG-ydeH1) and pHG-ydeH_{constitutive} (i.e., pHG-ydeH2). Km^r is the kanamycin resistance gene; rep is replication origin; mob is mobilization gene. In pHG-ydeH1, LacI^{q1}-P_{tac}-ydeH represents an inserted sequence, in which ydeH gene is under the control of an IPTG-inducible promoter P_{tac}. In pHG-ydeH2, ydeH gene is under the control of a constitutive promoter P_{arcA}.

The procedure in transforming the plasmid into *S. oneidensis* MR-1 was as following. The constructed vector was firstly transformed into *E. coli* WM3064. 1 ml donor cell (*E. coli* WM3064 with plasmid, with kanamycin and DPA), and 1 ml *S. oneidensis* MR-1 (without antibiotics) was cultured overnight,

respectively. Then 250 μ l *E. coli* WM3064 was centrifuged to remove the supernatant, washed once with 500 μ l LB, and re-suspended with the 150 μ l culture of *S. oneidensis* MR-1. Then, the mixture of the donor *E. coli* WM3064 cell and *S. oneidensis* MR-1 cell was spread onto an LB DPA plate (without kanamycin), and cultured for 8 hours at 30 °C. A wad of these cells was taken to streak onto a LB plate (with kanamycin, but without DPA), being cultured for 16 hours at 30 °C. Single colonies were then selected and screened by re-streaking on LB plates with antibiotics (Learman et al., 2009). Transforming the plasmid pHG-ydeH_{inducible} into wild-type *S. oneidensis* MR-1 strain led to the construction of the engineered strain HG1, in which the *ydeH* gene was under the control of an IPTG-inducible promoter P_{tac}. Transforming the plasmid pHG-ydeH_{constitutive} into wild-type *S. oneidensis* MR-1 strain led to the construction of the engineered strain HG2, in which the *ydeH* gene was under the control of a constitutive promoter P_{arcA} (i.e., no IPTG induction is needed for the expression of *ydeH* gene). The plasmid DNA sequence was further confirmed by sequencing after being extracted and purified from the strains HG1 and HG2, respectively.

3.3.3 Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was purified from the mid-log phase bacterial cultures using Purelink RNA Mini Kit (Invitrogen), according to the protocol of the manufacturer. Quantitative analyses of gene expression of *ydeH* were achieved using Power SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems). Gene *gyrB* was used for normalization. Samples were analyzed in triplicate using the listed primers (Table 3-2). Data was analyzed using the 2^{(-Delta Delta C(T))} method (Livak and Schmittgen, 2001).

Table 3-2 Primers used in RT-qPCR

qPCR primer name	Sequence (5' to 3')
ydeH-F	CCATCACACATTCTGAAGGGCATA
ydeH-R	GCTCTTCCAATGACCACATCCA
gyrB-F	GGAACGACGGCTACCAAGA

3.3.4 MFCs setup and Operations

Two-chamber MFCs with an inner size of 4.5 cm x 4 cm x 4 cm for each chamber were used in our experiments. Both anode and cathode have effective volume of 50 ml. The two chambers were separated by a proton exchange membrane (Nafion 117, from Gas Hub, Singapore). These proton exchange membranes were first immersed into 0.5 M sulfuric acid overnight and washed carefully with autoclaved water before use. Carbon cloth (from Gas Hub, Singapore) with the size of 2 cm x 2.5 cm was used for both anode and cathode. Cathode solution consists of 50 mM $K_3[Fe(CN)_6]$ and 50mM K_2HPO_4 and KH_2PO_4 . After inoculation with cells, the MFCs were continuously discharged under a constant loading of 2 k Ω . A digital multimeter (ESCORT 3146A) was used to record the output voltage. Once the output voltage was dropped under 50 mV, the anolyte was changed with fresh medium. Meanwhile, the catholyte was refreshed to guarantee a stable cathode performance.

S. oneidensis MR-1, HG1 and HG2 was used as the anode respiration bacteria, respectively. MFCs utilizing *S. oneidensis* MR-1 adding IPTG and HG1 without IPTG as inoculation were also tested in MFCs to eliminate the possible influence of IPTG and the background expression of *ydeH*. Triplicate MFCs were conducted for each experiment to guarantee our results highly repeatable.

3.3.5 Electrochemistry characterization

Polarization curves were measured by changing the external resistance at highest output voltage. A three-electrode system was used to perform the cyclic voltammetry (CV) analysis, where SCE was used as the reference electrode (+0.243 V vs. SHE), and the MFC's cathode as the counter electrode. The CV curves (at the scanning rate of 50 mV/s) were performed when the carbon source was exhausted (*i.e.*, non-turnover), while the CV curves (at a scanning

rate of 1 mV/s) were performed at turnover condition in order to study the bio-electrocatalytic behavior of the biofilms. CV curves taken before and after changing anolyte were also scanned at 1 mV/s.

Carbon cloth electrodes with pre-formed mature biofilm were used in measuring the current under poised-potential conditions. The experimental procedures were as following. Firstly, the carbon cloth electrodes were immersed in 100 mL inoculated LB medium (at aerobic conditions) to form biofilm for 24 h. Subsequently, the electrodes were then put in the MFC anodic chamber to continuously discharge in a three-electrode system (0 V *vs.* SCE) for five days. Finally, 18 mM lactate was added to the anode chamber of the MFCs. The current discharge process at poised potential of -0.2 V, 0 V and 0.2 V *vs.* SCE, were operated for 6000 seconds, respectively.

Linear scanning analysis of the MFC cathode was done at the beginning and the end of a MFC operation cycle. The MFCs' cathode, anode and the same SCE were used as working electrode, counter electrode, and reference electrode, respectively. The scanning range was from +0.7 V to -0.3 V *vs.* SCE with a scanning rate of 1 mV/s.

3.3.6 Biofilm characterization

Growth and analysis of static biofilm at aerobic conditions was performed as previously described (Merritt et al., 2005). Stationary phase cultures were diluted 1:100 in LB medium and 100 μ L of each diluted culture was transferred to each well of 96-well plate and then cultured at 30°C for 8h, 16h, 24h and 48h. Planktonic bacteria were removed after culture. 125 μ L of 0.1% crystal violet was added to each well and incubated for 10 minutes. The plates were washed 10 times to remove unstained crystal violet and dried. To solubilize the crystal violet in biofilm, 200 μ L 95% ethanol was added to each well and incubated 10 min at room temperature. 100 μ L of the crystal violet/ethanol solution from each well was transferred to a new plate accordingly. The optical density of each well was measured a wavelength of 595 nm.

Biofilms grown on the carbon cloth were observed by field emission scanning electron microscope (FESEM, JSM-6700F-FE-SEM, Japan). The sample preparation process was same with our former works (Yong et al., 2013b). In brief, a small piece of carbon cloth (2 mm x 2 mm) was cut from the anode and dipped in 1% Glutaraldehyde overnight. 0.85% NaCl solution was then used to wash the sample for three times. The samples were then rinsed with ethanol solutions in the sequence of concentrations of 30%, 50%, 70%, 80%, 90% and pure ethanol. Every step took about 15 minutes.

The quantification of biomass of the biofilms grown on carbon cloth electrodes was performed using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes). A small piece of cut off carbon cloth was washed with PBS to remove planktonic bacteria, and sonicated in 400 μ L PBS buffer for 8 seconds. The bacterial suspension was then stained by the Kit according to the recommendation of manufacturer and transferred to a 96-well plate. The fluorescence intensity was measured at a wavelength centered at 540 nm with the excitation wavelength of 485 nm. Complete removal of cells on carbon cloth was verified by SEM images of carbon cloth before and after sonication.

3.3.7 Quantification of riboflavin

Centrifuged (35,000g for 20 min) and filtered (0.4 μ m) samples were analyzed by using a Waters XBridge C8 column (2.1 * 100 mm; particle size, 3.5 μ m). Eluted compounds were detected by liquid chromatograph-tandem mass spectrometer (LC-MS/MS) (Shimadzu LCMS-8030) in positive ion mode.

3.4 Results and discussion

3.4.1 The expression of *ydeH* gene in *Shewanella* enhanced biofilm formation

To analyse the expression of *ydeH* in the engineered *Shewanella* strain HG1 induced by different concentrations of IPTG, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) technology was used to

quantify the *ydeH* cDNA abundance. The expression level of *ydeH* was enhanced with the increased IPTG concentration and reached the saturation level at 1mM IPTG (Fig. 3-3A). In particular, the *ydeH* cDNA abundance in HG1 with full IPTG induction ([IPTG]=1 or 10 mM) was ~ 15 times of its basal expression (in the absence of IPTG). Static biofilm analysis under aerobic conditions was further conducted to confirm that the *ydeH* expression would enhance biofilm formation (Fig. 3-3B). No obvious differences in the biomass of static biofilm between the wild-type strain MR-1 and HG1 (in the absence of IPTG induction) were observed. However, the biomass of the biofilm formed by the induced HG1 (in particular, with 1 mM IPTG induction) was significantly enhanced in comparison to MR-1 (Fig. 3-3B), *i.e.*, ~5.3 times' increase (after 24 hours) in biofilm mass was achieved under aerobic conditions upon the expression of *ydeH*.

Figure 3-3 Characterization of the *ydeH* gene' expression level and the biomass of biofilm formed by engineered *Shewanella* strain HG1 and HG2. (A) The relative *ydeH* mRNA abundance of HG1 upon induction by different IPTG concentrations and HG2. The control is the *ydeH* cDNA's basal expression in HG1 (in the absence of IPTG). The error bars were calculated from triplicate experiments. (B) The relative biofilm biomass of MR-1, HG1 upon induction by different IPTG concentrations, and HG2 at 8, 16, 24 and 48 hours in the static biofilm analysis under aerobic conditions, respectively. The control is the biofilm biomass of MR-1 (after 8 hours' incubation upon inoculation). The error bars were calculated from four parallel experiments.

To eliminate possible interferences of IPTG on cell physiology and extracellular electron transfer, we further constructed a engineered *Shewanella* strain HG2. In HG2, the *ydeH* gene was put under the control of P_{arcA} promoter (a constitutive promoter), enabling a constitutive expression of *ydeH* in HG2 (*i.e.*, no inducer is required for the expression of *ydeH*). The expression level of

ydeH in HG2 was even higher than that in HG1 (even with 1 mM IPTG induction) under aerobic conditions (Fig. 3-3A). Furthermore, the biomass of static biofilm formed by HG2 was comparable to that of the induced HG1 (Fig. 3-3B).

To confirm that the expression of *ydeH* could enhance biofilm formation of *Shewanella* on the surfaces of MFC anodes, field emission scanning electron microscopy (FESEM) images of the biofilms were taken from MFCs' anodes after MFC operation (Fig. 3-4). Biofilm of the strain HG1 with 1 mM IPTG could cover almost the entire surface of the carbon cloth (Fig. 3-4B), while the wild-type MR-1 barely covered the anode surface (Fig. 3-4A). Similar to the MFCs inoculated with the strain HG1 (in the presence of 1 mM IPTG), the MFC with HG2 exhibited similar coverage of bacterial cells on the carbon fiber surfaces. To quantify the relative biofilm mass of MR-1 and the engineered strains, a small piece of cut off carbon cloth was washed with PBS to remove planktonic bacteria, and sonicated in 400 μ L PBS buffer for 8 seconds. The suspension was then analyzed with LIVE/DEAD BacLight Bacterial Viability Kit. The biofilm formed by the strain HG1 (upon 1mM IPTG induction) on anode surfaces was ~7.9 times of that formed by MR-1 (Fig. 3-4D). A 5.3 times' increase was observed in the strain HG2 strain in comparison to that of the strain MR-1 (Fig. 3-4D). The successful removal of bacteria from anode surface was confirmed by comparison the SEM images before and after sonication (Fig. 3-5).

Figure 3-4 Characterization of MFC's anodic biofilms after 479 hours' operation. (A) SEM image of the biofilm of the wild-type strain MR-1, (B) SEM image of the biofilm of the strain HG1 (with the induction of 1 mM IPTG), (C) SEM image of anode from MFCs with the strain HG2, and (D) the relative biofilm mass of MR-1, HG1 and HG2, respectively. To calculate the relative biofilm mass of the HG-1 strain, the MR-1 biofilm biomass formed on the anode of MFC was used as the control (designated as "1"). The error bars were calculated from triplicate experiments.

Figure 3-5 SEM images of anode carbon cloth from MFCs with (A) MR-1, (B) HG1 and (C) HG2. (D), (E) and (F) are SEM images of sonicated anode carbon cloth from MFCs with MR-1, HG1 and HG2, respectively.

3.4.2 Enhanced biofilm improved bioelectricity generation

To investigate their bioelectrochemical capability, MFCs with different strains as inoculum were set up and operated under batch mode. The induced HG1 (1 mM IPTG) produced a higher maximum voltage (~ 400 mV) in MFC than that of the wild-type MR-1 (~ 230 mV) (Fig. 3-6A). Also, the addition of 1 mM IPTG had no obvious influence on the electrochemical behavior of MR-1 (Fig. 3-7). The performance of MFCs inoculated with HG1 (without IPTG) did not show obvious difference from that of MR-1 (Fig. 3-7), indicating the basal expression of *ydeH* in HG1 had negligible impact on its electrochemical behavior. As our MFCs were operated at batch mode, the total electrons harvested during the first batch (*i.e.*, during the time period from the initiation of MFC to the first replenish of fresh medium upon exhaustion of the carbon source lactate) were the integration of the temporal profile of the voltage output curve in Fig. 3A and then dividing the external resistance (2 k Ω in this work). A charge of 86.7 coulombs (in 300 hours) was transferred to the anode during the first batch of MR-1-innoculated MFCs, while it was 63.4 coulombs (in 142 hours) for the induced HG1-innoculated MFCs. Thus the apparent Coulombic efficiency calculated from lactate were 8.3% and 6.0% for MR-1 and HG1 (assuming lactate is oxidised to CO₂ and H₂O, so theoretically 12 electrons can be harvest from 1 lactate), which is similar to former works (Biffinger et al., 2008; Biffinger et al., 2007). As the cycle duration was much shorter for HG1 compared with MR-1 (142 hours to 300 hours), it is reasonable to conclude that the engineered strain HG1 consumed nutrients much faster than the wild-type MR-1, which is an advantage for potential industrial application (Rabaey and Verstraete, 2005). The slight decrease in Coulombic efficiency in the HG1 strain is probably due to its increased biomass on the anode. Similar trend (*i.e.*, increased substrate consumption rate and bioelectricity generation with a decrease in coulombic efficiency) was observed in former study on the aeration

effect on the power production in *Shewanella* inoculated bioelectrochemical systems (Rosenbaum et al., 2010). However, Coulombic efficiency in the above work was much lower (average 2.72% in MFC mode) due to preferential electron transfer to oxygen rather than electrode, although under aeration condition *Shewanella* could theoretically metabolize lactate to CO₂, achieving 12 electrons.

Figure 3-6 Bioelectricity generation of the MFCs inoculated with wild-type strain MR-1 and engineered *Shewanella* strain HG1, respectively. (A) Output voltage vs. operation time of MFCs with the strain HG1 (with 1 mM IPTG), (B) Output voltage vs. operation time of MFCs with the strain HG2.

Figure 3-7 Output voltage vs. operation time of MFCs inoculated with the wild-type strain MR-1 (adding 1mM IPTG) and the strain HG1 (without IPTG), respectively.

Polarization curve was done at highest output voltage to further investigate the bioelectricity generation capability of MFCs with MR-1, induced HG1 and HG2 (Fig. 3-8). V-j curve reflects the potential drop with the increase of current density (with external resistance decrease). To further verify that cathode performance had less impact on our results, we did linear scanning of MFCs cathode at the beginning and the end of a batch cycle. From Fig. 3-9, we found that when the cathodic current density was smaller than 10 A/m^2 , the linear scanning curves of catholyte before and after the batch cycle almost overlapped. Considering that the maximum output current density was ~ 0.2 to 0.4 A/m^2 , we conclude that the cathode potential was stable throughout the entire experiment. As the cathode potential change was rather small in the current density range of polarization curve, the difference in the V-j curve is anode originated. Fast potential drop near open circuit potential were observed for MR-1 and induced HG1, indicating an activation loss. The difference in the linear part of V-j curve became obvious when potential was below 600 mV. MFCs with MR-1 had much faster potential drop (*i.e.*, from 576 to 60 mV when the current density increases from 104 to 246 mA/m^2) than that with induced HG1 (potential drop

from 598 to 379 mV when the current density increases from 108 to 450 mA/m²). This difference was probably due to the improved electron transfer of MFCs with induced HG1. Thus less overpotential was required for HG1 to accomplish a high current density, as analyzed in our former work (Yu et al., 2015). In addition, a sharp potential drop was observed in the V-j curve of HG1 when the potential was below 379 mV (Fig. 3B), indicating diffusion loss became serious at high current density (Fan et al., 2008). The estimated corresponding short-circuit current densities were 267 ± 4.6 mA/m² for MR-1, and 609 ± 6.9 mA/m² for the induced HG1. Calculated from P-j curve in Fig. 3-7, the maximum power density achieved by the induced HG1 was 167.6 ± 3.6 mW/m², which was 2.8 times of that achieved by the wild-type MR-1 (61.0 ± 1.9 mW/m²).

Figure 3-8 Polarization curves of MFCs with MR-1, HG1 (with 1 mM IPTG) and HG2; solid: output voltage vs. current density (V-j curve), hollow: power density vs. current density (P-j curve).

Figure 3-9 Linear scanning curve of the MFCs' cathode before (blue) and after (red) nutrient depletion in the MFC anodic chamber. Insert arrow indicates the current value and its corresponding cathode voltage at steady MFCs discharge.

The MFCs inoculated with the strain HG2 had similar MFCs performance with that of induced HG1. A maximum output voltage ~ 400 mV was achieved in the MFCs inoculated with HG2 (Fig. 3-6B). The maximum power density and short-circuit current density achieved by HG2 were 177 mW/m^2 and 659 mA/m^2 (based on the polarization curves), respectively, which were comparable to that of induced HG1 (Fig. 3-8).

Recent studies showed that oxygen is favored for *Shewanella* biofilm formation and its MFC performance (Biffinger et al., 2008; Biffinger et al., 2007; Biffinger et al., 2009; Wu et al., 2013). We incubated the carbon cloth electrodes in LB broth with *Shewanella* inoculum at aerobic conditions for 24 hours to form biofilm. Interestingly, under aerobic conditions, HG1 (with 1 mM IPTG) and HG2 formed much thicker biofilm than MR-1 on carbon cloth electrodes (Fig. 3-10). Furthermore, these carbon cloth electrodes with aerobically pre-formed biofilm were used to measure current outputs under poised-potential conditions in bioelectrochemical systems (BES) (Fig. 3-10). HG1 and HG2 all generated higher current output than MR-1 when the anode was poised at -0.2 V , 0 V and 0.2 V vs. SCE. For example, the BES (at poised

potential of 0.2 V vs. SCE) with HG1 generated a stable current density of $\sim 900 \text{ mA/m}^2$, which was ~ 2.8 times of that with MR-1 (320 mA/m^2) (Fig. 3-11). For HG2, the steady current density (when the anode potential was poised at 0.2 V vs. SCE) can be as high as 1100 mA/m^2 .

Figure 3-10 SEM images of carbon cloth aerobically incubated in LB medium inoculated with (A) the strain MR-1, (B) the strain HG1 induced by 1 mM IPTG and (C) the strain HG2. (D) Their relative biofilm mass. The control is the carbon cloth electrode cultured in LB broth inoculated with MR-1, and the error bars are calculated from triplicate experiments.

Figure 3-11 Current discharge curves of the bioelectrochemical system under poised-potential conditions, i.e., -0.2V, 0V and 0.2V (vs. SCE), for the three *Shewanella* strains (MR-1, HG1 and HG2), respectively.

3.4.3 Flavins-mediated and contact-based EET increased by enhanced biofilm

Cyclic voltammetry (CV) analysis could provide useful information on the mechanism of EET. For MR-1, one pair of peaks centered at -0.32 V vs. SCE was identified in the CV under non-turnover condition (50 mV/s, Fig. 3-12). This pair of peaks corresponded to the outer membrane *c*-type cytochromes, whose exact redox peak position varies upon microenvironment differences (Baron et al., 2009; Carmona-Martinez et al., 2011; Deng et al., 2010; Peng et al., 2010b). For the strain HG1 (with 1mM IPTG induction), one pair of peaks centered at -0.36 V vs. SCE showed up in the CV (Fig. 3-12).

Figure 3-12 Cyclic voltammetry (CV) characterization of the MFCs with inoculations of *Shewanella* strain MR-1 and HG1 (with 1mM IPTG induction) under non-turnover condition, respectively. The scanning rate of the CV curves was 50 mV/s. Inset is the baseline-corrected anodic curves of the CVs (from -0.45 V to -0.1 V).

The relationship between peak current density with scanning rate was analyzed to further determine the nature of this redox species in HG1 biofilm (Fig. 3-13A). The height of the base line-corrected peaks current (centered at ~ -0.36 V vs. SCE) was linearly related with the scanning rate ($R^2=0.999$) (Fig. 3-13B), indicating a contact-based redox species, *i.e.*, the outer-membrane cytochromes (Baron et al., 2009; Laviron, 1979). We also observed the height of anodic peaks was smaller than the cathodic peaks (Fig. 3-13B), indicating the redox reaction on electrodes was partially irreversible (Baron et al., 2009; Huang et al., 2011a; Peng et al., 2010a).

Figure 3-13 (A) CV curves at different scanning rates for the MFCs inoculated with the engineered strain HG1 (with 1 mM IPTG); (B) relationship between peak current densities and scanning rates.

To investigate how different EET pathways may contribute to the electrochemical behavior of MR-1 and induced HG1 biofilm, CV analysis was further performed with a slow scanning rate of 1 mV/s under turnover condition (Fig. 3-14). For both MR-1 and HG1 biofilms, the anodic curve arose firstly at -0.46 V vs. SCE, generating a flavins-mediated catalytic current according to previous researches (Fig. 3-14)(Marsili et al., 2008; von Canstein et al., 2008). This shuttle-mediated catalytic current was $\sim 80 \text{ mA/m}^2$ (for MR-1), and $\sim 140 \text{ mA/m}^2$ (for induced HG1). Riboflavin concentration in the anolyte were further

analyzed with LC-MS/MS. The result demonstrated that riboflavin concentration in MFCs with induced HG1 was only slightly higher than that with MR-1 (0.63 and 0.45 μM , respectively) (Fig. 3-16). The difference in the riboflavin concentration can only partially explain the enhanced MET, which would be further discussed in discussion part. Furthermore, another catalytic current arose at $\sim -0.32\text{ V vs. SCE}$ were observed both in MR-1 and induced HG1, which was caused by the direct involvement of *c*-type cytochromes in the EET (Fig. 3-14). The current densities increased $\sim 350\text{ mA/m}^2$ and $\sim 660\text{ mA/m}^2$ for MR1 and HG1, respectively, when the potential increased from -0.32 V to $+0.3\text{ V vs. SCE}$. The higher increase of current density by HG1 was caused by the higher amount of cytochromes involved in the EET (discussed in detail in Discussion). We thus concluded that both the flavins-mediated and contact-based EET pathways were increased due to enhanced biofilm formation in our engineered HG1 strain.

Fig. 3-15A is the CV curve of the MFC inoculated with the strain HG2 at non-turnover condition. Three anodic peaks can be defined as indicated by the blue arrows. Under turnover conditions, multiple catalytic behaviors were observed in a broad potential window (Fig. 3-15B). Flavin-mediated electron transfer process was observed at $\sim -0.47\text{ V}$, while a few outer-membrane *c*-type cytochromes-based electron transfer processes at more positive potentials were observed.

Figure 3-14 CV curves of the MFCs inoculated with the *Shewanella* strain MR-1 and HG1 (with 1mM IPTG induction) under turnover condition, respectively, with a scanning rate of 1 mV/s.

Figure 3-15 (A) Cyclic voltammetry (CV) curves of MFCs with HG2 at non-turnover condition (50 mV/s). (B) CV curve of MFCs with HG2 at turnover condition (1 mV/s).

Figure 3-16 Riboflavin concentration of anolyte with MR-1, HG1 (with 1mM IPTG) and HG2.

Gene *ydeH* encodes a diguanylate cyclase to catalyze biosynthesis of the ubiquitous second messenger c-di-GMP. It was found that c-di-GMP influences many processes related to biofilm formation and disperse, including exopolysaccharide biosynthesis, cell cycle progression, flagellar genes, motility and secretion system at the transcriptional, translational, post-transcriptional and post-translational levels.(Hengge, 2009; Jenal and Malone, 2006; McDougald et al., 2012) The process controlled by c-di-GMP is complex and not completely unraveled. Our results (Fig. 1B & Fig. 2) demonstrated that overexpressing c-di-GMP synthesis gene *ydeH* enhanced *Shewanella* biofilm formation both under MFCs and aerobic conditions.

The improved MFCs performance and total extracellular electron transfer efficiency can be confirmed from polarization curve and CV analysis. To further understand the benefit of enhanced biofilm formation on bioelectrochemical performance, we did baseline corrected CV curves under non-turnover condition (insert, Fig. 5). The peak current density of induced HG1 was much higher than MR-1, indicating larger amount of redox species were involved in the EET in induced HG1. According to the Laviron equation

$I_p = \frac{n^2 F^2 A v \Gamma}{4RT}$, where v and I_p are the scanning rate and corresponding peak current; n is the electron transfer number (here $n=1$); A and Γ are the electrode area, outer-membrane cytochrome density (mole in unit area); and F , R and T are Faraday's constant, gas constant and experiment temperature, respectively, the average density (or activity) of the outer membrane cytochromes on the MFC anode with induced HG1 was calculated to be $1.79 \mu\text{mol}/\text{m}^2$ (based on anodic peak current density, insert in Fig. 5), which was 3.5 times of that of MR-1 ($0.51 \mu\text{mol}/\text{m}^2$) (Laviron, 1979). Thus we can conclude that the direct benefit of enhanced biofilm formation was that more OM cytochromes were involved in EET, explaining the improved MFC performance and enhanced DET based catalytic current form.

The observed increased OM cytochrome also explains why the flavins-mediated catalytic current was significantly enhanced (140 to $80 \text{ mA}/\text{m}^2$) while the riboflavin concentration was similar. As we known, flavins-mediated electron transfer mainly consists the cycle of reduce by the OM cytochrome and re-oxidize at anode (Brutinel and Gralnick, 2012; Coursolle et al., 2010). The fact that OM cytochromes (above -0.3 V vs. SCE) have more positive standard redox potential than flavins ($\sim -0.45 \text{ V vs. SCE}$), and low flavins concentration limit the electron transfer efficiency from reduced OM cytochromes to flvins. Direct adding riboflavin to anolyte has been proved to be an effective strategy to improve *Shewanella* EET efficiency (Baron et al., 2009). Increase the OM cytochrome amount on the electrode surface could have the same effect to improve the electron transfer efficiency from dynamics aspect. This calculated outer-membrane cytochromes density of induced HG1 is similar to a former work to modify anode with $\text{Au}/\text{Fe}_2\text{O}_3$ nanocomposite (Deng et al., 2010). This indicates that a potential combination of nanomaterial and genetic engineered bacteria to further enhance bioelectricity output.

3.5 Conclusions

In conclusion, heterologous expression of c-di-GMP biosynthesis gene *ydeH* in engineered *S. oneidensis* strains HG1 (IPTG-inducible expression of

ydeH) and HG2 (constitutive expression of *ydeH*) was accomplished in this work, leading to a significant enhancement in the biofilm formation of *Shewanella*. The maximum output voltages accomplished by MFCs inoculated with HG1 (with 1 mM IPTG induction) and HG2 were ~2.8 times of that inoculated with wild-type MR-1, which is caused by higher levels of bacterial cells in anode biofilm, and thus higher levels of cytochromes involved in the EET. Genetic engineering of exoelectrogens is a promising approach to enhance the efficiency of microbial bioelectrochemical systems and MFCs. A combination of engineered exoelectrogens with novel biocompatible electrode materials would be intriguing for further increasing the performance of MFCs and other microbial electrochemical systems.

CHAPTER 4 A SYNTHETIC MICROBIAL CONSORTIUM OF SHEWANELLA AND BACILLUS TO BOOST BIOELECTRICITY GENERATION²

4.1 Abstract

In this study, a synthetic microbial consortium containing exoelectrogen *Shewanella oneidensis* MR-1 and riboflavin producing strain, *Bacillus subtilis* RH33, was rationally designed and successfully constructed, enabling a stable, multiple cycles of MFC operations for more than 500 hours. The maximum power density of MFCs with this synthetic microbial consortium was 277.4 mW/m², which was 4.9 times of that with MR-1 (56.9 mW/m²) and 40.2 times of RH33 (6.9 mW/m²), separately. At the same time, the Coulombic efficiency of the synthetic microbial consortium (5.6%) was higher than MR-1 (4.1%) and RH33 (2.3%). Regardless the high concentration of riboflavin produced by RH33, the power density of RH33 was rather low and the low bioelectricity generation with RH33 inoculum can be ascribed to low efficiency in utilizing riboflavin for electron transfer. In the mixed-culture of MR-1 and RH33, it was found that both mediated and direct electron transfer efficiency were enhanced in mixed-culture. By exchanging the anolyte of MR-1 and RH33, it was confirmed that the improved MFC performance with the synthetic microbial consortium was because MR-1 could efficiently utilize the high concentration of riboflavin produced by RH33.

4.2 Introduction

Microbial electrochemical technologies, originated from microbial fuel cells (MFCs), are considered to be a seminal platform since they enable combination of waste treatment and energy recovery (Du et al., 2007; Franks and Nevin, 2010; Harnisch and Schroder, 2010; Logan and Rabaey, 2012; Rabaey and

² The findings described in CHAPTER 4 have been submitted. Liu, T., Yu, Y.Y., Chen, T., and Chen, W., A synthetic microbial consortium of *Shewanella* and *Bacillus* for enhanced generation bioelectricity. *Under review*.

Verstraete, 2005; Wang et al., 2015). Electroactive bacteria, termed exoelectrogens, are capable of oxidizing organic compounds and transferring electrons to electrode to generate electricity. *Shewanella oneidensis* MR-1 (*S. oneidensis* MR-1) attracts vast interest due to its multiple extracellular electron transfer (EET) mechanisms. There are two distinct EET mechanisms, which are not mutually exclusive, have been extensively studied for MR-1. One direct way to transfer electrons to the electrode is to via Mtr respiratory pathway. Five primary protein components: OmcA, MtrA, MtrB, MtrC and CymA have been identified in Mtr pathway, in which OmcA and MtrC are outer-membrane (OM) c-type cytochromes, contacting the electrode directly. Transferring electrons by electron shuttles (flavins for MR-1), which undergo redox cycling between the electrode and the cell, is an alternative way to direct-contact pathway.

Riboflavin and riboflavin-5'-phosphate (FMN) were found to be secreted by MR-1 to mediate EET, with riboflavin as the dominant component at biofilm mode (Marsili et al., 2008; von Canstein et al., 2008). It was found that proteins in the Mtr pathway, especially OM c-type cytochromes MtrC and OmcA, were essential for the activity of flavins-based electron shuttling (Coursolle et al., 2010; Okamoto et al., 2013). By analyzing a strain unable to secrete flavins, flavins-based electron shuttling plays a majority role in EET ability of MR-1 (Kotloski and Gralnick, 2013). Removal of flavins resulted in severe defect of EET of MR-1 and vice versa (Baron et al., 2009; Okamoto et al., 2013; Yong et al., 2013b). The relatively low concentration of secreted flavins restricted the EET efficiency of MR-1. It was an effective way to improve the EET efficiency of *Shewanella* by adding riboflavin to anolyte directly (Baron et al. 2009).

In this study, a synthetic microbial consortium, in which high concentration of riboflavin produced by *Bacillus subtilis* RH33 can be utilized by *S. oneidensis* MR-1 to boost bioelectricity generation, was rationally designed and successfully constructed (Fig. 4-1). *B. subtilis* RH33 is a genetically modified strain by genome shuffling and DNA recombination technologies, in which the riboflavin production can be as high as 12g/L in fed-batch fermentation (Chen et al., 2007). The maximum power density of MFCs with this synthetic

microbial consortium was 277.4 mW/m^2 , which was 4.9 times of that by MR-1 (56.9 mW/m^2) and 40.2 times of that by RH33 (6.9 mW/m^2), respectively. This microbial consortium enabled a stable, multiple cycles of MFC operations for more than 500 hours.

Figure 4-1 Schematic of a synthetic microbial consortium, in which high concentration of riboflavin produced by *Bacillus subtilis* RH33 can be utilized by *Shewanella oneidensis* MR-1 to enhance extracellular electron transfer. The maximum power density of MFCs with this synthetic microbial consortium was 277.4 mW/m^2 , which was 4.9 times of that by MR-1 (56.9 mW/m^2) and 40.2 times of that by RH33 (6.9 mW/m^2), respectively.

4.3 Materials and methods

4.3.1 MFCs setup and operation

S. oneidensis MR-1 and *B. subtilis* RH33 were first activated overnight in Lysogeny Broth (LB) medium after taken out from -80°C fridge. Then the strains were cultured in 100 ml LB medium, shaking at 220 rpm for 8 hours at 30°C . To remove the supernatant, the culture was centrifuged at $3000 \times g$ for 7 minutes. The cell pellets were dispersed into nitrogen bubbled (for 5 minutes) MFC anolyte after being washed with phosphate-buffered saline (PBS). Every 1 liter of anolyte contains 30 ml 1 M HEPES, 0.46 g NH_4Cl , 0.225 g K_2HPO_4 , 0.225 g KH_2PO_4 , 0.117 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.225 g $(\text{NH}_4)_2\text{SO}_4$, 10.8 g NaOH, 0.056 g CaCl_2 , 10 ml Wolfe's vitamin, 10 ml Wolfe's mineral, 200 μg L-arginine HCl, 200 μg L-glutamine, 400 μg DL-serine, and 18 mM sodium

lactate as carbon source. Catholyte consists of 50 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 50 mM K_2HPO_4 and KH_2PO_4 .

Sterilized proton exchange membrane (Nafion 117, from Gas Hub, Singapore) was used to separate the two chambers of MFC, with the inner size of 4.5 cm x 4 cm x 4 cm for each chamber. Carbon cloth (from Gas Hub, Singapore) with the size of 2 cm x 2.5 cm was used as both anode and cathode. After MFCs set-up, the MFCs were continuously discharged under external resistances (2 k Ω). Triplicate MFCs were conducted for each inoculum to guarantee the results highly repeatable. A digital multimeter (ESCORT 3146A) was used to record the output voltage. Once the output voltage was less than 20% of the maximum output voltage, the anolyte and catholyte were both changed with fresh electrolyte.

4.3.2 Electrochemical characterization

All electrochemical experiments were conducted using CHI 600D electrochemical workstation (CH Instruments, USA). Linear sweep voltammetry (LSV) method was used for polarization curve to estimate the maximum power density by using two-electrode mode in which the anode as the working electrode and the cathode as the reference as well as the counter electrode. When reaching the highest output voltages, MFC was open-circuit for 2 hours before LSV. LSV was carried out at the scanning rate of 1 mV/s from open-circuit voltage to 0 V.

A three-electrode system was used to perform the cyclic voltammetry (CV) analysis, where anode was used as the working electrode, cathode as the counter electrode, and saturated calomel electrode (SCE, +0.243 V vs. SHE)(ALS, Japan) as the reference electrode. Unless otherwise stated, all potentials in this article are relative to SCE. The CV analysis at a scanning rate of 1 mV/s was performed at turnover condition in order to study the bio-electrocatalytic behavior of the biofilms.

DPV Differential pulse voltammetry (DPV) was conducted from -0.7 V to +0.1 V, using 2.0-mV pulse increments, 20-mV pulse amplitude, 300-ms pulse width, and a 500-ms pulse period.

4.3.3 Quantification of riboflavin

The method for riboflavin quantification was as previous (Liu et al., 2015). The samples used for riboflavin quantification were filtered (0.4 μ m) after being centrifuged (35,000 $\times$ g for 20 min). Using a Waters XBridge C8 column (2.1 $\times$ 100 mm; particle size, 3.5 μ m), eluted compounds were detected by liquid chromatography-tandem mass spectrometer (LC-MS/MS) (Shimadzu LCMS-8030) in positive ion mode.

4.4 Results and discussion

4.4.1 Mixed-culture of *Shewanella* and *Bacillus* improved bioelectricity generation

Two-chamber MFCs were set up to investigate the potential difference of bioelectricity generation capability using MR-1, RH33 and mixed-culture as inocula, separately. MFC were operated under batch mode with consistent external resistance and the voltage output is presented in Fig. 4-2. It can be found that the difference in voltage output almost occurred upon the start-up. The voltage increase was much faster in mixed-culture compared with either of the pure cultures. The maximum voltage output for mixed-culture was 520 mV, which was 2.3 and 7.6 times of that for MR-1 (222 mV) and RH33 (68 mV), respectively. Meanwhile, it only took 48 hours for mixed-culture to reach the maximum voltage, which were 72 and 180 hours for MR-1 and RH33. The reduced start-up time is a valuable advantage.

Figure 4-2 Bioelectricity generation of MFCs with MR1 (blue), RH33 (red) and mixed-culture of MR-1 and RH33 (green), respectively. The error bars (mean $\pm$ standard error) were derived from triplicate experiments for each group of MFCs.

The anolyte and catholyte were both changed with fresh electrolyte when the output voltage was less than 20% of the maximum output voltage. From Fig. 4-2 we found that the cycle duration for mixed-culture was much shorter (88 hours in average) than those for MR-1 (138 hours in average) and RH33 (288 hours in average). Thus it is confirmed that MFCs with mixed-culture of MR-1 and RH33 could consume the substrate much faster than those with pure cultures of MR-1 and RH33. The total charge transfer from the bacteria to the electrode during the first cycle can be calculated by dividing the area that enclosed within the output voltage and operation time by the external resistance ($2K\Omega$). 57.8, 42.7 and 24.1 Coulombs electron were transferred in MFC with mixed-culture, MR-1 and RH33, with Coulombic efficiency as 5.6%, 4.1% and 2.3%, separately. The apparent increased Coulombic efficiency suggests that MFCs with mixed-culture can better convert the electron to the bioelectricity.

The polarization curve, which was frequently used to estimate maximum power density in MFC study, was conducted via linear sweep voltammetry (LSV) with scanning rate of 1 mV/s, as shown in Fig. 4-3. Fig. 4-3A and 4-3B

are voltage-current density (V-j) and power density-current density (P-j) curves. For MFCs with mixed-culture (green) and MR-1 (blue), the V-j curves showed similar trend as our precious work in which the electroactive performance of MR-1 and its genetic engineered strain HG1 was carefully compared.(Liu et al., 2015) The sharp voltage decrease from open circuit potential is ascribed to activation loss, as most of the previous work analyzed.(Fan et al., 2008) From the P-j curve, the maximum power densities were 277.4 mW/m² for mixed-culture, which was 4.9 times of that MR-1 (56.9 mW/m²) and 40.2 times of that by RH33 (6.9 mW/m²).

Figure 4-3 Polarization curves of MFCs with different strains. (A) Output

voltage vs. current density (V-j) curves and (B) Power density vs. current density (P-j) curves.

4.4.2 Both mediated and direct electron transfer efficiency were enhanced in mixed-culture

Differential pulse voltammetry (DPV) is one of the most powerful voltammetry techniques for detecting redox species in biological systems, due to the ability to detect traces amounts of substrates by largely decreasing non-faradaic current. The peak current of DPV is related to the abundance of the electroactive species in MFCs (Alper et al.). Fig. 4-4 shows the DPV curves of MFC with MR-1 (blue), RH33 (red) and mixed-culture (green), two peaks at redox potentials (E_p) of -0.460 ± 0.002 V and -0.308 ± 0.002 V (vs. SCE) were identified. The redox species at $E_p = -0.460$ V was attributed to flavins, while E_p at -0.308 V can be assigned to outer membrane c-type cytochromes, whose exact redox potential varies upon microenvironment (Marsili et al., 2008) (Meitl et al., 2009b; von Canstein et al., 2008). For RH33 and mixed-culture, the baseline subtracted peak current densities at $E_p = -0.460$ V were 288.3 and 214 mA/m², which were 7 and 5.1 times of MR-1 (41.3 mA/m²), respectively. This suggests that higher amount of riboflavin was present in RH33 and mixed-culture since the peak height of DPV is related to the abundance of electroactive species. Riboflavin concentration in the anolytes was further analyzed with LC-MS/MS. The results were consistent with the DPV. The riboflavin concentrations for MR-1, RH33 and mixed-culture were 0.55 ± 0.01 , 5.61 ± 0.04 and 5.32 ± 0.26 μ M, as indicated in Fig. 4-5. Meanwhile the baseline subtracted peak current density at $E_p = -0.308$ V was 12.8 mA/m² for mixed-culture, which is 1.7 times of that for MR-1 (7.6 mA/m²). It was found that flavins facilitated the process of EET by outer-membrane c-type cytochromes (Okamoto et al., 2013; Okamoto et al., 2014). It is reasonable that OM c-type cytochromes-based EET pathway was enhanced in the mixed-culture because MR-1 can utilize more riboflavin produced by RH33.

Figure 4-4 Differential pulse voltammetry curves for MR-1, RH33, mixed-culture of MR-1 and RH33.

Figure 4-5 Riboflavin concentration of analytes in the MFCs with MR1, RH33 and mixed-culture, separately.

Regardless of the high riboflavin concentration, the power density of RH33 was rather low compared with MR1 and mixed-culture. Considering the same MFC configuration and small cathode overpotential, the low bioelectricity generation with RH33 inoculum can be ascribed to low efficiency in utilizing

riboflavin for electron transfer. This phenomenon is not new in pure culture inoculum MFC, especially for those with *Pseudomonas* species. Although *Pseudomonas* species are well recognized in MFC microbial communities and are capable of synthesizing phenazines, which are efficient electron shuttles, bioelectricity in MFCs with pure culture of *Pseudomonas* species are usually very low. A previous work demonstrated a re-construction of mediated electron transfer pathway by expressing a heterologous porin protein in *E. coli* (Yong et al., 2013a). The result proved that for the electroactive microbes which rely on soluble shuttles to facilitate extracellular electron transfer, the electron transfer from central metabolism to biofilm interface may act as the rate limitation step. Under such situations, simply increase the electron shuttles concentration could not effectively improve the MET efficiency. Here we postulate that similar reason makes MFC with RH33 pure culture has much lower bioelectricity output.

To further compare the riboflavin utilization efficiency in MET by MR-1 and RH33, MFCs were first discharged under poised potential (0 V vs. SCE) for ten hours and then the anolyte of MR-1 and RH33 were exchanged. It is interesting that a dramatic increase in current density (from 249.8 to 1131.4 mA/m²) was observed instantly when anolyte of RH33 was added to anode with MR-1 biofilm, meanwhile difference was invisible for MR-1 anolyte with RH33 biofilm, as shown in Fig. 4-6. These results confirm that improved MFC performance with mixed culture inoculum was because MR-1 could utilize riboflavin produced by RH33. Considering the relative riboflavin concentration, it is reasonable to conclude that MR-1 could more effectively use riboflavin as electron shuttle, although RH33 has high capability of producing riboflavin.

Figure 4-6 Current discharge curves of the bioelectrochemical system under poised potential (0 V vs. SCE). MFCs were first discharged for ten hours and then the anolyte of MR-1 and RH33 were exchanged.

Cyclic voltammetry analyses at the scanning rate of 1 mV/s were conducted when MFCs were at their maximum voltage outputs and the CV curves thus stand the current response to the voltage change under turnover conditions, as shown in Fig. 4-7. The CV curves of MR-1 and mixed culture (blue and green curve) shows similar trends as in our previous work (Yong et al., 2013b; Yu et al., 2015). The anodic current increased from -0.5 V (vs. SCE), generating the catalytic current due to flavins-mediated electron transfer under turnover conditions. The sigmoid shape here indicates a dominant of turnover current, which is the evidence that flavins were involved in bioelectricity generation. Impressively, the calculated flavins-mediated catalytic current for mixed-culture was almost 11.7 times of that for MR-1 (74.6 and 874.3 mA/m²). For RH33, the CV curve at 1 mV/s is totally different. Instead of the sigmoid shape curve, one pair of highly reversible peak centered at -0.47 V can be observed. The peak clearly belongs to riboflavin, however the reversible peak shape here indicates that riboflavin hardly made any contribution to generate catalytic current, meaning RH33 could not effectively use riboflavin to facilitate extracellular electron transfer. Thus it is reasonable to conclude that flavins-

mediated electron transfer in mixed-culture was greatly enhanced because MR-1 could effectively utilize the riboflavin produced by RH33. Another interesting result is that increase rate of riboflavin in mixed culture to MR-1 is similar with that of flavins-mediated catalytic current (9.7 times vs. 11.7 times). This observation demonstrates that the efficiency of flavins-based MET is still limited by flavins concentration even the flavins concentration is increased by almost 10 times.

Figure 4-7 CV curves of the MFCs inoculated with MR-1, RH33 and mixed-culture under turnover condition, respectively, with a scanning rate of 1 mV/s.

The change in the slope from -0.33 V in CV curve indicates the participation of OM c-type cytochromes in extracellular electron transfer. The calculated DET based catalytic current for mixed-culture (508.4 mA/m^2) was 2.4 times of MR-1 (211.1 mA/m^2). This is consistent with the DPV results. Previous work confirmed that flavins bound to OM c-type cytochromes facilitate DET (Okamoto et al., 2013; Okamoto et al., 2014). Herein, we postulate similar effect derived from flavins-OM c-type cytochromes binding. The DET efficiency is enhanced with this binding because the active site of cytochromes would be more achievable to the electrode with the help of flavins.

Thus greatly increased riboflavin concentration also enhances the DET via OM cytochrome. More work is needed to verify this possibility in the future.

4.5 Conclusions

In conclusion, a synthetic microbial consortium, in which *S. oneidensis* MR-1 can utilize the high concentration of riboflavin produced by *Bacillus subtilis* RH33 to largely enhance bioelectricity generation, was rationally designed and successfully constructed. The maximum power density of MFCs with this synthetic microbial consortium was 277.4 mW/m^2 , which was 4.9 times of that by MR-1 (56.9 mW/m^2) and 40.2 times of that by RH33 (6.9 mW/m^2), respectively. It was found that both mediated and direct electron transfer efficiency were enhanced in mixed-culture.

CHAPTER 5 THE EFFECT OF EXTERNAL RESISTANCE ON BIOFILM FORMATION AND INTERNAL RESISTANCE IN SHEWANELLA INOCULATED MICROBIAL FUEL CELLS³

5.1 Abstract

External resistance is one of the important factors that affect the performance of microbial fuel cell (MFC). In this study, bioelectrochemical and biofilm characterization was conducted for *Shewanella oneidensis* MR-1 inoculated MFCs with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω , and 22 k Ω resistors. In overall, smaller external resistance resulted in higher maximum power density and more riboflavin secretion. Maximum power density of 136.8 ± 3.1 mW/m² was achieved when MFCs were operated with 500 Ω resistor, which was 3.7 times of that with 22 k Ω resistor. Electrochemical impedance spectra (EIS) analysis verified an increased internal resistance along with higher external resistance. Meanwhile more biofilm mass and extracellular polymer substances (EPS) were confirmed on MFC anode with higher external resistance.

5.2 Introduction

Microbial fuel cell (MFC), as a novel biotechnology which combines wastewater treatment and bioelectricity generation achieved vast attention during the past decades (Du et al., 2007; Franks and Nevin, 2010; Harnisch and Schroder, 2010; Logan, 2009; Lovley, 2006; Rabaey and Verstraete, 2005). Operation optimization like MFC configuration, substrate selection, strategy for anodic respiration community acclimation has successfully enhanced the power

³ The findings described in CHAPTER 5 have been published. Liu, T., Yu, Y.Y., Li, D., Song, H., Yan, X. and Chen, W.N., 2016. The effect of external resistance on biofilm formation and internal resistance in *Shewanella* inoculated microbial fuel cells. RSC Advances, 6(24), pp.20317-20323.

density of MFC for thousands of times compared with the initial stage (Cheng et al., 2006a, b; Kim et al., 2005b; Mohan et al., 2009; Parot et al., 2008; Rabaey et al., 2003; Rabaey et al., 2004; Reddy et al., 2010). Other parameters like temperature, pH were also proved to influence the MFC anode community physiology and then the MFC performance (Jadhav and Ghangrekar, 2009; Liu et al., 2005; Yong et al., 2013b). Among them, the necessity for external resistance and anode potential optimization stands out since they regulate the anode availability as an electron acceptor and thus are directly related with extracellular electron transfer (EET), the essential process in which anode respiration bacteria utilize special pathways to transfer the electron from internal metabolism to the anode (Aelterman et al., 2008b; Finkelstein et al., 2006; Marsili et al., 2010; Parot et al., 2008; Torres et al., 2009; Wagner et al., 2010; Wei et al., 2010).

Previous work found that different external resistance and anode potential would have significant impact on the anode biofilm composition, morphology and their corresponding MFC performance (Aelterman et al., 2008c; Finkelstein et al., 2006; Jung and Regan, 2011; Katuri et al., 2011; Lyon et al., 2010; Marsili et al., 2010; Premier et al., 2011; Ren et al., 2011; Rismani-Yazdi et al., 2011; Torres et al., 2009; Wei et al., 2010; Zhang et al., 2011). Generally, low start-up external resistance or higher anode potential means more microorganism species in mixed-culture could utilize the anode for electrode respiration, thus leading to high diversity anode biofilm community with more thickness, vice versa. However, the relationship between the external resistance with the power density of MFC was not consistent in previous works, although in most situation MFC operated at low external resistance had higher power density (Jung and Regan, 2011; Katuri et al., 2011; Lyon et al., 2010; Wei et al., 2010; Zhang et al., 2011).

Interestingly, compared with the mixed-culture MFC, the impact of external resistance on pure culture inoculated MFC were much less concerned and limited to several works (McLean et al., 2010; Wei et al., 2010). Mclean *et al.* calculated that cellular electron transfer rates on a per cell basis of *Shewanella oneidensis* MR1 at various external resistances were different. The cellular

electron transfer rate per cell was higher at larger external load. Also a mature thick biofilm was observed at larger external load, while a thin biofilm was developed at lower external load, which was different from the results of mixed-culture MFCs (McLean et al., 2010). Understanding the influence of external resistance on the pure culture inoculated MFC has important scientific significance, as it could provide us an opportunity for an observation on how bacteria would adjust their physiology and EET strategy without any interference with mixed culture.

Herein, the influence of external resistance on *Shewanella oneidensis* MR-1, a well-characterized anode respiration bacterium, inoculated MFC was carefully studied with five different external resistors. The MFC performance was compared and related electrochemical analysis was conducted to reveal the difference in EET mechanism and internal resistance. Riboflavin, an important endogenous electron shuttle secreted by *Shewanella* strains to facilitate the mediated electron transfer, was measured, together with the biofilm morphology imaging and relative biofilm mass and extracellular polymer substances (EPS) measurement, to compare the physiology change raised by different external resistance.

5.3 Materials and methods

5.3.1 MFCs setup and operation

S. oneidensis MR-1 used for MFCs' inoculum was first activated overnight in Lysogeny Broth (LB) medium after taken out from -80 °C fridge. Then the strain was cultured in 100 ml LB medium. After shaking at 220 rpm for 8 hours at 30 °C, the culture was centrifuged at 3000 g for 7 minutes to remove the supernatant. The cell pellet was washed with phosphate-buffered saline (PBS) and dispersed into nitrogen bubbled (for 5 minutes) MFC anolyte. Every 1 liter of anolyte contains 30 ml 1 M HEPES, 0.46 g NH₄Cl, 0.225 g K₂HPO₄, 0.225 g KH₂PO₄, 0.117 g MgSO₄•7H₂O, 0.225 g (NH₄)₂SO₄, 10.8 g NaOH, 0.056 g CaCl₂, 10 ml Wolfe's vitamin, 10 ml Wolfe's mineral, 200 µg L-arginine HCl, 200 µg L-glutamine, 400 µg DL-serine, and 18 mM sodium lactate as carbon

source (Atlas, 2010). Catholyte consists of 50 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and 50 mM K_2HPO_4 and KH_2PO_4 .

Two-chamber MFC architecture with an inner size of 4.5 cm x 4 cm x 4 cm for each chamber was used in this study. The proton exchange membrane (Nafion 117, from Gas Hub, Singapore) used to separate the two chambers was first immersed into 0.5 M sulfuric acid overnight and then washed with sterilized water before use. 2 cm x 2.5 cm carbon cloth (from Gas Hub, Singapore) was used for both anode and cathode. After MFCs set-up, the MFCs were continuously discharged under external resistances (250 Ω , 500 Ω , 2 k Ω , 6 k Ω , or 22 k Ω). Triplicate MFCs were conducted for each external resistance to guarantee the results highly repeatable. A digital multimeter (ESCORT 3146A) was used to record the output voltage. Once the output voltage was dropped under 20% of the maximum voltage, the anolyte and catholyte were both changed with fresh electrolyte.

5.3.2 Electrochemical characterization

Polarization curves were measured by changing the external resistances. When reaching the highest output voltages, MFC was open-circuit for 2 hours before polarization. A three-electrode system was used to perform the cyclic voltammetry (CV) analysis, where anode was used as the working electrode, cathode as the counter electrode, and saturated calomel electrode (SCE, +0.243 V vs. SHE) as the reference electrode. Unless otherwise stated, all potentials in this article are relative to SCE. The CV analysis at a scanning rate of 1 mV/s was performed at turnover condition in order to study the bio-electrocatalytic behavior of the biofilms.

Electrochemical impedance spectroscopy (EIS) analysis was carried out from 100 kHz to 0.001 Hz with an ac signal of 5 mV amplitude. EIS analysis for the MFC was conducted when the output voltage reached the maximum by using the two-electrode mode in which the anode as the working electrode and the cathode as the reference as well as the counter electrode. The applied voltage was set as the maximum output voltage.

5.3.3 Biofilm characterization

Anode biofilms were observed by field emission scanning electron microscope (FESEM, JSM-6700F-FE-SEM, Japan). The sample preparation process was same with former works (Liu et al., 2015; Yong et al., 2013b).

The quantification of biomass of the biofilms grown on carbon cloth electrodes was performed using the LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies, Carlsbad, CA) according to previous work (Liu et al., 2015). The characterization of extracellular polymeric substances (EPS) was modified according to previous study (Bales et al., 2013; Fang et al., 2002). A small piece of carbon cloth was cut off for EPS characterization. The carbon cloth was put in 800 μ L PBS buffer sonicated for 1 min. 200 μ L 1.1% formaldehyde was added to each 800 μ L PBS buffer. The mixture was incubated at 4 °C for 1 hour. After adding 0.4 mL 1 M NaOH to the mixture, the mixture was incubated at 4 °C for 3 hours. The mixture was then centrifuged at 4 °C, 20000 g for 30 min. The supernatant containing soluble EPS was filtered through a 0.2 μ m filter. The carbohydrate content of EPS in the extracted solution was measured using the phenol/sulfuric-acid method and the protein content using the Modified Lowry Kit for Protein Determination (Sigma-Aldrich, Singapore) (Dubois et al., 1956), (Lowry et al., 1951; Peterson, 1977).

5.3.4 Riboflavin quantification

The samples used for riboflavin quantification were first centrifuged ($35,000 \times g$ for 20 min) and then filtered (0.4 μ m). Eluted compounds were detected by liquid chromatography-tandem mass spectrometer (LC-MS/MS) (Shimadzu LCMS-8030) in positive ion mode using a Waters XBridge C8 column (2.1×100 mm; particle size, 3.5 μ m).

5.4 Results and discussion

5.4.1 Bioelectricity generation and internal resistance were affected by external resistance.

A series of MFCs were set up and operated with five different resistances (250 Ω , 500 Ω , 2 k Ω , 6 k Ω , or 22 k Ω) to study the potential influence of external resistance on bioelectricity capability of *Shewanella* inoculated MFC. The profile of output voltage during the batch mode operation is presented in Fig. 5-1. It can be observed that when MFCs were operated with high external resistances, the output voltage increase much faster than that with the lower ones. The maximum output voltage in the first batch cycle (from start to anode medium refresh) were 49.6, 98.7, 211.3, 393.3 and 588.1 mV for MFC with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω , and 22 k Ω resistors, respectively. The observation of increased output voltage with higher external resistance is consistent with former works (Aelterman et al., 2008a; Aelterman et al., 2008c; Katuri et al., 2011; Lyon et al., 2010; Premier et al., 2011; Rismeni-Yazdi et al., 2011).

Figure 5-1 Bioelectricity generation of MFCs with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω , and 22 k Ω resistors, respectively. The error bas (mean $\pm$ standard error) were derived from triplicate experiments for each group of MFCs.

The total charge transferred was calculated by dividing the area that enclosed within the output voltage and operation time in Fig. 5-1 by the corresponding external resistance. It can be confirmed that 58.1, 59.9, 42.6, 23.6 and 10.8 coulombs charge were transferred in MFCs with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω , and 22 k Ω external resistances during the first batch cycle.

Polarization curves were then conducted to evaluate the maximum power densities that can be achieved with different external resistances at the maximum output voltage in the second cycle. Fig 5-2A and 5-2B are voltage-current density (V-j) and power density-current density (P-j) curves. The maximum power density achieved was 42.4 ± 0.9 , 136.8 ± 3.1 , 50.1 ± 0.9 , 44.6 ± 1.0 and 36.5 ± 0.5 mW/m² for MFC with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω , and 22 k Ω resistors.

Figure 5-2 Polarization curves of MFCs with different external resistances. (A) Output voltage vs. current density (V-j) curves and (B) Power density vs. current density (P-j) curves.

V-j curves provide useful information on potential drop variation with increased current density. As 50 mM ferricyanide was used as the cathode solution and the corresponding overpotential change is really small within the current range in the polarization curves, the potential drop in V-j curve can be attributed to the anode potential change with increased current density (Liu et al., 2015). For all MFCs, a rapid potential drop was observed from 800 mV to 700 mV, representing activation loss.

The potential drop from 700 mV varied among MFCs with different external resistances. More rapid drop from 600 mV can be observed for MFC with 22 k Ω resistor compared with the small external resistance curves. The high output voltage of MFC with large resistance means low anode potential, which makes *Shewanella* inefficient to use those electrochemical active species with high redox potential, such as outer membrane (OM) c-type cytochromes, to facilitate the EET. One possible explanation is that *Shewanella* in these MFC adjusted their EET pathway to the low anode potential. As a result, rapid potential drop was observed since *Shewanella* lacked suitable redox species to carry on EET at more positive anode potential.

To reveal how different external resistance leads to difference MFCs performance, we conducted electrochemical impedance spectroscopy (EIS) analysis and cyclic voltammetry (CV) analysis. The results are presented in Fig. 5-3. The EIS data were presented in the form of Nyquist plots in which the real part of the impedance is represented in the x-axis and the negative of imaginary impedance is represented in the y-axis (Fig. 5-3A). The EIS data was fitted using Randles circuit in which the ohmic resistance (R_{ohm}) is in series with a parallel combination of the polarization resistance (R_p) and constant phase element (CPE) (Randles, 1947). CPE is used instead of a capacitor because of the non-ideal behavior of porous electrodes, such as MFC biofilms (Dominguez-Benetton et al., 2012; He and Mansfeld, 2009; Manohar and Mansfeld, 2009; Manohar et al., 2008; Ramaraja P Ramasamy, 2013). The fit parameters were showed in Table 1. The internal resistance (R_{int}) of MFC is usually defined as the summary of R_{ohm} and R_p . Since R_p is much larger than R_{ohm} , R_p can be treated as R_{int} . The EIS for MFC is used to measure R_{in} of the whole cell at an applied cell voltage. Here we used the maximum output voltage to simulate the status when MFC achieved its maximum output voltage. Fig.5-3 and Table 5-1 show that the internal resistance of MFC increased with external resistance.

Figure 5-3 (A) EIS (Nyquist plot) for MFCs with different external resistances at respectively maximum output voltages. (B) CV curves of MFCs with different external resistances under turnover conditions at a scanning rate of 1 mV/s.

Table 5-1 Fit parameters of EIS for MFC at maximum output voltage

External resistance (Ω)	R_{ohm} (Ω)	R_p (k Ω)	CPE	
			Q (μ Mho)	n
250	37.3	1.06	1237	0.704
500	99.0	1.66	735	0.794

2 k	58.4	5.04	583	0.939
6 k	97.8	12.3	513	0.922
22 k	41.3	32.3	662	0.945

Cyclic voltammetry analysis at low scanning rate (1 mV/s) was conducted at turnover conditions to reveal the details of the *Shewanella* catalytic behaviors (Fig. 5-3B). CV curves of MFCs under different resistances have similar trends. Two stages of catalytic behaviors, which individually started from -0.5 V to -0.38 V and from -0.38 V to 0.1 V, representing the flavins-based mediated electron transfer (MET) and OM c-type cytochromes-based direct electron transfer (DET), can be confirmed (Baron et al., 2009; Carmona-Martinez et al., 2011). Table 5-2 lists the value of MET and DET based catalytic current, which is calculated using the same method as in our former work (Yu et al., 2015). Consistent with polarization curve, MFCs with 500 Ω resistor has the highest MET (167.4 mA/m²) and DET (230.4 mA/m²) based catalytic current, followed by MFCs with 2 k Ω and 250 Ω resistances. MFCs operated with 6 k Ω and 22 k Ω resistors have much smaller catalytic current.

Table 5-2 Mediated electron transfer-based and direct electron transfer-based catalytic current density with different external resistances

External resistance (Ω)	MET (mA/m ²)	DET (mA/m ²)
250	130.5	130.0
500	167.4	230.3
2 k	123.8	149.2
6 k	59.01	37.3
22 k	45.55	33.40

Although *Shewanella* is capable of secreting flavins to facilitate its extracellular electron transfer, the secreted flavins concentration is usually low and limits its EET efficiency. As a result, increase of flavins concentration level in MFC using *Shewanella* always improves its bioelectricity output (Baron et al., 2009; Marsili et al., 2008; Yong et al., 2013b). Anolyte from different MFCs was taken to measure riboflavin concentration with LC-MS/MS. As presented in Fig. 5-4, the bulk riboflavin concentrations were 0.479 ± 0.075 ,

0.738 ± 0.057 , 0.354 ± 0.012 , 0.217 ± 0.035 and 0.159 ± 0.023 μM for MFCs with 250 Ω , 500 Ω , 2 k Ω , 6 k Ω and 22 k Ω external resistances, respectively. *Shewanella* secreted more riboflavin with smaller external resistance, especially with 500 Ω resistance. The higher riboflavin concentration explains the enhanced MFC performance and MET-based catalytic current in MFC with 500 Ω resistor, although the physiological mechanism needs further investigation.

Figure 5-4 Riboflavin concentration of analytes in the MFCs with different external resistances.

5.4.2 The external resistance affected the biofilm formation.

To understand how external resistance would influence the biofilm formation, we measured the relative biofilm mass, carbohydrate and protein contents of EPS on MFC anode with different external resistances (Fig. 5-5). It can be concluded that the anode biomass amount increased with external resistance. The biomass amount on anode in MFCs with 22 k Ω resistors was 7.1 times of that with 250 Ω resistors. The carbohydrate and protein contents of EPS on anode with different external resistances had similar trend with biomass amount. Considering the SEM images of anode (Fig. 5-6), we can conclude that higher external resistance leads *Shewanella* to form thicker biofilm on anode.

Figure 5-5 Characterization of anode biofilm with different external resistances. (A) The relative biofilm mass. To calculate the relative biofilm mass, the biofilm mass of anode with 250 Ω resistance was used as the reference (designated as “1”). (B) Carbohydrate and protein contents of EPS from anode biofilm.

Figure 5-6 SEM images of the biofilm on carbon cloth anode with (A) 250 Ω , (B) 500 Ω , (C) 2 k Ω , (D) 6 k Ω , and (E) 22 k Ω external resistances.

It was found that riboflavin play an essential role in *Shewanella* EET and direct addition of riboflavin to anolyte is an effective strategy to improve the EET efficiency of *Shewanella* (Baron et al., 2009; Marsili et al., 2008; Yong et al., 2013b). So it is reasonable that higher flavins-based MET catalytic current was observed from CV curves when MFCs was operated with lower external resistance because higher riboflavin concentration was confirmed by LC-MS/MS. There are only a few publications studying how riboflavin level was affected by different MFC operation parameters. Our previous work confirmed that *Shewanella* would secrete more riboflavin under slightly alkaline condition (optimal pH=9), leading to better MFCs performance (Yong et al., 2013b). Here, this is the first observation that riboflavin concentration in *Shewanella*-inoculated MFCs was varied due to different external resistances. The trend of riboflavin concentration change due to different external resistances was consistent with that of the total charge transfer in the first batch. As total charge transfer is the indication of electrochemical respiration, it means that easy access to electrochemical respiration might lead to high riboflavin synthesis and secretion. More work is required to further reveal the biochemical mechanism underlying the improved riboflavin concentration.

With much higher anode biofilm mass, MFCs with high resistances were supposed to be more efficient in DET as more microbe and OM c-type cytochromes would be involved in DET. However, the high external resistance resulted more negative anode potential (estimated from V-t curve, -0.3 V for 22 k Ω resistor and -0.1 V for 6 k Ω resistor). Previous study found that OM c-type cytochromes cannot be observed at the bacteria-electrode interface when the anode was posed at relative negative potential (Peng et al., 2010a). Electrochemical active bacteria that have multiple potential EET pathways are capable to adjust their EET strategies based on the anode potential (Busalmen et al., 2008a). Liu et al. found that the EET pathways can be switched from DET to MET when the anode potential is not favorable for cytochromes to transfer electron (Liu et al., 2010b). As the DET efficiency of *Shewanella* is low and large overpotential is required to achieve higher current density, DET was not the preferred choice for *Shewanella* when the anode potential was very negative. Taking all these results into consideration, despite the fact that there was more anode biofilm mass when the external resistance was high, the total amount of OM c-type cytochromes capable for EET was still limited. As a result, the OM c-type cytochromes-based DET catalytic current of MFCs with high external resistor was low when CV analysis was conducted.

5.5 Conclusions

In conclusion, external resistance has significant impact on bioelectrochemical property and biofilm formation of *Shewanella oneidensis* MR-1 on MFC anode. Generally speaking, MFCs with smaller external resistances achieved higher maximum power density and produced more riboflavin, especially MFCs with 500 Ω resistor. When higher external resistance results more negative anode potential, the MET is the preferred choice for *Shewanella*. It was found that the internal resistance of MFC, anode biofilm mass and EPS increased with external resistance. Taking all these facts into consideration, the best performance was achieved with 500 Ω resistor. This demonstrates that external resistance optimizing is of great importance for MFC set-up, especially for those with pure culture.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, different strategies were developed to improve the performance of MFCs by enhancing direct and mediated electron transfer pathways. The underlying mechanisms for the enhanced systems were studied through electrochemical and biofilm characterization. This thesis mainly consists of three works. Firstly, a significant enhancement in the electroactive biofilm formation of *Shewanella* was achieved via heterologous expression of c-di-GMP biosynthesis gene *ydeH* in engineered *S. oneidensis* strains HG1 (IPTG-inducible expression of *ydeH*) and HG2 (constitutive expression of *ydeH*). The MFCs inoculated with these engineered strains accomplished a maximum power density of $167.6 \pm 3.6 \text{ mW/m}^2$, which was ~ 2.8 times of that achieved by the wild-type MR-1 ($61.0 \pm 1.9 \text{ mW/m}^2$), caused by higher levels of bacterial cells in anode biofilm, and thus higher levels of cytochromes involved in extracellular electron transfer.

In the second work, I designed and constructed a synthetic microbial consortium, in which *S. oneidensis* MR-1 can efficiently utilize the high concentration of riboflavin produced by *Bacillus subtilis* RH33 to largely enhance bioelectricity generation. The maximum power density of MFCs with this synthetic microbial consortium was 277.4 mW/m^2 , which was 4.9 times of that by MR-1 (56.9 mW/m^2) and 40.2 times of that by RH33 (6.9 mW/m^2), separately. It was found that both mediated and direct electron transfer efficiency were enhanced in mixed-culture.

Furthermore, the effect of external resistance on extracellular electron transfer, biofilm formation, internal resistance and riboflavin production in *Shewanella* inoculated MFCs was studied in another work. Generally, smaller external resistance resulted in higher maximum power density and more riboflavin secretion. Maximum power density of $136.8 \pm 3.1 \text{ mW/m}^2$ was achieved when MFCs were operated with 500Ω resistor, which was 3.7 times of that with $22 \text{ k}\Omega$ resistor. Electrochemical impedance spectra analysis

verified an increased internal resistance along with higher external resistance. Meanwhile more biofilm mass and extracellular polymer substances were confirmed on MFC anode with higher external resistance.

6.2 Recommendations

The works in this thesis, while only a small portion in the field of microbial electrochemical systems, bring novel strategies to this field. Mutagenesis and recombinant DNA technology is a promising approach to engineer exoelectrogens, thus to enhance the efficiency of microbial electrochemical systems. A combination of engineered exoelectrogens with novel biocompatible electrode materials would be intriguing for further increasing the performance of MFCs and other microbial electrochemical systems. Rationally designed synthetic microbial consortium would offer better performance. This synthetic microbial consortium can be electron mediator-based mutualism, in which one type of bacterium could efficiently use electron mediators produced by another consortium bacterium to transport electrons to the electrodes, or metabolite-based mutualism, which enables broad spectrum of carbon sources (Song et al., 2014; Venkataraman et al., 2011; Wang et al., 2014; Yang et al., 2015).

There are still many remaining challenges for applying MFCs for practical applications. The efficiency and scale up of MFCs remain as a major challenge for their practical application, though the performance has been improved dramatically during the past decades. Despite the fact that the reactor costs have been largely reduced by replacing the expensive membranes and electrodes with cheaper alternative materials, it is still expensive to utilize MFCs for wastewater treatment. Studies about broadening the scope of microbial electrochemical systems from bioelectricity generation to a highly interdisciplinary platform technology enabling diverse applications have been initiated. Despite the fact that MFCs was initially developed for wastewater treatment, MFCs and technologies originated from MFCs have broad applications, such as hydrogen production, bioproduction, bioremediation, toxicity and biochemical oxygen demand sensors (Harnisch and Schroder, 2010; Knight et al., 2013; Logan, 2009; Logan and Rabaey, 2012). These

systems were summarized as MXCs, where X stands for different applications and systems (Harnisch and Schroder, 2010). But research about these systems is just in the beginning and mainly in lab scale. Microbial electrolysis cells (MECs) generate hydrogen gas in cathode by adding an external voltage on top of the potential produced by MFCs. Microbial electrosynthesis (MES) is an emerging microbial electrochemical technology, using cathode derived electrons to reduce CO₂ or other chemicals to produce a wide range of organic compounds, especially some precursors for high value chemicals (Lovley and Nevin, 2011; Rabaey and Rozendal, 2010). MES is capable of carbon sequestration and produce organic compounds at the same time. Furthermore, since MES can be driven by any kind of electricity, it also has the potential to address the problems related to storage, and distribution renewable but intermittent forms of energy, such as wind and solar, by converting them into desirable fuels or chemicals, which draws vast attention of researchers, which is the main research focus of MXCs. Overall, although there are several problems need to be addressed, it is reasonable to believe that booming research and rapid development about microbial electrochemical systems will lead this diverse platform technology becoming feasible to tackle several environmental and energy issues.

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LIST OF PUBLICATIONS

1. **Liu, T.**, Yu, Y.Y., Deng, X.P., Ng, C.K., Cao, B., Wang, J.Y., Rice, S.A., Kjelleberg, S., Song, H., 2015. Enhanced *Shewanella* biofilm promotes bioelectricity generation. *Biotechnol. Bioeng.* 112, 2051-2059.
2. **Liu, T.**, Yu, Y.Y., Li, D., Song, H., Yan, X. and Chen, W.N., 2016. The effect of external resistance on biofilm formation and internal resistance in *Shewanella* inoculated microbial fuel cells. *RSC Advances*, 6(24), pp.20317-20323.
3. **Liu, T.**, Yu, Y.Y., Chen, T., and Chen, W., A synthetic microbial consortium of *Shewanella* and *Bacillus* for enhanced generation bioelectricity. *Under review*.