Development of high-performance microbial fuel cells by enhancing extracellular electron transfer between bacteria and electrode materials

Yu, Yang-Yang

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Nanyang Technological University

School of Chemical and Biological Engineering

Thesis for Doctor of Philosophy

**Development of High-performance Microbial Fuel Cells by Enhancing
Extracellular Electron Transfer between Bacteria and Electrode Materials**

Submitted by: Yu Yang-Yang

Supervisor: Song Hao

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Abstract

The microbial fuel cell (MFC) is a novel biotechnology that combines wastewater treatment and energy harvest. Traditional MFCs usually consist of a bioanode in which bacteria community attached on the anode (known as biofilm) degrade organic carbon sources and transfer the electrons generated from intracellular metabolisms to electrodes. These electrons will pass through an external resistance and reach chemical or oxygen cathodes to complete electricity circuits. Many efforts have been done to improve the anode and cathode efficiency. For MFCs anode, material science development and reactor operation optimization greatly have improved the system performance in the past decade. Many studies on extracellular electron transfer (EET) mechanisms also have been done and two possible EET pathways were elucidated. The cathode development mainly focuses on investigating suitable materials and reactions to simultaneously meet the requirement on high performance and low cost. The recent development in biocathode opens the door to utilize electron generated from anode for more sophisticated application in bioremediation and bioproduction.

In this thesis, we implemented a number of researches aiming at the improvement of anode performances. Different strategies were developed to improve the extracellular electron transfer and thus bioelectricity and power output. In the first work, I rationally designed a conductive artificial biofilm (CAB) which immobilized *Shewanella oneidensis* MR-1 into the graphite and polypyrrole matrix. In this way the biomass loading amount was greatly increased compared with naturally occurring biofilms on carbon cloth electrode. Anode performance with such CAB-equipped MFCs was greatly enhanced. In the second work, we developed an anode modification with nitrogen doped carbon nanoparticle. Cyclic Voltammetry analysis reveals that the modified electrode has the capability to retention soluble mediator flavins compared with former reported

CNT. This greatly increased the MET in MFCs anode and also inspired rational design novel composite material for future applications. The bioelectrochemical behavior of *Pseudomonas aeruginosa* PAO-1 with global regulator mutation was studied in the third work. We demonstrate here that such upstream regulator random mutation is potential effective tool for improve the system performance. In the last work, we successfully developed synthesized ecosystem composes of *Shewanella oneidensis* MR-1 and *Pseudomonas aeruginosa* PAO-1. Maximum power density of 523.5 mW/m² was achieved, which was the best performance in all these works.

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Chapter 1: Introduction and Literature Review

1.1 Microbial fuel cells: Basic Concepts and knowledge

In recent years, as the shortage of fossil fuels and increasing demand for renewable alternative clean energy, biomass energy which benefiting from its large residue amount, has attracted great attention in the research area. Among many techniques in using biomass energy, microbial fuel cells (MFCs) emerge as a novel technology which combine energy harvest and wastewater treatment has achieved great achievement in the recent decade.(Rabaey et al., 2007) (Logan et al., 2006; Logan & Rabaey, 2012)

Regardless its rapid development in the past decade which was witnessed by the explosion number of published research articles, the concept of using microbes to generate current was not new at all. The first report that observed the combined bioelectricity release with the organic compound decomposition can be date back to 1911.(Potter, 1911) This interest regained its attention one century later when it was found that electricity can be harvested from wastewater and renewable biomass.(Bond et al., 2002) Various pure cultures which capable of current producing were isolated and studied and they covers from Firmicutes, acidobacteria, four of five classes of Proteobacteria and some yeasts strain.(Bond & Lovley, 2005; Borole et al., 2008; Holmes et al., 2004b; Kim et al., 1999; Park et al., 2001; Prasad et al., 2007; Rabaey et al., 2004; Walker & Walker, 2006; Zhang et al., 2008b) Principally, all of the MFCs systems involve microbes which capable of transferring the electron derived from inner metabolism to solid electrode (MFCs anode) rather than usual electron acceptor (O_2 , NO_3^- , etc.), knowing as extracellular electron transfer (EET). This process can happen via membrane associated components (*c*-type cytochrome, conductive pili and filament) and soluble electron

mediators.(Bond & Lovley, 2005; Gorby et al., 2006; Pham et al., 2008b; Rabaey & Verstraete, 2005; Reguera et al., 2006; von Canstein et al., 2008; Yi et al., 2009) Then the electron passes through out network and meets the final electron acceptor in the cathode to complete the whole circuit. Compared with the natural respiration pathway, MFCs spatially separate the intercellular metabolism with the final electron acceptor. Schematic 1.1A is a schematic for two chamber MFCs with supposed glucose as carbon source and oxygen as final electron acceptor. The individual half reactions are as following:

The anode reaction mainly involves the biodegradation of biomass and release the electron to the anode. Proton from intercellular metabolism is released to anode chamber and diffuses through separated proton exchange membrane (PEM) to cathode chamber. The most distinguished characterization of MFCs to other fuel cells lies here: in MFCs anode, bacteria are responsible for degrade and oxidize the substrate to generate electron, acting like a bio-catalyst (although not real catalyst as microbes have energy benefit from this step). There are at least two recognized inner metabolic pathways that involve EET and NADH probably work as the electron source for EET.(Rabaey & Verstraete, 2005) The standard potential of NADH (-0.32 V VS SHE) is usually regard as the minimum anode potential we can get theoretically, thus for the MFCs aiming at electricity output and energy harvest, the controlled anode potential are always higher than this value. For diverse Acetogenic Microorganisms as *Sporomusa ovate*, *Clostridium ljungdahlii*, *Clostridium aceticum*, and *Moorella thermoacetica*, when the electrode potential is controlled as low as -400 mV VS SHE, the above EET process can be reversed, meaning microbes would

consume the electron from the electrode rather than electron “generation”.(Nevin et al., 2011; Nevin et al., 2010) This contributes to the foundation of biosynthesis from carbon dioxide. However, the theoretical anodic potential at open circuit is determined by the species (ex. c-type cytochrome in DET and soluble shuttle in MET) that directly react at the anode surface. Oxygen is the final electron acceptor here and other reported final electron acceptors include NO_3^- , SO_4^{2-} , MnO_4^- , $\text{Fe}(\text{CN})_6^{3-}$, etc.(Clauwaert et al., 2009; Li et al., 2009; Rabaey et al., 2004; You et al., 2006) Schematic 1-1B indicates theoretical potential drop from MFCs cathode ($\text{O}_2/\text{H}_2\text{O}$) to MFCs anode (Glucose/ CO_2) where ΔE_1 represent the potential lose from electron donor (glucose here) to electron source for EET (NADH), ΔE_2 is the energy loss to drive the series intercellular electron transfer from the metabolic NADH to final electron carrier before reacts with electrode. ΔE_3 can be roughly viewed as the potential maximum energy we can get from MFCs although it is slightly different from Open circuit potential (OCP), the directly indication of the theoretical maximum potential we can get from a single MFC and is determined by the difference between potential of anode and cathode electrochemical active species under open circuit condition. Standard potential of mediator (soluble or fixed) depends on the mediator property, thus choose a mediator with standard potential as low as to that of NAD^+/NADH is a strategy that can potentially increase the energy harvest.(Feng et al., 2010b)

Although the energy which potentially can be harvested from MFCs is depended on the chosen EET pathways, microbes burden this process seems benefit from another aspect. Despite the long misunderstanding that microbes would get less energy benefit from anode respiration if more energy is harvested in MFCs anode, recent research highlighted that microbes gain the energy from anode respiration by pumping protons across the cell membrane to form a proton gradient,

this coupled “proton pump” works as driving force to synthesize ATP from ADP to facilitate microbe survive.(Lovley, 2008)

Compared with traditional wastewater treatment strategy, MFCs obviously benefit from their unique design. First, in traditional wastewater treatment aeration is usually a necessity for the high biomass conversion rate and efficiency. The energy cost for aeration account for the main part of operation fee in wastewater treatment.(Wang & Ren, 2013) MFCs offer a feasible method that can degrade the biomass under anaerobic conditions as that in aerobic conditions due to the electrode respiration. Second, MFCs are operated at room temperature and even in low temperature. This distinguishes it from other biomass treatment method. Third, MFCs are usually gas free and needs no post gas treatment. Fourth, the energy provided by MFCs can not only compensate the energy cost by wastewater treatment but also possible to power the remote and isolated human residence.(Franks & Nevin, 2010; Pham et al., 2006; Rabaey & Verstraete, 2005)

Schematic 1-1 (A) Schematic of two-chamber MFCs with glucose as anode fuel and oxygen as cathode fuel. (B) Potential increase from the anode substrate degradation to cathode reaction in bioelectricity generation.

1.1.1 Parameters to evaluate MFCs Performance

Although vast interest was taken during the past decade and explosion in the related publications were witnessed, there is somehow existed huge difference for the technology, terminology and parameter used in MFCs study. Multiple reasons contribute to this situation. The most distinguished one lies in that in different works the key factor as MFCs design, operation

conditions, electrode materials and inoculations differs with each other. This makes it very difficult to compare the work from different study in a comparable fair way. Meanwhile although MFCs retrieved its interest in the academic field by researchers from environmental engineering in the early of this century, researchers with other background like material science, bioengineering and analytical chemistry soon joined this field. They made new contributions to this subject with their original viewpoint, thinking method and analytical method as well. This made the outlook of research paper of MFCs even more versatile and different technology and parameters are easily observed in different works even though they may have similar research emphasize.

Thus the importance to establish terminology and technology to analysis this system have been put out and several review papers already taken their effort on the target.(Harnisch & Rabaey, 2012; Logan et al., 2006; Rabaey & Verstraete, 2005). The following part lists the basic parameters that are frequently used in studying the efficiency of MFCs.

Substrate consumption rate

Developed as an alternative wastewater treatment method beside traditional anaerobic and aerobic sludge process, the general efficiency of MFCs to treat the wastewater, or the substrate consumption rate, is as an important parameter to feature the system.(Logan et al., 2006) Chemical Oxygen Demand (COD) removal is the most frequently used parameter for this purpose as that in traditional wastewater treatment. This parameter indicates the how the substrate is completely consumed and used up. COD removal efficiency ϵ_{COD} is got by calculating the ratio between the removed and feeding COD. The value is important for the calculation of Coulombic Efficiency. For most MFCs, the substrate consumption rates are

between 0.1-10 kg COD/m³ per day.(Rabaey & Verstraete, 2005) The absolute value has no advantages compared original wastewater treatment technology like anaerobic sludge reactor.(Chang & Lin, 2004; Lettinga et al., 1980) However, MFCs stand out due to its capability to completely oxidize low concentration substrate, making it a promising supplementary method for traditional wastewater treatment method.

In most situations, anode respiration is only appeal to the bacteria that directly attached or closed to electrode surface. As a result, the MFCs configuration, effective anode area, substrate concentration, flow and feeding type would influence the total efficiency in substrate consumption rate. Thus this value provides good insight into the general performance of MFCs system.

Growth Yield

Growth yield is the ratio of substrate used for biomass growth to the total consumed substrate and can be described as:

$$Y = \frac{X}{\Delta COD}$$

Where X is the biomass produced in MFCs operation and ΔCOD is the total consumed substrate.(Logan et al., 2006) It is clear that high growth yield is not welcome in MFCs system as it would reduce the energy harvested at anode and more importantly, increase the operation cost due to the post-treatment of these biomass. Low growth yield in MFCs is one of the advantages compared with aerobic sludge process (0.07 - 0.22 vs. 0.4 g biomass/COD).(Rabaey & Verstraete, 2005)

Open circuit potential

Open circuit potential (OCP) is the potential difference between the cathode and anode when no current is passing through the out circuit. This is the potential under thermodynamic limitation we can get. OCP is an important parameter as it stands the maximum theoretical potential we can get from MFCs. More importantly OCP of MFCs could provide some instinct observations about the system performance. MFCs OCP clearly depended on the choice of anode and cathode reactions. The theoretical anode potential by substrate degrade (taken Glucose $\rightarrow$ CO₂, -0.42 V vs. SHE) cannot be reached in the experiment due to the existence of anode overpotential (current exist in measurement), electrochemical active species directly associated with the anode are more relevant to the measured OCP.

Anode and cathode overpotential

When electron transfers through the circuit, the overpotential both at MFCs anode and cathode becomes obvious. Polarization curve and cyclic voltammetry (CV) scanning at low rate provide useful information on how MFCs suffers from potential loss due to the overpotential at anode and cathode. In MFCs anode, activation loss is always observed.(Fan et al., 2008) For those MFCs that anode reaction is not fast enough, more overpotential is required to drive the MFCs to get higher current output, as a result more anodic current can be observed with the continuous increase in anode potential.(Baron et al., 2009) Higher electrode surface area and better anode material choice would partially reduce this overpotential. For MFCs cathode, especially those using oxygen as the final electron acceptor, cathode overpotential is an important concerns. Suitable catalysts and technology for oxygen reduction is required and balance between efficiency and cost is important. Chemical cathode like hexacyanoferrate solutions would greatly

reduce the cathode potential but its application in industry is not welcomed.(Park et al., 2000; Pham et al., 2004)

Internal resistance

Increase anode and cathode area or use novel electrode materials that is feasible for anode and cathode reaction happen would considerably reduce anode and cathode overpotential caused by the reaction. However the internal resistance account for another potential lose in MFCs operation. This internal resistance mainly composed of two parts: the resistance of material, electric circuit and their connections used in MFCs and resistance of ion immigration in anode and cathode chamber, as well as ion transfers across the membrane.(Gil et al., 2003; Logan, 2009) The potential loss by the internal resistance becomes dominant when MFCs is scaled up from lab level (several to several hundred ml in volume) to the future industry application level (tens of liters to cubic meters in volume). Beside the ohmic resistance of electrode material and circuit, low ion strength in the general wastewater clearly account one of the major obstacle for MFCs application when scaled up.

Coulombic Efficiency

Coulombic efficiency is the ratio of electron transferred to MFCs anode to the maximum theoretical amount by substrate degrades. This maximum theoretical amount typically refers to the electron released from the substrate when fully oxidized. In MFCs with organic compound as carbon source, it usually refers to carbon dioxide, regardless this carbon source is in the simple form like formate and acetate or complex compound like starch and cellulose. When fed with single carbon source, the calculation form is as following:

$$\epsilon_c = \frac{\int_0^t I dt}{FbV\Delta C}$$

I is the current measured by out circuit, F is Faradic constant, b is the number of electron transferred coupled with one substrate molecule completely oxidized, V is the anode volume and ΔC is the substrate concentration change. When using wastewater or other complex influent, coulombic efficiency can be calculated by change ΔC to ΔCOD , with multiplying necessity coefficient.(Logan et al., 2006)

Coulombic efficiency is among the most important parameters to evaluate the system performance as it directly indicates how EET contributes to microbe respiration. The ideal coulombic efficiency value is of course 100%, meaning anode respiration is the only process undergoes in the MFCs anode and substrate is completely oxidized. Up to now, only MFCs inoculated with pure culture *Geobacter* species and *Geothrix fermentans* fed with simple carbon source as acetate can approach this value.(Bond & Lovley, 2003; Bond & Lovley, 2005; Nevin et al., 2008) Biomass growth apparently reduces the coulombic efficiency and insufficiency substrate oxidizes account for another reason in low coulombic efficiency. The best example of later is *Shewanella oneidensis*, well-recognized anode respiration bacteria due to its multiple EET pathways. MFCs inoculated with pure culture *Shewanella oneidensis* only have coulombic efficiency around 20% (if calculated from lactate to CO_2) with lactate as carbon source. *Shewanella* can partially oxidize lactate to acetate, leaving 8 electrons in the form of acetate which originally derived from lactate.(Lanthier et al., 2008)

Output power density

Power output is another primary parameter that evaluates the MFCs performance beside coulombic efficiency. Harvested power is calculated by:

$$P = I * E_l$$

I is the current passing through the outer circuit and E_l is the voltage measured on the load. In lab works, a constant resist usually works as this load when operated as MFCs, as a result the output power can be calculated as:

$$P = \frac{E_l^2}{R_{ext}}$$

As different MFCs differ in MFCs construction and anode material choice, it is unfair to directly compare the power output by different MFCs systems. Reasonable way is to normalize the power output to anode surface area or the anode volume. The corresponding output power density is expressed in the form of mW/m^2 and W/m^3 . The later one is usually used when MFCs performance were studied as a whole, especially when to consider the real application future of the system. For researchers focus on new method and technology to develop the MFCs system board as a multiple-discipline, power density normalized to the area is favored, as it is easy to compare the results of different works.

Output power density is highly depended on the value of outer resist, maximum output power density may represent the “real capability” in power output for the system. Polarization curve helps to find out this output power limitation. In most MFCs system, polarization curve is done by change the outer resist and record the corresponding voltage on the load. Thus a relationship between MFCs loading voltage and output current density can get (V-j curve). Relationship of

output power density with output current density (P-j curve) can be derived from V-j curve and the maximum output density point can be found in P-j curve.

Energy Efficiency

Energy efficiency is another key parameter that evaluates MFCs system, similar with coulombic efficiency. It is the ratio of energy harvested from MFCs operation to the theoretical energy harvest by directly combusting the biomass to CO₂. It is calculated as following:

$$\epsilon_E = \frac{\int_0^t I * E_i dt}{\Delta H * m}$$

Where ΔH is heat combustion (enthalpy change when biomass combusted) and m is the biomass as influent. (Logan et al., 2006)

1.1.2 Principles of electrochemical analysis in MFCs

The distinguished character of MFCs is that they utilize live microbes as anode catalyst to oxidize the substrate (fuel) and transfer these internal metabolism released electron to the external circuit. As most MFCs either utilize membrane associated cytochrome and conductive pili or soluble small organic molecule to facilitate the electron transfer, side reaction is seldom observed, meanwhile most MFCs adopt non-corrosive material as the anode. As a result, the basic electrochemical process in MFCs can be simplified to the electron transfer from these electron carriers to the electrode. When assuming a surface reaction reaches equilibrium:

Based on surface Nernst equations, the relationship between electrode surface potential (E) and superficial concentration of O and R (Γ_O and Γ_R) can be expressed as:

$$E = E^0 + (RT/nF) * \ln(\Gamma_O / \Gamma_R) \quad (2)$$

Where E^0 is the standard potential of the species, R is the gas constant, T is the experimental temperature and F is Faraday Constant.

When the electrode potential changed under the control of a potentiostat, the above reaction deviates from the original equilibrium state. The potential difference provides a driving force to make the equilibrium shift and responsive current is thus observed. Generally speaking, most electrochemical analysis method involves either observation the responsive current with controlled potential or observation responsive potential change with controlled current.

In linear potential sweep, the potential E is given as:

$$E = E_i + vt \quad (3)$$

Where E_i is the initial potential, v is the sweep rate. If the species is firmly attached to the electrode, the responsive current can write as:

$$i = nFAk_s \{ \Gamma_O * \exp[-\alpha nF(E - E^0)/RT] - \Gamma_R * \exp[(1-\alpha)nF(E - E^0)/RT] \} \quad (4)$$

$$i = -nFAd\Gamma_O/dt \quad (5)$$

Where A is the electrode area, k_s is the rate constant of reaction (1), α is the constant which depends the reaction nature with a value from 0~1. If given:

$$\eta = \exp[nF/RT*(E - E^0)] \quad (6)$$

$$m = (RT/F)(k_s/nv) \quad (7)$$

$$\Gamma_T = \Gamma_O + \Gamma_R \quad (8)$$

Then the current expression can be written as:

$$\psi = i/(F^2/RT)n^2vA\Gamma_T = m[(\Gamma_O/\Gamma_T) \eta^{-\alpha} - (\Gamma_R/\Gamma_T) \eta^{1-\alpha}] \quad (9)$$

Where ψ is a dimensionless value.

From equation (9) we can see that the responsive current is depended on η , Γ_O and Γ_R , meaning the difference in electrode potential and standard potential, the surface concentration of O and R. If the potential is swept from the very negative potential, the electrochemical active species is almost totally in its reduced form in the initial stage. The increase of electrode potential drives the reaction happen and anodic current can be observed. The continuous increase in potential provides higher reaction driving force, thus increase in responsive current. However, the Γ_R decreased as the reaction and decrease in current is thus observed. In this way, peak occurs in the plot which describes the potential and current relationship.

Basic principle of cyclic voltammetry analysis is similar with linear potential sweep. As the most extensively used electrochemical analysis technique, cyclic voltammetry (CV) provides much information about the electron transfer process we are studying in MFCs. The peak location, peak current density, peak separation and their scanning rate dependence are the useful parameters which can get from CV curve. They could provide detailed information about the 1) standard potential of EET related electrochemical active species; 2) the relative abundance of

these species in the electrode and biofilm interface; 3) their electrochemical reversibility and 4) the diffusion type of these species.

The above linear potential sweep and CV analysis are done with non-catalytic assumption, in MFCs that means under substrate depletion conditions. The curve reflects the property of electrochemical active species rather than the MFCs anode performance. To study the catalytic potential of the live microbes in MFCs anode, CV must be done with substrate. With the presence of the substrate, live microbes continuously transfer the electron from internal metabolism to the electron carrier. When the system reaches its equilibrium, the discharge can be described with Nernst-Monod term:

$$j = j_{max} * \left(\frac{S}{K_s + S}\right) \left(\frac{1}{1 + \exp\left[-\eta \frac{F}{RT}\right]}\right) \quad (10)$$

Where j is the current density, S is the substrate concentration, K_s is the apparent half-maximum-rate concentration, $\eta = E - E^0$. This current is contributed by the “catalytic” activity of microbes. When CV analysis is done with the substrate, the responsive current is in fact the combination of “non-catalytic” and “catalytic” part. The most distinguished character is that the former is scanning rate dependent while the latter is not. Usually at medium and high scanning rate (50 mV/s or above), non-catalytic current contributes the main part in the total responsive current while at low scanning rate (1 mV/s or even slower) catalytic current domains. By changing the scanning rate, we can easily observe how different electrochemical active species really contribute to the anode performance.

1.1.3 MFCs configuration and operation conditions

The development in MFCs configuration and operation condition optimization achieved great success in the past decade, which improves the current density by 10000 times.(Franks & Nevin, 2010) Follow text gives a very brief introduction on this point.

MFC configuration

MFCs configuration is of great importance for good performance. MFCs configuration achieved great development in the pasting decade, together with development of whole field. A lot of factors needed to be considered for MFCs configuration design and basic ones attempting at good substrate diffusion and low internal resistance. Schematic 1-2 is shows the basic MFCs configuration with two chambers, single chamber and three chambers, respectively. For two-chamber MFCs, anode and cathode should theoretically be close as possible to reduce the internal resistance. When oxygen is used as the final electron acceptor, the large cathode potential usually accounts for the major potential loss in the system. Increase the cathode to anode volume ratio is a reasonable strategy to improve system performance. Several improved two- chamber MFCs design was brought out then and the most accepted designs are upflow MFCs (UMFC) and tubular MFC (TMFC).(He et al., 2005; Rabaey et al., 2005b)

When cathode solution is eliminated and the cathode directly face to the membrane and back to the air, we get the basic single-chamber MFCs. To avoid the water loss in anode chamber, gas diffusion layer is usually adopted to the air side. Catalysts for oxygen reduction are at membrane face side. Compared with two-chamber MFCs, the elimination of cathode solution reduces the internal resist and more importantly higher oxygen concentration is available now. The drawback is that expensive catalyst is required for oxygen reaction which increased the cost. The membrane can be further removed to assemble membrane-less MFCs. This would greatly reduce

the internal resist by ion transfer through the membrane. Anaerobic condition in anode is guaranteed as the dissolved oxygen is consumed near the cathode.

If anion exchange membrane (AEM) and cation exchange membrane (CEM) are simultaneously used, MFCs develop into desalination MFCs as schematic 1-2 (C). (Cao et al., 2009) When sea water is fed into the middle chamber, chloride anion transferred into anode chamber and sodium cation into cathode chamber due to the selectivity of AEM and CEM at both side. Distilled water is harvested then.

Schematic 1-2 different MFCs configuration. (A) two-chamber MFCs; (B) single-chamber MFCs; (C) middle chamber MFCs for desalination

Temperature

Although most of the lab scale MFCs operation are conducted under room temperature (around 25 °C) or slightly elevated temperature (30-35 °C), the scale up and future application require

that MFCs should work under a broad range of ambient temperature.(Michie et al., 2011a) Thus it is important to study the temperature influence on the MFCs performance and develop new strategy to design MFCs systems available of adjust to wide temperature change in application. Temperature has great influence of microbe activity thus is one of important consideration in conventional wastewater treatment as anaerobic digestion.(Liu et al., 2005a) However, it is interesting to find that when temperature was decreased from 32 °C to 20 °C, very limited performance decrease is observed (only 9% decrease in power density). Polarization curve indicates that even this 9% in power density is mainly due to the decreased cathode catalysts for oxygen reduction at lower temperature.(Liu et al., 2005a) Further study utilizing brewery wastewater as inoculation and pre-acclimation MFCs anode at 30 °C verifies that the mature system has small decrease in performance when temperature drops from 30 °C to 20 °C and reduced cathode performance contributes to the loss.(Feng et al., 2008) Biomass amount study reveals that when MFCs are operated at higher temperature, short start up time is needed and much thicker biofilm could form. However output power capability of MFCs acclimated at 10 °C, 20 °C and 35 °C are similar.(Michie et al., 2011b) Microbial diversity analysis indicates that when acclimated at above temperature, MFCs anode biofilm are composed of psychrophilic, psychrotolerant and mesophilic bacteria, respectively. MFCs acclimated at 20 °C seems can accustomed to the whole temperature change range.(Michie et al., 2011a)

It seems that temperature influence on the MFCs is mainly on the acclimation stage, as the dominant species in anode biofilm. For MFCs with mature biofilm, the temperature influences on the performance are less and the major is the increased cathode overpotential for oxygen reduction at low temperature.

Solution pH and ion strength

For most microbes, neutral pH is favor solution condition and necessary to keep high activity. In MFCs, pH condition is even more important due to spatially separation of cell respiration. Proton derived from the intercellular metabolism has to transfer through the anodic chamber, separated membrane (if PEM or CEM is used) and reaches cathode to complete the reaction to water. At high current density, the rate of proton transferred through the membrane is not comparable to rate generated at MFCs anode, leading proton accumulation in anode chamber and pH increase in cathode chamber. The former results in decreased microbe activity at anode and the latter increase the cathode overpotential for oxygen reduction. Even more serious situation is that with the growing of biomass on the anode, pH gradient forms inside the biofilm and proton diffuse rate between the anode biofilm and anode chamber becomes limitation factor for high current density get.(Torres et al., 2008b) To get a better MFCs performance, high buffer concentration is a good choice but this would greatly increase the operation cost when scale up.

As anode is easy to accumulate proton and oxygen reduction at cathode favors proton condition, many works have been reported on studying the effect of pH on both chamber in MFCs performance. Generally, a slight base anode solution (pH 8-9) would help to get higher current output.(Behera & Ghangrekar, 2009; He et al., 2008; Jadhav & Ghangrekar, 2009; Li et al., 2013a; Nimje et al., 2011) In some extreme cases, the best performed MFCs are operated at pH as high as 9.5.(Puig et al., 2010) An extra benefit for high anode pH is that methanogenesis are inhibited at alkaline conditions, which increase the coulombic efficiency of MFCs. (Zhuang et al., 2010) For MFCs with air-cathode, increase the cathode acidity would greatly reduce the cathode overpotential and increase the power output. (Erable et al., 2009; He et al., 2008; Zhuang et al.,

2010). Thus the theoretically MFCs should be operated under slightly base anode and acid cathode, if aiming at high power harvest.

The contradiction of required charge balance and the in fact proton accumulation at MFCs results in a higher internal resist due to exist of membrane. Former works demonstrate that even for Nafion membranes, which is supposed to support proton transfer, the transfer of other cation like Na^+ and K^+ contributes the more important part for charge balance in MFCs anode.(Rozendal et al., 2006) As a result, increase in the anode solution ion strength would greatly increase the conductivity and thus leads to better MFCs performance. For those MFCs fed with brewery wastewater, extra adding salts like NaCl is an effective way to increase performance.(Li et al., 2013b; Liu et al., 2005a)

Substrate

Substrate is one of the most important factor that influence the power output in MFCs systems.(Liu et al., 2009) The development of MFCs in recent years greatly board choice of the substrate used in MFCs and the potential for industry application is explored due to the use of versatile complex and industry wastewater as feeding.(Pant et al., 2010) In the lab research, substrates with simple structure are welcomed as it is easy to get high and repeatable performance with these simple substrates. Acetate is the most frequently used substrate as it is appeal to the most effective anode respiration bacteria *Geobacter* species.(Bond et al., 2002) MFCs fed with acetate could have very high coulombic efficiency, no matter it is inoculated pure *Geobacter* species or mixed culture. Glucose is another frequently used substrate in MFCs. When mixed culture is inoculated, due to the fermentation and methanogenesis, coulombic efficiency

would be lower. *Rhodoferrax ferrireducens* is exception, with which coulombic efficiency can reach 83% when fed with glucose.(Chaudhuri & Lovley, 2003)

More complex carbon source like cellulose and chitosan are explored their usage MFCs. For use of these complex substrates, anode community must be able to degrade them into simple saccharide for the further anode respiration. (Ren et al., 2007) One interesting application is that extra adding of chitin and cellulose to the sediment MFCs increases the power output in long time period.(Rezaei et al., 2007; Rezaei et al., 2009a)

Real wastewater includes domestic wastewater, brewery wastewater, starching process wastewater and dye wastewater has been used in MFCs feeding. Due to the low ion strength and lack of resistance to pH change, adding buffer solution or other salts are effective method to increase power output.

Anode potential and external resistance

Studying effect of anode potential is of great importance for understanding the MFCs performance. In MFCs conditions, when current is generated, overpotential due to reaction activation and mass transport occurs, leading potential increase in anode and decrease in cathode. From the point of energy harvest, this overpotential represents un-welcomed energy loss. However, for anode respiration bacteria, anode overpotential means the driving force for electron transfer and substrate transport from bulk medium to anode biofilm. Poised the anode potential to constant value is an effective strategy for current generation and studying of bacteria electrochemical behavior at anode.

It is easy to understand that the lower anode potential is the harder is for bacteria to facilitate electron transfer to anode. The increased difficulty in extracellular electron transfer to the anode means inhibited microbe respiration if electrode is the final electron acceptor. Meanwhile low potential increase the anode selectivity to available bacteria as it would be difficult to benefit from anode respiration for those possess electrochemical active species with high standard potential. One extreme example is *Desulfobulbus propionicus*, a sulfur-reducing microorganism whose optimal anode potential is 0.52 V (to SHE). *D. propionicus* is clearly capable of anode respiration, however the dependence on the high anode potential means it is almost no use in the real application for MFCs power generation. Finkelstein conduct an early study on how set anode potential would influence performance the sediment MFCs.(Finkelstein et al., 2006) In their work, three anode potential (-0.058, 0.103 and 0.618 V vs. Ag/AgCl electrode) were chose to enrich acetate reducing bacteria at anode. The most interesting observation of their work is that in all MFCs, the open circuit potential is 20-40 mV lower than the setting. They postulate that is because bacteria adjust their electron transfer strategy based on the anode potential set. Later in acetate fed MFCs, three potential (-0.2, 0, +0.2 vs. Ag/AgCl electrode) were set to study their effect on the MFCs performance.(Aelterman et al., 2008a) MFCs with three chose potential have similar power output after their anode community was acclimated. However, differ in biomass activity was observed. One former review provided a detailed investigation on how anode potential set would influence the MFCs performance.(Wagner et al., 2010) In most publications with mixed culture as inoculation when operated as BES, the higher anode potential usually results in shorter start up time, thicker and more versatile biofilm formation and higher electricity harvest. As the higher anode potential would make the anode respiration much easier. While pure culture like *G. sulfurreducens* were inoculated, the influence of anode potential

mainly focus on the choice of suitable EET pathways chose. The optimal anode potential in those BES varies based on the electrogens they use. Unlike those with mixed culture, higher anode potential not always presents higher performance.

Under MFCs operation, the choice of anode potential is achieved by using proper external resistance. Generally, the smaller external resistance is used, higher current density is harvested, meaning easier for bacteria to use anode to respiration. Higher current in turn leads to larger anode and cathode overpotential, thus increase in anode potential and drop in cathode potential. The influence of potential control and external resistance on the MFCs performance shows some similarity, however difference should be noted. Anode potential control is the straightforward way to influence the anode electron transfer, in the means of suitable electrochemical active species chose and rate control (if electron transfer is rate limited step in the whole anode respiration). External resistance choice regulates the anode performance in a very indirect way. Theoretically, microorganisms which possess completely extracellular electron transfer pathway and adopt electrochemical active species with lower standard potential of cathode reaction could benefit the energy from MFCs operation. Bioelectricity generated when the resistance is connected to MFCs anode and cathode. The smaller external resistance only means low obstacle for higher current density and the representative anode potential is determined by the corresponding current density.

When external resistance is high, the current density is inhibited by the resistance. Decrease in external resistance increase the current density achievement. However, when current density increases to a high level, the substrate transport rate and ion strength together with accumulated proton inside the biofilm suppress the further current density improve. Decrease the resistance

would no further increase the current density.(Gil et al., 2003) For mixed culture inoculated MFCs, the importance of external resistance choice is to regulate the anode community acclimation. When lower external resistance is load, the anode community shows less diversity and mainly composed of anode respiration bacteria, as benefit from electrode respiration is easier under this condition.(Katuri et al., 2012; Katuri et al., 2011) With low resistance, the COD removal efficiency and Coulombic Efficiency are usually higher than with high resistance.(Cheng et al., 2013; Jadhav & Ghangrekar, 2009; Li et al., 2013a; Song et al., 2010) In some works, MFCs acclimated at low external resistance clearly has higher output maximum power density. (Cheng et al., 2013; Katuri et al., 2012) While in some works, although different anode community is observed with different external resistance, the maximum output power density shows no significant difference.(Katuri et al., 2011; Lyon et al., 2010) One works using 10, 50, 250 and 1000 ohm resistance for studying find that the maximum power density is archived at 50 ohm. Further decrease the resistance to 10 ohm reduces the power density. Analysis reveals that at low resistance, biomass yield increased with enhanced EPS secretion, which reduces the electrochemical activity. (Zhang et al., 2011)

1.1.4 The bottleneck and future development

Although MFCs enjoys many advantages compared with traditional wastewater treatment method, there are still bottlenecks to be overcome before it can be utilized in the industry or domestic wastewater treatment.

It is believed that a power density of 1 kW/m³ is required for MFCs to compensate the energy used for operate the wastewater treatment.(Rinaldi et al., 2008) In fact, this power density has already been reached in the lab scale at 2008 and no breakthrough has been taken since then.(Fan

et al., 2007) However, this power density is reached in the lab conditions, meaning that the medium and all the other conditions are in the optimum for bioelectricity generation. When consider the industry or domestic wastewater, factors like the low pH buffer and ion strength will hamper the system performance out of question. The toughest problem that concerns industry or domestic wastewater treatment with MFCs is that most of the main anode respiration bacteria are critical for the carbon source they can use.(Pant et al., 2010) For example, the most powerful anode respiration bacteria *Geobacter* can only use non-fermentable acetate and *Shewanella*, an amphimicrobial bacteria that capable of multiple EET pathways, is prefer lactate as the sole carbon source. In mixed culture inoculated MFCs, glucose fed MFCs have already get the performance comparable to that of acetate fed ones. Community analysis reveals that there exist vast fermentation species that degrade the glucose to non-fermentable acetate for bioelectrogen like *Geobacter* use.(Cheng et al., 2007) When fed with even tough carbon source like starch and cellulose, the performance is greatly affected since the community usually lack the effective strategy to primary digest these carbon source for anode respiration bacteria use.(Rezaei et al., 2009b) How to use the complex carbon source in the real wastewater to facilitate the bioelectricity generation are the key problem lies in the future application and the progress are not cheerful until now.

After ten years of development, MFCs anode performance has reached a considerable level that its lab scale proficiency meets the industry application. For bioelectricity generation, the main bottleneck lies in the cathode reaction.(Rismani-Yazdi et al., 2008) In traditional two chamber MFCs, a chemical cathode (Fe(CN)_6^{3-} , MnO_4^-) or water soluble oxygen cathode are the usually choice. The former ones have the advantages in high reaction rate and low overpotential loss even at high current density, however the disadvantages is obvious as their advantages: these

chemicals cannot be re-oxidized by oxygen in the water and have to be replenished regularly, which is not preferred for long term operation. For the oxygen cathode, suitable catalysts for ORR are required and the cost is commercial used noble metal catalyst like Pt/C is the main concern.(Moon et al., 2006) Studying on the cheap catalyst with comparable effect is thus in urgent need and will be discussed in the later. Another problem concerns this kind of cathode is that the oxygen solubility in water is limited, thus bubbling is often a necessity in the published work and this will increase the operation cost if adopted in the future application. One chamber MFCs that directly use air cathode is another choice for MFCs design, in which the cathode is closely attached to the PEM and catalyst also the gas diffusion layers are fixed on the conductive supporting material in sequence.(Liu et al., 2004) However, the catalyst cost is still high and suitable substitute must be found. The long term stability of this cathode is also the problem due to the leakage of water from the anode.(Cheng et al., 2006) Recent development biocathode which utilize bacteria or enzyme as the cathode reaction catalyst is a good attempt but still there is a long way to go.

Scaling up is another serious problem that concerns MFCs application. The high power density is reached in the lab scale and researcher found that using small chamber MFCs are likely to get higher power density per volume. When scale up, the theoretical OCP of MFC (usually less than 1 V) would obviously not meet the requirement. How to stack the MFCs sequence and parallel with less energy loss is the problem researchers are struggling these years.(Aelterman et al., 2006; Kim et al., 2011; Oh & Logan, 2007) Also we should notice that the conductivity of carbon material (carbon felt, carbon cloth, carbon paper, etc.) can meet the lab scale experiment while would limit the performance when scale up. MFCs cost is always concerns the researchers in the past years. For set up the MFCs, nafion based PEM is in high performance while the cost is also

negligible (\$1000 per m²). Although Pt/C catalyst is effective for ORR in cathode, however, it is impossible to use them in large scale.

1.2 Anode development in microbial fuel cells

1.2.1 Microbial community study and anode respiration bacteria separation

In most sludge inoculated MFCs, it takes several weeks to months to evolve the anodic community to get the system reach its highest performance. Anodic respiration species, or otherwise named exoelectrogens, electrochemically active bacteria and electrigenes benefit great advantages compared with that unable to facilitate anode respiration.(Chang et al., 2006; Lovley, 2006; Rittmann et al., 2008) 16s rRNA analysis for a sludge inoculated MFCs reveals that the bacteria community in the bulk medium and on the electrode surface are greatly distinguished after 160 day's operation.(Rabaey et al., 2004) Anode community varies among MFCs with different inoculum and operation conditions. Proteobacteria (Gram negative) usually consists of major part in the whole community in MFCs although the ratio of α -, β -, γ -, and Δ -proteobacteria are largely different.(Schaetzle et al., 2008) Gram positive bacteria also exist in the whole community although they exhibit very limited capability in bioelectricity generation when inoculated in the MFCs alone.(Milliken & May, 2007) MFCs anode community shows vast variety meanwhile only limit species is observed high EET capacity without the exogenous mediator adding. Meanwhile most species isolated from acclimated MFCs anode have less capacity in power generation compared with the original mixed culture. These facts indicate bacteria in anode community may interact and benefit with each other for better anode respiration. Several reasons may contribute to this improved MFCs performance when microbes “work together”. The first one is that, as we mentioned when we discuss substrate utilization in

MFCs, mixed culture have much higher potential for power generation when complex substrate is fed. Use of electron shuttle secreted by another strain to improve the anode respiration is another benefit proved in the mixed culture MFCs anode.(Pham et al., 2008a; Pham et al., 2008b) Another possible interaction way is by cross-talk quorum sensing in anode community. One possibility lies in *Pseudomonas* strains. The secreted pyocyanin is not only can be used as the electron shuttle for *Pseudomonas* strains and other species in the community, but also up-regulate quorum sensing controlled genes.(Dietrich et al., 2006) Up to now, many studies have been put on the species that are capable of EET without exogenous mediator adding, although the role of most species in the community is still unclear.

Extra added electron shuttle is a necessity in early MFCs. Metal reducing bacteria *Shewanella putrifucens* is the first species that found to generate bioelectricity without exogenous mediator.(Kim et al., 1999) Many strains of this genus are then found their bioelectricity generation capability in mediator-less MFCs. *Shewanella* can use their c-type outer membrane cytochrome to facilitate the extracellular electron transfer to the electrode by direct contact. The confirmation of electrically conductive appendages, known as nanowire, makes the *Shewanella* EET mechanism more complex and debatable.(Gorby et al., 2006) *Shewanella oneidensis* MR-1 was further found be able secreted flavins (riboflavin and FMN) for mediated electron transfer, making it an interesting case for multiple EET pathway study.(Marsili et al., 2008) MFCs with pure culture *Shewanella oneidensis* MR-1 as anode respiration has much lower power density compared with that with acclimated wastewater as inoculation at same MFCs configuration and operation.(Watson & Logan, 2010) Interestingly, by optimization MFCs configuration and operation conditions, MFCs with strain *Shewanella oneidensis* DSP10 achieved a power density as high as 3 W/m² or 500 W/m³, which is comparable to the most effective MFCs systems with

mixed consortia as inoculation.(Ringeisen et al., 2006) *Shewanella* species is a class of facultative anaerobic bacteria, thus MFCs with pure culture *Shewanella* have very different response to the residue oxygen in the anode chamber, comparing to those with strictly anaerobic microbes as inoculation. Under micro oxygen conditions, the coulombic efficiency of MFCs decreases meanwhile the current density and power density is significantly increased. Multiple reasons contribute this interesting phenomenon, including better biofilm formation, increased respiration rate due to the synthesis of NADH with the existence and promoted substrate diversity.(Biffinger et al., 2008; Biffinger et al., 2007; Biffinger et al., 2009; Li et al., 2010b)

Another facultative anaerobic bacterium was isolated from acetate fed MFCs anode and was later nominated to *Aeromonas hydrophila* PA3 based on the 16S rDNA sequence analysis and DNA-DNA hybridization.(Pham et al., 2003) At anaerobic conditions, *A. hydrophila* PA3 can use Fe(III), Nitrate and sulfate as electron acceptor. An interesting observation is that, although the strain was separated from acetate fed MFCs anode, *A. hydrophila* PA3 cannot reduce Fe(III) when acetate is used as the sole substrate and no current increase was observed when acetate is adding. CV analysis reveals that *A. hydrophila* PA3 probably use OM cytochrome to facilitate the EET process, similar strategy observed by *Shewanella*. However, for *A. hydrophila* PA3, the cytochrome CV signal was only observed under anaerobic conditions with the existence of Fe(III). Exposure to the air lead reversibly also inactive its electrochemical activity.

Geobacter genus (*Geobacter metallireducens*, *Geobacter sulfurreducens*) is a group of anode respiration bacteria isolated from the lake sediment.(Bond & Lovley, 2003; Min et al., 2005) *Geobacter* is probably the most effective anode respiration bacteria in pure culture MFCs and its power density is comparable to those with acclimated wastewater. *Geobacter sulfurreducens*

KN400, a variant selected from original *Geobacter sulfurreducens* DL-1, have power density of 3.9 W/m² and is much higher than usually operated with mixed culture.(Yi et al., 2009) Higher electrochemical activity was observed and together with higher conductivity in nanowires. Meanwhile for KN400, the biofilm was thinner compared to strain DL-1 and less c-type OM-cytochromes was confirmed, indicating the increased electricity generation capability by the mutant strain. Later in strain DL-1, gene GSU 1240 which encoding proteins with Pilz domain was deleted and the nominated new strain CL-1 exhibits better biofilm conductivity and increased bioelectricity generation.(Leang et al., 2013) These impressive works provide new insight into the bottleneck of the system performance and gives new inspiration to future works.

Desulfuromonas acetoxidans is another strain isolated from sediment MFCs which belongs to Δ -proteobacteria, mostly from Geobacteraceae family.(Bond et al., 2002) This bacteria is capable of oxidize acetate and transfer electron to the Fe(III) to conserve energy for survive, very similar to *Geobacter metallireducens* and *Geobacter sulfurreducens*. Power density in two-chamber MFCs is 14 mW/m² and adding of anthraquinone-2,6-disulfonate (AQDS), humic acids analog which usually used as the electron acceptor and mediator, increase the power density by 24%.

Desulfobulbaceae is a group of sulfate-reducing bacteria enriched in sediment MFCs which belong to Δ -proteobacteria.(Holmes et al., 2004a) *Desulfobulbus propionicus* is the first sulfate-reducing microorganism that can conserve energy from extracellular electron transfer to solid iron oxide and graphite electrode to support their survive. *Desulfobulbus propionicus* have vast choice for electron donor include pyruvate, lactate, propionate and hydrogen. The maximum current density is 28 mA/m² when lactate is fed. Noticeably, the optimal electrode potential to facilitate *Desulfobulbus propionicus* is 0.52 V (to SHE). The dependence on this high electrode

potential makes it hardly used in MFCs system. Calculated coulombic efficiency was also lower than 25%, thus interest about this bacterium remains in the sediment MFCs mechanism study.

Rhodoferrax ferrireducens, a β -proteobacteria, is another dissimilatory Fe(III)-reducing microorganism separated from subsurface sediment. Unlike *Geobacter* species that can only use simple carbon source and is depended on fermentation bacteria when sugar and more complex substrate is fed, *R. ferrireducens* is capable of fully oxidizing the glucose to carbon dioxide when Fe(III) is used as the solo electron acceptor. Its capability in bioelectricity generation in MFCs is further verified.(Chaudhuri & Lovley, 2003) Current density of 31 mA/m² is achieved and coulombic efficiency is as high as 83%. Similar with *Geobacter* strains, *R. ferrireducens* use OM cytochrome to directly transfer the electron to electrode.(Schaetzle et al., 2008)

Rhodopseudomonas palustris DX-1, phototrophic purple nonsulfur bacteria isolated from acetate fed MFCs anode and belongs to α -proteobacteria, was reported its strong capability in power generation under MFCs conditions in 2008.(Xing et al., 2008) The maximum power density is 2720 mW/m² when brush anode is used, 56% higher than the mixed community in the same MFCs architecture and operation. CV analysis was done both at refresh medium without and with carbon source. Two pairs of peaks were identified which individually centered at -0.4 and -0.35 V (vs. Ag/AgCl electrode). Current was restored immediately after medium refresh, indicating solution medium is not important for current generation. However, the work fails to identify whether the corresponding peaks belong to contact based direct electron transfer or biofilm absorbed water-insoluble mediators. Unlike *Shewanella*, *Geobacter* and *Rhodoferrax ferrireducens* that belongs to dissimilatory metal-reducing bacterium, *Rhodopseudomonas palustris* DX-1 was first known for their ability to generate hydrogen. Meanwhile, *R. palustris*

DX-1 could use a vast range of substrate for current generation, series of volatile acids, yeast extract and thiosulfate.

The concept of extracellular electron transfer is mainly ascribed to gram-negative bacteria.(Pham et al., 2008a) However in some acclimated MFCs system, gram-positive bacteria include *Brevibacilli*, *Enterococcus faecium* and *Clostridium sp.* are the dominant species.(Aelterman et al., 2006; Park et al., 2001; Rabaey & Verstraete, 2005) *Clostridium butyricum* EG3 is the first isolated gram-positive bacterium that is proved to produce bioelectricity when inoculated as the pure culture in MFCs.(Park et al., 2001) The nominated strain exhibits highest similarity (98%) in 16S rRNA analysis and microbe physiology with *Clostridium butyricum*. However, only strain EG3 is observed the direct electrochemical activity in CV analysis and one pair of peaks centered at -0.175 V (to Ag/AgCl electrode) was observed. The irreversibility between the anode and cathode peaks together with the similar peak location as in lead to the postulation that EG3 adopts OM cytochrome for extracellular electron transfer.

Although MFCs are generally operated at neutral or slightly base solution, MFCs with one acidophile, *Acidiphilium cryptum* as anode inoculation was developed and bioelectricity was harvested at low pH ($\text{pH} \leq 4$). (Borole et al., 2008) Iron is used as the electron shuttle and maximum power density is 12.7 mW/m^2 , calculated based on polarization curve. *Acidiphilium cryptum* is temporary the only acidophile capable of electron transfer to electrode.

Pseudomonas is another important genus bioelectrogen in MFCs study. However, their importance is not because of their strong ability in power generation as a pure culture, but of their well-recognized capability to extracellular secretion phenazines, a group of substituted heterocyclic compounds which is originally used as antibiotics and signal molecules for

Pseudomonas species. These phenazines are later proved to improve the electricity generation by mixed culture and gram-positive species *Brevibacillus* sp. PTH1 inoculated MFCs.(Pham et al., 2008a; Pham et al., 2008b; Rabaey et al., 2005a) Metabolite-based mutualism between *Pseudomonas aeruginosa* PA14 and *Enterobacter aerogenes*, with their role in bioelectricity generation was studied in the artificial ecosystem.(Venkataraman et al., 2011) This works gives an interesting glimpse on the complex mutualism in MFCs anode community.

Escherichia coli cannot produce bioelectricity without the adding of extra mediators. However, after long term operation, *E. coli* was evolved at MFCs anode and their direct electrochemical behavior was observed without any extra mediators.(Qiao et al., 2008b) It is demonstrated that self-secreted hydroquinone derivatives is used as the electron shuttle and improved permeability in evolved strains accounts for the improved performance.

In conclusion, versatile microorganisms are found to be capable of facilitate extracellular to support survival and growth under electron acceptor limited conditions. However, there still a lot of work to do to explore the choice anode respiration bacteria. Most of proved anode respiration bacteria are metal reducing bacteria which can naturally reduce the metal oxide in the environment. *Geobacter sulfurreducens* is the most effective electrochemical active microbes up to now. The isolated variant CL-1 proves the unreached end of their capability in anode performance. However the strictly anaerobic requirement partially limits its application. *Shewanella* species represent another potential choice; high power density is got under with optimization of operation condition. However the critical on the substrate choice greatly limit its application and meanwhile the Coulombic Efficiency is too low as under anode respiration condition the lactate metabolism stops at acetate. Work on the individual role of different

microbes in anode community and their mutualism are very limited up to now and only few works using synthetic ecosystem to studying this complex role and relationship.(Venkataraman et al., 2011) Molecular microbiology is very effective tools in bioengineering and related areas. However its application in MFCs only begins in the recent years. These two directions are probably two important futures for anode respiration bacteria studying.

1.2.2 Electron transfer mechanism study in MFCs anode

Under anaerobic conditions, bacteria metabolism can be categories into two main pathways: anaerobic respiration and fermentation. For anaerobic respiration, electron acceptors are needed to balance the charge inside bacteria. Commonly used electron acceptors are NO_3^- , SO_4^{2-} , fumarate and metal oxide like iron oxide and MnO_x . In most situations, MFCs are operated under anaerobic conditions and anode acts as the sole electron acceptor. Unlike those soluble electron acceptors as nitrate and fumarate, bacteria must use elaborative strategies to overcome the physical obstacles, i.e. its nonconductive cell membrane and cell walls, to make the electron from inner metabolism accessible to the solid anode. In MFCs and related systems, which are generally nominated as bioelectrochemical systems (BES), well-recognized extracellular electron transfer (EET) pathways are divided into two groups. Direct electron transfer (DET) pathways utilize outer membrane (OM) associated compound, like c-type cytochrome to facilitate the EET by direct contact to the electrode. Mediated electron transfer (MET) using soluble primary or secondary metabolism achieve the electron transfer from bacteria to electrode .(Rabaey & Verstraete, 2005) Schematic 1-3 illustrates the most basic processes for these two kinds of EET pathways.

Schematic 1-3 (A) DET by cytochrome in MFCs. (B) DET by conductive pili in MFCs. (C) MET in MFCs.

Direct electron transfer (DET) occurs when the membrane bonded electrochemical active species directly attached to MFCs electrode surface. This pathway is utilized by various dissimilatory metal-reducing bacterium include *Geobacter* species (*Geobacter metallireducens* and *Geobacter sulfurreducens*), *Rhodoferrax ferrireducens* and *Shewanella* species (*Shewanella putrifaciens* and *Shewanella oneidensis*), just as introduced in the previous chapter. These dissimilatory metal-reducing bacteria are commonly isolated from marina and lake environment, where accessible electron acceptor is the limitation for microorganism respiration.(Chaudhuri & Lovley, 2003)

Novel strategies are developed by these bacteria to use like iron oxide, which is abundant in

sediment environment, to respire. Among them, the anaerobic respiration of *Shewanella* species with the help of OM c-type cytochromes is well studied and documented by series research articles.(Beliaev & Saffarini, 1998; Myers & Myers, 1997a; Myers & Myers, 1997b) Schematic 1-4 (A) is the general three-dimensional c-type cytochrome with a heme coordinating iron atom. The electron transfer by OM cytochrome is accomplished by the valence change of coordinated iron atom. Anodic current will be observed when Fe(II) changes to Fe(III) and release the electron to the electrode. Previous study using surface enhanced Raman scattering (SERS) confirms that in *Shewanella oneidensis* MR-1, heme represented SERS spectra is only observed when insoluble electron acceptor is used. Heme signal missed when changed to a soluble electron acceptor. AFM observation also confirms that MR-1 membrane surface is more un-even when solid electron acceptor is used.(Biju et al., 2007) The relationship between observed OM cytochrome at *Shewanella oneidensis* MR-1 membrane surface and solubility of final electron acceptor proves that OM cytochromes directly involved in EET to metal oxides or electrode. *Shewanella* is later found to possess conductive membrane appendage, which known as nanowire.(Gorby et al., 2006) Similar with OM cytochromes, *Shewanella* synthesize nanowire in the absence of oxygen, leading to the postulation that these species could also use conductive nanowire to facilitate the EET, as indicated in schematic 1-3 (B). However the actual role of these nanowires in the anode EET remains question and no research is reported that the *Shewanella* biofilm could use conductive nanowire to facilitate the long distance EET as *Geobacter* species.(Malvankar & Lovley, 2012) The metal reducing and bioelectricity capability by mutant lacking genes encoding c-type cytochrome MtrC and OmcA is severely damaged.(Bouhenni et al., 2010; Carmona-Martinez et al., 2011; Myers & Myers, 2001) Schematic 1-4 (B) shows the detailed electron transfer pathways in *Shewanella oneidensis* MR-1.

Electron released from intercellular metabolism is first passed to Menaquinone, a soluble mediator, and then to a periplasmic cytochrome CymA. Reduced CymA will then transfer the electron to c-type cytochrome MtrA and MtrB in sequence and finally to the outer membrane cytochrome MtrC and OmcA. Then the anode will re-oxidize the reduced MtrC and OmcA to complete the EET.(Carmona-Martinez et al., 2011) The involved cytochromes with multi-heme structures are believed to transfer the electron one by one via oxidize and reduce of the central iron chelated in the heme. The electron transfer rate between these cytochromes are less reported but the EET rate between the OM cytochrome OmcA is calculated to be as high as 300 s^{-1} when individually attached on the carbon electrode surface.(Firer-Sherwood et al., 2008) This rate constant may vary in live cell and under different MFCs operation conditions which is greatly related with the electrode properties and reaction microenvironment. DET requires the related OM cytochrome directly contacted with electrode as cytochromes can only transfer the electron for some nanometers. As a result, only the mono layer of bacteria that directly contact on the electrode surface can make contribution to the bioelectricity production. Electrochemical analysis of DET based single layers biofilm attached electrode show catalytic current density is in $\mu\text{A}/\text{cm}^2$, much lower than that in the real MFCs operation (Kim et al., 2002). *Shewanella* are then supposed to be accomplish the long distance DET by conductive pili which work as a metal wire and transfer the electron from reduced OmcA and MtrC to far away anode, as showed in Schematic 1-4 (B). (Gorby et al., 2006; Reguera et al., 2005) This would greatly facilitate long distance EET for those DET based bacteria and increase the bioelectricity generation. However, such long distance electron transfer with conductive pili in *Shewanella* biofilm still lack the experiment support, although the conductive pili have been known for years.

Schematic 1-4 (A) c-type cytochrome with heme coordinated iron atom; (B) electron transfer pathways from inner membrane (IM) to electrode in *Shewanella oneidensis* MR-1, left is the DET and MET via series c-type cytochromes and right is supposed DET and MET via conductive pili

Bacteria that are not directly contact with the electrode surface suffer spatial obstacle for electrode respiration. A smart strategy is to allow certain “carriers” taking the electron from the metabolism and then travelling to the electrode to find a final electron acceptor. This strategy is nominated as mediated electron transfer (MET) pathways. Here the used compound, or electron shuttle, should have proper standard redox potential value to guarantee easily reduced by the bacteria and oxidized by the anode. The idea of use soluble electron shuttle to facilitate the EET is not new to the researchers at all. Before *Desulfuromonas acetoxidans* and *Geobacter metallireduces* proves their capability in bioelectricity generation, researchers already known sediment strain could use humic acids like anthraquinone-2,6-disulfonate (AQDS) as the final electron acceptor or to assist the metal oxide reduction. Adding AQDS directly to the sediment MFCs improve the current production by 24%. (Bond et al., 2002) Early MFCs need extra adding electron shuttle to facilitate the electron transfer. The shortcoming of extra shuttle required

MFCs system is that these shuttles would lose during the MFCs in continuous flow operation, that's greatly increase the operation cost if they are repeatedly added to MFCs. Some shuttles are even toxic to the environment, extra environmental concern arises if they are used in application.

“Mediatorless” MFCs development attracts key focus in the early of this century. Anode respiration bacteria were first found to be capable of DET pathways for anode respiration. Then several strains found to synthesize and secrete the electron shuttle to facilitate the EET. *Pseudomonas* species are found to secrete phenazines (pyocyanin, phenazine-1-carboxamide (PCN), phenazine-1-carboxylic acid (PCA) and 1-hydroxyphenazine (OHPHZ)) to improve their anode electron transfer efficiency.(Rabaey et al., 2005a) In the pure *Pseudomonas* culture inoculated MFCs, extra adding pyocyanin impressively increase the power output and phenazine gene inactive mutants have much low performance compared with wild type, indicating phenazines are directly involved in *Pseudomonas* anode electron transfer. Interesting fact about phenazines is that although *Pseudomonas* strains can use them to facilitate the electron transfer, MFCs with pure *Pseudomonas* species are usually in very low current and power density. These antibiotic based compounds are later find to increase the electricity generation in mixed culture and other pure culture inoculated MFCs.(Pham et al., 2008a; Pham et al., 2008b; Venkataraman et al., 2011) These founding partially gives view on why mixed culture inoculated MFCs have better performance than pure culture. *Geothrix fermentans*, which is the reported organism capable of bioelectricity generation capability out of *Proteobacteria* family, also use self-secreted electron shuttles to facilitate EET. Evidence is that more 50% current drop was observed when anode medium is refreshed and gradually recovered in 10 days operation.(Bond & Lovley, 2005) However, this shuttle is not defined in the reported work.

Shewanella was later found to synthesize and secrete flavins (riboflavin and flavin mononucleotide (FMN)) to facilitate their anode respiration.(Marsili et al., 2008; von Canstein et al., 2008) Later Mtr pathway is found to be essential both in flavin reducing and electrode respiration, meaning flavin based MET in *Shewanella* is also associated with OM cytochromes.(Coursolle et al., 2010) Flavins clearly act as an important supplementary for direct electron transfer from OM cytochromes to electrode by overcome the critical spatial and dynamics requirement for direct electron transfer. Noticeably flavins have much lower standard potential than that of OM cytochromes *Shewanella* used in DET, thus matured *Shewanella* biofilm with electrochemical activity would generate bioelectricity in lower anode potential with the presence of flavins (-0.46 V vs. -0.36 V, to SCE).(Baron et al., 2009) This obviously makes *Shewanella* more adjustable to more board anode potential range and outcome from anode competitions in mixed community.

The multiple EET pathways adopted by *Shewanella* make it an interesting case for EET mechanism study. Schematic 1-4 (B) also exhibit the recognized flavin based MET with OM cytochrome and supposed conductive pili MET in *Shewanella*. The behavior of flavins directly transferring through the outer membrane for reducing is currently not confirmed in *Shewanella*. The accepted MET involves the electron shuttle cycles between microorganism and the electrode, where diffusion and reaction can be individually described to study the bottleneck of whole process. When electron shuttle lose its electron to the electrode it diffuse back to microorganism and then refreshed through membrane bonded compound (situations in *Shewanella*) or directly transported through microbe outer membrane (as suggested in evolved *E. coli*), as indicated in Schematic 1-3 (C). Little observation was provided on whether electron shuttles transport through the membrane is a totally negative diffusion or need certain membrane assisted. Work in

our lab demonstrates that heterologous expression *Pseudomonas* originated membrane transport protein oprF in *E. coli* would increase its bioelectricity generation in MFCs. The protein is found to selective to the mediator added, providing one view on how MET works in anode respiration.(Yong et al., 2013b) Due to the recycle of electron shuttle between microorganism and electrode, one molecule shuttle will transfer more than one electron. As a result small amount of extra adding shuttle to the system will greatly increase the MFCs performance.

Expanded concept of shuttles would include primary or secondary metabolic. Besides secondary metabolic with highly reversible electrochemical activity, another small group belong to primary metabolic also attracts attention in the early study. One of them is hydrogen. The hydrogen based MFCs usually involves the glucose fermentation under anaerobic conditions in the anode and then independent oxidize of hydrogen at anode.(Bullen et al., 2006) The fuel cell reactions here are in fact the same like those in chemicals fuel cells and it is necessary to use special material in the anode to facilitate the hydrogen oxidize. Although these kind of MFCs can get very high power density compared with the former ones,(Niessen et al., 2004) it is still not the preference since the glucose fermentation is far too energy cost.

In conclusion, anode respiration bacteria developed several novel strategies to facilitate extracellular electron transfer. They can be primarily grouped into membrane associated compound based direct electron transfer and soluble shuttle based mediated electron transfer.

1.2.3 Anode material development

The burst development in MFCs could leave out the development of material science. Vast efforts have been made in the past decade to optimize the electrode choice, witnessed by the

larger number of publication on this direction. The most basic consideration for the choice of anode material is to facilitate biofilm formation, excellent structure for mass transfer and favored EET. The choice of anode material is usually considered from following aspects: high conductivity, large surface area, good biocompatibility, not corrosive and also economical for large scale application.(Wei et al., 2011) Versatile kinds of carbon material are applied in most MFCs systems, due to their good conductivity under lab experiment size and excellent biocompatibility. The now utilized carbon material include carbon paper, graphite plate, carbon cloth, carbon mesh, granular graphite, granular active carbon, carbon felt, reticulated vitrified carbon and carbon brush.(Aelterman et al., 2008b; He et al., 2005; Logan et al., 2007; Rabaey et al., 2005b; Wang et al., 2009; Zhang et al., 2009b). MFCs anode can be categorized into plane structure and packed structure based on the anode type. Used carbon paper, carbon cloth and even three-dimensional carbon felt can be viewed as plate structure since the volume ratio of electrode to the whole chamber is very small. The packed structure which is usually consists of granular graphite could have quite high surface area for biofilm growth and can get high volume energy density.(Li et al., 2010a) However, the death corner of the reactor is inevitable and longer term operation can leads reactor clog.(Logan et al., 2007) The plane structure has a limited effective area however is less concerned with diffusion limitation. Most research articles aiming at anode material studying generally use power density nominated to area (actual sometimes actual area like graphite rod, or sometimes project area if the structure is complex) to compare the relative performance among different works. However, power density by volume is more close to the further industry application. High anode surface area/anode volume ratio is an indeed consideration. In this situation, carbon brush anode, which is fabricated from carbon fibers,

stands out among the carbon materials (theoretical surface area is 18, 200m²/m³, using commercially 7.2 μm fiber with 95% porosity).(Logan et al., 2007)

To further improve the anode efficiency, a lot of study is done on the functionalization and modification these carbon materials. Heat treatment of carbon material in the NH₃ gas is one of these successful attempts. When carbon cloth is treated with 5% NH₃ gas at 700 °C, the maximum power density of corresponding MFCs is 20% higher than the untreated carbon cloth and shorten the startup time by 50%.(Cheng & Logan, 2007) The performance improvement is explained due to the increase of surface charge, which makes bacteria easier attached to the electrode and improved the electron transfer rate at high current density (>0.5 mA/cm²). Reflux in the sulfate acid and heat in the air to form carboxyl group is another vast developed carbon material surface treatment method and can increase the power density by 8-25%.(Feng et al., 2010c) Besides surface treatment, coating is another strategy which is rapidly developed in the past years. Versatile materials have been tested and reported include Carbon Nanotube (CNT), graphene, conductive polymer, metal oxide and also soluble mediator. CNT attracts most attention due to their high conductivity and surface area. (Liang et al., 2011; Peng et al., 2010; Qiao et al., 2007; Thepsuparungsikul et al., 2012; Xie et al., 2012a) CV analysis reveals that CNT has the capability to increase the DET by cytochrome by increasing the interaction between cytochrome active site and CNT.(Peng et al., 2010) Conductive polymer like PANI and polypyrrole also attracted vast interest due to their high surface area and different functional group.(Feng et al., 2010a; Li et al., 2011; Qiao et al., 2008a; Yuan & Kim, 2008a; Yuan & Kim, 2008b; Zou et al., 2010; Zou et al., 2008) Community analysis reveals that the anode community is distinguishly changed when using anode modified with these conductive polymer.(Li et al., 2011) Adding electron shuttles could impressively improve the system performance. However,

the shuttle loss in the continuous flow mode hinders its application. Several articles reported the attempt of physically or chemically immobilize the electron shuttles to the electrode surface in improve the electron transfer.(Adachi et al., 2008; Feng et al., 2010b; Park et al., 2000; Wang et al., 2011a) Impressive progress is achieved.

The most recent development in material science application in MFCs cathode mainly focus on the re-design three-dimensional porous material to overcome the diffusion problem in packed anode and poor surface area in plane structure. These materials composed of conductive or non-conductive three-dimensional base with several tens of micrometer porosity and functional material like CNT and graphene for electron collect.(Xie et al., 2011a; Xie et al., 2011b; Xie et al., 2012a; Xie et al., 2012b) The direct construct of three dimensional architecture anode is also tried.(Yong et al., 2012a) This area is just beginning on the way and more progress is anticipated in the near future.

1.2.4 Computer modeling study

Computer modeling study is effective method to access the role of operation parameters in multiple kinds of reactors. During the MFCs development in recent years, some researchers have already tried to build the modeling to study this system. These works are quite impressive and give deep insight into the key bottlenecks about the novel system.

The first MFCs modeling study can date back to 1995. (Zhang & Halme, 1995) This modeling greatly simplify the model description by assume that only bulk medium bacteria involved and the rate limited step in the whole process are biomass degrade, which is ruled by Michaelis-Menten equation. Their modeling is somehow fit with their own experiment data, which is

probably due to high concentration shuttle HNQ is added to the anode chamber. To more accurately access the parameters in MFCs operation, biofilm must be carefully considered.

Piciooreanu and his collaborator published one work in 2007 based on their former biofilm modeling.(Piciooreanu et al., 2007) This model fully considers the mass balance in the anode chamber and a developing biofilm as the MFC operation is described. Also this model assumes the MFCs are MET based and mediator diffusion inside the biofilm is thus the key limited factor as a nature result. This model depicts a system more similar to the real MFCs compared with former work and are fitted with typical published work. At the same year, Rittman and his group member published a developing biofilm based modeling.(Marcus et al., 2007) Their modeling is DET based. The key assumption is that biofilm is totally conductive and bacteria accomplish the EET by transfer the electron among the conductive biofilm network. The key factors that determine the whole system is the biofilm conductivity, potential descending along the biofilm and substrate diffusion. This model is quite fit with those biofilm and DET based MFCs although the key evidence for how bacteria successfully accomplish the EET along the biofilm still not efficient. Further, based on their model, they published several more works which reflect that in the mature biofilm in MFCs anode, the key limitation is the substrate diffusion and their work reflect that biofilm in MFCs anode can as high conductive as 0.5 mS/cm.(Lee et al., 2009; Torres et al., 2008b)

1.3 Cathode development in microbial fuel cells

1.3.1 Material science development in abiotic MFCs cathode

It is believed that a power density of 1000 W/m^3 is required for the application of microbial fuel cells in the industry.(Rabaey & Verstraete, 2005) The reported energy density have already exceeded this value at the lab scale level.(Fan et al., 2007) Although the successful scale up of microbial fuel cells still remains a problem, the combination of experiment and modeling study by Rittman and his cooperator indicates that we are in fact reaching the limit of anode efficiency.(Torres et al., 2008b) Proton acclimation in anode chamber and low solution ion strength, result in the main potential loss in MFCs anode when the current density is high.(Lee et al., 2009; Marcus et al., 2007; Torres et al., 2008a) When MFCs anode is in the research focus, stable and high efficiency cathode is used to better compare the performance of different MFCs anode. Chemical cathode, represented by potassium ferricyanide have very small overpotential even in the high anode current density and is thus preferred for lab use in anode studied.(Rabaey et al., 2004) However, potassium ferricyanide is not sustainable and have to be refreshed once it is depleted, making it impossible for industry application. Oxygen is the most important final electron acceptor in most fuel cells and is used in the MFCs in the form of air-cathode and water-cathode, both of which require an effective chemical catalyst for oxygen reduction reaction (ORR). Cathode for oxygen usually consists of a conductive base material and catalyst for oxygen reduction. Platinum catalysts have already been commercialized and are vast used in MFCs. However, the noble metal used will greatly increase the MFCs set up cost. Meanwhile, due to the complex biochemical component and reactions, noble metal catalysts in MFCs cathode is easily to be contaminated, further increase the operation cost due to the need of cathode catalyst replacement. Thus, noble metal catalyst based air cathode would not be a favor choice for the MFCs industrial applications. Generally, cathode construction and operation cost

account for the major part of total MFCs operation cost and is estimated to be as high as 47%.(Lu & Li, 2011)

Cathode reactions between MFCs and other fuel cells are similar when chemical catalyst is used to facilitate oxygen reduction. However, the power density of MFCs is much lower than other chemical fuel cells ($\text{mW}\sim\text{W}/\text{m}^2$ compared with KW/m^2 based on the anode projected area), meaning more carefully balance between the efficiency and cost is needed. Thus, in the part years, one of the important developments in MFCs cathode is to find cheaper and effective oxygen reduction catalysts to substitute the noble metal catalyst used in former works. Many works have been done in the lab research and the progress is quite impressive and promising. In the earlier years, researchers mainly focus on looking for inexpensive metal or metallic compound to replace the Pt/C catalyst. These substitutes mostly are transition metal compound include MnO_x .(Li et al., 2010c; Liu et al., 2010; Roche et al., 2010; Zhang et al., 2009a) iron compound (FePC , FeAc , ClFeTMMP , PFeEDTA/C)(Birry et al., 2011; Harnisch et al., 2009a; Harnisch et al., 2009b; Saito et al., 2010; Wang et al., 2011b; Yu et al., 2009; Zhao et al., 2005) and CoTMMP (Cheng et al., 2006; HaoYu et al., 2007; Zhao et al., 2005). These published substitutes usually can achieve the performance in the similar level as Pt/C catalysts and the reached power density of $1500 \text{ mW}/\text{m}^2$ is comparable to that of anode efficiency, indicating their possible role as an alternative choice in the future use. The next generation of cathode catalyst for ORR is the so-called metal free catalysts, as its development in other fuel cell study. The already published works involves the use of polypyrrole/C composite, nitrogen-doped carbon nanoparticle and nitrogen-doped graphene.(Feng et al., 2011a; Feng et al., 2011b; Yuan et al., 2010)

1.3.2 Development in biocathode.

Besides the effort to synthesize cheap chemical catalyst for ORR as the substitute of expensive metal catalyst, a lot of works have been done to develop the MFCs cathode with biocatalyst, composing a so-called biocathode. The development of biocathode greatly extends the concept and application potential of MFCs, result in more versatile system capable of bioelectricity generation with more other functions.

Similar with MFCs abiotic cathode, oxygen is the most frequently used electron acceptor in MFCs with biocathode. Based on the “biocatalyst” type, MFCs’ biocathode could generally divide into two groups which individually use enzyme and live microorganism as cathode catalyst. Laccase, an efficient oxygen reduction enzyme has been used as the cathode catalyst to facilitate oxygen reduction in MFCs system.(Schaetzle et al., 2009) The power density is 10 times of MFCs that use Platinum based catalyst as cathode when mediator facilitate the electron transfer between electrode and Laccase is used. The improvement power density mostly benefits from increased open circuit potential when enzyme is used (1.1 V vs. 0.8 V), as the oxygen reduction, Laccase has much smaller overpotential than Platinum catalyst. Although the efficiency is high, the cost and short durability of enzyme based cathode not favored for application. Better choice is to display the active enzyme at the surface of microbes, which would greatly reduce the cost, increase the system duration with the keep of high performance. However, only limited works are reported on successfully development such kind of novel systems.(Fishilevich et al., 2009)

Using microbes to catalyze the oxygen reduction at MFCs cathode achieved great progress in the recent years. Both pure culture and mixed culture biocathode for oxygen reduction are designed

and reported. Several metal oxidizing bacteria have been proved their role as biocatalyst to reduce oxygen reduction in the cathode. Schematic 1-5 (A) shows the basic oxygen reduction process by *Leptothrix discophora* in the presence of Manganese compound as mediator, which is the first report on this topic.(Rhoads et al., 2005) When electrons reach cathode, mineralized MnO_2 accepts two electrons in sequence and first reduced to MnOOH then to Mn^{2+} . Mn^{2+} is further re-oxidized by the *L. discophora* with the presence of Oxygen. The concept of using Manganese bio-recycle for cathode reaction was further verified by the re-design of sediment MFCs to power wireless sensor in which corrosive Manganese cathode was used.(Shantaram et al., 2005) Later similar system is developed using ferrous-oxidizing bacteria *Acidithiobacillus ferrooxidans* to reduce the oxygen in MFCs cathode in the assist of ferric ion. The report work demonstrates that the iron oxidizing rate by the microorganism is fast enough to support MFCs current density as high as 4.4 A/m^2 .(Ter Heijne et al., 2007)

Sediment MFCs (as indicated in Schematic 1-5 (B), (Lowy et al., 2006)) are probably among the first MFCs with mixed community biocathode. Microbe on the cathode was found to improve the oxygen reduction rate.(Hasvold et al., 1997) Removal the biofilm on the cathode from sediment MFCs significantly reduces the system performance.(Bergel et al., 2005) Later, biocathode achieves fast development with sludge and wastewater as inoculation.(Clauwaert et al., 2007b; Freguia et al., 2008; Rabaey et al., 2008; You et al., 2009; Zhang et al., 2008a) Compared with chemical catalyst, the overpotential for oxygen reduction for those well-developed biocathode is even lower, indicating microorganism is very good “catalyst” for reduction.(Chen et al., 2008; You et al., 2009) Compared with one-chamber MFCs with chemical catalyst, oxygen solubility in biocathode is a severe problem for high current density get, thus aerostation is always needed, increasing its operation cost.

Schematic 1-6 (A) Biocathode use *L. discophora* for oxygen reduction, Manganese as electron shuttle; (B) Sediment MFCs

As that in MFCs anode, microorganisms at biocathode are believed to facilitate the electrode electron transfer by direct and mediated pathway.(Huang et al., 2011a; Sharma & Kundu, 2011). However, the pathways for electron transfer out and transfer in maybe different, as that in *Geobacter*.(Dumas et al., 2007) Meanwhile the way microorganisms benefit from the oxygen reduction is not clear as that of with anode respiration. Interestingly, most of the reported biocathode for ORR that are stable in long operation are autotrophic nutrition, meaning no extra carbon source is added in the cathode medium.(Huang et al., 2011a; Liu et al., 2011; Zhang et al., 2012) Using anode reflux to feed the biocathode reduces the system performance, due to the excessive COD lead to an aerobic heterotrophic biofilm.(Behera et al., 2010) Different from that in anode, biofilm in cathode with better performance seems should be thin and stack, thus the electrode material choice is also different with MFCs anode.(Behera et al., 2010) Stainless mesh is the most frequently used biocathode material and large surface area material brush anode also proved their value.(Huang et al., 2011a) Generally, although biocathode performance has been

greatly improved, fundamental knowledge about electron transfer mechanism and microbe physiology still lacks. More works on the biocathode is anticipated in the future.

In conclusion, for oxygen reduction at MFCs cathode, noble metal based catalysts are with high efficiency but expensive. Feasibility of one-chamber MFCs with cathode directly face to the air eliminates the requirement of aeration as in general aerobic bioreactors. Enzyme based catalyst could have high efficiency but the cost and durability remains problem. Microorganism biocathode is cheap for startup and could have high efficiency after successful acclimation. However, aeration is need which greatly increase the operation cost.

1.3.3 From MFC to MXC: explore potential applications with bioelectricity

The concept of MXC is developed with MFCs expanded its function from bioelectricity generation to more applications like bioremediations, desalination and bioproduction.(Harnisch & Schroder, 2010) “X” has different meanings when the system is developed for different purpose. Most of these MXC systems share similar anode with that of MFCs and the main function is to degrade the substrate and release the electron to the electrode. Most of these “X” function is accomplished in the cathode. Different with MFCs which current is spontaneously generated due to potential difference between cathode and anode, potential control is usually adopted in MXC to control and sometimes provide the power to drive the reaction happen. In the early studies, chemical catalysts were used in the cathode to facilitate the different purpose. The development of biocathode expands the catalyst choice for those MXCs cathode and brings new development in the related area. The following part gives brief introduction about bioremediation and bioproduction in MXC systems.

1.3.3.1 Bioremediation in MXC

Bioremediation is biocatalyst assisted contaminant remove process. The flexibility of using electrode both as electron acceptor (anode) and electron donor (cathode) making MFCs are ideal choice for versatile contaminants removal. Bioremediation in anode mainly involves degrade stubborn and environmental toxic substrate as electron donor. Petroleum contaminants have been reported to be degraded by MFCs for electricity generation, with a maximum power density 120 mW/m^2 .(Morris & Jin, 2008) Further work verifies that MFCs greatly improve diesel degrade rate compared with the open circuit operation conditions, indicating the importance of extracellular electron transfer.(Morris et al., 2009) Applying U-tube MFCs improve the petroleum hydrocarbons degrading rate by 120%.(Wang et al., 2012) Besides petroleum contaminants, phenol was also successfully used in MFCs as the solo substrate and power density of 9.1 W/m^3 was achieved.(Luo et al., 2009) Similar with diesel as fuels, MFCs operation condition greatly improve the phenol degrade rate compared with open circuit cell conditions. Extra adding glucose as the substrate improves the maximum power density to 28.3 W/m^3 . Azo dyes are also successfully degraded in MFCs as sole substrate.(Sun et al., 2009)

Although there is increasing development in MFCs anode for bioremediation, basic anode working mold limits its application, especially for those contaminants that are in oxidized form and require reducing force to remove. MFCs cathode thus provide an ideal choice supplementary choice, primary using these contaminants as the substituted electron acceptor of oxygen.

Oxygen is good choice for abiotic MFCs cathode due to its abundance and relatively easy to reduce. Nitrate have high redox potential (0.73 V , from nitrate to nitrogen gas) as oxygen reduction. Meanwhile nitrogen remove is an important issue due to the environment concern.

First report on MFCs with nitrate reduction in the cathode is probably in sediment MFCs system.(Gregory et al., 2004) Publication number within this effort is rapidly increasing from 2007 when Clauwaert reported their work on successfully combine power generation with denitrification (NO_3^- to NO_2^- , NO, N_2O and N_2). (Clauwaert et al., 2007a) Complete nitrogen removal with nitrification (NH_4^+ to NO_3^-) and denitrification in MFCs was also studied by using a loop configuration.(Virdis et al., 2010; Virdis et al., 2008) Although the detail mechanism of nitrogen remove is still not clear, the remove efficiency in MFCs system is believed satisfactory.(Lu & Li, 2012)

Heavy metal reducing is another group of contaminants remediated by MFCs biocathode. *Geobacter* has been found to remove the uranium contaminants in underground water by reducing soluble U(VI) to insoluble U(IV).(Gregory & Lovley, 2005) The reduced U(IV) compound is stable on *Geobacter* harbored electrode until re-exposure to oxygen. 87% of the reduced Uranium in the underground water is recovered on the electrode. Perchlorate, dichromate and chromate are also reduced on the biocathode similar way as U(VI).(Butler et al., 2010; Tandukar et al., 2009; Wang et al., 2008)

1.3.3.2 Bioproduction in MXC cathode

Although performance have been greatly increased in the past years in lab scale. Targeting at bioelectricity as the main purpose still remains a not favor choice for future application. The expanded research using MFCs as the basic platform greatly boarded the application potential in related system. Among them, producing valuable chemicals seems promising.(Harnisch & Schroder, 2010)

Hydrogen is one of such promising products generated from MXC systems. Early MXC system for hydrogen production adopt bioanode for substrate degrade and electron generation, working with platinum catalyst harbored cathode in buffer solution.(Liu et al., 2005b) The role of microorganisms in this system is to provide supplementary energy for hydrogen recovery. This microorganism assisted process reduces the energy cost compared with direct electrochemical reducing for hydrogen production and proves potential effective strategy to the supplement to direct glucose fermentation for hydrogen. Following work demonstrates that with proper acclimation strategy, microbial biocathode could facilitate the hydrogen production when the potential is poised at -0.7 V.(Rozendal et al., 2008) The corresponding current density is 1.2 A/m² while the abiotic cathode which works as the control only have a current density of 0.3 A/m². This verifies the role cathode microbes play in the hydrogen and eliminating of noble metal catalyst greatly brings up its application potential.

The discovery that acetogenic strain *Sporomusa ovata* could reduce CO₂ to acetate with the assist of electrode opens the door and interest to carbon dioxide immobilization at biocathode.(Nevin et al., 2010) 85% of the electron input from the cathode is turned into the final product, indicating very high turnover efficiency. The impressive advantage in using microorganisms to reduce CO₂ is that much smaller overpotential is required to drive CO₂ reduced to acetate compared with chemical catalyst assisted carbon dioxide reduction (-0.4 V vs. -0.7 ~ -2 V).(Kapusta & Hackerman, 1984; Rasmussen et al., 1990) More important about this work is that acetate is formed from acetyl coenzyme A, which is a central intermediate for bioproduction of various compounds, implying vast potential for biosynthesize more complex carbohydrate in the future. Further works found that more strains include *Clostridium ljungdahlii*, *Clostridium aceticum*, and *Moorella thermoacetica* which belong to acetogenic microorganism could facilitate the CO₂

reduction by consuming the current.(Nevin et al., 2011) Mixed autotrophic community was later developed in which CO₂ is used the sole carbon source. Acetate accumulated to a concentration of 28.5 mM after 12 days operation, demonstrating its potential use in the future.(Marshall et al., 2012)

In conclusion, bioremediation and bioproduction represent two application directions for MFCs and its derivative systems. Study on the attempt to directly use the bioelectricity generated from MFCs will continuous, while more efforts would be placed on more promising field like CO₂ immobilization with electricity and microorganisms.

1.2 Overall Objective

After decade's development, MFCs achieved great improvement in both anode and cathode performance and their application potential. However, there is still much work need to be done and to expand the system with new concept and deeper understanding. Material science development and reactor operation optimum tremendously increase MFCs performance compared with early years. Our attempt here would mainly focus on the exploration of new concepts, designs and studying the combination of different strategy improved MFCs anode efficiency. The left part of this manuscript is organized is as following.

Chapter two provide basic information about the chemicals and materials used in the experiment.

Chapter three to Chapter six are four individual works which are separately described by introduction, method, result & discussion and conclusion. In Chapter three, we describe an artificially prepared biofilm using graphite particle, polypyrrole (PPy) to chemical immobilize *Shewanella oneidensis* MR-1 onto carbon cloth electrode. The enhanced MFCs performance of

this artificial biofilm is studied. In Chapter four we use nitrogen doping carbon nanosphere to modify carbon cloth and use them as MFCs cathode, the doping material proves not only increase the direct electron transfer as that with other carbon material, but also exhibit high flavins retention capability which increase the mediated electron transfer in *S. oneidensis* as well. Chapter five is work with *Pseudomonas aeruginosa* PAO 1. Two global regulator gene knock out strains are used and their corresponding MFCs behavior are studied after the “random mutant”. From Chapter four and Chapter five, we confirms that soluble mediator greatly facilitate extracellular electron transfer and bioelectricity generation is *S. oneidensis* while for *P. aeruginosa*, although the capability of soluble mediator phenazines synthesize and secretion is high, could not effectively use these mediators for electron transfer. We successfully develop an ecosystem consist of *S. oneidensis* and *P. aeruginosa*. By optimization the medium condition, we find that both cultures co-exist in the anode when phenazines synthesize by *P. aeruginosa* is partially inhibits. Meanwhile, MFCs with this mixed culture has a maximum power density of 523.5 mW/m², which is almost 10 times of the pure culture *S. oneidensis* inoculated.

Chapter Two: Chemicals, Materials and Instruments

2.1 Chemicals and mediums

Proton Exchange Membrane Nafion 117 (PEM): Gas Hub, Singapore

Carbon Cloth: Gas Hub, Singapore

LB Broth: BD Inc.

LIVE/DEAD BacLight Bacterial Viability Kits L7012: Molecular Probes, Inc.

Pyrrole: Sigma Aldrich, Singapore. Pyrrole is distilled before use.

Other chemicals are purchased from Sigma Aldrich, Singapore or Merck, Singapore and directly used without further purification.

LB medium: 25 g LB broth (containing 10 g peptone, 5 g yeast extract and 10 g NaCl) is dissolved in 1L DI water and sterilized for 20 min at 121 °C.

M9 salt medium (per liter contains): 8.8 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3 g KH_2PO_4 , 0.5 g NaCl, 1 g NH_4Cl . Sterilize as LB broth. Extra adding 1mM MgSO_4 , 0.1 mM CaCl_2 and carbon source before use.

SM1 medium (per liter contains; used in Chapter six): 30 ml 1 M HEPES (Sigma-Aldrich), 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. Sterilize as LB broth. Extra adding 0.5 mM CaCl_2 , 10 ml Wolfe's vitamin, 10 ml Wolfe's mineral, 10 ml amino acid solution and 18 mM sodium lactate (carbon source) before use.

Wolfe's Vitamin (per liter contains): 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine HCl, 5.0 mg riboflavin, 5.0 mg thiamine, 5.0 mg nicotinic acid, 5.0 mg pantothenic acid, 0.1 mg cyanocobalamin, 5.0 mg p-aminobenzoic acid, and 5.0 mg thioctic acid. Filter with 0.2 μ m membrane.

Wolfe's Mineral (per liter contains): 2.14 g nitrilotriacetic acid (NTA), 0.1 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.3 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.17 g $\text{CoCl}_2 \cdot \text{H}_2\text{O}$, 0.2 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mg $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 5mg of H_3BO_3 , 0.09 g Na_2MoO_4 , 0.11 g $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, and 0.02 g of $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$. Filter with 0.2 μ m membrane.

Amino Acid solution (per liter contains): L- arginine HCl (20 μ g/ml), L-glutamine (20 μ g/ml), and DL-serine (40 μ g/ml). Filter with 0.2 μ m membrane.

Concentrated Sodium lactate solution: lactic acid is dissolved in DI water and neutralized to pH=7 using 1M NaOH. The final storage concentration is 900 mM and is diluted for 50 times when used in MFCs.

MFCs cathode solution: The cathode medium used here consists of 50 mM $\text{Fe}(\text{CN})_6^{3-}$, 50 mM K_2HPO_4 and KH_2PO_4 . The storage cathode solution is 3 times concentrated than the used one.

2.2 Instruments

MFCs configuration: all of the work here use a two chamber MFCs manufactured with acrylic. Two sizes chambers are used. Large one has an inner size of 6 cm * 6 cm * 5 cm for each chamber and effective volume in the MFCs operation is 140 ml. Small one have an inner size of 4.5 cm * 4.5 cm * 4 cm for each chamber and effective volume is 50 ml. Anode and cathode is

separated by PEM with circle cross section (diameter of 2.5 cm). O-ring with suitable size is used between the anode and cathode to prevent the water leakage.

Electrochemical Workstation and electrode: CHI 660D electrochemical workstation (CHI, China) is used to do electrochemical analysis when needed. Saturated Calomel Electrode (SCE, 0.207 V and 0.243 V vs. SHE, which will be separately indicated in the corresponding chapter) and platinum electrode are also bought from CHI, China.

Field Emission Scanning Electron Microscope: FESEM, JSM-6700F-FE-SEM, Japan.

Fluorescence Confocal Scanning Microscopy: Zeiss LSM 510 Meta confocal microscope, Japan

Four Probe Measurements: Systems consist of Summit series probe station (Cascade Microtech, Beaverton, OR, USA), E5270B semiconductor analyzer (Agilent, Santa Clara, CA, USA) and optical microscope (Olympus BX51, QES. PTE LTD., Singapore).

High-performance liquid chromatography: Agilent HPLC/DAD, 1100 series.

Column for HPLC analysis: Agilent Eclipse XDB-C18, 5 μm , 4.6 * 250 mm.

2.3 experiment preparation

Carbon Cloth Electrode: the purchased carbon cloth is first immersed in the acetone overnight and washed with DI water repeatedly; then these carbon cloths is immersed in the 1 M HCl overnight again and washed with DI water repeatedly. The clean carbon cloth is then dry in the oven at 60 °C. The treated carbon cloth is cut into well-defined size and connects to copper wire. Epoxy resin glue is then used to seal connection part to separate the wire from water.

PEM treatment: PEM is put in 0.5 M H_2SO_4 water solution overnight and water with vast sterilized water before use.

Fuel Cells chamber treatment: Clean chamber is immersed into 1 M HCl water solution overnight. Water with vast sterilized water and then put into for biosafety cabinet with UV sterilize for more than 1 hour.

Chapter 3: Conductive Artificial Biofilm Dramatically Enhances Bioelectricity Production in *Shewanella*-Inoculated Microbial Fuel Cells*

Abstract

In this work, we developed a novel strategy to electrochemically immobilize electrogen, *Shewanella oneidensis* MR-1, into graphite and polypyrrole (PPy) matrix, forming conductive artificial biofilm (CAB). MFCs with this CAB as anode achieved a maximum power density of 207 mW/m², which was almost 12 times of MFCs with natural biofilm grow on the carbon cloth.

3.1 Introduction

Biofilm, as a structured microbial community adhered to MFCs' anode, plays a determinant role in the EET via two categories of mechanisms: direct electron transfer by outer membrane cytochromes and/or conductive pili, and mediated electron transfer by soluble mediators.(Bond & Lovley, 2003; Bond & Lovley, 2005; Chaudhuri & Lovley, 2003; Marsili et al., 2008; Reguera et al., 2006; Schroder, 2007) Biofilm can facilitate bacterial electron transfer efficiency in MFCs primarily due to several reasons, including much higher biomass densities within biofilm than in planktonic cells and higher bacteria viability due to anode respiration.(Bakken & Olsen, 1983; Bond & Lovley, 2005; Read et al., 2010b) However, natural anodic biofilms usually have limited thickness varying from several to tens of microns, which are mainly caused by the diffusion limitation of nutrients and insufficient interaction of bacteria with anode materials.(Lee et al., 2009; Nevin et al., 2008; Read et al., 2010b) Therefore, optimizing morphology and thickness of the anodic biofilms to increase the power output would be a preferable strategy in engineering MFCs. However, altering biofilm structures is not a trivial task, because the underlying mechanisms of biofilm formation of many anode respiration bacteria and microbial consortia

remain ambiguous. Efforts were thus made recently on the fabrication and functional decoration of anode materials to enhance anode performance.(Chen et al., 2011; Xie et al., 2011a; Zhao et al., 2010) For example, several 3-dimensional mesoporous anode materials were developed to allow for an efficient diffusion of nutrients and enhance biofilm growth into the deep inside of electrode matrixes.(Chen et al., 2011; Xie et al., 2011a; Zhao et al., 2010) However, the limitation of naturally occurring biofilms might impede further improvement of energy output of advanced materials in MFCs.

Herein, we employed a strategy of electrogen immobilization to construct a conductive artificial biofilm (CAB), which offered several advantages over natural biofilms, including facilitated EET. *Shewanella oneidensis* MR-1, a dissimilatory metal reducing bacterium, was used as a model electrogen.

3.2 Experiments

3.2.1 Bacteria culturing and CAB preparation

Shewanella Oneidensis MR-1 (*S. Oneidensis*, MR-1, got from Max-Planck-Institut für Terrestrische Mikrobiologie, Germany) was used as the anode respiration bacteria. After incubated in LB broth for 12 hours at 37 °C, the bacteria was inoculated in 120 ml LB broth containing 1.2 g graphite at 37 °C, 210 rpm for 24 h (OD₆₀₀=2 when no graphite incubated together). They were then kept at rest (without shaking) at 37 °C for 6 hours, allowing further attachment of bacteria to the graphite powder. Finally, they were harvested by centrifuge at 9000 rpm for 5 min.

After centrifuge, the precipitate was redispersed in 3 ml of medium to obtain a mixture of graphite and bacteria. To prepare the CAB, 100 μ L of the composite was spread onto the unsealed side of the carbon cloth and medially dried under room temperature. Polypyrrol (PPy) was then electrochemically polymerized onto the composite of bacteria/graphite from a solution containing 0.2 M pyrrole and 0.1 M PSS under the constant potential of 0.8 V (*vs.* SCE, +0.209 V *vs.* SHE) and 0.5C electricity quantity except that for the outmost layer where 2C was used. These processes were repeated to fabricate CAB with different thickness. As for the CAB-1 in the text, it was fabricated for one time with 100 μ L composite and 2C electricity quantity in total; for CAB-2 was fabricated for three times of electrical polymerization processes with 300 μ L composite and 0.5C+0.5C+2C electricity quantity in total; similarly, CAB-3 was fabricated with five times' electrical polymerization processes. The PPy monomer solution was purge with pure nitrogen for 15 min and filtered with 0.2 μ m membrane before polymerization.

After the CAB fabrication, part of the CAB is cut off for FESEM measurement. Final anode area is 1.5cm², which is used to calculate the current density and power density in the text.

3.2.2 MFCs operation

Two chamber MFCs with size of 6 cm * 6 cm * 5 cm is used here. Cathode solution is described as in the Chapter Two. Mixed anode medium is used with M9 salt medium and LB broth in a ratio of 9:1. Extra 18 mM sodium lactate is added as carbon source. The MFCs is operated under batch mode and Medium is refreshed when the output voltage drops to as low as 50 mV, which indicate the depletion of anode nutrient.

3.2.3 CAB and biofilm characterization

To get the SEM image of anode after operation, a small piece of modified and un-modified carbon cloth after MFC operation is peeled off and dipped into 1% glutaric dialdehyde solution overnight to immobilize the bacteria on the electrode surface. Then it is rinsed in gradient ethanol solution with a concentration of 30%, 50%, 60%, 70%, 80%, 90% and pure ethanol every 10 minutes.

Clean and treated carbon cloth, graphite, bacteria/graphite composite and CAB were observed with a JEOL field emission electron microscope before and after the MFC operation.

To investigate the viability of bacteria in the CAB after the MFC operation, the CAB-2 was cut into several pieces with same size. One piece was directly used, and a certain layer of the composite material was removed from the surface of CAB-2 to expose inner layer of CAB. The samples were dyed with the LIVE/DEAD BacLight Bacterial Viability Kit which labels live cell with green color and dead cell with red color and proceeded to confocal microscopy observation with Zeiss LSM 510 Meta confocal microscope.

The conductivity of the CAB was measured using a four probe measurement system, which consisted of Summit series probe station and E5270B semiconductor analyzer Result was calculated using the uniform thin sheet model:

$$\rho = \frac{V}{I} * \frac{\pi t}{\ln 2}$$

where t was the thickness of the CAB and approximately be determined to be 0.45mm under optical microscope measurement.

3. 3 Results and Discussions

3.3.1 CAB construction and characterization

As shown in Fig. 3-1 (A), the CAB was designed as a conductive matrix interlocked with the conductive polymer chains of polypyrrole (PPy), within which *S. oneidensis* was wrapped with micro-sized graphite (< 20 μm in diameter). Thus, electrons generated by the *S. oneidensis*' metabolism of nutrients can easily access and be transferred to either the neighboring graphite particles or the PPy polymeric chains by EET (purple arrows, Fig. 3-1 (A)), *i.e.*, via either c-type cytochromes (direct contact-based EET) or riboflavin (shuttle-mediated EET). Subsequently,

Fig. 3-1 (A) Schematic of the CAB. Red arrows indicate the possible electron transfer pathways from the CAB to electrode. Black color represents the graphite particles. (B-C) FESEM images of the CAB. (C) is the magnification of the framed area in (B). Arrow in (C) shows the conductive PPy polymer chains on the cell membrane.

electrons transferred through the CAB conductive matrix to the carbon cloth anode (red arrows in Fig. 3-1(A)). To construct the CAB, graphite particles were firstly incubated with *S. oneidensis*, following by centrifugation to obtain the graphite and bacteria composite. This composite was then spread onto carbon cloth and dried. Subsequent electrochemical polymerization of PPy was performed to form the CAB (see details in the method). Optimizing the immobilization procedure (*e.g.*, controlling the degree of PPy polymerization, the ratio of graphite and immobilized cells), the thickness and morphology of the CAB can be controlled to facilitate nutrients' diffusion into the interior of the biofilm with less resistance. Such immobilization of electrogens formed a mesoporous structure of conductive matrixes (a 3D artificial biofilm), providing a large number of electron transfer channels between electrons generated by bacteria and the anode.

Field emission scanning electron microscopy (FESEM) images of the chemically treated carbon cloth (Fig. 3-2 (A)) and graphite particles (Fig. 3-2 (B)) showed no impurities on surfaces. After the incubation of *S. oneidensis* with graphite particles for 24 hours, *S. oneidensis* attached to the graphite surface and grew on top of it (Fig. 3-2C). Fig. 3-1 (B) and 3-1 (C) showed the CAB morphology, in which *S. oneidensis* covered almost the entire surface area of the graphite particles with a high density. Thin PPy fibers stretching out from the cell membrane were observed (Fig. 3-1 (C)), providing the possible electron transfer pathways. The CAB exhibited a number of microchannels (Fig. 3-2 (D)), providing nutrients' exchange tunnels for the bacteria deep inside the CAB, which is favorable for the nutrition diffusion and the viability of cells inside the CAB.

Fig. 3-2 (A) FESEM image of carbon cloth, (B) graphite, (C) graphite and bacteria composite after centrifugation, and the cross section view of the CAB-2 before (D) and after (E) the MFC operation.

3.3.2 MFCs performance

To test the electrochemical performance of the CAB in MFCs, the CABs with various thicknesses were prepared. The CAB 1-3 had the thickness of 200 μm , 450 μm and 600 μm respectively while sharing the same density of immobilized cells. The MFC with a natural biofilm grown on carbon cloth was considered as a control. All the four MFCs were operated under the batch mode with an external load of 2 $\text{k}\Omega$. To test the long-term behavior of the CABs, the MFCs were run for three cycles (*i.e.*, two times of anode nutrient depletion and refresh new medium, 630 hours in total). Medium was refreshed when the voltage dropped off to a low level, as indicated by the red arrows in Fig. 3-3 (A). After two cycles, 18 mM sodium lactate was added to the anode chamber to keep the performance of the MFCs for the subsequent polarization measurement of the CABs. Fig. 3-3 (A) shows the voltage discharge profiles (V vs. t)

of the four MFCs, respectively. In the first cycle (*i.e.*, from MFC start-up to nutrients depletion, 230 hours), the carbon cloth with a natural biofilm generated output voltage of 28 mV, while the output voltage peaks of the CAB 1-3 were 250 mV, eight-fold higher than that of the natural biofilm. After medium refresh at 230 h, the MFCs with the CAB 1-3 immediately recovered their output voltage, while 80 hours was taken for the control. Reasons for this difference may lie in that bioelectricity generation in MFCs with CABs is not highly dependent on the soluble mediators (riboflavin) synthesized by *Shewanella*. (Bond & Lovley, 2005; Rabaey et al., 2007) PPy polymer chains might have a stronger Van der Waals interaction with the flavins due to the attraction between the aromatic rings of PPy and the conjunctive heterocycle of flavin, resulting in higher flavin retention after medium refresh.

To better understand the MFCs' performance, we measured the polarization curves of these MFCs (*i.e.*, the output voltage (V) and the power density vs. current density (j), Fig. 3-3 (B)) at 460 hour. The polarization curves of the MFCs showed that the CAB 1-3 had an output power density of 111, 207 and 180 mW/m², separately, while the natural biofilm had only 18 mW/m². About 11 times' improvement of power output of the CAB was achieved over the natural biofilm. Calculated from the slope of linear part of the V-j curves, the CAB 1-3 exhibited a very similar total ohmic resistance of 4.6 k Ω ; however, the natural biofilm showed an ohmic resistance of 49 k Ω . Four probe measurements revealed that after the MFC operation the CAB had a conductivity of 3.2 mS/cm. According to the ohmic law $R = l / \sigma S$, where S is the surface area of the CAB (1.5 cm²) and l is the thickness of the CAB (~0.45 mm according to microscopic images), the ohmic resistance (R) of the CAB was 9.6 Ω , much smaller than the total ohmic resistance of the CAB. Such high conductivity of the CAB suggested that electrons could be easily transferred

through the CAB's conductive matrix to the anode with very little potential loss, fulfilling our design purpose of achieving a conductive biofilm.(Lee et al., 2009)

Our CAB structure is highly flexible in the immobilization of different amounts of cells and graphite particles, making it convenient to optimize the conductivity of the CAB and bacteria viability to enhance MFCs' performance. To further study the role of PPy and graphite in the CABs, we set up another round of MFCs with different CAB construction (Fig. 3-4 (A)). From its curve in the running cycle, we noticed that the artificial biofilm without PPy showed a low power output and is similar to the natural biofilm mainly due to the lower conductivity and poor stability of such artificial biofilms. This result verifies that PPy plays an essential role in the structural integrity and the high conductivity of the CAB. Similarly, the CAB, in which *S. oneidensis* was directly immobilized by PPy (*i.e.*, no graphite in the CAB) failed to start up (Fig.3-4 (A)), indicating that tight PPy warping on the cell membrane might lead to an

Fig. 3-3 Electrochemical performance of the CAB in MFCs. (A) Voltage-time (V-t) curves of the four MFCs for 630 hours' MFC operation under batch mode. The Control is the MFC with natural biofilm on carbon cloth. CAB-1 to -3 are the CABs with different thickness, respectively. Two red arrows indicate the times at which the medium is refreshed; black arrow indicates the time when 18mM sodium lactate is added. (B) Polarization curves of the four MFCs after 460 hours' operation.

unfavorable cell respiration and impair cellular viability. Based on above result, CAB with decreased graphite amount of was also constructed and tested in MFCs system (Fig. 3-4 (B)). Similar with that of original design, CAB-1 and CAB-2 in Fig. 3-4 (B) also refer to CAB constructed by single and triplicated fabrication process. The CAB-1 with less immobilized graphite failed to generate power output (Fig. 3-4 (B)), as that of directly immobilize bacteria on the carbon cloth with PPy. CAB-2 with less graphite has much lower electricity generation (110 mV *vs.* original 250 mV as the maximum value) in the first cycle. Current is partially restored after medium refresh however the maximum voltage is even lower, only 76 mV. Taking these results in conclusion, we can see that both PPy and graphite play important role in CAB performance and stability. Reducing the graphite amount or omit the PPy use both brings decrease in MFCs performance, even fails to start up.

Fig. 3-4 (A) Voltage output of the CAB-2 (blue), the natural biofilm on carbon cloth anode (black), the CAB without graphite (*i.e.*, directly coated the PPy onto the bacteria on the surface of the carbon cloth, red) and the CAB without PPy (*i.e.*, using the graphite and bacteria composite to directly spread onto the carbon cloth as CAB, green). (B) Voltage output of the CABs with less graphite. When preparing these CABs with less graphite, only 0.24g graphite (1/5 amount of the original CABs) was inoculated in a 120ml LB broth. Blue arrow showed the time at which the medium was refreshed.

On the other hand, the amount of bacteria immobilized in the CAB is another vital factor in determining the CAB's power output. As expected, since CAB-1 has less bacterial immobilization amount than CAB-2 and -3, the power output of CAB-1 dropped to a low level after two cycles of operation, failing to maintain its power output in the long term. The sharp potential decrease in the range of low current density in polarization curve (*i.e.*, less than 150 mA/m² in the V-j curve, Fig. 3-3 (B)) is due to the charge transfer limitation, resulting from poor microbial catalysis.(He et al., 2006) The slopes of the V-j curves in this low range of current density shows that the CAB-1 has a larger charge transfer resistance than that of the CAB-2 and -3, which indicates that a larger amount of bacteria immobilization could reduce the charge transfer resistance of the CAB.

3.3.3 Biofilm characterization after MFCs operation

FESEM was further used to characterize the architecture of the natural biofilm on the anode and the CAB after the MFC operation. We found that the natural biofilm formed on the carbon cloth anode was rather thin and did not fully cover the carbon fibers (Fig. 3-5 (A)). Such limited number of bacteria on the carbon cloth explains the poor performance of the MFC with the natural biofilm. The CABs showed a much higher number of cells immobilized on the graphite particles than that of the natural biofilm (Fig. 3-5 (B)). By comparing the FESEM images of the CAB before and after the MFC operation (Fig. 3-2 (D) and 3-2 (E)), we also found that the mesoporous structure of the CAB was maintained after 630 hours' MFC operation. Such high bacterial density and the sustained porous structures of the CAB accounted for the higher power output of the MFC with the CAB.

The viability of the tethered bacteria has always been a main concern in the cell immobilization.(Gutierrez et al., 2007; Klein et al., 2009; Luckarift et al., 2010) To characterize the cell variability in the CAB, the CAB was stained with fluorescence dyes, and subjected to fluorescence confocal scanning microscopy (FCSM) measurement. Fig. 3-5 (C) and 3-5 (D) showed the FCSM images of the cells in the exterior and interior of the CAB, respectively. Dead

Fig. 3-5 Characterization of the morphology and structure of the CAB after 600 hours' MFC operation. (A-B) FESEM images of the natural biofilm grown on the surface of carbon fibers (A) and the CAB (B). (C-D) FCSM images of exterior (C) and interior (D) of the CAB stained by fluorescence dyes. Green fluorescence indicated live bacteria, while red fluorescence indicated dead bacteria.

bacteria stained with red fluorescence were barely visualized both in the interior of the CAB, implying a high bacterial viability through the entire CAB. While in the exterior of CAB, red stains can be observed, which indicating decline in viability when microbes were away from the electrode. The above phenomenon was similar with former works.(Read et al., 2010a) The bacteria near electrode were easy to facilitate EET to achieve anode respiration, which provides extra benefit for bacteria survival.

3.3.4 Advantages of CAB in MFCs application

The CAB clearly possesses several advantages over the natural biofilm. Firstly, the CAB structure takes advantage of the possible EET mechanisms of electrogens, including direct electron transfer from the c-type cytochromes to the anode, and shuttle-mediated electron transfer via riboflavin (the soluble mediator of *S. oneidensis*). Secondly, a high biomass loading in the CAB could be achieved. Thirdly, the morphology and thickness of the CAB could be regulated to enhance nutrients' diffusion and bacterial respiration. The CAB construction procedure offers a great deal of flexibility in controlling viable biofilm structures and morphologies by proper selection of the amounts of electrogen cells and scaffolding materials, respectively or collectively. Recent research revealed that many nanomaterials could enhance the direct electron transfer between *S. oneidensis* and anode materials. (Deng et al., 2010b; Huang et al., 2011b; Nakamura et al., 2009; Peng et al., 2010) It would be interesting to immobilize different nanomaterials into our CAB matrix to study if EET and power output could be further enhanced. Meanwhile, developing novel strategies of diffusible shuttles' immobilization in the CAB would further promote bioelectricity generation in MFCs. Conductive polymers with better biocompatibility or branching structures that could improve direct-contact electron transfer

between cytochromes on the cell membrane and the polymer chains deserved further study for the optimization of the CAB.

3.4 Conclusion

In conclusion, we developed a new immobilization strategy of electrogens to construct as conductive artificial biofilm (CAB). The fabricated CABs provided much flexibility in controlling the amount and viability of cells, as well as morphology and thickness of the biofilm. MFCs with optimized CAB have a maximum power density of 207 mW/m^2 , which is almost 11 times of that with natural biofilm when carbon cloth is used as anode. Both PPy and graphite plays essential role in CAB stability and their corresponding MFCs performance. Omitting use of PPy or reduce the graphite use amount both greatly reduce the system performance. Our work here provides an novel strategy to improve the system performance and is also potential for further development as a platform.

* We acknowledge RSC for their providing copyright to the materials used in this chapter.

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Chapter 4: Nitrogen Doped Carbon Nanoparticles Enhanced Extracellular Electron Transfer for High-Performance Microbial Fuel Cells Anode

Abstract

Nitrogen doped carbon nanoparticles (NDCN) were applied to modify the carbon cloth anodes of microbial fuel cells (MFCs) inoculated with *Shewanella oneidensis* MR-1, one of the well-studied exoelectrogens. Experimental results demonstrated that the use of NDCN increased anodic absorption of flavins (*i.e.*, the soluble electron mediator secreted by *S. oneidensis* MR-1), facilitating shuttle-mediated extracellular electron transfer. In addition, we also found that NDCN enabled enhanced contact-based direct electron transfer *via* outer-membrane c-type cytochromes. Taken together, the performance of MFCs with the NDCN-modified anode was enormously enhanced, delivering a maximum power density 3.5 times' higher than that of the MFCs without the modification of carbon cloth anodes.

4.1 Introduction

Microbial fuel cell (MFC) attracts much attention in recent decade as a sustainable and green energy technology that integrates wastewater treatment and bioelectricity harvest (Logan, 2009; Rabaey & Verstraete, 2005). Organic substances are oxidized in MFC's anode chambers by the metabolism of exoelectrogens, and the generated electrons are then transferred to external circuits *via* multiple extracellular electron transfer (EET) pathways. These electrons are further transferred to the cathode and consumed by the cathodic reactions to enable a closed electrical circuit, by which the electricity energy can be harvested. Low anode performance remains a major bottleneck in restricting practical applications of MFCs. Many efforts have thus been made

to increase the anodes' electron transfer efficiency and performance, including optimization of operation conditions and electroactive microbial communities in anodic chambers, genetic engineering of exoelectrogens and modification of anode materials to increase EET efficiency (Liu et al., 2012; Rinaldi et al., 2008; Yong et al., 2013a; Yong et al., 2012a; Yong et al., 2011; Yong et al., 2012b; Yong et al., 2013b). Among various approaches, development of new anode materials showed much success in improving the MFCs' performance. The crucial requirements for the high-performance anode material include excellent conductivity, high surface area for bacteria attachment and growth, and surface modification for enhanced biofilm formation and EET efficiency (Rinaldi et al., 2008). A number of three-dimensional architectures modified with functional nanomaterials favoring electroactive biofilm formation were recently constructed as high-performance MFC anodes (Nguyen et al., 2013; Xie et al., 2011a; Xie et al., 2012b; Yong et al., 2012a). Further rational design and development of high-performance MFCs anode materials should combine some of these advantages and properties.

Herein, we synthesized and utilized nitrogen-doped carbon nanoparticles (NDCN) to modify the commonly used carbon cloth anode to improve the bioelectricity output of MFCs. *Shewanella oneidensis* MR-1 was used as the anode inoculation because it is one of the most well-established exoelectrogens that has been extensively used in MFCs. *S. oneidensis* MR-1 has well-characterized EET pathways that include shuttle-mediated electron transfer (MET) *via* flavins, and contact-based direct electron transfer (DET) *via* outer-membrane c-type cytochromes. (Baron et al., 2009; Marsili et al., 2008) With the anode modification by NDCN, an increase of 3.5 times' maximum power density output in MFCs was achieved in comparison to that of the MFCs with carbon cloth anodes. The mechanism of the enhanced MFC performance was also elucidated, wherein the efficiency of both MET and DET were improved

by this anode modification. To the best of our knowledge, our finding is the first demonstration of MET enhancement *via* electrode modification.

4.2 Materials and Methods

4.2.1. Doped and un-doped carbon nanoparticles' synthesis and characterizations

Both doped and un-doped carbon nanoparticles were synthesized by using a chemical vapor deposition method. To prepare doped carbon nanoparticles, a liquid mixture containing iron pentacarbonyl, methanol and N-doping chemical of pyrrole was pyrolyzed in a quartz pipe furnace under argon protection at 900 °C. Then, the black powder was collected and refluxed in concentrated HCl. To fabricate un-doped carbon nanoparticles, the same procedure as that of doped ones was used but without the N-doping chemical. As-prepared materials were characterized by field emitted scanning electron microscopy (FESEM, JSM-6700F), transmission electron microscopy (TEM, JEM-2100F), and X-ray photoelectron spectroscopy (XPS). We greatly appreciate Dr. Guo Chunyang for his effort on providing material and related characterization.

4.2.2 Anode preparation

To modify the carbon cloth anode, doped and non-doped carbon nanoparticles were dispersed in the DI water (Millipore, Singapore), respectively, with a concentration of 2 mg/ml by sonication. 25 μ l of dispersed ink was evenly added to a carbon cloth electrode (1 x 1.5 cm²) with a syringe and dried in the air. This process was repeated for 15 times and the amount of material loading is $\sim$ 0.5 mg/cm².

4.2.3 MFC operation and CV analysis

Two chamber MFCs with inner size of 4.5 cm x 4.5 cm x 4 cm for each chamber and separated by proton exchange membrane (Nafion 117 PEM, from Gas Hub, Singapore) were constructed. Carbon cloth with a size of 2 cm x 4 cm was used as cathode. The effective volume for each chamber was 50 ml. MFCs with intact carbon cloth, non-doped and doped carbon nanoparticles modified carbon cloth as anode and are denominated as CC, CC-C and CC-CN, respectively.

Wild-type *S. oneidensis* MR-1 was firstly activated overnight, then grown in LB medium to the steady state before inoculation into MFCs. The inoculation was adjusted to a starting OD₆₀₀ of 0.4 for all MFCs. All MFCs were operated under batch mode with a consistent resistance of 2 kΩ. The anode medium was a mixture of 90% M9 salt medium and 10% LB broth. 18 mM sodium lactate was used as carbon source for the growth of *S. oneidensis* MR-1. Cathode solution is the same as the description in Chapter two. When nutrients in anode chambers were exhausted, both the anode medium and cathode solution were refreshed, thus to start up a new batch cycle.

CV analyses were conducted by CHI 660D electrochemical workstation (CH Instrument, China) in a three-electrode system. SCE (+0.243 vs. SHE, according to the manufacture) was used as the reference electrode, and the MFC cathode as counter electrode. When nutrients were depleted, MFCs were firstly opened circuited overnight, and then the CVs with different scanning rates were done from -0.7 to 0.1 V to get the CV curves at the non-turnover conditions. CV curves with the turnover current were obtained by adding sodium lactate (the carbon source) when the output voltage was low. All voltages reported here were in comparison to SCE.

4.2.4 Biofilm imaging with FESEM

After MFCs dissembled, small piece of MFCs anode (CC and CC-CN) was cut off and dipped into 1% glutaric dialdehyde solution overnight to immobilize bacteria on the electrode surfaces. Then, it was washed with 0.85% NaCl solution for three times and followed by rinsing in gradient ethanol solution with concentration of 30%, 50%, 60%, 70%, 80%, 90% and pure ethanol every 10 minutes. Such pretreated biofilms were imaged by FESEM.

4.3 Results and Discussions

4.3.1 Characterizations of doped and non-doped carbon nanoparticles

The FESEM image in Fig. 4-1 (A) showed that the nitrogen doped carbon nanoparticles had particle-like structures with quite uniform size. TEM image illustrated that the nitrogen-doped carbon nanoparticles had a hollow nanostructure (Fig. 4-1(B)). In comparison, the non-doped carbon nanoparticles had a similar hollow nanostructure as that of the nitrogen doped ones (Fig. 4-1 (C)). The success of nitrogen doping was confirmed by the XPS N 1s spectrum in Fig. 4-1 (D). The XPS peak locating at around 400.5 eV corresponded to a pyrrolic N-doped structure.

Fig. 4-1 Image and spectrum characterization of the anode materials. (A) FESEM image of Nitrogen doped carbon nanoparticles. (B) TEM image of Nitrogen doped carbon nanoparticles. (C) TEM image of undoped carbon nanoparticles. (D) XPS N 1s spectrum of Nitrogen doped and undoped carbon nanoparticles.

4.3.2 Enhanced MFCs performance with modified anodes

MFCs equipped with carbon cloth anode (CC), non-doped carbon nanoparticles modified carbon cloth anode (CC-C) and nitrogen-doped carbon nanoparticles modified carbon cloth anode (CC-CN) were set up, and the individual performance of these anode materials in MFCs was investigated in detail. Fig. 4-2 (A) showed the voltage output (V-t curve) of these MFCs in the two cycles' discharge (with an external resistance of 2 k Ω). MFCs with CC delivered a steady output voltage of 150 mV, while that for MFCs with CC-C and CC-CN were 230 and 270 mV,

respectively, showing an impressive improvement on the bioelectricity output by the carbon cloth decoration with nitrogen-doped carbon nanoparticles. We also noticed that for the MFCs with CC-CN, the steady state time (wherein the output voltage was at the plateau) was longer than that of the MFCs with CC-C, enabling a higher Columbic efficiency.

Polarization curves were performed at steady state by changing the external resistance from 112 to 2 k Ω to identify the maximum output power density of MFCs. As shown in Fig 4-2 (B), the maximum power density achieved by the CC, CC-C and CC-CN anode were 66.2, 218.9 and 298.0 mW/m², respectively. Thus, with anode modification, an increase of ~2.3 times (for CC-C) and 3.5 times (CC-CN) of the maximum power density output were obtained compared with that of MFCs with carbon cloth anode. The polarization curves (V-j, *i.e.*, output voltage *vs.* current density, Fig. 4-2 (B)) showed that the open circuit potential (OCP) for the modified electrodes had a significant increase, *i.e.*, 791 mV for CC-C, 826 mV for CC-CN, and only 648 mV for CC. The V-j curves of the CC-C and CC-CN anodes could be divided into three linear parts based on their slopes. The linear part that is higher than 700 mV is dominated by the activation potential. The other two linear parts located between 700-550 mV and 550 mV below, which probably belonged to two EET pathways rather than the accepted the "ohmic losses" and "concentration losses" in the former study (Fan et al., 2008). The main difference between the CC-C and CC-CN anodes was that the potential drop for the CC-CN anode was slower in the range of 700-550 mV than that of the CC-C anode, which partially explained the higher current output by nitrogen doping.

Fig. 4-2 The performance of MFCs equipped with the anode made of clean carbon cloth (CC), carbon cloth with non-doped carbon nanoparticles modification (CC-C), and carbon cloth with nitrogen doped carbon nanoparticles (CC-CN), respectively. (A) The profiles of voltage output of MFCs (with an external resistance of 2 k Ω), *i.e.*, V-t curve; (B) Polarization curves.

4.3.3. Mechanism on the EET of MFC anode with CC-CN modification

To explore the mechanism underlying the improved MFCs performance, cyclic voltammetry (CV) analyses were further carried out. Figure 4-3 (A) showed the CV curves of the MFCs at the scanning rate of 1 mV/s upon the depletion of nutrients. It could be concluded that the anode capacitance was greatly enhanced upon electrode modification with the CC-C and CC-CN anodes. To elucidate the electrochemical active species in the MFCs systems, the CV curves after baseline subtraction were constructed and presented in Fig. 4-3 (B). For MFCs with the CC-C anode, four pairs of peaks show up (Fig. 4-3 (B)). The biggest peaks centered at -0.46 V corresponded to the self-synthesized flavins that dictate the MET processes. The two pairs of peaks centered at -0.32 V and -0.15 V were associated with the outer-membrane c-type cytochromes that control the DET processes. In addition, there appeared a pair of highly reversible peaks centered at -0.56 V, which was not reported before. For the MFCs with the CC anode, only two pair of peaks could be identified, which were associated with flavins (centered at -0.46 V), and outer-membrane c-type cytochromes (centered at -0.28 V). The DET-based peaks of the CC-C anode was highly reversible in comparison to that of the CC, which was also previously observed in a carbon-based nanomaterials that could facilitate the DET efficiency *via* outer-membrane c-type cytochromes (Peng et al., 2010) . For MFCs with the CC-CN anode, three pairs of peaks are defined, *i.e.*, one pair of peaks associated with flavins, and two pairs of peaks corresponding to the outer-membrane c-type cytochromes. Strikingly, the peaks associated with flavins in the CC-CN anode have much higher responding current than that of the CC and CC-C anodes (Fig. 3B), meaning that the relative activity of favins in the CC-CN anode was much higher than that in the CC and CC-C anodes.

Fig. 4-3 (A) CV curves at non-turnover conditions, with a scanning rate of 1 mV/s. (B) CV curves after baseline subtract.

To further investigate how these electrochemical active species contributed to the total EET, lactate was added to the medium upon its depletion, and CV was carried out at the scanning rate of 1 mV/s to achieve the turnover current curve. The CVs with the turnover current were compared with those of non-turnover current for the three MFC anodes, *i.e.*, the CC, CC-C and CC-CN anodes (Fig. 4). We found the MET-based catalytic current increased from -0.46 V,

while the DET-based catalytic current increased around -0.32 V. Table 4-1 summarized the catalytic currents *via* the MET and DET for the three different anodes, which were calculated by subtracting the current densities between two points where the slope in anodic curve was enormously changed. From the amplitude of the catalytic currents (Table 1), we concluded that the DET *via* outer-membrane c-type cytochromes was enhanced in the MFC with the CC-C anode, while the MET *via* flavins was not. Another interesting observation of the CC-C anode was that the peak centered at -0.56 V did not exhibit catalytic current although its apparent electrochemical activity was good.

Anode	MET ($\mu\text{A}/\text{cm}^2$)	DET ($\mu\text{A}/\text{cm}^2$)
CC	15.9	10.2
CC-C	14.8	25.5
CC-CN	48.5	35.0

Table 4-1 The amplitude of the MET- and DET-based catalytic current in the MFC anode made of carbon cloth (CC), carbon cloth with non-doped carbon nanoparticles modification (CC-C), and carbon cloth with nitrogen doped carbon nanoparticles (CC-CN), respectively.

However, both the MET- and DET-based catalytic currents were increased in the MFC with the CC-CN anode, which explained its enhanced MFC performance, and was consistent with the V-j polarization curves in Fig. 2B. Strikingly, the shuttle-mediated electron transfer between flavins and anode surfaces was enhanced in the MFC with the CC-CN anode. In the CV curve of the CC-CN anode with the turnover current, the anodic current arose at ~ -0.5 V and reached the plateau at ~ -0.41 V, in which only 90 mV overpotential was needed for the shuttle-mediated redox reaction to happen. A well-shaped sigmoid curve occurred, which distinguished the MET process from the DET process at the higher voltage. However, for the MFCs with the CC and CC-C anodes, although the MET process was clearly observed, much higher overpotentials were required to reach the current plateau and their DET and MET processes cannot be easily

Fig. 4-4 The comparison of CV curves with and without turnover current for the MFCs with anode of (A) CC, (B) CC-C, and (C) CC-CN.

distinguished. Based on the previous work that the overpotential for flavin-mediated MET was not sensitive to flavin concentration (Baron et al., 2009), it can be confirmed that this decreased overpotential was due to enhanced flavin electrochemical activity at the surfaces of the CC-CN anode.

4.3.4. Selective absorption of flavins to CC-CN

CV analyses revealed a high flavin activity at the CC-CN electrode interface, thus enabling an enhanced MET based on the calculation of turnover currents (Fig. 4-4). To further investigate the electrochemical performance of flavins and calculate the concentration of flavins, CVs at different scanning rates were implemented in the MFCs with the CC-CN anode without turnover current (Fig. 4-5 (A)), and linear fits of anode peak current density vs. scanning rate were performed at both low and high ranges of scanning rates, respectively, as shown in Fig. 4-5 (B) and 5 (C). Our results (Fig. 4-5) demonstrated very interesting results that at lower scanning rates flavins acted like an attached electroactive species, while at higher scanning rate (above 40 mV/s here) flavins-mediated EET turned to the diffusion mode (Nicholson & Shain, 1964; Richter et al., 2009). The total amount of the attached species can be calculated based on Laviron equation:(Laviron, 1979)

$$I_p = \frac{n^2 F^2 A v \Gamma}{4RT} = \frac{n F Q v}{4RT}$$

where v and I_p are the scanning rate and corresponding peak current density; n is the electron transfer number ($n=2$ here); A , Γ and Q are the electrode area, absorbed flavins molar concentration and total charge involved in electron transfer, respectively; and F , R and T are Faradic constant, gas constant and experiment temperature, respectively. Using the anodic peak

current density at 20 mV/s (Fig. 4-5 (B)), we estimated the "attached" amount of flavins was 2.17 nmol/cm². Since the bulk flavin concentration is ~0.2 mM and the anode chamber was 50 ml, we thus concluded the CC-CN anode was able to absorb a higher concentration flavins (Marsili et al., 2008; von Canstein et al., 2008).

Fig. 4-5 (A) CV curves at different scanning rates for the CC-CN anode without turnover current. **(B)** The relationship of anode peak current density vs. scanning rate at a low range of scanning rate. **(C)** The relationship of anode peak current density vs. scanning rate at a high range of scanning rate. **(D)** The CV curves of the clean carbon cloth (CC) and the modified carbon cloth (CC-CN) at cathode solution, with a scanning rate of 10 mV/s.

Carbon electrode and biofilm interface was known to have potential to absorb soluble mediators (Marsili et al., 2008). To exclude the possibility that the enhanced flavin absorption in the CC-CN anode was merely due to the increased surface areas of carbon cloth after CC-CN modification, we scanned the CVs for clean CC and CC-CN anodes in a 50 mM $\text{K}_3\text{Fe}(\text{CN})_6$ (with phosphate buffer) at a scanning rate of 10 mV/s (Fig. 4-5 (D)). We estimated the surface area of the CC-CN anode was only 2 times of that of the CC anode. Meanwhile, for the CC-CN anode, both the observed flavin activity and flavin-based catalytic current were similar to those of CC. We thus concluded that nitrogen doping played a key role in flavin absorption in the carbon-based anode materials.

Compared with non-doped carbon nanoparticles, the doped carbon nanoparticles could absorb more flavins on the electrode and biofilm interfaces. Flavins were also in absorption and desorption equilibrium.. Meanwhile we noticed that the flavin reaction activity on the CC-CN anode was the highest. Both facts might be explained by the structure of flavin and its reduced form (shown in Schematic 4-1). It had been recognized that flavins or other soluble mediators can be absorbed at the interfaces of carbon materials and biofilms (Richter et al., 2009). In our former work, we found that the π - π interaction between material and heterocyclic structure of flavins would lead to a high flavin retention at the interfaces of electrodes and biofilms, which explained the fast recover of output voltage upon medium change (Yu et al., 2011). Here, with the modification of carbon materials by nitrogen doped carbon nanoparticles, the π structure of the carbon cloth electrode was more polarized. It can be anticipated that the π - π interaction was enhanced, which led to a higher absorbed concentration. Since flavin reduction only involved the nitrogen substituted quinone structure, the structure similarity between electron donor (substituted quinone structure of flavin) and electron acceptor (nitrogen doped carbon

nanoparticles) would probably favor the electron transfer. Thus, the lower overpotential in the CV curve of the CC-CN anode could be rationalized.

Schematic 4-1 Chemical reduction of riboflavin (structure is the riboflavin molecule)

FESEM images of biofilms formed on the anodes after two cycles of MFC operations were taken (Fig. 4-6). For both MFCs with the CC and CC-CN anodes, a single layer of biofilm was formed and no significant difference was observed. Thus, nitrogen doping did not lead to much difference in biofilm formation, which was not the reason for the better MFC performance in the CC-CN anode.

Fig. 4-6 FESEM images of the biofilms formed on (A) the CC anode, and on (B) the CC-CN anode

Previous research showed that anode modifications using nanomaterials could increase the electrochemical activity of outer-membrane c-type cytochromes, leading to the enhanced DET in *S. oneidensis* MR-1 (Deng et al., 2010a; Huang et al., 2011b; Peng et al., 2010). Meanwhile, several efforts were also made on the physical and chemical immobilization of electron shuttles directly onto electrode to improve the shuttle-mediated EET efficiency (Adachi et al., 2008; Feng et al., 2010b; Wang et al., 2011a). Here, our work implied that it was possible for improved physical absorption of electron shuttles on electrode interfaces by nitrogen doped modification on electrode materials, providing a new approach in the rational design of novel electrode materials for high-performance MFCs and other bioelectrochemical systems.

4.4 Conclusions

In summary, we utilized nitrogen doped carbon nanoparticles-modified carbon cloth (CC-CN) as the MFCs anode. CV analyses revealed that the efficiency of EET in the MFC with the CC-CN anode was much higher than that of the un-modified carbon cloth. Our result showed that the enhanced MET was due to the increased flavin absorption on the interfaces of the CC-CN anode and biofilm, thus increasing the shuttle-mediated MET rate between flavins and the modified electrodes. The maximum power density output of the MFC with the CC-CN anode was 4.5 times' of the MFC with carbon cloth anode.

Chapter 5: Knocking out Sigma factors promotes bioelectroactivity and extracellular electron transfer of *Pseudomonas aeruginosa*

Abstract

Herein we demonstrate a random mutation strategy to search for anode respiration strains with improved electrochemical activity with *Pseudomonas aeruginosa* PAO-1 as model type. Two single global regulator mutant strains from *P. aeruginosa* PAO-1 were tested in MFCs. Strain Δ rhoS exhibit 40% increase in bioelectricity generation while that of strain Δ rhoN is 22% compared with wild type PAO-1 under MFCs conditions. One new EET pathway is ascribed to explain the enhanced bioelectricity generation with strain Δ rhoS and Δ rhoN benefit from much higher PCA secretion.

5.2 Introduction

Microbial fuel cells (MFCs) enabling wastewater treatment and electricity energy harvest have attracted wide interest in the last decade.(Logan, 2009; Rabaey & Verstraete, 2005) Microbe consortia formed on anode surfaces, known as biofilm, plays central roles in transferring electrons released from bacterial metabolism to anodes via multiple extracellular electron transfer (EET) pathways. Two EET pathways have been identified: direct electron transfer (DET) by outer-membrane c-type cytochromes and conductive pili, and mediated electron transfer (MET) by soluble shuttles (e.g., phenazines in *Pseudomonas aeruginosa*, and flavins in *Shewanella oneidensis*).(Marsili et al., 2008; Rabaey et al., 2005a; Reguera et al., 2006; Richter et al., 2009)

Anode efficiency is a one of limiting factor in restricting the bioelectricity output in most pure culture MFCs. (Adachi et al., 2008; Feng et al., 2010b; Liu et al., 2012; Yong et al., 2013a; Yong et al., 2011; Yong et al., 2012b) Thus, developing new strategies for improving EET efficiency is of highly demand. Since EET involved both electrodes and bacteria, which are the two targets to be engineered to improve EET. Most previous efforts focused on developing electrode materials that could facilitate biofilm formation, thereby the efficiency of EET.(Deng et al., 2010a; Huang et al., 2011b; Peng et al., 2010) Meanwhile, strain engineering, *i.e.*, developing enhanced electroactive bacteria strains, gained much interest in the past few years. (Kouzuma et al., 2010; Leang et al., 2013; Yi et al., 2009) For example, we recently engineered *Escherichia coli* (a non-electrogenic bacteria strain) to be electroactive. Upon genetically knockout *ldhA* gene responsible for the lactate biosynthesis, the intracellular NADH level of *E. coli* was increased, which was further redirected to electrodes to increase bioelectricity production in MFCs. ((Yong et al., 2012b)). We also genetically knocked out a global inhibitor of the tricarboxylic acid (TCA) cycle (*i.e.*, *arcA* gene) in *E. coli*, thus much intracellular electrons could be released to electrodes and the bioelectrocatalytic activity of *E. coli* was significantly promoted (Liu et al., 2012). *Pseudomonas aeruginosa* PAO-1 is a well-established anode respiration bacteria strain that can use phenazines as electron shuttles in its EET in MFCs. We overexpressed the *rhl* quorum sensing (QS) system to overproduce phenazines, which effectively enhanced EET and bioelectricity production in *P. aeruginosa*-inoculated MFCs. (Yong et al., 2011)

Sigma factors of RNA polymerase, as one category of global factors, recognize promoter regions of genes, and control the initiation and expression of a large number of downstream genes, being responsible for the determination of various cellular activities and phenotypes. (Alper & Stephanopoulos, 2007; Grossman et al., 1984; Klein-Marcuschamer & Stephanopoulos, 2008;

Liu et al., 2012; Tanaka et al., 1993; Yu et al., 2008) Genetic engineering σ factors were used to improve bacterial strains' tolerance to toxins and control biofilm formation.(Fux et al., 2005; Heydorn et al., 2002; Klein-Marcuschamer et al., 2009) However, no investigation has been reported on how global regulators influence bioelectrocatalytic activities of electrogenic bacteria. To this end, we investigated the knockout effects of global regulators (*rpoS* and *rpoN*) in controlling electroactivity of *P. aeruginosa* and its bioelectricity output in MFCs. RpoS (SigmaS, σ^S , or σ^{38}) is involved in the *Pseudomonas* quorum sensing systems by negatively regulating the transcription of *rhII*, thus controlling the response of *Pseudomonas* to cell density and environmental stress. RpoN (SigmaN, σ^N , or σ^{54}) positively regulate *rhII* synthesis. RpoN also plays key role in pili and flagella formation, which are crucial for biofilm formation. We found the either knocking out RpoS or RpoN could all lead to an enhanced bioelectricity output in the MFCs, but with different mechanisms. The biofilm formation on anodes of the *rpoS*-knockout strain was enhanced, leading to an increased bioelectroactivity in MFCs. Meanwhile evidence of new EET pathways adopted by strain Δ rpoS is confirmed by CV analysis. On the other hand, *rpoN* knockout increased biosynthesis of phenazines. Our research in engineering global regulators provided a new strategy in increasing the bioelectrocatalysis activity and EET efficiency of anodic respiration bacteria and bioelectricity production in MFCs.

5.2 Materials and Methods

5. 2.1 Strains and culturing

Wild type *Pseudomonas aeruginosa* PAO-1 (*P. aeruginosa* PAO-1) Single mutation of global regulator *rpoS* and *rpoN* (strain Δ rpoS and Δ rpoN) are used as the anode respiration bacteria. All of these strains are get from Prof. Yang Liang (Singapore Centre on Environmental Life

Sciences Engineering (SCELSE), Singapore). Both strains were constructed as former works.(Schuster et al., 2004; Thompson et al., 2003)

0.5 ml overnight bacteria (both wild type and mutants) are inoculated into 50 ml LB medium and incubated in the shaker at 37 °C and 200 rpm. The bacteria are harvested by centrifuge (5000 rpm, 7 min) after eight hours. The bacteria residue is then suspended by M9 and diluted to 1 ml. 400 µl suspension is then inoculated for each MFCs anode chamber.

5.2.2 MFC setup and electrochemical measurement

Dual-chamber MFCs with an inner size of 4.5 cm * 4.5 cm * 4 cm for each chamber are used in the experiment and is separated by Nafion 117 membrane. The Nafion membrane is first dipped into 1 M sulfuric acid solution overnight and then washed with vast sterile water before use. Carbon cloth (Gas Hub, Singapore) with a size of 2 cm * 3 cm is used both as anode and cathode. Carbon cloth is treated and connected to a copper wire as Chapter II. Saturated calomel electrode (CH Instrument, Shanghai China) is inserted into anode chamber as the reference electrode. The standard potential of this SCE is 0.243 V according to the handbook. M9 medium with 4 g/L glucose is used as the anodic medium as before and the catholyte consists of 50 mM $K_3Fe(CN)_6$, 50 mM K_2HPO_4 and 50 mM KH_2PO_4 . Both anode and cathode have effective volume of 60 ml. All the MFCs are operated anaerobically and three parallel MFCs are set up for each strain.

After inoculation, MFCs continuously discharge with 2 K Ω out resist. Output potential is recorded by a digital Multimeter (ESCORT 3146A). Cyclic Voltammetry (CV) analysis is done on the 8th day in a three-electrode system with CHI 660D electrochemical workstation (CH Instrument, China), when all the MFCs begin to steadily discharge. Anode is worked as the

working electrode and the cathode as counter electrode. To do the CV analysis, the MFCs is first open circuit for 3 hours and then scanned at 50 mV/s repeatedly until the response current become stable; followed by slow scan analysis with 1 mV/s is done. All the CV analysis is scanned from -0.7 V to 0.3 V.

5.2.3 Phenazines measurement

Phenazines of all strains are analyzed with high performance liquid chromatography (HPLC) as the former work with slight variation. Briefly, 1.5 ml sample is centrifuged by 15 min at 13000 rpm under room temperature. The supernatant is filtered by 0.2 μ m membranes before analyzed by an Agilent HPLC system equipped with Agilent C18 analytical column (250 mm * 4.6 mm with 5 mm particle size) and a photodiode array detector (CA, USA). Phenazines are separated by gradient of water-0.1% acetic acid (solution A) and acetonitrile-0.1% acetic acid (solution B) at the flow rate of 1 ml/min. Gradient variation is in the following sequence: from 0-2 min, linear gradient of solution B from 0 to 15% ; from 2-22 min, linear gradient of solution B to 83%; from 22-25min, drop back to 100% solution A. Final step using pure solution A for 5 minutes. Three wavelengths include 280 nm (for PYO), 250 nm (for PCA and PCN) and 262 nm (for 1-hydroxyphenazine (1-OHPHZ)) are chosen for photodiode array detector. Standard PYO (Sigma-Aldrich, Singapore) is used after filtered with a 0.2 μ m membrane.

5.2.4 Biofilm Characterization

The morphology and relative density of biofilm for each strain is qualitatively characterized by a JEOL field emission scanning electron microscope (FESEM, JSM-6700F-FE-SEM, Japan). The FESEM sample is prepared as follows: a small piece (around 2 mm * 2 mm) of carbon cloth

anode is cut off and the bacteria are immobilized by emerging into 1% glutaric dialdehyde solution overnight. After that the carbon cloth is washed with 0.85% NaCl solution for three times and rinsed in a gradient ethanol solution (in the sequence of 30%, 50%, 70%, 80%, 90% and pure ethanol solution) to totally remove the water in the sample.

5.3 Results

5.3.1 Enhanced bioelectrocatalytic activity of *rpoS* and *rpoN* knockout strains than wild-type

The *rpoS* and *rpoN* knockout strains are denoted as $\Delta rpoS$ and $\Delta rpoN$, respectively. After 24 hours' aerobic incubation, the culture of $\Delta rpoS$ is dark green, wild type PAO-1 is light green, while $\Delta rpoN$ is yellow, indicating $\Delta rpoS$ synthesizes higher level of pyocyanin (PYO) (Fig. 5-1 (A)). This was further validated by HPLC analysis (Fig. 5-2). Rhl QS system in *Pseudomonas aeruginosa* up-regulates PYO synthesis. RpoN is proved to be a positive regulator its gene expression under certain medium conditions while *rpoS* negatively regulates the transcription of *rhlI*. (Thompson et al., 2003) Thus the strain $\Delta rpoN$ is impaired in PYO synthesis while in strain $\Delta rpoS$ PYO synthesis is enhanced, consistent with HPLC result (Fig. 5-2). Interestingly, obviously increased phenazine-1-carboxylic acid (PCA) concentration is observed in $\Delta rpoN$ (14.3 min). PCA, the precursor of PYO, can be sufficiently synthesized by either gene cluster of *phzA1-G1* or *phzA2-G2*. A *las*-box is located at the upstream of *phzA1-G1* (Whiteley et al., 1999). RpoN is reported to act as a repressor for LasR-I QS system at low cell density and thus the *rpoN* deletion may result in the increased PCA synthesis. (Heurlier et al., 2003) This partially explains the why in $\Delta rpoN$ PYO synthesis is impeded while the PCA synthesis is enhanced. The most important indication of the phenazines synthesis with different strains

under aerobic conditions is that phenazines are in fact under very complex regulation system and it is very difficult to predict the result after single sigma factor is deleted.

Several redox peaks were clearly observed in the CV plots of the *P. aeruginosa* strains soon after they are inoculated into MFCs.(Fig. 5-1 (B)) These signals are clearly belong to the residue electrochemical active species from bacteria culturing. The redox peaks centered around -0.23 V (vs. SCE) correspond to PYO, and one centered around -0.31 V to PCA.(Wang & Newman, 2008) The HPLC and CV analyses showed that $\Delta rpoS$ synthesized more PYO, while $\Delta rpoN$ synthesized more PCA under aerobic culture conditions.

Fig. 5-1 (A) PAO-1 and mutants after aerobic culturing, left to right: $\Delta rpoN$, $\Delta rpoS$ and PAO-1; (B) CV analysis of MFCs after inoculation, scanning rate is 50 mV/s

Fig 5-2 HPLC result of supernatant after aerobic culturing: (A) PAO-1; (B) $\Delta rpoS$; (C) $\Delta rpoN$.

Small window in (B) is the full wavelength UV absorption at 6.1 min

Wild type PAO-1, $\Delta rpoS$ and $\Delta rpoN$ *P. aeruginosa* strains were then tested in MFCs (with 9.6 k Ω external resistances) for continuous discharge by 320 hours (three parallels for each strain). Fig. 5-3 shows the profiles of the current output in MFCs. The current density output of PAO-1 reached a plateau of 6.6 ± 0.2 mA/m² after 85 hours (with an increase rate of 1 mA/m² per day). $\Delta rpoS$ exhibited the highest electricity output, reaching the plateau current density of 9.3 ± 0.4 mA/m² at 50th hour (with an increase of 2.8 mA/m² per day), and steadily discharged for more

than one week. Unexpectedly, although ΔrpoN synthesized the highest PCA level, its initial current density is only as low as $\sim 1 \text{ mA/m}^2$, and it took ~ 240 hours to reach its plateau of $8.1 \pm 0.2 \text{ mA/m}^2$. In all, the ΔrpoS and ΔrpoN knockout strains of *P. aeruginosa* showed an increased current density output than the wild-type strain PAO-1.

Fig. 5-3 i-t curve of MFCs inoculated different *P. aeruginosa* strains

5.3.2 Differential mechanism for enhanced bioelectricity generation in ΔrpoS and ΔrpoN inoculated MFCs

Although ΔrpoS and ΔrpoN generated higher bioelectricity output than PAO-1, the differential time profiles of current output by these strains (Fig. 5-3) implies a complex mechanism on the regulation of bioelectrochemical activity upon deletion of global regulators. To elucidate the underlying mechanism, CV analysis (with scanning rate of 50 mV/s and 1 mV/s) were performed on these MFCs after 240 hours' discharge (Fig. 5-4). At the scanning rate 50 mV/s , the contribution of catalytic current to the whole responding current is negligible, and the peak area can be viewed as the relative activity of redox species. The well-defined peaks centered at ~ -0.28

V in the CV plot correspond to PCA. From the peak areas, we concluded that Δ rpoN produced highest level of PCA, while Δ rpoS produced the lowest PCA under MFCs conditions (Fig. 5-4 (A)).

At a lower scan rate of 1 mV/s (Fig. 5-4 (B)), catalytic current by bacteria respiration had more contribution to the responding current, and CV shows a sigmoid shape if this responding electrochemical active species exhibit good catalytic (Baron et al., 2009). Although PAO-1 secreted slightly more phenazines, MFCs with Δ rpoS can better utilize this phenazine for bioelectricity generation, leading to the similar corresponding catalytic current with PCA (estimated by subtract the current density at -0.2 V with -0.3 V in anodic curve). Later SEM image confirms that the better biofilm is formed in MFCs with Δ rpoS (Fig. 5-6), this explains why Δ rpoS have better PCA efficiency for bioelectricity generation.

MFCs with strain Δ rpoN synthesize most phenazines at 50 mV/s. When scanned at 1 mV/s, the peak mostly remains its shape as that at 50 mV/s. Compare the peak anodic current of Δ rpoN and PAO-1 at 50 mV/s and 1 mV/s, we find this difference enlarges from about 2 times ($115 \mu\text{A}/\text{cm}^2$ vs. $55 \mu\text{A}/\text{cm}^2$) to 4 times ($8 \mu\text{A}/\text{cm}^2$ vs. $2 \mu\text{A}/\text{cm}^2$). Former works prove that carbon material has a physical absorption to flavin, thus they show linear relationship with scanning rate when scanned at low rate (Marsili et al., 2008). Thus, we can tell that phenazine reaction at CV is more diffusion controlled in MFCs with Δ rpoN compared with PAO-1, indicating phenazine is “saturated” for absorption in Δ rpoN MFCs. Regardless the great phenazine concentration difference between Δ rpoN and PAO-1, the estimated catalytic current is about 2 times. The worst biofilm in Δ rpoN is one of the explanations as in Fig. 5-7.

The shape of peaks centered near 0.05 V rarely changes from 50 mV/s to 1 mV/s and almost no catalytic behavior is observed for all three plots. We thus speculate that this electrochemical active species make no contribution to the bioelectricity generation. More interestingly, we observed a linear slope from -0.1 V to 0 V in $\Delta rpoS$ at 1 mV/s while the plots of the other two are very small peaks. This phenomenon is highly repeatable in different batches experiments (Not shown here). Considers the plateau responding cathodic current from -0.05 V to -0.2 V, we rule out the possibility of capacity discharge and point it as catalytic current from a new EET pathways. The small peaks in the PAO-1 and $\Delta rpoN$ curves indicate that this is not a new electrochemical active species for PAO-1. But only $\Delta rpoS$ could use it to facilitate the electron transfer, thus increase the bioelectricity generation. From CV curve at 1mV/s, we can confidently conclude that both mutants have better performance than PAO-1, although they use a different strategy. The detailed biomolecule mechanism is unknown.

Fig. 5-4 CV analysis of MFCs with different *P. aeruginosa* strains at steady state: (A) 50 mV/s; (B) 1 mV/s

After CV analysis, MFCs anode supernatant is immediately taken for HPLC analysis and the result is listed in Figure 5-5. We can see that the phenazine in all MFCs anode is PCA, and the

relative concentration revealed by HPLC result and CV are consistent with each other. PYO, which is in high concentration when strain Δ rpoS is aerobic cultured, is not detected under MFCs operation conditions. It is a reasonable result since oxygen is required for the PYO synthesis from PCA while PCA synthesis is not affected under anaerobic conditions (Dietrich et al., 2006). Further compared the HPLC result between sample from aerobic culturing and MFCs, we find that under MFCs conditions, extracellular metabolic of Δ rpoN is greatly reduced. It can be anticipated that bacteria metabolism is greatly influenced after global regulator deletion as showed by microarray analysis in former work (Whiteley et al., 2000). Bacteria metabolism regulation is proved a possible strategy for enhancing bioelectricity generation in reported work (Yong et al., 2012b), which may be another benefit for this random mutation.

A small piece of electrode is cut and post treated to do FESEM. Fig. 5-6 shows the representative SEM images of biofilm formed with all strains. Remarkable difference is observed between these strains, especially the Δ rpoS one. Branch-like structure occurs between carbon fiber in electrode from Δ rpoS inoculated MFCs (indicated in the insertion of Fig. 5-6 (B)). At high resolutions, we can clearly find that these branches are totally composed of bacteria and they shall be the similar mushroom structure as in the *P. aeruginosa* biofilm formed on the plain wall, indicating a mature biofilm in MFCs with Δ rpoS (Klausen et al., 2003). Both PAO-1 and Δ rpoN have biofilm closely attached to carbon fiber and Δ rpoN have an even poor coverage (qualitative result). The biofilm formation trend for these three strains is in the sequence of Δ rpoS, PAO-1 and Δ rpoN, which is consistent with regulation network analysis as in Schematic 5-1. We believe this is the main reason why MFCs with these strain are different in time-cost to reach the current plateau as in Fig. 5-3.

Fig 5-2 HPLC result of supernatant MFCs operation: (A) PAO-1; (B) $\Delta rpoS$; (C) $\Delta rpoN$. Small window in (B) is the full wavelength UV absorption at 14.3 min

Fig. 5-6 FESEM images of anode biofilm with different *P. aeruginosa* strains: (A) PAO-1; (B) $\Delta rpoS$; (C) $\Delta rpoN$

5.4 Discussions

5.4.1 Global regulator *rpoS* and *rpoN* deletion enhance bioelectricity generation in *P. aeruginosa* inoculated MFCs

Lovely, etc. first reported HgtR as a global regulator in biosynthesis and energy recovery in *Geobacter* strains (Ueki & Lovley, 2010). However, to the best of our knowledge no literature has been reported on how global regulator mutation would influence the system performance. Here, we studied the effect of two global regulator RpoS (σ^{38}) and RpoN (σ^{54}) deletion on bioelectrochemical activities of *P. aeruginosa*.

Schematic 5-1 exhibits the regulation network of genes closely related with bioelectricity generation in *P. aeruginosa*. Involved genes are related with phenazine synthesis (phzA1-G1, A2-G2, H, M and S) and biofilm formation (pil gene cluster and fle gene cluster) (Mavrodi et al., 2001; Rabaey et al., 2005a). Rhamnolipid plays an important role in the biofilm formation of *P. aeruginosa* (Pham et al., 2008b; Wen et al., 2010). Membrane channel proteins (OprDEF) are also involved in EET based on the former work from our group.(Yong et al., 2013b) A group of Xcp proteins are crucial for extracellular synthesis of type IV pili responsible for biofilm formation. Most of these genes are regulated by the upstream lasR/lasI and rhlR/rhII QS systems, which are further regulated by RpoS (σ^{38}) and RpoN (σ^{54}), the two global regulators in *P. aeruginosa*. The RpoS and RpoN knockout mutants exhibited fundamental differences in metabolism and biofilm formation, and bioelectrochemical behavior. Strain Δ rpoN produces more PCA and thus benefit in bioelectricity generation although its biofilm formation seems greatly inhibited compared with wild type. As for strain Δ rpoS, the enhanced bioelectricity generation comes from a new EET pathway centered near -0.05 V, which has not been reported by yet as our knowledge. Strain Δ rpoS is the only one exhibit mature biofilm under MFCs operation. However we lack the direct evidence between this better biofilm formation with enhanced bioelectricity generation and assumed new EET pathway. We believe it would be worth to use such strategy for biofilm regulation in bacteria with high anode respiration capability.

Schematic 5-1 General regulation network of global regulator *rpoS* and *rpoN*, indicating parts are that possibly related with bioelectricity generation in *P. aeruginosa*

5.4.2 Phenazine based MET is currently not an efficient EET pathway for *P. aeruginosa*

Phenazines are a group of famous electron shuttle in MFCs since it is bacteria self-secreted and is proved can be utilized by bacteria in other species, which provide interesting case studying for how mixed community mutual benefit with each other.(Pham et al., 2008a) However, *P. aeruginosa* itself are not compatible anode respiration bacteria like those *Geobacter* and *Shewanella* species. The c-type outer membrane cytochromes (OMCs) in *S. oneidensis* enables complete EET via an electron transfer chain in the sequence of membrane bonded cytochrome → OMCs → flavin → electrode.(Carmona-Martinez et al., 2011) However, no similar mechanism is reported in *P. aeruginosa*. It is possible that the process for electron carry to transport through the bacteria membrane will be rate limit step during the whole EET under such situations. Here, in the strain $\Delta rpoS$, we provide new insight into this assumption. This recognized new EET

pathway is obviously limited in its total electrochemical activity (roughly estimated by the peak current density at high scanning rate) while it makes considerable contributions for the bioelectricity generation. We can conclude that although its relative amount is much smaller than PCA, the contribution is similar, deduced from the respective catalytic current calculated from CV curve at 1 mV/s. Though the molecular microbiology mechanism is not clear yet, this implies more complex EET pathway potentially available and possible to turn this electrochemical low efficient bacteria to efficient one.

5.4.3 Global regulator mutation is an effective tool for engineering anode respiration bacteria

In our early works, we mainly focus on engineering bacteria in a well-recognized way, regardless it is in the bacteria metabolism, electron shuttle synthesis or its diffusion pathway engineering. Here, we try to study the system performance in a global view sight and the result is quite interesting. Besides the general acknowledged aspects that influence bioelectricity generation like mediator synthesis and biofilm formation, we also find a postulated new EET pathway in strain $\Delta rpoS$. This attempt, at first, gives a general tool for vast filtering the knowing EABs in MFCs anode since RpoS (σ^{38}) and RpoN (σ^{54}) homologue are quite universal. For another part, the new recognized EET pathway here implies bacteria are more feasible for anode respiration as we supposed, giving more possibility to board EABs choice. For bacteria, their gene expression is controlled under complex networks with multiple global regulator cross-talks with each other. Unlike the deletion of gene with single or limited functions, deletion one of such global regulator seldom means totally deficient in one specific function. It seems sophisticated way to overexpress

another global regulator in the network to compensate the partial functional defect, gives more unpredictable effect on the whole system.

5.5 Conclusions

In conclusion, we construct two single global regulator mutation strains for *P. aeruginosa* and tested in MFCs. Strain Δ rhoS exhibit a 40% increase in bioelectricity generation while that of strain Δ rhoN is 22%. One new EET pathway is ascribed to explain the enhanced bioelectricity generation with strain Δ rhoS and Δ rhoN benefit from much higher PCA concentration, meanwhile we should notice vast difference lies in metabolism, biofilm formation and electrochemical behavior among these three strains. Here, we verify the suitable global regulator mutation is a meaningful strategy for this system.

Chapter 6: Synthesized Ecosystem with *Shewanella oneidensis* and *Pseudomonas aeruginosa* Greatly Improve the Microbial Fuel Cells Performance

Abstract

Novel ecosystem consists of *Pseudomonas aeruginosa* PAO-1 and dissimilatory metal-reducing bacterium *Shewanella oneidensis* MR-1 is designed and applied as the anode respiration community in microbial fuel cells (MFCs) to improve the system performance. MFCs with this anode ecosystem steadily works for 280 hours by refresh the anode medium when the nutrient is depleted. Calculated based on the polarization curve, the maximum power density of MFCs with co-cultures is 523.5 mW/m², which is round 13 and 854 times of MFCs use MR-1 and PAO-1 pure culture as inoculation, separately. Slow rate cyclicvoltammetry verifies that improved performance is resulted from increased phenazines mediated electron transfer.

6.1 Introduction

After rapid development in the past decade, performance in microbial fuel cells (MFCs) has greatly improved. Most high efficiency MFCs adopts mixed culture community as anode respiration species by using wastewater and sludge as inoculation. However, the underlying mechanism how bacteria mutual with each other in the anode community is still a black box for researchers and only limited works published during the past years to give some glimpses into this important issue.(Pham et al., 2008a; Pham et al., 2008b; Venkataraman et al., 2011) Meanwhile, more and more research interest is turning to biocathode for bioremediation and bioproduction.(Harnisch & Schroder, 2010; Wang & Ren, 2013), which increase the demand for

better understanding the mechanism of biomolecule of corresponding species and their relative role in mixed community.

Only limited microorganisms like *Geobacter* species and *Rhodopseudomonas palustris* were reported to have comparable bioelectricity generation capability to the mixed culture inoculated MFCs.(Xing et al., 2008; Yi et al., 2009) Dissimilatory metal-reducing bacterium *Shewanella oneidensis* attracts great research interest in MFCs and related fields due to its multiple extracellular electron transfer (EET) pathways.(Logan, 2009) However, most works using *Shewanella* species as anode respiration bacteria are primary to elucidate the corresponding EET and biomolecule mechanisms, to verify the possible interaction of novel materials with anode respiration bacteria and to expand the technology potentially useful in the related area.(Baron et al., 2009; Carmona-Martinez et al., 2011; Deng et al., 2010a; Peng et al., 2010) However, *Shewanella* is seldom used as pure culture inoculation to targeting a high performance MFCs, due to its critical to substrate (prefer lactate) and relative low efficiency compared with *Geobacter* species.

MFCs with *Shewanella oneidensis* DSP 10 is reported to achieved high power density as 2 W/m² by optimization MFCs configuration and operation.(Ringeisen et al., 2007) Electrochemical co-immobilize polypyrrole (PPy) and electron shuttle anthraquinone-2,6-disulphonic disodium salt with (PPy/AQDS) onto the anode leads to 1300 mW/m² power density achievement in *Shewanella* inoculated MFCs under short period operation.(Feng et al., 2010b) By redesign the anode constructor, work in our group verifies that MFCs with *Shewanella* as pure inoculation could reach a power density around 800 mW/m², approaching mixed culture MFCs.(Yong et al., 2014; Yong et al., 2012a) On the other hand, *Shewanella* has multiple

electron transfer pathways; the EET efficiency is limited due to several reasons. Extra adding flavins greatly increase the system performance even at low level. This indicates although *Shewanella* is capable of secrete soluble flavins as electron shuttle, the concentration is low and cannot fully support MET in *Shewanella*. Meanwhile, modify the electrode with nanomaterial is possible to greatly increase the direct electron transfer (DET) efficiency in *Shewanella*.(Deng et al., 2010a; Huang et al., 2011b; Peng et al., 2010) Above result indicate that *Shewanella* has potential to achieve high power output as that of *Geobacter* species and mixed culture in MFCs. Overcome the barrier on electron transfer is important to accomplish this target.

Pseudomonas aeruginosa attract vast interest in MFCs as they can synthesize electrochemical active phenazines to be used as electron shuttle by *Pseudomonas* and other species in the mixed culture.(Rabaey et al., 2005a) Meanwhile phenazines are group of antibiotics. The reported pure cultures that use phenazines facilitate EET belong to Gram-positive bacteria that originally co-exist in the community with *P. aeruginosa*.(Pham et al., 2008a; Venkataraman et al., 2011) No works on the long-term stable *S. oneidensis* and *P. aeruginosa* has been reported according to our knowledge.

Here in the work, novel ecosystem consists of *S. oneidensis* MR-1 and *P. aeruginosa* PAO-1 is successfully developed. MFCs with this co-culture anode stably runs for 280 hours with repeatedly anode medium refresh when the nutrient is depleted. The fast voltage restore after medium change demonstrate a high activity anode ecosystem formed on the anode. MFCs with this co-culture as anode inoculation achieve maximum power density of 523.5 mW/m², which is 13 and 854 times for MFCs with sole *S. oneidensis* and *P. aeruginosa* under the same operation conditions. CV analysis confirms that the improved performance is mainly due to the phenazines

based mediated electron transfer, indicating *Shewanella* could effectively use phenazines to facilitate EET.

6.2 Materials and Methods

6.2.1 Strains and culturing

Both *S. oneidensis* MR-1 and *P. aeruginosa* PAO-1 were activated overnight and amplified in LB broth for 8 eight hours at 30 °C and 200 rpm, followed by centrifuged at 4300 rpm for 7 minutes to harvest the bacteria. Supernatant was then removed and bacteria were redispersed in SM1 salt medium and inoculated into MFCs anode chamber. *S. oneidensis* was inoculated in the ratio of 1:5 to anode effective volume while for *P. aeruginosa* this ratio is 1:50.

6.2.2 MFCs configuration and operation

Two chamber with the inner size of 4.5 cm * 4.5 cm * 4 cm for each chamber was used, separated by Nafion 117 membrane. Carbon cloth was used both as anode and cathode. Anode size is 1 * 2 cm² while for cathode is 2* 3 cm². Cathode solution was the same as work in previous chapters. Well defined anode medium is used and consists of basic SM1 salt medium, 0.5 mM CaCl₂, Walfe's vitamin, Walfe's, amino acids and 18 mM sodium lactate as carbon source. The details of the medium are listed in Chapter II. Three parallel MFCs were set up for each condition.

MFCs were discharged under batch mode with a constant external resistance 2 kΩ. Output voltage was measured with digital multimeter (ESCORT 3146A). Anode medium was totally

refreshed when the potential drops for MFCs with MR-1 and mixed culture. In the presented operation period, anode medium for PAO-1 inoculated MFCs were not changed.

6.2.3 Electrochemical measurement

Polarization curve was done when the output voltage reaches its maximum by decrease suitable external resistance. For MFCs with mixed culture, MFCs is open circuit for half an hour before doing the polarization curve while for MFCs with MR-1 the open circuit time is 4 hours and overnight for MFCs with PAO-1.

Cyclicvoltammetry (CV) analysis was done with CHI 660D electrochemical workstations and Saturated Calomel Electrode (SCE, +0.243 V vs. SHE) as reference electrode. CV analysis was done when output potential drops to 20 mV to get the non-turnover CV curve and done at highest output voltage to get CV curve under turnover conditions. For MFCs with PAO-1, only with turnover condition CV analysis was done

6.2.4 FESEM image of anode biofilm

FESEM sample preparation and imaging were done the same as former chapters.

6.3 Result and Discussion

6.3.1 Improved MFCs performance with co-culture inoculated MFCs

Fig. 6-1 shows the V-t curve during MFCs operation under batch mode. For MFCs inoculated with MR-1, it takes around 15 hours to reach the steady state and then stably discharged for 60 hours before potential drop is observed. The maximum output voltage is 74 mV. For MFCs with

mixed culture, the output voltage is similar with that of MR-1 at first 15 hours. Rapid increase in output voltage is observed then and reaches the maximum value of 373 mV within 10 hours. Slow potential drop is observed, indicating depletion of nutrient. After anode medium refresh, MFCs with mixed culture restores its performance and reaches a maximum output voltage 475 mV. It is interesting to find that from the second batch, the cycle become very short and however, successfully reaches a maximum output voltage around 450 mV. For MFCs with MR-1, the performance also recovered after medium refresh with much longer time compared with the first

Fig. 6-1 Voltage output during MFCs operation: (A) V-t curve for MFCs with MR-1 (red) and co-culture as inoculation; (B) V-t curve for MFCs with PAO-1

cycle. The long time for performance restore after nutrient refresh indicating that in both conditions, soluble electron shuttle may plays essential in EET. For MFCs with PAO-1, maximum output voltage of 6.3 mV is reached. Then drops to around 4 mV and steadily discharges.

Polarization curve is done when the MFCs reaches its maximum output voltage (Fig. 6-2). The maximum power density is 523.5, 39.6 and 0.613 mW/m² for MFCs with mixed culture, MR-1 and PAO-1 based on the P-j curve, separately. For MFCs with mixed culture, 12 and 853 times

Fig. 6-2 Polarization curves of MFCs with (A) MR-1 (red) and mixed culture (blue); (B) PAO-1

improvement compared with MR-1 and PAO-1 is thus confirmed. V-j curve in Fig. 6-2 (A) provides interesting sight about the mechanism underlies the improved performance in mixed culture. We can find that open circuit potential for MR-1 and mixed culture are almost the same meanwhile the corresponding potential are also similar when the current density reaches around 100 mA/m^2 . These results demonstrate that MFCs with MR-1 and mixed culture should have similar performance when the anode potential is low, including a similar activation loss if we regard the whole EET process only involves single reaction. The difference become visible when potential drops under 630 mV and rapid increase in current density is observed from 630 mV to 450 mV.

As the electron transfer in *Shewanella* is comparable slow, thus the accepted ohmic loss due to the internal resistance revealed in the linear part of V-j curve in fact contains more components when we discuss similar curves in *Shewanella* inoculated MFCs. At least partial of these “ohmic loss” is due to the overpotential to drive the cytochromes to facilitate electron transfer to higher current density. This can be verified by the electrochemical studying in *S. oneidensis* system where continuously increase in turnover current is observed when potential increase at OM cytochrome dominated interregional.(Baron et al., 2009) It is reasonable to conclude that the greatly increased current density is mainly benefitted from enhanced EET, which is reflected by the linear part in V-j curve. At higher current density (1240 mA/m^2) rapid potential drop is observed. MFCs inoculated at similar conditions could have a maximum current density as high as 5000 mA/m^2 use *Shewanella* as inoculation, the rapid drop here cannot be due to a diffusion limitation as in most works.(Fan et al., 2008) We thus ascribe it to the limitation in substrate turnover rate.(Harnisch & Schroder, 2010) Increase the biomass amount may overcome this limitation and further to even higher performance.

6.3.2 *S. oneidensis* can effectively use phenazines as electron shuttle to facilitate EET

To further investigate the mechanism underlying the improved MFCs performance, CV analysis was done at non-turnover (substrate depletion) and turnover conditions. Fig. 6-3 (A) is the CV curve with a scanning rate of 50 mV/s at non-turnover conditions. For MFCs with MR-1 (red), two separate pairs of peak can be defined. One couple centered around -0.44 V belong to *S. oneidensis* synthesized flavins and another smaller one centered at -0.31 V belong the OM based direct electron transfer. For PAO-1, one pair peaks centered at -0.28 V can be defined, which probably belong to PCA as in Chapter V. For MFCs with mixed culture, two pair of peaks can be identified at the similar location of flavins and PCA. It is interesting to find that in the mixed culture, the PCA peak current density is even higher than PAO-1 wild type, although MFCs with mixed culture has refresh the medium for several times when CV is done while PAO-1 MFCs does not refresh the medium in the whole operation. It is reasonable to conclude that PAO-1 has higher activity in the mixed culture compared with in pure culture. For the flavins based peak, the cathodic peak current density is similar for MR-1 and mixed culture if an imagined baseline is subtracted. However, the anodic peak in mixed culture is more obvious and more importantly, catalytic behavior is observed. Considering the CV is done under substrate depletion conditions, we postulate this catalytic behavior result from electron transfer from PCA to flavins.

Fig. 6-2 (B) exhibits the CV curve at 1 mV/s with turnover current. The catalytic current of PAO-1 inoculated MFCs almost cannot be observed. For MR-1, both flavins based MET (from -0.48 V) and OM cytochrome based DET (from -0.25 V) catalytic current can be confirmed. MFCs with mixed culture has similar flavin mediated catalytic current beginning from -0.48 V and the value is almost the same (two line almost overlapped when potential is negative than -

0.36 V) Difference occurs from -0.36 V and sigmoid shape indicates catalytic current dominated the responding current at these potential period. Compared with CV curve of pure cultures and under non-turnover CV curves, it is reasonable to conclude this catalytic current is PCA based, used by *S. oneidensis* to facilitate its electron transfer. The corresponding catalytic current is $81 \mu\text{A}/\text{cm}^2$ (calculated from anodic current, -0.32 V to -0.13 V). Another interesting phenomenon should be noted is that continuous potential increase no longer increases the current density, meaning no direct electron transfer can be observed. This is totally different from former

Fig. 6-3 CV analysis of MFCs with different inoculation: (A) 50 mV/s without turnover current; (B) 1 mV/s with turnover current

publication and also different from result in former chapters.(Baron et al., 2009) However, this observation is totally fit with sharp potential drop at high current density in polarization curve (Fig. 6-3 (A)) Limitation in substrate turnover rate is reasonable explanation.

To further prove that MR-1 could use phenazines as electron shuttle, 1 μM PYO was directly added into a continuous discharging MR-1 inoculated BES. The results were shown in Fig. 6-4. Immediately current increase was observed after PYO adding and the current density was increased from 13 $\mu\text{A}/\text{cm}^2$ to 25 $\mu\text{A}/\text{cm}^2$. CV analysis confirms an increased EET beginning from -0.3 V, indicating MR-1 could effectively use PYO as electron shuttle.

Although *S. oneidensis* MR-1 can utilize OM-cytochromes to facilitate the direct electron transfer from microbe to electrode, its efficiency on the unmodified carbon electrode is rather low. Flavins would greatly enhance its EET efficiency as they act as the “bridge” between the OM cytochromes and electrode, as indicated in Schematic 1-4(B). In this process, flavins do not need to transport through the microbe membrane and greatly increased the flavin utilization efficiency. Phenazines have similar redox active center as flavins, thus it is not surprising that they could transfer electron with OM cytochromes in the same way as flavin. Meanwhile, phenazines have much more positive standard potential than flavins (-0.2 V vs. -0.42 V), which means it is easier to transfer electron from reduced OM cytochrome to phenazines which is in oxidized state.

Fig. 6-4 (A) MR-1 discharge curve before and after 1 μM PYO adding, poisoning at 0 V vs. SCE; (B) MR-1 CV curve before and after 1 μM PYO adding

6.3.3 FESEM image of anode biofilm

Fig. 6-5 shows the anode biofilm image of different inoculation. Partially covered biofilm formation can be observed in any case and no mature biofilm like ΔrpoS in Chapter V exists. PAO-1 and mixed culture based anode seems have more extracellular secretions which makes bacteria well covered. However whether this difference would make significant performance difference remains further study.

Fig. 6-5 FESEM image of anode biofilm: (A) PAO-1; (B) MR-1; (C) Mixed culture

6.4 Conclusions

In synthetic ecosystem consist of *S. oneidensis* MR-1 and *P. aeruginosa* PAO-1 is successfully developed and long-term stability is confirmed according to the restored MFCs after anode medium refresh. The maximum power density with mixed culture is 13 times of MR-1 and 854 times of PAO-1. CV analysis verifies that PCA (one kind of phenazines) based MET is the reason for the improvement. From the polarization curve and CV curve, we postulate limited substrate turnover rate is the new barrier for higher current density achieve, which is the first report of such phenomenon in *Shewanella* inoculated MFCs according to our knowledge. The new limitation implies the system still have large potential for further improve in performance as in this work only base carbon

cloth and wild type MR-1 is used. Improve the biofilm formation would probably explore this potential, which is our next stage work.

Chapter 7: Conclusions and Future Development

The last decade witnessed the rapid development of microbial fuel cells: from whole cell operation condition optimizing to detailed mechanism studying; from MFCs anode to cathode; from a chemical view like electrode material, solution condition and mass transport to a biological view like electrochemical active species isolation, biofilm development, metabolic and extracellular electron transfer pathways studying.

In my four years' PhD study, my research focus is deriving from bioelectrochemistry: the bioelectrochemical behavior of electrogens like *Shewanella* and *Pseudomonas* strains. By developing new strategies, I successfully improve performance of the above pure culture inoculated microbial fuel cells. With the help of electrochemical analysis, I did detailed analysis to reveal the mechanisms underlying the improved system performance, which also exploring some interesting bioelectrochemical behavior that has not been observed in the past work. The presented thesis mainly consists of four individual works which are aiming at developing novel strategies to broaden the research view of MFCs.

In the first work, I tried to use electrochemical method to directly immobilize *S. oneidensis* MR-1 into graphite and polypyrrole matrix, forming a "Conductive Artificial Biofilm (CAB)". Compared with naturally formed biofilm, these CABs are easy to form and much larger biofilm mass. When MFCs were equipped with these CABs, it took very short time for the system to reach its steady discharge state from MFCs start up and after medium refresh compared with naturally formed biofilm. The maximum power density achieved from polarization curve was 207 mW/m², which is 11 times of that with natural biofilm. Either reducing the graphite amount or totally abandon the polypyrrole used in the CAB leads to decline in MFCs performance and

stability. This work is one of the pioneer attempts which aimed to explore the utilization of synthesized biofilm in the field of microbial fuel cells.

In the second work, I used nitrogen doped carbon nanoparticle (DNCN) to modify the carbon cloth anode. MFCs with these DNCN modified carbon cloth anode had much better performance compared with unmodified carbon cloth anode. 3.5 times higher power density was achieved from polarization curve. Cyclic voltammetry analysis revealed that DNCN modification greatly enhanced direct electron transfer (DET) from OM cytochrome to MFCs anode. Similar phenomenon were observed in the former works which using carbon nanotubes and nanoparticles. Meanwhile the nitrogen doping resulted in much higher soluble flavin absorption and enhanced mediated electron transfer (MET). According to my knowledge, this work is the first one demonstrates that suitable anode modification could also improve the MET in microbial fuel cells by soluble electron shuttle absorption.

Besides the “chemical” effort I made in the first two works, in the presented third work, I try to develop novel biological strategy to bring new insight to the MFCs. Herein, I carefully studied the how random mutation of sigma factor in *Pseudomonas aeruginosa* would influence the those pure culture inoculated MFCs performance. *P. aeruginosa* PAO-1 mutant separately with upstream global regulators rpoS and rpoN deletion were compared with wild type. 40% and 22% increase in bioelectricity were achieved. Mechanism study revealed that strain Δ rpoS has a new EET pathway and Δ rpoN have higher soluble shuttle phenazines secretion, which separately explained their enhanced MFCs performance. The enhancement in MFCs performance was not comparable to the first two works. However, as the similar global regulators are found in many other microbes, this “bio” attempt clearly represents new possibility for the future works.

From the second works, we can say that increase the soluble shuttle concentration at biofilm and electrode interface would greatly increase the MFCs performance with *S. oneidensis*, meanwhile the third work demonstrate that although *P. aeruginosa* have high capability in shuttle secretion, their utilization efficiency is quite low. In my last work, I tried to develop an ecosystem which consists of *S. oneidensis* and *P. aeruginosa*. Highest MFCs performance (523.5 mW/m^2) and long term stability were achieved with this ecosystem. CV analysis confirms that *S. oneidensis* could effectively use phenazines as electron shuttle, which explained the great improved MFCs performance.

The presented work in this thesis only consist a small part in MFCs development during the past decade. They bring new concepts and strategies to this field and would be great inspiration for the future works. However, the key problems and bottlenecks still remain which require continuous enthusiasm and contribution. The essential concern for MFCs in the near future is to find out its industry applications.

MFCs were first developed by the scientists in environmental engineering. By harvesting the extra bioelectricity, MFCs was supposed to act as new biotechnology to compensate the huge electricity consumption in wastewater treatment, which is estimated to be 1 kW/m^3 . This power density has been achieved in lab scale MFCs, however the scale up of MFCs to the industry level still remain a huge challenge. The low pH buffer and ion strength of the real wastewater, increased internal resistance when MFCs are scaled up, lack of suitable cathode choice and high operation cost all hinder the application of scaled up MFCs. Besides these technical difficulties, bioelectricity is not favored as the only product of MFCs, if we consider from “economic” aspect. Future application of this biotechnology requires much more valuable products.

Applying MFCs as biosensor to monitor the wastewater quality is one of the promising applications. The publications in the topic are rapidly increasing in recent years. Most of these work focus on using MFCs as chemical oxygen demand (COD) or biological oxygen demand (BOD) sensor.(Di Lorenzo et al., 2014; Hsieh & Chung, 2014; Kim et al., 2003) When the electro-active anode biofilm is well established, the output current is positively related with substrate concentration if MFCs are operated under nutrient limitation conditions. For mature MFCs system, it only takes tens of minutes to response to the change of organic compound concentration in the wastewater. Such MFCs based BOD biosensor has been commercialized now. When pure culture is inoculated as the electrogen, it is possible to detect some specific substrate. One example is utilizing *Shewanella putrefaciens* based MFCs as lactate sensor.(Kim et al., 1999)

Bioremediation represents another MFCs potential application. The spatial separation of “oxidation” and “reduction” process in MFCs provides great advantages for bioremediation, especially in MFCs cathode. Most reported work in this topic aiming at using the “reducing force” at cathode to degrade pollutants like nitrate, nitrite, perchlorate, heavy metals like U (VI) and Gr (VI). Bioproduction may be the most promising MFCs application in the future. Transform the bioelectricity from MFCs to energy in other forms like hydrogen and fatty acid are attracting more and more attentions, which is the main research focus of next generation MXC.

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Y. Y. Yu, H. L. Chen, Y. C. Yong, D. H. Kim and H. Song, Conductive artificial biofilm dramatically enhances bioelectricity production in *Shewanella*-inoculated microbial fuel cells, *Chemical Communications*, 2011, **47**, 12825-12827.

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Under Preparation

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Y. Y. Yu, T. Liu, J. Y. Wang and H. Song, Synthesized Ecosystem with *Shewanella oneidensis* and *Pseudomonas aeruginosa* Greatly Improve the Microbial Fuel Cells Performance (manu preparation)

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