A Study on Thermophilic Bioelectrochemical Systems for CO_2–to–Methane Conversion Technology

（二酸化炭素−メタン変換技術の開発へ向けた好熱性バイオ電気化学的システムの研究）

東京大学大学院工学系研究科

システム創成学専攻

博士(工学) 学位論文

付　　乾

Abstract

Bioelectrochemical systems (BESs) are promising technologies which use living microorganisms as biocatalysts to drive the reactions at the electrodes and have wide applications in wastewater treatment, electricity generation, H_2 production, biosensor and bioelectronics. Recently, it has been reported that CO_2 could be converted to CH_4 in a process called "electromethanogenesis", in which methanogens attached on the cathode act as biocatalyst. Based on this technology, our lab proposed a promising application for BESs: to build a sustainable carbon cycle system by combining BESs and CO_2 dioxide capture and storage (CCS) technology together, aiming to convert the CO_2 stored in CCS reservoir to CH_4. Up to now, however, BESs still cannot be commercialized due to their low performance, not even the sustainable carbon cycle systems. Operating BESs at elevated temperatures is one measure to improve the performance of BESs, but there are only a few studies investigated thermophilic BESs. Compared with more than 20 species of mesophilic electrochemically-active microorganisms, there are only two species of thermophilic electrochemically-active microorganisms, *Thermincola potens* JR and *T. ferriacetica*, were reported to be capable of transferring electrons to anode. Therefore, expanding our knowledge of thermophilic BESs is desirable to improve the performance of thermophilic BESs. My thesis is mainly focused on the fundamental studies of thermophilic BESs, including the exploration of novel thermophilic electrochemically-active microorganisms and their application on H_2 production and CO_2 conversion to CH_4.

Firstly, to study the mechanism of CO_2 conversion to CH_4, we built a mesophilic single-chamber BES reactor aiming to produce CH_4 by inoculating the effluent of a mesophilic microbial fuel cell and adding 1.0 V voltage into the circuit. After inoculation, the current was generated in the single-chamber BES reactor, while there was no current observed in the abiotic control reactor with 1.0 V voltage. After one batch cycle, only CH_4 was produced in the inoculated BES reactor while there was no CH_4 or H_2 was observed in the abiotic control reactor with 1.0 V voltage and

inoculated control reactor (without applied voltage). The results showed that CH_4 was probably produced due to the electromethanogenesis reaction:

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O \qquad \text{(Eq. 1)}$$

We also presented the first comprehensive phylogenetic analysis of both the biocathodic and bioanodic communities by constructing 16S rRNA clone libraries. The results showed that the composition of the cathodic microorganisms was significantly different with that in a previous report: no methanogen of the *Methanobacteriales* class was detected, and instead, a methanogen closely related to *M. bavaricum* of the *Methanomicrobia* class was the dominant methanogen. Moreover, it was suggested that an exoelectrogenic bacteria, *G. sulfurreducens*, was enriched on the biocathode. These observations indicated the possibility that diverse species of methanogens could catalyze electromethanogenesis on the biocathode. It has been shown that *G. sulfurreducens* is also capable of catalyzing hydrogen production using an electrode (cathode) as the electron donor. Thus, we think it is possible that, in the following stage of incubation, *G. sulfurreducens* established a cooperative relationship with the methanogen for the electromethanogenic reaction by first receiving electrons from the cathode for hydrogen formation and then providing the resulting H_2 to the methanogens for hydrogenotrophic methanogenesis. Alternatively, because it has recently been shown that *G. sulfurreducens* and related *Geobacter* species can directly transfer electrons to other microorganisms (including methanogens), it is also possible that *G. sulfurreducens* provided electrons (not molecular H_2) directly to the methanogen, which utilized the electrons in the electromethanogenic reaction (Eq. 1). The detailed mechanism needs to be further investigated by using pure culture as inoculum.

Secondly, to explore and identify novel thermophilic electrochemically-active microorganisms, two-chamber microbial fuel cells were built in this study. Thermophilic microorganisms from various sources, including thermophilic digestive sludge and oilfield formation water under different temperature, were used as the inoculum. These MFCs started up successfully and showed substantial power density

generation, suggesting that electrochemically-active microorganisms (exoelectrogens) were enriched in the anode chambers of these microbial fuel cells. The maximum power density was obtained in the thermophilic MFC inoculated with Yabase oilfield formation water (1003 mW m^{-2}), higher than those reported with thermophilic MFCs in several previous studies (generally ≦ 400 mW m^{-2}) and comparable to that of a thermophilic MFC under continuous mode of operation (1030 ± 340 mW m^{-2}). The electron transfer mechanisms between the electrochemically-active microorganisms and anodes were investigated by using medium exchange experiment and electrochemical methods (cyclic voltammetry). The results showed that all the electron transfer mechanisms (except the hyperthermophilic bioanodes) were direct electron transfer. The microbial analyses of the bioanode in each reactor was analyzed by constructing gene-clone libraries. The results showed that *Firmicutes* and *Deferribacteres* phylum accounted for the majority in the microbial analyses of bioanodes. Based on the microbial analyses, two novel thermophilic exoelectrogens, *Caloramator australicus* strain RC3 and *Calditerrivibrio nitroreducens* Yu37-1, were tested in the experiment and proven to be capable of transferring electrons to anodes. Furthermore, a hyperthermophilic MFC was successfully started up by inoculating the hyperthermophilic microorganisms from the produced water of an oilfield. As the hyperthermophilic MFC could operate at the elevated temperature range between 75℃ and 98℃, it has a potential application in industrial processes under extreme conditions. The microbial analysis showed that *Caldanaerobacter subterraneus* (subspecies *subterraneus* and *tengcongensis*, respectively) are the dominating bacteria. These results largely expanded our knowledge of thermophilic electrochemically-active microorganisms.

Thirdly, a thermophilic biocathode capable of H_2 production was for the first time built in this study. A single-chamber microbial electrolysis cell (MEC) reactor was firstly started up by inoculating the effluent of a thermophilic MFC inoculated with the thermophilic digestive sludge. At an applied voltage of 0.8 V, H_2 was produced in the inoculated single-chamber MEC reactor, while there was no H_2 measured in the

abiotic control reactor (with 0.8 V) and the inoculated control reactor (without applied voltage), suggesting both the microorganisms and voltage are needed for the H_2 production. The cyclic voltammogram of the biocathode showed that the cathodic current of the cathode was significantly more negative than that of the anode, suggesting that the cathode have a relatively higher catalyzing activity for H_2 production. Thus the cathode in the single-chambered MEC was transferred into a two-chamber MEC reactor and further analyzed by using electrochemical methods. The linear sweep voltammetry (LSV) showed that the biocathode had a significant higher reducing activity than the control electrodes (bioanode or non-inoculated electrode). At the potential of –0.8 V vs. SHE, the thermophilic biocathode produced a current density of 1.28 A m^{-2} and an H_2 production rate of 376.5 mmol day^{-1} m^{-2}, which were around 10 times higher than those of the non-inoculated electrode, with the cathodic H_2 recovery of *ca*. 70 %. The molecular-phylogenetic analysis of the bacteria on the biocathode indicated that the community was comprised of six phyla, in which *Firmicutes* was the most populated phylum (77% of the clones in the 16S rRNA library). It was the first report of thermophilic biocathode capable of producing H_2, largely expanding our knowledge of thermophilic BESs.

Last, a thermophilic biocathode capable of converting CO_2 to CH_4 was for the first time built and its electron transfer mechanisms was investigated in this study. This biocathode was firstly started up in a single-chamber reactor using the effluent of a thermophilic MFC inoculated with Yabase oilfield formation water as the inoculum. After start-up, the maximum CH_4 production rate of the biocathode was around 1103 mmol day^{-1} m^{-2}, which was much higher than that in previous studies (lower than 656 mmol day^{-1} m^{-2}) and the mesophilic biocathode (450 mmol day^{-1} m^{-2}) reported in this study. In addition, the current to CH_4 conversion efficiency was around 100% in the single-chamber BES reactor, suggesting a directly electron transfer mechanism. Then the biocathode was transferred into a two-chamber reactor for further analysis. At a set potential of –0.7 V vs. SHE, the biocathode was capable of converting CO_2 to CH_4 with an abiotic anode as the courter electrode and CO_2 as sole carbon source.

The cyclic voltammogram (CV) of the biocathode showed a catalytic wave with a midpoint potential of –0.34 V vs. SHE in the range of –0.6 V ~ –0.3 V vs. SHE. In contrast, there was no significant peaks observed in the CV of the cell-free spent medium of the biocathode and the abiotic control electrode. In addition, the biocathode can produce CH_4 at a rate of 14 mmol day^{-1} m^{-2} with CO_2 as the sole carbon source at a set potential of –0.4 V vs. SHE. As the theoretical redox potential for H_2 production was –0.456 V at pH 7 at 55 ℃ and no CH_4 or H_2 was detected in the absence of CO_2, it suggested that the H_2 evolution was not necessary for the conversion of CO_2 to CH_4 and the electron transfer was in a direct manner. Correspondingly, the midpoint potential of –0.34 V vs. SHE was responsible for the CO_2 reduction, which was probably due to the redox components (e.g. enzyme) on the surfaces of microorganisms. The morphology of the biocathode was also analyzed by the scanning electron microscopy (SEM), which showed that a thin layer of biofilm with relative homogeneous shape of microbial cells was formed on the biocathode. The microbial analyses showed that *Methanothermobacter thermautotrophicus* and *Thermincola ferriacetica* were the dominant species of archaea and bacteria, respectively. To investigate the functional role of the pure culture methanogen, *M. thermautotrophicus* was inoculated into a two-chamber BES reactor and the result showed that this pure culture was capable of accepting electrons from the cathode for CO_2 reduction by itself. However, the CH_4 production rate was lower than that of the mixed culture, which was probably due to the lack of supporting functions of other microorganisms, such as exoelectrogens.

Acknowledgment

First of all, I would like to thank my advisor, Professor Kozo Sato, for giving me this valuable opportunity to do my Ph. D research in the University of Tokyo. I really appreciate his guidance in my research and his tolerance of my fault in the lab. In the past three years in Professor Sato's lab, I have not only learnt the scientific knowledge, but also the attitude towards the research, which will be my priceless treasure in my whole life.

Second, I would like to express my deep appreciation to Associate Professor Hajime Kobayashi, without whom I cannot finish my doctoral thesis, especially on the microbial analysis part. I really appreciate his help and encouragement in my research and daily life in Japan. I also want to thank my vice-advisor in GMSI program, Professor Shigeo Maruyama, for his suggestion and encouragement on my research.

I am grateful to all my colleagues in my lab for their assistance. I especially want to thank Professor Yoshihiro Masuda, Dr. Javier Vilcaez, Goda, Zhengguo Li, Saito, Onaka, Kuramochi, Hara, Tanaka, Jing Xu, Umeda, Tamamura, Omer, for their help in my research and daily life. My Ph. D research was supported by the MEXT (Ministry of Education, Culture, Sports, Science, and Technology) scholarship and I thank this agency for his support.

Finally, I want to thank my family and all my friends for their consistent support and encouragement. Without their love and support, I could have never achieved this honorable milestone in my life.

Qian Fu

June, 2013

Table of Content

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Energy Demand and Global Warming Issue

As we all know, energy issue is one of the biggest issues concerning humanity world now. Since the industry revolutionary, the annual world energy consumption has been increasing year by year. According to a recent report of IEA, the total world energy supply increased from 102, 569 TWh in 1990 to 117, 687 TWh in 2000 and 143, 851 TWh in 2008 (Muncuk 2012). Among the energy structures, fossil fuels are still the dominating energy supply for the primary sources of world energy. In 2008, the fossil fuels account for more than 81% share in primary energy consumption in the world, which consisted of oil 33.5%, coal 26.8% and natural gas 20.8% (Muncuk 2012). However, we all know that these fossil fuels are non-renewable and will be consumed up in some day. Based on the current proved reserves and flows, the years of fossil fuel production left in the ground are 43 years for oil, 164 years for coal and 64 years for natural gas (Shafiee and Topal 2009). Therefore, developing alternative energy is urgent and necessary to meet the increasing energy needs.

Besides, the environmental issue (e.g. Climate change) is another big issue facing the humanity. In the past 200 years, we emitted uncountable tons of greenhouse gases (GHG) (mainly CO_2) into the atmosphere due to the fossil fuel combustion, causing the average surface temperature of the earth rise in response (i.e. global warming issues). Fig 1.1 shows the CO_2 concentration variation verse time, which shows that the CO_2 concentration increased drastically in past 50 years. According to the 2007 Intergovernmental Panel on Climate Change (IPCC) 4th Assessment Report IPCC report, the rise in CO_2 emission could lead to a temperature increase in the range of 4 ~ 7℃ by 2050 if no CO_2 emission reduction measures will be performed, causing significant negative effects on the environment and human activity (Parry 2007). It has been widely agreed that reducing the CO_2 emissions is needed by 2050 to limit the expected temperature increase to less than 2℃. To achieve this goal, 50% to 80% cuts in global CO_2 emissions by 2050 compared to the 2000 level will be needed to limit the long-term global mean temperature rise to 2℃ (Parry 2007).

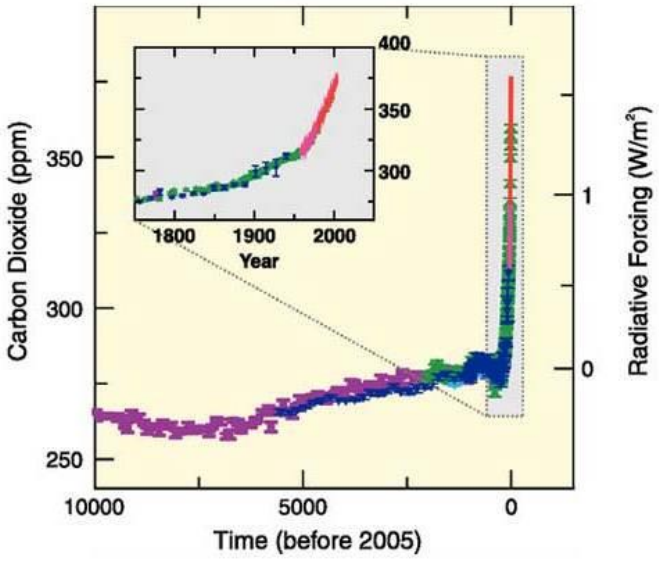


Figure 1.1 The concentration of CO_2 variation verse time (Parry 2007)

Table 1.1 The Relation between Emissions and Climate Change According to the IPCC 2007 Assessment Report (Bennaceur *et al*. 2008; Parry 2007)

Temperature increase	All GHGs	CO_2	CO_2 emissions 2050 (% of 2000 emissions)
(℃)	(ppm CO_2 equivalent)	(ppm CO_2)	(%)
2.0 – 2.4	445 – 490	350 – 400	−85 to −50
2.4 – 2.8	490 – 535	400 – 440	−60 to −30
2.8 – 3.2	535 – 590	440 – 485	−30 to +5
3.2 – 4.0	590 – 710	485 – 570	+10 to +60

1.2 Renewable Energy and CO_2 Capture and Storage Technology

1.2.1 Renewable Energy

Developing renewable energy is one promising and urgent solution to meet the increasing energy demand and reduce greenhouse gas emission. Renewable energy refers to the energy comes from natural resources which are renewable, for example, sunlight, wind, tides, biomass, and geothermal heat. These renewable energies are zero carbon emission (e.g. solar, wind, tides and geothermal energies) or carbon-neutral (e.g. biomass energy), thus they can contribute significantly to the CO_2 emission reduction. According to the IEA's blue scenario (Fig. 1.2), to achieve 50% reduction in GHG emission by 2050, renewable energy needs to contribute around 21% of the GHG emission reduction (Bennaceur *et al*. 2008).

Furthermore, Zerta *et al.* reported that the renewable energy will become the dominating energy resource in the future, which will begin to substitute the role of fossil fuels since 2030 and replace fossil fuels by 2100 (Zerta *et al*. 2008). However, up to now, only 16% of global final energy consumption comes from renewable energy (Martinot and Sawin 2011). Among these renewable energies, traditional biomass energy, which is mainly used for heating directly, accounts for the largest portion due to its low price and ease of storage.

1.2.2 CO_2 Capture and Storage Technology (CCS)

To achieve the goal of reducing greenhouse gases (GHG) emissions by 50% by 2050, CO_2 capture and storage (CCS) technology is also necessary except developing renewable energies (Metz 2005). CCS is a promising technology to mitigate CO_2 emissions from the large-scale fossil fuel use. The concept of CCS is shown in Fig 1.3, which can be divided into two parts: capture and storage processes. The capture process involves capturing the CO_2 released from the combustion of fossil fuels, such

as power generation, the preparation of fossil fuels and natural-gas processing (Metz 2005). Then the captured CO_2 will be transported to a storage site (usually depleted oil or natural gas reservoirs and saline formation water) where it will be stored away from the atmosphere for a very long time (Metz 2005).

Based on the IEA's CCS roadmap, to achieve 50% reduction in greenhouse gases emission by 2050, CCS needs to contribute around 20% on the reduction. Therefore, the CCS storage reservoirs must be large enough relative to annual CO_2 emissions. Furthermore, it had been suggested that at least one hundred CCS projects need to be globally deployed by 2020 and over 3000 projects by 2050 (Metz 2005). However, the deployment of CCS over the world is limited to only eight fully integrated operations until now (i.e. Sleipner, Snohvit, In Salah, Weyburn, Shute Creek, Val Verde, Enid Fertilizer and Century projects) (Global 2011; Sato *et al*. 2013). Such sluggish development is attributed to several factors, such as, legal and regulatory aspects, public acceptance, and financial issues (Sato *et al*. 2013).

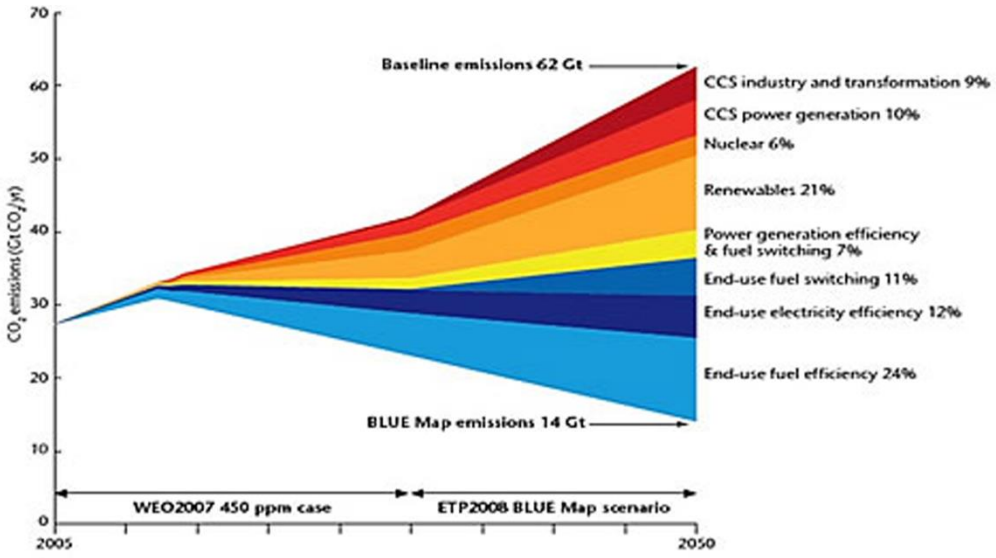


Figure 1.2 IEA blue scenario roadmap to reduce CO_2 emission by 50% by 2050 (Bennaceur *et al*. 2008).

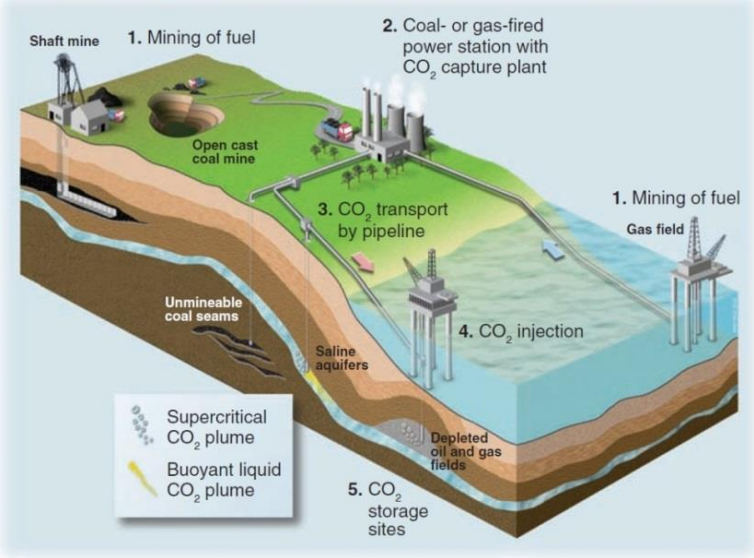


Figure 1.3 The schematic of CO_2 capture and storage (Haszeldine 2009).

1.3 Opportunity of Bioelectrochemical Systems (BESs)

1.3.1 Overview of BESs

Bioelectrochemical systems (BESs) are promising renewable technologies which can provide alternative solutions to meet energy demand and solve global warming issues. Unlike the traditional electrochemical systems, BESs use living microorganisms as biocatalysts to drive the oxidation and reduction reactions on the electrodes and use the organic materials in wastewater as substrates. Therefore, they can be used as electricity generation while treating wastewater (Logan *et al*. 2006), CO_2 reduction (Cheng *et al*. 2009), production of chemicals (Lovley 2012; Rabaey and Rozendal 2010) and bioremediation and bioelectronics.

Among these applications, the most widespread application of BESs is microbial fuel cell (MFC), which aims to harvest electricity from the organic materials in wastewater while simultaneously treating it. As we know, wastewater treatment is an

energy intensive process, which needs a lot of energy input. For instance, the wastewater treatment processes are responsible for 2% of electricity consumption in U. S. and $45 billion in capital expenditure is needed to keep up with the increasing population in the next 20 years (Logan 2005). However, the wastewater contains a lot of energy in the form of biodegradable organic matter. It was estimated that there was around 9.3 times as much as energy in the wastewater than that was used to treat the wastewater (Shizas and Bagley 2004). Therefore, if we could recover 10% of this energy from the wastewater, we could make the wastewater treatment self-sufficient (Logan 2008). In this regard, MFC is a promising renewable technology, as it can use microorganisms as biocatalysts to produce electricity directly using the biodegradable organic matter as substrates.

Another promising BESs technology is the microbial electrosynthesis cell, which use microorganisms as biocatalysts to convert CO_2 to CH_4 or other valuable chemicals (e.g. acetate, alcohol) with electricity as energy input (Rabaey and Rozendal 2010). Like MFCs, microbial electrosynthesis uses living microorganisms as biocatalysts to drive the reduction reaction, which not only reduces the cost but also increases the electricity conversion efficiency (Rabaey and Rozendal 2010). As the CO_2 reduced in microbial electrosynthesis comes from atmospheric, therefore, the microbial electrosynthesis is essentially a carbon-neutral technology. As for the electricity, it comes from clean energy like wind and solar energy, which always produce more electricity than that can be used or stored. In 2008, Cheng *et al.* reported the first microbial electrolysis, which is also called as "electromethanogenesis", aiming to convert CO_2 to CH_4 using electricity as the energy input (Cheng *et al*. 2009). In their study, a thick biofilm containing methanogens were observed on the cathode surface and the current to CH_4 conversion efficiency was higher than 96%. The authors concluded that the methanogens could accept electrons from the cathode directly (Eq. 1.1). One pure culture methanogen, *Methanobacterium palustre*, was reported to be capable of accepting electrons directly from cathodes:

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O \qquad \text{(Eq. 1.1)}$$

Although microbial electrosynthesis technology is promising, the study of microbial electrosynthesis is still in its infancy. The species of microorganisms capable of converting CO_2 to CH_4 are largely limited, as well as the electron transfer mechanisms.

1.3.2 Sustainable Carbon Cycle System

1.3.2.1 The Key Concept

Although CCS is a promising technology to reduce the CO_2 emissions, the deployment of CCS cannot be easily commercialized due to its high cost. Thus, it requires financial support or value-added options to offset the high cost of CCS operations (Sato *et al*. 2013). Several value-added options have been proposed, such as enhanced oil recovery (EOR), enhanced gas recovery (EGR), and enhanced coal-bed methane (ECBM) recovery, which are applicable to oil fields, gas fields, and coal beds, respectively (Oldenburg *et al*. 2001; Ross *et al*. 2009; Rubin and de Coninck 2005; Solomon *et al*. 2008). However, these storage sites have limited storage capacity and geographic distribution (Bachu 2008). In contrast, it has been reported that saline aquifers have the largest storage capacity, which exist all over the globe (Rubin and de Coninck 2005), but no value-added options have been proposed for them. Therefore, it is desirable to develop a means to add substantial value to CO_2 storage in saline aquifers (Sato *et al*. 2013).

Recently, our lab proposed a concept of "sustainable carbon cycle system", aiming to convert the CO_2 stored in saline aquifers to CH_4 (Sato *et al*. 2013). The concept of the sustainable carbon cycle system is shown in Fig. 1.4, in which the CO_2 emitted from the power plant will be first captured and stored in saline aquifer, and then converted to CH_4 by BESs with excess renewable energy (such as solar, wind energy) or other intermittent electrical energy which cannot be stored as the energy input. As the BESs could convert electricity into CH_4 at a very high conversion efficiency (ca.

96%) (Cheng *et al*. 2009), this technology can also be considered as using the CO_2 storage reservoirs as energy-reserving tanks.

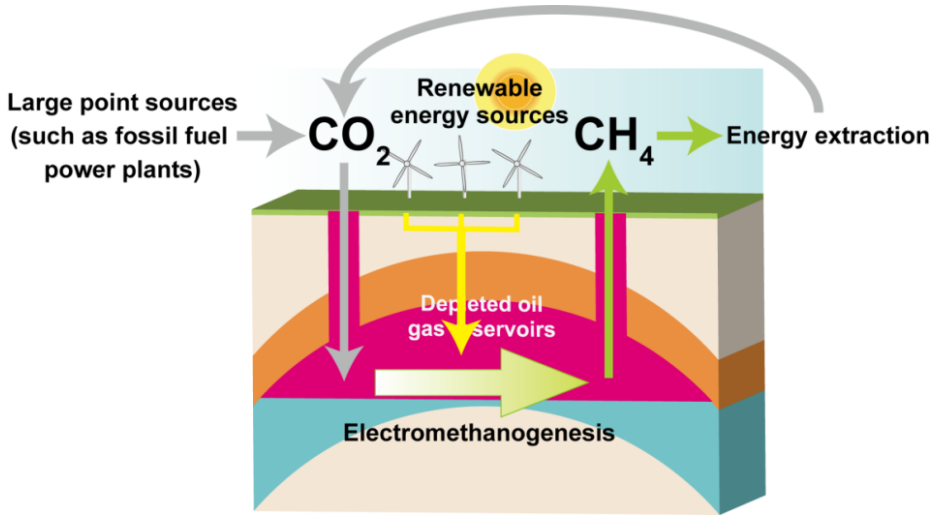


Figure 1.4 The schematic of sustainable carbon cycle system (Sato *et al*. 2013)

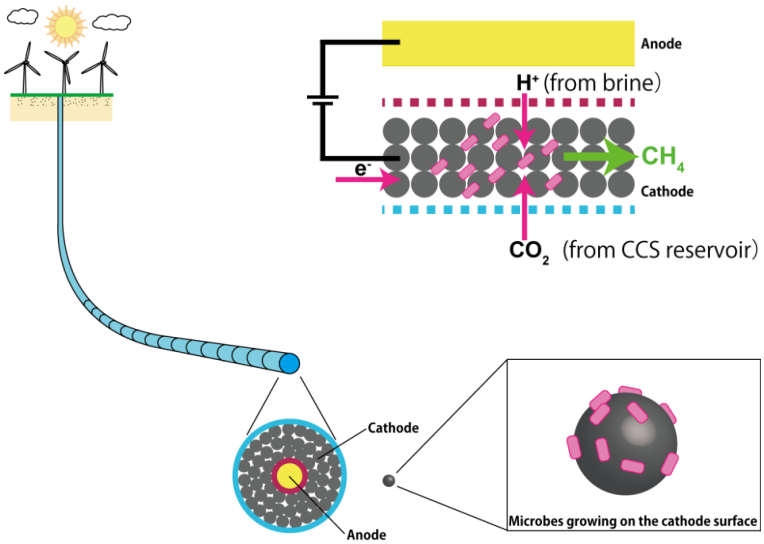


Figure 1.5 The schematic of electrode well for sustainable carbon cycle (Sato *et al*. 2013)

In this sustainable carbon cycle system, the CO_2 conversion process will be realized by electrode wells, which is shown in Fig. 1.5. The electrode well will be constructed by using a horizontal well as the vessel for electrodes, which will be filled with graphite granules as the electrode material (Fig. 1.5). The cathode and anode are separated by a membrane, which will be made of electrical nonconductive materials and has high fluid (ion) permeability (e.g., cloth or felt made of plastic or glass microfiber) (Sato *et al*. 2013). The casing of the well is reticulated or has slits, allowing exchange of fluids and gases between the interior (the electrodes) and exterior (the reservoir) of the well (Sato *et al*. 2013). On the surface of graphite granules in the cathode compartment, methanogens (either indigenous to the reservoir or exogenously injected) utilize electrons from the electrode and protons from the reservoir brine to reduce CO_2, which is dissolved in the brine, to CH_4 (Eq. 1.1) (Sato *et al*. 2013).

1.3.3.2 Comparison With Other Carbon Cycle Systems

There are also several other concepts of carbon cycle systems aiming to convert CO_2 to fuel or other valuable chemicals. For example, Audi company is building a plant capable of converting CO_2 to CH_4 using excess renewable energy (such as solar and wind energy) as the energy input (Audi 2011). The high amount of excess renewable energy in Germany, which grew from 150 GW-hours per year to 1000 GW-hours per year in two years, make this technology commercially possible (Bullis 2013). The schematic of this concept was shown in Fig 1.6. This technology consists of two mature technologies: electrolysis, which splits water to H_2 and O_2, and methanation, which combines H_2 with carbon from CO_2 to make CH_4. The major drawback of this technology is the low efficiency of converting electricity to CH_4, which was only around 40%.

In 2012, Li *et al.* reported a method to store electrical energy as chemical energy in higher alcohols, which can be used as liquid transportation fuels (Li *et al*. 2012). Their technology also consists of two part reactions: firstly, CO_2 is converted to

formate by traditional electrochemical reaction using solar cells as energy input, and then the formate is used by a genetically engineered lithoautotrophic microorganism, *Ralstonia eutropha* H16, to produce isobutanol and 3-methyl-1-butanol. Although this technology opens the possibility of electricity-driven bioconversion of CO_2 to commercial chemicals, the production rate was still low and energy conversion efficiency was quite low due to two separate reactions. Moreover, their reactor design is also needed to be improved.

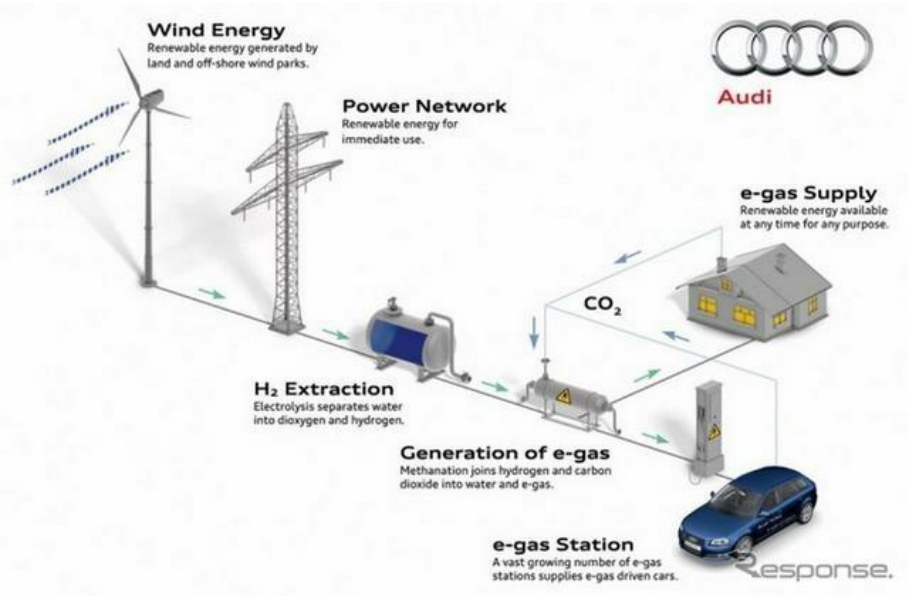


Figure 1.6 The schematic of sustainable CH_4 production of Audi company (Audi 2011).

Compared with these two techniques, there are several advantages for our sustainable carbon system. First, the conversion efficiency in our system is higher than that of these two techniques, as the CO_2 can be directly converted to CH_4 by using microorganisms as biocatalysts. In a reported study, the conversion efficiency of electricity to CH_4 in electromethanogenesis is around 96% (Cheng *et al*. 2009). Second, the other techniques are intended to be implemented on the surface of ground,

which will occupy a large area. In contrast, our system will be implemented in CCS reservoir, which will not occupy a large area on the ground.

1.3.3.3 Limitations of the Sustainable Carbon Cycle System

Currently, the major limitation of the sustainable carbon cycle system proposed by our lab is the relatively slow rate of electromethanogenesis and the limited knowledge of thermophilic electromethanogenesis, especially the electron transfer mechanisms between the cathodes and microorganisms. Up to now, all the studies of electromethanogenesis are focused on the mesophilic conditions, however, the temperature in CCS reservoir is usually higher than 40℃ and the mesophilic electromethanogenesis cannot be used in CCS reservoir. In addition, it has been reported that the electromethanogenic activity of a mesophilic microbial consortium, which was enriched in an electrochemical cell and consisted of multiple microbial species, was at least 30-fold higher than that of the purely cultured methanogen (Cheng *et al*. 2009). Therefore, exploring and identifying new natural thermophilic consortium (or consortia) with higher electromethanogenic activity is desirable for the development of the sustainable carbon cycle system.

Moreover, some other technical matters remain to be settled for practical deployment of electrode wells, including well intervention operations for electrode-material installation, maintenance, and monitoring purposes (Sato *et al*. 2013). In particular, considering that a carbon plantation takes a few decades before the product (CH_4) can be harvested, electrode well maintenance needs to be executed as needed without posing any difficulty for sustained electromethanogenesis (Sato *et al*. 2013).

1.4 This Research on BESs

1.4.1 The Objective of Thesis

The first objective of my research is to explore and identify novel thermophilic electrochemically-active microorganisms aiming to improve the performance of

BESs. Although BESs have been recognized as promising technologies to meet energy demand and reduce CO_2 emission, the research of BESs are still mainly in laboratory now due to their low performance. One limiting factor is the species of electrochemically-active microorganisms. Up to now, the studies of BESs are mainly in room temperature, thus the species of electrochemically-active microorganisms are limited in mesophilic microorganisms, which contain more than 20 species of electrochemically-active microorganisms (Logan 2009). Actually, it has been reported that operating BESs at elevated temperature could improve the performance of BESs (Jong *et al*. 2006). However, there are only few studies reported that thermophilic BESs and only two species of thermophilic electrochemically-active microorganisms, *Thermincola potens JR* (Wrighton *et al*. 2008) and *T. ferriacetica* (Marshall and May 2009), are identified in previous studies. Therefore, to explore and identify novel thermophilic electrochemically-active is desirable and necessary.

The second objective of my research is to build thermophilic biocathodes capable of producing H_2 and converting CO_2 to CH_4, as well as to study the electron transfer mechanisms in the biocathodes. Although the studies of thermophilic MFCs had been reported (Jong *et al*. 2006; Marshall and May 2009; Mathis *et al*. 2008), there is no report about the study of thermophilic MEC and thermophilic biocathodes for H_2 production and CO_2 reduction. In addition, for the sustainable carbon cycle system we proposed in last section, the mesophilic BESs cannot be used due to the high temperature in CCS reservoir (usually higher than 40 ℃). Therefore, establishing thermophilic biocathodes for H_2 production and CO_2 reduction is also of interest and necessary. In addition, to improve the CH_4 production rate, the electron transfer mechanisms in biocathodes also need to be investigated.

1.4.2 The Structure of Thesis

My Ph. D research is mainly focused on the study of BESs under thermophilic conditions which consists of four components.

First, I investigated the mechanism of the CH_4 production in a mesophilic electromethanogenic reactor. In this study, a mesophilic biocathode capable of converting CO_2 to CH_4 was produced, and its electromethanogenic activity was comparable to that reported in a previous study (Cheng *et al*. 2009). I presented the first comprehensive phylogenetic analysis of both the biocathodic and bioanodic communities. The results suggested that the CH_4 production in this mesophilic biocathode was probably mediated by exoelectrogenic bacteria and methanogens together.

Second, I exploited and enriched novel thermophilic electrochemically-active microorganisms using microbial fuel cell (MFC) and different sources of thermophilic microorganisms as inoculum. The results showed that the thermophilic electrochemically-active microorganisms are ubiquitous and exist in various environments. I also identified two novel thermophilic electrochemically-active microorganisms, *Caloramator australicus* RC3 and *Calditerrivibrio nitrrreducens* Yu37-1, were proven to be capable of transferring electrons to anodes.

Third, I examined the potential of thermophilic microorganisms as biocatalysts on the cathode of MEC. To achieve sustainable hydrogen production by microbial electrolysis cell (MEC) without precious metal catalysts, a biocathode was first developed in a single-chambered MEC operated at 55℃ and further analyzed in a two-chambered reactor. At the potential of –0.8 V vs. SHE, the thermophilic biocathode produced a current density of 1.28 A m^{-2} and an H_2 production rate of 376.5 mmol day^{-1} m^{-2}, which were around 10 times higher than those of the non-inoculated electrode, with the cathodic H_2 recovery of ca. 70%. The molecular-phylogenetic analysis of the bacteria on the biocathode indicated that the community was comprised of six phyla, in which *Firmicutes* was the most populated phylum (77% of the clones in the 16S rRNA library).

My last investigation was the thermophilic biocathodes capable of converting CO_2 to CH_4. A thermophilic biocathode was built in a single-chamber BES reactor using the thermophilic microorganisms enriched in Chapter 4. This biocathode was capable

of converting CO_2 to CH_4 at a set potential of –0.7 V vs. SHE with an abiotic anode as the courter electrode and CO_2 as sole carbon source. The current to CH_4 conversion efficiency was around 100% in a single-chamber bioelectrochemical reactor. The cyclic voltammetry analysis of the biocathode showed a clear catalytic behavior while no clear current was observed for the abiotic control cathode. The scanning electron microscopy (SEM) analysis showed that a thin layer of biofilm was formed on the biocathode. These data suggest that the biofilm directly accepts electrons from the cathode and convert CO_2 to CH_4. The microbial analyses showed that *Methanothermobacter thermautotrophicus* and *Thermincola ferriacetica* were the dominant species of archaea and bacteria.

Chapter 2

Literature Review: Fundamentals of Bioelectrochemical Systems (BESs)

This chapter reviews the important aspects of bioelectrochemical systems (BESs) that are useful in this study including the classification of BESs, biocatalysts and their electron transfer mechanisms, electrochemical background, as well as the previous studies of BESs under thermophilic conditions.

2.1 Introduction

Bioelectrochemical systems (BESs) refer to the electrochemical systems which use whole living microbial cells as biocatalysts to drive the oxidation and reduction reactions at solid electrodes (Aelterman and Verstraete 2009; Rabaey and Rozendal 2010). Compared with the electrochemical systems using purified enzymes or other organelles, the BESs using whole microbial cells have several advantages, including self-regeneration of the biocatalysts, flexibility in substrate use and higher versatility for product formation or conversion pathway (Rabaey and Rozendal 2010). In BESs reactions, an oxidation process and a reduction process occur at the anode and the cathode, respectively. By separating the oxidation and reduction process, a wide range of applications could be possible, such as electricity generation, wastewater treatment, biofuel production, bioremediation, biosensors and bioelectronics (He and Angenent 2006; Logan and Regan 2006; Rabaey and Rozendal 2010).

2.2 Classification of BESs

Based on the operating modes, BESs can be generally divided into three kinds of systems: microbial fuel cells (MFCs), microbial electrolysis cells (MECs) and microbial bioelectrosynthesis systems (Rabaey and Rozendal 2010), which will be separately introduced in the following sections.

2.2.1 Microbial Fuel Cells (MFCs)

Microbial fuel cells (MFCs) are the most investigated bioelectrochemical systems, aiming to directly convert the chemical energy of organic matters in wastewater to

electricity through biological, electrochemical, and electrical reactions (Logan 2008). Because MFCs can produce electricity while treating wastewater simultaneously, they are considered as a promising technologies for wastewater treatment (Rabaey and Verstraete 2005).

Fig. 2.1 shows the schematic of the working principle of MFCs. A typical MFC reactor consists of an anode chamber and a cathode chamber, which are separated by a proton exchange membrane. The anode chamber is the place where the microorganisms grow and the cathode chamber is the place where the electrons react with the catholyte. In the anode chamber, certain microorganisms degrade organic matters to release electrons and protons. The released electrons are transferred to the anode by the microorganisms (via a direct or indirect pathway) and pass through the circuit to the cathode. On the cathode, the electrons react with oxygen and protons which migrate from the anode chamber to produce water. The electrical energy is harvested when the protons pass through the load in the external circuit.

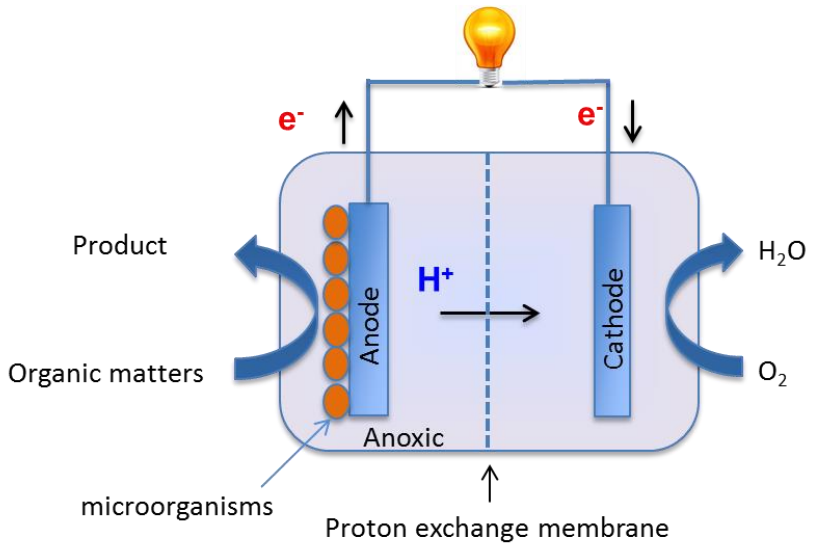


Figure 2.1 The schematic of microbial fuel cells

To separate the oxidation and reduction process, there must be no or little amount of electron acceptors in the anode chamber. Therefore, oxygen is removed from the anode chamber and anaerobic microorganisms are used in the anode chamber. On the other hand, the catholyte is usually sparged with air to provide enough dissolved oxygen to act as electron acceptors. When oxygen is used as terminal electron acceptor, metal-based catalysts (e.g. Platinum) are commonly used on the cathode to reduce the activation energy of oxygen. To avoid using expensive catalysts and improve the reaction on the cathode, some other soluble electron acceptors with high oxidative ability are also used, including potassium ferricyanide ($K_3[Fe(CN)_6]$) (Min *et al*. 2005), potassium permanganate ($KMnO_4$) (You *et al*. 2006) and triiodide (I_3^-) (Fu *et al*. 2010).

2.2.2 Microbial Electrolysis Cells (MEC)

Microbial electrolysis cell (MEC) is a modified type of MFC, aiming to produce H_2 from wastewater using electrical energy as energy input (Liu *et al*. 2005; Logan *et al*. 2008; Rozendal *et al*. 2008b).

Fig. 2.2 shows the schematic of a typical two-chamber MEC reactor, of which the structure was same with an MFC reactor except that an external power supply was added into the circuit. In MFCs, oxygen is commonly used as electron acceptor in the cathode chamber, whereas it is excluded from the cathode chamber and the protons act as the sole electron acceptor in MEC. The anodic reaction in MEC is same with that in MFC, the exoelectrogens on the anode oxidize the organic matters in the anode chamber to release protons and electrons. The electrons are transferred to the anode by the exoelectrogens and pass through the circuit to the cathode. The protons migrate from the anode chamber to the cathode chamber, and combine with the electrons which pass through the circuit on the cathode to produce hydrogen.

The electricity generation in a microbial fuel cell is thermodynamic favorable (*i.e.* Spontaneous), whereas the reaction in a microbial electrolysis cell is not. As we know, the equilibrium potential for proton reduction at biological suitable conditions (pH 7,

25 ℃) is –0.414 V (vs. Standard hydrogen electrode, in short, SHE) (Brett and Brett 1993), and the anodic potential of acetate oxidation by exoelectrogens is usually –0.3 V (Logan 2008). The potential produced by the microorganism on the anode is not negative enough to drive the hydrogen production on the cathode. Therefore, theoretically, –0.114 V (negative means voltage input) is needed to add into the circuit as driving force to make the H_2 production possible. Practically, more than 0.4 V is usually added into the system to make a sustainable hydrogen production due to the overpotential on the cathode, but this amount of voltage is still quite lower than that needed for the traditional water electrolysis which was usually higher than 2.3 V (Call *et al*. 2009).

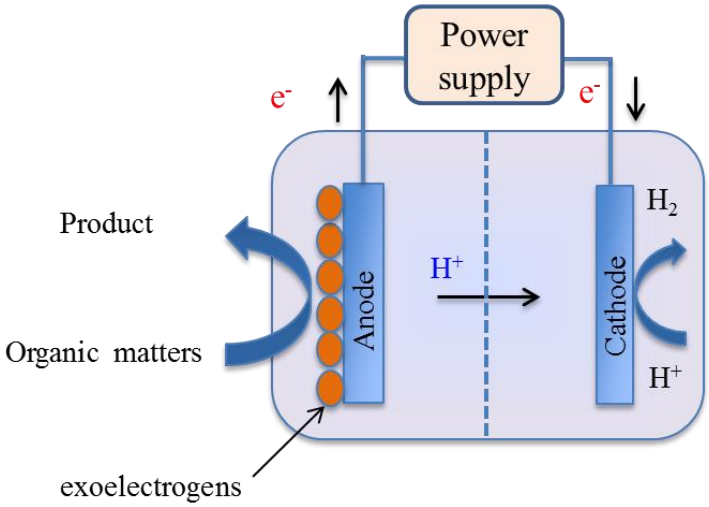


Figure 2.2 The schematic of a typical two-chamber microbial electrolysis cell

To reduce the overpotentials on the cathode, metal-based catalysts (e.g. platinum) are commonly used on the cathode (Lee *et al*. 2010). Although these metal-based catalysts can reduce the overpotentials on the cathodes and significantly improve the H_2 production rate of MEC, their high cost largely limits their application in MEC. Besides, these metal-based catalysts are also easily to be poisoned by the sulfide in the wastewater and thus need periodically replacement (Rasmussen *et al*. 2006).

Viewed in this light, microbial biocathodes, in which live microorganisms act as biocatalysts to catalyze cathodic reactions, are of particular interest, because they are inexpensive, self-renewable and not susceptible to corrosion(Pisciotta *et al*. 2012; Rozendal *et al*. 2008b). The concept of biocathodes will be further introduced in the section 2.3.3.

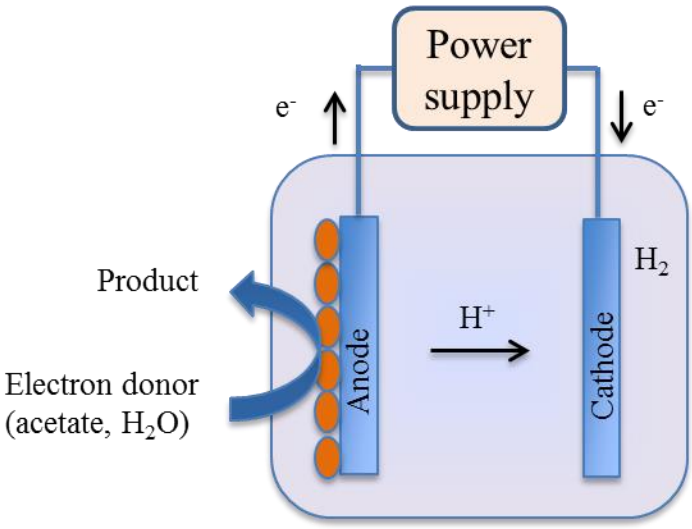


Figure 2.3 The schematic of a single-chamber microbial electrolysis cell

In traditional water electrolyzers, membranes are needed to prevent oxygen and hydrogen gases mixing and reacting. Membranes are also commonly used in MECs, presumably to prevent H_2 utilization by the bacteria in the anode chamber and ensure high concentration of H_2 (Liu *et al*. 2005; Logan *et al*. 2008). However, the commonly used membranes (e.g. Nafion membrane) are not only expensive, but also can increase the internal resistance and hence reduce the H_2 production rate of MEC (Hu *et al*. 2008). Because the anolyte and catholyte used in MECs are usually the same and no oxygen is produced in the anode chamber (Liu *et al*. 2005; Liu *et al*. 2008), therefore, the membranes are not necessary for MEC to prevent oxygen and hydrogen mixing. Based on this concept, single-chamber microbial electrolysis cells were reported in several studies (Call and Logan 2008; Hu *et al*. 2008; Logan *et al*.

2008), which largely reduced the internal resistance and improving the H_2 production rate of microbial electrolysis cells. Fig. 2.3 showed the schematic of a single-chamber microbial electrolysis cell. The single-chamber reactor has been widely used as a common structure for microbial electrolysis cells.

2.2.3 Microbial Electrosynthesis Cells

Microbial electrosynthesis is a recently reported bioelectrochemical system, which use electricity as the energy input for microbial reduction of CO_2 to chemical products (e.g. acetate and CH_4) (Desloover *et al*. 2012; Nevin *et al*. 2011; Nevin *et al*. 2010; Rabaey *et al*. 2011; Zhang *et al*. 2013). Recently, the term of "microbial electrosynthesis" not only mean the electricity-driven reduction of CO_2, but also include the electricity-driven reduction or oxidation of other organic feedstocks (Rabaey and Rozendal 2010).

The reactors of microbial electrosynthesis systems are same with the MEC reactors, containing two-chamber and single-chamber reactors. Additionally, the working principles are also similar: electrons are produced at the anode and pass through the circuit to the cathode where they are consumed by the certain microorganisms to produce chemical products (Fig. 2.4). These certain microorganisms which are capable of capturing electrons from the cathode are collectively called as "electrotrophs" (Pisciotta *et al*. 2012), as a counterpart to "exoelectrogens" which can donate electrons to anodes. The reaction in microbial electrosynthesis is also not thermodynamic-favorable, thus a small amount of voltage needs to be added into the circuit. The only different point between microbial electrosynthesis and MEC is the species of electrotrophs used on the cathode. The products of microbial electrosynthesis are dependent on the species of electrotrophs functioning on the electrosynthetic biocathode.

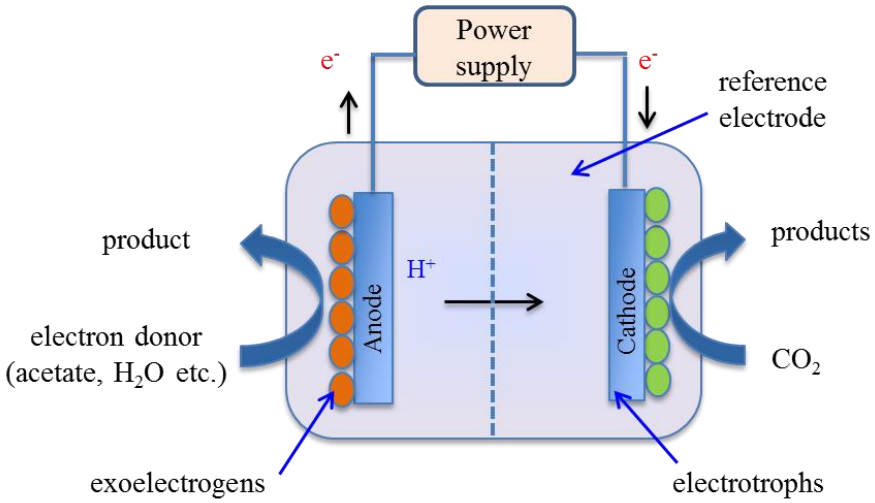


Figure 2.4 The schematic of microbial electrosynthesis

Up to now, diverse microbial electrosynthesis systems aiming to produce different products (e.g. CH_4, acetate, 2-oxobutyrate) have been reported (Cheng *et al*. 2009; Nevin *et al*. 2011; Nevin *et al*. 2010; Steinbusch *et al*. 2010). In 2009, Cheng *et al* built a biocathode capable of converting CO_2 to CH_4 and named this system as "electromethanogenesis". The linear sweep voltammetry showed that the current density of the biocathode was substantially higher than that of an abiotic cathode, which produced only small H_2. A thick biofilm containing methanogens were observed on the cathode surface. The microbial analysis and fluorescence in situ hybridization (FISH) showed that the methanogens were the dominant microorganisms on the biocathode. The authors concluded that both the increased current density and very small H_2 production rates by a plain cathode support a mechanism of methane production directly from current and not from H_2 gas. It was also suggested a pure culture methanogen, *Methanobacterium palustre*, was capable of accepting electrons directly from cathodes:

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O \qquad \text{(Eq. 2.1)}$$

However, the direct electron transfer pathway is still speculative and need to be further verified (Rabaey and Rozendal 2010).

2.3 Biocatalysts in BESs

2.3.1 Exoelectrogens Transferring Electrons to Anodes

The essential of bioanodes of BESs is the utilization of microorganisms capable of transferring electrons to anodes as biocatalysts. These special microorganisms capable of transferring electrons to anodes are collectively called as "exoelectrogens" (Logan *et al*. 2006). The "exo-" refers to exocellular and "electrogens" refers to the ability to directly transfer electrons to a chemical or material that is not the immediate electron acceptor (Logan 2008). In other previous studies, exoelectrogens are also called as electrochemically active bacteria (Chang *et al*. 2006), anode respiring bacteria (Rittmann *et al*. 2008) and electricigens (Lovley 2006).

It had been reported that many anaerobic microorganisms can transfer electrons to soluble compounds such as nitrate and sulfate which diffuse into the cell by crossing the cell membrane. However, exoelectrogens are different with these anaerobic microorganisms because they can transfer electrons outside of the cells to solid electron acceptors (i.e. electrodes). As the process of electron transfer from microorganisms to electrodes and to insoluble iron (III) are similar, it has been found that most of the bacteria capable of dissimilatory iron reduction can also be capable of directly transferring electrons to anodes (Logan 2009), suggesting the importance of exocellular electron transfer.

In MFC, exoelectrogens always attach on the surface of anode in a form of biofilm, which was usually thicker than 80 μm (Lovley 2012). Usually, the bioanode inoculated with mixed culture possess higher performance than the bioanode inoculated with pure culture (Logan *et al*. 2006). The microbial analysis of the mixed culture inoculated bioanode always showed a diverse microbial population on the anode. On the other hand, pure cultures were widely used to investigate the electron transfer mechanisms between the exoelectrogens and anode.

The studies of pure cultures have confirmed that many different bacteria in the anodic biofilm are capable of transferring electrons to anodes. To date, there are more than 20 microbial species, most of which are mesophilic Gram-negative bacteria affiliated with the phylum *Proteobacteria*, have been reported to possess exoelectrogenic activity (Fedorovich *et al*. 2009; Logan 2009; Lovley 2012). Among them, *Geobacter sulfurreducens* (Bond and Lovley 2003; Holmes *et al*. 2006; Holmes *et al*. 2008; Reguera *et al*. 2005; Reguera *et al*. 2006; Richter *et al*. 2009) and *Shewanella oneidensis* (Bretschger *et al*. 2007; El-Naggar *et al*. 2010; Firer-Sherwood *et al*. 2008; Gorby *et al*. 2006; Marsili *et al*. 2008; Von Canstein *et al*. 2008) have been extensively studied as model exoelectrogens, and different mechanisms for electron transfer have been proposed, which will be reviewed in the section 2.4.

2.3.2 Electrotrophs Accepting Electrons from Cathodes

The microorganisms capable of accepting electrons from the cathode are collectively called as "electrotrophs" (Pisciotta *et al*. 2012). In the beginning, the term was used to describe the microorganisms capable of accepting electrons from the cathode for H_2 production, now it was expanded to describe all the microorganisms capable of accepting electrons from cathode for bio-production.

The concept of microbial biocathode for H_2 production was firstly developed based on an immobilized pure culture of *Desulfovibrio vulgaris* with methyl viologen (MV) as a redox electron-shuttling mediator (Lojou *et al*. 2002; Tatsumi *et al*. 1999). However, as redox electron-shuttling mediators are not sufficient enough for H_2 production and need replacement periodically, mediator-less microbial biocathodes with direct electron transfer are of particular interest.

Recently, Rozendal *et al*. developed a biocathode for catalyzing H_2 production based on the reversibility of hydrogenases (Rozendal *et al*. 2008b). In their study, an acetate- and H_2-oxidizing bioanode was firstly developed (in an MFC mode), and was then converted to an H_2-producing biocathode by reversing the polarity of the

electrode. At –0.7 V vs. SHE, the "anode-converted" biocathode produced an average current of 1.1 A m^{-2} and 0.63 m^{-3} H_2 m^{-3} cathode liquid volume day^{-1}, significantly higher than that of the control electrode. In biocathodes, microbial biocatalysts are thought to accept electrons directly from the cathode and use the electrons to produce H_2. Up to now, however, only few attempts have been made to characterize the microbial community of biocathodes as well as the mechanisms of microbial electron uptake from biocathodes (Croese *et al*. 2011). Several hydrogenase-containing bacteria, *Desulfovibrio caledoniensis* (Yu *et al*. 2011), *Desulfovibrio paquesii* (Aulenta *et al*. 2012), *Desulfovibrio* sp. *G11* (Croese *et al*. 2011), *Desulfitobacterium* sp. (Villano *et al*. 2011), and *Geobacter sulfurreducens* (Geelhoed and Stams 2011), which are all mesophilic (25 ~ 40 ℃) microorganisms, had been shown to be capable of electron uptake from the cathode.

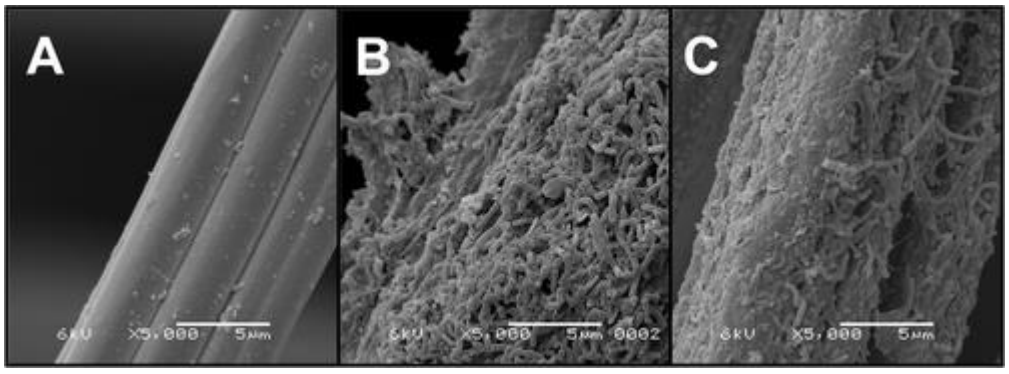


Figure 2.5 Scanning electron micrographs of biocathodes for H_2 production: (A) unused electrode, (B) original biocathode that had been operated as a biocathode for over 2000 h, and (C) former control electrode that had been operated as a biocathode for less than 600 h. (Rozendal *et al*. 2008b)

In 2010, Nevin *et al.* used an acetogenic microorganism *Sporomusa ovata* as biocatalyst on the cathode to convert CO_2 to acetate (Nevin *et al*. 2010). The results showed that biofilms of *S. ovata* growing on the cathode surfaces consumed electrons with the reduction of CO_2 to acetate and small amounts of 2-oxobutyrate. Electrons

appearing in these products accounted for over 85% of the electrons consumed. In 2011, Nevin *et al.* reported that several other acetogenic bacteria, including two other *Sporomusa* species, *Clostridium ljungdahlii* and *Clostridium aceticum* and *Moorella thermoacetica*, were also capable of consuming electrons with the production of acetate and 2-oxobutyrate (Nevin *et al*. 2011). In addition, the electrons capture efficiency for *S. sphaeroides*, *C. ljungdahlii*, and *M. thermoacetica* were higher than 80%. These results expanded the known range of microorganisms capable of electrosynthesis, providing multiple options for the further optimization of this process. However, the detailed electron transfer mechanisms are still not well elucidated.

2.4 Mechanisms of Electron Transfer

2.4.1 Mechanisms of Electron Transfer from Exoelectrogens to Anodes

The ability of electron transfer from microorganisms to solid electrodes was first reported by M. C. Potter in 1910 (Potter 1911). After one century, although the power density of MFC has been improved by several orders, MFCs still cannot be commercialized due to their low power density. One limiting factor is the limited understanding of electron transfer mechanisms between microorganisms and electrodes. Basically, there are two kinds of mechanisms for microorganisms transferring electrons to anodes: indirect electron transfer and direct electron transfer.

2.4.1.1 Indirect Electron Transfer

The indirect electron transfer pathway involves the participation of so-called electron shuttling mediators. The working principle is shown in Fig. 2.5: firstly, the oxidized mediators penetrate the microbial cell membrane to accept electrons inside the microbial cells and become reduced mediators; secondly, the reduced mediators

are transferred out from the microbial cells and arrive at the anode to donate the electrons to the anode; then the oxidized mediators repeat the two steps. Therefore, an ideal electron shuttling mediator must be a low molecular weight water-soluble redox compound which possess a high reactivity and could harvest most of the potential energy available in a given electron transfer process.

The electron shuttling mediators can be divided as exogenous and endogenous mediators based on their sources. The exogenous mediators refer to the artificial mediators, which are easy to add and adjust the redox potential by careful design the molecule of mediators, and the natural redox mediators in the subsurface environment. The commonly used artificial mediators include anthraquinone 2-6-disulfonate (AQDS) (Logan *et al*. 2006), neutral red (Park and Zeikus 2000) and resazurin (Logan *et al*. 2006). These artificial mediators are non-specific, thus can be used for a wide range of microorganisms. However, they are not very stable and can be toxic to the microorganisms. On the other hand, the natural redox mediators in the subsurface environment, such as humic acids (HA) (Thygesen *et al*. 2009), cysteine (Logan *et al*. 2005), MnO_2/Mn_2^+ (Schroeder 2007) and Fe^{3+}/Fe^{2+} (Schroeder 2007), are more stable and less toxic. But their concentration is usually not high enough to sustain a high power MFC power density. The endogenous mediators refer to the secondary metabolites produced by the microorganisms in MFCs, for instance, phenazines (Price-Whelan *et al*. 2006), flavins (Newman and Kolter 2000) and quinones (Keck *et al*. 2002). It had been reported that many bacteria, including the "non-electroactive bacteria", are able to excrete endogenous redox shuttling mediators. It has been suggested that the electron transfer between microorganisms and electrodes is a very general energy-conserving strategy. However, from an energy-harvesting efficiency point, electron transfer through endogenous mediators is not a very economical pathway, as part of energy during the metabolism of microbial cells is used to synthesize the endogenous mediator molecules (Logan 2008). In addition, these soluble mediators can be easily washed out during the medium exchange process.

Therefore, the direct electron transfer using electron shuttling mediators is more attractive and promising.

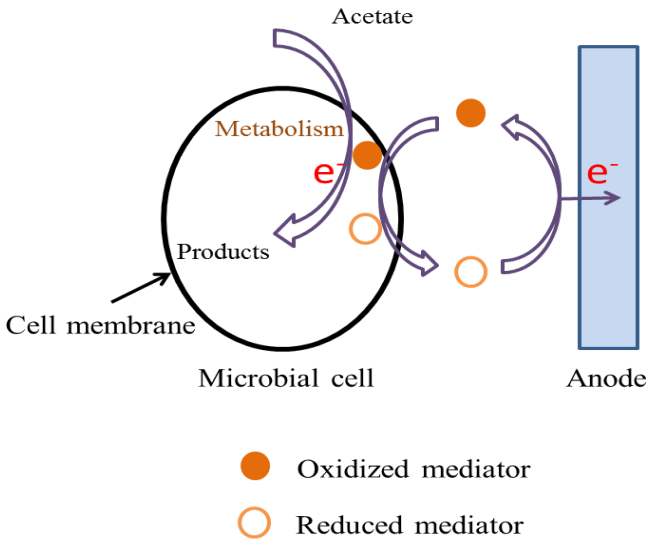


Figure 2.6 The schematic of indirect electron transfer mechanism using electron shuttling mediators

2.4.1.2 Direct Electron Transfer

The second mechanism for anodic electron transfer is the direct electron transfer mechanism, i.e., no involvement of the diffusion of electron shuttling mediators. The current knowledge about extracellular electron transfer by microorganisms in MFCs has been primarily investigated in mesophilic Gram-negative bacteria (especially, *Proteobacteria*), which are dominated in the microbial population analysis of bioanodes. By far the two most extensively studied mesophilic Gram-negative bacteria are *Geobacter* spp. and *Shewanella* spp. (Marshall and May 2009; Rabaey and Rozendal 2010). Consequently, the prevalent theories of extracellular direct electron transport have been developed based on the experiments of these two genera of microorganisms, and two pathways for direct electron transfer were proposed.

Biochemical and genetic studies have indicated that *G. sulfurreducens* can transfer electrons to an anode surface directly by using membrane bound c-type cytochromes (Holmes *et al*. 2006; Holmes *et al*. 2008; Richter *et al*. 2009) and conductive pili called nanowires (Reguera *et al*. 2005; Reguera *et al*. 2006), as shown in Fig 2.7. The studies of *S. oneidensis* showed that it contained membrane bound c-type cytochromes (Bretschger *et al*. 2007; Firer-Sherwood *et al*. 2008) and conductive pili (El-Naggar *et al*. 2010; Gorby *et al*. 2006), but it was also capable of transferring electrons to electrodes by using self-secreted mediator molecules (Marsili *et al*. 2008; Von Canstein *et al*. 2008).

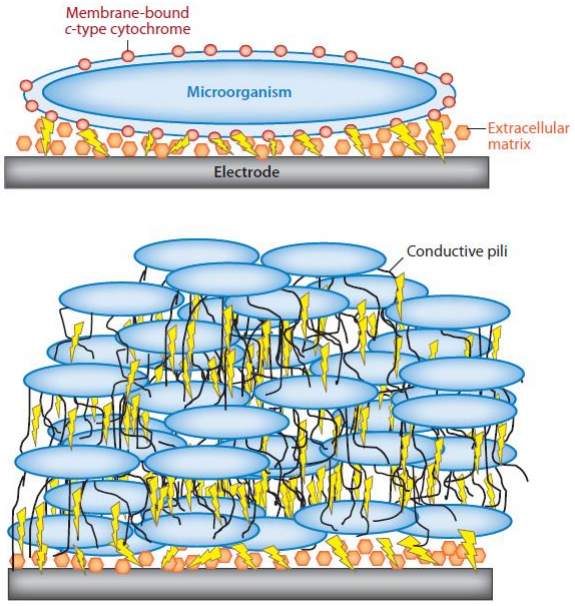


Figure 2.7 The schematic of direct electron transfer from the microorganisms to anodes: (A) Direct electron transfer through membrane-bound c-type cytochrome; (B) Direct electron transfer through conductive pili. (Lovley 2012)

2.4.2 Mechanisms of Electron Transfer in Biocathodes for H_2 Production

2.4.2.1 Indirect Electron Transfer Through Mediators

The concept of microbial biocathode for H_2 production was firstly developed based on an immobilized pure culture of *Desulfovibrio vulgaris* with methyl viologen as a redox electron-shuttling mediator (Lojou *et al*. 2002; Tatsumi *et al*. 1999). However, as redox electron-shuttling mediators are not sufficient enough for H_2 production and need replacement periodically, mediator-less microbial biocathodes with direct electron transfer are of particular interest.

2.4.2.2 Direct Electron Transfer Through Hydrogenase

It is usually accepted that hydrogenases play a pivotal role in the H_2 production by biocathodes. Diverse microorganisms capable of producing hydrogen had been found in various environments and usually possess hydrogenases that catalyze the reversible reaction: $2H^+ + 2e^- \leftrightarrow H_2$ (Croese *et al*. 2011). In addition, purified hydrogenases have been successfully used as catalysts on cathode for hydrogen production ((Cracknell *et al*. 2008; Lamle *et al*. 2003)). The drawback of these systems is that the enzymes are relatively unstable and easily lose catalytic activity over time. The use of whole cells can help in maintaining enzyme stability. It has been suggested that hydrogenase is required to catalyze the conversion of electrons and protons into H_2, because the H_2 production significantly decreased when the hydrogenase was inhibited by carbon monoxide (Rosenbaum *et al*. 2011). Several hydrogenase-containing bacteria, *Desulfovibrio caledoniensis* (Yu *et al*. 2011), *Desulfovibrio paquesii* (Aulenta *et al*. 2012), *Desulfovibrio* sp. *G11* (Croese *et al*. 2011), *Desulfitobacterium* sp. (Villano *et al*. 2011), and *Geobacter sulfurreducens* (Geelhoed and Stams 2011), which all are mesophilic (25 ~ 40 ℃) microorganisms, had been shown to be capable of electron uptake from the cathode.

2.4.3 Mechanisms of Electron Transfer in Biocathodes for Microbial Electrosynthesis

Compared with the mechanism for electron transfer to electrodes, little is known about the mechanisms of electron transfer from electrodes to microorganisms, especially for microbial electrosynthesis. In addition, the electron transfer mechanisms also vary from different products. Generally, it was thought that there are four kinds of pathway for the electron transfer from electrodes to microorganisms (Rabaey and Rozendal 2010).

2.4.3.1 Indirect Electron Transfer through H_2

For microbial electrosynthesis, one common electron transfer pathway is through intermediate products (e.g. H_2 and formate), which can readily be produced at cathodes and serve as substrates for lots of microorganism (Clauwaert and Verstraete 2009; Rabaey and Rozendal 2010; Villano *et al*. 2010). Take H_2 For example, it had been reported that methane could be produced in microbial electrolysis cells due to the hydrogenotrophic methanogenesis by methanogens ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$) (Call and Logan 2008; Clauwaert and Verstraete 2009; Wagner *et al*. 2009; Wang *et al*. 2009). However, there are two disadvantages for the electron transfer through H_2. First, the low solubility of H_2 will make it difficult to achieve by the microorganisms. The second is the fact the H_2 production accompanies with a high overpotentials at non-catalyzed electrodes, which means suitable catalysts (e.g. Platinum) are necessary for notable current density. Therefore, electron transfer through H_2 is not a suitable pathway for effective cathodic bio-production.

2.4.3.2 Indirect Electron Transfer through Mediators

The second pathway for cathodic indirect electron transfer is through electron-shuttling mediators like for anodic electron transfer. Neutral red, methyl viologen and thionion are the commonly used electron-shuttling mediators. (Hongo and Iwahara

1979; Kim and Kim 1988; Lithgow *et al*. 1986). Although these electron-shuttling mediators could be dissolved at a higher concentration than H_2 and decrease the overpotential at the electrode, their limited stability, possible toxicity on microorganisms and loss in flow through systems largely limited their application.

2.4.3.3 Direct Electron Transfer

The last and the most attractive pathway for cathodic electron transfer is direct electron transfer, which means microorganisms on the cathode accept electrons directly from the electrode for bio-production. It has been reported that biofilms were attached on the electrosynthetic biocathodes, in which the biofilms may directly accept electrons to produce chemicals. For instance, Nevin *et al* reported that *Sporomusa ovate* could act as biocatalysts on the cathode and convert CO_2 to acetate and Oxo-butyrate (Nevin *et al*. 2010). Because the microorganisms were attached to an electrode with an applied potential of around –0.4 V (vs. SHE), which is higher than the theoretical redox potential for H_2 production, the electron transfer between the cathode and microorganisms was suggested as a direct electron transfer. In addition, Cheng *et al.* reported a methanogen was capable of accepting electrons to convert CO_2 to CH_4, however, the electron transfer mechanism was only speculative (Rabaey and Rozendal 2010).

2.5 Electrochemical Backgrounds

In this section, the fundamental theory of electrochemistry and the methods to evaluate the performance of BESs are summarized and reviewed.

2.5.1 Theoretical Electrode Potentials

The reactions occurring in the BESs can be analyzed in terms of the half-cell reactions, i.e., the separate reactions occurring at the anode and the cathode. According to the IUPAC convention, standard potentials (at 298 K, 1 bar, 1 M) are

reported as a reduction potential, i.e., the reaction is written as consuming electrons (Bard *et al*. 1985; Logan *et al*. 2006). Take MFC for instance, the reaction of acetate oxidation by bacteria at the anode of MFCs can be written as:

$$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2O \qquad \text{(Eq. 2.2)}$$

Based on the Nernst Equation (Logan 2008), the anode potential (E_{An}) can be calculated as

$$E_{An} = E_{An}^0 - \frac{RT}{8F}\ln\left(\frac{[CH_3COO^-]}{[HCO3^-]^2[H^+]^9}\right) \qquad \text{(Eq. 2.3)}$$

where,

E_{An}^0 = standard electrode potential of acetate oxidation relative to the standard hydrogen electrode (SHE);

R = ideal gas constant (8.31447 J/mol-K)

T = operating temperature (K)

n = number of electros transfer in the reaction

F = Faraday's constant (96485 C/mol)

For the theoretical cathode potential, E_{cat}, if oxygen is used as the electron acceptor for the reaction, the reaction equation can be written as

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \qquad \text{(Eq. 2.4)}$$

Similar to the anode potential, the cathode potential can be written as

$$E_{cat} = E_{cat}^0 - \frac{RT}{4F}\ln\left(\frac{1}{pO_2[H^+]^4}\right) \qquad \text{(Eq. 2.5)}$$

where pO_2 is the pressure of the oxygen.

In practice, a variety of catholytes have been used as alternative catholytes due to the sluggish kinetics of oxygen on the cathode. For example, potassium permanganate (You *et al*. 2006) and ferricyanide (Min *et al*. 2005) have been used .

The cell voltage can be calculated using the potential different across the anode and cathode electrodes as follow.

$$E = E_{cat} - E_{an} \quad \text{(Eq. 2.6)}$$

2.5.2 Power Density

The performance of MFCs is usually evaluated by the power (P) production of the MFC, which is shown as follow (Logan 2008):

$$P = UI \quad \text{(Eq. 2.7)}$$

Where U represents the voltage of the external resistance of MFCs, and I represents the current passing through the external resistance. As the MFCs structures and the sizes of the electrodes are usually different, it is difficult to compare the performance of MFCs in term of power. Usually, the MFCs with larger electrode surface areas tend to produce higher power than the MFCs with smaller surface areas. Therefore, the power density (PD) (power subjected to the electrode area) is always used to evaluate the performance of MFCs, which is shown as follows:

$$PD = P/A = UI/A; \quad \text{(Eq. 2.8)}$$

where A is the square surface of electrodes or proton exchange membrane. Usually, we use the power density subjected to anode surface.

In the case that the surface area of electrodes (e.g. porous electrodes) is difficult to distinguish, power density subjected to reactor volume is also used, which is shown as:

$$PD = P/V = UI/V; \quad \text{(Eq. 2.9)}$$

where V represents the volume of the reactor.

2.5.3 Cathodic Efficiency for MEC and Microbial Electrosynthesis

As introduced in section 2.2, voltage input is needed for the production of H_2 and other chemicals in MECs and microbial electrosynthesis. Theoretically, all the electrons passing through the circuit to the cathode should be consumed for H_2 or other chemicals production. In practice, however, not all the electrons could be used for H_2 or other chemicals due to the losses. Therefore, cathodic capture efficiency, which is the ratio of the actual H_2 produced to the theoretical H_2 produced based on the current, is one parameter to evaluate the performance of MECs and microbial electrosynthesis. Take MECs for example, the theoretical number of moles of H_2 that can be produced based on the current is shown as (Logan 2008)

$$n_{CE} = \frac{\int_{t=0}^{t} Idt}{nF} \qquad \text{(Eq. 2.10)}$$

where,

n_{CE} = Theoretical H_2 produced based on the current (mol)

I = System current (A)

n = The number of electrons per mole of H_2 (mol e^- / mol H_2)

F = Faraday's constant (96485 C / mol e^-)

The cathodic capture efficiency for the microbial electrosynthesis is similar to that of MEC, except the value of n is different.

2.5.4 Energy Efficiency for MEC and Microbial Electrosynthesis

The energy efficiency is used to compare the energy obtained with the energy added into the BESs. Basically, the energy efficiency can be evaluated in two ways based on the energy input: electricity input and the total energy input. The energy efficiency based on the electricity input only takes into account the electrical input energy as input energy, while the total energy efficiency takes into account the

electrical energy input and the chemical energy contained in the substrate. The calculations of the two kinds of energy efficiency are shown as follows (Logan 2008).

2.5.4.1 Energy Efficiency Based on The Electrical Energy Input

The total electrical energy input can be calculated by integrating the product of the current in the circuit and the voltage added into the system during the H_2 or other chemicals production period, which can be shown as:

$$W_{ps} = \int_0^t IUdt \qquad \text{(Eq. 2.11)}$$

where

W_{ps} = the total input electrical energy (W);

I = the current in the circuit;

U = the voltage added into the system by the power source;

t = the operating time.

Usually, the current in the circuit is not constant due to the unpredictable activity of biocatalysts in BESs in actual experiments. Therefore, the total electrical energy can also be calculated by

$$W_{ps} = \sum IU\Delta t \qquad \text{(Eq. 2.12)}$$

In the real experiment, a small external resistance is always added into the circuit for measuring the current. A small part of electrical energy is consumed in Joule heat by the external resistance, which can be shown as:

$$W_r = \sum IU_r\Delta t \qquad \text{(Eq. 2.13)}$$

Because this part of energy cannot be used for H_2 or other chemicals production, therefore, this part of energy is usually subtracted from the total input electrical energy. Hence, the input electrical energy can be written as:

$$W_{in} = W_{ps} - W_r = \sum IU\Delta t - \sum IU_r \Delta t \qquad \text{(Eq. 2.14)}$$

The energy contained in the H_2 or other chemicals can be calculated based on the entropy:

$$W_{output} = n_{H_2} \Delta H_{H_2} \qquad \text{(Eq. 2.15)}$$

where

n_{H_2} = the mole of the H_2 or other chemicals produced by the BESs;

ΔH_{H_2} = the combustion entropy of the chemicals produced by the BESs

Therefore, the energy efficiency based on the input electrical energy can be calculated as:

$$\eta_W = \frac{W_{H_2}}{W_{in}} = \frac{n_{H_2} \Delta H_{H_2}}{\sum IU\Delta t - \sum IU_r \Delta t} \qquad \text{(Eq. 2.16)}$$

2.5.4.2 Energy Efficiency Based on Substrate and Electricity Input

In MECs and microbial electrosynthesis systems, substrate is usually required for the growth of biocatalysts, especially for the biocatalysts on the anode. The energy input by the substrate can be calculated by (Logan 2008):

$$W_s = n_s \Delta H_s \qquad \text{(Eq. 2.17)}$$

where

W_s = the chemical energy contained in the substrate;

ΔH_s = the heat of combustion for the substrate (KJ/mol);

n_s = number of moles of substrate

Therefore, the energy efficiency based on substrate and electricity input (η_{w+s}) can be written as:

$$\eta_{W+S} = \frac{W_{H_2}}{W_s + W_{in}} = \frac{n_s \Delta H_s}{n_s \Delta H + \sum IU\Delta t - \sum IU_r \Delta t} \qquad \text{(Eq. 2.18)}$$

To distinguish energy efficiency based on the electrical energy input, it also called as "energy ratio" in this study.

2.6 Reviews of Thermophilic BESs

Up to now, most of the studies of BESs are operated at room temperature, which are probably due to the diversity of mesophilic electrochemically-active microorganisms and the easy-handling of MFCs at room temperature (Logan *et al*. 2008; Logan *et al*. 2006). However, the elevated operating temperature may be more suitable for BESs in contrast to the room temperature. Theoretically, the higher temperature could reduce the anode potential in the anode chamber, consequently resulting in a higher voltage generation in MFCs. In addition, for biocathodes, the high temperature could help in assisting the energy input to the endothermic reaction in the cathode for H_2 production and other chemicals production, hence, resulting in a lower voltage input.

Take MFCs for example, Ahn and Logan tested MFCs for domestic wastewater treatment at two different temperature ranges (23 ± 3℃ and 30 ± 1℃) (Ahn and Logan 2010). The results showed that the higher temperature resulted in a higher power density. In addition to higher power density at higher operating temperature, Jadhav *et al.* also reported that higher temperature range (25-35℃) resulted in a higher COD removal efficiency than the lower temperature range (8-22℃) (Jadhav and Ghangrekar 2009).

Although the recent studies showed that higher operating temperature did result in higher MFC performance, the mesophilic biocatalysts cannot sustain the higher operating temperature. Patil *et al.* operated MFCs at different operating temperatures and concluded that MFC performance increased with operating temperature when operating temperature was lower than 40℃. When operating temperature was higher

than 40 ℃, on the other hand, MFC current generation decreased drastically and became around zero at the operating temperature higher than 50 ℃ (Patil *et al*. 2010). Therefore, using thermophilic microorganisms (45 ~ 122 ℃) which can sustain elevated temperatures as biocatalysts in BESs is one pathway to increase their performance. The increase of temperature not only increase the diffusion coefficient of substrates, but also enhance the metabolic activity of microorganisms (Niehaus *et al*. 1999). In addition, the bioavailability of hardly biodegradable and insoluble environmental pollutants can also be improved a lot at elevated temperature (Niehaus *et al*. 1999).

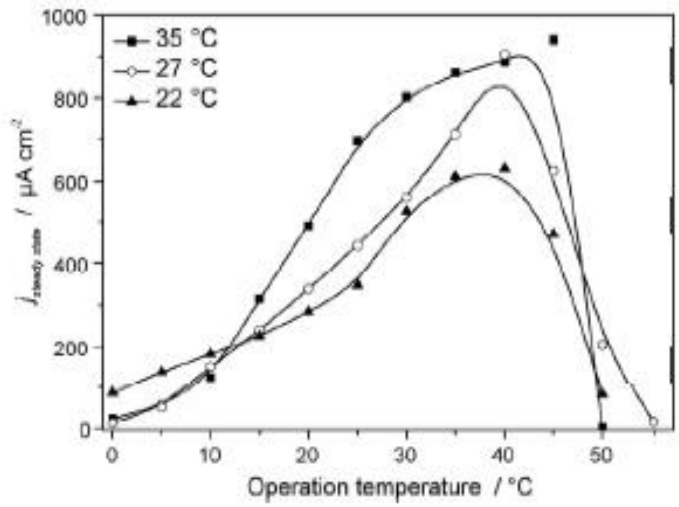


Figure 2.8 The effect of operating temperature on the current generation in MFCs (Patil *et al*. 2010)

2.6.1 Thermophilic MFC Using Electron-Shuttling Mediators

In 2004, Choi *et al.* constructed the first thermophilic MFC with thermophilic microorganisms, *Bacillus licheniformis* and *Bacillus thermoglucosidasius*, and got a substantial amount of electricity in the presence of an exogenous redox electron-

shuttling mediator. The MFCs showed a good performance in the range of 30 to 60 ℃, and got the best efficiency at 50- 60 ℃. A rapid deterioration of the MFC performance was resulted when the temperature was higher than 70 ℃. It was also noticeable that efficiency and discharge pattern strongly depended on the kind of carbon sources used in the initial culture medium. In the case of *B. thermoglucosidasius*, glucose alone was utilized as a substrate in the microbial fuel cell irrespective of use carbons sources. When *B. licheniformis* was cultivated with lactose as a carbon source, best charging characteristics were recorded.

Like the mesophilic MFCs using electron-shuttling mediators, the thermophilic MFCs using mediators also need to replenish the mediators periodically, and the performances are usually lower than that of the mediatorless thermophilic MFCs. Therefore, the studies of the thermophilic MFC are also focused on the mediatorless thermophilic MFC.

2.6.2 Mediatorless Thermophilic MFC

Jong *et al.* built the first mediatorless thermophilic MFC by inoculating the effluent collected from a thermophilic anaerobic digester, aiming to treat the thermophilic agricultural processing wastewater (e.g. oil palm and sago) in tropical area (e.g. Malaysia, Indonesia, Thailand, etc.) (Jong *et al*. 2006). A maximum power density of 1030 ± 340 mW m^{-2} was generated continuously at 55 ℃ with an anode retention time of 27 min (11 mL h^{-1}) and continuous pumping of air-saturated PBS buffer into the cathode chamber. Direct 16S rDNA analysis showed that the dominant bacteria representing 57.8% of total population in anode was phylogenetically very closely related to an uncultured clone E4. However, they did not isolate this pure culture.

In 2008, Mathis *et al* built a thermophilic mediatorless MFC using thermophilic microorganisms from marine sediment as inoculum (Mathis *et al*. 2008). The results showed that the current generation of the thermophilic MFC at 60 ℃ (209 to 254 mA m^{-2}) was much higher than that of the mesophilic MFC at 22 ℃ (10 to 22 mA m^{-2}). The maximum power density of the thermophilic MFC was around 207 mW m^{-2}. The

microbial analysis showed that the dominant species was closely related to a Gram-positive thermophile, *Thermincola carboxydophila* (99% similarity). However, they also didn't isolate a pure culture capable of transferring electrons to anodes or test one pure culture if it could produce electricity or not.

In 2008, Wrighton *et al.* reported the first thermophilic exoelectrogen, *Thermincola* sp. strain JR, which belongs to a Gram-positive phylum *Firmicutes* (Wrighton *et al*. 2008). The authors firstly started up the MFC using the effluent of an operational thermophilic methanogenic anaerobic digester as inoculum, and then isolated this thermophilic exoelectrogen from the anode mixed culture. In 2009, Marshall *et al* investigated the electricity-generating ability of a pure culture, *Thermincola ferriacetica* strain Z-0001, and studied its electron transfer mechanism using cyclic voltammetry (Marshall and May 2009). The results showed that *Thermincola ferriacetica* strain Z-0001 could be able to transfer electrons to anodes in a direct electron manner. The maximum power density of 146 mW m^{-2} was obtained by this pure culture.

In contrast to the mesophilic species, however, thermophilic members of exoelectrogens remain underexploited and have so far been largely limited to Gram-positive species affiliated with the phylum *Firmicutes.* The current generating ability of these thermophilic exoelectrogen is lower than the reported mesophilic exoelectrogens. Therefore, novel thermophilic exoelectrogens possessing high electricity generating ability are needed to exploit in future studies.

Table 2.1 Comparison of the performance of thermophilic MFCs in previous studies

Sources	Temperature (℃)	Power density (mW m^{-2})	Inoculum	Electron donors	Operation mode
Choi *et al.* 2004	50 ~ 60	--	*Bacillus licheniformis, Bacillus thermoglucosidasius*	Glucose	Fed-batch
Jong *et al.* 2006	55	1030	Mixed culture	Acetate	Continuous
Wrighton *et al*. 2008	55	37	Mixed culture	Acetate	Fed-batch
Mathis *et al.* 2008	60	207	Mixed culture	Acetate	Fed-batch
Marshall *et al.* 2010	60	146	*Thermincola*	Lactate	Fed-batch

"--" represents not applicable.

Chapter 3

Study of Mesophilic Electromethanogenic CH_4-Producing Biocathodes

3.1 Introduction

Bio-electrochemical systems (BESs) are emerging energy-generation/conversion technologies that are applicable to renewable-energy generation, wastewater treatment, biosensors and bioremediation (Lovley 2011a). The key feature of BESs is the utilization of microorganisms to catalyze electrochemical reactions at solid electrodes. For example, in a typical microbial fuel cell (the most investigated BES), some certain microorganisms on the anode degrade organic matters to release electrons and protons. Then the electrons are transferred to the anode by these special microorganisms and pass through the external circuit to the cathode, where they combine with oxygen and protons which migrate from the anode to produce water (Logan 2009; Logan *et al*. 2006; Rabaey and Verstraete 2005). These special microorganisms capable of transferring electrons to anodes are collectively called as "exoelectrogens" (Logan *et al*. 2006).

"Electromethanogenesis" is a recently reported bio-electrochemical reaction in which methanogenic microorganisms act as biocatalysts to convert CO_2 to CH_4 at the BES cathode using electrical current as a reducing power source (Cheng *et al*. 2009). It has been reported that the conversion efficiency of the electrons (current) consumed at the cathode into CH_4 was as high as 96%. Therefore, this technology is promising to convert CO_2 to CH_4 using the excess renewable or other intermittent electrical energy which cannot be stored in a high conversion efficiency.

However, the molecular mechanisms of this reaction, especially the electron transfer mechanisms, has yet to be elucidated (Lovley 2011b; Rabaey and Rozendal 2010; Rosenbaum *et al*. 2011). Basically, there are two kinds of electron transfer mechanisms for the CH_4 production. The first one, which is also the most attractive one, is the direct electron transfer between the microorganisms and cathodes. Several observations have supported the hypothesis that some certain methanogenic archaea can directly accept electrons from the cathodes to react with protons and CO_2 (Eq. 3.1) (Cheng *et al*. 2009).

$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$ (Eq. 3.1)

The second one is electron transfer through H_2. Although significant evolution of H_2 was not detected during the electromethanogenic process (Cheng *et al*. 2009), there is still a possibility that the process involves *de novo* formation of molecular H_2 (Eq. 3.2), which is then immediately used for hydrogenotrophic methanogenesis (Eq. 3.3) by methanogens (Villano *et al*. 2010). The formation of molecular hydrogen (Eq. 3.2) can occur as an abiogenic electrochemical reaction and/or can be microbially catalyzed (Geelhoed and Stams 2011).

$2H^+ + 2e^- \rightarrow H_2$ (Eq. 3.2)

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (Eq. 3.3)

The "electromethanogenic" BES is an attractive technology for applications in energy storage. Because of the biocathode capable of electromethanogenesis yields CH_4 from the electrical current with high efficiency, it allows for the storage of electrical energy in a stable form, CH_4. Such technology can be particularly useful for the storage of intermittent electrical energy from renewable power sources (such as solar and wind) (Rabaey and Rozendal 2010). Accordingly, as an innovative step toward the implementation of electromethanogenic BESs, we investigated the microbial composition of an electromethanogenic biocathode. A single-chamber electromethanogenic BES was built, and its electrochemical and microbial properties were analyzed. The population analysis of the microbial consortia on the biocathode, as well as that on the current-producing bioanode, suggested an electromethanogenesis pathway mediated by exoelectrogenic bacteria.

3.2 Materials and Methods

3.2.1 Construction of BES reactors

Single-chamber BES reactors were constructed using 250 ml serum bottles (Maruemu, Osaka, Japan), as shown in Fig. 3.1. The anode and cathode were both composed of plain carbon felt (42 cm^2; Tsukuba Materials Information Laboratory, Tsukuba, Japan) and were connected to the circuit via titanium wires (0.5 mm in diameter; Alfa Aesar, Ward Hill, MA, USA). The resistances between the titanium wires and carbon felts were lower than 3 Ω.

3.2.2 Inoculation and Start-up

One hundred milliliters of anoxically prepared *Methanobacterium* medium (medium #1067; NITE Biological Resource Center, Chiba, Japan) excluding Na_2S was aliquoted into each reactor. The medium consisted of the following in 1 L of deionized water: 0.8 g $NaCH_3COO$, 0.136 g KH_2PO_4, 0.54 g NH_4Cl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.147 g $CaCl_2 \cdot 2H_2O$, 2.5g $NaHCO_3$, 0.2 g yeast extract, 1 ml resazurin, 10 ml of wolfe's vitamin and mineral solutions. The mineral solution consisted of the following ingredients in 1 L deionized water: 12.8 g Nitrilotriacetic acid, 1.35 g $FeCl_3 \cdot 6H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.024g $CoCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $ZnCl_2$, 0.025 g $CuCl_2 \cdot 2H_2O$, 0.01g H_3BO_3, 0.024 g $Na_2MoO_4 \cdot 2H_2O$, 1g NaCl, 0.12 g $NiCl_2 \cdot 6H_2O$, 0.004 g Na_2SeO_4, 0.004 g Na_2WO_4, 0.02 g $KAl(SO_4)_2 \cdot 12H_2O$. The wolfe's vitamin consisted of following ingredients in 1 L deionized water: 2 mg Biotin, 2 mg Folic acid, 10 mg Pyridoxine-HCl, 5 mg Thiamine-HCl, 5mg Riboflavin, 5 mg Nicotinic acid, 5 mg Ca-pantothenate, 1 mg *p*-Aminobenzoic acid, 0.01 mg Vitamin B_{12}.

A microbial consortium originating from a mesophilic digestive sludge was inoculated into each reactor except the abiotic control reactor. The reactors were then sealed with butyl-rubber stoppers and incubated anaerobically with a gas mixture of 80% N_2: 20% CO_2 at 28 ℃ without agitation. During the startup process, a constant

voltage of 1.0 V was applied to the inoculated reactor using a digital power supply (Array 3645A; Array Electronics, Nanjing, China) with the positive pole connected to the anode and the negative pole connected to the cathode.

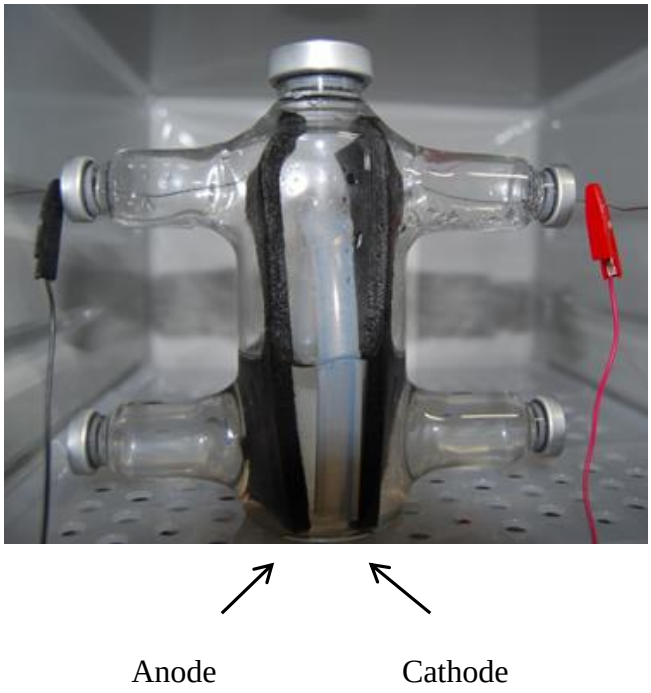


Figure 3.1 The photograph of single-chambered BES reactor for CH_4 production

3.2.3 Data Acquisition and Calculations

The gas composition in the headspace of reactors was analyzed by a gas chromatograph [GC-2014 with a Shincarbon ST column (6 m × 3 mm ID); Shimadzu, Kyoto, Japan]. The concentration of acetate was quantified with an LC-20 liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an electric conductivity detector and an SPR-H column (250 mm×7.8 mm ID, Shimadzu). A fixed external

resistance (1.0 Ω) was applied between the anode and the cathode. To measure the current produced in the reactor, the voltage across the fixed external resistance was monitored using a multimeter (34970A; Agilent Technologies, Santa Clara, CA, USA). The reactors were operated in a fed - batch mode, in which the media were exchanged with fresh media after CH_4 production ceased.

3.2.4 Electrochemical Analysis

Linear Sweep Voltammetry (LSV) was employed using a potentiostat (HSV-110; Hokuto Denko, Tokyo, Japan) with a standard three-electrode system. The cathode and the anode acted as the working electrode and the counter electrode, respectively. An Ag/AgCl reference electrode was inserted into the reactor as the reference electrode. The LSV was conducted in the potential range from – 0.1 to – 0.8 V (vs. SHE) at a slow scan rate of 1.0 mV s^{-1}.

3.2.5 Microbial Analysis

For microbial analysis, community DNA was extracted from 250 mg of aseptically crushed electrodes using a PowerMax Soil DNA isolation kit (MO BIO laboratories, Carlsbad, CA). The extracted DNA (20 ng) were used as templates for PCR with the primers 8F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTACGACTT-3') (Grabowski *et al*. 2005). The pooled PCR amplicons were cloned into pCR4-TOPO using TOPO TA cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were purified using High Pure Plasmid Isolation kit (Roche Applied Science, Indianapolis, IN) and sequenced with T3 and T7 primers. The assembled sequences were aligned with NAST aligner programs in Greengenes (http://greengenes.lbl.gov/) with the closest sequence relatives from NCBI database on October 2012. The alignments were then manually improved in MEGA ver. 4.0.2. (Tamura *et al*. 2007). Sequences of 90 and 162 clones from the cathodic archaeal and bacterial libraries, as well as nine and 88

clones from the anodic archaeal and bacterial libraries, respectively, were analyzed until Good's coverage estimator reached at least 90% (Good and Toulmin 1956).

3.2.6 Accession Number

The nucleotide sequences obtained in this study were deposited in GenBank with the following accession numbers: JX462513-JX462552.

3.3 Results and Discussion

3.3.1 CH_4 production in BES Reactors

Electromethanogenic BESs were built using mesophilic sludge-derived consortium as the inoculum. The reactors were initially incubated with an applied voltage of 1.0 V. In the first cycle of the fed-batch process (Fig. 3.2A), H_2 was produced at the early incubation stage. Subsequently, the H_2 concentration decreased to background levels in 13 h, and CH_4 production was initiated. CH_4 was produced at a maximum rate of 93 mmol day^{-1} m^{-2} (subjected to the cathode geometric surface area) with a current-to-CH_4 conversion efficiency as low as approximately 60% (data not shown). Thus, the CH_4 production observed in the first cycle was likely due to hydrogenotrophic methanogenesis by consuming the H_2 produced by the cathode rather than the electromethanogenesis (i.e. Eq. 3.2 + Eq. 3.3).

In the second cycle of the fed-batch process (after the medium exchange: Fig. 3.2A), CH_4 was produced in an applied-voltage-dependent manner. The CH_4 production rate (the maximum rate is 182 mmol-CH_4 day^{-1} m^{-2}) in the second fed-batch cycle was higher than that of the first fed-batch cycle. The current generation was nearly proportional to the CH_4 production rate and depended on the applied voltage (Fig. 3.2B), suggesting that the current produced in the circuit was consumed during the CH_4 production. The current-to-CH_4 conversion rate was improved to values as high as approximately 95% (data not shown), suggesting that the electromethanogenic microbial population was enriched during the fed-batch cycles.

No significant gas production was observed in the open-circuit control reactor which was inoculated with microorganisms (Fig. 3.3). Exclusion of the possible electron carriers (i.e. resazurin, cysteine and yeast extract) from the medium did not significantly affect the CH_4 production (data not shown), suggesting that the consortium did not require exogenous mediators to achieve electromethanogenic activity. Thus, resazurin, cysteine and yeast extract were thereafter excluded from the media. The microbial consortium was further enriched by repeating the fed-batch operation and was then used in the following studies.

3.3.2 Effect of Applied Voltage on the CH_4 Production

To further examine the effect of applied voltage on the production of CH_4, a range of voltages (from 0.5 to 1.5 V) was applied to the electromethanogenic reactors (Fig. 3.4). At an applied voltage of 0.5 V, the CH_4 production rate was indistinguishable from the control (no applied voltage). At applied voltages of 0.75 to 1.5 V, the CH_4 production rate was significantly higher than that of the control (with microorganisms but without applied voltage). The current-to-CH_4 conversion rates were ≧95%, regardless of the applied voltage levels. However, the relationship between the applied voltage level and the CH_4 production rate was not linear. For example, the CH_4 production rate with an applied voltage of –1.25 V was higher than that with an applied voltage of –1.5 V, which suggests that applied voltages higher than –1.25 V could be harmful to the microorganisms.

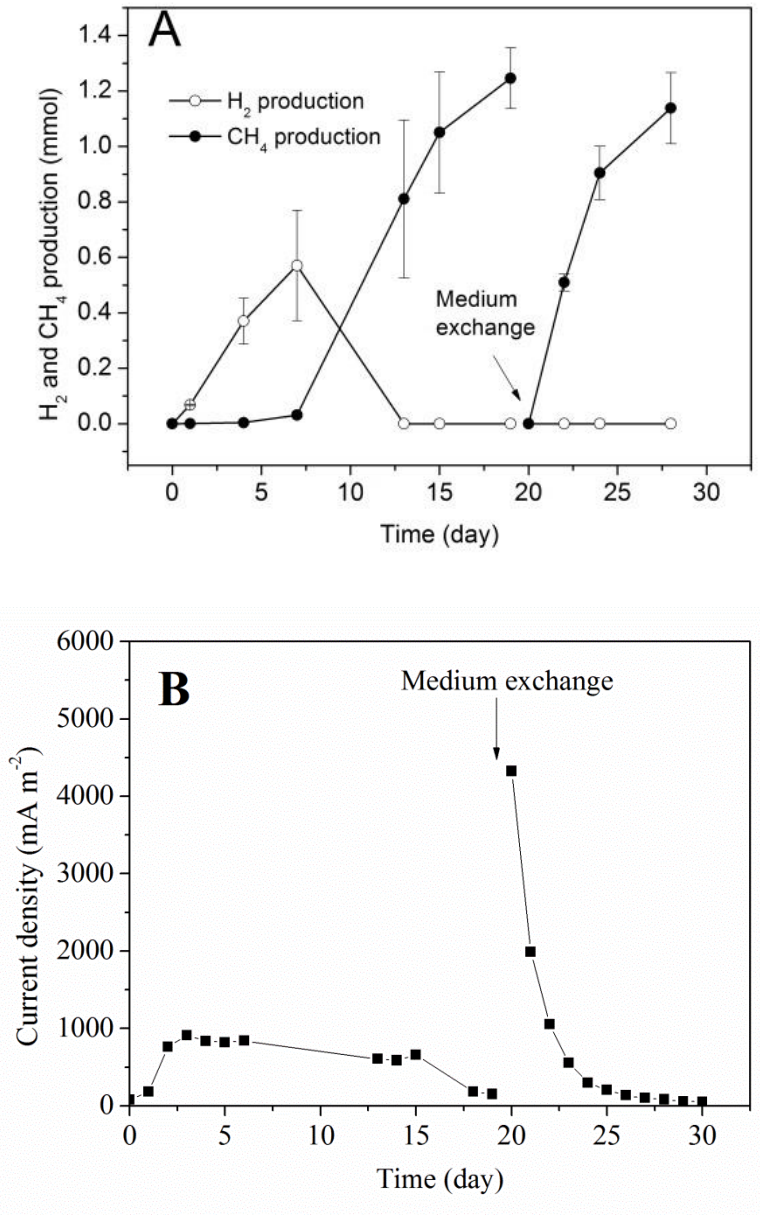


Figure 3.2 (A) CH_4 (the closed circles) and H_2 (the open circles) production in the BES reactors with an applied voltage of 1.0 V during the first cycle (before the medium exchange) and the second cycle (after the medium exchange) of the batch-fed process. (B) Current production of the BES reactor with an applied voltage of 1.0 V.

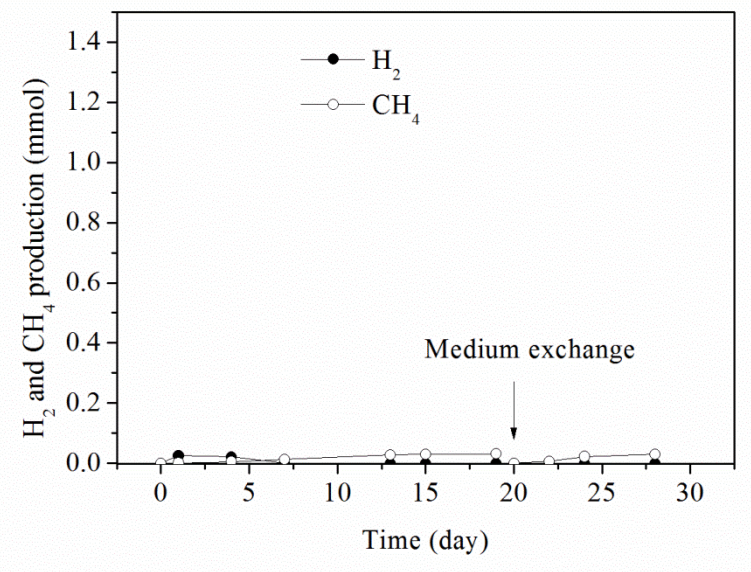


Figure 3.3 CH_4 (the open circles) and H_2 (the closed circles) production in the open-circuit control reactor which was inoculated with microorganisms

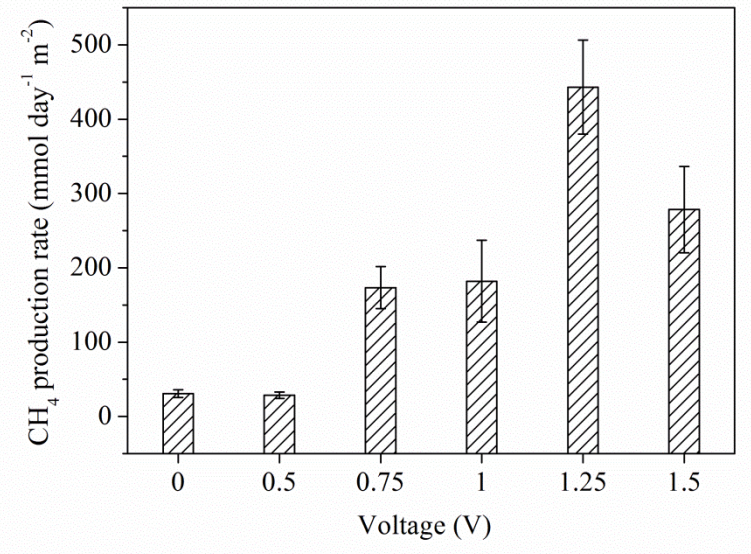


Figure 3.4 The maximum CH_4 production rates of the electromethanogenic reactors with various applied voltages [0.5, 0.75, 1.0, 1.25, 1.5 V and no voltage applied (0 V)]. The maximum CH_4 production rates were calculated after four to eight days of incubation.

3.3.3 Electrochemical Analysis of the Biocathode

To examine the possible current densities in the reactor in the presence and absence of the enriched microorganisms, linear sweep voltammetry (LSV) was performed on the biocathode. The resulting voltammogram of the cathode with the enriched consortium showed more current in comparison to the values obtained with a non-inoculated electrode and the electrode in the open-circuit control (Fig. 3.5). Thus, LSV indicated that electron consumption at the cathode was significantly dependent on the presence of the enriched consortium.

Because the reactors used in this study were single-chamber type, the anode also harbored a microbial consortium, which was derived from the same source as that of the cathodic consortium. However, because the anode served as an electron acceptor, the exoelectrogenic microorganisms were likely enriched on the anode (also reported on the anodes of other single-chamber BESs) (Kiely *et al*. 2011; Liu *et al*. 2010). The CH_4 production coincided with the consumption of acetate (Fig. 3.6A). Moreover, no significant CH_4 production was observed in the reactors without acetate (Fig. 3.6B). Thus, our observations suggested that the anodic exoelectrogenic consortium oxidized acetate and released electrons, which were transferred via the circuit and consumed by the cathodic consortium in the electromethanogenic reactors; this observation is consistent with the results of a previous study (Cheng *et al*. 2009).

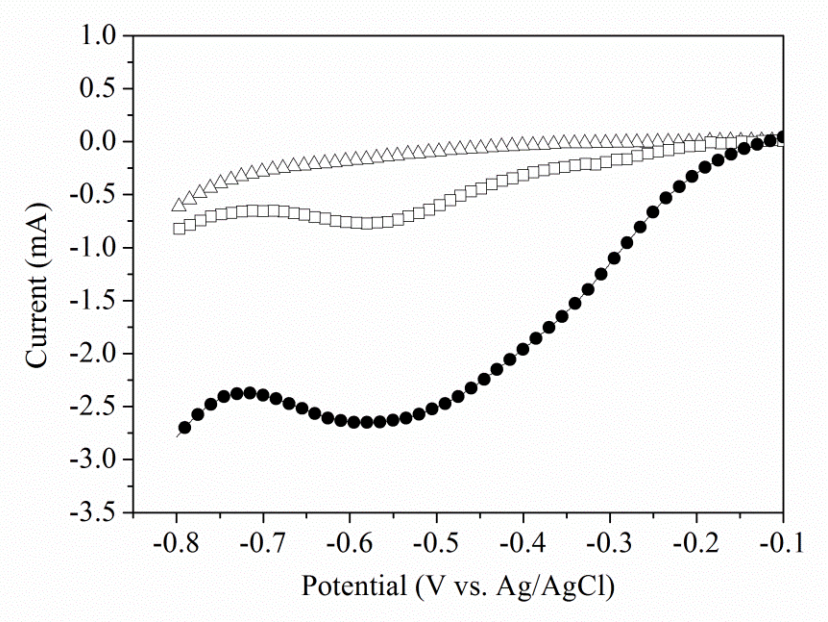


Figure 3.5 Linear sweep voltammograms of the biocathode with the enriched consortium (the closed circles), a non-inoculated electrode (open triangles) and the electrode of the open-circuit control (the open squares).

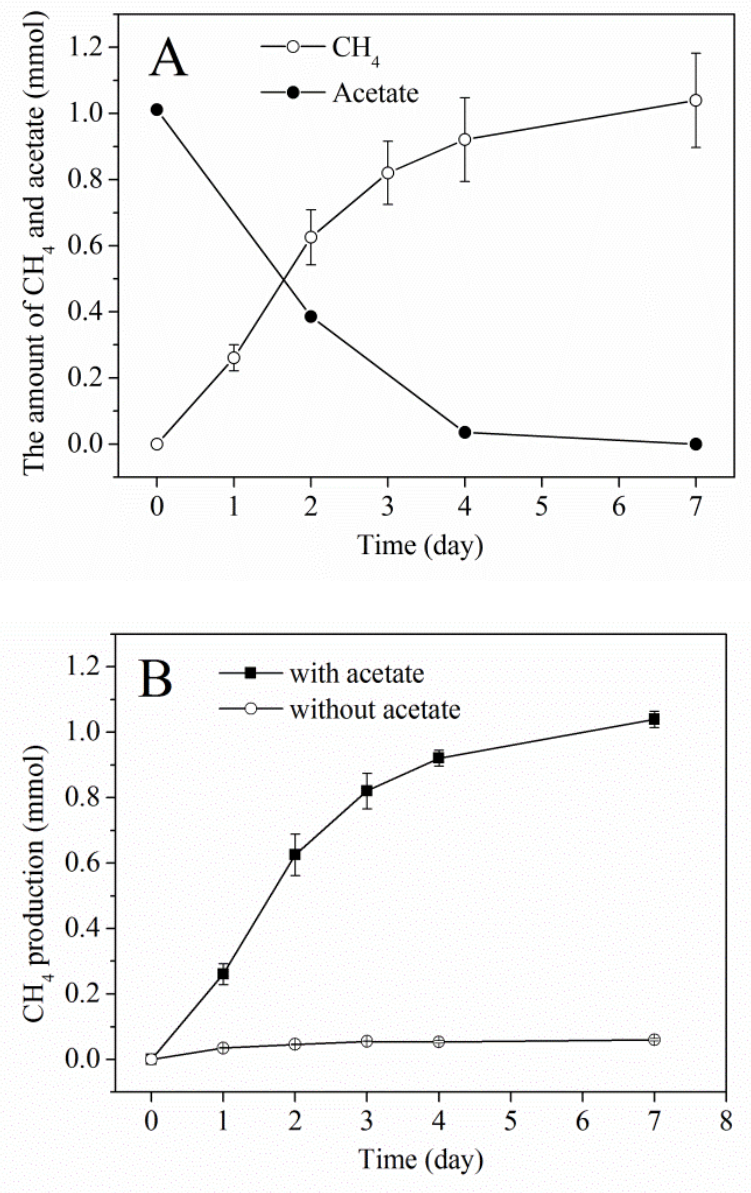


Figure 3.6 The acetate consumption and requirement for electromethanogenesis in the BES reactors (D, E): (D) The amounts of CH_4 (the open circles) and acetate (the closed circles) in the BES reactors with an applied voltage of –0.75 V. (E) CH_4 production in the BES reactors with 10 mM acetate (the closed squares) or without acetate (the open circles) with an applied voltage of –0.75 V.

3.3.4 Microbial Population Analysis of the Electrodes

To understand the microbial basis of the electromethanogenic reaction, the microbial composition of the consortium enriched on the cathode was investigated by constructing 16S rRNA gene-clone libraries. The clones from the archaeal libraries represented, in total, six unique phylotypes belonging to the phylum *Euryarchaeota* (Fig. 3.7). All six phylotypes were detected in the cathode, while the anodic population contained four of the archaeal phylotypes. Among these phylotypes, four (NSArc1-4) were related to methanogens affiliated with the *Methanomicrobia* class. All methanogens in this class can produce CH_4 via hydrogenotrophic methanogenesis (as in Eq. 3.3), while the *Methanosarcina*-affiliated species (NSArc4) were also capable of acetoclastic and methylotrophic methanogenesis. In a previous study, *Methanobacterium palustre*, which is a methanogen of the *Methanobacteriales* class, was found to dominate the microbial population in the electromethanogenic biocathode and has been proposed to be capable of direct electromethanogenesis (as in Eq. 1) (Cheng *et al*. 2009). In this study, however, no *Methanobacteriales*-affiliated species (including *M. palustre*) were detected in either the cathode or the anode. To determine the semi-quantitative contributions of the detected phylotypes, the relative abundances of clones representing the phylotypes were compared. In the library of the anodic archaeal population, the four detected phylotypes were represented by a relatively even number of clones. In the cathodic library, however, the majority (77%) of archaeal clones represented the single phylotype NSArc3, which is closely (98% sequence identity) related to the *Methanocorpusculum bavaricum* strain SZSXXZ, suggesting that this methanogen played an important role in the electromethanogenic biocathode.

The bacterial 16S rRNA gene-clone libraries contained a total of 34 phylotypes belonging to the *Firmicutes*, *Elusimicrobia*, *Synergistetes*, *Proteobacteria*, *Bacteroidetes* and *Spirochaetes* phyla. The cathodic and anodic libraries contained 25 and 24 phylotypes, respectively; 15 of these phylotypes were commonly detected in both libraries. In the cathodic bacterial library, *Proteobacteria* was the most populous

phylum (54% of the clones in the library) (Fig. 3.8). Additionally, 27% of the clones in the library were represented by the phylotype NSBac25, which is closely related to *Geobacter sulfurreducens* str. PCA, a widely studied exoelectrogen (Logan 2009; Lovley 2012; Malvankar *et al*. 2012; Reguera *et al*. 2007; Strycharz *et al*. 2011). This finding was in contrast to the results of a previous study, in which no proteobacterial or exoelectrogen-related sequences were detected in the biocathode (Cheng *et al*. 2009). In the anodic bacterial library, *Firmicutes* was the most populous of exoelectrogens (Lovley 2012; Xing *et al*. 2010). Although the exoelectrogenic activity of the reference species in Fig. 3.8 has not previously been examined, it is possible that those phylotypes correspond to exoelectrogens. Because the phylotypes related to those potential exoelectrogens (NSBac17-19 and 22) were populated at a level similar to that of the *Geobacter*-related phylotype NSBac25 in the anodic library, it is possible that multiple exoelectrogenic species contributed to the generation of current at the anode. At the cathode, however, *Geobacter*-related bacteria played an important role.

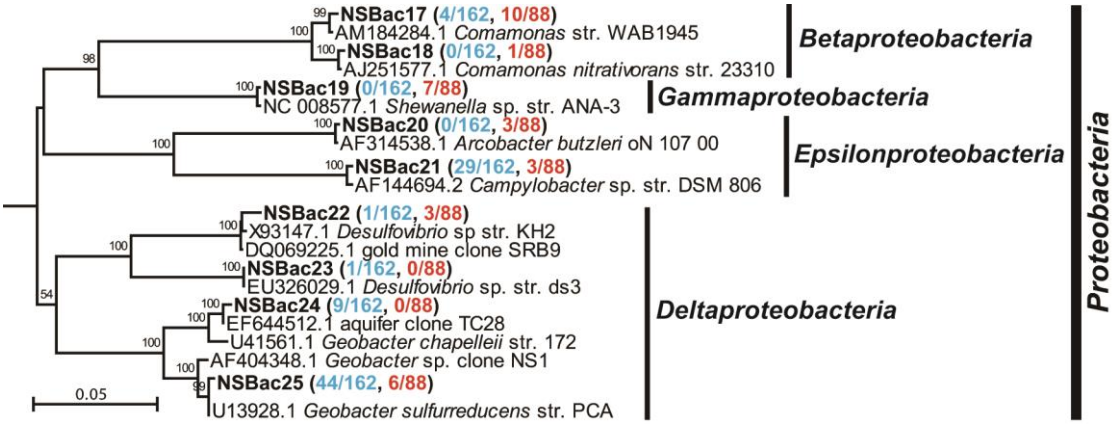


Figure 3.7 The phylogenetic trees of the archaeal 16S rRNA gene sequences obtained from the electrodes of the electromethanogenic BES reactor. The trees were constructed using the neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≧ 50% are shown above the branches. The scale bars represent the number of changes per nucleotide position. *Thermotoga lettingae* strain TMO (CP000812.1) was used as an outgroup (not shown). The numbers of clones representing each phylotype / the numbers of total clones in each library derived from the cathode (blue) and anode (red) are shown in parentheses.

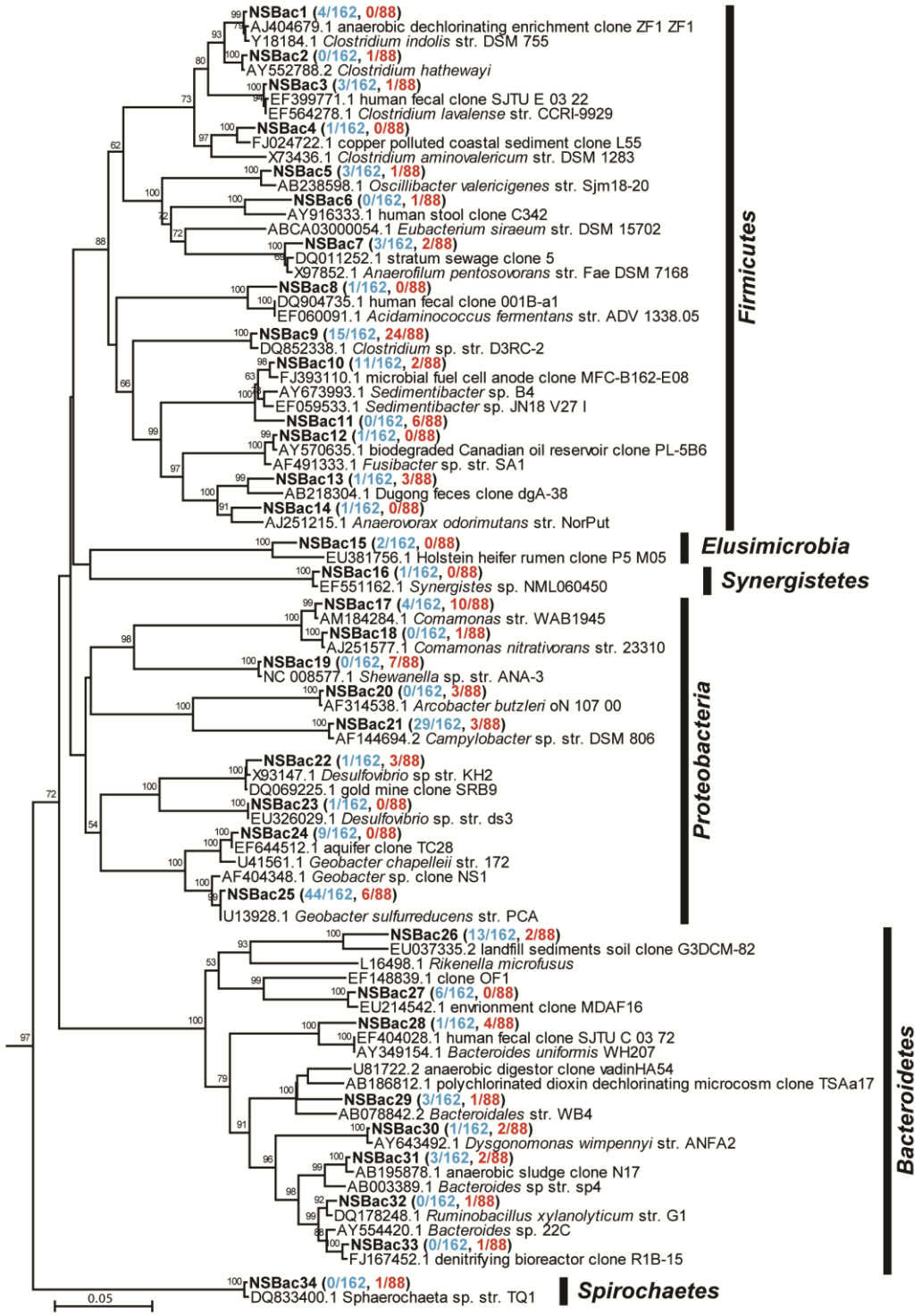


Figure 3.8 The phylogenetic trees of the bacterial 16S rRNA gene sequences obtained from the electrodes of the electromethanogenic BES reactor. The trees were constructed using the

neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≧50% are shown above the branches. The scale bars represent the number of changes per nucleotide position. *Methanococcus maripaludis* strain S2 (BX957219.1) was used as an outgroup (not shown). The numbers of clones representing each phylotype / the numbers of total clones in each library derived from the cathode (blue) and anode (red) are shown in parentheses.

3.4 Conclusion

In this study, we built a biocathode with an electromethanogenic activity comparable to that reported in a previous study (Cheng *et al*. 2009). We also presented the first comprehensive phylogenetic analysis of both the biocathodic and bioanodic communities. It is interesting to note that the composition of the cathodic microorganisms was significantly different from that detailed in a previous report: no methanogen of the *Methanobacteriales* class was detected, and instead, a methanogen closely related to *M. bavaricum* of the *Methanomicrobia* class was suggested to be the dominant methanogen. Moreover, it was suggested that an exoelectrogenic bacteria, *G. sulfurreducens*, was enriched on the biocathode. These observations indicated the possibility that diverse species of methanogens could catalyze electromethanogenesis on the biocathode. It has been shown that *G. sulfurreducens* is also capable of catalyzing hydrogen production (as in Eq. 3.3) using an electrode (cathode) as the electron donor (Geelhoed and Stams 2011). The hydrogen production observed in the first fed-batch cycle was likely due to the hydrogen-producing activity of *G. sulfurreducens*.

Thus, we think it is possible that, in the following stage of incubation, *G. sulfurreducens* established a cooperative relationship with the methanogen for the electromethanogenic reaction by first receiving electrons from the cathode for hydrogen formation (as in Eq. 3.2) and then providing the resulting H_2 to the

methanogens for hydrogenotrophic methanogenesis (Eq. 3.3), which was shown in Fig. 3.9A. Alternatively, because it has recently been shown that *G. sulfurreducens* and related *Geobacter* species can directly transfer electrons to other microorganisms (including methanogens) (Kato *et al*. 2012a, b), it is also possible that *G. sulfurreducens* provided electrons (not molecular H_2) directly to the methanogen, which utilized the electrons in the electromethanogenic reaction (Eq. 1), which was shown in Fig. 3.9B. Thus, our results suggest an electromethanogenic pathway mediated by exoelectrogenic bacteria ("Eq. 3 + Eq. 3" or "Eq. 1 by using electrons from exoelectrogenic bacteria, which receives electrons from the cathode"). In the future, BES reactors using co-cultures of exoelectrogens (*G. sulfurreducens* and other species) and methanogens will be constructed to examine this hypothesis.

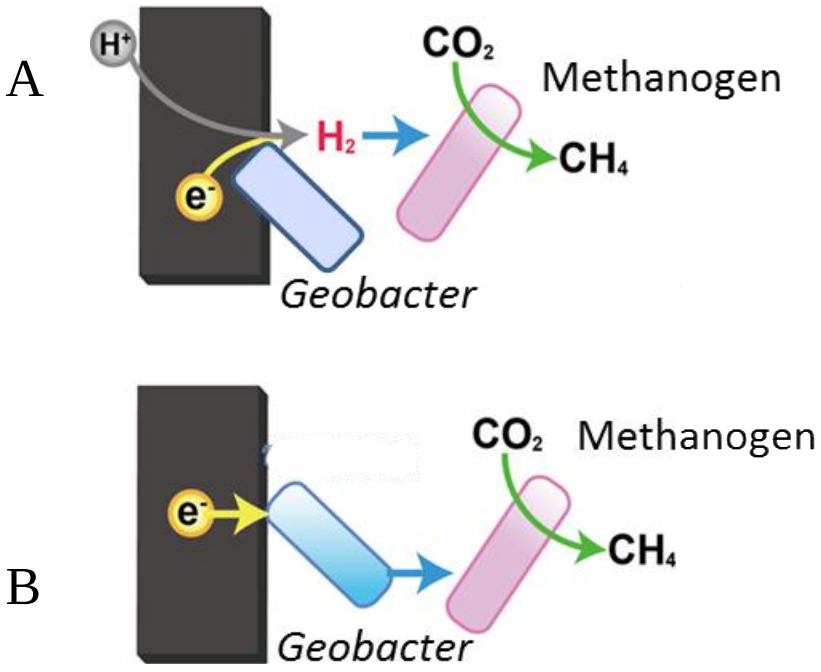


Figure 3.9 The pathway of CH_4 production: (A) interspecies H_2 transfer between *Geobacter* and *Methanogens* for CH_4 production; (B) Interspecies electron transfer between *Geobacter* and *Methanogens*.

Chapter 3

This chapter was published in Journal of Bioscience and Bioengineering under the following reference:

Hajime Kobayashi, Naoki Saito, **Qian Fu**, Hideo Kawaguchi, Javier Vilcaez, Tatsuki Wakayama, Haruo Maeda, and Kozo Sato. Bio-electrochemical property and phylogenetic diversity of microbial communities associated with bioelectrodes of an electron methanogenic reactor. Journal of Bioscience and Bioengineering, 2013, 116 (1), 114-117. (The first three authors contributed equally)

Chapter 4

Explorations of Thermophilic Electrochemically-Active Microorganisms Using Microbial Fuel Cells

4.1 Introduction

As we described in chapter 1, one promising application of bioelectrochemical systems (BESs) is to combine them with CO_2 capture and storage (CCS) technology together to establish a sustainable carbon cycle system. In last chapter, we built a mesophilic electromethanogenic CH_4-producing biocathode being capable of converting CO_2 to CH_4 with a high conversion rate at 28 ℃, and concluded that both methanogens and exoelectrogens involved in the CH_4 production.

However, the mesophilic electromethanogenic system reported in chapter 2 cannot be directly used in CCS reservoir due to the high temperature in CCS reservoir environment (usually 40℃~60℃). Generally, the electrochemical activity of the common mesophilic electrochemically-active microorganisms, including exoelectrogens and methanogens, drop drastically when the temperature is higher than 40 ℃ and lose at the temperature higher than 50℃ (Patil *et al*. 2010).

Furthermore, it has been suggested that thermophilic bioelectrochemical systems (BESs) is potentially superior to mesophilic BESs in performance, wih higher reaction activity, limited biomass yields, greater durability and wider substrate range (Jong *et al*. 2006; Mathis *et al*. 2008). Therefore, using thermophilic electrochemically-active microorganisms as biocatalysts is one pathway to improve the performance of BESs and overcome the limitation of temperature on the mesophilic exoelectrogens.

Although diverse thermophilic methanogens had been reported in previous studies (Huber *et al*. 1982; Jones *et al*. 1983), the studies on exoelectrogens are mainly focusing on mesophilic exoelectrogens (Logan 2009). To date, there are more than 20 microbial species, most of which are mesophilic Gram-negative bacteria affiliated with the phylum *Proteobacteria*, has been reported to possess exoelectrogenic activity (Fedorovich *et al*. 2009; Logan 2009; Lovley 2012). Among them, *Geobacter sulfurreducens* (Bond and Lovley 2003; Holmes *et al*. 2006; Holmes *et al*. 2008; Reguera *et al*. 2005; Reguera *et al*. 2006; Richter *et al*. 2009) and *Shewanella*

oneidensis (Bretschger *et al*. 2007; El-Naggar *et al*. 2010; Firer-Sherwood *et al*. 2008; Gorby *et al*. 2006; Marsili *et al*. 2008; Von Canstein *et al*. 2008) have been extensively studied as model exoelectrogens, and different mechanisms for electron transfer have been proposed. Biochemical and genetic studies have indicated that *G. sulfurreducens* can directly transfer electrons to an anode surface using membrane-bound c-type cytochromes (Holmes *et al*. 2006) and conductive pili(Reguera *et al*. 2005; Reguera *et al*. 2006) Similarly, membrane-bound c-type cytochromes (Bretschger *et al*. 2007; Firer-Sherwood *et al*. 2008) and conductive pili (El-Naggar *et al*. 2010; Gorby *et al*. 2006) have also been characterized in *S. oneidensis*, however, the extracellular electron transfer of *S. oneidensis* is also mediated by redox-active flavin compounds (Marsili *et al*. 2008; Von Canstein *et al*. 2008), which are secreted by the bacteria and function as electron shuttles.

In contrast to the mesophilic species, thermophilic members of exoelectrogens remain unexploited and have so far been largely limited to Gram-positive species affiliated with the phylum *Firmicutes*. Two species of thermophilic exoelectrogens, *Thermincola potens* strain JR (Wrighton *et al*. 2008) and *Thermincola ferriacetica* (Marshall and May 2009), were shown to be capable of directly transferring electrons to an electrode. A recent study has suggested that multiheme c-type cytochromes localized to the cell envelope of *T. potens* strain JR, which in contrast to the Gram-negative envelope lacks an outer membrane and instead has a cell wall, are implicated in the electron transfer (Carlson *et al*. 2012).

Molecular phylogenetic analyses revealed a high possibility that more diverse species of thermophilic bacteria share exoelectrogenic activity. In the thermophilic MFCs inoculated with the marine-sediment- and digester-sludge-derived consortia, although *Firmicutes* (particularly, the genus *Thermincola*)-affiliated species dominated the anodic microbial communities, sequences related to other phyla (including *Deferribacteres, Proteobacteria, Spirochaeta, Nitrospira, Thermotoga* and *Coprothermobacter*) were also detected (Mathis *et al*. 2008; Wrighton *et al*. 2008). Moreover, in the anode of MFC inoculated with the digester effluent, no *Firmicutes*-

related sequence was detected and the dominant species belonged to the phyla *Deferribacteres* and *Coprothermobacter* (Jong *et al*. 2006). However, the exoelectrogenic activity of such non-*Firmicutes* thermophilic bacteria has never been examined.

Concerning possible applications under diverse conditions, however, availability of more biocatalysts (other than *Thermincola*-affiliated species) will contribute to maximize the performance of thermophilic BESs. This chapter was therefore intended to identify new thermophilic exoelectrogens using microbial fuel cells by inoculating with inoculum from diverse sources. Thermophilic anaerobic digestive sludge and oilfield formation water under different temperatures were chosen as inoculum in this chapter. The performance of MFCs and electron transfer mechanisms were analyzed by electrochemical methods. The microbial composition of the bioanodes was separately analyzed by constructing 16S rRNA clone libraries. The pure culture of *Caldterrivibrio nitroreducens* and *Caloramator australicus* were inoculated into MFCs, and were proven to be capable of current generation, expanding members of thermophilic exoelectrogens to Gram-negative species.

4.2 Materials and Methods

4.2.1 MFCs Construction

Two-chamber MFCs were used in the experiment, each consisting of two glass bottles (300 ml volume) separated by a proton exchange membrane (Nafion 117, DuPont Co.), as shown in Fig. 4.1. The proton exchange membranes were pre-treated as described previously (Li *et al*. 2009). Both anode and cathode were made of plain carbon cloth (2 cm×10 cm, TMIL Ltd). Titanium wires (0.5 mm, Alfa Aesar) were used to connect electrodes to circuits. The internal resistances between the electrodes and titanium wires were less than 3 Ω. All reactors were sealed with butyl rubber stoppers and aluminum seals to maintain anaerobic condition. During the start-up process, a fixed external resistance (100 Ω) was connected between the anode and

cathode. The voltage (U) across the external resistance was monitored automatically by an Agilent 34970A data acquisition unit (Agilent Technologies, Santa Clara, CA, USA) every 5 minutes. Each reactor was operated in a fed-batch mode continuously stirred by a magnetic stir bar and incubated at 55℃ except the room temperature control.

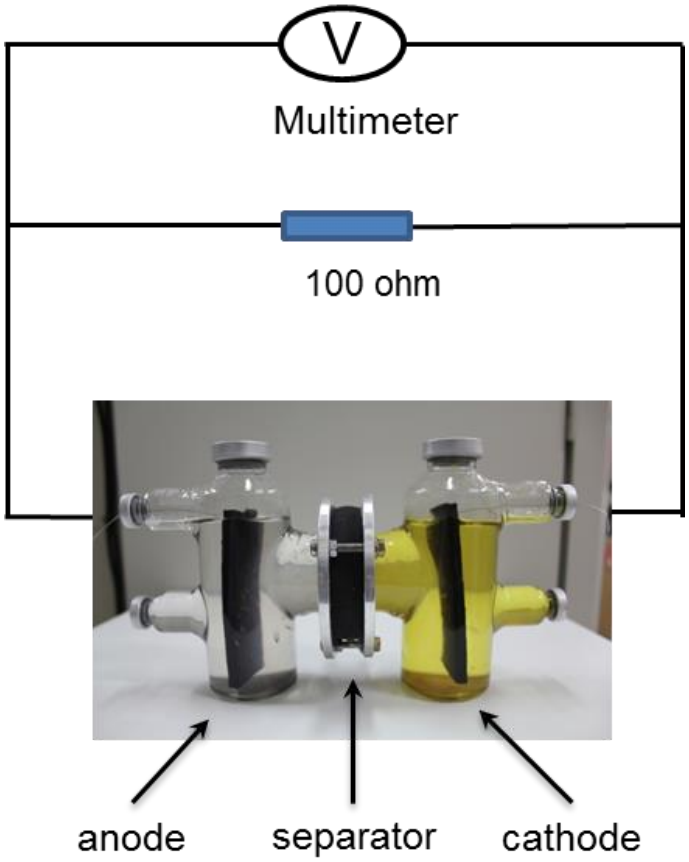


Figure 4.1 Experimental system of the two-chamber MFC reactor

4.2.2 Inoculum and Inoculation

The experiments of exploring novel thermophilic electrochemically-active microorganisms in this chapter are divided into four sections with different experimental conditions. The detailed inoculum and nutrient conditions in each section are shown in shown in Table. 4.1.

Table 4.1 Inoculum and nutrient conditions of each experiment

Experiments number	Inoculum source	Nutrient conditions (electron donors)	Temperature (℃)
1	Thermophilic digestive sludge	1067 medium (Acetate, Yeast extract)	55
2	Thermophilic digestive sludge	Modified 1067 medium (Acetate)	55
3	Yabase oilfield formation water	Modified 1067 medium (1067)	55
4	Minami Aga oilfield formation water	684 medium (Glucose, Yeast extract, Tryptone)	75~98

For experiment #1, the inoculum source is thermophilic digestive sludge from a thermophilic wastewater treatment facility. During the inoculation process, 25 ml of anaerobic digestive sludge was inoculated into anode chamber of each MFC in an anoxic chamber supplementing with 225 ml anaerobic pre-sterilized 1067 medium. The 1067 medium contains: 2.7 g $NaCH_3COO$, 0.136 g KH_2PO_4, 0.54 g NH_4Cl, 0.2 g $MgCl_2$ $6H_2O$, 0.147 g $CaCl_2$ $2H_2O$, 2.5g $NaHCO_3$, 0.2 g yeast extract, 1 ml resazurin, 10 ml of wolfe's vitamin and mineral solutions per liter. The mineral solution consists of the following ingredients in 1 L deionized water: 12.8 g Nitrilotriacetic acid, 1.35

g $FeCl_3 \cdot 6H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.024g $CoCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $ZnCl_2$, 0.025 g $CuCl_2 \cdot 2H_2O$, 0.01g H_3BO_3, 0.024 g $Na_2MoO_4 \cdot 2H_2O$, 1g NaCl, 0.12 g $NiCl_2 \cdot 6H_2O$, 0.004 g Na_2SeO_4, 0.004 g Na_2WO_4, 0.02 g $KAl(SO_4)_2 \cdot 12H_2O$. The wolfe's vitamin consists of following ingredients in 1 L deionized water: 2 mg Biotin, 2 mg Folic acid, 10 mg Pyridoxine-HCl, 5 mg Thiamine-HCl, 5mg Riboflavin, 5 mg Nicotinic acid, 5 mg Ca-pantothenate, 1 mg *p*-Aminobenzoic acid, 0.01 mg Vitamin B_{12}. To remove the soluble oxygen, the medium was firstly boiled for 10 minutes, then sparged with N_2/CO_2 (80/20) for at least 1 hour, and finally pressurized to 150 kPa with H_2/CO_2 (80/20) before autoclaving. 50 mM potassium ferricyanide solution supplemented with 2.5 g/L bicarbonate was used as the catholyte. The operating temperature was 55 ℃.

For experiment #2, the inoculum was same with experiment #1 (thermophilic digestive sludge), but the nutrient conditions for the inoculum are different. Because yeast extract, resazurin and vitamin solution may act as electron-shuttling mediators in MFC and affect the microbial community on the bioanode, they were removed from the nutrient in experiment #2. The medium preparation process and the inoculation process are same with experiment #1. The operating temperature was 55 ℃.

For experiment #3, thermophilic microorganisms from formation water in an petroleum reservoir (located in Akita, Japan) was used as inoculum. The reservoir is a formation of tuffaceous sandstone of Miocene-Pliocene age, located around 1293 to 1436 m under the surface, with in situ temperature of 40-82 ℃. The formation water sample was first acclimated for 2 weeks in pre-sterilized anaerobic medium which was same with experiment #2 to enrich the thermophilic microorganisms. Then it was used as inoculum and inoculated into the anode chamber of MFC, the inoculation process was same with experiment #1. The operating temperature was 55 ℃.

For experiment #4, formation water from an oilfield (located in Nigata, Japan) was used as inoculum. The reservoir is located around 2000 m under the surface with *in situ* temperature of 98 ℃. Microorganisms in the water sample were first augmented

by pre-cultivation in pre-sterilized anaerobic 684 medium at 80℃ for 2 weeks. The medium contains 0.75 g KH_2PO_4, 1.5 g KH_2PO_4, 0.9 g NaCl, 0.9 g NH_4Cl, 0.2 g $MgCl_2 \cdot 6H_2O$, 5 μl 10% $FeSO_4 \cdot 7H_2O$, 2.0 g tryptone, 2.0 g yeast extract, 2.0 g glucose, 1.0 g resazurin, 10 ml Wolfe's mineral solution per liter (Balch *et al*. 1979; Patel *et al*. 1985). Then it was inoculated into the anode chamber of MFC, the inoculation process was same with experiment #1. The operating temperature for startup was 80 ℃.

4.2.3 Electrochemical Analyses

The polarization and power density curves of MFCs were obtained by altering the external resistance of a variable resistance box (10000 Ω ～ 30 Ω) connected into the circuit. The voltage (U) across each external resistance was measured by a data acquisition unit. The current (I) was calculated by Ohm's low: I＝U/R, and power density (PD) was calculated according to PD = UI/A, where R (Ω) represents the external resistance and A (m^2) represents the surface area of the anode.

Cyclic voltammetry (CV) was conducted by using a potentiostat (HSV-110, Hokuto Denko, Japan) with a standard 3-electrodes system. The anode, cathode and an Ag/AgCl reference electrode inserted into the anodic chamber were acted as the working electrode, counter electrode and reference electrode, respectively. In turnover conditions, the parameters for CV were as follow: equilibrium time 99 s, scan rate 1 mV s^{-1}, and scan range –0.4 V ～ 0.2 V vs. SHE. The medium excluding soluble redox compounds was used in the anode chamber. CVs with cell-free spent medium and non-inoculated control medium were also performed with a pre-sterilized electrode in the same reactor. The spent medium was collected from the anode chamber and then filtered with pre-sterilized filter in an anoxic chamber to remove the planktonic cells. The filtrates were then analyzed to determine whether soluble electron shuttles were present in the spent medium. In non-turnover conditions, the biofilm-attached anode was first washed three times with acetate-free

fresh medium and then poised at –0.1V vs. SHE until the current fell to near background (2 μA cm^{-2}), then CV was performed with increasing scan rates from 1 mV s^{-1} to 1 V s^{-1} (scan range: –0.5 V to 0.3 V).

4.2.4 Characterization of Anodic Bacterial Population

For scanning electron microscopy, anodes were aseptically sliced and fixed by 2.5 % (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer solution (pH= 7.4).

For microbial population analysis, community DNA was directly extracted from 250 mg of the crushed electrode using PowerSoil DNA isolation kit (MO BIO laboratories, Carlsbad, CA, USA). The extracted DNA (20 ng) were used as templates for PCR with the primers 8F (5'- AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTACGACTT-3') (Grabowski *et al*. 2005). The pooled PCR amplicons were cloned into pCR4-TOPO using TOPO TA cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were purified using High Pure Plasmid Isolation kit (Roche Applied Science, Indianapolis, IN) and sequenced with T3 and T7 primers. The assembled sequences were aligned by NAST aligner program in Greengenes (http://greengenes.lbl.gov/) with the closest sequence relatives from Greengenes database on March 2012. The alignments were then manually improved in MEGA ver. 4.0.2. (Tamura *et al*. 2007). Phylogenetic trees were constructed on the basis of the Tamura-Nei model and the evolutionary history was inferred using the Neighbor-Joining method (Tamura *et al*. 2004). The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site (Tamura *et al*. 2004).

4.2.5 Pure Culture Tests

4.2.5.1 Caloramator australicus strain RC3

Caloramator australicus strain RC3 was obtained from Japan Collection of Microorganisms (RIKEN bio-resource center, Saitama, Japan) and pre-cultured in anaerobic TYEG 684 medium (Patel *et al*. 1985) for one night. The medium contains: 0.75 g KH_2PO_4, 1.5 g K_2HPO_4, 0.9 g NaCl, 0.2 g $MgCl_2$ $6H_2O$, 0.9 g NH_4Cl, 9 ml wolfe's trace element solution, 10 ml wolfe's vitamin solution, 5 μl 10% $FeSO_4$ $7H_2O$ solution, 2.0 g tryptone, 2.0 g yeast extract, 2.0 g glucose and 1.0 mg resazurin per liter. The composition of trace element solution and vitamin solution was same with the trace element and vitamin solution described in 4.2.2.

Because the anodic reactions between the exoelectrogens and anode are same in the anode of MFC and microbial electrolysis cell (MEC), we used a single-chamber MEC reactor which can be easily kept strictly anaerobic condition to test the exoelectrogenic activity of pure culture. The reactor consisted of a glass bottle (300 ml vol) containing both anode and cathode (made of 4 cm×10 cm carbon cloth) in one chamber without a separator membrane. The reactors were added with 200 ml of PL media (Ogg and Patel 2011) excluding ammonium ferric citrate. As an electron donor, although both yeast extract and acetate were used in the sludge-inoculated MFC, only yeast extract [0.2% (w/v)] was added to the single-chamber reactors, because it had been shown that *C. australicus* strain RC3 can utilize yeast extract but not acetate for the growth substrate (Ogg and Patel 2009). The reactors were anaerobically (N_2) sealed with butyl-rubber stoppers and pre-sterilized. Constant voltage (0.75 V) was applied on the reactors with the positive pole connected to the anode and the negative pole to the cathode using a digital power supply (Array 3645A: Array Electronics, Nanjing, China).

4.2.5.2 Calditerrivibrio nitroreducens strain Yu37-1

Calditerrivibrio nitroreducens strain Yu37-1 (NBRC101217) was obtained from NITE Biological Resource Center (Chiba, Japan). The pure culture was firstly propagated in serum bottles with anaerobic *Deferribacter* medium for 5 days at 55 ℃, then inoculated to two-chambered MFC reactors. The *Deferribacter* medium contains: 2.7 g $NaCH_3COO$, 0.136 g KH_2PO_4, 0.535 g NH_4Cl, 0.204 g $MgCl_2 \cdot 6H_2O$, 0.147 g $CaCl_2 \cdot 2H_2O$, 2.52 g $NaHCO_3$, 0.85 g $Na_2S \cdot 9H_2O$, 0.85 g $NaNO_3$, 1 mg resazurin, 1ml Wolfe's vitamin solution, and 1ml Wolfe's mineral solution per liter. The vitamin solution and the mineral solution used here are same with the solution used in section 3.2.2.

During the pure-culture test, the two-chamber MFC reactors used were same with the reactors inoculated with mixed culture. For inoculation, the enriched pure culture in serum bottles was firstly centrifuged (15, 000 × g) for 30 minutes at room temperature. Then the supernatant was removed from the serum bottles in an anoxic chamber. Finally, the cells in the serum bottles were washed with fresh *Deferribacter* medium (excluding $NaNO_3$, resazurin and $Na_2S \cdot 9H_2O$) and inoculated to MFC reactors in an anoxic chamber. In contrast, only fresh *Deferribacter* medium were used as anlyote in the anode chamber of control reactor. 50 mM potassium ferricyanide solution supplemented with 2.5 g/L bicarbonate was used as the catholyte. To maintain anaerobic condition in the anodic chamber, 0.05 g/l of $Na_2S \cdot 9H_2O$ was added to the medium during the first three cycles. For subsequent cycles, $Na_2S \cdot 9H_2O$ was replaced by continuous sparging with N_2/CO_2 (80/20). To investigate the effect of nitrate on current generation by *C. nitroreducens* strain Yu37-1, 1 ml of anoxic 2 M $NaNO_3$ solution (or anoxic distilled water as the control) was injected to the anodic chamber of MFC producing stable current. Reproducibility was evaluated in at least three independent MFC operations. The other analyzing methods are same with the MFCs inoculated with mixed culture.

4.3 Results and Discussion

4.3.1 MFC Inoculated with Thermophilic Digestive Sludge #1

4.3.1.1 Electricity Generation

Fig. 4.2A showed the current generation of the sludge-inoculated MFC and the abiotic control reactor verse operating time. The initial current of the inoculated MFC and the abiotic control reactor were both around 0.3 mA, which was mainly due to the residual reducing activity of the medium compounds (i.e. Na_2S and cysteine). The current of the abiotic control reactor decreased to a background level (nearly zero mA) soon after the inoculation. For the sludge-inoculated MFC, on the other hand, the current generation exponentially increased with operating time and obtained the first maximum current generation (2.1 mA) at around 20 hours post inoculation. This exponentially increases manner was probably due to the exponential growth of the exoelectrogenic microorganisms on the anode.

Each time when the produced current of the MFC decreased to *ca.* 0.5 mA, which was likely due to the substrate consumption, the media in both chambers were exchanged with fresh media, (arrows in Fig. 4.2A). After the medium exchange, the current generation of the sludge-inoculated MFC recovered to the maximum level in less than three hours. Most of the suspended microbial cells and possible soluble electron-shuttling mediators were removed during the medium exchange process. Thus, it concluded that the electricity generation of the MFC was mainly due to the microorganisms attached on the anode, rather than the planktonic cells in the medium or the soluble electron-shuttling mediators.

Fig. 4.2B showed the polarization and power density curve of the sludge-inoculated MFC as a function of current. The open circuit potential and maximum power density of the sludge-inoculated MFC were around 0.64 V and 436 mW m^{-2} (subjected to the anode surface area), respectively, which were comparable to those of thermophilic MFCs in previous studies (Mathis *et al*. 2008; Wrighton *et al*. 2008).

The polarization showed a linear relationship with the current, suggesting that the dominant limiting factor for this MFC was ohmic resistance. Based on the slope of the polarization curve, the internal resistance of this MFC was around 68 Ω.

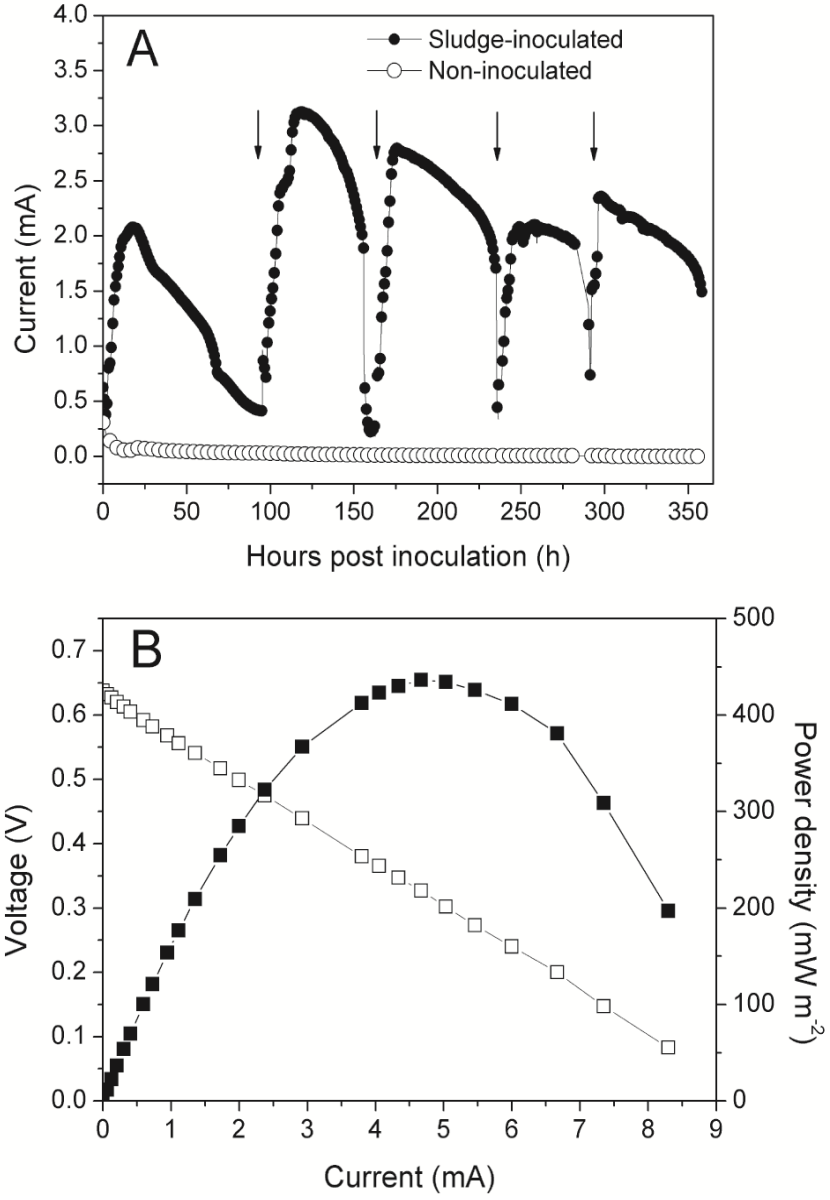


Figure 4.2 Start-up and performance of the sludge-inoculated thermophilic MFC. (A) Startup curve of the sludge-inoculated thermophilic MFC (closed circles) and the abiotic control

(open circles). Each arrow represents an exchange of medium. (B) Polarization curve (open square symbols) and power density curve (close square symbols) of the sludge-inoculated thermophilic MFC as a function of current.

4.3.1.2 Cyclic Voltammetry Analysis

To investigate the mechanism of electron transfer between the anode and the anode-attached microorganisms, the electrochemical property of the anode was analyzed using cyclic voltammetry (CV) in a MFC stably producing electricity for more than 300 hours.

Fig. 4.3A showed the cyclic voltammogram of the bioanode in the MFC and Fig. 4.3B showed the derivative of the cyclic voltammogram for further analysis. When the potential lower than –0.3 V (vs. SHE), the anodic current was around zero, suggesting no reaction was happened. As the potential rise above –0.3 V, the positive anodic current increased continuously with the increase of potential, reflecting continuous oxidation of acetate and transfer of electrons to the anode. Correspondingly, the first derivative curve of the cyclic voltammogram showed two symmetrical peaks, which ranged from –0.3 V to –0.25 V and centered at around –0.15 V. In this narrow region (between –0.3 V to –0.25 V), the capacity of microbial cells to oxidize acetate and generate electrons is in excess, but the electron transfer rate from the microbial cells to the anode was limited by the kinetics at the interface. As the kinetics could be accelerated by the driving force, thus the current increased with the increase of potential. However, as the working potential rise above –0.25 V, the anodic current got a plateau no matter how the potential increased. This was because the enzymatic reaction got the maximum rate. Therefore, this sigmoidal wave shape represented a typical catalytic behavior, in which proteins on the anode contribute to the electricity generation.

The CV profile of the anode was retained after exchanging the anode-chamber medium with fresh medium. In addition, no catalytic behavior was detected in the filter-sterilized spent medium (data not shown) or on the anode of the non-inoculated

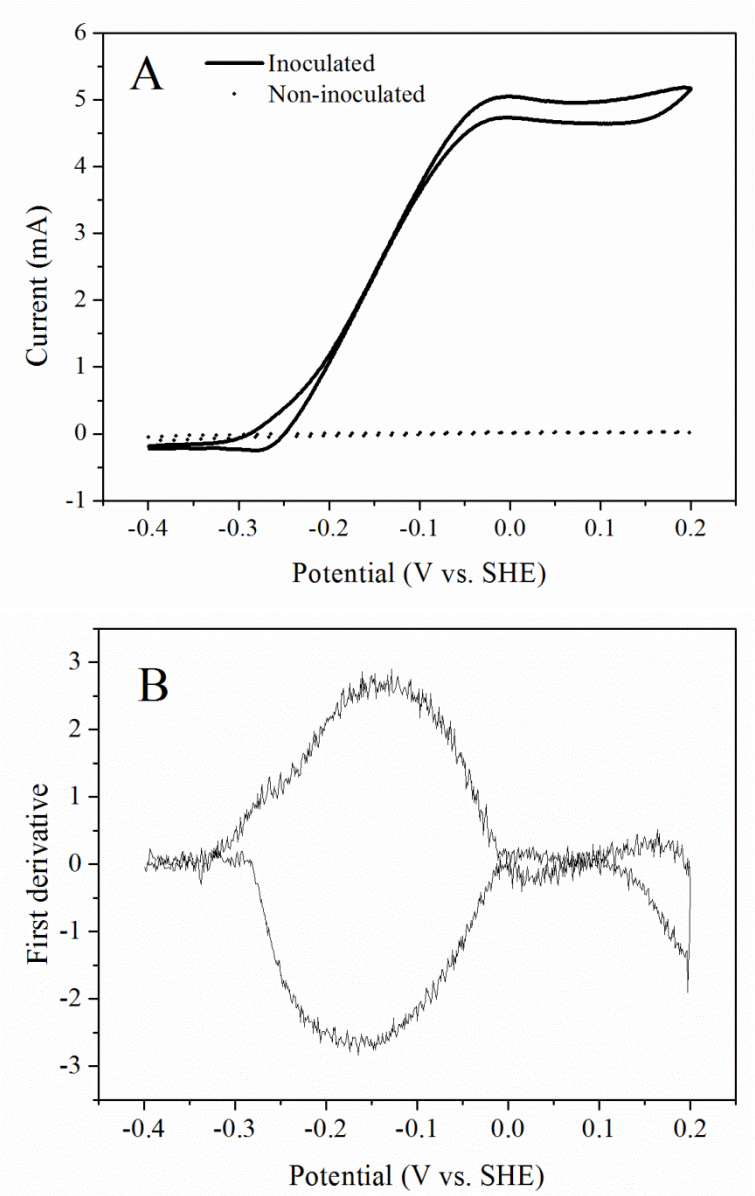


Figure 4.3 Cyclic voltammetry analysis. (A) Cyclic voltammograms of the anodes of the sludge-inoculated MFC (solid lines) and the non-inoculated control (dotted line). (B) The first derivative of cyclic voltammogram of the sludge-inoculated anode.

reactor (Fig. 4.3A). Thus, CV analyses suggested that the main redox-active compound was tightly associated with the anode-attached microorganisms and no evidence for soluble electron shuttles endogenous or exogenous to the microorganisms. Taken together, our results suggested that thermophilic exoelectrogens enriched on the anode and generated current via direct electron transfer.

4.3.1.3 Microbial Analysis of the Anode

To explore the possible diversity of thermophilic exoelectrogens, we investigated the phylogenetic diversity of the anodic bacterial populations in the MFC, which was shown in Fig. 4.4. The bacterial 16S rRNA gene-clone library contained ten phylotypes belonging to *Firmicutes*, *Synergistetes*, *Coprothermobacteria* and *Chloroflexi* phyla. Among them, *Firmicutes* was the most populous phylum which accounted for around 87.5% of the clones in the library and was represented by seven phylotypes (TA2-B1 to TA2-B7). Such dominance of *Firmicutes* was in agreement with a previous study (Wrighton *et al*. 2008), in which a functional role of *Firmicutes* in the current production of a thermophilic MFC was documented. In particular, the phylotype TA2-B5, which was closely related to *Thermincola* species, was the most abundant sequence in the library. This observation suggested a contribution of this species to the electricity generation in the sludge-inoculated thermophilic MFC and also implied that the *Thermincola*-related bacteria were widely-distributed exoelectrogens.

On the other hand, the genus *Caloramator*, which was represented by two phylotypes TA2-B1 and TA2-B2, was the second most populous genus. The members of the genus *Caloramator* are known to be thermophilic, strict anaerobic, chemo-organoheterotrophs and have been detected in various thermophilic environments, such as hot springs, subsurface aquifer and digester sludge (Ogg and Patel 2009). So far, no *Caloramator*-related bacterium has been detected in thermophilic MFCs in previous studies. Interestingly, at least two species of the

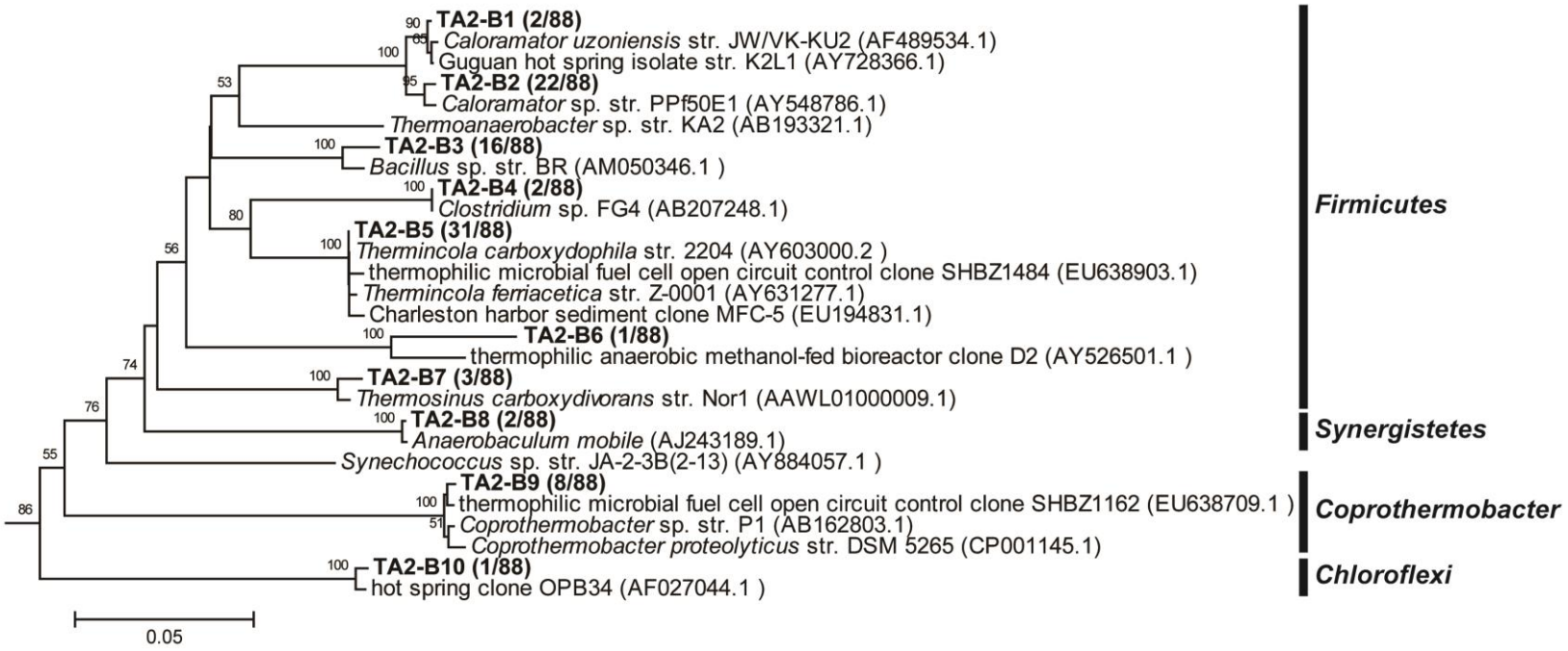


Figure 4.4 Phylogenetic tree illustrating the phylogenetic position of 16S rRNA gene clones generated from the anode of the sludge-inoculated thermophilic MFC. The tree was constructed using the neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≧50% are shown above the branches. The scale bar represents the number of changes per nucleotide position. *Thermotoga lettingae* strain TMO (CP000812.1) was used as an outgroup (not shown). Numbers of clones representing each phylotype/numbers of total clones are shown in parentheses.

genus (*C. australicus* and *C. proteoclasticus*) were shown to have the ability to reduce various metals, such as iron (III), manganese (IV) and vanadium (V) (Ogg and Patel 2011). Taken together with our detection of *Caloramator*-related species in the anodic consortium, it seemed plausible that *Caloramator*-affiliated bacteria have exoelectrogenic activity.

4.3.1.4 Pure Culture Test

To verify if *Caloramator*-affiliated bacteria have exoelectrogenic activity, a purely-cultured *Caloramator* species was examined. *C. australicus* strain RC3 (JCM15081) was chosen for the analysis, as the metal-reducing activity and the draft genome of the strain has been reported.

C. australicus was a strictly anaerobic thermophilic bacterium (Ogg and Patel 2009); thus, the two-chambered MFC reactor used for the sludge-inoculated MFC was not available due to the easy low-level contamination of oxygen from the membrane-holding joint between two chambers. As the anodic reactions between the exoelectrogens and anode are same in the anode of MFC and microbial electrolysis cell (MEC) (Logan *et al*. 2008), we used a single-chamber MEC reactor which can be easily kept strictly anaerobic condition to test the exoelectrogenic activity of pure culture.

The exoelectrogenic activity of *C. australicus* strain RC3 was examined in the single-chamber reactors by monitoring the current generation, as the voltage across an external resistance (1.0 Ω resistor) connected between the anode and cathode. The initial current of the *C. australicus*-inoculated reactors and the non-inoculated reactors were both around 0.1 mA due to the residual reducing activity of the inoculum (i.e. Na_2S). Then the current quickly decreased upon the onset of the voltage-applied incubations (Fig. 4.5). While no further current generation was observed in the non-inoculated control, the *C. australicus*-inoculated reactors began to produce current in an exponential manner from four hours post inoculation, with the maximum produced current of 0.16 mA at 10 hours post inoculation. The

current level was then gradually attenuated and maintained steadily at 0.08 mA, which was yet significantly higher than that of the non-inoculated control, until the end of the analysis (around 70 hours post inoculation). The experiment was repeated three times and similar current generation profiles were obtained (data were not shown). This result indicated that *C. australicus* strain RC3 has exoelectrogenic activity, likely coupling the oxidation of organic substances in yeast extract to the reduction of the electrode.

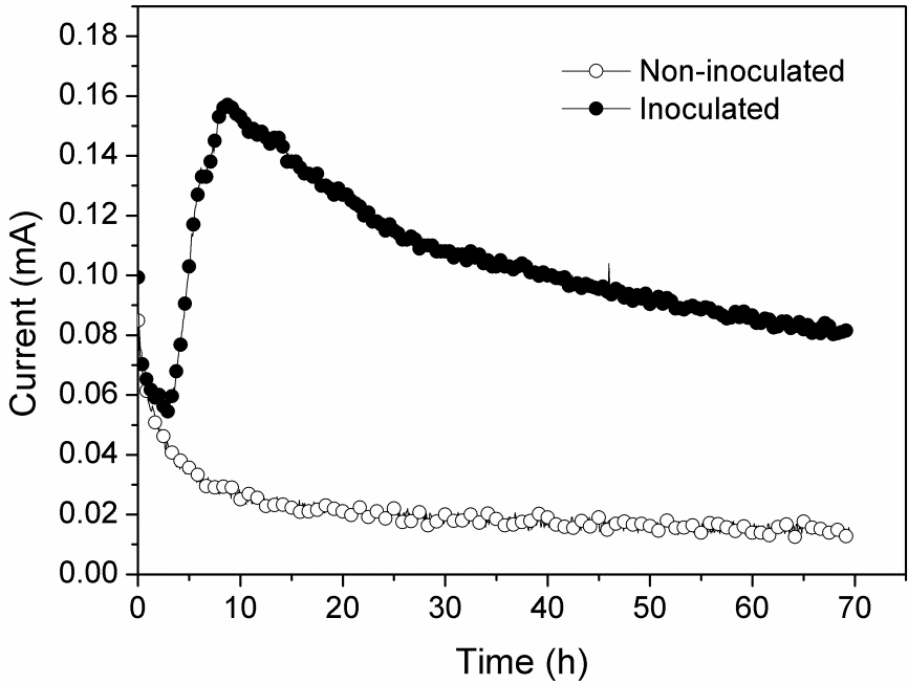


Figure 4.5 Representative profiles of the current generation in the single-chamber electrochemical reactor inoculated with *C. australicus* strain RC3 (closed circles) and the non-inoculated control (open circles) an applied voltage of 0.75 V.

Thus, in this study, we showed that *C. australicus* strain RC3 is a new thermophilic exoelectrogen and suggested possible contribution of *Caloramator*-related bacteria to the electricity generation in a thermophilic MFC. It is possible, however, that *Caloramator*-related bacteria was not the main exoelectrogenic species in the sludge-inoculated MFC, as *Thermincola*-related species were also detected. Yet, *C. australicus* RC3 is so far the second thermophilic *Firmicutes*, in which electricity-generating capability is documented. Our finding contributes not only to our understanding of the potential diversity of thermophilic exoelectrogens but also to develop new applications of thermophilic MFC in industrial processes, as *Caloramator*-related species have ability to utilize various organic substrates including cellulose, starch (biomass substances) and glycerol (a byproduct of the bio-diesel production process). Utilization of various substrates by *Caloramator*-related bacteria in MFC will be examined in future studies.

4.3.2 MFC inoculated with thermophilic digestive sludge #2

4.3.2.1 Electricity Generation

In section 4.3.1, we concluded that the electron transfer mechanism between the microorganisms and the anode was mainly in a direct electron transfer. As resazurin and cysteine could be acted as electron-shuttling mediators (Grayc and Headc 2005; Sund *et al*. 2007), their existence in the medium may affect the microbial population, and hence affect the performance of the MFC. Therefore, these possible electron-shuttling mediators were removed from the medium hereafter.

The electricity generation in MFCs operated at 55℃ (thermophilic MFC) and 25℃ (mesophilic MFC) were examined (Fig. 4.6). Both of the MFCs were inoculated with thermophilic digestive sludge and with acetate as electron donor. After inoculation, the thermophilic MFC started to generate current faster and produced higher current density than the mesophilic MFC. The current produced in

thermophilic MFC began to increase exponentially after 30 h post inoculation, which meant that the lag period for the thermophilic MFC was around 30 h. On the other hand, the lag period of the mesophilic MFC was 330 hours, 11 times longer than that of the thermophilic MFC. In the thermophilic MFC, the first maximum current generation (3.45 mA) was observed at around 48 hours after inoculation, and then decreased gradually, indicating the consumption of electron donor. In the mesophilic MFC, on the other hand, a maximum current generation (1.41 mA) was obtained at 460 hours post inoculation.

The media in both chambers of the thermophilic MFC were exchanged with fresh media when the current decreased to 0.1 mA except the third circle. After changing the medium, the current generation of thermophilic MFC recovered to the maximum level in less than 2 hours without addition of any exogenous electron-shuttling mediators. As the planktonic cells and possible soluble electron mediators in the spent medium were removed during the medium exchange process, it can be concluded that the electricity generation was mainly due to the microorganisms attached on the anode, rather than the planktonic cells and the soluble electron-shuttling mediators in the medium. Thus, it proved that the thermophilic MFC in this study was mediatorless MFC.

The power density generated by the thermophilic MFC was around 823 mW m^{-2}, whereas it was only 102 mW m^{-2} for the mesophilic MFC (Fig. 4.7). The power density generated by the thermophilic MFC was also higher than those reported with thermophilic MFCs in several previous studies (generally $\leqq$ 400 mW m^{-2}) (Carver *et al*. 2011; Mathis *et al*. 2008; Wrighton *et al*. 2008) and comparable to that of a thermophilic MFC under a continuous mode of operation (1030 $\pm$ 340 mu m^{-2}) (Jong *et al*. 2006). Considering that the MFC reactor used in this study was not optimized for performance (which was operated in a fed-batch mode and had a relatively high internal resistance due to the large electrode spacing), we concluded that microorganisms with high exoelectrogenic activity were successfully enriched on the anode.

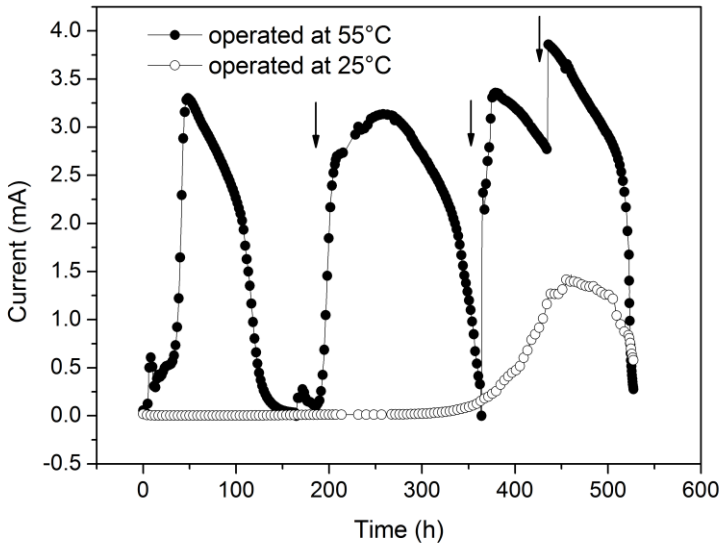


Figure 4.6 current production by MFCs inoculated with thermophilic digestive sludge and operated at 55℃ (solid symbols) or 25 ℃ (open symbols). Arrows represent media exchanges.

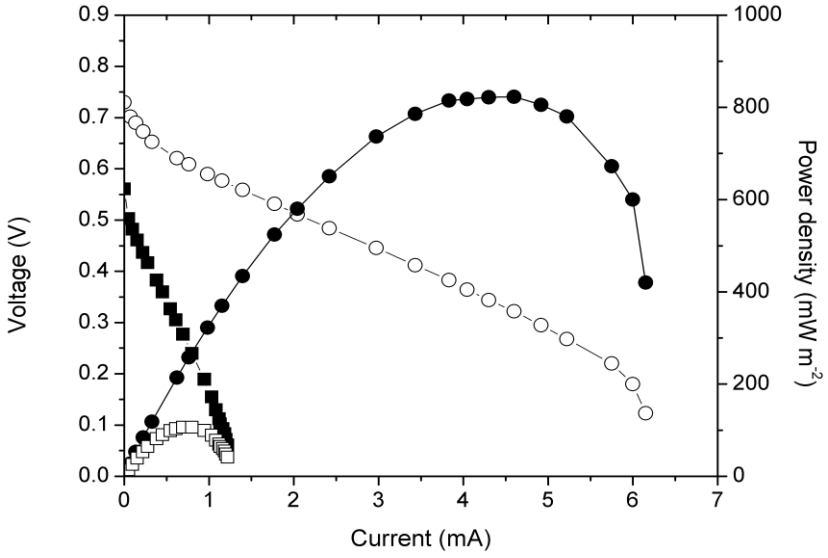


Figure 4.7 Polarization and power density curve of MFCs operated at different temperatures (the triangle symbols represent the thermophilic MFC, and the square symbols represent the MFC operated at room temperature; the open symbols represent the polarization curve and the solid symbols represent the power density.)

4.3.2.2 Phylogenetic Analysis of the Anodic Microbial Population

The microbial community on the anode surface was analyzed by constructing 16S rRNA gene clone library, which was shown in Fig. 4.8. Most (84 clones, 88%) of the clones in the library (95 clones in total) were represented by a phylotype TA-B1, which was closely related to sequences affiliated with the *Calditerrivibrio* genus in the Deferribacteres phylum

As two species of the *Deferribacter genus* (a genus in the *Deferribacteres* phylum, closely related to *Calditerrivibrio*), *Deferribacter thermophilus* (Greene *et al*. 1997) and *Deferribacter abyssi* (Miroshnichenko 2003), have been reported to be capable of reducing insoluble iron, it is possible that more diverse bacteria affiliated to the *Deferribacteres* phylum also have exoelectrogenic activity.

In previous studies, *Calditerrivibrio*-related sequences had also been detected in two thermophilic mediator-less MFCs (inoculated with effluent from an anaerobic digester and marine sediment, respectively) (Jong *et al*. 2006; Mathis *et al*. 2008), leading us to hypothesize that *Calditerrivibrio*-affiliated bacteria have exoelectrogenic activity. Among the related sequences, the uncultured clones 1A162 and 1B62 (DQ424915 and DQ424925) had been detected in a thermophilic mediator-less MFC, which was continuously fed with effluent from a brewery-wastewater-treating anaerobic digester, and partly dominated the anodic microbial populations. Similar sequences had also been detected in a thermophilic MFC inoculated with microorganisms originated from marine sediment (Mathis *et al*. 2008). Such a common presence (in some cases, dominance) of the *Calditerrivibrio*-related sequences in several thermophilic MFCs suggested that *Calditerrivibrio*-affiliated bacteria played an important role in electricity generation.

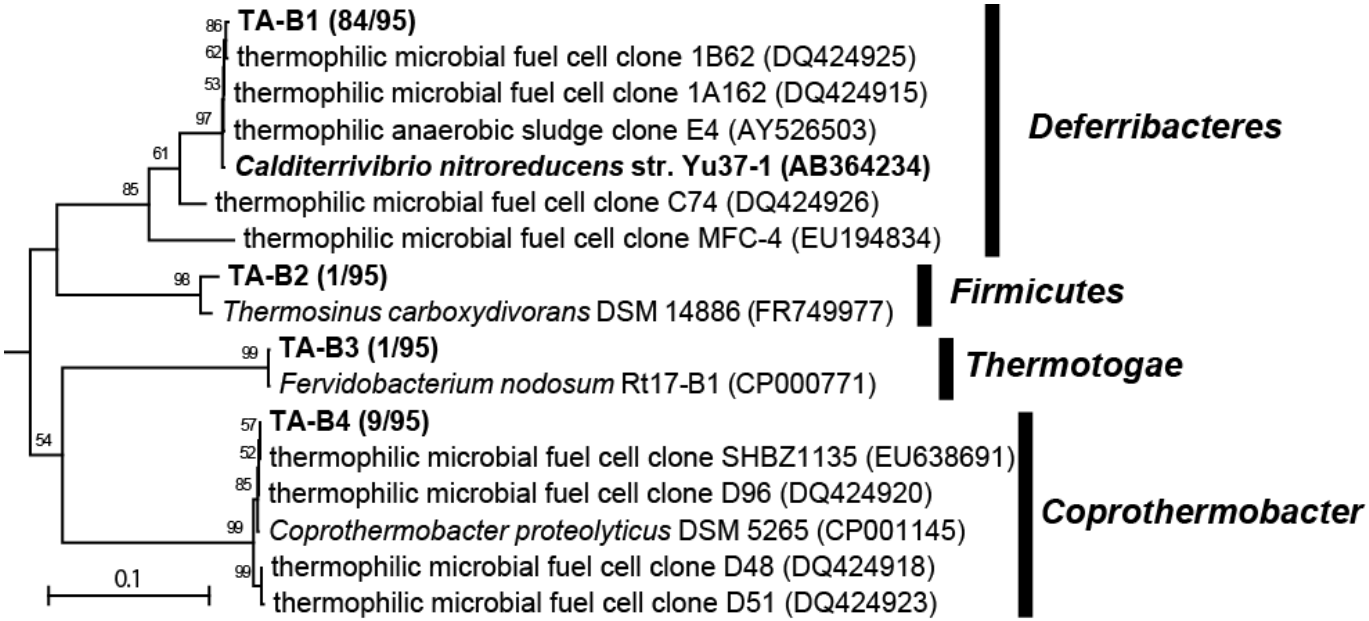


Figure 4.8 Phylogenetic tree illustrating the relationship of the phylotype TA-B1 detected in the thermophilic MFC anode relative to *C. nitroreducens* strain Yu37-1 and other 16S rRNA gene sequences within the *Deferribacteres* phylum. The trees were constructed using the neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≧50% are shown above the branches. The scale bar represents the number of changes per nucleotide position. *Thermotoga lettingae* strain TMO (CP000812.1) was used as the outgroup (not shown).

4.3.2.3 Electricity Generation by *C. nitroreducens* strain Yu37-1

To date, the *Calditerrivibrio* genus contains a single cultivated species, *C. nitroreducens* strain Yu37-1, which was originally isolated from hot-spring water (Iino *et al*. 2008). Nitrate is the only electron acceptor shown to be utilized by *C. nitroreducens* strain Yu37-1 (thus, the strain is nitrate reducer) (Iino *et al*. 2008). However, exoelectrogenic activity of the strain had never been investigated.

To determine whether *Calditerrivibrio*-affiliated bacteria was new thermophilic exoelectrogen, a pure culture of the strain was inoculated into MFCs. Because *C. nitroreducens* strain Yu37-1 is a strictly anaerobic bacterium that could not grow under microaerobic conditions (Iino *et al*. 2008), the anodic medium was supplemented with a low concentration (0.05 g/l) of Na_2S $9H_2O$ during the initial three fed-batch cycles to support anodic bacterial colonization. After the third cycle, the reducing agent was omitted from the medium, instead anaerobic conditions were maintained by continuous sparging of the medium with N_2/CO_2 (80/20). Thus, the medium did not contain possible exogenous redox mediators (such as reducing agents, vitamins, and resazurin).

For more than 400 h of incubation, a current of *ca.* 2.5 mA was sustainably generated (Fig. 4.9A), indicating that the *C. nitroreducens* strain Yu37-1 can generate electricity in MFC without requiring exogenous mediator compounds. Each time the medium was replaced with fresh medium, the current level generated by MFC immediately recovered to the original level, suggesting that the electricity generation was caused by the microorganisms attached to the anode rather than to planktonic cells or soluble mediators in the medium. Moreover, current generation by the *C. nitroreducens* strain Yu37-1 was strongly inhibited by 20 mM $NaNO_3$ (Fig. 4.9B). The current level recovered immediately when the medium was replaced with fresh media without nitrate. No inhibition was observed when the same amount of anoxic distilled water was injected (data not shown). Such nitrate inhibition of electricity generation had been reported in MFC inoculated with the *Comamonas denitrificans* strain DX-4, a mesophilic

nitrate-reducing exoelectrogen (Xing *et al*. 2010). These results indicated that the *C. nitroreducens* strain Yu 37-1 is a new thermophilic exoelectrogen.

The polarization curve and power density curve of the MFC inoculated with pure culture was shown in Fig. 4.10. It can be seen that the open circuit and maximum power density of the MFC was around 0.66 V and 274 mW m^{-2}. The polarization curve showed a linear relationship with the current when the current was lower than 3 mA, suggesting that the ohmic resistance was dominant resistance at the low current region. On the other hand, when the current was higher than 3.5 mA, the voltage of the MFC decreased drastically with the increasing current, suggesting that the mass transfer limitation was the dominating factor limiting the performance of MFC.

Moreover, as previously reported with mesophilic exoelectrogens (Nevin *et al*. 2008), the maximum current produced by *C. nitroreducens str*. Yu 37-1 was lower than that of the mixed consortia (823 mW m^{-2}). This is probably due to the lack of the supporting features of biofilm and/or because the strain is not an actual isolate from the MFC. Yet, the current-generating activity of *C. nitroreducens* Yu37-1 was comparable to those of the known thermophilic exoelectrogens, *T. potens* strain JR and *T. ferriacetia* (Marshall and May 2009; Mathis *et al*. 2008; Wrighton *et al*. 2008).

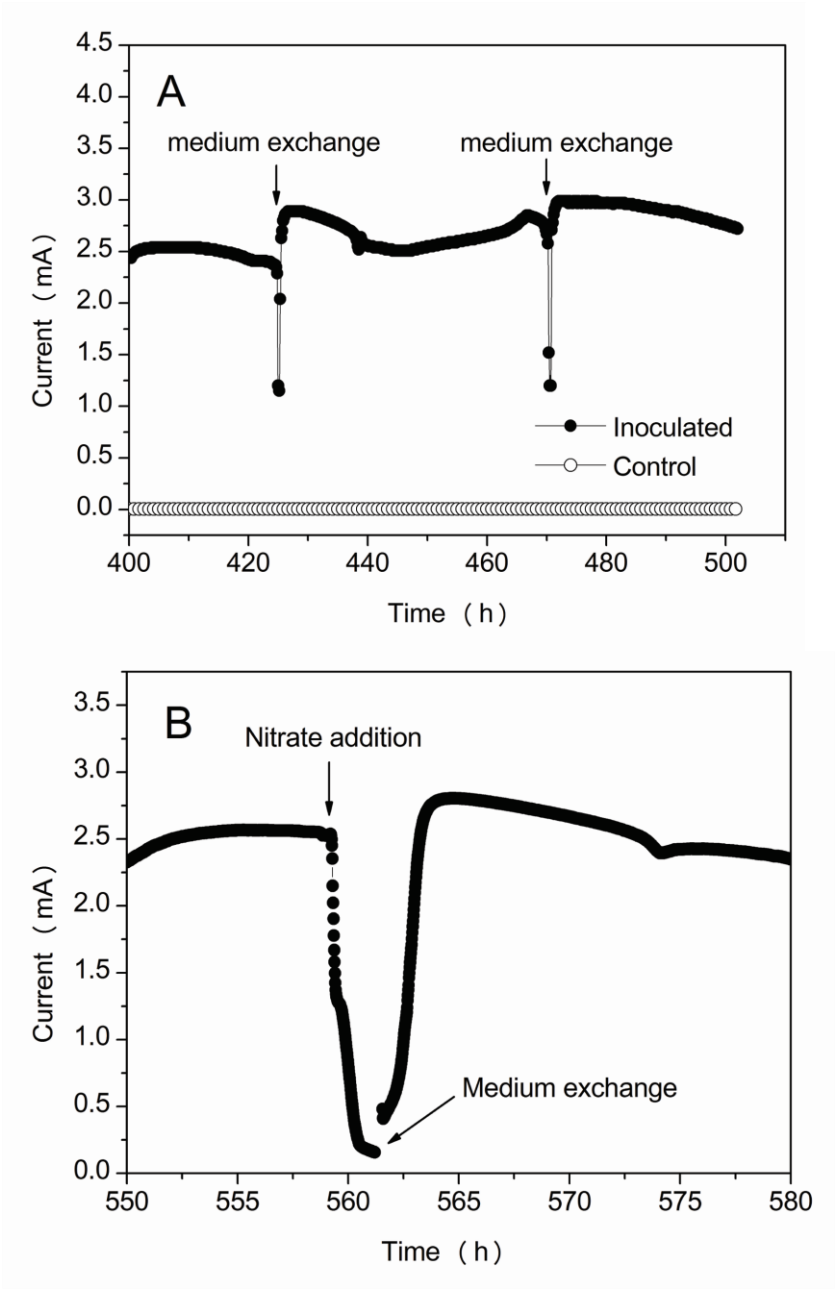


Figure 4.9 (A) Electricity generation by the pure-cultured *C. nitroreducens* strain Yu37-1after 400 hours incubation; (B) The effect of nitrate on the electricity generation of pure culture.

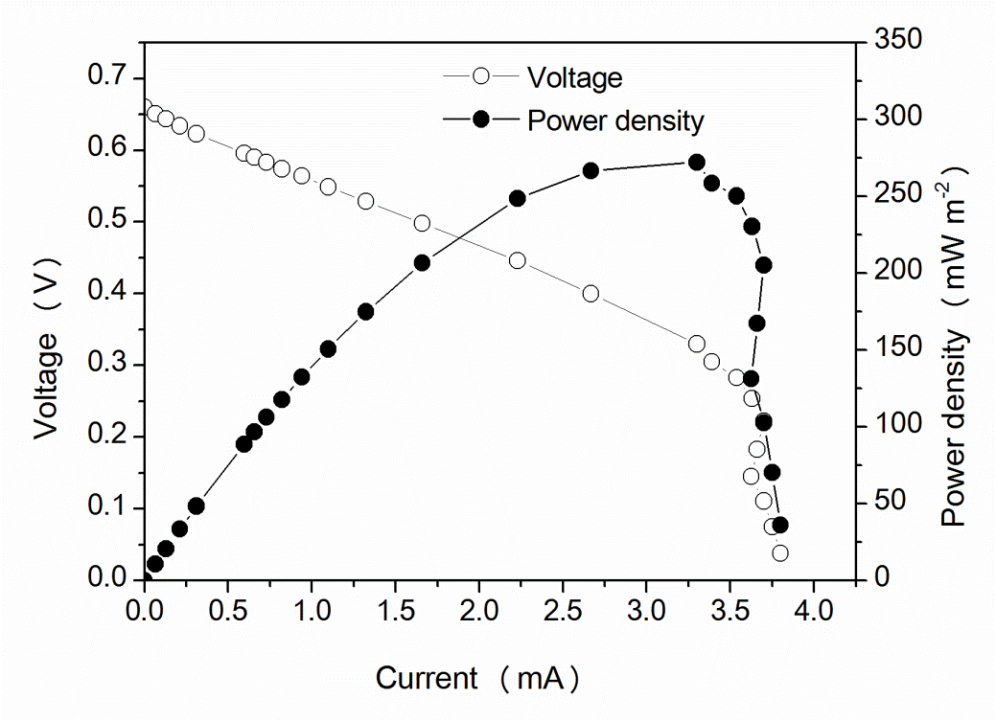


Figure 4.10 The performance of thermophilic MFC inoculated with pure culture *C. nitroreducens* Yu37-1

4.3.2.4 Cyclic Voltammetry Analyses

To investigate the mechanism of electron transfer between the anode and thermophilic microorganisms attached on the electrode surface, cyclic voltammetry (CV) was performed on the themophilic MFCs inoculated with mixed culture and pure culture, respectively. Both the thermophilic MFCs were incubated for more than 400 hours and produced stable currents. Representative turnover cyclic voltammogram of the anode inoculated with mixed culture was shown in Fig. 4.11A. The voltammogram showed a typical catalytic wave with a midpoint potential near –0.135 V, which can be identified in the first derivative plot of the voltammogram (Fig. 4.11B). The CV of non-inoculated control reactor (Fig. 4.11A) and spent medium of thermophilic MFC (data was not shown) were also measured. No catalytic current was produced in the CV of non-inoculated control reactor and spent medium, thus, it can be concluded that there was no apperent mediator in the spent medium and the current generted by the bio-anode was mainly due to the microorganisms on the anode.

In the presence of electron donors, the catalytic current of CV represents multiple turnovers of each redox species, and the high catalytic current may obscure signals from individual redox species (Marsili *et al*. 2010). Therefore, to investigate individual redox species, non-turnover CV of the thermophilic MFC anode in the absence of substrate was also performed. A representative non-turnover cyclic voltammogram at a slow scan rate of 1 mV s^{-1} was shown in Fig. 4.12A. Four major reversible peaks (respectively centered around –0.38 V, –0.22 V, –0.14 V and 0.14 V) were distinguished in the voltammogram (marked as E_1, E_2, E_3, and E_4). Based on the heright of peak current, the system E_2 and E_3 appeared to contribute more to the electricity generation than system E_1 and E_4. By calculating the first derivative of the turnover CV, the formal potenial of electron-transfer sites involved in the current generation were around –0.22 V and –0.135 V (which were shown as a small peak and major peak in the first derivative plot, respectively). As the formal potenial of electron-transfer sites revealed in the first derivative plot

were similar to that of system E_2 (–0.22 V) and system E_3 (–0.14 V) in the non-turnover voltammogram, it can be concluded that system E_3 is the main redox system functioning in the bio-anode inoculated with mixed culture, while E_2 is a secondary redox system.

Additionally, the catalytic votammetric behaviour showed a strong dependence on the scan rate. When the scan rate increased to 10 mV s^{-1}, the system 2 and 3 overlaped together, appeared as only one redox peak ($E_{2,3}$), while system 4 also became obscure (Fig. 4.12B). Fig. 4.13A and B depict the dependency of peak current of E_3 on the scan rate (v) and the root square of scan rate ($v^{1/2}$), respectively. The plots showed that peak current was linear up to a threshold scan rate of 10 mV s^{-1}, suggesting a thin film behavior. At scan rate faster than 10 mV s^{-1}, however, the peak current was proportional to the $v^{1/2}$, indicating a diffusion controlled regime. This bimodal behavior was also found in the CV of wild type *Geobacter sulfurreducens*, which was due to the confinement of electron transfer mediators in biofilm, whether bound (insoluble) or unbound (soluble) (Richter *et al*. 2009). We speculated that it was the same reason in our study. This kind of bimodal behavior not only indicated the increasing importance of charge-balancing ion-transfer at higher scan rates, but also showed the complexity of the mass-transfer conditions, even when studing the archetypal direct electron transfer (Harnisch and Freguia 2012). All of the four reversible peaks were reproduced each time immediately after medium exchange with fresh acetate-free medium. None of the redox peak was obtained in the spent medium (data was not shown) or fresh medium with a pre-sterilized electrode, eliminating the possibility of the contribution of extracellular mediators. Thus, it can be concluded that all redox peaks observed in CV were due to redox compounds in the biofilm on the anode surface.

The cyclic voltammetry of MFC inoculated with *C. nitroreducens* Yu37-1 was also performed, which was shown in Fig. 4.13. Similar to the turnover CV of sludge-derived biofilm, the turnover CV of pure culture derived biofilm (Fig. 4.11A) also showed a typical catalytic behavior. The first derivative analysis (inserted in

Fig. 4.11A) proved that the redox system was at around –0.17 V, which was consistent with the position of redox system in the non-turnover CV (marked as system E_c in Fig. 4.11B). In contrast to the sludge-derived biofilm, the peak current of system E_c was proportional to the scan rate (Fig. 4.11C). This relationship revealed a typical thin film behavior, indicating that the rate of any electron transfer to the cell-electrode interface (i.e. from the cell interior to the cell surface) was faster than the rate of interfacial electron transfer (i.e. from cell surface to electrode surface) (Armstrong *et al*. 2000; Baron *et al*. 2009; Strycharz *et al*. 2011). The reversible peak with the thin-film behavior was reproduced immediately after changing medium with fresh acetate-free medium. Additionally, no redox peak was obtained in the spent medium or fresh medium with a pre-sterilized electrode, eliminating the possibility of the contribution of extracellular mediators. Thus, it can be concluded that *C. nitroreducens* Yu37-1 has the exoelectrogenic activity and the exoelectrogenic ability was mainly due to redox compound which centered around –0.17 V on the anode surface.

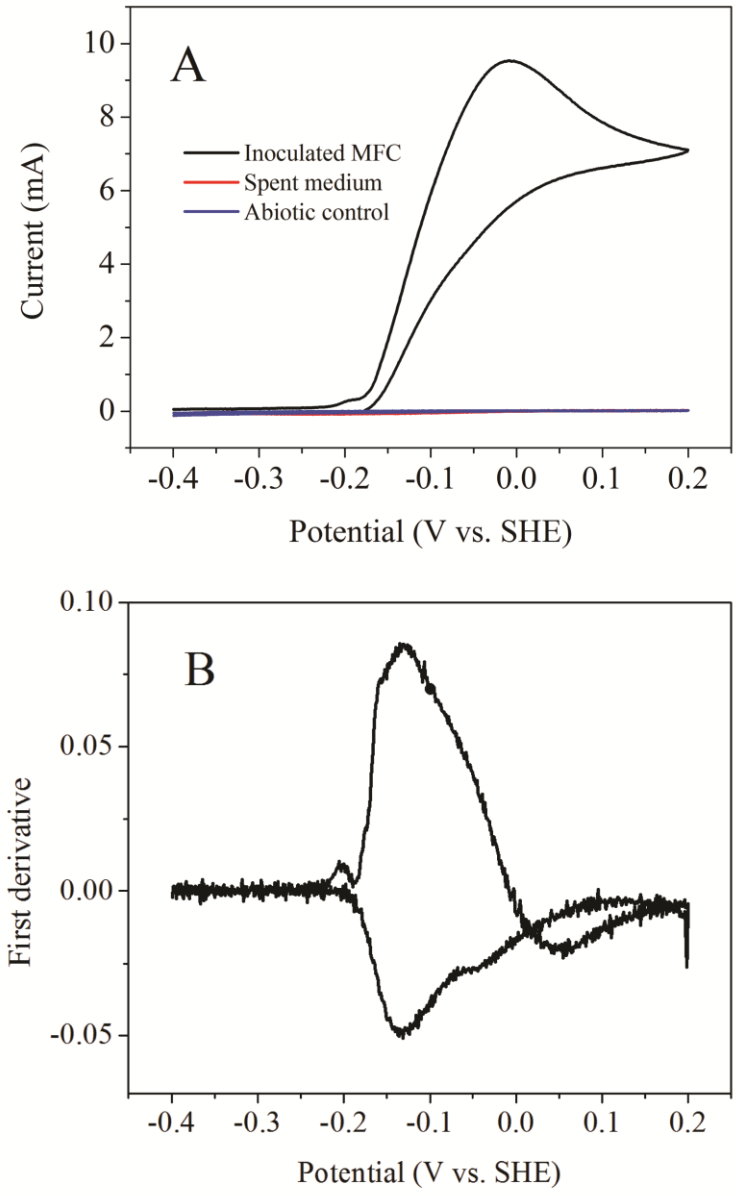


Figure 4.11 Turnover CV of the thermophilic bioanode (scan rate: 1 mV s^{-1}); (B) the first derivative of the turnover CV of the thermophilic bioanode

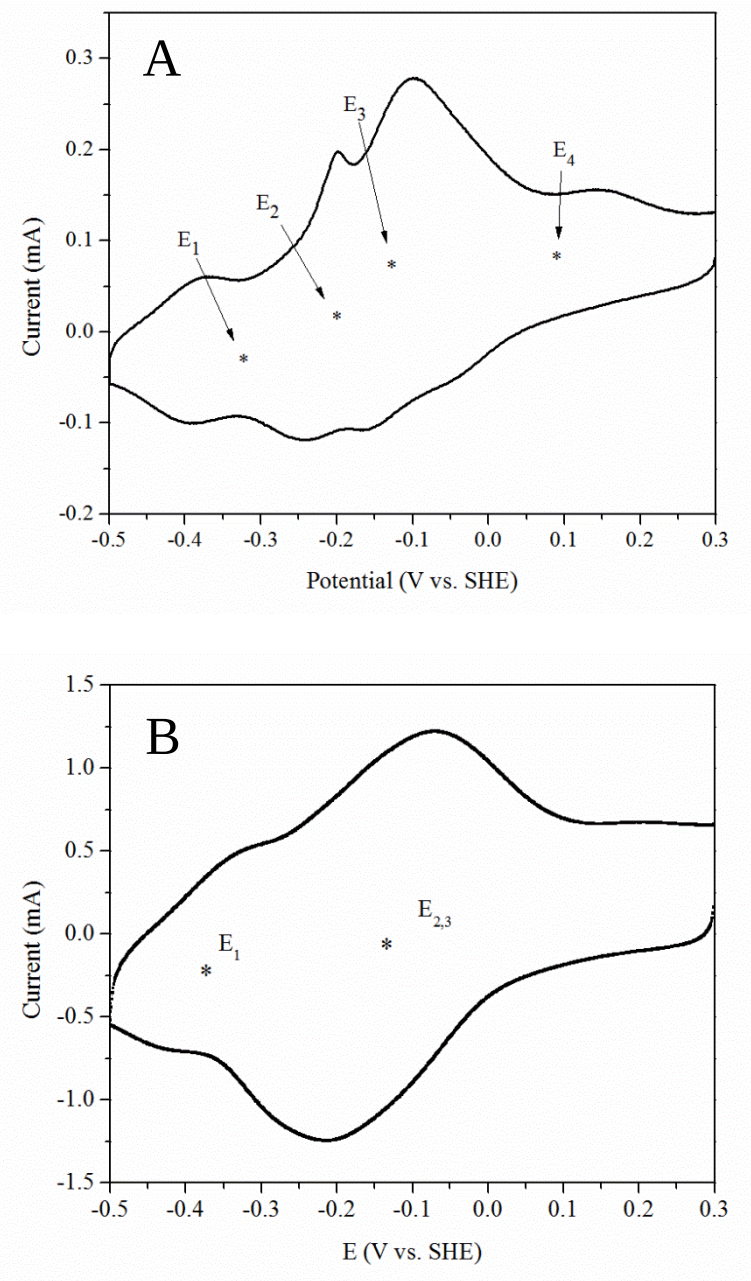


Figure 4.12 Non-turnover CV of the thermophilic bioanode at different scan rate: (A) 1 mV s^{-1}, (B) 10 mV s^{-1}

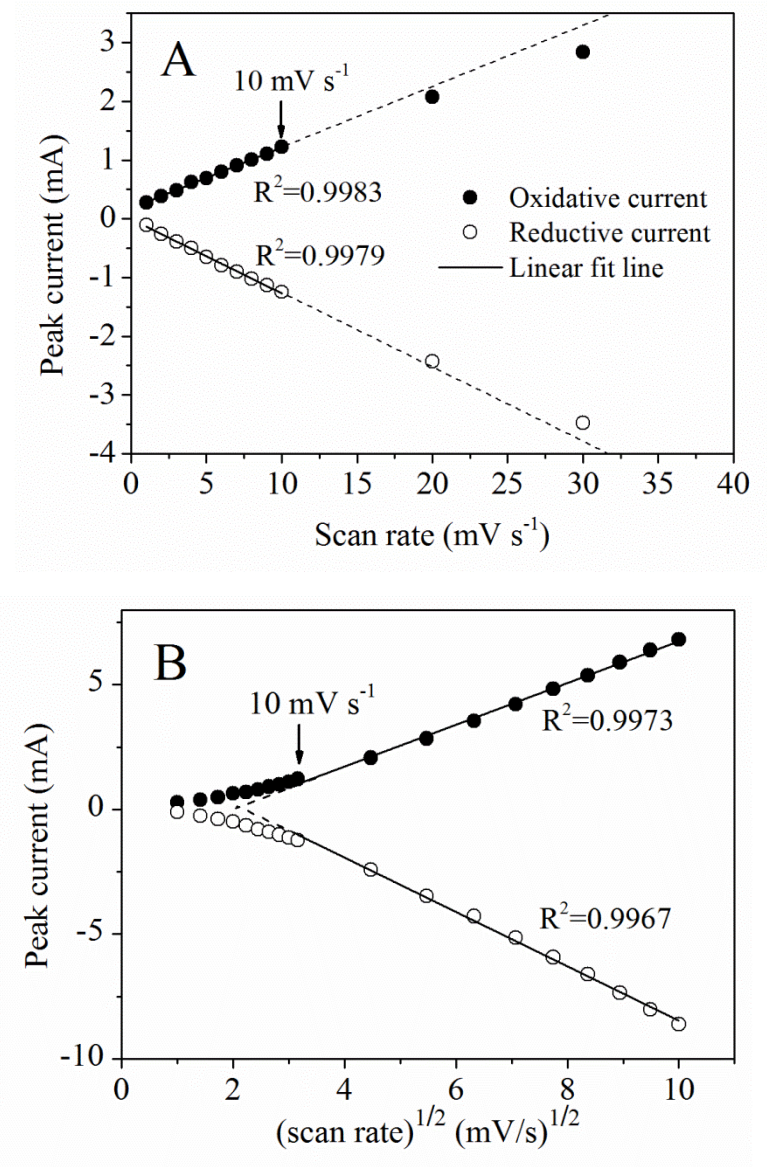


Figure 4.13 Scan rate analysis of system E_3 in the non-turnover CV of the thermophilic bioanode: (A) Peak current as a function of scan rate; (B) Peak current as a function of the square root of scan rate.

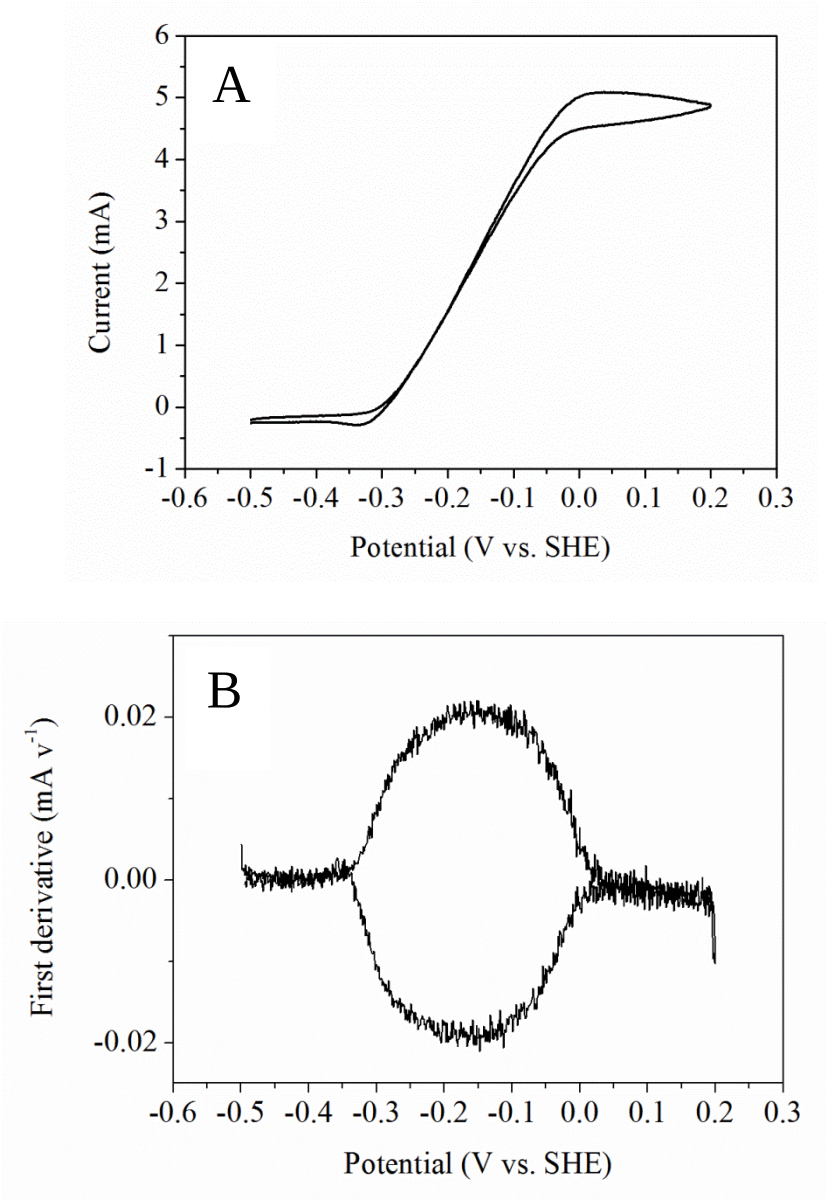


Figure 4.14 Cyclic voltammetric measurement of MFC inoculated with *C. nitroreducens* strain Yu37-1. (A) Turnover CV using scan rate of 1 mV s^{-1}; (B) the first derivative of the turnover CV.

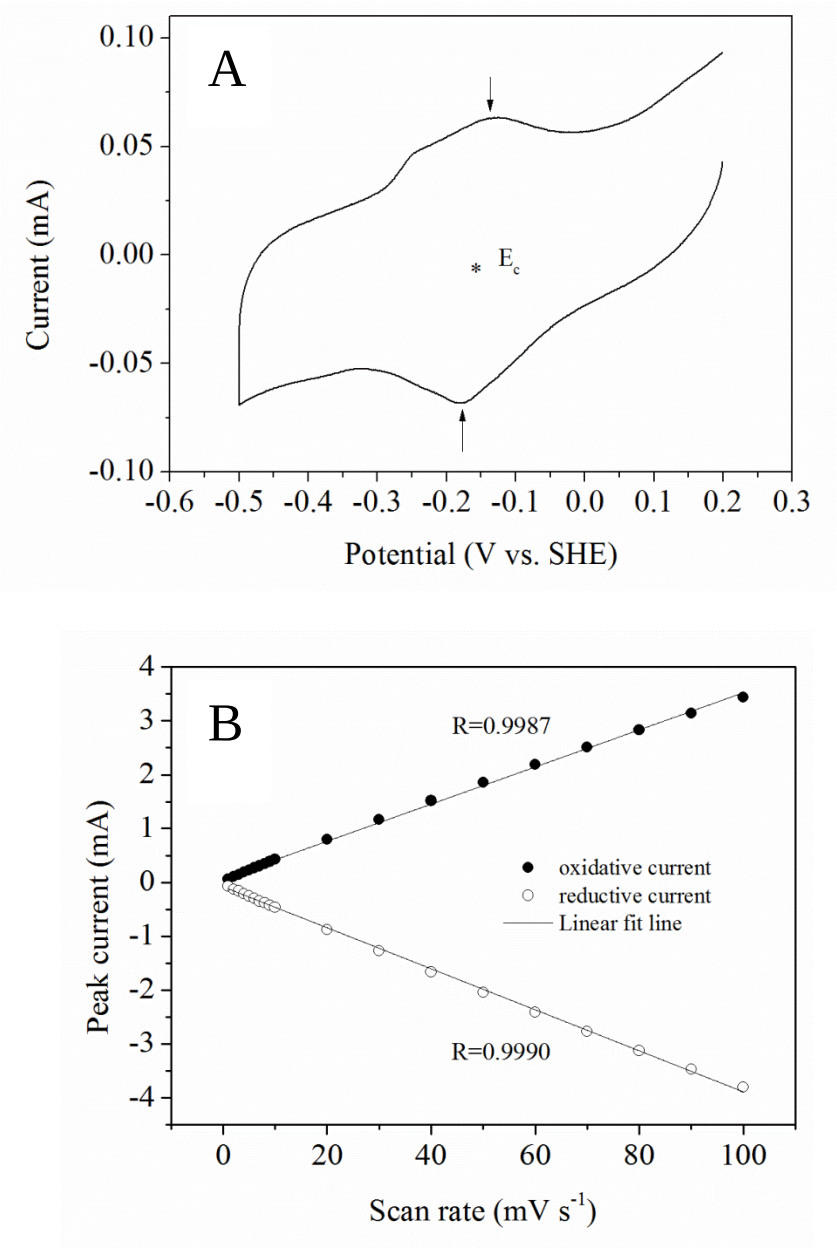


Figure 4.15 (A) non-turnover CV of MFC inoculated with *C. nitroreducens* strain Yu37-1., scan rate: 1 mV s^{-1}; (B) scan rate analysis of the non-turnover CV

4.3.2.5 Microscopic characterization of microorganisms on the anode surfaces

As reported in last section, the maximum current produced by *C. nitroreducens* strain Yu 37-1 was lower than that of the sludge-derived consortium. This difference was probably because the strain was not an actual isolate from the MFC and/or reflected the distinct physiological conditions in the microbial consortium and the pure culture. To get an deep insight into the electron transfer mechanisms between the microorganisms and anode, the morphologies of microorganisms on the anode surfaces were examined in the thermophilic MFCs inoculated with the sludge-originated microorganisms and *C. nitroreducens* strain Yu37-1, respectively, by scanning electron microscopy (SEM) (Fig. 4.16).

In the sludge-inoculated MFC, a dense biofilm was formed on the anode surface (Fig. 4.16C, D). The biofilm relatively uniformly consisted of vibroid-shaped cells, some of which were filamented as long as over 30 μm in length. These vibroid-shaped cells intertwined together and formed multilayered matrix with little extracellular polymeric substances. In the MFC inoculated with *C. nitroreducens* strain Yu37-1, however, vibroid-shaped cells sparsely attached on the anode surface, rather than in the form of biofilm (Fig. 4.16E, F). The cell density was significantly lower than that of the sludge-inoculated anode. This was probably because the pure culture was propagated exclusively in the defined medium, which contained limited concentration of acetate as the sole carbon source. On the other hand, the digestive sludge (inoculum) initially contained various organics as well as auxiliary microorganisms (such as fermentative bacteria), which could facilitate the biofilm development. The lower cell density of *C. nitroreducens* strain Yu37-1 on the anode likely reflected the current-generating activity of the pure-culture inoculated MFC, which is relatively lower than that of the sludge-inoculated MFC.

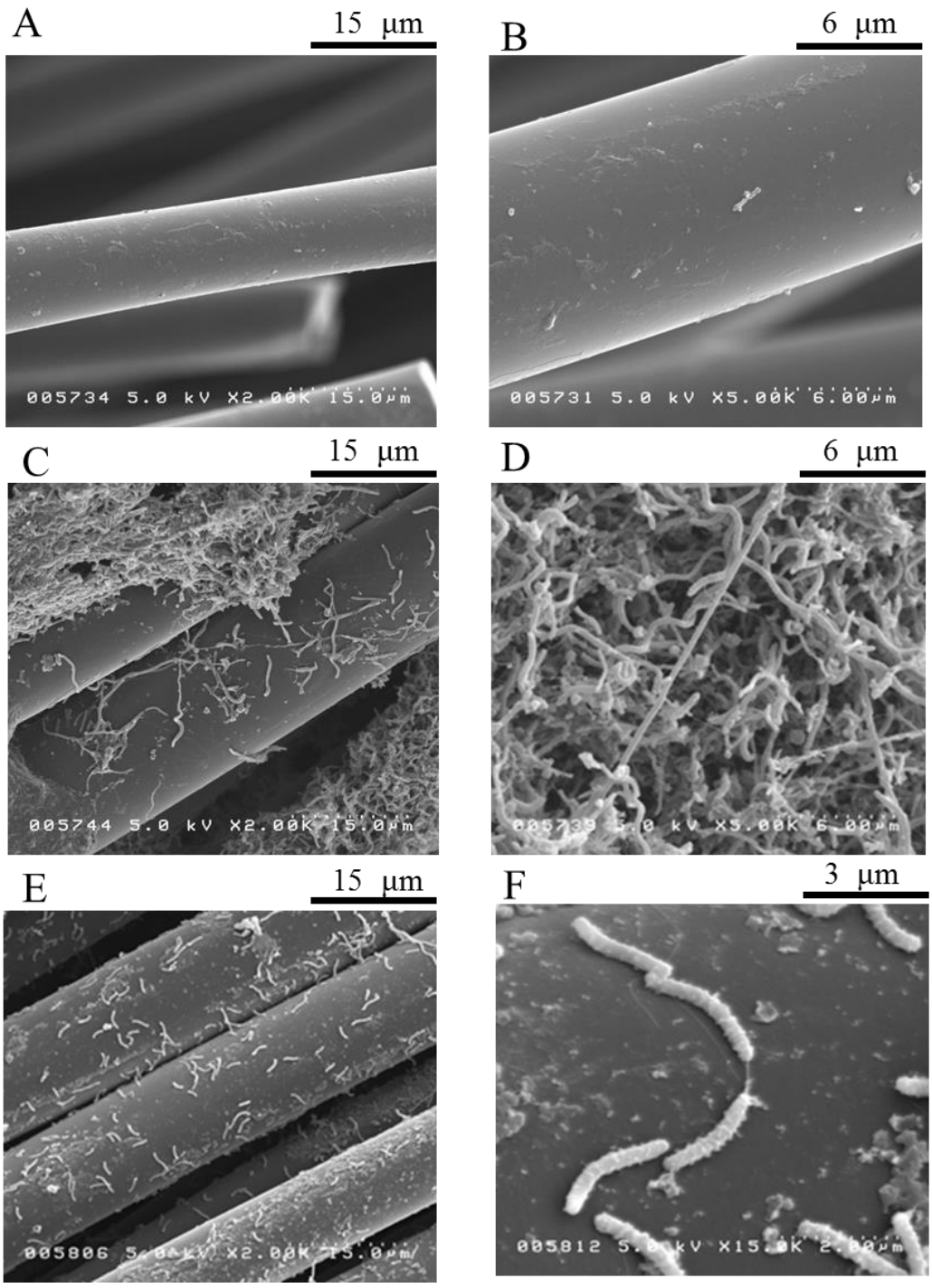


Figure 4.16 Scanning electron microscopy of: (A) (B): control electrode; (C) (D): sludge-inoculated bioanode; (E) (F): pure culture inoculated bioanode

4.3.3 MFC Inoculated with Thermophilic Microorganisms from Formation Water

As we know, the environments in petroleum reservoirs are usually high temperature (higher than 40℃) and anoxic, thus, are appropriate for anaerobic microorganisms. Basically, there are four major anaerobic metabolic groups microorganisms in the natural habitats of petroleum reservoir: iron (III)-reducing bacteria, sulfate-reducing bacteria, methanogens and acetogens (Zinder 1993). In habitats where the electron donors is limited, iron (III)-reducing bacteria outcompete other microorganisms if their electron acceptor is present. As we know, most of the exoelectrogens reported until now belong to iron (III)-reducing bacteria. Therefore, formation water from the petroleum reservoir may be a good source for thermophilic exoelectrogen.

4.3.3.1 Electricity Generation

Fig. 4.17A showed the electricity generation of thermophilic MFC inoculated with microorganisms from formation water and the non-inoculated control reactor. It can be seen that no electricity generation was observed in the non-inoculated control reactor during the experiment. For the inoculated reactor, the current was nearly zero until 25 hours post inoculation, and then it began to increase slowly. From 30 h post inoculation, the current of the inoculated reactor began to increase drastically in an exponential manner and reached to the maximum value of 2.75 mA at 70 h. The exponentially-increased current generation was mainly due to the exponential growth of electrochemical-active microorganisms attached on the anode. The current stably remained at the high level for 30 h and then began to decrease gradually, probably due to the consumption of electron donor (i.e. acetate). When the current was reduced to *ca.* 1.0 mA, both the anolyte and catholyte were exchanged with fresh solutions. The current generation recovered to the original or even higher level in several hours each time after the medium replacement. During the medium exchange, planktonic microbial cells and endogenous electron shuttling

mediators (if present) in the anode chamber was thrown out with the spent medium. Thus, we concluded that the electricity generation of the MFC reactors was mainly attributed to the microorganisms attached on the anode surface rather than the planktonic cells in the media.

Fig. 4.17B plotted the polarization and power density curve of the thermophilic MFC. The open circuit potential and maximum power density of the thermophilic MFC were around 0.76 V and 1003 mW m^{-2} (subjected to anode surface). The polarization curve showed that the voltage decreased linearly with the increasing current when the current was lower than 5.5 mA, indicating that ohmic resistance was the main rate limiting factor. Based on the slope of the polarization curve, the ohmic resistance was around 71.4 Ω. When the current was higher than 5.5 mA, the voltage decreased drastically with the increasing current, representing that the mass transfer limtation from bulk medium to anode was the controlling factor at high current density. The maximum power density was around 1003 m^{-2}, higher than those reported with thermophilic MFCs in several previous studies (generally ≦ 400 mW m^{-2}) (Carver *et al*. 2011; Mathis *et al*. 2008; Wrighton *et al*. 2008) and comparable to that of a thermophilic MFC under continuous mode of operation (1030 ± 340 mW m^{-2}) (Jong *et al*. 2006). However, it should be noticed that the MFC reactor used in this study was operated in a fed-batch mode and had a relatively high internal resistance which was due to the large electrode spacing. Overall, we concluded that microorganisms with high exoelectrogenic activity were successfully enriched on the bio-anode.

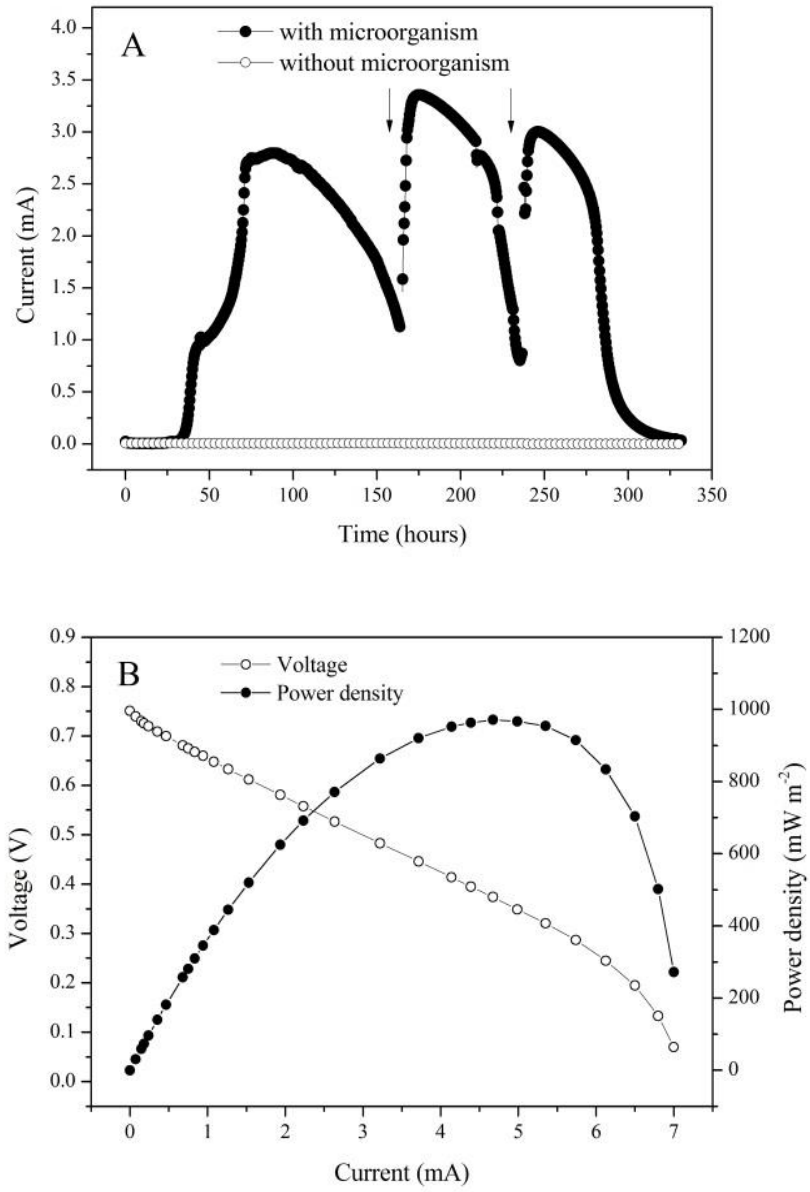


Figure 4.17 (A) electricity generation by thermophilic MFC inoculated with formation water sample (arrow represent medium exchange); (B) polarization and power density curve of the thermophilic MFC.

4.3.3.2 Electrochemical Analyses

Based on the medium replacement experiments, the electron transfer mechanism between the microorganisms and the anode was probably in a direct manner, as the current of MFC could recover to the origianl level immediately after changing fresh medium. To further investigate the electron transfer mechanism, cyclic voltammetry (CV) was performed on a themophilic MFC stably producing current (*ca.* 400 hours post inoculation).

Fig. 4.18A showed representative cyclic voltammograms of the bio-anode and the spent medium in the presence of acetate (turnover conditions). The voltammogram of the bio-anode showed a typical catalytic wave (sigmoidal behavior). The current was around zero at the starting potential, and then began to increase gradually with the increasing potential, suggesting microbes on the bio-anode began to oxidize acetate and transfer electrons to the anode. The current increased sharply when the working potential increased higher than –0.15 V vs. SHE until 0.05 V. This was because the capacity of the anodic microbes to generate electrons was in excess, while their capacity to transfer electrons to the anode (kinetics at the interface) is limited. As the interfacial reactions can be accelerated by increases in driving force (described by Butler-Volmer equation) and the enzymatic reactions have a characteristic maximum rate, the current got a plateau when the working potential was higher than 0.05 V. Fig. 4.15B showed the first derivative analysis of the voltammogram of the bio-anode as a function of potential. Based on the first derivative analysis of the voltammogram, two catalytic sites were identified by the reversible peaks (which one located *ca.* –0.18 V vs. SHE and the other one located *ca.* –0.8 V vs. SHE). As no any catalytic current was observed in the CV of the spent medium, it suggested that there was no soluble electron shuttle in the spent medium. Thus, we concluded that the electricity generation was due to the microorganisms on the anode in a direct electron-transferring manner.

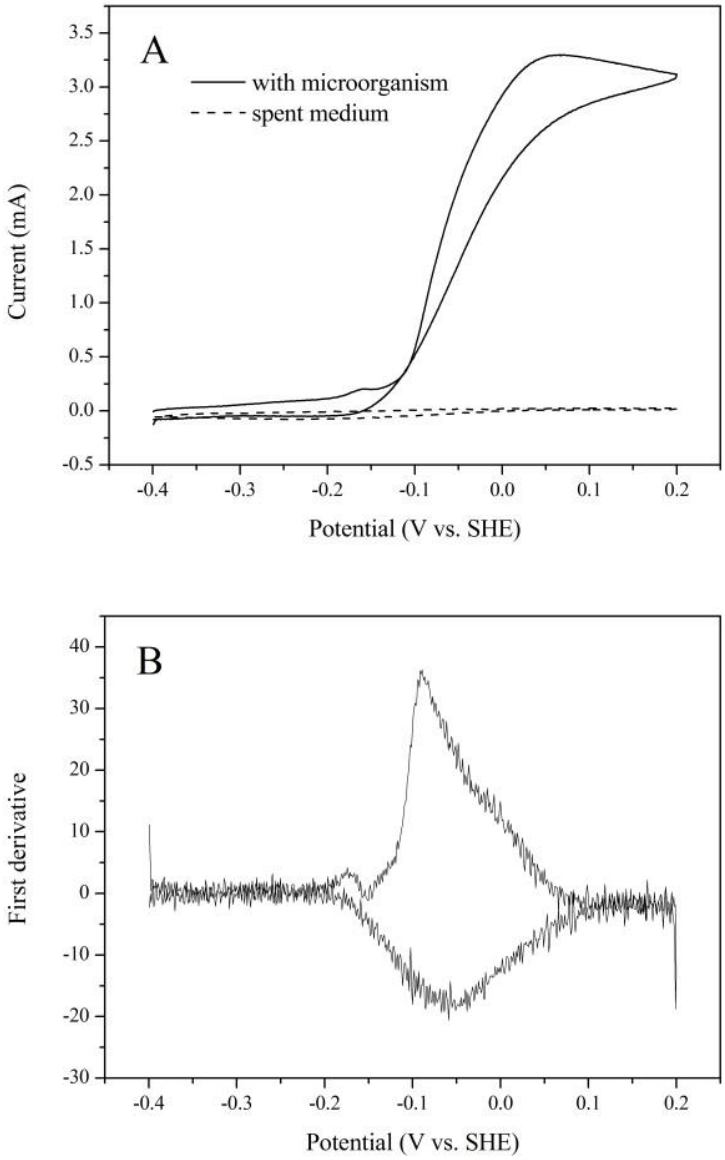


Figure 4.18. (A) turnover cyclic voltammetry of thermophlic MFC; (B) first derivative analysis of turnover cyclic voltammetry

In the presence of electron donors, the catalytic current of CV represents multiple turnovers of each redox species, and the high catalytic current may obscure signals from individual redox species (Fricke *et al*. 2008; Richter *et al*. 2009). Therefore, to investigate individual redox species, non-turnover CV of the thermophilic-MFC anode in the absence of substrate was also performed. A representative non-turnover cyclic voltammogram at a slow scan rate of 1 mV s^{-1} was shown in Fig. 4.19A. One reversible peak centered around –0.1 V vs. SHE was observed in the non-turnover cyclic voltammogram. It represented that a redox site centered around –0.1 V vs. SHE. Fig. 4.19B showed that the peak current of the redox site was proportional to the scan rate, indicating a thin-film behavior (Richter *et al*. 2009). This behavior suggested that the interfacial reaction (the final hop of electrons from redox poteins to anodes) was lower than the reactions responsible for bringing electrons through the biofilms to this interface. No redox peak was obtained in the spent medium (data was not shown) or fresh medium with a pre-sterilized electrode, eliminating the possibility of the contribution of extracellular electron shuttles.

The reversible peak with thin-film behavior can be reproduced each time immediately after medium exchange with fresh acetate-free medium. In addition, the position of the reversible peak with thin-film behavior (*ca.* –0.1 V vs. SHE) was nearly consistant with the position of midpoint potential of the turnover voltammogram (*ca.* –0.08V vs. SHE). Thus, it can be concluded the redox peaks observed in CV were due to redox compounds, such as cytochromes, associated with thermophilic microorganisms on the anode surface.

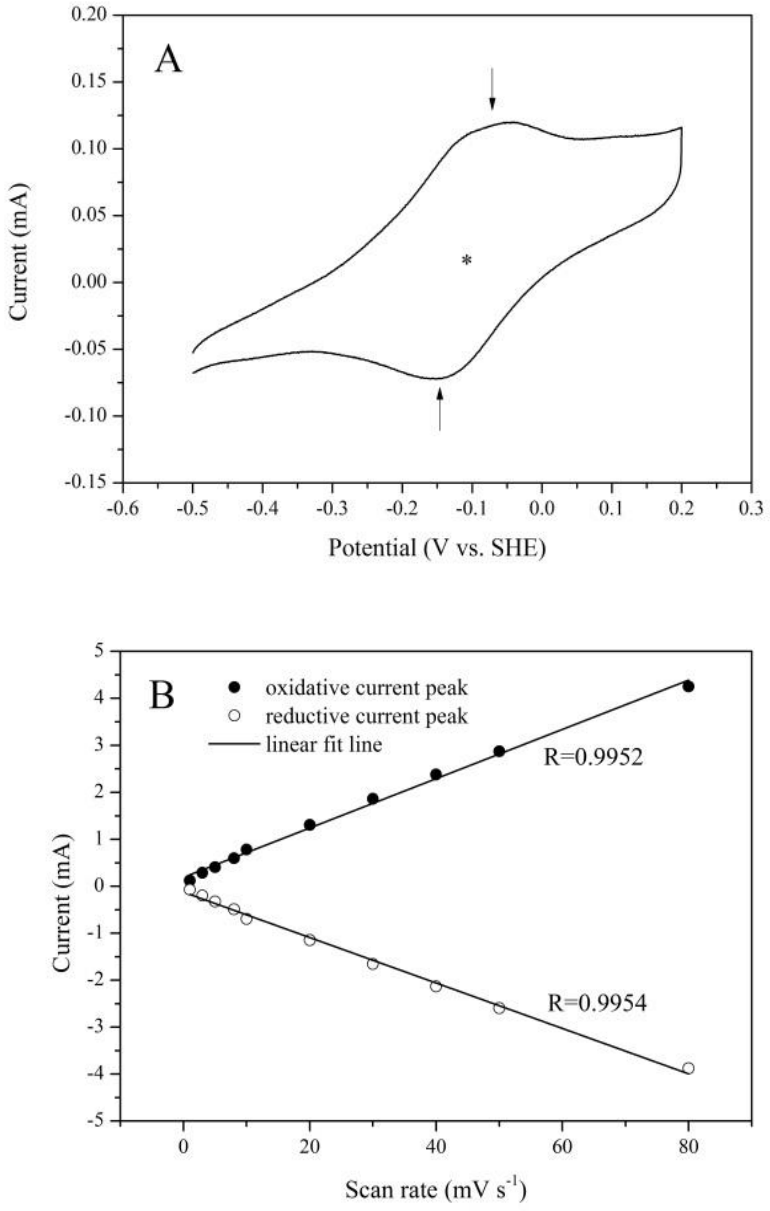


Figure 4.19 (A) non-turnover cyclic voltammetry of thermophlic MFC; (B) the relationship between current peaks and scan rate

4.3.3.3 Microbial Population Analysis

The microbial community on the bio-anode surface was analyzed by construcing 16S rRNA gene clone library (Fig. 4.20). All of the clones (67 clones) analyzed so far belonged to *Firmicutes*. No *Proteobacteria*, which was usually abundant in the anodic microbial community of mesophilic MFC, was detected. Among the related sequences, the clones OR-TA-B3 was the dominating species, which was closely related to *Anoxybacillus* sp. strain DR02, suggesting its possible contribution to the electricity generation of the MFC. However, this species had never been detected in previous studies of MFC and its electrochemical activity remained to be examined. On the other hand, a phylotype closely affiliated to the genus *Thermincola*, *T. carboxydophla*, was also detected in the anodic microbial community. So far two *Thermincola*-related species were shown to be capable of electricity generation, *T. potens* JR and *T. feriacetica* (Marshall and May 2009; Wrighton *et al*. 2008). Therefore, *T. carboxydophla* is also likely an exoelectrogen, which needs to be further verified.

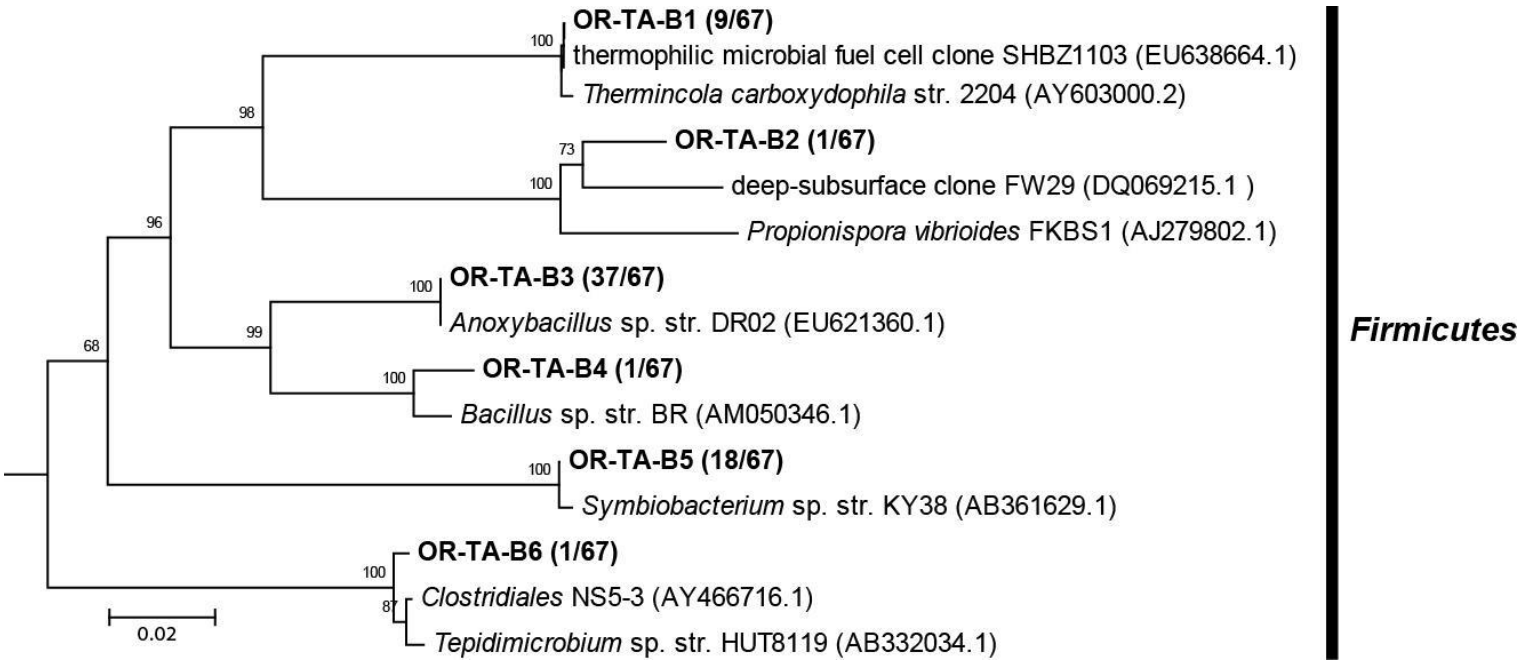


Figure 4.20. Phylogenetic trees of the bacterial 16S rRNA gene sequences obtained from the bio-anode of the thermophilic MFC. The tree was constructed using the neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≧50% are shown above the branches. The scale bars represent the number of changes per nucleotide position. *Methanococcus maripaludis* strain S2 (BX957219.1) was used as an out group (not shown). Numbers of clones representing each phylotype / numbers of total clones in each library are shown in parentheses.

4.3.4 Hyperthermophilic MFC

4.3.4.1 Electricity Generation at 80℃

The MFC operated at 80℃ produced electricity soon after inoculating with the microorganisms indigenous to the formation water from a high-temperature petroleum reservoir (Fig. 4.21). The current produced by the MFC increased to near 0.5 mA in few hours post inoculation. The current began to increase in an exponential manner at 25 h and reached the first maximum value of 1.3 mA at 45 h. Such exponential increase in current generation was typically due to the exponential growth of exoelectrogenic microorganisms on the anode surface. The current of the MFC stably sustained at the maximum level for around 10 hours and then began to decrease gradually, probably due to the consumption of electron donors. When the current dropped to around 0.5 mA, both the anolyte and catholyte were exchanged to fresh ones. The current recovered to the maximum level soon after the media exchange. Such phenomenon was observed each time after the media exchange. As the planktonic microbial cells were largely removed by the media exchange, it can be concluded that the electricity generation by the MFC was mainly attributed to the microorganisms attached on the anode surface. On the other hand, the non-inoculated control reactor only produced a low level of current which gradually decreased to nearly the background level (Fig. 4.21).

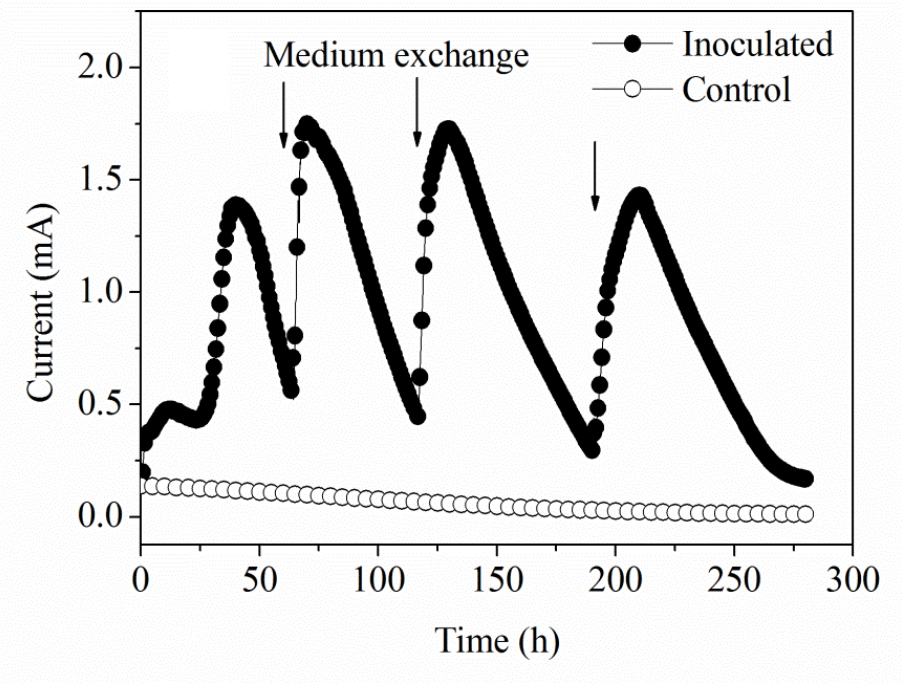


Figure 4.21 Startup curve of hyperthermophilic MFC operated at 80℃

4.3.4.2 Effect of Operating Temperatures on the MFC Performance

The effect of operating temperatures on the MFC performance was investigated (Fig. 4.22). In the range of 75℃ to 95℃, the maximum power density of MFC was improved with the increase in the operating temperature, from 41.0 mW m^{-2} at 75℃ to 165.3 mW m^{-2} at 95℃. At the operating temperature of 98℃, however, the maximum power density decreased to 156.3 mW m^{-2}. Correspondingly, the internal ohmic resistance decreased from 199 Ω at 75℃ to 80 Ω at 95℃ and then increased to 90 Ω at 98℃ (Table. 4.2).

In previous studies, no electricity generation in MFC had been reported above 90℃. The highest operating temperature of MFC so far reported was 75℃ with a thermophilic MFC inoculated with marine sediment (Mathis *et al*. 2008). However, although the MFC could produce a current density of *ca.* 50 mA m^{-2} at 75℃, the performance was optimal at 60℃ (Mathis *et al*. 2008). Thus, the MFC developed in this study represented the first example of "hyperthermophilic" MFC, in which the current generation was catalyzed by hyperthermophilic biocatalysts having the optimal activity at 95℃. The highest power density produced by the hyperthermophilic MFC (165.3 mW m^{-2} at 95℃) was comparable to those of thermophilic MFCs inoculated with marine sediment (207 mW m^{-2} at 60℃) (Mathis *et al*. 2008) and *T. ferriacetica* (146 mW m^{-2} at 60℃) (Marshall and May 2009). However, it should be noticed that, as this study was intended as a first proof-of-concept study of hyperthermophilic MFC, the reactor used here was not optimized for the performance. For example, no magnetic stirrer was used in our reactor, thus the performance may be limited by the diffusion of substrate from the bulk liquid to the electrode surface.

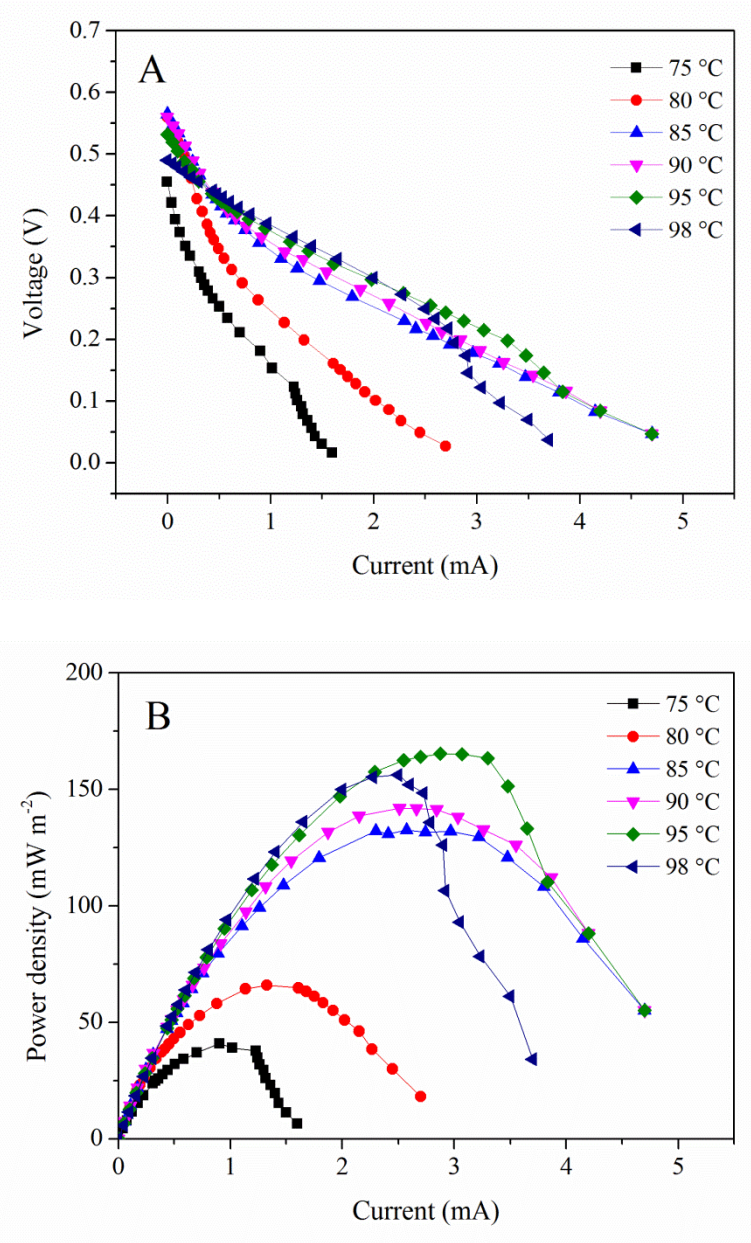


Figure 4.22 The polarization curve (A) and power density curve (B) as a function of current.

Table 4.2 Maximum power density and internal resistance of hyperthermophilic MFC at different temperatures.

Temperature (℃)	Power density (mW m^{-2})	Internal resistance (Ω)
75	41.0	199
80	66.0	138
85	132.3	84
90	141.9	83
95	165.4	80
98	156.3	90

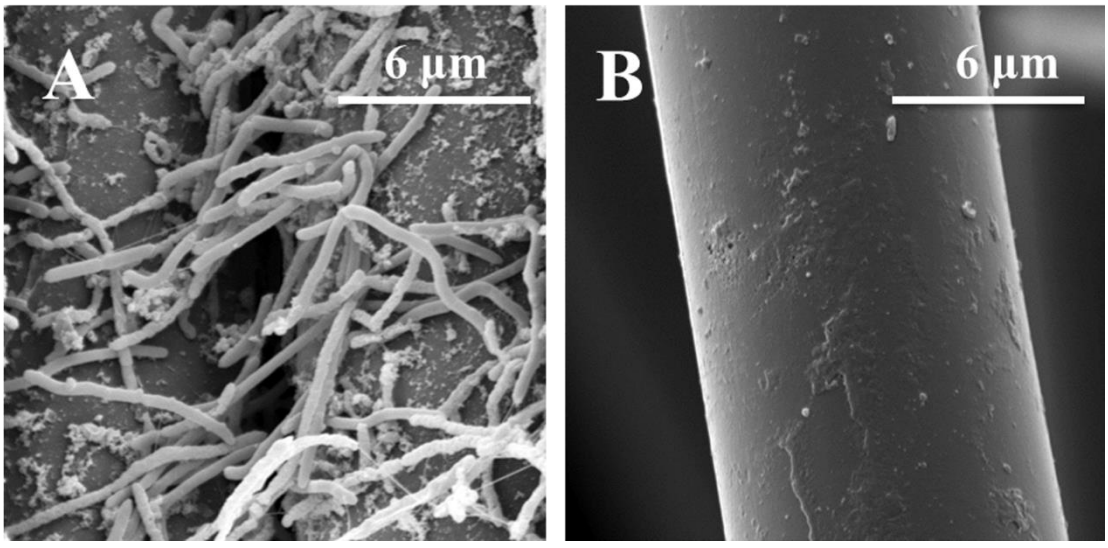


Figure 4.23 The scanning electron microscopy images of (A) hyperthermophilic bioanode and (B) control electrode.

4.3.4.3 SEM Analysis of the Anode Surface

The morphologies of microorganisms on the anode surface were examined at one month post inoculation by SEM, which was shown in Fig. 4.23). A mostly single-layered biofilm was observed on the inoculated anode surface. The microbial cells composing the biofilm were relatively homogeneous and filamentous shape, some of which were elongated as long as over 10 μm. In contrast, there was no microbial cell detected on the non-inoculated control electrode. Compared with the mesophilic biofilm in previous reports (Zhang *et al*. 2011), the biofilm of the hyperthermophilic MFC anode was relatively thinner and contained visibly no pili and extracellular matrix. This was probably the reason of the relative lower performance.

4.3.4.4 Molecular Phylogenetic Analysis of the Anodic Bacterial Population

The phylogenetic diversity of the bacterial community on the anode was analyzed by constructing 16S rRNA gene clone library (Fig. 4.24). The library (58 clones in totoal) contained three phylotypes belonging to two different phyla, *Firmicutes* (52 clones, 90% of clones analyzed) and *Thermodesulfobacteria* (6 clones, 10% of clones analyzed). The *Firmicutes*-affiliated phylotypes, HyTMFC-AB-1 and HyTMFC-AB-2, were closely related to *Caldanaerobacter subterraneus* (subspecies *subterraneus* and *tengcongensis*, respectively) which was isolated from thermophilic anaerobic environments (a petroleum reservoir and hot spring, respectively) (Fardeau *et al*. 2000; Xue *et al*. 2001). Both *C. subterraneus* subsp. *subterraneus* and *tengcongensis* are fermentative, producing acetate, H_2 and CO_2 as the products of glucose fermentation (Fardeau *et al*. 2000; Xue *et al*. 2001). The remaining phylotype HyTMFC-AB-3 was closely related to *Thermodesulfobacterium commune*, a hyperthermophilic dissimilatory sulfate-reducing bacterium using H_2 as the electron donor.

Interestingly, *T. commune* has the ability to reduce poorly crystalline Fe (III) oxide, thus the bacterium is also a dissimilatory metal-reducing bacteria (DMRB)

(Kashefi *et al*. 2002; Zeikus *et al*. 1983). It has been reported that many DMBR have the ability to extracellularly transfer electrons to a solid electrode, based on either direct contact by outer-membrane c-type cytochromes or indirectly via redox mediator(s) (Logan 2009). Indeed, *T. commune* contains c-type cytochromes (Hatchikian *et al*. 1984), which have been suggested to mediate the direct electron transfer to electrodes in *T. potens* strain JR (a thermophilic exoelectrogen) (Wrighton *et al*. 2011). The electrochemical study of c-type cytochrome in *T. commune* revealed that the cytochrome system behaved like a reversible system, in which four redox potential values at Eh1= –0.14V ± 0.010 V, Eh2 = Eh3 = Eh4 = –0.28 ± 0.010 V were determined (Hatchikian *et al*. 1984). Thus, in the hyperthermophilic MFC, current generation was possibly mediated by *T. commune* transferring electrons to the anode with H_2 as the electron donor, which was syntrophically produced by *C. subterraneous*-related species via glucose fermentation. This hypothesis will be examined in future study with hyperthermophilic MFC inoculated with co-cultures of isolated representatives.

4.4 Conclusion

In this chapter, to explore and identify novel thermophilic electrochenmcially-active microorganisms, two-chamber microbial fuel cells were built and inoculated with thermophilic microorganisms from various sources (thermophilic digestive sludge and formation water under different temperature) with different nutrient conditions. These inoculated MFCs started up successfully and showed substantial power density generation, suggesting that exoelectrogens were enriched in the anode chambers and the inoculums are good sources for thermophilic exoelectrogens.

The maximum power density of thermophilic MFC was obtained in a thermophilic MFC inoculated with Yabase oilfield formation water, which was around 1003 mW m^{-2}. This value was higher than those reported with thermophilic MFCs in several previous studies (usually lower than 450 mW m^{-2}) and comparable

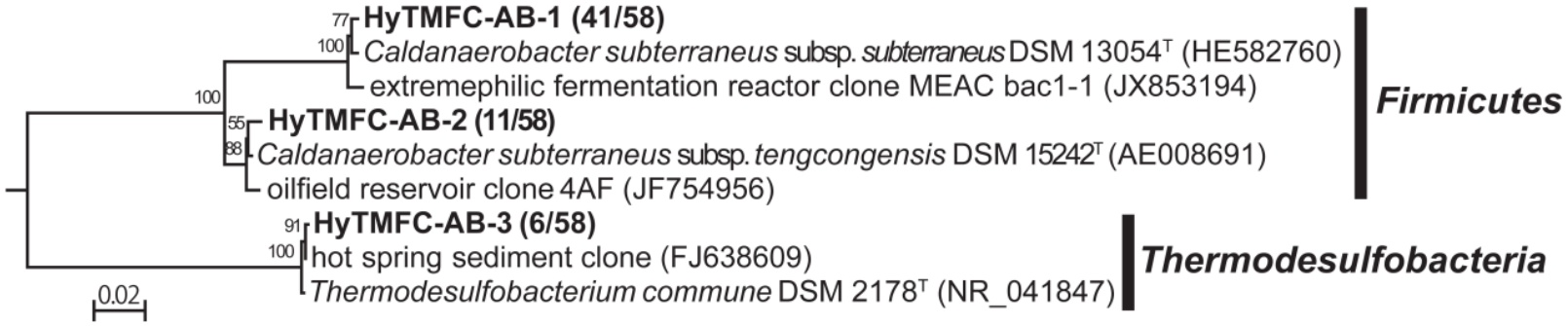


Figure 4.24 Phylogenetic tree illustrating the relationship of the phylotype detected in the hyperthermophilic bioanode. The trees were constructed using the neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≥50% are shown above the branches. The scale bar represents the number of changes per nucleotide position. Thermotoga lettingae strain TMO (CP000812.1) was used as the outgroup (not shown).

to that of a thermophilic MFC under continuous mode of operation (1030 ± 340 mW m^{-2}).

The electron transfer mechanisms between the electrochemically-active microorganisms and anodes were also investigated by using electrochemical methods (cyclic voltammetry). The results showed that all the electron transfer mechanisms on the bioanodes (except the hyperthermophilic bioanodes) were direct electron transfer. The MFC inoculated with thermophilic digestive without any exogenous mediators was extensively studied by non-turnover CV analyses. The result showed that there were four redox components on the outer membrane surface were observed, of which system E_3 centered around –0.14 V played the major functional role.

Two novel thermophilic exoelectrogens, *C. australicus* strain RC3 and *C. nitroreducens* Yu37-1, were tested in the experiment and proven to be capable of transferring electrons to anodes, largely expanding our knowledge of thermophilic exoelectrogens.

A hyperthermophilic MFC was successfully started up by inoculating the hyperthermophilic microorganisms from the produced water of an oilfield. As the hyperthermophilic MFC could operate at the elevated temperature range between 75℃ and 98℃, it has a potential application in industrial processes under extreme conditions. The current-generating mechanism and ability to utilize diverse substrates will be examined in future work.

Chapter 4

The contents in this chapter were published in four papers under the following reference:

Qian Fu, Hajime Kobayashi, Hideo Kawaguchi, Javier Vilcaez, Tatsuki Wakayama, Haruo Maeda, and Kozo Sato. Electrochemical and phylogenetic analyses of current-generating microorganisms in a thermophilic microbial fuel cell. *Journal of Bioscience and Bioengineering***,** 2012, 115 (3), 268-271.

Qian Fu, Hajime Kobayashi, Hideo Kawaguchi, Javier Vilcaez, Tatsuki Wakayama, Haruo Maeda, and Kozo Sato. *Calditerrivibrio nitroreducens*, a thermophilic Gram-positive nitrate-reducing bacterium, is capable of electricity generation. Environmental science and technology. (under review)

Qian Fu, Hajime Kobayashi, Hideo Kawaguchi, Javier Vilcaez, Kozo Sato. Identification of new microbial mediators for electromethanogenic reduction of geologically-stored carbon dioxide. *11th International Conference on Greenhouse Gas Control Technologies conference*, Kyoto, Japan, 2012.11.

Qian Fu, Hajime Kobayashi, Tatsuki Wakayama, Haruo Maeda and Kozo Sato. Electricity generation by a microbial fuel cell operated under hyperthermophilic conditions. (In preparation)

Chapter 5

Construction of a Thermophilic Biocathode Catalyzing Sustainable Hydrogen Production

5.1 Introduction

Microbial electrolysis cell (MEC) is an emerging technology for the production of H_2 from biodegradable materials (Harnisch and Schroder 2010; Lovley 2012; Rozendal *et al*. 2006; Rozendal *et al*. 2008a). In a typical MEC, exoelectrogenic microorganisms in the anode chamber oxidize organic matters and transfer electrons to an anode. The electrons pass through the circuit to the cathode where they combine with protons to produce H_2. However, the reaction in MEC is not spontaneous. Under standard conditions at pH 7, the equilibrium potential of H_2 production from protons is –0.42 V vs. SHE, and the anode potential of acetate oxidation by exoelectrogenic microorganisms is around –0.28 V vs. SHE (Rozendal *et al*. 2006). Thus, a small external voltage (theoretically, 0.14 V) is required to make the H_2 production possible in MEC (Rozendal *et al*. 2006). In practice, at least 0.22 V or larger are needed due to the overpotentials of the electrodes. Yet, this input is still substantially less than the average of 2.3 V required for water electrolysis (Call *et al*. 2009).

To reduce the overpotentials on the cathode, metal catalysts (e.g. platinum) are commonly used as the catalyst on the cathode. However, due to their high costs, instability and environmental toxicity, such precious metal catalysts are not practical for actual implementations. Viewed in this light, microbial biocathodes, in which microorganisms act as biocatalysts to catalyze cathodic reactions, are of particular interest as they are inexpensive, self-renewable and not susceptible to corrosion (Jeremiasse *et al*. 2010; Jeremiasse *et al*. 2012; Pisciotta *et al*. 2012; Rozendal *et al*. 2008b). Rozendal *et al*. developed the first biocathode for MEC based on the reversibility of hydrogenases (Rozendal *et al*. 2008b). In their study, an acetate- and H_2-oxidizing bioanode was firstly developed, and was then converted to an H_2-producing biocathode by reversing the polarity of the electrode. At –0.7 V vs. SHE, the "anode-converted" biocathode produced an average current of 1.1 A m^{-2} and 0.63 m^{-3} H_2 m^{-3} cathode liquid volume day^{-1}, significantly higher than that of the control electrode (Rozendal *et al*. 2008b).

In biocathodes, microbial biocatalysts, collectively called electrotrophs (Pisciotta *et al*. 2012), are thought to accept electrons directly from the cathode and use the electrons to produce H_2. Up to now, however, only few attempts have been made to characterize the microbial community of biocathodes as well as the mechanisms of microbial electron uptake from biocathodes (Croese *et al*. 2011). It has been suggested that hydrogenase is required to catalyze the conversion of electrons and protons into H_2, as the H_2 production significantly decreased when the hydrogenase was inhibited by carbon monoxide (Rosenbaum *et al*. 2011). Several hydrogenase-containing bacteria, *Desulfovibrio caledoniensis* (Yu *et al*. 2011), *Desulfovibrio paquesii* (Aulenta *et al*. 2012), *Desulfovibrio* sp. *G11* (Croese *et al*. 2011), *Desulfitobacterium* spp. (Villano *et al*. 2011), and *Geobacter sulfurreducens* (Geelhoed and Stams 2011), which all are mesophilic (25 ~ 40 ℃) microorganisms, had been shown to be capable of electron uptake from the cathode.

Thermophilic biotechnological processes using thermophilic microorganisms had several advantages over mesophilic processes in performance, such as higher reaction activity, greater durability and wider substrate range (Kato *et al*. 2001; Niehaus *et al*. 1999). The bio-availability of hardly biodegradable and insoluble environmental pollutants can also be improved at elevated temperature (Niehaus *et al*. 1999). Recently, several studies reported that thermophilic microbial fuel cells (MFCs) using thermophilic microorganisms as biocatalysts on the anodes showed substantial performances (Jong *et al*. 2006; Marshall and May 2009; Mathis *et al*. 2008; Wrighton *et al*. 2008). As a type of modified thermophilic MFC, thermophilic MECs may also be promising to improve the efficiency of the H_2 production without expensive metal-based catalysts and treat thermophilic agricultural processing wastewater in trophic regions (Jong *et al*. 2006). However, to our best knowledge, there was no report on thermophilic MEC as well as the utilization of thermophilic microorganisms as cathodic biocatalysts.

Thus, this study was intended to construct a thermophilic biocathode for sustainable H_2 production in MEC. Single-chambered MECs were firstly built by inoculating the effluent from a thermophilic MFC operated for more than three

months. A thermophilic biocathode was developed by transferring the cathode of the single-chambered MEC into the cathodic chamber of a two-chambered MEC (thus, in contrast to the mesophilic "anode-converted" biocathode (Rozendal *et al*. 2008b), the biocathode in this study was a "cathode-converted" biocathode). The biocathode produced a higher current density and H_2 production rate than the non-inoculated control electrode. The bacterial community of the thermophilic biocathode was also analyzed by constructing a 16S rRNA gene clone library.

5.2 Materials and Methods

5.2.1 MEC Construction

Nine single-chambered MEC reactors were constructed by using glass bottles (250 ml vol; Maruemu, Osaka, Japan) (Fig. 5.1A). All the electrodes were made of plain carbon cloth (4 cm×10 cm, TMIL Ltd, Ibaraki, Japan) and connected to the circuit via titanium wires (0.5 mm in diameter, Alfa Aesar, Ward Hill, MA, USA). The internal resistances between the electrodes and titanium wires were less than 3.0 Ω. An Ag/AgCl reference electrode was inserted into the reactor to measure the anodic and cathodic potential.

The two-chambered MEC reactors were composed of two glass bottles (300 ml vol; Maruemu, Osaka, Japan) which were separated by a proton exchange membrane (12.5 cm^2, Nafion 117, DuPont Co., Wilmington, USA) (Fig. 5.1B). The proton exchange membranes were pre-treated as described previously (Li *et al*. 2009). The electrodes were same with the electrodes used in the single-chambered MEC. An Ag/AgCl reference electrode was inserted into the cathodic chamber for electrochemical analysis. All reactors were sealed with butyl rubber stoppers and aluminum seals to maintain anaerobic condition.

5.2.2 Start-up of Single-chambered MEC Reactors

Six single-chambered MEC reactors were inoculated with 25 ml effluent of a thermophilic MFC operated for 3 months in our lab and 125 ml anaerobic pre-sterilized medium (Fu *et al*. 2013). The medium contained 0.8 g $NaCH_3COO$,

0.136 g KH_2PO_4, 0.54 g NH_4Cl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.147 g $CaCl_2 \cdot 2H_2O$, 2.5 g $NaHCO_3$ and 10 ml Wolfe's mineral solution per liter (Balch *et al*. 1979). During start-up process, three of the inoculated single-chambered MEC reactors were applied with 0.8 V. In contrast, the other three inoculated reactors were not applied without voltage, and another three abiotic control reactors (without microorganisms) was applied with 0.8 V. The headspaces of reactors were flushed with N_2/CO_2 (80/20) to maintain anaerobic condition. A constant voltage of 0.8 V was applied to the reactor using a digital power supply (Array 3645A, Array Electronics, Nanjing, China) with the positive pole connected to the anode and the negative pole connected to the cathode during the start-up process. To measure the current produced in each reactor, a fixed external resistance (1.0 Ω) was connected to each circuit, and the voltage across the fixed external resistance was recorded by using a multimeter (34970A, Agilent Technologies, Santa Clara, CA, USA) every 1 minute. The anodic and cathodic potential were also recorded during the start-up process. The reactors were incubated at 55 ℃ operated in fed-batch mode, in which the media were exchanged with fresh media when the current of MEC decreased.

5.2.3 Development of Biocathode in Two-Chambered MEC

To obtain a biocathode, both the anode and cathode of the H_2-producing single-chambered MEC were anaerobically collected from the reactor and gently rinsed with anoxic distilled water. Each electrode was then transferred into the cathodic chamber of each individual two-chambered MEC reactor, respectively. In contrast, an abiotic fresh electrode act as a cathode in a control two-chambered MEC reactor. The same medium used in single-chambered MECs excluding sodium acetate was used as the catholyte. Fresh abiotic electrodes were used as anodes in the two-chambered MEC reactors, and 50 mM potassium ferrocyanide ($K_4[Fe(CN)_6]$) solution supplemented with 2.5 g $NaHCO_3$ was used as the anolyte (Rozendal *et al*. 2008b). The initial pH values of anolyte and catholyte were 6.8. During the biocathode start-up process, the cathodes of the two-chambered MECs were poised at –0.7 V (vs. SHE) by a potentiostat (HSV-110, Hokuto Denko,

Japan). The reactors were operated in a fed-batch mode at 55℃. The H_2 and current produced in the two-chambered MECs were measured under different poised potentials.

5.2.4 Analysis and Calculation

The headspace volume (V) of MECs was maintained at a constant volume. The H_2 percentage (C) in the headspace of MECs was analyzed by using a gas chromatography [GC-2014 equipped with a Shincarbon ST column (6 m × 3 mm ID); Shimadzu, Kyoto, Japan]. The pressure (P) in the headspaces of the reactors was measured by a digital pressure sensor (AP-C40 series, Keyence, Osaka, Japan). The amount of H_2 (n) was calculated from the ideal gas theory: n=PVC/RT, where R was the ideal gas constant (8.31 m^3 Pa mol^{-1} K^{-1}), and T was the operating temperature (328 K in this study).

Cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were conducted by using a potentiostat (HSV-110, Hokuto Denko, Japan) with a standard three-electrode system. The performance of MEC was evaluated in terms of: H_2 production rate (subjected to the cathode surface, mmol-H_2 m^{-2} day^{-1}), current density (A m^{-2}) and cathodic H_2 recovery (r_{cat}). The cathodic H_2 recovery is the fraction of electrons that are recovered as H_2 gas from the total number of electrons that reach the cathode, as described previously (Logan *et al*. 2008).

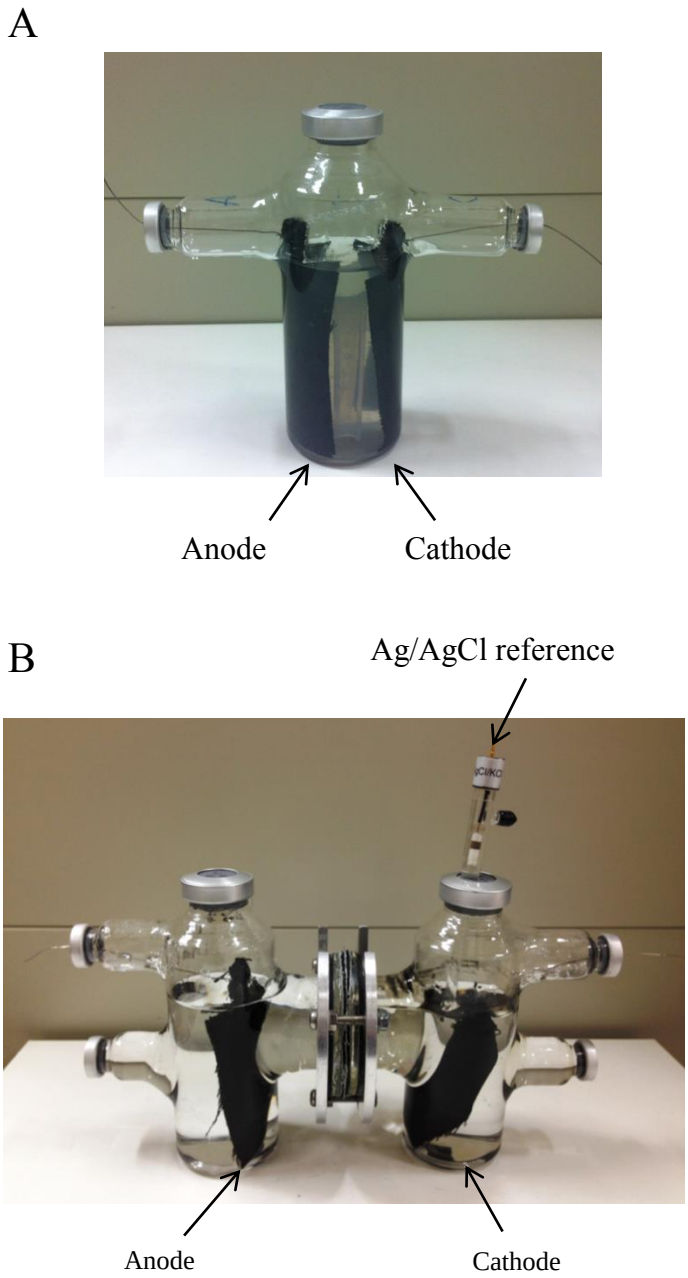


Figure 5.1 Photographs of single-chambered MEC reactor (A) and two-chambered MEC reactor (B).

5.2.5 Bacterial Population Analysis

Community DNA was directly extracted from the aseptically-crushed biocathode (250 mg) by using a PowerSoil DNA isolation kit (MO BIO laboratories, Carlsbad, CA, USA). The extracted DNA (20 ng) was used as the template for PCR with the primers 8F (5'- AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTACGACTT-3') (Grabowski *et al*. 2005). The pooled PCR amplicons were cloned into pCR4-TOPO using TOPO TA cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were purified using High Pure Plasmid Isolation kit (Roche Applied Science, Indianapolis, IN) and sequenced with T3 and T7 primers. The assembled sequences were aligned with the NAST aligner program in Greengenes (http://greengenes.lbl.gov/) with the closest sequence relatives from NCBI database (http://www.ncbi.nlm.nih.gov/) on August 2012. The alignments were then manually improved in MEGA ver. 4.0.2. (Tamura *et al*. 2007). Phylogenetic tree was constructed on the basis of the Tamura-Nei model and the evolutionary history was inferred using the Neighbor-Joining method (Tamura *et al*. 2004). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura *et al*. 2004).

5.3 Results and Discussion

5.3.1 Construction of Single-Chambered Thermophilic MEC

Three inoculated single-chambered thermophilic MEC reactors were started up at an applied voltage of 0.8 V. The current generation, anodic and cathodic potential variation of one representative reactor during the start-up process were shown in Fig. 5.2A and Fig. 5.2B. The initial current produced in the single-chambered MEC after the inoculation was around 0 mA and the initial anodic and cathodic potential were 0.5 V vs. SHE and –0.3 V vs. SHE, respectively. The current generation began to increase exponentially at 10 hours post inoculation and reached a stable level of 3.5 mA at 15 h (Fig. 5.2A), coinciding with the

simultaneous exponential drop of the anodic and cathodic potential, which reached a stable level of –0.18 V and –0.98 V at 15 h (Fig. 5.2B), respectively. Such exponential decrease/increase behavior was likely due to the exponential growth of electrochemically-active microorganisms on the electrodes, either on the anode or the cathode. In contrast, negligible current was detected in the non-inoculated control reactor with an applied voltage of 0.8 V, likely due to an abiotic electrochemical reaction, and the anodic and cathodic potential of control reactor kept stable at 0.44 V vs. SHE and –0.36 V vs. SHE, respectively.

H_2 was detected in the headspace of the inoculated reactor (with 0.8 V) (Fig. 5.2C) with the average H_2 production rate was *ca*. 46 mmol day^{-1} m^{-2}. The cathodic H_2 recovery (i.e. the measured H_2 production compared to the expected H_2 production based on the cumulative charge production) was *ca*. 20 % (Fig. 5.2C). The other two inoculated MEC reactors (with applied voltage) showed a similar current generation and H_2 production (data were not shown). On the other hand, no detectable H_2 was produced in the non-inoculated reactors (with an applied voltage of 0.8 V) or the inoculated reactors with no applied voltage (Fig. 5.2C).

The current production in the single-chambered MEC began to decrease gradually from 33 h. Simultaneously, the anodic and cathodic potential of the MEC also increased gradually, probably due to the consumption of electron donors (i.e. acetate) in the medium. When the current in MEC dropped to around 1.5 mA, the medium was exchanged with fresh pre-sterilized medium. The current and the anodic and cathodic potentials recovered to near the original level in several hours after changing medium. As planktonic microbial cells and possible electron-shuttling mediators were almost removed by the medium exchange, it was concluded that the current generation and H_2 production in the MEC was mainly due to the electrochemically-active thermophilic microorganisms attached on the electrodes.

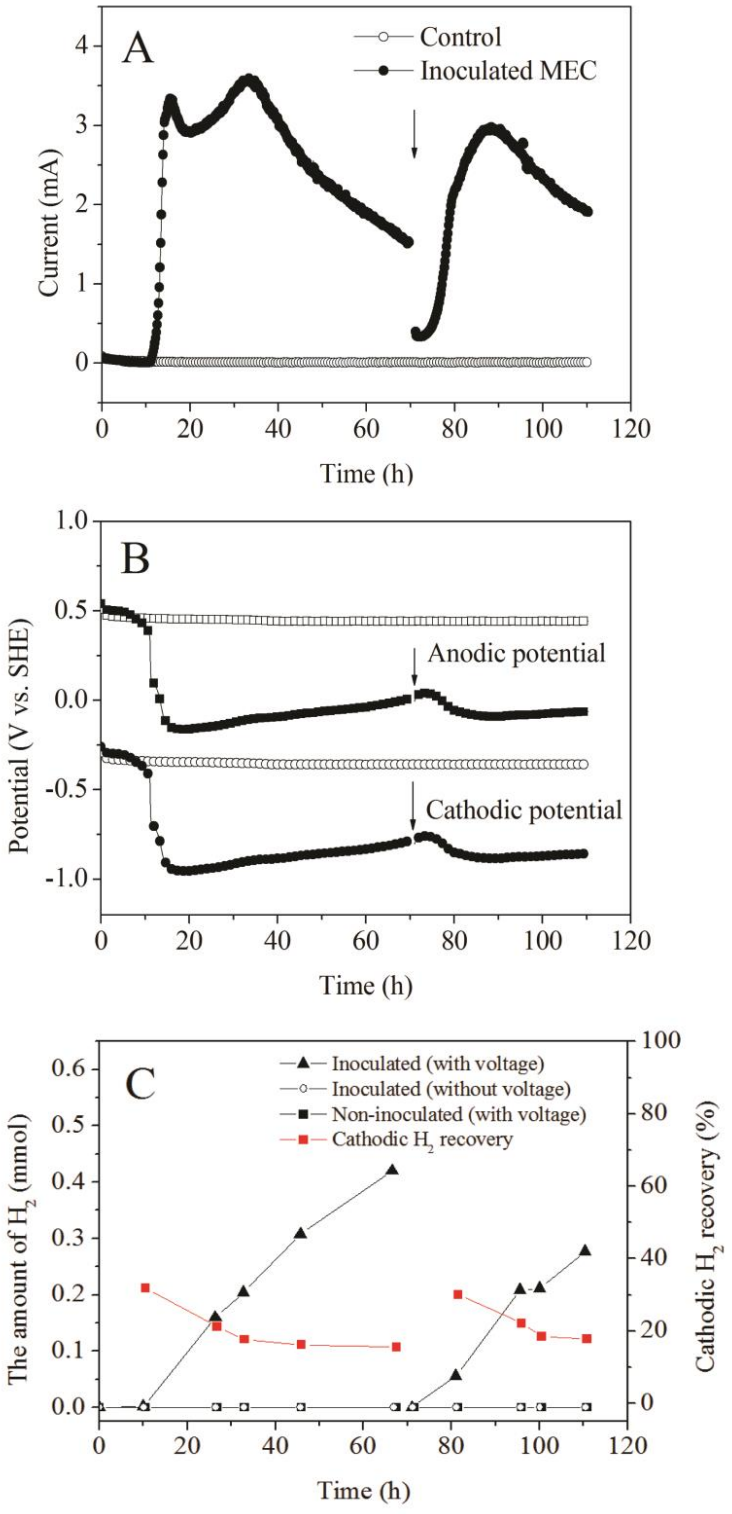


Figure 5.2 (A) Current generation profile during the single-chambered MEC startup; (B) The anodic and cathodic potential variation during the MEC startup; (C) H_2 production during the MEC startup.

5.3.2 Cyclic Voltammetry Analysis of the Single-Chambered MEC

To investigate the electrochemical characteristics of the anode and cathode in MEC, cyclic voltammetry (CV) was performed on the anode and cathode of the single-chambered MEC, respectively (Fig. 5.3).

As expected, the cyclic voltammogram of anode showed a sigmoidal shape of current, which increased from –0.3 V vs. SHE and became stable (around 1.5 mA) at around – 0.1 V vs. SHE. This shape of current was consistent with a previous CV analysis of a bioanode in a mesophilic MEC, suggesting a catalytic behavior (Call *et al*. 2009). Conversely, no clear sigmoidal shape was observed in the cyclic voltammogram of the cathode, and the anodic current of the cathode was much lower than that of the anode, indicating the cathode did not have the ability to oxidize acetate.

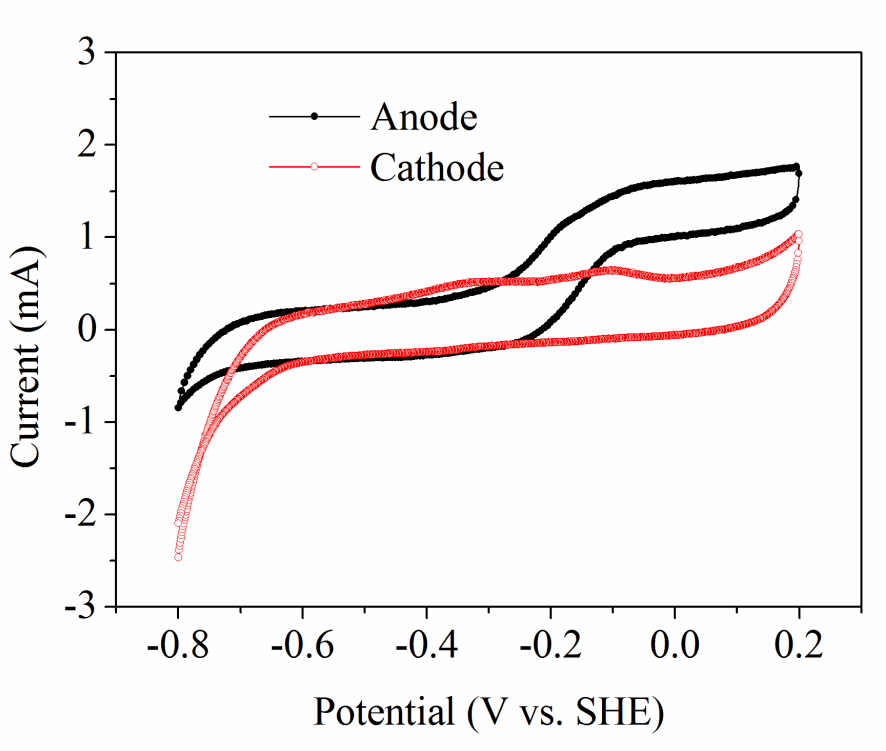


Figure 5.3 Cyclic voltammetry of the anode and cathode in the single-chambered MEC

On the other hand, the cathodic current of the cathode was significantly lower than that of the anode at the potential lower than –0.6 V vs. SHE. The more negative the cathodic current, the higher the reducing ability is. Thus, the CV analysis indicated that the cathode of the single-chambered thermophilic MEC had a catalyzing activity for H_2 production higher than that of the anode.

5.3.3 Analyses of the Biocathode in Two-Chambered MEC

In the single-chambered reactors, the low H_2 production rate and cathodic efficiency was likely because H_2 produced on the cathode was simultaneously consumed by the anodic H_2-oxidizing microorganisms (Lee and Rittmann 2010). To analyze the H_2-producing activity of the cathode independently of the anodic activity, the cathode of the single-chambered MEC was transferred to the cathodic chamber of a two-chambered MEC reactor. In the anodic chamber of the two-chambered MEC reactors, abiotic fresh electrodes were used as anodes and potassium ferrocyanide ($K_4[Fe(CN)_6]$) was used as sole electron donor in a fed-batch mode.

The cathode, hereafter designated as CC ("cathode-converted")-cathode, was poised at –0.7 V vs. SHE using a potentiostat. The current density produced by the CC-cathode quickly decreased, soon after being poised at –0.7 V vs. SHE, probably due to the gradual acclimation of enzyme(s) or other redox compounds responsible for the H_2 production on the cathode (Fig. 5.4A). The current density increased gradually after one hour, but it remained the level around four times lower than that of the non-inoculated control electrode. In the two-chambered MEC system used here, potassium ferrocyanide acted as the sole electron donor in the anodic chamber and no proton was therefore produced in the anodic reaction ($[Fe(CN)_6]^{4-} \rightarrow [Fe(CN)_6]^{3-} + e^-$). Thus, the cathodic H_2 production likely consumed protons, which were mainly produced via the bicarbonate buffering reaction ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$). Accordingly, the pH of the catholyte was increased from 6.8 to 7.5 during the experiment, and the CO_2 concentration in the headspace of cathodic chamber also dropped from 20% to near zero. Therefore, the increasing current was probably due to the proton

depletion in the cathodic chamber. After changing the catholyte, the current density of the CC-cathode recovered to the level below –0.3 A m^{-2} (data were not shown).

In previous studies, H_2-producing biocathodes were mainly obtained by the polarity inversion of bioanodes (Croese *et al*. 2011; Jeremiasse *et al*. 2010; Pisciotta *et al*. 2012; Rozendal *et al*. 2008b). Thus, to compare the biocathodic activity, the anode of the single-chambered MEC was also transferred to the cathodic chamber of a two-chambered MEC reactor and hereafter designated as AC ("anode-converted") cathode. It had been observed that the current density produced by the AC-cathode decreased gradually after being poised at –0.7 V vs. SHE in the previous study (Rozendal *et al*. 2008b). In our study, however, the current density in the AC-cathode increased to the level significantly higher than that of the CC-cathode and similar to that of the non-inoculated control electrode soon after polarity inversion (Fig. 5.4A). The AC-cathode was operated in a fed - batch mode for more than one week, and no decrease in current density was observed, suggesting that it had no catalyzing activity as a biocathode.

Fig. 5.4B showed the linear sweep voltammograms of the CC- and AC-cathodes in two-chambered reactors. The abiotic control cathode did not reveal significant catalytic current, while the AC-cathode showed a small cathodic current when the potential was lower than –0.7 V vs. SHE. For the CC-cathode, on the other hand, a large cathodic current, which started from –0.62 V vs. SHE and most likely corresponded to the H^+ reduction to H_2 (Aulenta *et al*. 2012), was obtained during the sweep. Although the potential required to produce H_2 was substantially lower than that of Pt-based cathode (usually around –0.4 V vs. SHE at pH 7), it was higher than the previously reported biocathode with *D. paquesii* (–0.75 V vs. SHE) (Aulenta *et al*. 2012)and stainless steel mesh cathodes (–0.67 V vs. SHE) (Zhang *et al*. 2010). Thus, the LSV analyses of the electrodes further indicated that the CC-cathode was the first thermophilic H_2-producing biocathode, of which the catalyzing activity was at least comparable to or higher than the mesophilic biocathodes. Moreover, it was the first example of biocathode

obtained by transferring a cathode from a single-chambered MEC without polarity inversion, providing an alternative method to develop a biocathode.

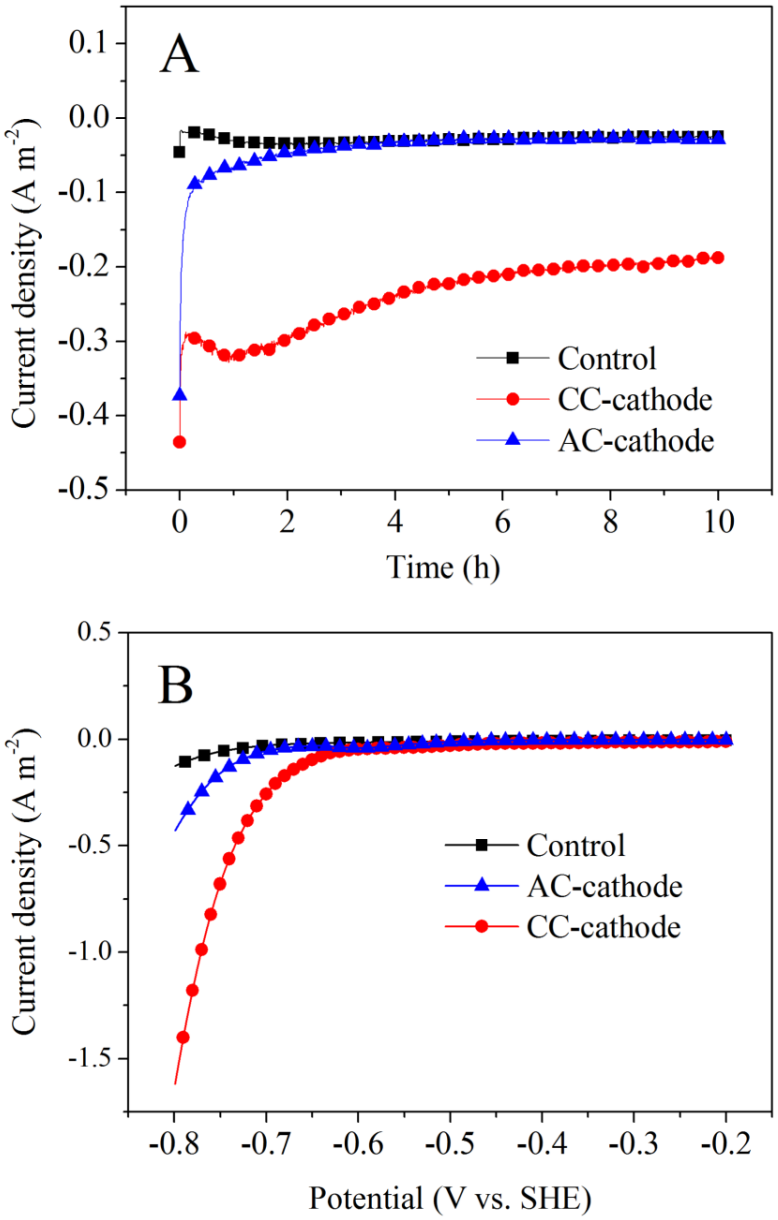


Figure 5.4 (A) Current variation of the cathodes poised at a constant potential of –0.7 V vs. SHE in two-chambered MEC reactors; (B) Linear sweep voltammetry analysis of the cathodes in two-chambered MEC reactors.

5.3.4 H_2 production by the Biocathode

The average current density and H_2 production rate of the thermophilic biocathode (i.e. CC-cathode) under different potentials were shown in Fig. 5.5. The thermophilic biocathode showed a current density and an H_2 production rate significantly higher than those of the abiotic control cathode. In addition, the current density and H_2 production rate of biocathode showed a potential-dependent manner, increasing with the decreasing potential. When the potential was –0.7 V vs. SHE, the current density and H_2 production rate of the biocathode was –0.32 ± 0.08 A m^{-2} and 107.25 ± 22.82 mmol day^{-1} m^{-2}, respectively, higher than those of the abiotic control (–0.03 ± 0.01 A m^{-2} and 7.83 ± 5.58 mmol day^{-1} m^{-2}). As the potential decreased to –0.8 V vs. SHE, the current density and H_2 production of the biocathode became –1.28 ± 0.15 A m^{-2} and 376.5 ± 73.42 mmol day^{-1} m^{-2}, around 10 times higher than that of the control electrode (–0.14 ± 0.06 A m^{-2} and 32.84 ± 11.64 mmol day^{-1} m^{-2}). The current density and H_2 production level were comparable to the mesophilic biocathode inoculated with *Geobacter sulfurreducens*, which was around –2.4 A m^{-2} and 261.8 mmol day^{-1} m^{-2} (Geelhoed and Stams 2011). However, the reducing ability of the biocathode was still one order lower than that of Pt-based catalyst (Kundu *et al*. 2013).

The cathodic H_2 recovery of the biocathode in the two-chambered MEC was around 70% (except at –0.65 V vs. SHE, where the applied potential was likely not sufficient to promote effective H_2 production) (Fig. 5.5B). This was in contrast to the low cathodic H_2 recovery in the single-chambered MEC (ca. 20%), supporting our hypothesis that the H_2 produced in the single-chambered MEC was oxidized by the exoelectrogens on the anode and contribute to the electricity generation as reported in the mesophilic single-chambered MEC (Lee and Rittmann 2010).

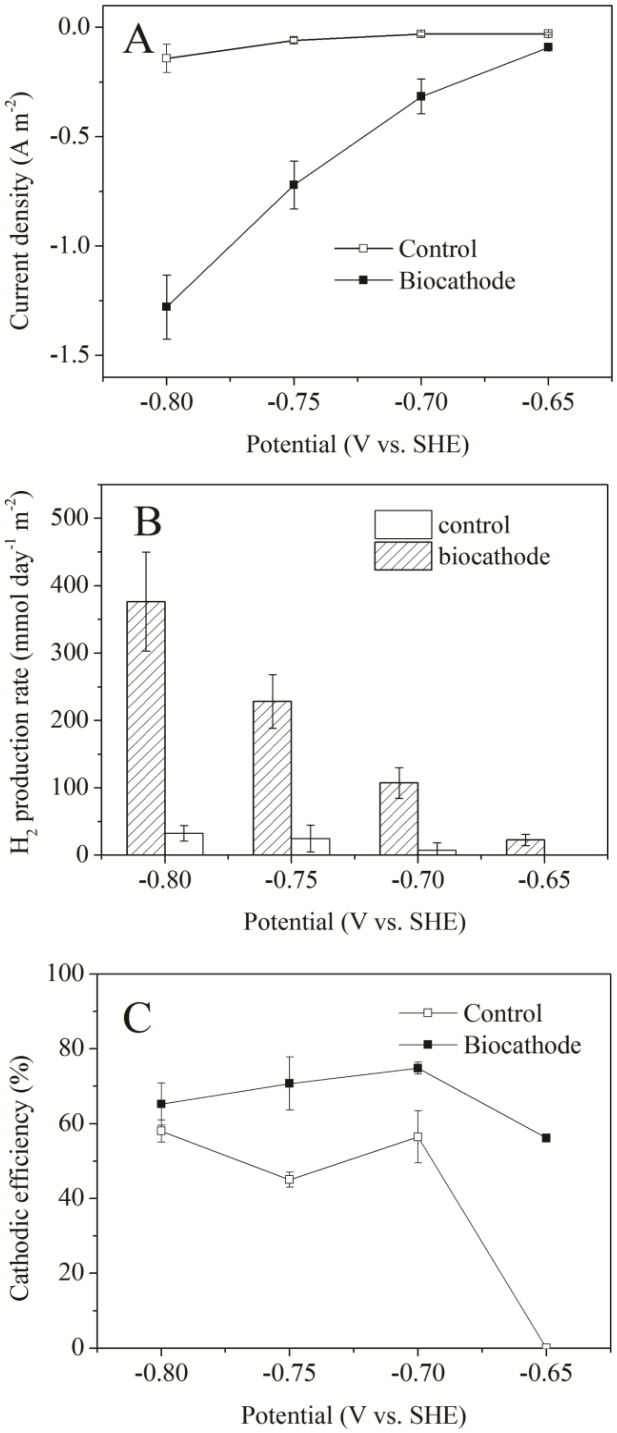


Figure 5.5 (A) Current density production of the biocathode under different poised potential; (B) H_2 production rate of the biocathode under different poised potential; (C) cathodic H_2 recovery of the biocathode under different poised potential.

5.3.5 Microbial Population Analyses of the Biocathode

The phylogenetic diversity of the bacterial community on the biocathode (i.e. CC-biocathode) was analyzed by constructing 16S rRNA gene clone library (Fig. 5.6). The library (177 clones) contained, in total, 21 phylotypes belonging to six different phyla, in which *Firmicutes* (137 clones, 77.4%) was the dominant phylum and followed by *Coprothermobacter* (35 clones, 19.8%). The other five clones were phylogenetically related to *Nitrospirae* (one clone), *Chloroflexi* (one clone), *Thermotogae* (two clones) and the candidate division OP10 (one clone). In contrast to the previous report of mesophilic biocathodes (Croese *et al*. 2011), no *Proteobacteria* was detected in the thermophilic biocathode. The electrochemical activity has never been studied on microbes related to most of the phylotypes detected on the biocathode, suggesting possible diversity of thermophilic electrotrophs. One exception was the phylotype H2TBiocatB-6 (represented by two clones), which was closely related to *Thermincola potens* strain JR, a known thermophilic exoelectrogen. Although not much is known about the biochemical mechanisms of electron uptake from a cathode (Rosenbaum *et al*. 2011), it was commonly suggested that c-type cytochromes and hydrogenases involved in the electron uptake and H_2 production. As it has been shown that *T. potens* strain JR contains surface multiheme *c*-type cytochromes and several periplasmic hydrogenase subunits (Carlson *et al*. 2012), it is possible that, regardless of the low abundance, *T. potens* contributed to the H_2 production in the biocathode. Yet, although it has been shown that *T. potens* strain JR has acetate-oxidizing activity on the anode of MFC (Marshall and May 2009; Wrighton *et al*. 2008), our CV analysis in the single-chambered MEC reactor did not detect significant acetate-oxidizing activity on the biocathode, suggesting that the *Thermincola*-related bacteria were not primary biocatalysts on the biocathode. Among the phylotypes, the H2TBiocatB-5, 10 and 20 were dominant phylotypes, suggesting their major contribution to the biocathodic catalyzing activity. Role(s) of individual microorganisms on the biocathode will be assessed in future study.

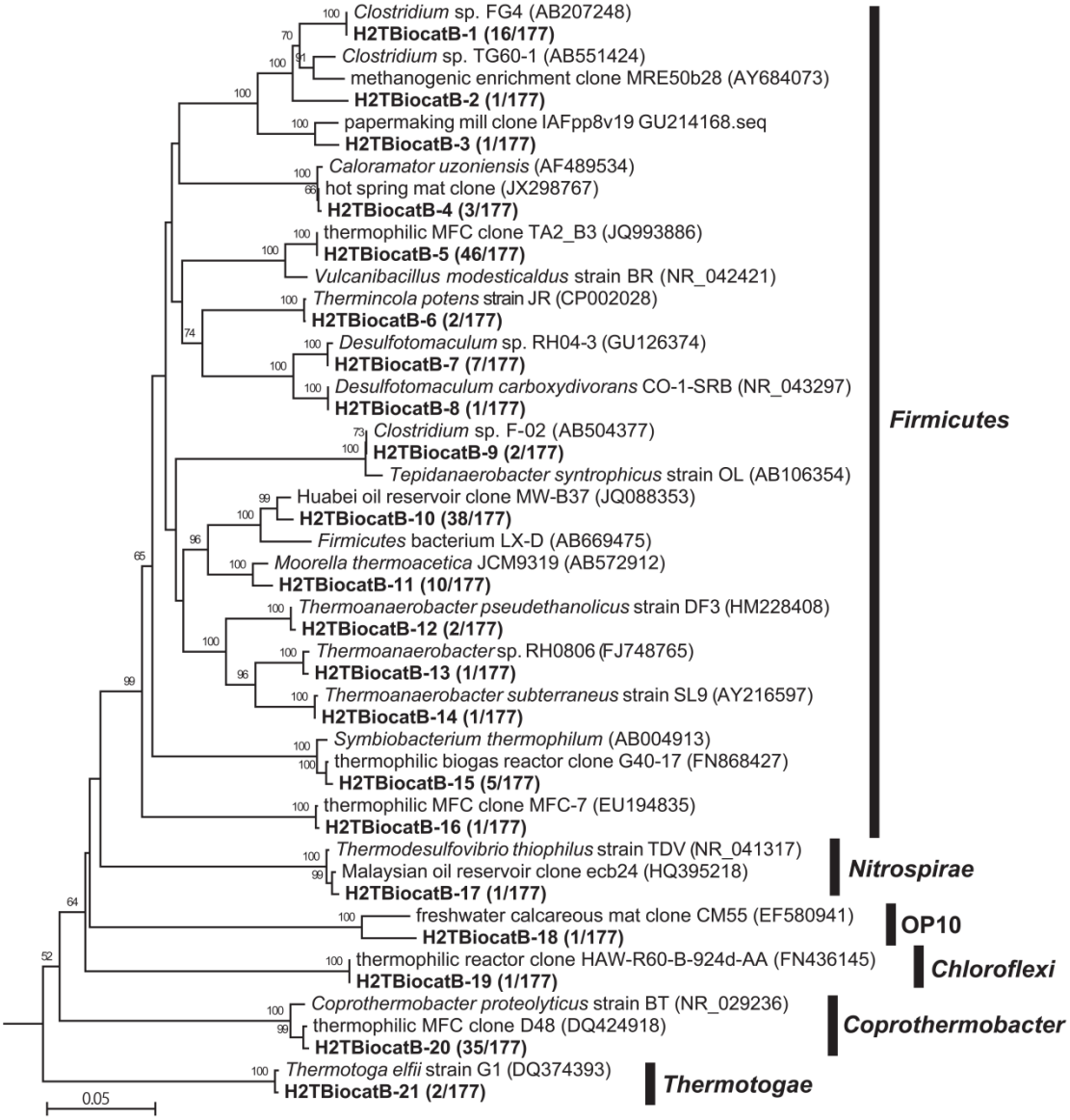


Figure 5.6 Phylogenetic tree illustrating the relationship of the phylotype detected in the thermophilic biocathode. The trees were constructed using the neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≧50% are shown above the branches. The scale bar represents the number of changes per nucleotide position. *Thermotoga lettingae* strain TMO (CP000812.1) was used as the outgroup (not shown).

5.4 Conclusions

A thermophilic biocathode capable of catalyzing H_2 production in MEC was for the first time established in this study. Cyclic voltammogram of the biocathode showed that the cathodic current of the cathode was significantly more negative than that of the anode, suggesting that the cathode have a relatively higher catalyzing activity for H_2 production. The H_2 production by the biocathode was in a potential-dependent manner with the cathodic H_2 recovery of *ca.* 70%. The linear sweep voltammetry (LSV) showed that the biocathode had a significant higher reducing activity than the control electrodes (bioanode or non-inoculated electrode). At the potential of –0.8 V vs. SHE, the thermophilic biocathode produced a current density of 1.28 A m^{-2} and an H_2 production rate of 376.5 mmol day^{-1} m^{-2}, which were around 10 times higher than those of the non-inoculated electrode. The microbial community on the biocathode was largely dominated by bacteria affiliated with the *Firmicutes* phylum, suggesting their possible contribution to the biocathodic activity.

Chapter 5

This chapter was published in Journal of bioscience and bioengineering under the following reference:

Qian Fu, Hajime Kobayashi, Yoshihiro Kuramochi, Jing Xu, Tatsuki Wakayama, Haruo Maeda and Kozo Sato. Bioelectrochemical analyses of a thermophilic biocathode catalyzing sustainable hydrogen production. *International Journal of Hydrogen Energy*. (accepted)

Chapter 6

Study of Thermophilic Biocathodes Capable of Converting CO_2 to CH_4

6.1 Introduction

Microbial electrosynthesis is a promising biotechnology which can be used to store intermittent renewable energy (such as wind and solar energy) as stable chemical bonds (Lovley 2011a; Lovley 2012; Marshall *et al*. 2012; Nevin *et al*. 2011; Rabaey and Rozendal 2010; Zhang *et al*. 2013). One key feature of microbial electrosynthesis is the use of certain microorganisms as biocatalysts on the cathode to reduce CO_2 and other organic feedstocks to valuable chemicals by accepting electrons from the cathode (Rabaey and Rozendal 2010). The products of microbial electrosynthesis were dependent on the microbial species on the electrosynthetic biocathode. Up to now, CH_4 (Cheng *et al*. 2009), acetate (Nevin *et al*. 2011; Nevin *et al*. 2010; Zhang *et al*. 2013) and ethanol production (Steinbusch *et al*. 2010) by microbial electrosynthesis have been reported.

Electromethanogenesis (EM) is one of the microbial electrosynthesis technologies, in which methanogenic microorganisms accept electrons from cathodes to convert CO_2 into CH_4 (Cheng *et al*. 2009; Villano *et al*. 2010). It has been reported that the conversion efficiency of the electrons consumed at the cathode into CH_4 was as high as 96% with mixed culture. However, the mechanisms of electron transfer from cathode to microorganisms are still poorly understood (Lovley 2011b; Rosenbaum *et al*. 2011). Generally, there are two common opinions for the cathodic electron transfer: direct electron transfer and indirect electron transfer through H_2 or other electron-shuttling mediators.

The direct electron transfer mechanism was first proposed by Cheng *et al.* (Cheng *et al*. 2009). In their study, they reported that CH_4 can be directly produced by a biocathode containing methanogens at the potential negative than –0.7 V vs. Ag/AgCl. The linear sweep voltammetry (LSV) showed that the current density of the biocathode was substantially higher than that of an abiotic cathode, which produced only small H_2. The authors concluded that both the increased current density and very small H_2 production rates by a plain cathode supported a mechanism of CH_4 production directly from current and not from H_2 gas (Eq. 6.1).

A pure culture, *Methanobacterium palustre*, was also tested and reported to be capable of accepting electrons directly from cathodes.

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O \quad \text{(Eq. 6.1)}$$

However, the direct electron transfer pathway is still speculative, because there is still one possibility that molecular H_2 is first produced (Eq. 6.2), which is then immediately consumed for hydrogenotrophic methanogenesis (Eq. 6.3) by methanogens (Kobayashi *et al*. 2013; Lovley 2011b; Lovley 2012; Rabaey and Rozendal 2010; Villano *et al*. 2010). In 2010, Villano *et al* reported a biocathode, based on a hydrogenophilic methanogenic culture, capable of reducing CO_2 to CH_4 (Villano *et al*. 2010). By comparing the gas production of the biocathode and an abiotic cathode under different poised potentials, the authors concluded that the CH_4 was produced at potentials negative than –0.65 mV vs. SHE, both via abiotically produced H_2 gas (Eq. 6.1 and Eq. 6.2) and via direct extracellular electron transfer (Eq. 3). Additionally, in other previous studies, it has been reported microorganisms possessing hydrogenases are able to directly accept electrons from the cathode to produce H_2 (Pisciotta *et al*. 2012; Rozendal *et al*. 2008b; Villano *et al*. 2011). Therefore, there is also one possibility that the CH_4 production was due to the interspecies H_2 transfer between H_2-producing microorganisms and hydrogenophilic methanogens in the biofilm.

$$2H^+ + 2e^- \rightarrow H_2 \quad \text{(Eq. 6.2)}$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \text{(Eq. 6.3)}$$

Electromethanogenesis is a promising technology to reduce CO_2 emission and meet the energy demand. However, the lack of understanding of the electron transfer mechanisms largely limits the improvement of CH_4 production rate. Therefore, the purpose of this chapter is aiming to gain a deeper understanding of the mechanisms of electron transfer from the cathode to microorganisms and find methods to improve the CH_4 production rate. A thermophilic biocathode being capable of CH_4 production was firstly established in this study. Cyclic voltammetry (CV) was performed on the thermophilic biocathode and the results suggested that the electron transfer between the electrode and microorganisms

was in a direct electron transfer manner which is not involved with interspecies H_2 transfer and exogenous mediators. The microbial analysis of the thermophilic biocathode showed that the *Methanobacterium thermautotrophicus* and *Thermincola ferriacetica* were the dominant methanogen and exoelectrogen, respectively. The pure culture, *M. thermautotrophicus*, was also inoculated into the reactor to test if it was capable of accepting electrons from the cathode.

6.2 Methods and Materials

6.2.1 Reactors Construction

Several single-chamber MEC reactors were constructed using glass bottles (250 ml vol; Maruemu, Osaka, Japan). The electrodes were made of plain carbon cloth (4 cm×10 cm, TMIL Ltd, Ibaraki, Japan) except in the last part experiments of the study of the effect of different electrode materials on the CH_4 production rate. All the electrodes were connected to the circuit via titanium wires (0.5 mm in diameter, Alfa Aesar, Ward Hill, MA, USA). The internal resistances between the electrodes and titanium wires were less than 3.0 Ω.

The two-chamber MEC reactors consisting of two glass bottles (300 ml vol; Maruemu, Osaka, Japan) separated by a proton exchange membrane (12.5 cm^2, Nafion 117, DuPont Co., Wilmington, USA). The proton exchange membranes were pre-treated as previously described (Li *et al*. 2009). The electrodes were the same with the electrodes used in the single-chamber MECs. One Ag/AgCl reference electrodes was inserted into each cathodic chamber for electrochemical analysis. All the reactors were sealed with butyl rubber stoppers and aluminum seals to maintain anaerobic condition.

In the experiment to investigate the effect of different materials on the CH_4 production rate, carbon cloth and graphite felt were used as electrode materials. The same sizes of the electrodes (40 m^2) were used in the single-chamber reactors. The inoculum and other operating conditions were same except 0.75 V was applied on the reactor with graphite felt electrode and 0.7 V was applied on the reactor with carbon cloth electrode.

6.2.2 Start-up of Single-Chamber Reactor

Single-chamber MEC reactors were inoculated with 25 ml effluent of a thermophilic MFC operated for 3 months in our lab and filled with 125 ml anaerobic pre-sterilized medium. The medium contained 0.8 g $NaCH_3COO$, 0.136 g KH_2PO_4, 0.54 g NH_4Cl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.147 g $CaCl_2 \cdot 2H_2O$, 2.5 g $NaHCO_3$ and 10 ml Wolfe's mineral solution per liter . In contrast, the control reactors were only filled with 150 ml anaerobic pre-sterilized medium. The headspaces of reactors were flushed with N_2/CO_2 (80/20) to maintain anaerobic condition. A constant voltage of 0.7 V was applied to the reactor using a digital power supply (Array 3645A, Array Electronics, Nanjing, China) with the positive pole connected to the anode and the negative pole connected to the cathode during the start-up process. To measure the current produced in each reactor, a fixed external resistance (1.0 Ω) was connected to the circuit, and the voltage across the fixed external resistance was recorded by using a multimeter (34970A, Agilent Technologies, Santa Clara, CA, USA) every 1 minute. The reactors were incubated at 55 ℃ in a fed-batch mode, in which the media were exchanged with fresh media when the current ceased.

6.2.3 Calculation

The H_2 concentration (C) in the headspace of reactors was analyzed by using a gas chromatography [GC-2014 equipped with a Shincarbon ST column (6 m × 3 mm ID); Shimadzu, Kyoto, Japan]. The headspace volume (V) of reactors was maintained at a constant volume, and the pressure (P) in the headspaces of the reactors was measured by a digital pressure sensor (AP-C40 series, Keyence, Osaka, Japan). The amount of H_2 (n) was calculated from the ideal gas theory: n=PVC/RT, where R was the ideal gas constant (8.314 J mol^{-1} K^{-1}), and T was the operating temperature (328 K in this study). The performance of the electromethanogenic reactors was evaluated in terms of: CH_4 production rate (subjected to cathode surface, mmol- CH_4 m^{-2} day^{-1}), current density (A m^{-2}) and cathodic CH_4 recovery (r_{cat}). The cathodic CH_4 recovery is the fraction of

electrons that are recovered as CH_4 gas from the total number of electrons that reach the cathode, as previously described (Cheng *et al*. 2009).

6.2.4 Electrochemical Analyses

During the cyclic voltammetry (CV) analysis, the biocathode in the single-chamber reactor was transferred into a two-chamber reactor, in which a fresh abiotic electrode act as an anode and bicarbonate buffer excluding soluble redox compounds act as anolyte. CV was conducted using a potentiostat (HSV-110, Hokuto Denko, Japan) with a standard three-electrode system. The anode, cathode and an Ag/AgCl reference electrode inserted into the cathode chamber were acted as the working electrode, counter electrode and reference electrode, respectively. CVs with cell-free spent medium of the biocathode and non-inoculated control medium were also performed with a pre-sterilized electrode in a same reactor. The spent medium of the biocathode was collected from the cathode chamber and then filtered with pre-sterilized filter in an anoxic chamber to remove the planktonic cells. The filtrates were then analyzed by CV to determine whether soluble electron shuttles were present in the spent medium. The parameters for CV were as follow: equilibrium time 99 s, scan rate 1 mV s^{-1}, and scan range –0.7 ∼–0.2 V vs. SHE.

6.2.5 Microbial Population Analysis

The community DNA was directly extracted from the aseptically-crushed biocathode (250 mg) by using a PowerSoil DNA isolation kit (MO BIO laboratories, Carlsbad, CA, USA). The extracted DNA (20 ng) was used as the template for PCR with the primers 8F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTACGACTT-3') (Grabowski *et al*. 2005). The pooled PCR amplicons were cloned into pCR4-TOPO using TOPO TA cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were purified using High Pure Plasmid Isolation kit (Roche Applied Science, Indianapolis, IN) and sequenced with T3 and T7 primers. The assembled

sequences were aligned with the NAST aligner program in Greengenes (http://greengenes.lbl.gov/) with the closest sequence relatives from NCBI database (http://www.ncbi.nlm.nih.gov/) on August 2012. The alignments were then manually improved in MEGA ver. 4.0.2. (Tamura *et al*. 2007). Phylogenetic tree was constructed on the basis of the Tamura-Nei model and the evolutionary history was inferred using the Neighbor-Joining method (Tamura *et al*. 2004). The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site (Tamura *et al*. 2004).

6.2.6 Pure Culture Test

Methanobacterium thermautotrophicus was obtained from NITE Biological Resource Center (Chiba, Japan). The pure culture was firstly propagated in serum bottles with anaerobic *Methanothermobacter* medium for 2 days at 65 ℃, and then inoculated into two-chambered MFC reactors. The *Methanothermobacter* medium contains: 0.136 g KH_2PO_4, 0.535 g NH_4Cl, 0.204 g $MgCl_2$ $6H_2O$, 0.147 g $CaCl_2$ $2H_2O$, 2.52 g $NaHCO_3$, 0.85 g Na_2S $9H_2O$, 1ml Wolfe's vitamin solution and 1ml Wolfe's mineral solution per liter. The wolfe's mineral solution consisted of the following ingredients in 1 L deionized water: 12.8 g Nitrilotriacetic acid, 1.35 g $FeCl_3$ $6H_2O$, 0.1 g $MnCl_2$ $4H_2O$, 0.024g $CoCl_2$ $6H_2O$, 0.1 g $CaCl_2$ $2H_2O$, 0.1 g $ZnCl_2$, 0.025 g $CuCl_2$ 2H2O, 0.01g H_3BO_3, 0.024 g Na_2MoO_4 $2H_2O$, 1g NaCl, 0.12 g $NiCl_2$ $6H_2O$, 0.004 g Na_2SeO_4, 0.004 g Na_2WO_4, 0.02 g $KAl(SO_4)_2$ $12H_2O$. The wolfe's vitamin consisted of following ingredients in 1 L deionized water: 2 mg Biotin, 2 mg Folic acid, 10 mg Pyridoxine-HCl, 5 mg Thiamine-HCl, 5mg Riboflavin, 5 mg Nicotinic acid, 5 mg Ca-pantothenate, 1 mg *p*-Aminobenzoic acid, 0.01 mg Vitamin B_{12}.

To remove the soluble oxygen, the medium was firstly boiled for 10 minutes, then sparged with N_2/CO_2 (80/20) for at least 1 hour, and finally pressurized to 150 kPa with H_2/CO_2 (80/20) before autoclaving. To remove soluble oxygen in the medium, the mix ingredients except vitamin solution and Na_2S $9H_2O$ were first autoclaved under a H_2/CO_2 atmosphere (80/20). The concentrated sodium

sulfide solutions are sterilized under a N_2 atmosphere in tightly closed vessels. Prior to inoculation, add the filter-sterile vitamin solution and $Na_2S \cdot 9H_2O$. Pressurize the inoculated vessels to 150 kPa with H_2/CO_2 (80/20).

During the pure-culture test, the two-chamber MFC reactors used were same with the reactors inoculated with mixed culture. Both the anode and cathode are fresh abiotic electrodes, which were same with the electrodes used in mixed culture tests. The fresh *Methanothermobacter* medium was used as electrolyte for both anode chamber and cathode chamber. 10 ml pre-cultured *Methanobacterium thermautotrophicus* solution was inoculated into the cathode chamber. During the startup process, the cathode was poised at –0.7V vs. SHE using a potentiostat (HSV-110, Hokuto Denko, Japan) with a standard three-electrode system. The anode acted as the counter electrode, and an Ag/AgCl reference electrode inserted into the cathode chamber acted as the reference electrode. The headspace of the cathode chamber was kept constant at 70 ml, and the pressure (P) in the headspace was measured by a digital pressure sensor (AP-C40 series, Keyence, Osaka, Japan). The gas composition in the headspace was analyzed using a gas chromatography [GC-2014 equipped with a Shincarbon ST column (6 m × 3 mm ID); Shimadzu, Kyoto, Japan].

6.3 Results and Discussion

6.3.1 Startup of a CH_4-producing Biocathode

To establish a CH_4-producing biocathode, a single-chamber thermophilic BES reactor was first started at an applied voltage of 0.7 V, in which the effluent of a thermophilic MFC inoculated Yabase oilfield formation water was used as the inoculum. The current generation and gas production were shown in Fig 6.1. It can be seen that both the initial current of the inoculated reactor and the control reactor were around zero after inoculation. Then the current of the inoculated reactor began to increase exponentially at around 10 h post inoculation and reached a stable level of 12.5 mA at 25 h, while the current of the control reactor kept around zero during all the operating process. The exponentially increasing

behavior of the current in the inoculated reactor was likely due to the exponential growth of electrochemically-active microorganisms at the electrodes, either on the anode or the cathode. In response, CH_4 was produced in the inoculated reactor at a rate consistent with current generation and no H_2 was detected in the headspace of the inoculated reactor (Fig. 6.1B). In contrast, there was only background level current detected in the non-inoculated control reactor (with an applied voltage of 0.7 V), and no detectable H_2 or CH_4 was produced in the non-inoculated reactor (with an applied voltage of 0.7 V) or the inoculated reactor without applied voltage (Fig. 6.1B).

The CH_4 production rate in the single-chamber BES reactor under different applied voltages was also measured, which was shown in Fig. 6.2. It can be found that the CH_4 production rate showed a voltage-dependent manner, which increased gradually with the increase of applied voltage. The maximum CH_4 production rate was obtained at the applied voltage of 0.8 V, which was around 1103 mmol day^{-1} m^{-2} (subjected to the cathode surface area). This CH_4 production rate in thermophilic single-chamber BES reactor was higher than that in the previous studies, which was shown in Table 6.1. In addition, the current-to-CH_4 conversion efficiency of the thermophilic BES reactor was around 100%, indicating that nearly all the electrons were converted to CH_4. However, there is another possibility that a small portion of CH_4 was produced by acetoclastic methanogenesis in the single-chamber reactor.

To investigate if acetoclastic methanogenesis contributed to the CH_4 production in the single-chamber BES reactor, the cathode in the single-chamber BES reactor was transferred to a two-chamber BES reactor, in which a pre-sterilized fresh electrode was used as the anode and only bicarbonate buffer was used as electrolyte in both chambers. At a poised potential of –0.7 V, the biocathode produced only CH_4 with a production rate of 68.9 mmol day^{-1} m^{-2} (the current generation curve was not shown). Since acetate was removed from the medium, acetoclastic methanogenesis cannot happen in the reactor. In addition, there was no H_2 production detected in the headspace of both the inoculated reactor and control reactor. Therefore, the CH_4 production was due to electrochemical

reactions (Eq. 6.1) on the biocathode, rather than the acetoclastic methanogenesis or soluble mediators.

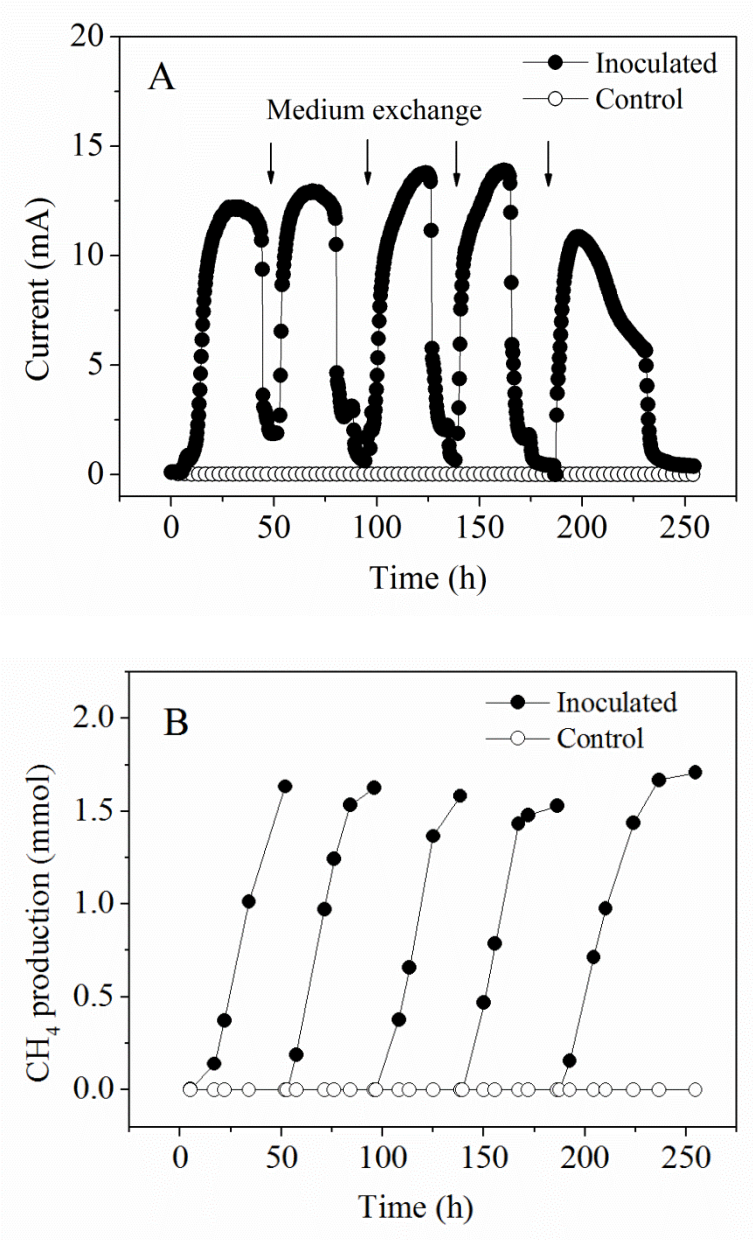


Figure 6.1 Startup curve of single-chamber BES reactor for CH_4 production (A) The current generation of the single-chamber reactor; (B) CH_4 production in the reactor

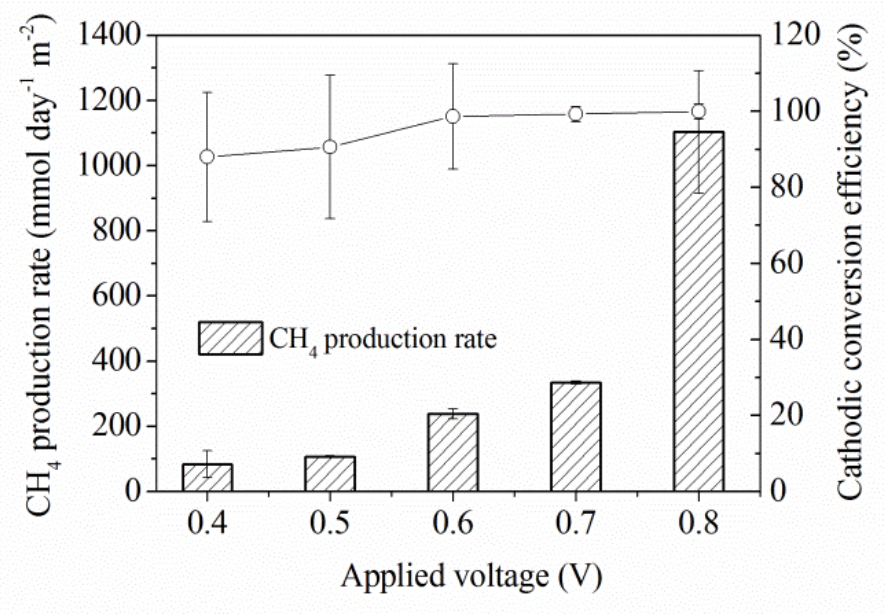


Figure 6.2 CH_4 production rate of single-chamber reactor under different applied voltages

Table 6.1 Comparison of CH_4 production rate with previous studies

Authors	Operating temperature (℃)	Maximum CH_4 production rate (mmol day^{-1} m^{-2})	Inoculum
Cheng *et al.*	25	656	Mixed culture
Villano *et a.l*	25	69	Pure culture
Chapter 3 in this study	28	450	Effluent of a mesophilic MFC
This study	55	1103	Effluent of a thermophilic MFC inoculated with Yabase oilfield formation water

6.3.2 Cyclic Voltammetry Analyses of the Biocathode

To further investigate the electron transfer mechanisms between the microorganisms and the cathode, cyclic voltammetry was performed on the biocathode in a two-chamber reactor, which was shown in Fig. 6.3. In the potential range of –0.6 V ~ –0.3 V, the biocathode produced a catalytic current with an onset potential near –0.32 V and plateaued at the potential lower than –0.5 V (Fig. 6.3A). Based on the first derivative of the voltammogram (Fig. 6.3B), the midpoint potential of the catalytic current was around –0.37 V, which only varied ± 0.01 V between replicates. Because the midpoint potential was higher than the standard equilibrium potential for H_2 production which was –0.456 V at pH 7 at 55℃), the redox active component which centered –0.37 V was probably responsible for the CH_4 production. At the potential lower than –0.6 V vs. SHE, the current of the biocathode decreased drastically, which was likely due to the proton reduction. In contrast, the current of the abiotic control electrode (Fig. 6.3, blue line) and the cell-free supernatant (Fig. 6.3, red line) were just background level, and no redox peaks were found in the voltammograms, suggesting there was no electron-shuttling mediators in the medium. Therefore, the electron transfer between the microorganisms and cathode was likely direct electron transfer.

To further investigate the electron transfer mechanism between the biofilm and cathode, CV was performed on the biocathode after immediately changing with fresh pre-sterilized medium (Fig. 6.4A). The voltammogram measured after immediately changing the medium showed a similar shape with the voltammogram in Fig. 6.3A, although the current was a little higher than that before changing medium (Fig. 6.3A). Additionally, the midpoint potential of the voltammogram after changing medium was around –0.36 V (Fig. 6.4B), which was also similar to that in the voltammogram before changing medium. Since all the possible mediators were removed during the medium exchange, it was strongly suggested that the electron transfer between the biofilm and cathode was regulated by the redox compounds in the biofilm.

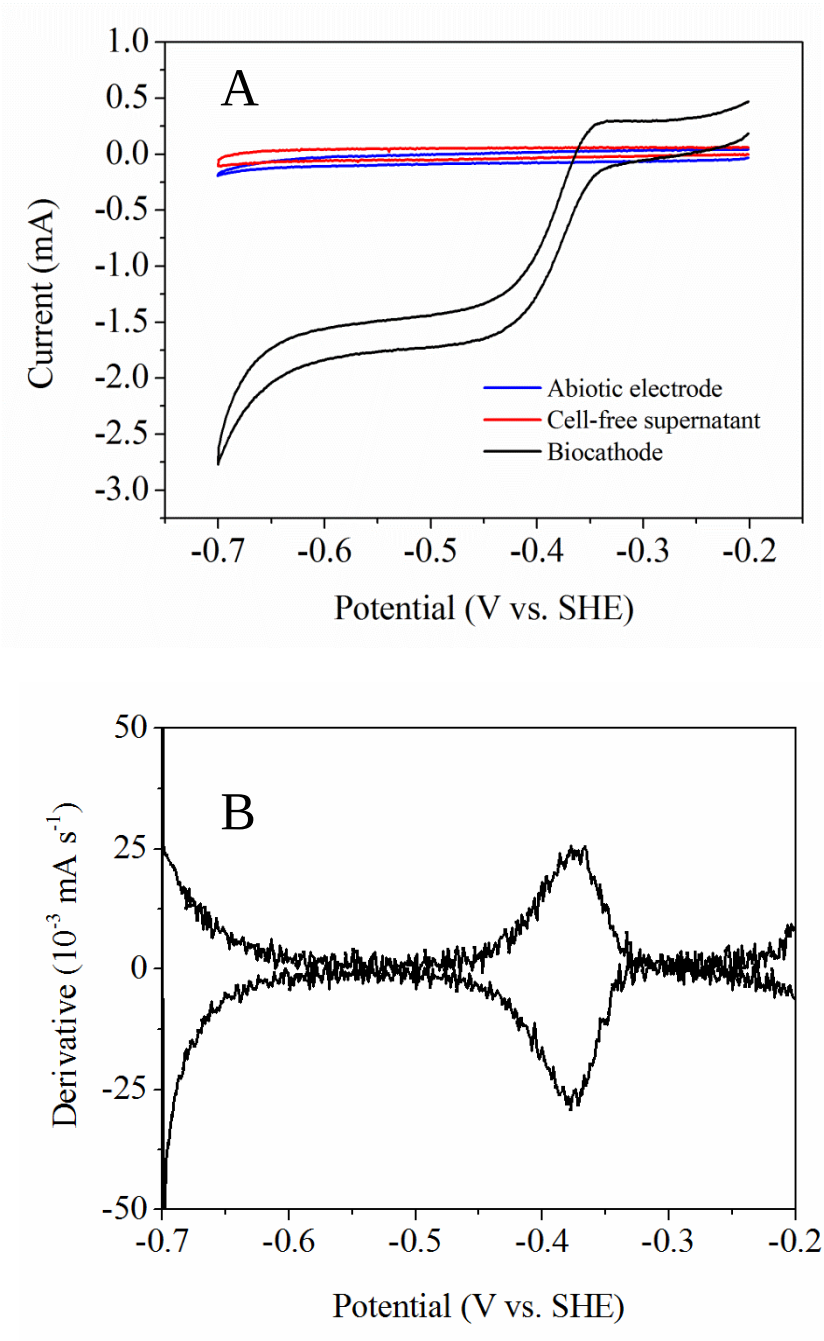


Figure 6.3 (A) Cyclic voltammetry on the non-inoculated control electrode (red line), cell-free supernatant (blue line) and the biocathode before changing the medium (black line). Scan rate, 1 mV s^{-1}; (B) The first derivative of the CV of biocathode.

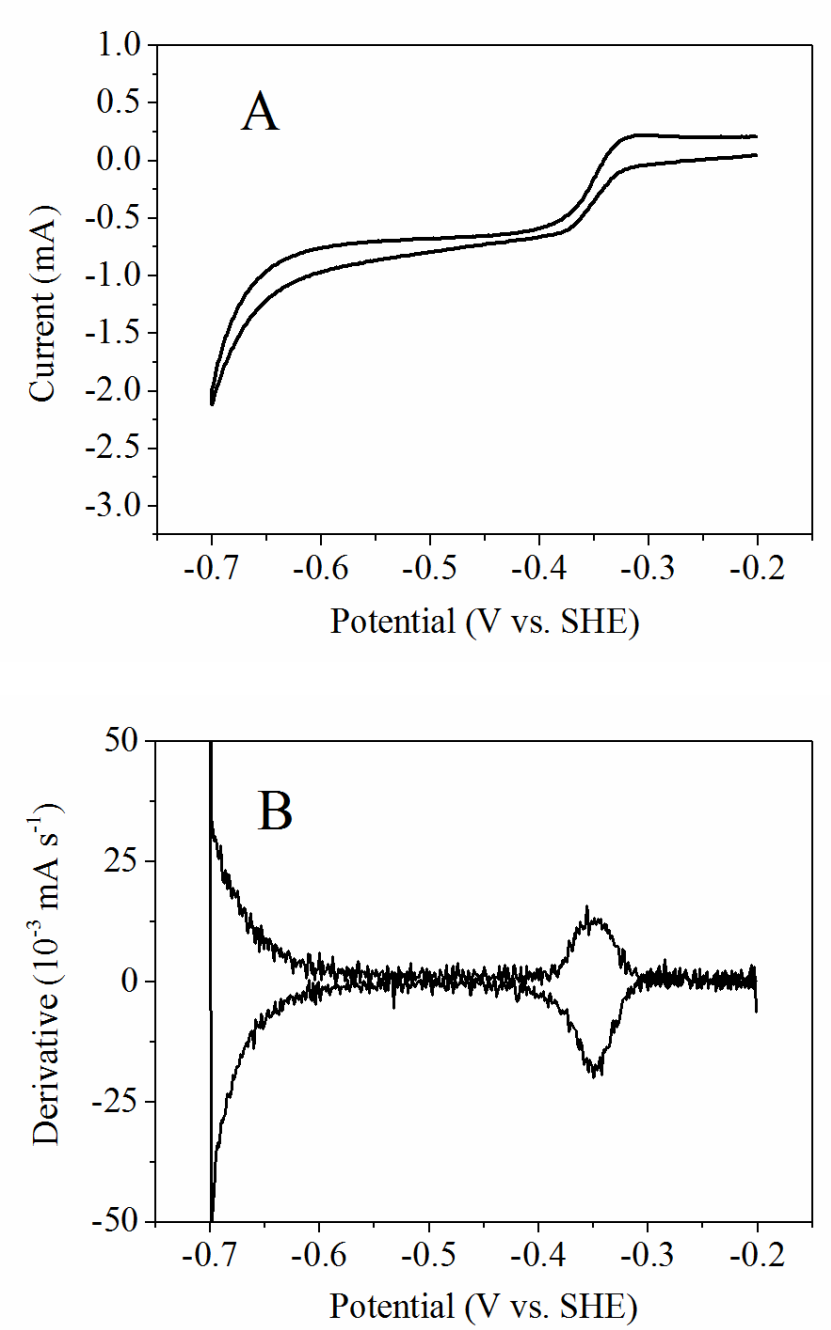


Figure 6.4 (A) Cyclic voltammetry of the biocathode after changing the medium. Scan rate, 1 mV s^{-1}; (B) The first derivative of the CV of biocathode.

6.3.3 Chronoamperometry Analyses of the Biocathode

As it has been reported that some hydrogenase-containing microorganisms could directly accept electrons from cathode to produce H_2, it still one possibility that H_2 was involved in the CH_4 production. To verify if H_2 evolution was necessary for the CH_4 production and the possible redox compound centered around –0.37 V was responsible for the CH_4 production, the biocathode was operated at –0.4 V for around 30 hours.

In the presence of CO_2, the biocathode produced an average current of –0.9 mA and only CH_4 at an average production rate of 18.4 mmol day^{-1} m^{-2} at –0.4 V. Conversely, the abiotic control electrode produced only background level current, and no H_2 or CH_4 gas was detected at –0.4 V. To test if H_2 could be produced by the biocathode in the absence of CO_2, the biocathode was firstly gently washed for three times with pre-sterilized phosphate buffer solution and sparged with high pure N_2 for more than 20 h, then was poised at the potential of –0.4 V. The current of the biocathode increased to around 0.2 mA, and no CH_4 or H_2 was detected (Fig. 6.6). As the theoretical redox potential for H_2 production was –0.456 V at pH 7 at 55℃, thus it indicated that the H_2 evolution was not necessary for the CH_4 production.

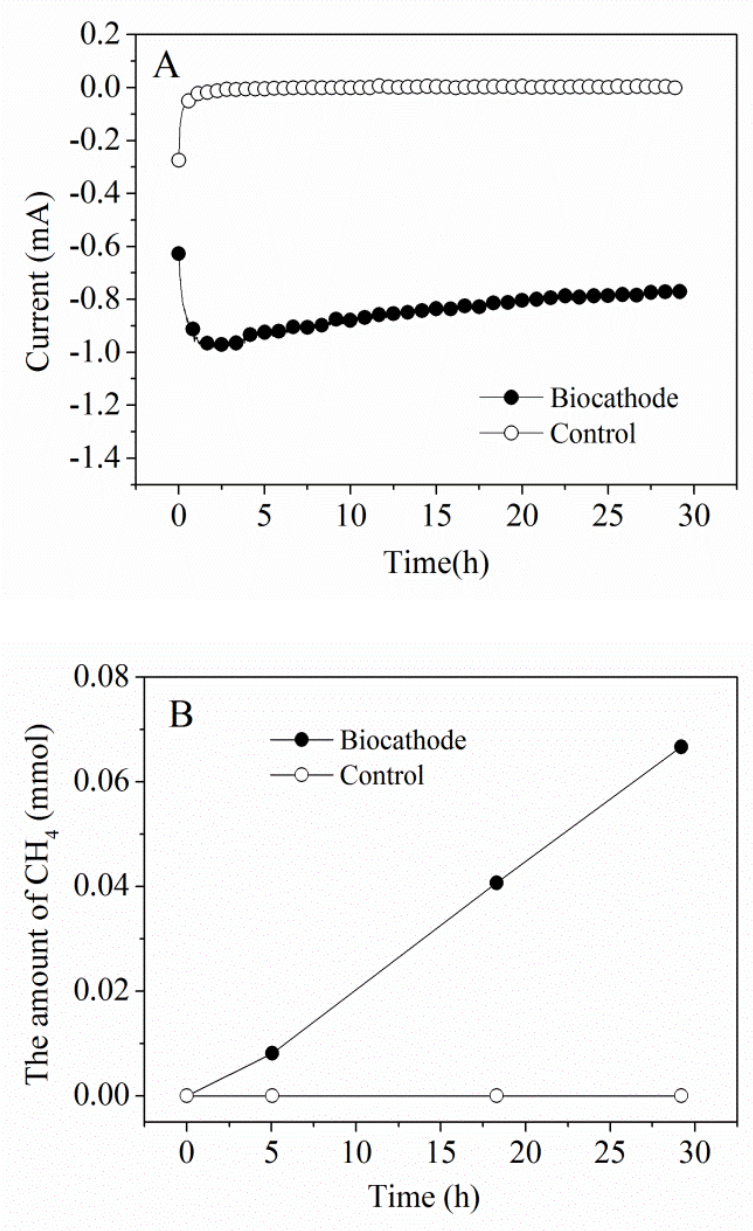


Figure 6.5 Chronoamperometry analyses of the biocathode：(A) Current generation; (B) CH_4 production

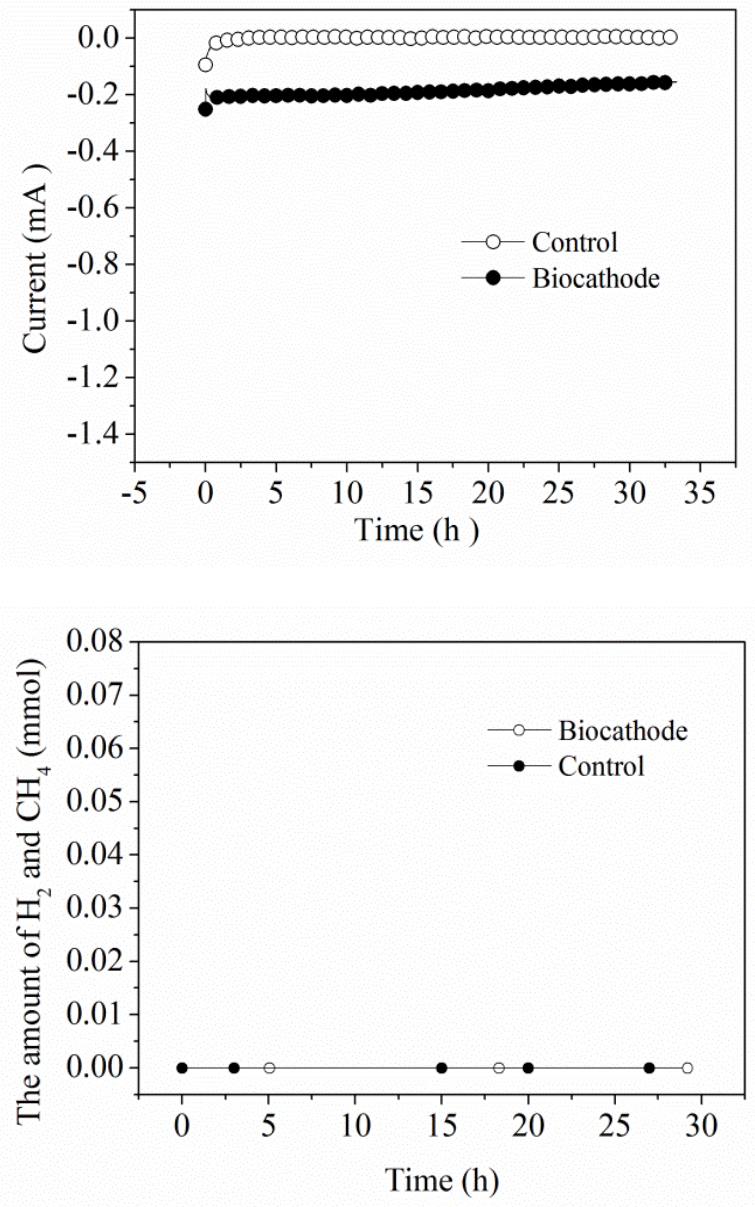


Figure 6.6 Current production (A) and CH_4 production (B) of the control electrode under –0.4 V vs. SHE.

6.3.4 Startup of a New Biocathode with the Spent Medium

To investigate if the spent medium in the biocathode chamber could be inoculum to start a new biocathode, the spent medium from the biocathode chamber was collected and inoculated into a new two-chamber BES reactor. During the startup process, the new cathode was poised at –0.7 V vs. SHE

Fig 6.7 showed the start-up curve of the new biocathode. It can be shown that the current of the inoculated-cathode decrease continuously and got a relative stable current of –0.4 mA at 10 h after inoculation. In contrast, the non-inoculated control reactor only decreased a little in the beginning 5 h and then increased continuously and became stable (–0.1 mA) at around 5 h. The current of the inoculated cathode was 4 times lower than the non-inoculated cathode. As the negative of the current, the better the cathodic reactions are, thus the inoculated cathode had a better cathodic performance than the non-inoculated cathode. Additionally, CH_4 was measured in the headspace of the inoculated cathode, and no H_2 was detected in the headspace of the inoculated cathode chamber. In contrast, no H_2 or CH_4 was measured in the non-inoculated control reactor. Therefore, the inoculated cathode is a new biocathode capable of converting CO_2 to CH_4, probably in a direct electron transfer manner.

However, both the current and the CH_4 production rate of the new biocathode were lower than that of the original CH_4-producing biocathode. It was probably because that the biofilms responsible for the CH_4 production were not easily formed on the cathode, especially in the absence of organic carbon source (e.g. acetate). It had been reported that adding acetate in the cathode chamber could accelerate the formation of the biofilm capable of producing H_2 on the cathode. Based on this information, starting a biocathode in a single-chamber BES reactor with acetate as organic carbon source is the best start-up method for the CH_4-producing biocathode.

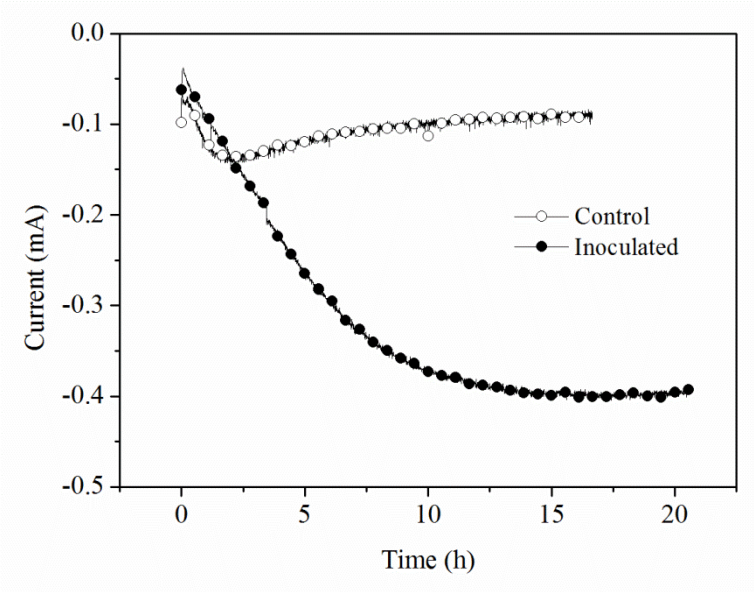


Figure 6.7 Start-up curve of a new biocathode using the spent medium from the original biocathode

6.3.5 Morphologies of Electrodes

To get an insight of the CH_4 production mechanism, the morphologies of the CH_4-producing biocathode as well as the bioanode in single-chamber reactor and the control electrode were analyzed by the scanning electron microscopy (SEM).

Fig. 6.8 showed the SEM images of the electrodes in the single-chamber thermophilic BES reactor and the control electrode. It can be seen that there was no microbial cells on the control electrode. On the other hand, biofilms were observed on both the bioanode and the biocathode capable of CH_4 production. It can be seen that a thick biofilm with diverse shapes of microbial cells was formed on the anode surface. The biofilm on the bioanode consisted of vibroid shapes, of which some were as long as more than 30 μm, and rod shapes which was less than 1 μm. These diverse morphologies of microbial cells mixed and intertwined together and contributed to the electricity generation on the bioanode. In contrast, a thin biofilm consisting of cells with relatively homogeneous morphology was formed on the cathode surface. The dominating shape of the microbial cells was vibroid shape, which was similar to methanogens.

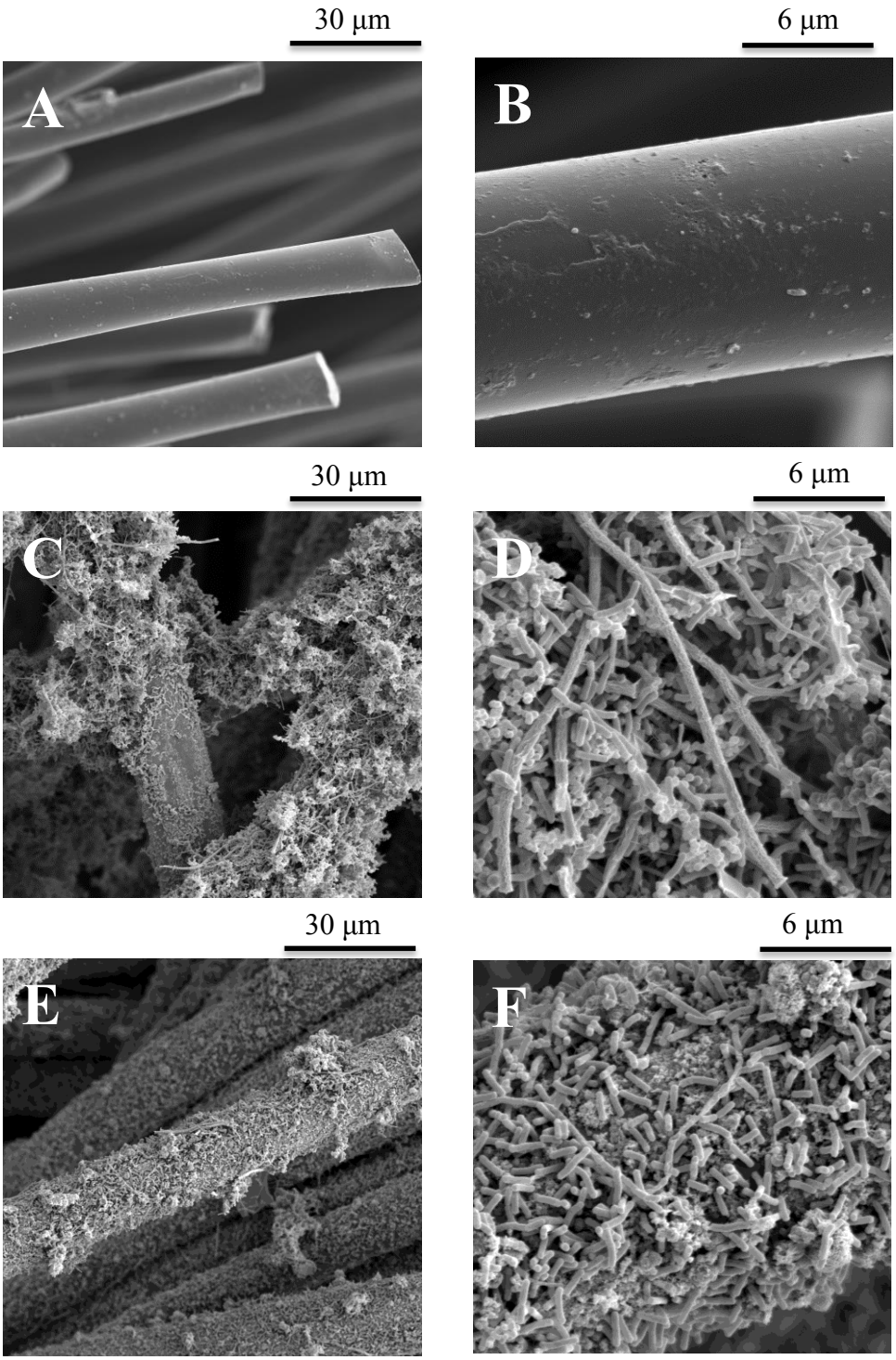


Figure 6.8 Scanning electron microscopy images of electrodes. (A) (B): control electrode; (C) (D): Anode; (E) (F): Cathode.

6.3.6 Phylogenetic Analysis of the Biocathode

To understand the microbial basis of the electromethanogenic reaction, the microbial composition of the consortium enriched on the cathode was investigated by constructing 16S rRNA gene-clone libraries.

The majority (32 clones, 97% of clones analyzed) of the clones in the archaeal library were closely related to the sequences affiliated to the *Methanobacterium thermautotrophicus*, suggesting that this methanogen played an important role in the electromethanogenic biocathode (Fig. 6.9). In a previous study, *Methanobacterium palustre*, which is a methanogen of the *Methanobacteriales* class, was found to dominate the microbial population in a mesophilic electromethanogenic biocathode and has been proposed to be capable of direct electromethanogenesis (as in Eq. 6.1) (Cheng *et al*. 2009). In this study, however, no *Methanobacteriales*-affiliated species (including *M. palustre*) were detected in the thermophilic biocathode.

Additionally, there were also several phylotypes of the *Bacteria* domain present in the biocathode, which belonged to the *Firmicutes*, *Coprothermobacteria*, *Thermotogae* phyla. Among them, *Firmicutes* was the most populous phylum (54% of the clones in the library) (Fig. 6.10). Additionally, 35% of the clones in the library were represented by the phylotype YEMCa-B2, which is closely related to *Thermincola ferriacetica*, a widely studied exoelectrogen capable of transferring electrons to anode (Marshall and May 2009; Wrighton *et al*. 2008). This finding was in contrast to the results of a previous study, in which no exoelectrogen-related sequences were detected in the biocathode.

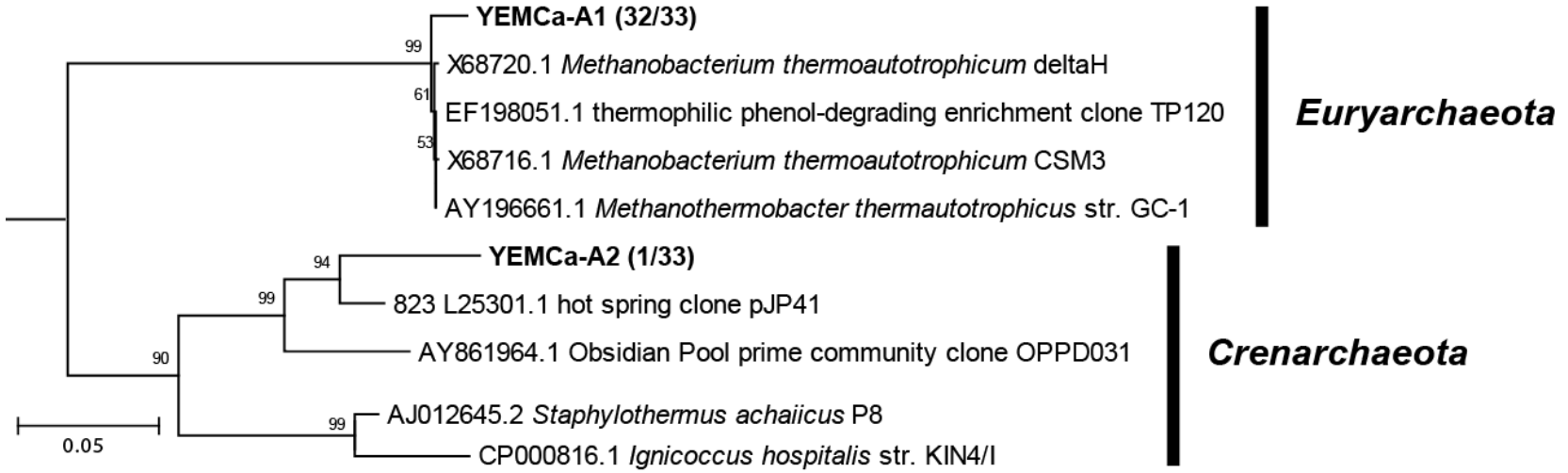


Figure 6.9 The phylogenetic trees of the archaeal 16S rRNA gene sequences detected in the thermophilic biocathode. The trees were constructed using the neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≧50% are shown above the branches. The scale bar represents the number of changes per nucleotide position. *Thermotoga lettingae* strain TMO (CP000812.1) was used as the outgroup (not shown).

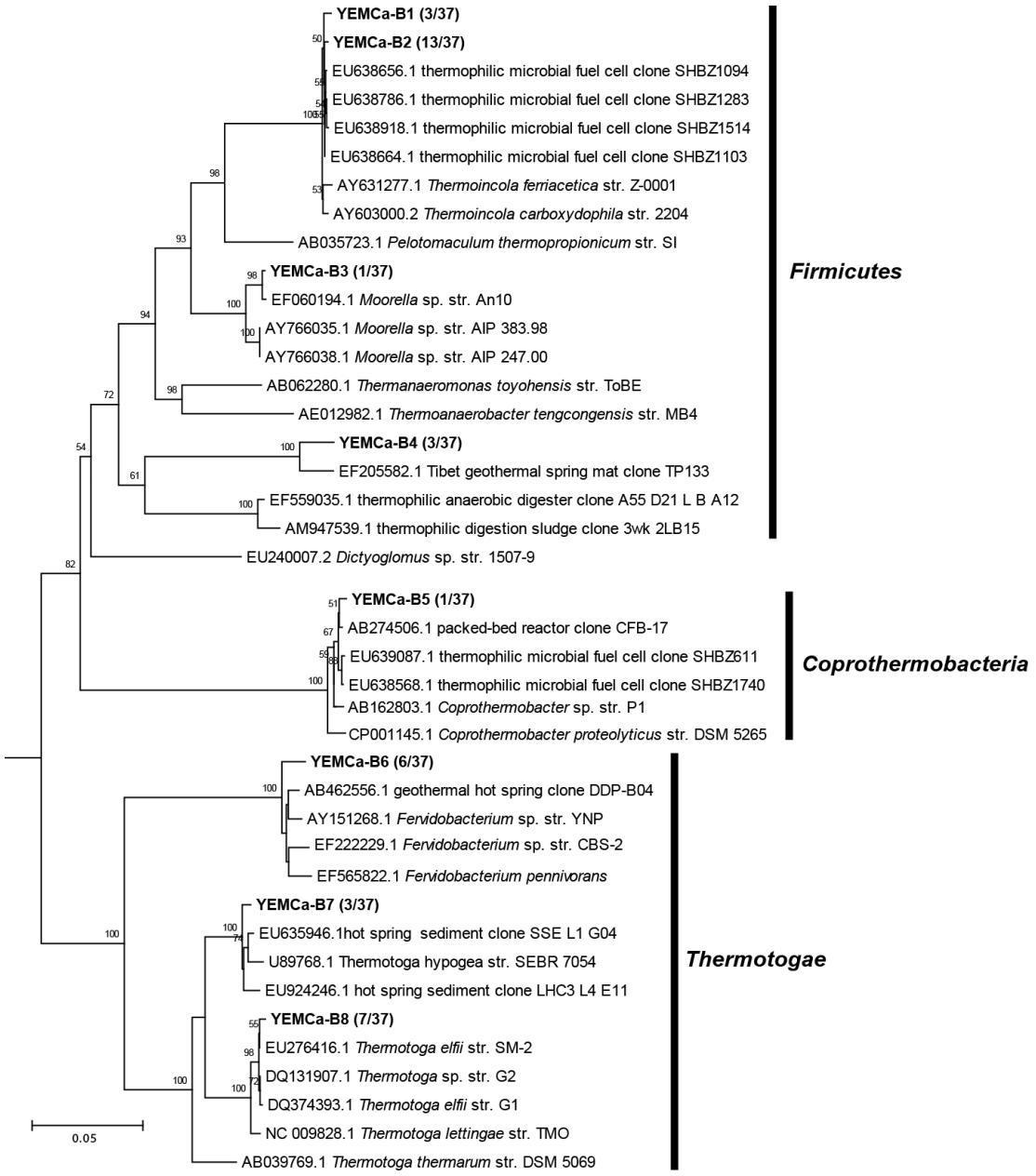


Figure 6.10 The phylogenetic trees of the bacterial 16S rRNA gene sequences detected in the thermophilic biocathode. The trees were constructed using the neighbor-joining method. Bootstrap values (n = 2000 replicates) of ≧50% are shown above the branches. The scale bar represents the number of changes per nucleotide position. *Thermotoga lettingae* strain TMO (CP000812.1) was used as the outgroup (not shown).

6.3.7 Pure Culture Test

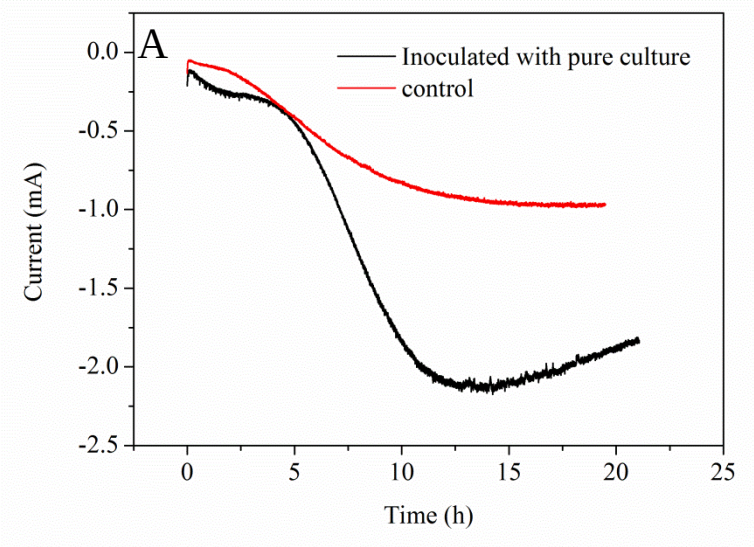
As *M. thermautotrophicus* was the dominating archaeal species in the biocathode, it was possible that this species can be capable of directly accepting electrons from the cathode for CH_4 production. To investigate the electromethanogenic activity of this *M. thermautotrophicus*, this pure culture was inoculated into the cathode chamber of a new two-chamber BES reactor and the cathode was poised at –0.7 V vs. SHE.

Fig. 6.11A showed the current generation of the two-chamber reactor inoculated with *M. thermautotrophicus*. It can be seen that the current of the cathode inoculated with *M. thermautotrophicus* decreased continuously after inoculation and got a relative stable current of 2.2 mA at around 12.5 h. Then the current increased a little slowly, which was due to the proton consumption. In contrast, the current of the abiotic cathode was only half of that of the inoculated cathode. The current generated in the abiotic electrode was probably due to the residual reducing agents in the medium. Correspondingly, CH_4 production (12.7 mmol day^{-1} m^{-2}) was observed in the headspace of the cathode chamber inoculated with *M. thermautotrophicus*, while there was no CH_4 or H_2 detected in the control electrode. Therefore, it concluded that the pure culture, *M. thermautotrophicus*, was capable of producing CH_4 through electromethanogenesis reaction.

To further investigate the electron transfer mechanism between this pure culture and the cathode, cyclic voltammetry was performed, which was shown in Fig. 6.11B. It can be seen that the cathodic current of the control electrode was quite low (almost zero). On the other hand, the cathodic current of the cathode inoculated with *M. thermautotrophicus* was much lower than that of the control electrode. It decreased gradually from –0.3 V and became relative stable at the range of –0.5 V to –0.6 V vs. SHE. The CV pattern between –0.6 V ~ –0.3 V vs. SHE looked like a sigmoidal shape, representing a catalytic behavior for the cathode inoculated with the pure culture. In another word, the CV pattern suggested that the electron transfer was

probably due to direct electron transfer at the potential range of –0.6 V ~ –0.3 V vs. SHE. When the potential was lower than –0.6 V vs. SHE, the current of the cathode inoculated with *M. thermautotrophicus* decreased drastically. One possible reason was the abiotic protons reduction on the cathode, because the potential was lower than the standard potential of H_2 production at pH 7 at 55 ℃ (–0.456 V vs. SHE). However, for the abiotic control electrode, the cathodic current was around zero at the potential lower than –0.6 V vs. SHE, thus it excluded the possibility of abiotic protons reduction. It has been reported that the presence of methanogens on the surface of steel could accelerate the corrosion by consuming the protons on the surface of steel, which was called as "depolarization" (Daniels *et al*. 1987). Therefore, the most possible reason was "depolarization", in which *M. thermautotrophicus* accelerate the consumption of protons to produce CH_4.

The CH_4 production rate of the biocathode inoculated with pure culture (12.7 mmol day^{-1} m^{-2}) was lower than that of mixed culture inoculated biocathode (68.9 mmol day^{-1} m^{-2}). This phenomenon was common for the biocatalyst comparison of mixed culture and pure culture, not only for the CH_4 production (Cheng *et al*. 2009), but also for the electricity generation in MFC (Logan 2008). The main reason was because the diverse microbial cells on the mixed cultured inoculated biocathode can provide supporting role on the CH_4 production. For example, the presence of exoelectrogen *T. ferriacetica* on the mixed culture inoculated bequeathed may accept electrons from the cathode and then transfer them to *M. thermautotrophicus* for CH_4 production. However, the detailed cooperative relationship between the exoelectrogens and methanogens need to be further investigated using two model pure cultures.



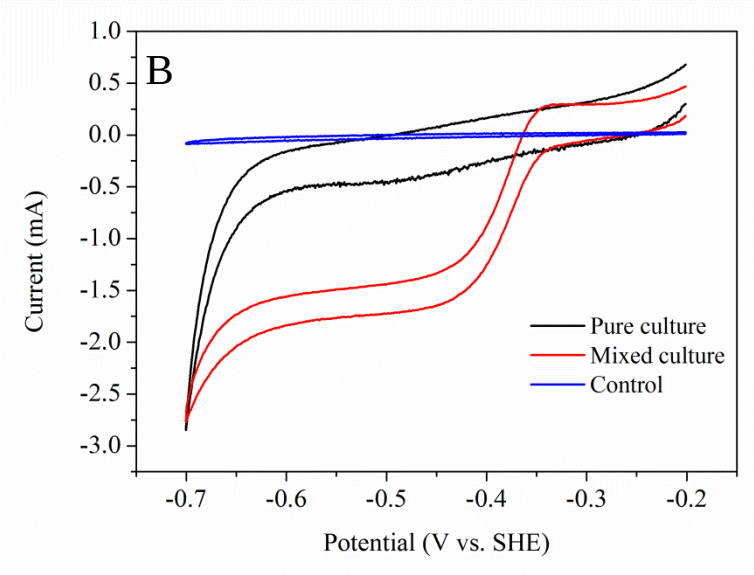


Figure 6.11 (A) Start-up curve of the biocathode inoculated with *M. thermautotrophicus* (black line) and control electrode (red line); (B) CV of the biocathode inoculated with *M. thermautotrophicus* (black line), mixed culture (red line) and control (blue line)

6.3.8 Effect of Electrode Materials on the CH_4 Production Rate

The effect of electrode materials on the CH_4 production rate was investigated in this study, in which carbon cloth and graphite felt were used as electrode materials and the result was shown in Fig 6.12.

The reactor with graphite felt electrode was started up with an applied voltage of 0.75 V, of which the current density generation was lower than 2500 mA m^{-2}. Correspondingly, the CH_4 was generated at a rate of 111.4 mmol day^{-1} m^{-2}. In contrast, the current density of the reactor with carbon cloth electrode was around 3000 mA m-2 and the CH_4 production rate was 204 mmol day^{-1} m^{-2}. It also should be noticed that the voltage applied on the reactor with carbon cloth electrode was only 0.7 V, lower than that applied on the reactor with graphite felt electrode. Therefore, carbon cloth was a better electrode material for CH_4 production in electromethanogenesis. This was probably because that the pores in graphite felt electrode were much smaller than that in carbon cloth and the graphite felt used in this study was thicker than carbon cloth. Thus, the pores in graphite felt could be easier to be clogged than that in carbon cloth.

6.4 Conclusion

A thermophilic biocathode capable of converting CO_2 to CH_4 was for the first time established in this study. This biocathode was capable of converting CO_2 to CH_4 at a set potential of –0.7 V vs. SHE with an abiotic anode as the courter electrode and CO_2 as sole carbon source. The current to CH_4 conversion efficiency was around 100% in a single-chamber bioelectrochemical reactor. The cyclic voltammetry (CV) of the biocathode showed a typical catalytic behavior with a midpoint potential of –0.37 V vs. SHE, and no significant peaks were found in the spent medium of biocathode and abiotic control electrode. The chronoamperometry (CA) showed that the biocathode could produce CH_4 at a poised potential of –0.4 V vs. SHE, which was higher than

the theoretical redox potential for H^+/H_2. Therefore, the electrochemical analyses showed that the electron transfer from the cathode to the microorganisms was in a direct electron transfer manner without the involvement of soluble mediators and H_2. The microbial analyses showed that *M. thermautotrophicus* and *T. ferriacetica* were the dominant species of archaea and bacteria. The pure culture test also showed *M. thermautotrophicus* was capable of accepting electrons from the cathode for CO_2 reduction by itself.

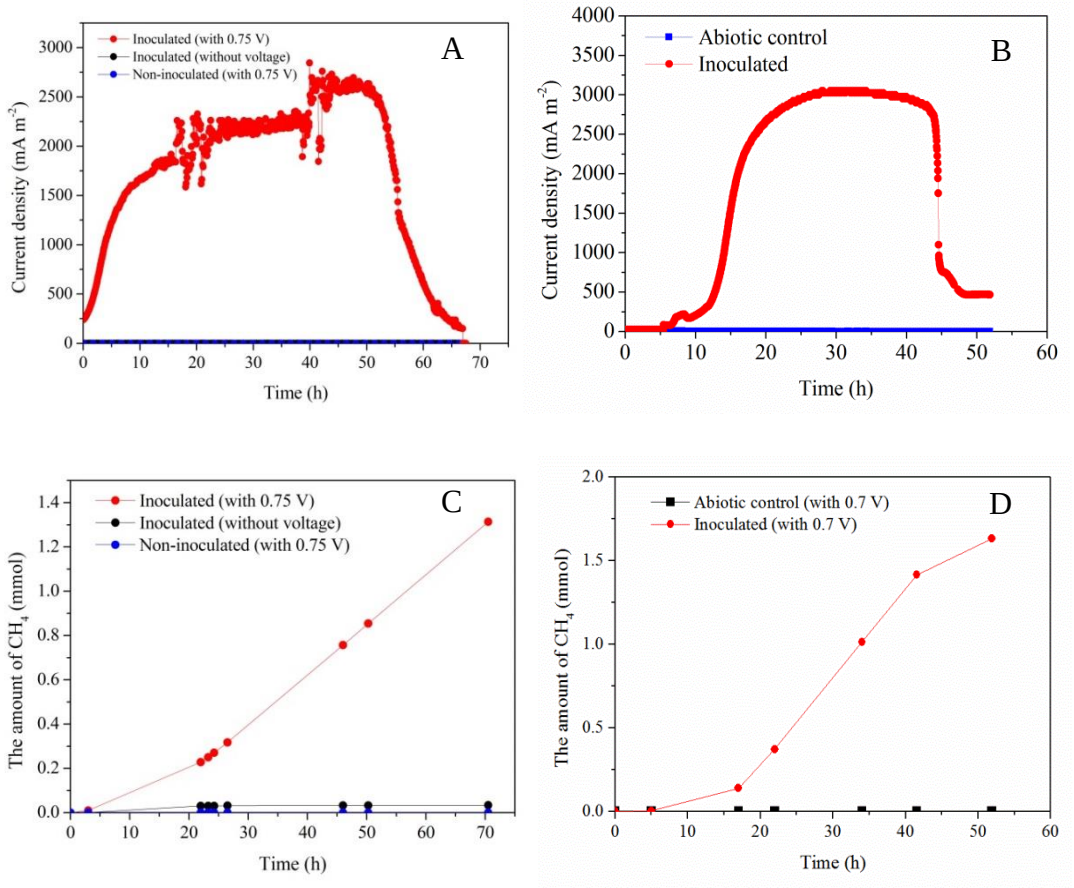


Figure 6.12 The effect of electrode materials on CH_4 production: (A) Current generation with graphite felt as electrode; (B) current generation with carbon cloth as electrode; (C) CH_4 production with graphite felt as electrode (D) CH_4 production with carbon cloth as electrode.

Chapter 7

Conclusion and Future Directions

7.1 Conclusion

7.1.1 Overview of My Research

Bioelectrochemical systems (BESs) are promising technologies which have wide applications in wastewater treatment, electricity generation, H_2 production, biosensor and bioelectronics. The key feature of BESs is the utilization of microorganisms as biocatalysts on the electrodes. Recently, it has been reported that CO_2 could be converted to CH_4 by BESs (also called "electromethanogenesis") (Cheng *et al*. 2009), in which methanogens attached on the cathode act as biocatalysts. Based on this technology, our lab proposed a promising application for BESs: to build a sustainable carbon cycle system by combining BESs and CO_2 dioxide capture and storage (CCS) technology together, aiming to convert the CO_2 stored in CCS reservoirs to CH_4 (Sato *et al*. 2013).

Up to now, BESs still cannot be commercialized due to their low performance, not even the sustainable carbon cycle systems. In addition, the recent studies of BESs are mainly focused on mesophilic BESs (20 ~ 45℃), which cannot be used in CCS reservoirs where the temperatures are usually higher than 45℃. It has been reported that operating BESs at elevated temperatures is one measure to improve the performance of BESs. However, only a few studies of thermophilic BESs, which mainly focused on thermophilic microbial fuel cells (MFCs), and no thermophilic electromethanogenesis are reported now. Compared with more than 20 species of mesophilic electrochemically-active microorganisms, there are only two species of thermophilic electrochemically-active microorganisms, *Thermincola portions* JR and *T. ferriacetica*, reported to be capable of transferring electrons to anodes. Therefore, expanding our knowledge of thermophilic BESs, especially thermophilic electromethanogenesis, is desirable to improve the performance of thermophilic BESs and realize the sustainable carbon cycle system.

In this regard, my thesis mainly focuses on the fundamental studies of thermophilic BESs, aiming to expand our knowledge of thermophilic BESs and improve the

performance of thermophilic BESs (especially thermophilic methanogenesis). To achieve this objective, I did four parts of investigation in this thesis, which will be summarized in the following part.

7.1.2 Conclusions of the Study of a Mesophilic CH_4-producing Biocathode

Firstly, to improve the performance of thermophilic methanogenesis, the mechanism of CH_4 production needs to be well understood. Although it had been reported that methanogens could accept electrons directly from the cathode for CO_2 conversion to CH_4, there is still one possibility that H_2 was firstly produced on the cathode and then immediately used for hydrogenotrophic methanogenesis to produce CH_4. Therefore, my first investigation was to build a mesophilic CH_4-producing BES reactor and investigate its CH_4 production mechanism. The conclusions are as follows:

1. A mesophilic single-chamber BES reactor aiming to produce CH_4 was started up by inoculating the effluent of a mesophilic microbial fuel cell and applying 1.0 V voltage. In the first batch cycle, only H_2 was produced in the beginning post inoculation. Then the amount of H_2 continuously decreased, accompanying with an increase of amount of CH_4. The results suggested that CH_4 production was probably due to H_2 production and hydrogenotrophic methanogenesis together in the first batch cycle (i.e. Eq. 7.1 + Eq. 7.2)

   $2H^+ + 2e^- \rightarrow H_2$ (Eq. 7.1)

   $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (Eq. 7.2)

2. In the second batch cycle, only CH_4 (no H_2) was produced in the inoculated BES reactor, while there was no CH_4 or H_2 was observed in the abiotic control reactor with 1.0 V voltage and inoculated control reactor (without applied voltage). The results showed that CH_4 was probably produced due to the electromethanogenesis reaction (Eq. 7.3) in the second batch cycle:

   $CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$ (Eq. 7.3)

3. We presented the first comprehensive phylogenetic analysis of both the biocathodic and bioanodic communities in the single-chamber reactor by constructing 16S rRNA clone libraries. The results showed that the composition of the cathodic microorganisms was significantly different from that detailed in a previous report: no methanogen of the *Methanobacteriales* class was detected, and instead, a methanogen closely related to *M. bavaricum* of the *Methanomicrobia* class was the dominant methanogen. Moreover, it was observed that an exoelectrogenic bacteria, *G. sulfurreducens*, was enriched on the biocathode. These observations indicated the possibility that diverse species of methanogens could catalyze electromethanogenesis on the biocathode.
4. As *G. sulfurreducens* is also capable of catalyzing hydrogen production (as in Eq. 7.1) using an electrode (cathode) as the electron donor (Geelhoed and Stams 2011). Thus, it is possible that *G. sulfurreducens* established a cooperative relationship with the methanogen for the electromethanogenic reaction by first receiving electrons from the cathode for hydrogen formation (as in Eq. 7.1) and then providing the resulting H_2 to the methanogens for hydrogenotrophic methanogenesis (Eq. 7.2), as shown in Fig. 7.1A.
5. Alternatively, because it has recently been shown that *G. sulfurreducens* and related *Geobacter* species can directly transfer electrons to other microorganisms (including methanogens) (Kato *et al*. 2012a, b), it is also possible that *G. sulfurreducens* provided electrons (not molecular H_2) directly to the methanogen, which utilized the electrons in the electromethanogenic reaction (Eq. 7.3), as shown in Fig. 7.1B. Overall, both the methanogens and exoelectrogens need for a high rate CH_4 production.

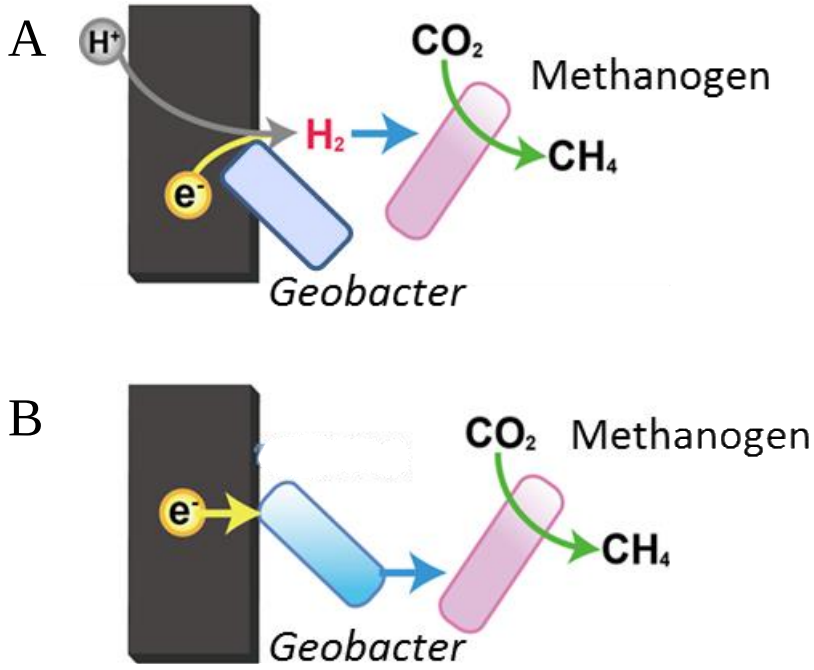


Figure 7.1 The pathway of CH_4 production: (A) interspecies H_2 transfer between *Geobacter* and Methanogens for CH_4 production; (B) Interspecies electron transfer between *Geobacter* and Methanogens.

7.1.3 Conclusions of Exploration of Thermophilic Electrochemically-Active Microorganisms

My second investigation was identification and enrichment of thermophilic electrochemically-active microorganisms using MFCs. Based on the conclusion of my first investigation, both methanogens and exoelectrogens are needed for a high rate CH_4 production in electromethanogenesis. It has been reported that thermophilic methanogens are ubiquitous in global and only two species of thermophilic exoelectrogens (*T. potens JR* and *T. ferriacetica*) are reported. Therefore, my second investigation was mainly focused on identification and enrichment of thermophilic exoelectrogens. In this study, two-chamber microbial fuel cells were used as the reactors and thermophilic microorganisms from various sources, including

thermophilic digestive sludge and oilfield formation water under different temperatures (Fig. 7.2), were used as the inoculum. The conclusions are as follows:

1. These inoculated MFCs started up successfully and showed substantial power density generation, suggesting that exoelectrogens were enriched in the anode chambers and the inoculums are good sources for thermophilic exoelectrogens.
2. The maximum power density of thermophilic MFC was obtained in a thermophilic MFC inoculated with Yabase oilfield formation water, which was around 1003 mW m^{-2}. This value was higher than those reported with thermophilic MFCs in several previous studies (usually lower than 450 mW m^{-2}) and comparable to that of a thermophilic MFC under continuous mode of operation (1030 ± 340 mW m^{-2}), as shown in Table 7.2. It also should be noticed that the MFC reactor used in this study was not optimized for performance, which was operated in a fed-batch mode and had a relatively high internal resistance due to the large electrode spacing.
3. A thick biofilm was formed on the bioanode in the MFC inoculated with thermophilic digestive sludge, while no microbial cells was observed on the control electrodes, suggesting that the biofilm contribute significantly to the electricity generation.
4. The electron transfer mechanisms between the microorganisms and anodes were investigated using medium exchange experiments and electrochemical methods. The results showed that the electron transfer mechanisms in these thermophilic MFCs (except the hyperthermophilic bioanodes) were direct electron transfer. The MFC inoculated with thermophilic digestive without any exogenous mediators was extensively studied by non-turnover CV analyses. The result showed that there were four redox components on the outer membrane surface were observed, of which system E_3 centered around –0.14 V played the major functional role.
5. The microbial analyses of the bioanode in each reactor were analyzed by constructing gene-clone libraries. The results showed that *Firmicutes* and

Deferribacteres phylum accounted for the majority in the microbial communities of bioanodes operated at 55 ℃. The dominant species in each MFC was shown in Table 7.1.

6. Two novel thermophilic exoelectrogens, *Caloramator australicus* strain RC3 and *Calditerrivibrio nitroreducens* Yu37-1, were tested in the experiment and proven to be capable of transferring electrons to anodes in a direct electron transfer manner, largely expanding our knowledge of thermophilic exoelectrogens .
7. A hyperthermophilic MFC, which can operate between 75 ℃ and 98 ℃, was successfully started up by inoculating the hyperthermophilic microorganisms from the produced water of an oilfield. The microbial analysis showed that *Caldanaerobacter subterraneus* (subspecies *subterraneus* and *tengcongensis*, respectively) are the dominating bacteria in the hyperthermophlic MFC.

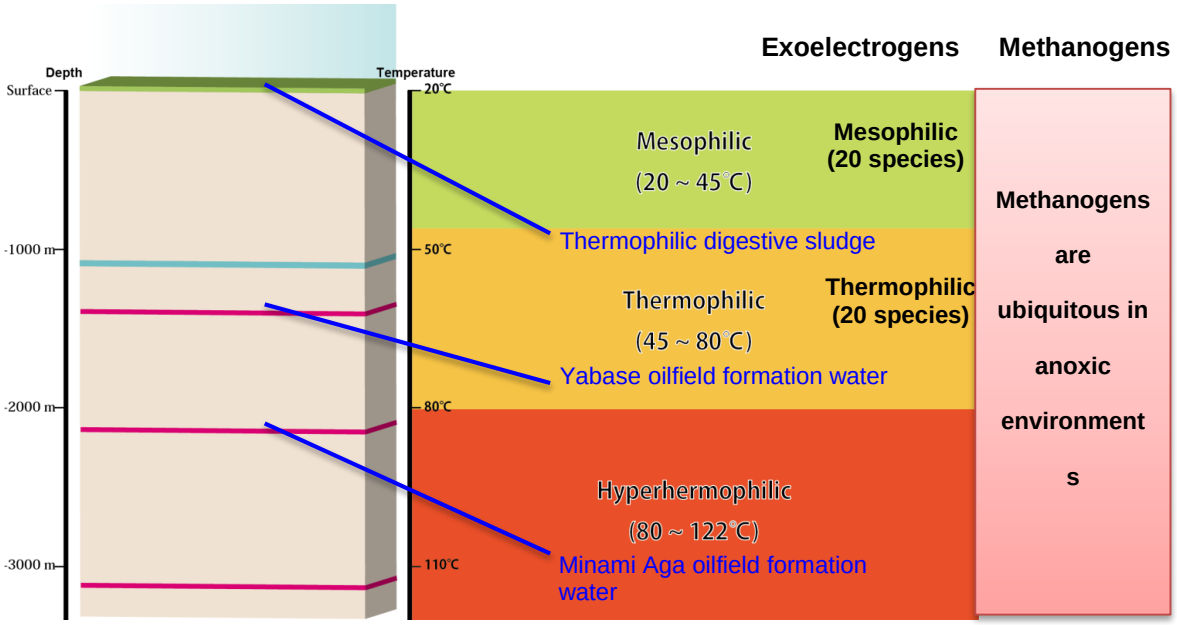


Figure 7.2 Distributions of exoelectrogens and methanogens in various environments

Table 7.1 Comparison of thermophilic MFCs in previous studies

Authors	T (℃)	Maximum power density (mW m^{-2})	Inoculums	Electron donors	Dominant species closely related
Wrighton *et al*. 2008	55	24	Mixed culture	Acetate	*Thermincola potens JR*
Mathis *et al.* 2008	60	207	mixed culture	Acetate	*Thermincola carboxydophila*
Marshall *et al*. 2010	60	146	*Thermincola*	Lactate	*Thermincola*
Jong *et al*. 2006	55	1030	Mixed culture	Acetate	Unknown species
This study ①	55	823	Thermophilic digestive sludge	Acetate	*Calditerrivibrio nitroreducens*
This study ②	55	450	Thermophilic digestive sludge	Acetate and yeast extract	*Caloramator australicus*
This study ③	55	1004	Yabase oilfield formation water	Acetate	*Anoxybacillus* sp. strain DR02
This study ④	95	165	Minami Aga oilfield formation water	Glucose, yeast extract, tryptone	*Caldanaerobacter subterraneus*

7.1.4 Conclusions of the Study of a Thermophilic H_2-producing Biocathode

My third investigation was to build a thermophilic biocathode capable of H_2 production by using the enriched thermophilic microorganisms in my second investigation. In this study, a thermophilic biocathode was first started up in a single-chamber BES reactor and then analyzed by transferring into a two-chamber reactor. The conclusions are as follows:

1. At an applied voltage of 0.8 V, H_2 was produced in the inoculated single-chamber MEC reactor, while there was no H_2 measured in the abiotic control reactor (with 0.8 V) and the inoculated control reactor (without applied voltage), suggesting both the microorganisms and voltage are needed for the H_2 production.
2. The cyclic voltammogram of the biocathode showed that the cathodic current of the cathode was significantly more negative than that of the anode, suggesting that the cathode have a relatively higher catalyzing activity for H_2 production.
3. The cathode in the single-chambered MEC was transferred into a two-chamber MEC reactor and further analyzed by using electrochemical methods. The linear sweep voltammetry (LSV) showed that the biocathode had a significant higher reducing activity than the control electrodes (bioanode or non-inoculated electrode).
4. At the potential of –0.8 V vs. SHE, the thermophilic biocathode produced a current density of 1.28 A m^{-2} and an H_2 production rate of 376.5 mmol day^{-1} m^{-2}, which were around 10 times higher than those of the non-inoculated electrode, with the cathodic H_2 recovery of *ca*. 70 %.
5. The molecular-phylogenetic analysis of the bacteria on the biocathode indicated that the community was comprised of six phyla, in which *Firmicutes* was the most populated phylum (77% of the clones in the 16S rRNA library).

It was the first report of thermophilic biocathode capable of producing H_2, largely expanding our knowledge of thermophilic BESs.

7.1.5 Conclusions of the Study of a Thermophilic CH_4-producing Biocathode

My last investigation was to establish a thermophilic biocathode capable of converting CO_2 to CH_4 and study its electron transfer mechanisms. This biocathode was firstly started up in a single-chamber reactor using the effluent of a thermophilic MFC inoculated with Yabase oilfield formation water as the inoculum. The conclusions are as follows:

1. The maximum CH_4 production rate of the biocathode was around 1103 mmol day^{-1} m^{-2}, which was much higher than that in previous studies (lower than 656 mmol day^{-1} m^{-2}) and the mesophilic biocathode (450 mmol day^{-1} m^{-2}) reported in this study, as shown in Table 7.2.
2. The current to CH_4 conversion efficiency was around 100% in the single-chamber BES reactor, suggesting a directly electron transfer mechanism for CH_4 production.
3. The biocathode was transferred into a two-chamber reactor for further analysis. At a set potential of –0.7 V vs. SHE, the biocathode was capable of converting CO_2 to CH_4 with an abiotic anode as the courter electrode and CO_2 as the sole carbon source.
4. The cyclic voltammogram (CV) of the biocathode showed a catalytic wave with a midpoint potential of –0.34 V vs. SHE in the range of –0.6 V ~ –0.3 V vs. SHE, suggesting a direct electron transfer mechanism for CH_4 production. In contrast, there was no significant peak observed in the CV of the cell-free spent medium of the biocathode and the abiotic control electrode.
5. The biocathode can produce CH_4 at a rate of 14 mmol day^{-1} m^{-2} with CO_2 as the sole carbon source at a set potential of –0.4 V vs. SHE. Because the theoretical redox potential for H_2 production was –0.456 V at pH 7 at 55℃

and no CH_4 or H_2 was produced by the biocathode in the absence of CO_2, we concluded that the H_2 evolution was not necessary for the conversion of CO_2 to CH_4 and the electron transfer was in a direct manner.

6. The morphology of the biocathode was also analyzed by the scanning electron microscopy (SEM), which showed that a thin layer of biofilm with relative homogeneous shape of microbial cells was formed on the biocathode. The microbial analyses showed that *Methanothermobacter thermautotrophicus* and *Thermincola ferriacetica* were the dominant species of archaea and bacteria, respectively.
7. *M. thermautotrophicus* was inoculated into a two-chamber BES reactor and the result showed that this pure culture was capable of accepting electrons from the cathode for CO_2 reduction by itself. However, the CH_4 production rate was much lower than that of the mixed culture, which was probably due to the lack of supporting functions of other microorganisms, such as exoelectrogens. Therefore, there is still one possible direct electron transfer pathway: the exoelectrogens accept electrons directly from the cathode and then transfer them to the methanogens. Different electrode materials were also used to investigate their effect on the CH_4 production. The results showed that the carbon cloth was a better material for CH_4 production than graphite felt, which was probably because that carbon cloth was not easily to be clogged compared with graphite felt.

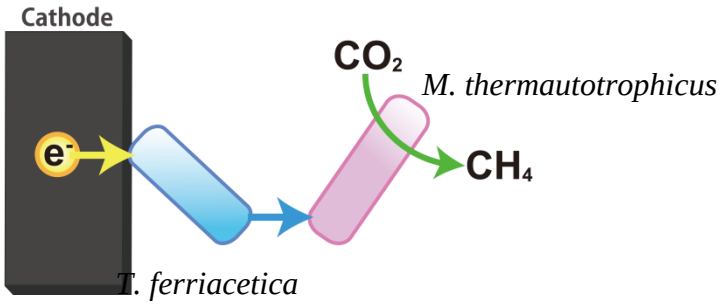


Figure 7.3 The assumption of the cooperative relationship between *T. ferriacetica* and *M. thermautotrophicus.*

Table 7.2 Comparison of the performance of electromethanogenesis

Authors	T (℃)	CH_4 production rate (mmol day^{-1} m^{-2})	Inoculums	Dominant species	Proposed electron transfer mechanism
Cheng *et al*. (2009)	25	656	Mixed culture from	*Methanobacterium palustre*	Direct
Villano *et al*. (2010)	25	69	Anaerobic sludge from a packed bed biofilm reactor fed	--	Direct and Indirect
This study chapter 3	25	450	Effluent of a mesophilic MFC	*Methanomicrobia bavaricum*	Direct and Indirect
This study chapter 6	55	1103	Effluent of a thermophilic MFC inoculated with Yabase oilfield formation water	*Methanothermobacter thermautotrophicum*	Direct

"--" represent not applicable

7.2 Future Directions

To improve the performance of thermophilic BESs and realize the deployment of sustainable carbon cycle system, the future research directions of thermophilic BESs are shown as follows:

1. The species of thermophilic electrochemically-active microorganisms, including the thermophilic exoelectrogens and methanogens, still need to be expanded. It is because that there are only four species of thermophilic exoelectrogens (including the two species identified in this study) and one species of thermophilic methanogen (*M. thermautotrophicus*) were reported.
2. The CH_4 production mechanisms, especially the mechanism of electrons transfer from cathode to the microorganisms, need to be further investigated. For example, the cooperative relationship between the exoelectrogens and methanogens for CO_2 conversion to CH_4, which will give insightful information for the design of the system, need to be verified by the co-culture experiment.
3. The reactors of thermophilic BESs need to be further designed to increase the performance of thermophilic BESs. The electrodes materials should be well studied and designed to decrease the contact resistance between the microorganisms and the electrodes.
4. New cheap electrode materials which are suitable for CH_4 production need to be further exploited. The methods of electrode modification also need to be investigated, for example, heating at high temperature and treatment with ammonia gas.
5. As it has been proven that the pure culture, *M. thermautotrophicus*, is capable of accepting electrons from the cathode to reduce CO_2 to CH_4. The simulation based on this model culture is also desirable in future study.
6. The current studies of BESs are only focused on laboratory scales, to meet the real situation, large scales of BESs reactor need to be investigated.

References

Aelterman, P., Verstraete, W., 2009. Bioanode performance in bioelectrochemical systems: recent improvements and prospects. Trends in Biotechnology 27(3), 168-178.

Ahn, Y., Logan, B.E., 2010. Effectiveness of domestic wastewater treatment using microbial fuel cells at ambient and mesophilic temperatures. Bioresource Technology 101(2), 469-475.

Armstrong, F.A., Camba, R., Heering, H.A., Hirst, J., Jeuken, L.J.C., Jones, A.K., Le′ger, C., McEvoy, J.P., 2000. Fast voltammetric studies of the kinetics and energetics of coupled electron-transfer reactions in proteins. Faraday Discussions 116, 191-203.

Audi, 2011. Audi balanced mobility – The route to CO_2 neutral mobility. Audi press release 13.5.

Aulenta, F., Catapano, L., Snip, L., Villano, M., Majone, M., 2012. Linking bacterial metabolism to graphite cathodes: electrochemical insights into the H_2-producing capability of *Desulfovibrio* sp. ChemSusChem 5(6), 1080-1085.

Bachu, S., 2008. CO_2 storage in geological media: Role, means, status and barriers to deployment. Progress in Energy and Combustion Science 34(2), 254-273.

Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R., Wolfe, R.S., 1979. Methanogens: reevaluation of a unique bological group. Microbiological Reviews 43(2), 260-296.

Bard, A.J., Parsons, R., Jordan, J., 1985. Standard potentials in aqueous solution. CRC press.

Baron, D., LaBelle, E., Coursolle, D., Gralnick, J.A., Bond, D.R., 2009. Electrochemical measurement of electron transfer kinetics by *Shewanella oneidensis* MR-1. The Journal of Biological Chemistry 284(42), 28865-28873.

Bennaceur, K., Gielen, D., Kerr, T., Tam, C., 2008. CO_2 capture and storage: a key carbon abatement option. OECD.

Bond, D.R., Lovley, D.R., 2003. Electricity production by *Geobacter sulfurreducens* attached to electrodes. Applied and Environmental Microbiology 69(3), 1548-1555.

Bretschger, O., Obraztsova, A., Sturm, C.A., Chang, I.S., Gorby, Y.A., Reed, S.B., Culley, D.E., Reardon, C.L., Barua, S., Romine, M.F., Zhou, J., Beliaev, A.S., Bouhenni, R., Saffarini, D., Mansfeld, F., Kim, B.H., Fredrickson, J.K., Nealson, K.H., 2007. Current production and metal oxide reduction by *Shewanella oneidensis* MR-1 wild type and mutants. Applied and Environmental Microbiology 73(21), 7003-7012.

Brett, C.M., Brett, A.M.O., 1993. Electrochemistry: principles, methods, and applications. Oxford University Press Oxford.

Bullis, K., 2013. http://www.technologyreview.com/news/510066/audi-to-make-fuel-using-solar-power/.

Call, D., Logan, B.E., 2008. Hydrogen production in a single chamber microbial electrolysis cell lacking a membrane. Environmental Science & Technology 42(9), 3401-3406.

Call, D.F., Wagner, R.C., Logan, B.E., 2009. Hydrogen production by *Geobacter species* and a mixed consortium in a microbial electrolysis cell. Applied and Environmental Microbiology 75(24), 7579-7587.

Carlson, H.K., Iavarone, A.T., Gorur, A., Yeo, B.S., Tran, R., Melnyk, R.A., Mathies, R.A., Auer, M., Coates, J.D., 2012. Surface multiheme c-type cytochromes from *Thermincola potens* and implications for respiratory metal reduction by Gram-

positive bacteria. Proceedings of the National Academy of Sciences of the United States of America 109(5), 1702-1707.

Carver, S.M., Vuoriranta, P., Tuovinen, O.H., 2011. A thermophilic microbial fuel cell design. Journal of Power Sources 196(8), 3757-3760.

Chang, I.S., Moon, H., Bretschger, O., Jang, J.K., Park, H.I., Nealson, K.H., Kim, B.H., 2006. Electrochemically active bacteria (EAB) and mediator-less microbial fuel cells. Journal of Microbiology and Biotechnology 16(2), 163-177.

Cheng, S., Xing, D., Call, D.F., Logan, B.E., 2009. Direct Biological Conversion of Electrical Current into Methane by Electromethanogenesis. Environmental Science & Technology 43(10), 3953-3958.

Clauwaert, P., Verstraete, W., 2009. Methanogenesis in membraneless microbial electrolysis cells. Applied Microbiology Biotechnology 82(5), 829-836.

Cracknell, J.A., Vincent, K.A., Armstrong, F.A., 2008. Enzymes as working or inspirational electrocatalysts for fuel cells and electrolysis. Chemical Reviews 108(7), 2439-2461.

Croese, E., Pereira, M.A., Euverink, G.J., Stams, A.J., Geelhoed, J.S., 2011. Analysis of the microbial community of the biocathode of a hydrogen-producing microbial electrolysis cell. Applied Microbiology and Biotechnology 92(5), 1083-1093.

Daniels, L., Belay, N., Rajagopal, B.S., Weimer, P.J., 1987. Bacterial methanogenesis and growth from CO_2 with elemental iron as the sole source of electrons. Science 237(4814), 509-511.

Desloover, J., Arends, J.B.A., Hennebel, T., Rabaey, K., 2012. Operational and technical considerations for microbial electrosynthesis. Biochemical Society Transactions 40, 1233-1238.

El-Naggar, M.Y., Wanger, G., Leung, K.M., Yuzvinsky, T.D., Southam, G., Yang, J., Lau, W.M., Nealson, K.H., Gorby, Y.A., 2010. Electrical transport along bacterial

nanowires from *Shewanella oneidensis* MR-1. Proceedings of the National Academy of Sciences of the United States of America 107(42), 18127-18131.

Fardeau, M.L., Magot, M., Patel, B.K.C., Thomas, P., Garcia, J.L., Ollivier, B., 2000. *Thermoanaerobacter subterraneus* sp nov., a novel thermophile isolated from oilfield water. International Journal of Systematic and Evolutionary Microbiology 50, 2141-2149.

Fedorovich, V., Knighton, M.C., Pagaling, E., Ward, F.B., Free, A., Goryanin, I., 2009. Novel electrochemically active bacterium phylogenetically related to *Arcobacter butzleri*, isolated from a microbial fuel cell. Applied and Environmental Microbiology 75(23), 7326-7334.

Firer-Sherwood, M., Pulcu, G.S., Elliott, S.J., 2008. Electrochemical interrogations of the Mtr cytochromes from *Shewanella*: opening a potential window. Journal of Biological Inorganic Chemistry 13(6), 849-854.

Fricke, K., Harnisch, F., Schröder, U., 2008. On the use of cyclic voltammetry for the study of anodic electron transfer in microbial fuel cells. Energy & Environmental Science 1(1), 144-148.

Fu, Q., Kobayashi, H., Kawaguchi, H., Vilcaez, J., Wakayama, T., Maeda, H., Sato, K., 2013. Electrochemical and phylogenetic analyses of current-generating microorganisms in a thermophilic microbial fuel cell. Journal of Bioscience and Bioengineering 115(3), 268-271.

Fu, Q., Li, J., Zhu, X., Liao, Q., Ye, D., Zhang, L., 2010. An MFC capable of regenerating the cathodic electron acceptor under sunlight. Science China-Technological Sciences 53(9), 2489-2494.

Geelhoed, J.S., Stams, A.J.M., 2011. Electricity-assisted biological hydrogen production from acetate by *Geobacter sulfurreducens*. Environmental Science & Technology 45(2), 815-820.

Global, C., 2011. Institute. The global status of CCS: 2011. Global CCS Institute, Canberra, Australia.

Good, I.J., Toulmin, G.H., 1956. The Number of New Species, and the Increase in Population Coverage, When a Sample Is Increased. Biometrika 43(1-2), 45-63.

Gorby, Y.A., Yanina, S., McLean, J.S., Rosso, K.M., Moyles, D., Dohnalkova, A., Beveridge, T.J., Chang, I.S., Kim, B.H., Kim, K.S., Culley, D.E., Reed, S.B., Romine, M.F., Saffarini, D.A., Hill, E.A., Shi, L., Elias, D.A., Kennedy, D.W., Pinchuk, G., Watanabe, K., Ishii, S., Logan, B., Nealson, K.H., Fredrickson, J.K., 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proceedings of the National Academy of Sciences of the United States of America 103(30), 11358-11363.

Grabowski, A., Nercessian, O., Fayolle, F., Blanchet, D., Jeanthon, C., 2005. Microbial diversity in production waters of a low-temperature biodegraded oil reservoir. FEMS Microbiology Ecology 54(3), 427-443.

Grayc, N.D., Headc, I.M., 2005. Electricity generation from cysteine in a microbial fuel cell. Water Research 39, 942-952.

Greene, A.C., Patel, B.K.C., Sheehy, A.J., 1997. *Deferribacter thermophilus* gen nov, sp nov, a novel thermophilic manganese- and iron-reducing bacterium isolated from a petroleum reservoir. International Journal of Systematic Bacteriology 47(2), 505-509.

Harnisch, F., Freguia, S., 2012. A basic tutorial on cyclic voltammetry for the investigation of electroactive microbial biofilms. Chem Asian J 7(3), 466-475.

Harnisch, F., Schroder, U., 2010. From MFC to MXC: chemical and biological cathodes and their potential for microbial bioelectrochemical systems. Chem Soc Rev 39(11), 4433-4448.

Haszeldine, R.S., 2009. Carbon capture and storage: how green can black be? Science 325(5948), 1647-1652.

Hatchikian, E.C., Papavassiliou, P., Bianco, P., Haladjian, J., 1984. Characterization of cytochrome c_3 from the thermophilic sulfate reducer *Thermodesulfobacterium commune* Journal of Bacteriology 159(3), 1040-1046.

He, Z., Angenent, L.T., 2006. Application of bacterial biocathodes in microbial fuel cells. Electroanalysis 18(19-20), 2009-2015.

Holmes, D.E., Chaudhuri, S.K., Nevin, K.P., Mehta, T., Methe, B.A., Liu, A., Ward, J.E., Woodard, T.L., Webster, J., Lovley, D.R., 2006. Microarray and genetic analysis of electron transfer to electrodes in *Geobacter sulfurreducens*. Environmental Microbiology 8(10), 1805-1815.

Holmes, D.E., Mester, T., O'Neil, R.A., Perpetua, L.A., Larrahondo, M.J., Glaven, R., Sharma, M.L., Ward, J.E., Nevin, K.P., Lovley, D.R., 2008. Genes for two multicopper proteins required for Fe(III) oxide reduction in *Geobacter sulfurreducens* have different expression patterns both in the subsurface and on energy-harvesting electrodes. Microbiology 154(Pt 5), 1422-1435.

Hongo, M., Iwahara, M., 1979. Electrochemical studies on fermentation. 1. Application of electro-energizing method to L-glutamic acid fermentation. Agricultural and Biological Chemistry 43(10), 2075-2081.

Hu, H., Fan, Y., Liu, H., 2008. Hydrogen production using single-chamber membrane-free microbial electrolysis cells. Water Research 42(15), 4172-4178.

Huber, H., Thomm, M., Konig, H., Thies, G., Stetter, K.O., 1982. *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. Archives of Microbiology 132(1), 47-50.

Iino, T., Nakagawa, T., Mori, K., Harayama, S., Suzuki, K., 2008. *Calditerrivibrio nitroreducens* gen. nov., sp. nov., a thermophilic, nitrate-reducing bacterium isolated from a terrestrial hot spring in Japan. International Journal of Systematic and Evolutionary Microbiology 58(Pt 7), 1675-1679.

Jadhav, G.S., Ghangrekar, M.M., 2009. Performance of microbial fuel cell subjected to variation in pH, temperature, external load and substrate concentration. Bioresoure Technology 100(2), 717-723.

Jeremiasse, A.W., Hamelers, H.V., Buisman, C.J., 2010. Microbial electrolysis cell with a microbial biocathode. Bioelectrochemistry 78(1), 39-43.

Jeremiasse, A.W., Hamelers, H.V., Croese, E., Buisman, C.J., 2012. Acetate enhances startup of a H_2-producing microbial biocathode. Biotechnology and Bioengineering 109(3), 657-664.

Jones, W.J., Leigh, J.A., Mayer, F., Woese, C.R., Wolfe, R.S., 1983. *Methanococcus jannaschii* sp. nov., an extremely thermophilic Methanogen from a submarine hydrothermal vent. Archives of Microbiology 136(4), 254-261.

Jong, B.C., Kim, B.H., Chang, I.S., Liew, P.W.Y., Choo, Y.F., Kang, G.S., 2006. Enrichment, performance, and microbial diversity of a thermophilic mediatorless microbial fuel cell. Environmental Science & Technology 40(20), 6449-6454.

Kashefi, K., Holmes, D.E., Reysenbach, A.L., Lovley, D.R., 2002. Use of Fe(III) as an electron acceptor to recover previously uncultured hyperthermophiles: Isolation and characterization of *Geothermobacterium ferrireducens* gen. nov., sp nov. Applied and Environmental Microbiology 68(4), 1735-1742.

Kato, S., Hashimoto, K., Watanabe, K., 2012a. Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals. Environmental Microbiology 14(7), 1646-1654.

Kato, S., Hashimoto, K., Watanabe, K., 2012b. Microbial interspecies electron transfer via electric currents through conductive minerals. Proceedings of the National Academy of Sciences of the United States of America 109(25), 10042-10046.

Kato, T., Haruki, M., Imanaka, T., Morikawa, M., Kanaya, S., 2001. Isolation and characterization of long-chain-alkane degrading *Bacillus thermoleovorans* from deep

subterranean petroleum reservoirs. Journal of Bioscience and Bioengineering 91(1), 64-70.

Keck, A., Rau, J., Reemtsma, T., Mattes, R., Stolz, A., Klein, J., 2002. Identification of quinoide redox mediators that are formed during the degradation of naphthalene-2-sulfonate by *Sphingomonas xenophaga* BN6. Applied and Environmental Microbiology 68(9), 4341-4349.

Kiely, P.D., Cusick, R., Call, D.F., Selembo, P.A., Regan, J.M., Logan, B.E., 2011. Anode microbial communities produced by changing from microbial fuel cell to microbial electrolysis cell operation using two different wastewaters. Bioresource Technology 102(1), 388-394.

Kim, T.S., Kim, B.H., 1988. Electron flow shift in *Clostridium acetobutylicum* fermentation by electrochemically introduced reducing equivalent. Biotechnology Letters 10(2), 123-128.

Kobayashi, H., Saito, N., Fu, Q., Kawaguchi, H., Vilcaez, J., Wakayama, T., Maeda, H., Sato, K., 2013. Bio-electrochemical property and phylogenetic diversity of microbial communities associated with bioelectrodes of an electromethanogenic reactor. Journal of Bioscience and Bioengineering. 114-117.

Kundu, A., Sahu, J.N., Redzwan, G., Hashim, M.A., 2013. An overview of cathode material and catalysts suitable for generating hydrogen in microbial electrolysis cell. International Journal of Hydrogen Energy 38(4), 1745-1757.

Lamle, S.E., Vincent, K.A., Halliwell, L.M., Albracht, S.P.J., Armstrong, F.A., 2003. Hydrogenase on an electrode: a remarkable heterogeneous catalyst. Dalton Transactions(21), 4152-4157.

Lee, H.-S., Rittmann, B.E., 2010. Significance of Biological Hydrogen Oxidation in a Continuous Single-Chamber Microbial Electrolysis Cell. Environmental Science & Technology 44(3), 948-954.

Lee, H.S., Vermaas, W.F.J., Rittmann, B.E., 2010. Biological hydrogen production: prospects and challenges. Trends in Biotechnology 28(5), 262-271.

Li, H., Opgenorth, P.H., Wernick, D.G., Rogers, S., Wu, T.-Y., Higashide, W., Malati, P., Huo, Y.-X., Cho, K.M., Liao, J.C., 2012. Integrated electromicrobial conversion of CO_2 to higher alcohols. Science 335(6076), 1596-1596.

Li, J., Fu, Q., Liao, Q., Zhu, X., Ye, D.D., Tian, X., 2009. Persulfate: A self-activated cathodic electron acceptor for microbial fuel cells. Journal of Power Sources 194(1), 269-274.

Lithgow, A.M., Romero, L., Sanchez, I.C., Souto, F.A., Vega, C.A., 1986. Interception of the electron-transport chain in bacteria with hydrophilic redox mediators. 1. Selective improvement of the performance of biofuel cells with 2,6-disulfonated thionine as mediator. Journal of Chemical Research-S(5), 178-179.

Liu, H., Grot, S., Logan, B.E., 2005. Electrochemically assisted microbial production of hydrogen from acetate. Environmental Science & Technology 39(11), 4317-4320.

Liu, W.-z., Wang, A.-j., Ren, N.-q., Zhao, X.-y., Liu, L.-h., Yu, Z.-g., Lee, D.-j., 2008. Electrochemically assisted biohydrogen production from acetate. Energy & Fuels 22(1), 159-163.

Liu, W.Z., Wang, A.J., Cheng, S.A., Logan, B.E., Yu, H., Deng, Y., Van Nostrand, J.D., Wu, L.Y., He, Z.L., Zhou, J.Z., 2010. Geochip-Based Functional Gene Analysis of Anodophilic Communities in Microbial Electrolysis Cells under Different Operational Modes. Environmental Science & Technology 44(19), 7729-7735.

Logan, B., 2005. Generating electricity from wastewater treatment. Water environment research: a research publication of the Water Environment Federation 77(3), 211.

Logan, B.E., 2008. Microbial fuel cells. Wiley-Interscience.

Logan, B.E., 2009. Exoelectrogenic bacteria that power microbial fuel cells. Nature Reviews Microbiology 7(5), 375-381.

Logan, B.E., Call, D., Cheng, S., Hamelers, H.V., Sleutels, T.H., Jeremiasse, A.W., Rozendal, R.A., 2008. Microbial electrolysis cells for high yield hydrogen gas production from organic matter. Environ Sci Technol 42(23), 8630-8640.

Logan, B.E., Hamelers, B., Rozendal, R.A., Schrorder, U., Keller, J., Freguia, S., Aelterman, P., Verstraete, W., Rabaey, K., 2006. Microbial fuel cells: Methodology and technology. Environmental Science & Technology 40(17), 5181-5192.

Logan, B.E., Murano, C., Scott, K., Gray, N.D., Head, I.M., 2005. Electricity generation from cysteine in a microbial fuel cell. Water Research 39(5), 942-952.

Logan, B.E., Regan, J.M., 2006. Microbial fuel cells-challenges and applications. Environmental Science & Technology 40(17), 5172-5180.

Lojou, E., Durand, M.C., Dolla, A., Bianco, P., 2002. Hydrogenase activity control at Desulfovibrio vulgaris cell-coated carbon electrodes: Biochemical and chemical factors influencing the mediated bioelectrocatalysis. Electroanalysis 14(13), 913-922.

Lovley, D.R., 2006. Bug juice: harvesting electricity with microorganisms. Nature Reviews Microbiology 4(7), 497-508.

Lovley, D.R., 2011a. Live wires: direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. Energy & Environmental Science 4(12), 4896-4906.

Lovley, D.R., 2011b. Powering microbes with electricity: direct electron transfer from electrodes to microbes. Environmental Microbiology Reports 3(1), 27-35.

Lovley, D.R., 2012. Electromicrobiology. Annual Review of Microbiology 66, 391-409.

Malvankar, N.S., Tuominen, M.T., Lovley, D.R., 2012. Biofilm conductivity is a decisive variable for high-current-density *Geobacter sulfurreducens* microbial fuel cells. Energy & Environmental Science 5(2), 5790-5797.

Marshall, C.W., May, H.D., 2009. Electrochemical evidence of direct electrode reduction by a thermophilic Gram-positive bacterium, *Thermincola ferriacetica*. Energy & Environmental Science 2(6), 699-705.

Marshall, C.W., Ross, D.E., Fichot, E.B., Norman, R.S., May, H.D., 2012. Electrosynthesis of commodity chemicals by an autotrophic microbial community. Applied and Environmental Microbiology 78(23), 8412-8420.

Marsili, E., Baron, D.B., Shikhare, I.D., Coursolle, D., Gralnick, J.A., Bond, D.R., 2008. *Shewanella* secretes flavins that mediate extracellular electron transfer. Proceedings of the National Academy of Sciences of the United States of America 105(10), 3968-3973.

Marsili, E., Sun, J., Bond, D.R., 2010. Voltammetry and growth physiology of *Geobacter sulfurreducens* biofilms as a function of growth stage and imposed electrode potential. Electroanalysis, 865-874.

Martinot, E., Sawin, J.L., 2011. Renewables 2011, Global Status Report. Renewable Energy Policy Network for the 21st Century REN21 2011.

Mathis, B.J., Marshall, C.W., Milliken, C.E., Makkar, R.S., Creager, S.E., May, H.D., 2008. Electricity generation by thermophilic microorganisms from marine sediment. Applied Microbiology and Biotechnology 78(1), 147-155.

Metz, B., 2005. Carbon Dioxide Capture and Storage: Special Report of the Intergovernmental Panel on Climate Change. Cambridge University Press.

Min, B., Kim, J.R., Oh, S.E., Regan, J.M., Logan, B.E., 2005. Electricity generation from swine wastewater using microbial fuel cells. Water research 39(20), 4961-4968.

Miroshnichenko, M.L., 2003. *Deferribacter abyssi* sp. nov., an anaerobic thermophile from deep-sea hydrothermal vents of the Mid-Atlantic Ridge. International Journal of Systematic and Evolutionary Microbiology 53(5), 1637-1641.

Muncuk, U., 2012. Design optimization and implementation for RF energy harvesting circuits. Emerging and Selected Topics in Circuits and Systems, IEEE Journal 2(1), 24 - 33

Nevin, K.P., Hensley, S.A., Franks, A.E., Summers, Z.M., Ou, J., Woodard, T.L., Snoeyenbos-West, O.L., Lovley, D.R., 2011. Electrosynthesis of organic compounds from carbon dioxide is catalyzed by a diversity of acetogenic microorganisms. Applied and Environmental Microbiology 77(9), 2882-2886.

Nevin, K.P., Richter, H., Covalla, S.F., Johnson, J.P., Woodard, T.L., Orloff, A.L., Jia, H., Zhang, M., Lovley, D.R., 2008. Power output and columbic efficiencies from biofilms of *Geobacter sulfurreducens* comparable to mixed community microbial fuel cells. Environmental Microbiology 10(10), 2505-2514.

Nevin, K.P., Woodard, T.L., Franks, A.E., Summers, Z.M., Lovley, D.R., 2010. Microbial electrosynthesis: feeding microbes electricity to convert carbon dioxide and water to multicarbon extracellular organic compounds. MBio 1(2): e00103-10. doi:10.1128/mBio.00103-10..

Newman, D.K., Kolter, R., 2000. A role for excreted quinones in extracellular electron transfer. Nature 405(6782), 94-97.

Niehaus, F., Bertoldo, C., Kahler, M., Antranikian, G., 1999. Extremophiles as a source of novel enzymes for industrial application. Applied Microbiology and Biotechnology 51(6), 711-729.

Ogg, C.D., Patel, B.K., 2009. *Caloramator australicus* sp. nov., a thermophilic, anaerobic bacterium from the Great Artesian Basin of Australia. International Journal of Systematic and Evolutionary Microbiology 59(Pt 1), 95-101.

Ogg, C.D., Patel, B.K.C., 2011. Draft gnome squence of *Caloramator australicus* srain RC3(T), a *Thermoanaerobe* from the great Artesian basin of Australia. Journal of Bacteriology 193(10), 2664-2665.

Oldenburg, C., Pruess, K., Benson, S.M., 2001. Process modeling of CO_2 injection into natural gas reservoirs for carbon sequestration and enhanced gas recovery. Energy & Fuels 15(2), 293-298.

Park, D.H., Zeikus, J.G., 2000. Electricity generation in microbial fuel cells using neutral red as an electronophore. Applied and Environmental Microbiology 66(4), 1292-1297.

Parry, M.L., 2007. Climate Change 2007: Impacts, Adaptation and Vulnerability: Working Group I Contribution to the Fourth Assessment Report of the IPCC. Cambridge University Press.

Patel, B.K.C., Morgan, H.W., Daniel, R.M., 1985. A simple and efficient method for preparing and dispensing anaerobic media. Biotechnology Letters 7(4), 277-278.

Patil, S.A., Harnisch, F., Kapadnis, B., Schroder, U., 2010. Electroactive mixed culture biofilms in microbial bioelectrochemical systems: The role of temperature for biofilm formation and performance. Biosensors and Bioelectronics 26(2), 803-808.

Pisciotta, J.M., Zaybak, Z., Call, D.F., Nam, J.Y., Logan, B.E., 2012. Enrichment of microbial electrolysis cell biocathodes from sediment microbial fuel cell bioanodes. Applied and Environmental Microbiology 78(15), 5212-5219.

Potter, M.C., 1911. Electrical effects accompanying the decomposition of organic compounds. Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character 84(571), 260-276.

Price-Whelan, A., Dietrich, L.E.P., Newman, D.K., 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. Nature Chemical Biology 2(2), 71-78.

Rabaey, K., Girguis, P., Nielsen, L.K., 2011. Metabolic and practical considerations on microbial electrosynthesis. Current Opinion in Biotechnology 22(3), 371-377.

Rabaey, K., Rozendal, R.A., 2010. Microbial electrosynthesis - revisiting the electrical route for microbial production. Nature Reviews Microbiology 8(10), 706-716.

Rabaey, K., Verstraete, W., 2005. Microbial fuel cells: novel biotechnology for energy generation. Trends in Biotechnology 23(6), 291-298.

Rasmussen, S.B., Kustov, A., Due-Hansen, J., Siret, B., Tabaries, F., Fehrmann, R., 2006. Characterization and regeneration of Pt-catalysts deactivated in municipal waste flue gas. Applied Catalysis B-Environmental 69(1-2), 10-16.

Reguera, G., McCarthy, K.D., Mehta, T., Nicoll, J.S., Tuominen, M.T., Lovley, D.R., 2005. Extracellular electron transfer via microbial nanowires. Nature 435(7045), 1098-1101.

Reguera, G., Nevin, K.P., Nicoll, J.S., Covalla, S.F., Woodard, T.L., Lovley, D.R., 2006. Biofilm and nanowire production leads to increased current in *Geobacter sulfurreducens* fuel cells. Applied and Environmental Microbiology 72(11), 7345-7348.

Reguera, G., Pollina, R.B., Nicoll, J.S., Lovley, D.R., 2007. Possible nonconductive role of *Geobacter sulfurreducens* pilus nanowires in biofilm formation. Journal of Bacteriology 189(5), 2125-2127.

Richter, H., Nevin, K.P., Jia, H., Lowy, D.A., Lovley, D.R., Tender, L.M., 2009. Cyclic voltammetry of biofilms of wild type and mutant *Geobacter sulfurreducens* on fuel cell anodes indicates possible roles of OmcB, OmcZ, type IV pili, and protons in extracellular electron transfer. Energy & Environmental Science 2(5), 506-516.

Rittmann, B.E., Krajmalnik-Brown, R., Halden, R.U., 2008. Pre-genomic, genomic and postgenomic study of microbial communities involved in bioenergy. Nature Reviews Microbiology 6(8), 604-612.

Rosenbaum, M., Aulenta, F., Villano, M., Angenent, L.T., 2011. Cathodes as electron donors for microbial metabolism: which extracellular electron transfer mechanisms are involved? Bioresour Technol 102(1), 324-333.

Ross, H.E., Hagin, P., Zoback, M.D., 2009. CO_2 storage and enhanced coalbed methane recovery: Reservoir characterization and fluid flow simulations of the Big George coal, Powder River Basin, Wyoming, USA. International Journal of Greenhouse Gas Control 3(6), 773-786.

Rozendal, R., Hamelers, H., Euverink, G., Metz, S., Buisman, C., 2006. Principle and perspectives of hydrogen production through biocatalyzed electrolysis. International Journal of Hydrogen Energy 31(12), 1632-1640.

Rozendal, R.A., Hamelers, H.V., Rabaey, K., Keller, J., Buisman, C.J., 2008a. Towards practical implementation of bioelectrochemical wastewater treatment. Trends in Biotechnology 26(8), 450-459.

Rozendal, R.A., Jeremiasse, A.W., Hamelers, H.V.M., Buisman, C.J.N., 2008b. Hydrogen production with a microbial biocathode. Environmental Science & Technology 42(2), 629-634.

Rubin, E., de Coninck, H., 2005. IPCC special report on carbon dioxide capture and storage. UK: Cambridge University Press. TNO (2004): Cost Curves for CO_2 Storage, Part 2.

Sato, K., Kawaguchi, H., Kobayashi, H., 2013. Bio-electrochemical conversion of carbon dioxide to methane in geological storage reservoirs. Energy Conversion and Management 66, 343-350.

Schroeder, U., 2007. Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. Physical Chemistry Chemical Physics 9(21), 2619-2629.

Shafiee, S., Topal, E., 2009. When will fossil fuel reserves be diminished? Energy Policy 37(1), 181-189.

Shizas, I., Bagley, D.M., 2004. Experimental determination of energy content of unknown organics in municipal wastewater streams. Journal of Energy Engineering 130(2), 45-53.

Solomon, S., Carpenter, M., Flach, T.A., 2008. Intermediate storage of carbon dioxide in geological formations: A technical perspective. International Journal of Greenhouse Gas Control 2(4), 502-510.

Steinbusch, K.J.J., Hamelers, H.V.M., Schaap, J.D., Kampman, C., Buisman, C.J.N., 2010. Bioelectrochemical Ethanol Production through Mediated Acetate Reduction by Mixed Cultures. Environmental Science & Technology 44(1), 513-517.

Strycharz, S.M., Malanoski, A.P., Snider, R.M., Yi, H., Lovley, D.R., Tender, L.M., 2011. Application of cyclic voltammetry to investigate enhanced catalytic current generation by biofilm-modified anodes of *Geobacter sulfurreducens* strain DL1 vs. variant strain KN400. Energy & Environmental Science 4(3), 896-913.

Sund, C.J., McMasters, S., Crittenden, S.R., Harrell, L.E., Sumner, J.J., 2007. Effect of electron mediators on current generation and fermentation in a microbial fuel cell. Applied Microbiology and Biotechnology 76(3), 561-568.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24(8), 1596-1599.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences of the United States of America 101(30), 11030-11035.

Tatsumi, H., Takagi, K., Fujita, M., Kano, K., Ikeda, T., 1999. Electrochemical study of reversible hydrogenase reaction of *Desulfovibrio vulgaris* cells with methyl viologen as an electron carrier. Analytical Chemistry 71(9), 1753-1759.

Thygesen, A., Poulsen, F.W., Min, B., Angelidaki, I., Thomsen, A.B., 2009. The effect of different substrates and humic acid on power generation in microbial fuel cell operation. Bioresource Technology 100(3), 1186-1191.

Villano, M., Aulenta, F., Ciucci, C., Ferri, T., Giuliano, A., Majone, M., 2010. Bioelectrochemical reduction of CO_2 to CH_4 via direct and indirect extracellular electron transfer by a hydrogenophilic methanogenic culture. Bioresourc Technology 101(9), 3085-3090.

Villano, M., De Bonis, L., Rossetti, S., Aulenta, F., Majone, M., 2011. Bioelectrochemical hydrogen production with hydrogenophilic dechlorinating bacteria as electrocatalytic agents. Bioresource Technology 102(3), 3193-3199.

Von Canstein, H., Ogawa, J., Shimizu, S., Lloyd, J.R., 2008. Secretion of flavins by *Shewanella* species and their role in extracellular electron transfer. Applied and Environmental Microbiology 74(3), 615-623.

Wagner, R.C., Regan, J.M., Oh, S.E., Zuo, Y., Logan, B.E., 2009. Hydrogen and methane production from swine wastewater using microbial electrolysis cells. Water Research 43(5), 1480-1488.

Wang, A., Liu, W., Cheng, S., Xing, D., Zhou, J., Logan, B.E., 2009. Source of methane and methods to control its formation in single chamber microbial electrolysis cells. International Journal of Hydrogen Energy 34(9), 3653-3658.

Wrighton, K.C., Agbo, P., Warnecke, F., Weber, K.A., Brodie, E.L., DeSantis, T.Z., Hugenholtz, P., Andersen, G.L., Coates, J.D., 2008. A novel ecological role of the *Firmicutes* identified in thermophilic microbial fuel cells. The ISME Journal 2(11), 1146-1156.

Wrighton, K.C., Thrash, J.C., Melnyk, R.A., Bigi, J.P., Byrne-Bailey, K.G., Remis, J.P., Schichnes, D., Auer, M., Chang, C.J., Coates, J.D., 2011. Evidence for direct electron transfer by a Gram-positive bacterium isolated from a microbial fuel cell. Applied and Environmental Microbiology 77(21), 7633-7639.

Xing, D., Cheng, S., Logan, B.E., Regan, J.M., 2010. Isolation of the exoelectrogenic denitrifying bacterium *Comamonas denitrificans* based on dilution to extinction. Applied Microbiology and Biotechnology 85(5), 1575-1587.

Xue, Y.F., Xu, Y., Liu, Y., Ma, Y.H., Zhou, P.J., 2001. *Thermoanaerobacter tengcongensis* sp nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tengcong, China. International Journal of Systematic and Evolutionary Microbiology 51, 1335-1341.

You, S., Zhao, Q., Zhang, J., Jiang, J., Zhao, S., 2006. A microbial fuel cell using permanganate as the cathodic electron acceptor. Journal of Power Sources 162(2), 1409-1415.

Yu, L., Duan, J., Zhao, W., Huang, Y., Hou, B., 2011. Characteristics of hydrogen evolution and oxidation catalyzed by *Desulfovibrio caledoniensis* biofilm on pyrolytic graphite electrode. Electrochimica Acta 56(25), 9041-9047.

Zeikus, J.G., Dawson, M.A., Thompson, T.E., Ingvorsen, K., Hatchikian, E.C., 1983. Microbial ecology of volcanic sulfidogenesis. Isolation and characterization of *Thermodesulfobacterium commune* gen. nov. and sp. nov. Journal of General Microbiology 129(APR), 1159-1169.

Zerta, M., Schmidt, P.R., Stiller, C., Landinger, H., 2008. Alternative World Energy Outlook (AWEO) and the role of hydrogen in a changing energy landscape. International Journal of Hydrogen Energy 33(12), 3021-3025.

Zhang, L., Zhu, X., Li, J., Liao, Q., Ye, D.D., 2011. Biofilm formation and electricity generation of a microbial fuel cell started up under different external resistances. Journal of Power Sources 196(15), 6029-6035.

Zhang, T., Nie, H., Bain, T.S., Lu, H., Cui, M., Snoeyenbos-West, O.L., Franks, A.E., Nevin, K.P., Russell, T.P., Lovley, D.R., 2013. Improved cathode materials for microbial electrosynthesis. Energy & Environmental Science 6(1), 217-224.

Zhang, Y., Merrill, M.D., Logan, B.E., 2010. The use and optimization of stainless steel mesh cathodes in microbial electrolysis cells. International Journal of Hydrogen Energy 35(21), 12020-12028.

Zinder, S.H., 1993. Physiological ecology of methanogens. Methanogenesis, pp. 128-206. Springer.