Comprehensive Study of Biocathode in Bio-electrochemical System (BES) for Energy Harvesting and CO_2 Conversion

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Abstract

The focus of this thesis is studying the mixed community biocathode to solve the bottlenecks for the scale-up and commercialisation of Bio-electrochemical Systems (BESs).

First, the utilisation of aerobic iron-oxidising bacteria enriched from natural environment and Fe^{2+} in Microbial fuel cell (MFC) equipped with gas diffusion electrodes was studied as an efficient alternative to Pt-based catalysts for MFCs with unlimited oxygen diffusion. Controlling the system pH during continuous operational mode, MFCs produced maximum power density of 0.251 mW cm^{-2}, compared to 0.134 mW cm^{-2} for MFCs equipped with Pt catalyst (0.5 mg cm^{-2} Pt on carbon paper) indicating advantages of fully microorganism catalysed system, and importance of operational parameters in such systems.

In microbial electrosynthesis (MES) converting CO_2 to various organic compounds, optimisation of operational parameters, such as applied potential on the cathode, pH, and operational mode were investigated. The community analysis revealed different dominant communities on the electrode and in solution suggesting an electro-active biofilm was formed on the cathode. It also suggested the importance of cathodic potentials corresponding to sufficient energy provided for MES and inorganic carbon sources (CO_2 and HCO_3^-) on biofilm formation and composition of bacterial community in biofilm responsible for MES, respectively. It was found that -1.0 V (Ag/AgCl) was required for MES, and CO_2 was preferred inorganic carbon source for microorganisms for MES. Mechanisms of MES and chain elongation were studied. The hypothesis on hydrogen mediated electron transfer mechanism was confirmed from cyclic voltammetry showing a positive shift on hydrogen evolution onset potential with optimum conditions. The role of cathode on providing electrons was explored comparing the systems operated at open circuit potential (OCP) and applied potential. In addition, two different strategies of 1) impact of continuous operational mode and, 2) addition of external electron donors were investigated to promote chain elongation through acetate, triggering crucial parameters affecting production through MES.

Acetate concentration reached the maximum of 6.8 g L^{-1} over fed-batch mode with a production rate of 660 ppm day-1 (maximum columbic efficiency~69%), while the highest acetate production rate in this study was reached over continuous operational mode (803 ppm day^{-1}, maximum columbic efficiency~90%). Over longer operational time and providing extra electron donors, longer chain organic acids (Butyrate) and alcohols (Butanol and Hexanol) were produced, concluding MES is a promising technology alternative to petrochemical production.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ADP Adenosine diphosphate

AEM Anion exchange membrane

Ag/AgCl Sliver/Silver Chloride Reference Electrode

ANOVA Analysis of Variance

ARB Anode respiring bacteria

ASV Amplicon sequence variant

ATP Adenosine triphosphate

BES Bio-electrochemical system

BID Barrier Discharge Ionization

BLAST Basic Local Alignment Search

BOD Biological oxygen demand

bp base pairs

CA Chronoamperometry

CDE Carbon dioxide equivalent

CE Counter electrode

CE% Columbic efficiency

CEM Cation exchange membrane

CH_4 Methane

CO_2 Carbon dioxide

CO Carbon monoxide

COD Chemical oxygen demand

CoTMMP Cobalt-based materials

CV Cyclic voltammetry

D Depth

DET Direct electron transfer

DNA Deoxyribonucleic acid

EDTA Ethylenediaminetetraacetic acid

EET Extracellular electron transfer

EF Electro-fermentation

EIS Electrochemical impedance spectroscopy

EPS Extracellular polymeric substance

Fe Iron

FePc Iron phthalocyanine

FSC Forward scatter

GDE Gas diffusion electrode

GDL Gas diffusion layer

Gt Gigatonne

H Height

H_2 Hydrogen

HCO_3^- Bicarbonate

HER Hydrogen evolution reaction

HRT Hydraulic Retention Time

IC Inorganic carbon

IET Indirect electron transfer

IOB Iron oxidising bacteria

MANOVA Multivariate analysis of variance

MEC Microbial electrolysis cell

MFC Microbial fuel cell

MES Microbial electrosynthesis

N_2 Nitrogen

NAD^+ Nicotinamide adenine dinucleotide

NCBI National centre for biotechnology information

NMDS Non-metric multidimensional scaling

O_2 Oxygen

OCP Open circuit potential

ORR Oxygen reduction reaction

OTU Operational taxonomic unit

PBS Phosphate buffer solution

PCR Polymerase chain reaction

Pt Platinum

Pt-C Platinum on carbon

PTFE Polytetrafluoroethylene

RDP Ribosomal Database Project

RE Reference electrode

RNA Ribonucleic acid

rRNA Ribosomal RNA

rrnDB Ribosomal RNA operon database

SEM Scanning electron microscopy

PI Propidium iodide

SMFC Sedimentary microbial fuel cell

TC Total carbon

TOC Total organic carbon

VFA Volatile fatty acid

W Width

WE Working electrode

WL Wood-Ljungdahl

Publications

Articles

- P.Izadi, J.M. Fontmorin, L. F. L. Fernandez, S. Cheng, I. Head, E. H. Yu, "High performing gas diffusion biocathode for microbial fuel cell (MFC) using acidophilic oxidising bacteria" *Frontiers in Energy Research*, 2019.
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- P.Izadi, J.M. Fontmorin, A. Godain, E. Yu, I. Head, "Cathodic potential is key to physical properties of cathodic biofilm developed during microbial eleoctosynthesis (MES), but its composition is controlled by inorganic carbon source" (in preparation)
- P. Izadi, J.M. Fontmorin, I. Head, E. Yu, "Strategies for increasing long chain bio-products from CO_2 by Microbial electrosynthesis (MES)" (in preparation)
- Beate Christgen, P.Izadi, Edward Milner, Clare McCann, E. Yu, I. Head, "Effect of different types of inoculum and substrate on the performance of microbial fuel cells (MFCs) " (in preparation)

Conferences

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- P.Izadi, J.M. Fontmorin, E. Yu, I. Head, " Critical factors affecting biocathode development and bio-production from CO_2 reduction in Microbial electrosynthesis (MES)" 16th IWA World Conference on Anaerobic Digestion (AD 16), June 2019, Delft, The Netherlands.
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- P. Izadi, J.M. Fontmorin, E. Yu, I. Head, "Microbial electrochemical synthesis of carbohydrates from carbon dioxide reduction" European Fuel Cell (EFC) Conference, December 2017, Naples, Italy.
- P. Izadi, E. Milner, E. Yu, I. Head, " Investigating the effect of inoculum and substrate type on microbial fuel cells (MFCs) performance" in European International Society for Microbial Electrochemistry and Technology (EU-ISMET) conference, September 2016, Rome, Italy.

Chapter 1. Introduction

1.1 Background and context

Over the last few decades, fossil fuels have been used as the main source of energy for human and industrial activities. Apart from the risk of depletion of fossil fuels in near future, the intense consumption of these resources has led to the horrendous effects on environment such as global warming and climate change. Carbon dioxide (CO_2) emission to atmosphere from burning the fossil fuels is believed as the main reason for the dreadful consequences of climate change (DNV, 2011). Production of CO_2 from combustion of fossil fuels has been estimated to reach 15 Gt $year^{-1}$ in the world by 2035 (DNV, 2011). The UK had set a target to cut greenhouse gas emissions by 80 % relative to 1990 levels, by 2050 through Climate Change Act (2008), which in June 2019 the new target has set requiring the UK to bring the greenhouse gas emissions to net zero by 2050 (Banister, 2019). Over the same time, the population of the UK is expected to grow by 15-30% associated with increase in water and energy use in both domestic and industry. This raises the demand on new technologies to reduce the emission and capture of CO_2 produced from various processes. Different methods have been suggested and studied in order to either capture or utilize CO_2 from atmosphere (DNV, 2011). Apart from CO_2, global release of carbon-rich liquid and solid wastes and municipal wastewater has brought serious concerns on environmental and public health (Jiang et al., 2018). Novel technology is in urgently need to tackle the challenges. Bio-electrochemical systems (BESs) are the promising technologies combining electrochemistry, microbiology and biotechnology. Due to this versatility, they have various applications of wastewater treatment, electricity generation and resource recovery, as well as conversion of CO_2 to valuable products, attracting academic and industrial attentions. The primary interest in BESs includes the ability of these systems in degradation of organics in wastes using microorganisms, conversion of chemical energy to electricity and also conversion of electricity into favourable chemical energy (Rabaey and Rozendal, 2010). Microbial fuel cells (MFCs) are the important type of BESs, able to treat

wastewater using microorganisms in anode compartments and produce energy simultaneously (Seelam et al., 2018). Conventional MFCs have commonly two separate compartments of anode and cathode. Due to the high redox potential of oxygen, the common reaction in cathode compartments are the oxygen reduction reaction (ORR) (Oliveira et al., 2013). Other possible cathodic reactions can be metal recovery or denitrification (Milner, 2015). Alternatively, under anaerobic condition, CO_2 can be a terminal electron acceptor and can be converted to organic products using microorganisms. Due to these promising applications of BESs and their potential utilization in industrial scales in future, more fundamental studies on BESs are required to gain better understanding of these systems.

1.2 Limitations, hypothesis, aim and objectives

One of the promising applications of BESs is simultaneous wastewater treatment and electricity generation. However, low value of power produced in MFCs is still a major bottleneck for the scale-up and commercialisation of MFCs. It has been shown that cathode is one of the limiting factors in receiving the electrons transferred from anodic compartment and using them in selective cathodic reactions. In order to gain higher power production in MFCs, strong selective cathodic reactions are required. Different methods have been suggested to improve the reduction reaction at the cathode. Using different electron acceptors with high reduction potential such as permanganate or ferricyanide showed improvement in terms of electricity generation (Oh et al., 2004, Kong et al., 2010, Oliveira et al., 2013). However, due to the irreversibility of these reactions, catholyte should be replaced regularly. Therefore, this method is not economically favourable particularly at larger scale. So far, ORR at the cathode catalysed by chemical catalysts has been the most favourable and convenient cathodic reactions in MFCs, as carbon based electrodes are not efficient catalysts for such reactions. However, one of the biggest cost of MFCs arises from the chemical catalysts such as Platinum (Pt), transition metal-based catalysts, metal oxide catalysts or activated carbon, particularly for larger scale MFCs (HaoYu et al., 2007, Liew et al., 2014, Burkitt et al., 2016). Apart from the cost, using these chemicals are associated with sustainability issues and lack of robustness. Biological catalysts have different advantages such as they are completely free, stable in long term and environmentally sustainable. Aerobic biocathode using microorganisms seems a proper substitute to chemical catalysts in catalysing ORR at the cathode (Milner et al., 2016). Despite the advantages of aerobic biocatalysts for ORR, a long start-up of such biocathodes (more than 50 days) is one of the main difficulties of their development (Milner et al., 2016).

In addition, the focus on BESs has been extended to other possible reactions in cathodic compartments than ORR offering another unique application. Microbial electrosynthesis (MES) is another type of BESs able to convert CO_2 using microorganisms to value-added products of organic acids and alcohols (Rabaey et al., 2011). It was suggested that CO_2 utilisation can be performed with different methods through three major pathways: 1) Conversion to chemical feedstocks 2) Conversion to chemical fuels 3) Using as solvent and working fluids (DNV, 2011). In case of CO_2 conversion to organic fuels different chemical, biological and electrochemical methods has been suggested. CO_2 is a strong molecule and catalysts are required to reduce it. Although electrochemical methods offer efficient techniques in CO_2 sequestration, the catalysts used in these methods are typically metals which are expensive and not robust over long term operations. Bio-electrochemical method is the combination of biological and electrochemical methods with significant advantages compared to other methods. The benefits of BES over other methods are due to the facts that biocatalysts responsible for CO_2 reduction offer a cheap, sustainable and robust technology with ambient operational conditions such as temperature and pressure (Liew et al., 2014, He and Angenent, 2006, Milner et al., 2014). Under anaerobic conditions, bacterial community in these biocatlaysts are able to reduce CO_2 and produce other valuable products. Depending on operational conditions and bacterial community products of MES vary. The nature of biocatalysts involved in the reduction of CO_2 in MES plays a key role in the selectivity of products due to different pathways of different bacterial communities (Jiang and Zeng, 2018b). The unique capability of such biocatalysts in production of value-added products such as short and long chain organic acids and alcohols from CO_2 in BESs makes this technology of interest. Several studies reported different types of products such as acetate, butyrate, ethanol, butanol, methane and longer chain products such as caproate and caprylate (Van Eerten-Jansen et al., 2013a) and in different operational conditions, biopolymer (Chen et al., 2018b, Bajracharya et al., 2016a).

Although MES is an emerging technique with promising potential application, it is still little understood about biocathode development in MES, biofilm formation and its responsibility in MES, growth and composition of biofilm, role of cathode in MES processes and the mechanism of electron transfer between microorganisms and cathodes. To seek whether such technology has a potential of commercialization, a deep understanding of the processes and parameters affecting them is essential. Therefore, the main aim of this thesis is to study aerobic and anaerobic biocathode to gain fundamental understanding concerning their development and function in BES. The first target was developing aerobic biocathode catalysing ORR comparable with chemical catalysts in enhancing energy harvesting. The second target was

developing a low cost BES to convert CO_2 to value-added chemicals or liquid fuels as energy storage medium or chemical feedstock using developed anaerobic biocathode.
Specifically, the objectives of this thesis are described as below:

1. Develop a high performing aerobic biocathode from mixed culture of microorganisms with a quick start-up

- Despite the various advantages of aerobic biocathodes, their development in BESs are very slow and time-consuming. However, it was hypothesized that aerobic biocathodes using mediator could accelerate electron transfer and subsequently start-up of the system. Gas diffusion electrode (GDE) reactor configuration was designed to provide the sufficient oxygen for acidophilic iron oxidising bacteria enriched from natural environmental samples, with the purpose of accelerating biocathode development and enhancing MFC performance. The eventual target was operating a low cost MFC coupled with mediated iron oxidising biocathode to achieve the power production comparable with that in MFC equipped with chemical catalysts as cathodes.

2. Develop an active anaerobic biocathode for the reduction of CO_2 and synthesis of valuable chemicals

- Enrichment of desired CO_2 reducing bacteria from mixed culture of microorganisms can be challenging, due to presence of different types of bacteria competing in consuming CO_2. Before transferring the inoculum to BESs, it was important to enrich the desired bacteria from mixed culture of microorganisms and later grow them as a biocathode in BESs. As organic acids were the evident more desirable products than methane, enrichment stage was done to suppress the competitive bacterial community of methanogens in the system, and biocathode was developed in the BES for further studies. The effect of different cathodic potentials and inorganic carbon sources were investigated on development of biocathode, bacterial growth and start-up of the system to calrify the best condition for MES processes.

3. Assess the possibility of biofilm formation at the surface of a cathode during MES processes, and its role in production through MES

- The possibility of biofilm formation at the surface of the cathodes were compared at different cathodic potentials and inorganic carbon sources. Physical and electrochemical properties of biofilms at different conditions were characterised

using bio-imaging analysis of confocal microscopy and scanning electron microscopy (SEM), and electrochemical analysis of cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), respectively, while bacterial composition of biofilms were studied through community analysis of 16S. In addition, to clarify the bacteria responsible for MES, whether MES tends to happen in suspension or at the electrode, the community analysis from the biocathode at the electrodes were compared by that in suspension.

4. Examine the effect of continuous feeding regime in BESs on production

- It was hypothesised that providing constant fresh nutrients and inorganic carbon for biocathode and adjusting the catholytes pH could enhance the production particularly towards longer chain products. In this direction, BESs were operated in continuous operational mode and the effect of different hydraulic retention times (HRTs) on the production of system was assessed. Community analysis was performed from the suspension to compare the bacterial community before and after continuous operational mode applied.

5. Gain better understanding in production of longer chain organic acids and alcohols

- Acetate is the base unit of chain elongation (production of long chain organic acids and alcohols). It was reported that cathode as a sole energy source is not sufficient for chain elongation from acetate and another source of electron is required. To study the effect of different electron donors on production in the experiment, formate and ethanol were supplied in BESs in addition to polarised cathodes. Community analysis was performed from the suspension to compare the bacterial community before and after supplying electron donors. Importantly, the function of cathode in MES processes and chain elongation was also investigated by repeating the same experiments without providing the polarised cathodes for developed CO_2 reducing biocathode (under open circuit potential (OCP) condition).

1.3 Outline of chapters

This thesis is structured with 8 chapters, including 3 experimental results chapters. The outline of chapters are briefly described:

1. Introduction

- Main aims, hypothesis, objectives and content of this thesis has been explained briefly.

2. Literature Review on Aerobic and Anaerobic Biocathodes
   - The information and previous studies about two main applications of bio-electrochemical systems (BESs) in electricity generation and reduction of CO_2, and different parameters affecting their performance are gathered in this chapter.
3. Materials and Methods
   - The materials, experimental designs and setups used in this thesis are described in details in this chapter.
4. Development of a Gas Diffusion Electrode (GDE) Aerobic Biocathode Using Acidophilic Iron Oxidisers; A Rapid and Reproducible Method to Develop Highly Active Biocathodes for Oxygen Reduction Reaction
   - Iron oxidising bacteria was enriched from natural environmental samples and used to develop an active aerobic biocathode. Development of iron mediated aerobic biocathode was performed in half-cell GDE designed BES at cathodic potential of 200 mV vs. Ag/AgCl (all the potentials in this thesis are reported against Ag/AgCl). Active aerobic biocathode was then transferred to a complete MFC coupled with active bioanode. The effect of operational conditions on the maintenance of anodic and cathodic biocatalysts in MFC and also optimising the MFC in terms of energy harvesting was studied.
5. Production of Valuable Organic Chemicals from CO_2 Using Microbial Electrosynthesis (MES)
   - Different methods for enrichment of acetogens and suppression of methanogens from mixed culture of microorganisms were established. Development of anaerobic biocathode able to reduce CO_2 and produce acetate at different operational conditions was studied. The effect of different cathodic potentials of -0.7, -0.8, -0.9 and -1.0 V with two different types of inorganic carbon source (CO_2 and HCO_3^-) provided on bacterial cells growth and production of acetate was investigated. After developing an active biocathode from mixed culture of microorganisms, formation of biofilm at the cathodes, composition of biofilm and its role in CO_2 reduction was studied using various fundamental electrochemical and biological techniques.
6. Strategies for Production of Long Chain Organic Chemicals Through Optimized MES and Parameters Affecting the Optimization

- Different strategies on active CO_2 reducing biocathode were studied to steer the production towards longer chain organic acids and alcohols. Continuous operational mode was applied on reactors with two different flow rates. Furthermore, two different chemical electron donors were supplied as energy sources for biocathode in addition to polarised cathodes. The function of polarised cathodes in production of acetate from CO_2 and chain elongation through acetate was studied in this chapter.

7. Conclusions and Future Work
8. Appendices

Chapter 2. Literature Review on Aerobic and Anaerobic Biocathodes

This chapter gathers the information and previous studies about two main applications of bio-electrochemical systems (BESs) in electricity generation and reduction of CO_2 and different parameters affecting their performance. Therefore, first subsection describes the overviews and principles about BES and its different applications. Second subsection summarizes electricity generation in microbial fuel cells (MFCs) and the key role of bioanode. Third subsection describes the role of aerobic bioacathode in catalysing oxygen reduction in MFCs. This includes electron transfer mechanism between electrodes and biocatalyst and the effect of such biocatalysts in enhancing power production in MFCs. Fourth subsection describes anaerobic biocathode in CO_2 reduction in BESs. This treats on bacteria responsible for reduction of CO_2 for production of organic chemicals, enrichment of anaerobic biocathode from mixed culture of microorganisms, biofilm formation during the process and its composition, and the mechanism of electron transfer between bacterial cells and polarised cathodes. Last subsection focuses on chain elongation and production of longer chain products from CO_2 in BESs.

2.1 Principles of BES

The principle of electrochemical system derives from the oxidation and reduction reactions occur in two different electrodes of anode and cathode in one system resulting in potential difference between two electrodes and subsequently electricity generation. More specifically, oxidation and reduction reactions occur at anodic and cathodic electrodes, respectively. Principally, electrons flow from an electrode with a low redox potential to an electrode with a high potential.

The ideal potential in biological processes can be identified using Gibbs free energy change (ΔG) of reactions (Amend and LaRowe, 2019). Gibbs free energy quantifies the thermodynamic equilibrium of redox reactions based on electromotive force according to Equation (2.1):

$$\Delta G = \Delta G° + \frac{RT}{zF} \ln \frac{Cox}{Cred} \quad (2.1)$$

Where $\Delta G°$ is the Gibbs free energy at the standard condition, R is the molar gas constant (8.314 J mol^{-1} K^{-1}), T is temperature in K, z is the mole number of the electrons transferred, F is Faraday's constant (96485 C mol^{-1}), C_{ox} is the concentration of oxidised species and C_{red} is the concentration of reduced species. The value of ΔG determines the favourability of the reaction and whether the reaction is spontaneous. If $\Delta G < 0$ the reaction is spontaneous, while $\Delta G > 0$ shows that the reaction is not spontaneous. In electrochemical system, ΔG can be related to the electrochemical potential according to Equation (2.2):

$$\Delta G° = - zF \Delta E° \quad (2.2)$$

Since $\Delta G = - zF \Delta E$, reactions potential or the redox potential of each electrode can be quantified using Nernst equation (Bard et al., 2002):

$$E = E° + \frac{RT}{zF} \ln \frac{Cox}{Cred} \quad (2.3)$$

Where E° is the reduction potential at the standard condition. Although the difference sign is removed in Nernst equation, E still shows potential difference, which in case of electrochemical analysis shows the potential of a complete cell (E_{cell}). E_{cell} comprising anodic and cathodic electrodes is calculated as in Equation (2.4):

$$E_{cell} = E_{cathode} - E_{anode} \quad (2.4)$$

$E_{cathode}$ is the redox potential of the reduction half-reaction at cathode and E_{anode} is the redox potential of the oxidation half-reaction at anode. When $E_{cell} > 0$, the electrons flow leading to electricity generation, and when $E_{cell} < 0$ external power supply is required to derive the reactions (Scott and Yu, 2015).

2.2 Applications of BES

BES is the multidisciplinary technique combining electrochemistry and biology which has different types with particular applications (Jiang and Zeng, 2018a). A typical BES consists of two compartments of anode and cathode. MFC is an important type of BES able to produce electricity and treat wastewater, simultaneously. To be more specific, microorganisms degrade the organics in wastes in an anodic compartment leading to release and transfer of electrons to a cathode and subsequently electricity generation. As in MFC $E_{cell} > 0$, anodic and cathodic reactions are spontaneous. However, when $E_{cell} < 0$ the reactions are not thermodynamically favourable. Therefore, another source of energy is required to derive the reactions. Such system is known as microbial electrolysis cell (MEC) (Scott and Yu, 2015). Degradation of organics in wastes at anodic compartments approaches chemical oxygen demand (COD) removal, a key step in wastewater treatment, which this application has been examined in pilot-scale MFCs (Ge et al., 2015b, Dong et al., 2015b). In addition, removal of other contaminants in wastes such as sulphide is another application of MFCs through anodic compartments (Izadi et al., 2015a, Izadi et al., 2015b). Soil remediation is another application of MFCs related to anodic compartments by accelerating oxidation reactions in soil compared to natural soil bioremediation through providing electron acceptor of anode. Successful organics removal such as hydrocarbons or COD from sediment in MFCs has been reported (Cruz Viggi et al., 2015, An et al., 2013). BES has another application able to assess the quality of (waste) water by measuring biological oxygen demand (BOD). BOD is dissolved oxygen which is required for biodegradation and oxidation of organic compounds in wastewater. MFC can be used as a biosensor which the current generated in MFCs can correlate the biodegradable organics concentrations in wastewater (Spurr et al., 2018).

BES has other applications involving cathodic compartments. One of these applications is resource recovery. In case of resource recovery, oxygen in the cathode compartment can be replaced by metals as electron acceptors such as copper and zinc. Traditional methods in removal of metals from wastes faced a problem of low efficiency, as the metal concentration less than 100 ppm could not be removed. However, this is not a limit in MFCs (Mathuriya and Yakhmi, 2014). In addition to recovery of useful compounds from the wastes, toxic compounds can be removed from wastes at cathodic compartments. Removal of toxic chemicals in wastes such as dye or chlorine through cathodic reactions was reported in MFCs (Thung et al., 2015, Foley et al., 2010). Denitrification is another application of MFCs (Puig et al., 2011). In this context, nitrite and nitrate can be used as terminal electron acceptors in cathodic compartments and removed from wastewater. Nitrate has the lower redox potential and higher solubility in

liquid compared to oxygen. Removal of nitrate catalysing by biocathode was reported as the efficient method for denitrification using MFCs (Clauwaert et al., 2007a).

BES can also be used for the abiotic or biotic production of hydrogen. In this case, proton in the cathodic compartment accepts the electrons derived from the anode and reduces to hydrogen (Yossan et al., 2013). It has been observed that hydrogen evolution reaction (HER) on carbon-based electrode requires high overpotential; however, microorganisms can also be efficient biocatalysts catalysing HER (Rozendal et al., 2007).

Another important application of BES attracted scientists' attentions recently is CO_2 conversion to valuable chemicals known as microbial electrosynthesis (MES) (Rabaey and Rozendal, 2010). In MES, microorganisms in the cathodic compartment uptake the electrons derived from the anode and use CO_2 as an electron acceptor. Based on bacterial community and the metabolic pathway involved for reduction of CO_2, products can be different such as volatile fatty acids (VFAs) and alcohols.

2.3 Microbial Fuel Cell (MFC)

MFC is one of the most important type of BES offering different applications, which usually comes in single or dual chamber configurations. Figure 2.1 shows a schematic of a typical dual chamber MFC consists of two anodic and cathodic compartments, separated by an ion exchange membrane. Anodic compartment is filled with a solution (anolyte) contains electroactive bacteria known as anode respiring bacteria (ARB) and bacterial growth medium under anaerobic condition. Bacterial growth medium is a conductive solution, generally includes vitamins, minerals and organic substrate such as sugars and adjusts around pH 7.0 using buffers such as phosphate buffer. Carbon-based electrodes are usually used as anodes due to their conductivity, biocompatibility and cheap price. In case of abiotic reaction in cathodic compartment, cathodic compartment is usually filled with a matrix electrolyte such as phosphate buffer and a cathodic electrode such as a metal or carbon-based electrode. Under anaerobic condition ARBs in anodic compartments oxidise the organic substrate (in wastewater), release the electrons from the organics and use the anode as a sink of electrons. By the donation of electrons to anode, bacteria attach and grow on the electrode making biofilm. Electrons produced in this reaction are transferred from anode to cathode through the external circuit connecting these two electrodes thus generating electricity (Logan, 2008, Oliveira et al., 2013).

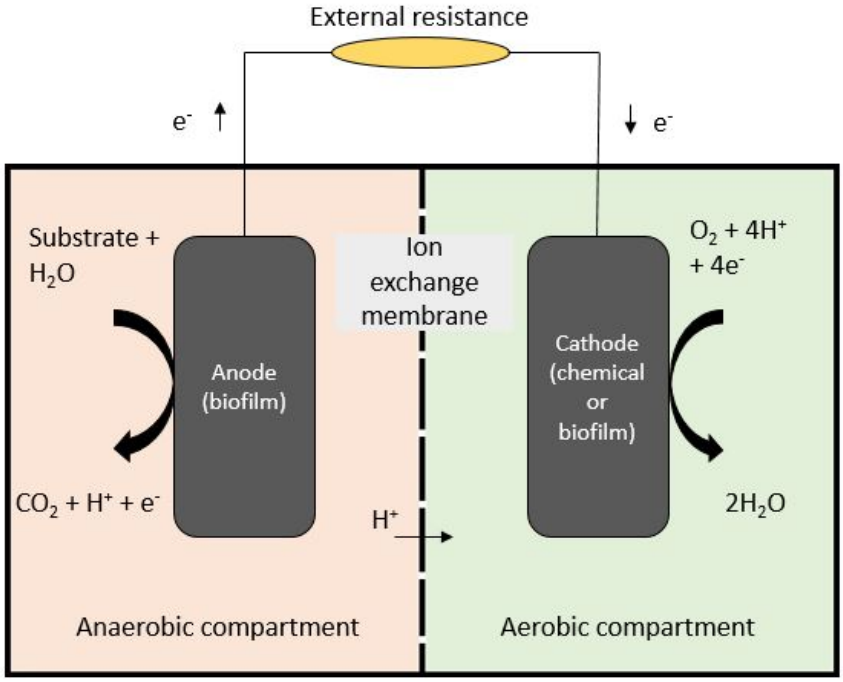


Figure 2.1. Schematic of dual chamber microbial fuel cell (MFC).

The most common reaction at the cathode is oxygen reduction reaction (ORR), usually catalysed by chemical catalysts such as Pt on carbon electrodes. The possibility of the electron transfer from an anode to a cathode strongly depends on the redox potential difference (E_{cell}) between these two electrodes. In case of oxygen as a terminal electron acceptor in cathodic compartments, the high redox potential of ORR makes the transfer of electrons from anodes to cathodes thermodynamically favourable. Therefore, this technology, known as a renewable energy source, enables the simultaneous wastewater treatment (organic degradation) and electricity generation. Considering the typical MFC using acetate as the carbon source for bioanode and ORR in the cathode, the acetate oxidation reaction is:

$$CH_3COO^- + 4\,H_2O \rightarrow 2\,HCO_3^- + 9\,H^+ + 8e^- \quad (2.5)$$

And in case of reduction of oxygen to water, 4 electrons are needed from the anode to reduce one molecule of oxygen:

$$O_2 + 4\,H^+ + 4\,e^- \rightarrow 2\,H_2O \quad (2.6)$$

Thus, the overall reaction is:

$$CH_3COO^- + 2\ O_2 \rightarrow 2\ HCO_3^- + H^+ \quad (2.7)$$

The theoretical MFC potential (E_{cell}) in this system ($[CH_3COO^-] = [HCO_3^-] = 10$ mM, pH: 7, 298.15 K, $pO_2 = 0.2$ bar) is 0.60 V - (-0.51 V) = 1.1 V, according to Equations (2.3) and (2.4). Therefore, as $E_{cell} > 0$, the reactions in this MFC are thermodynamically favourable. This potential is an electromotive force driving the reactions, transferring electrons from the anode to cathode and thus generating electricity. However, in the practical working condition, the actual potential is lower than 1.1 V due to different parameters affecting the anode and cathode potentials such as anode and cathode materials or bacterial community in bioanode (Logan, 2008).

Although dual chamber MFC is the most common design, different configurations have been designed and used as MFCs according to the operational condition required for different studies. In dual chamber designs, solubility of oxygen in catholytes is one of the parameters limiting the performance of MFC. To tackle this limit, gas diffusion electrode (GDE) design has been used in recent studies. GDE can be used in both single and dual chamber MFC providing constant available dissolved oxygen in catholyte by allowing the passive oxygen mass transfer from the air to the cathode surface (Kadier et al., 2016). In case of single chamber GDE, membrane can be directly pressed on the surface of GDE that faces the solution. GDE design will be discussed in Section 2.4.1.

2.4 Microbial electrosynthesis (MES)

The potential application of CO_2 reduction and production of value-added products has made MES one of the important applications of BES. As shown in Figure 2.2, MES occurs in a similar configuration of MFC, but under anaerobic condition provided in a cathodic compartment.

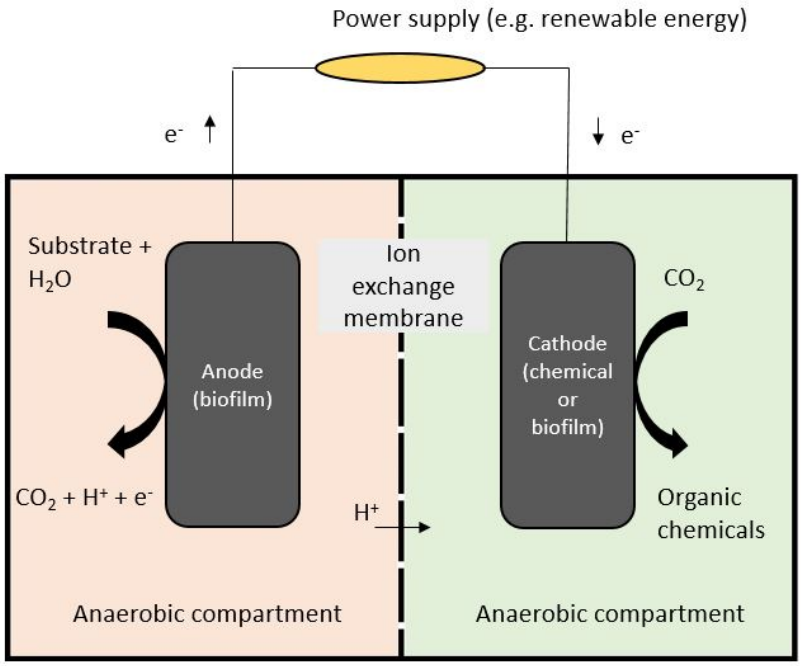


Figure 2.2. Schematic of dual chamber bio-electrochemical system (BES) with microbial electrosynthesis (MES) in cathodic compartment.

Anaerobic condition is required, because CO_2 reducing bacteria for production of value-added products are generally anaerobic. In addition, presence of oxygen at the cathode leads to ORR as it is thermodynamically more favourable than CO_2 reduction. Considering the BES using acetate as the carbon source for ARBs in anodic compartment and CO_2 reduction by biocathode ($[CH_3COO^-] = [HCO3^-] = 10$ mM, pH: 7, 298.15 K), the oxidation reaction is similar to Equation (2.5). In case of reduction of CO_2 to acetate, the reduction reaction is:

$$2\,HCO_3^- + 9\,H^+ + 8e^- \rightarrow CH_3COO^- + 4\,H_2O \qquad (2.8)$$

In this BES, the theoretical BES potential (E_{cell}) is 0.0 V. This potential in the practical condition is even less than 0.0 V due to the possible losses such as electrodes overpotentials. Therefore, another source of energy as an electromotive force is required to drive the reactions. This energy can be supplied by other types of renewable energy such as wind power. Half-cell design known as 3-electrode configuration is another method to derive MES reactions. In this design, cathodic and anodic electrodes are set as working and counter electrodes, respectively and the focus is on working electrode by applying the desired potential for CO_2 reduction at a working electrode using potentiostat. Galvanostatic control can also be used for 3-electrode configuration by fixing the current at working electrodes. As carbon-based electrodes are cheap, biocompatible,

conductive with high surface area, they are used as cathodes in MES. In case of half-cells, anodic compartments can be operated in abiotic condition (Rabaey and Rozendal, 2010). The common abiotic anodic reaction in 3-electrode configuration is water electrolysis as shown in Equation (2.9):

$$2\ H_2O \rightarrow O_2 + 4\ H^+ + 4\ e^- \qquad (2.9)$$

To provide enough number of electrons transferred from an anode through Equation (2.9) to a cathode for the reduction of CO_2, metal electrodes such as Pt are usually used as anodes in laboratory scale experiments. Dual chamber reactor is the most common and simple designs used for MES. Particularly, separation of anodic and cathodic compartments using dual chamber design is required to prevent the diffusion of oxygen produced at an anode through Equation (2.9) to cathodic compartment.

2.5 Role of bioanodes in MFCs

2.5.1 *Role of bioanode in electricity generation in MFCs*

Through different methods, fermentation for instance, microorganisms degrade complex organic compounds such as glucose to the simple compounds such as acetate. Acetate is then converted to acetyl coenzyme for anabolism (through tricarboxylic acid cycle) and to adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) for catabolism. ATP is an organic chemical supplies energy for bacterial activities. NADH is a reducing agent able to donate an electron to the terminal electron acceptor and change to NAD^+. In case of MFCs, the anode is used as a terminal electron acceptor. Anodic chamber can support the growth of bioanode in form of both biofilm at the anode surface and planktonic cells in the anolyte (Logan, 2008). Electrons can be transferred from bacteria to the anode through two mechanisms: direct electron transfer (DET) and indirect electron transfer (IET). IET is also known as mediated electron transfer and the mediators involved can be excreted from bacterial cells or artificial mediators between the bacteria and anode. DET mechanism occurs by the direct interact of bacterial cells and electrodes using membrane-bound cytochromes, conductive pili or both. Inside the bacterial cell, the electron released from oxidation process reduces NAD^+ to NADH and is pulled away from bacterial membrane using bacterial membrane-bound enzymatic complex called NADH dehydrogenase (Proft and Baker, 2009). The electron is donated to

series of cytochromes or conductive pili of bacteria and then to the anode. Donation of electrons to the anode forms a conductive matrix developing biofilm at the surface of the electrode. Concentration of reduced cytochromes are higher in the outer side of the biofilm compared to that at the surface of the anode. Different types of cytochromes involved in DET has been identified such as OmcB and OmcS (Nevin et al., 2009). *Geobacter sulfurreducens* and *Shewanella oneidensis* are the well-known electro-active bacteria able to transfer electron to the anode through DET mechanism using membrane-bound cytochromes and conductive pili or nanowires and form a biofilm at the surface of the anode. The important role of membrane-bound cytochromes and nanowires in electron transfer of *G. sulfurreducens* and *S. oneidensis* has been broadly studied and reported in previous studies (Tremblay et al., 2011, Gorby et al., 2006, Shi et al., 2009). Nanowires are pilus-like conductive extracellular appendages of bacteria connecting bacterial cells together and to the anode and facilitating the electron transfer between bacterial cells and the anode. Depending on growth condition the size pf nanowires can be different, but it was reported that they have the average length of 6 µm and width of 7 – 12 nm (Eaktasang et al., 2016). It was proposed that nanowires were not directly involved in DET mechanism but in organising outer membrane cytochromes (Snider et al., 2012). However, comparing two different strains of *Geobacter*, it was observed that the better performing strain formed thicker biofilm involving higher density of nanowires and lower outer membrane cytochromes (Yi et al., 2009). DET mechanism was also reported in many other bacteria such as *Desulfobulbus propionicus*, *K. pneumoniae* and *Thermincola ferriacetica* (Matysik and Borisov, 2014).

Electron transfer between anodes and bacterial cells can also be facilitated using external mediators known as IET mechanism. Microorganism that either cannot donate the electrons directly to the anodes or are present in the solutions as planktonic cells use mediators as shuttles between cells and the anode through IET mechanism. Such mediators should be non-toxic for bacteria, electrochemically reversible and have sufficient positive redox potentials. These mediators can be artificially added to the anolyte or produced by microorganisms (Lovley, 2008).

2.5.2 *Development of electro-active bioanode from mixed culture of bacteria in MFCs*

According to the key role of bioanode in MFCs, the types of microorganisms forming the biofilm is an important parameter affecting the electron transfer between the bacterial cells and

the anode and therefore the electricity generation. Wide range of bacteria showed the ability to use anode as an electron acceptor. Extensive studies were executed on electricity generation using pure culture of *G. sulfurreducens* or *S. oneidensis* in MFCs. However, using pure culture for bioanode has its own challenges such as requirement of sterile conditions. In addition, higher power production was observed in MFCs using mixed culture of microorganisms in anodic compartments due to the synergetic interactions of diverse bacterial communities. Mixed cultures are generally preferred because of their availability at large scale and their compatibility in different situations (Bajracharya et al., 2016a).

Wide types of bacteria exist in mixed culture sources of microorganisms. Selection of desired bacteria to form bioanode depends on the conditions provided in anodic compartment. Imposing the proper conditions for mixed culture of microorganisms by optimizing the relevant parameters is considered as the main target of many studies. Substrate is an important factor providing carbon and energy source for bacteria. Apart from the direct influence of substrate on the composition of the bacterial community, it affects the MFC performance in terms of power production (Liu et al., 2009). ARBs have shown the ability to consume different types of substrates ranging from pure forms to complex organics in wastewater. The effect of various types of substrate on bioanode development and electricity generation in MFCs was investigated in many studies. Glucose has been used as a substrate in many studies, however, due to its fermentable nature columbic efficiency reported in these studies was not significant (Xing et al., 2009). Glucose selects not only electrochemically active bacteria, but also different fermenters or methanogens leading to loss of electrons caused by competition between different bacterial community and decrease in system's efficiency. Acetate has been used as the substrate of choice in many studies on MFCs. Acetate is the simple carbon source which can be produced as the end-product through degradation of complex organics in bi-processes such as fermentation, and different metabolic pathways. When acetate is provided as a sole organic substrate, it tends to select *Geobacter*, known electro-active bacteria, form various types of bacteria. Comparing with other complex substrates, acetate shows the consistency in MFC performance in terms of electricity generation. Liu et al reported 66% higher power production in acetate-fed MFCs (506 mW m^{-2}) compared to that in butyrate-fed MFCs (305 mW m^{-2}) (Liu et al., 2005). In a previous study, the effect of acetate, propionate, butyrate and glucose on MFC performance was investigated. It was reported that acetate could enrich more electro-active bacterial community resulting to the best performance of MFC in terms of efficiency in reactors fed by acetate (72.3%), compared to 43.0%, 36.0% and 15.0% fed by butyrate, propionate and glucose, respectively (Chae et al., 2009). In addition, the abundance of *G. sulfurreducens* in a

bioanode developed in MFCs fed by acetate (31.7%) was the highest compared to other reactors with different substrates. The effect of using acetate as a substrate on MFC performance was also compared that with more complex source. It was reported that acetate-fed MFCs produced 2 times higher power than MFCs fed by protein-rich wastewater (Liu et al., 2009). Due to the ease of consumption and MFC performance consistency, acetate was selected and used as an anodic substrate for growth and development of bioanode when required in this thesis.

2.6 Aerobic biocathode in BESs

2.6.1 *Aerobic biocathodes and their effect on enhancing the performance of MFCs*

MFCs comprise of organic compounds oxidation in anodic compartments and commonly oxygen reduction reactions in cathodic compartments. Currently, Pt is the most common electrode effectively catalysing ORR in MFCs. However, to use the MFC technology in larger scales, the costs need to be lowered. Pt is an expensive metal which is not environmentally sustainable. Other metal-based electrodes such as iron phthalocyanine (FePc) and cobalt-based materials (CoTMMP) or carbon-based electrodes have also been used as cathodes in MFCs to catalyse ORR (Milner et al., 2016). However, metal-based electrodes still have cost and sustainability issues. In addition, carbon materials are poor in catalysing ORR. Mixed culture aerobic biocathode, however, is the free and sustainable biocatalyst showing the potential application in catalysing ORR and offering the proper substitute to high-cost and non-sustainable chemical electrodes. Developing mixed culture aerobic biocathode and using it in MFCs has recently attracted researchers' attention. Much of interest in this area has begun almost from 2007. In 2007, Clauwart et al reported the operation of MFC combining an acetate oxidising bioanode and aerobic biocathode (Clauwaert et al., 2007b). In their study, cathodic compartment was inoculated by mixed sludge and sediment inocula and the maximum power density of 83 ± 11 W m^{-3} was achieved on batch operational mode (Clauwaert et al., 2007b). Soon after, Rabaey et al reported three times increase in MFC power density, comparing the MFC with aerobic biocathode and non-inoculated MFC (Rabaey et al., 2008). In their study, the mixture of sludge, sediment and rusted metal poles were used as an inoculum for aerobic biocathode (Rabaey et al., 2008). Similar to bioanode, two mechanisms of electron transfer between biocathode and cathodes has been proposed, direct electron transfer (DET) and indirect electron transfer (IET). Different fundamental investigations were carried out to clarify the

mechanisms of electron transfer between the bacterial community responsible for ORR and the cathode, which will be discussed in next sections. Regardless of the mechanism involved for uptaking electrons from cathodes (DET or IET), different types of bacteria are able to catalyse ORR in cathodic compartment such as manganese oxidising bacteria (MOB), iron oxidising bacteria (IOB), nitrite oxidising bacteria (NOB) and many more (He and Angenent, 2006). Most of these bacteria consume inorganic carbon in CO_2, known as autotrophic bacteria, through Calvin cycle (Milner, 2015). These bacteria produce proton motive force and ATP by coupling oxygen reduction and substrate oxidation. Reducing power in Calvin cycle comes from NADH and the reduction potential for substrate oxidation by these bacteria is higher than that for NAD^+/NADH, therefore ATP and yield is quite low as the proton motive force is consumed over reverse electron transport (Milner, 2015). Many studies reported the enrichment of aerobic biocathode in BESs using mixed culture of microorganisms. The community composition of these biocathodes determined through community 16S analysis were different depending on the inoculum source, or operational conditions provided in cathodic compartments. The examples of bacteria dominated in aerobic biocathode developed from mixed culture in previous studies are *Gammaproteobacterium*, *Pseudomonas*, *Sphingobacterium*, and *Acidovorax* species (Rabaey et al., 2008, Reimers et al., 2006, Milner et al., 2016).

Many studies have focused on aerobic biocathodes by studying the key parameters enhancing their performance in MFCs. Enhancing ORR in cathodic compartment leads to uptake more electrons from anodic side and therefore increase in power production by MFC. One of the important factor that affects the devolvement and performance of bicathode is cathodic poised potential. According to equation (2.4), ORR occurs at the potential of 0.60 V. However, this is different in practical conditions due to the potential losses such as activation loss or mass transfer. Applying different potentials of 50, 150 and 250 mV applied at graphite plates as cathodes in half-cell configuration inoculated by activated sludge were investigated by Heijne et al, which the maximum current and most rapid biocathode enrichment were achieved at the potential of 150 mV (Ter Heijne et al., 2010). Similarly, Liang et al operated half-cells using carbon felt with activated sludge, compared the applied potentials of -63, 37, 137, 237 and 337 mV for 27 and reported the maximum current density at the potential of 37 mV (Liang et al., 2009). Comparing two different potentials of -100 and 200 mV during enrichment in half-cells at graphite felt inoculated by activated sludge, it was reported by Milner et al that the cathodic poised potential during the enrichment had the direct effect on the composition of the bacterial community enriched from mixed culture of microorganisms (Milner et al., 2016). In their study, cathodic poised potential of -100 mV could successfully enrich the aerobic biocathode with DET mechanism. More significant redox peaks appeared in CV performed from biocathode

poised at -100 mV compared to that at 200 mV, showing the higher electrochemical activities of biocathodes developed at -100 mV. The authors reported the increase in power density up to nine-fold in MFCs using the developed biocathode compared to MFC using plain electrode as a cathode (Milner et al., 2016). In addition, number of studies focused on engineering the system. For instance, anode effluent was used as cathode inlet for biocathode to solve the pH difference between two compartments, however the organic compounds in anodic effluent influenced the performance of biocathode (Freguia et al., 2008). There were also other studies that used organic electron donors of acetate or azo dye in addition to cathodes (Sun et al., 2011, Cheng et al., 2009a, Blanchet et al., 2014). However, this can decrease the efficiency of the system as bacteria tends to use the organics rather than the cathode as an electron donor.

Furthermore, the solubility and therefore availability of oxygen in the catholyte is a crucial parameter in aerobic biocathode performance. Particularly, in case of presence of biofilm of aerobic biocathode at the electrode, oxygen mass transfer can limit ORR by microorganisms. This is because oxygen should diffuse through the biofilm and reach the outer membrane of each bacterial cell. Commonly, dissolved oxygen has been provided for aerobic biocathode using air pump directly in the solution. In a recent study, Milner et al supplied dissolved oxygen in catholyte compartment using air pump with different flow rates (Milner and Yu, 2018). In their study, MFCs were operated using aerobic biocathode from activated sludge at graphite felts as cathodes. After formation of aerobic biocathode biofilm, the effect of different flow rates of air pump was investigated on the MFCs' current generation. The linear correlation between air flow rate and current density strongly showed the impact of oxygen mass transfer on biocathode performance (Milner and Yu, 2018). This was also reported by Heijne et al that increase in the flow rate of air pump led to decrease in mass transfer resistance measured through EIS analysis (Ter Heijne et al., 2011b). Although temperature affects the solubility of oxygen and more oxygen molecules dissolve in solutions at lower temperature, the operational condition should be also in the appropriate range for bacteria growth and activities. The effect of different operational temperature (31, 27, 24, 20 and 14 °C) was investigated on the solubility of oxygen aerated in the catholyte, and it was reported that decrease in temperature decreased the bacterial activity, and therefore decrease in ORR by microorganisms (Strik et al., 2013). Active aeration using air pump to supply oxygen has few disadvantages. First, in case of long-term experiments it increases the energy cost required for the pump. In addition, in case of dual chamber MFC it pressurizes or even breaks the membrane. High flow rate can also damage the biofilm at the cathode. Passive aeration using GDE design has been an appealing alternative to active aeration solving the oxygen mass transfer limitations and the issues associated with

active aeration (Wang et al., 2013). The common design for GDE is dual chamber of anode and cathode separated by membrane which the cathode is faced to the air known as air-breathing cathode as demonstrated in Figure 2.3. The electrode used for this design should have a hydrophobic surface to keep the liquid in the reactor from leakage. This hydrophobic surface is usually provided by brushing polytetrafluoroethylene (PTFE) to one side of carbon-based electrodes, which gas can diffuse through this surface inside the reactor but keeps the liquid from leakage. Commonly, the commercial carbon paper is used for this reason which has one hydrophilic surface of carbon to face the solution and a hydrophobic surface of PTFE to face the air (Xia et al., 2012). Using this design, oxygen is constantly diffused through the gas diffusion layer providing available dissolved oxygen for ORR by aerobic biocathode. Xia et al reported the higher current generation in MFCs coupled with aerobic biocathode in GDE design (maximum 1.0 A m^{-2}) than that in dual chamber MFC (maximum 0.49 A m^{-2}) (Xia et al., 2013). The superior performance using GDE was reported comparable to MFC with Pt as a cathode in this study (Xia et al., 2013).

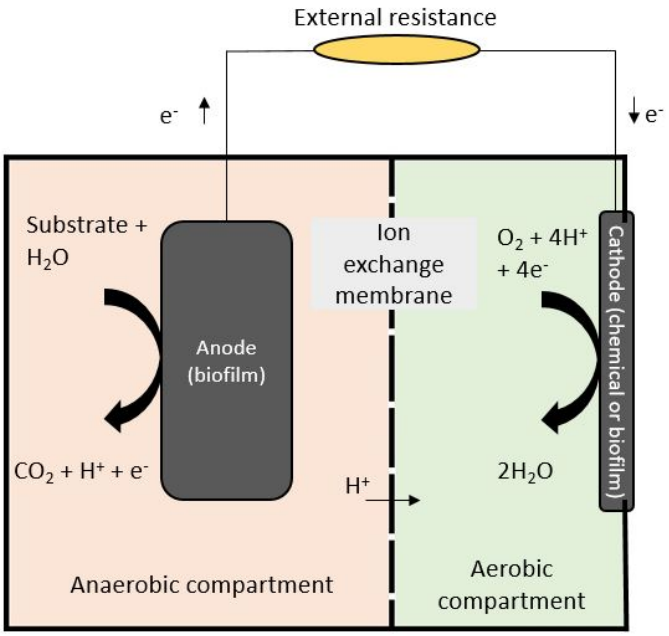


Figure 2.3. Schematic of dual chamber MFC with aerobic cathodic compartment with gas diffusion electrode (GDE) design.

2.6.2 *Catalysing oxygen reduction reaction (ORR) using direct electron transfer between bacteria and cathode*

Catalysing ORR in cathodic compartments using aerobic biocathode can occur directly. Using this mechanism bacteria take electrons directly from the electrode for their growth and metabolism. DET mechanism of electron transfer between anodes and bioanodes proved by previous extensive studies has raised the hypothesis that biocathodes can take electrons directly from cathodes (Matysik and Borisov, 2014). In case of bioanode, DET occurs either by direct interact of outer-membrane cytochromes or conductive pili such as nanowires to give electrons to the anode (Lovley, 2008). *G. sulfurreducens* and *S. oneidensis* are the known bacteria using these mechanism for electron transfer to the anode. It was shown that *Geobacter* species not only can use anode as an electron acceptor, but can uptake electrons from cathodes for respiration. For instance, *G. sulfurreducens* was able to convert nitrate to nitrite using cathode as an electron donor (Gregory et al., 2004). C-type cytochromes seem to be electrochemically reversible shown on CV analysis, therefore the electrons are more likely taken from the poised cathode using these enzymes. It is also believed that in IOB soluble Fe^{2+} oxidises in outer-membrane of the cell which the electron is transferred to inner membrane (Castelle et al., 2008). The advantage of DET mechanism is the lack of need for chemical redox mediators leading to simple and low cost MFC operation. However, one of the challenges with DET mechanism in aerobic biocathode is that biofilm formation for ORR requires long time. In the previous study, aerobic biocathode enriched from mixed culture of bacteria after almost 50 days in half-cell BESs poised at -100 mV. This was even longer in the study by Chen et al. In their study, MFCs operated by inoculating both anode and cathode compartments and it was reported that the start-up time for developing both biocatalysts was 2 months (Chen et al., 2008). DET mechanism of electron transfer of pure culture of IOB, *Acidithiobacillus ferrooxidans*, was reported by Carbajosa et al (Carbajosa et al., 2010). In this study, IOB was enriched using Fe^{2+} in medium and it was removed from the solution using paper filter before transferring to electrochemical cell, which it was reported that after 20 days cathodic current began to generate slowly (Carbajosa et al., 2010).

2.6.3 *Catalysing oxygen reduction reaction (ORR) using the mediated electron transfer between bacteria and cathode*

Another mechanism for electron transfer between aerobic biocathode and cathode is known as IET. In this mechanism, electrons will be transferred from cathodes to bacterial cells by intermediates such as different chemicals. The utilisation of a chemical mediator seems to help overcoming the long start-up associated with DET mechanism, as some bacteria are able to

reduce oxygen by using mediators as electron donors. Many bacteria, particularly metal oxidising bacteria are known to follow this mechanism to catalyse ORR (Choi and Sang, 2016). Acidophilic IOB is one of these bacteria. Although acidophilic IOB are able to derive energy directly from cathodes, the presence of Fe^{2+} as a mediator significantly improves the kinetics of cathodic reactions and makes the biocathode much faster to develop (Ter Heijne et al., 2006). Moreover, iron is one of the most abundant metal in environment. Acidophilic IOB can be found in environment such as sediments with lower pH. The enrichment of acidophilic IOB from mixed culture of microorganisms were carried out through a simple method by providing high concentration of $FeSO_4$ (20 g L^{-1}) under low pH condition (Fontmorin and Sillanpaa, 2015).

Acidophilic IOB such as *acidithiobacillus ferrooxidans* are autotrophs that use CO_2 as the carbon source, and in case of presence of Fe^{2+} in catholyte they derive energy from the oxidation of Fe^{2+} coupled with reduction of oxygen at extremely low pH condition (typically less than 3.0). Fe^{3+} produced from the biological oxidation is then regenerated at the cathode to Fe^{2+} (Lovley, 1991). Therefore, the reactions occur in cathodic compartments are as below:

Biological: $$8\ Fe^{2+} + 8\ H^{+} + 2\ O_2 \rightarrow 8\ Fe^{3+} + 4\ H_2O \quad (2.10)$$

Electrochemical: $$Fe^{3+} + e^{-} \rightarrow Fe^{2+} \quad (2.11)$$

The schematic of such electron transfer in a GDE design MFC is presented in Figure 2.4. Oxidising ferrous iron and reducing oxygen by the iron oxidisers in an acidic condition yields almost 30 KJ/mole energy for their growth (Hedrich et al., 2011). Using Calvin cycle to fix CO_2, it has been estimated that *acidithiobacillus ferrooxidans* oxidises 71 mole Fe^{2+} to fix one mole CO_2 (Hedrich et al., 2011). Although at neutral pH, neutrophilic IOB are able to oxidise Fe^{2+}, the reduction of oxygen, and thus the energy gain for the bacteria, is more favourable at pH 2.0 than at pH 7.0 (Ferguson and Ingledew, 2008). Redox potential of iron ($Fe^{2+} \rightarrow Fe^{3+} + e^{-}$) at pH 2.0 is 0.57 V, higher than that in neutral pH. In addition, Fe^{3+} and in smaller extent Fe^{2+} are soluble in lower pH. Due to these advantages, acidophilic IOB in presence of ferrous iron seemed as a proper alternative to chemical catalysts for catalysing ORR. Maximum MFC power density of 0.86 w m^{-2} at a current density of 4.5 A m^{-2} was reported by Heijne et al using iron-mediated acidophilic aerobic biocathode (Ter Heijne et al., 2006). Due to the pH difference between anodic and cathodic compartments, bipolar membrane was used in their study to separate the compartments and prevent the pH shifts in each compartment (Ter Heijne et al., 2006). Afterwards, similar setup but with immobilized microorganism *acidithiobacillus*

ferrooxidans in the cathodic compartment showed the higher power density of 1.2 W m^{-2} (Ter Heijne et al., 2007). A successful scaled-up MFC using acidophilic iron oxidisers at the cathode at pH 2.0 and acetate oxidation at the anode compartment through a bioanode biofilm resulted in a high power density of 2.0 W m^{-2} (Ter Heijne et al., 2011a). The scaled-up MFC using acidophilic iron oxidisers in their study was operated for 37 days. The authors discussed that the further increase in iron concentration in the medium resulted in higher cathode potential and consequently higher MFC potential while the precipitation of iron should also take into consideration (Ter Heijne et al., 2011a). In all these studies, the iron-mediated mechanism improved the start-up of the cathodic reactions, however expensive materials were used such as a bipolar membrane to overcome the pH shifts in both compartments as well as costly Pt- and Ir-coated electrodes.

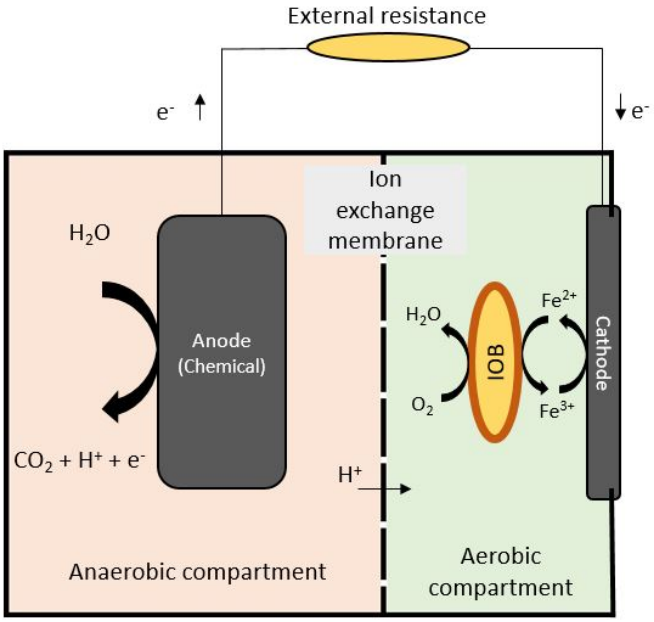


Figure 2.4. Schematic of dual chamber MFC with aerobic iron oxidising bacteria (IOB) in presence of Fe^{2+} as an intermediate in cathodic compartment with gas diffusion electrode (GDE) design.

2.7 CO_2 reducing biocathode in BESs

2.7.1 *Anaerobic autotrophic bacteria*

Heterotrophic microbes, fermenters for instance, are the microorganisms that take nutrition and derive energy from organic compounds, whereas autotrophic microbes are the microorganisms consume inorganic sources to live (Ebeling et al., 2006). Anaerobic autotrophs are the microorganisms able to consume inorganic carbon such as CO_2 known as carbon fixation mechanism and some of them synthesize organic compounds under anaerobic condition. There are different types of anaerobic autotrophs in the environment with different pathways to consume inorganic carbon (Madigan et al., 2010, Quayle, 1961). Depending on the pathways, end products can be different. Methanogens are the known autotrophic microbes belong to the domain of *archaea* able to produce methane. They usually live abundantly in environment particularly in soil, sludge and marine sediments. The terms methanogenesis is used to describe the process of methane production by methanogens (Balch et al., 1979). Methanogens are generally recognised as two main types of autotrophs and heterotrophs. The autotrophic methanogens (known as hydrogenotrophic methanogens) are able to produce methane from CO_2 and H_2 (Equation 2.12) as an inorganic carbon and energy sources, respectively both under strictly anaerobic condition.

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O\ (\Delta G_r = -131\ kj\ mole^{-1}) \qquad (2.12)$$

Acetogens are another well-known H_2-oxidising autotrophs able to synthesize mainly acetate from CO_2 and H_2 through Wood-Ljungdahl (WL) pathway, which the process is known as acetogenesis (Equation 2.13).

$$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O\ (\Delta G_r = -95\ kj\ mole^{-1}) \qquad (2.13)$$

WL pathway, also known as acetyl Co-A pathway is one of the most important metabolism in fixing carbon and generating energy. The term acetogen has been used before for any acetate producing microorganisms such as *Enterococcus* that produce acetate from lactate in presence of oxygen (Tholen et al., 1997). However, the term homoacetogen has been often used for the bacteria producing acetate through WL pathway (Drake et al., 2006). In this thesis, production of acetate from CO_2 through WL pathway is emphasized and the term acetogen is used for the bacteria catalysing such synthesis. For the reduction of CO_2, acetate is the more valuable product than methane in market. Besides, acetate is the key intermediate in degradation of

organic compounds through bacterial anaerobic digestion. Therefore, its formation is more of the interest for microbiologists and biochemists.

WL pathway is a linear process includes of two reductive branches of methyl branch and carbonyl branch (Figure 2.5). Via this pathway, acetogens can reduce CO_2 through both methyl and carbonyl branches, synthesize acetyl Co-A, conserve energy, build cell carbon by fixing CO_2 (anabolism) and produce acetate (catabolism) (Drake et al., 2006).

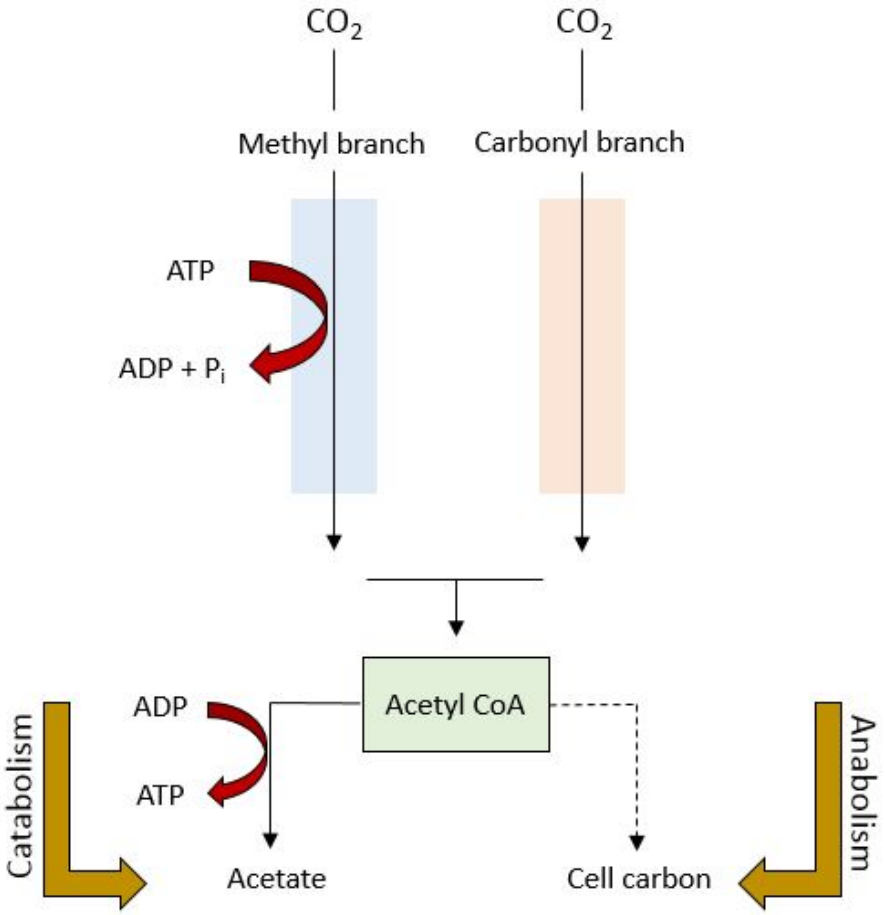


Figure 2.5. Schematic of Wood-Ljungdahl pathway, adapted from (Drake et al., 2006).

The whole process involves of different enzymes such as dehydrogenase, formate dehydrogenase, carbon monoxide dehydrogenase, corrinoid protein and methyltransferase (Ragsdale, 2008). Hydrogenase is evidently the important enzyme in WL with the key function of oxidising H_2 as an energy source in the autotrophic growth of acetogens on CO_2/H_2. Electrons generated from H_2 oxidation diffuses through the cell membrane for the reduction of CO_2 and synthesize of acetate, while protons are used for the generation of ATP (Ljungdhal, 1986). The concept of electron transfer in acetogens and providing energy for the acetate synthesize is illustrated in Figure 2.6.

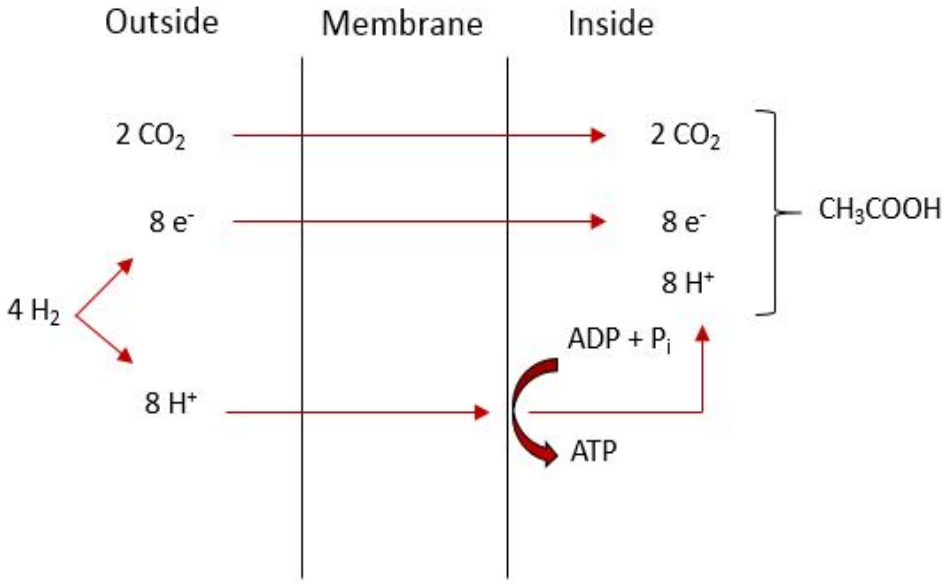


Figure 2.6. Schematic of the concept of electron transfer, CO_2 reduction and acetate production in acetogens growing on CO_2/H_2, adapted from previous study of acetogens (Ljungdhal, 1986).

The cultivation medium for acetogens should be anaerobic. Although acetogens are considered as anaerobic bacteria, most of them can tolerate small quantity of oxygen. Selenium, tungsten and trace metals such as nickel, zinc and iron are the important components of several type of enzymes in WL pathway, therefore the presence of these compounds in solution is essential (Ljungdhal, 1986). As CO_2 is the final electron acceptor, its presence in the medium is evidently vital. However, its availability in the medium can vary for different acetogens as some of them cannot grow unless CO_2 is promptly accessible (Drake et al., 2006). The medium should also contain supplemental vitamins. To date, different pH values favourable for acetogenic growth has been reported, but it is usually between 5.8 and 8.0 (Drake et al., 2006, Abubackar et al., 2011). It's worth mentioning that for the growth of actogens, methanogens need to be suppressed as they can be competitors to acetogens in CO_2 reduction. Due to Equations 2.11 and 2.13 and comparing their Gibbs free energy, synthesize of acetate from CO_2 is thermodynamically less favourable than methane production from CO_2.

In addition to heterotrophic methanogens, acetoclastic methanogens produce methane from acetate (produced from acetogens). Therefore, acetogens and methanogens usually co-exist in nature. Thus, to enrich the acetogens from mixed culture of microorganisms and enhance their activity the suppression of methanogens should be taken into consideration (Berg, 2011). Different methods have been used to suppress the methanogens, such as low pH, pre-heating the inoculum and using chemicals such 10-50 mM bromoethanesulfonate (Jadhav et al., 2018).

Acetogens has wide range of species. To date, acetogens have been allocated to almost 21 genera with different properties. Among them, 2 genera of *Clostridium* and *Acetobacterium* have the highest number of acetogenic species. Besides the autotrophic growth, acetogens can also grow heterotrophically. However, CO_2 fixing process is still involved in the heterotrophic growth of acetogens (Drake et al., 2006). For instance, heterotrophic growth of *Moorella thermoacetica* on the organic compound of glucose leads to production of 3 molecules of acetate. 2 molecules of acetate produces from the conversion of glucose and the third molecule synthesizes from 2 molecules of CO_2 (Drake and Daniel, 2004).

Although the term acetogens implies that acetate is the sole end product of the process, products can be varied depending on the cultivation methods and the substrates used for the growth and enrichment. As described, the main product of acetogens growing on CO_2/H_2 is acetate, but in case of presence of other compounds the products can be more diverse then acetate (Drake, 1994). Acetogens have diverse metabolic capabilities which various types of substrates (electron donors) can be oxidised and supply reductant to the WL pathway and synthesize acetate and also other products. Electron donors or growth-supportive compounds varied depending on different acetogenic species which can be CO, formate, glucose, methanol, ethanol, amino acids, pyruvate, lactate and etc. The examples of these species are *Clostridium formicoacetium, Clostridium acetium, Moorella thermoacetica, Acetobacterium malicum and Acetobacterium carbinolicum* (Drake et al., 2008). Furthermore, depending on the type of electron donors, cultivation method and operational condition, products through acetogenic process can be different. Propionate, butyrate, hexanoate, ethanol, iso propanol, butanol and hexanol are the examples of the diverse products from acetogens (Drake et al., 2006). This diverse metabolic activity of acetogens and their remarkable ability of production of valuable chemicals and solvents from CO_2 makes them one of the most interesting type of bacteria attracting the attention of many scientists.

2.7.2 *Microbial electrosynthesis (MES) and conversion of CO_2 to valuable organic chemicals using anaerobic biocathode*

Over the last few decades, the excessive consumption of fossil fuels led to the most destructive problems such as danger of irreversible fossil fuels depletion in near future and global warming which is the consequence of emissions of greenhouse gases (Lovley, 2006). It was estimated that 86% of CO_2 emission is caused by fossil fuels combustion through industrial activities, and the rest 14% is from other activities such as forestry activities. Carbon dioxide equivalent (CDE)

is a term used to measure the involvement of CO_2 (equivalent quantity or concentration) in global warming. The unit used for CDE is ''Gt CO_2 eq'' which is equal to n × 10^9 tonnes of CO_2 equivalent (Gohar and Shine, 2007). Annual CDE is measured at approximately 52 Gt CO_2 eq $year^{-1}$. This huge emission makes CO_2 as a major contributor for global warming and climate change (Bajracharya et al., 2017a). To reduce the load of CO_2 in atmosphere, two main approaches have been considered. 1. Utilizing renewable sources of energy as an alternative to fossil fuels 2. Capturing and utilizing CO_2 in environment. The first approach has been put into action in many countries by using the energy generated by solar or wind power. The second approach raises the demand on new technologies to reduce CO_2 emission and improve its capture with various processes. In order to sequestrate CO_2, different physical, chemical and electrochemical methods have been suggested recently (Kondratenko et al., 2013); however, the electrochemical method is considered as the most promising due to the moderate operation conditions such as temperature, pressure and even the cost of operation. The electrocatalytic reduction of CO_2 to liquid fuels, chemical feedstocks and valuable chemicals has attracted growing interest in CO_2 capture and utilization in the past few years (Zhao et al., 2012). The electrochemical processes offer good reaction selectivity and enhance CO_2 reduction reaction kinetically which is one of the notable advantage of this method. However, due to the extreme stability of CO_2 molecule, the overpotential for electrochemical reduction of CO_2 is relatively high and thus the energy required for the process could be large (Rabaey and Rozendal, 2010). Attempts to commercialize CO_2 reduction techniques have been met with limited success despite the vast amount of research undertaken. This has been largely as a result of high capital and operating costs. Regarding previous research studies, overpotential is one of the main concerns in electrochemical system caused by potential losses such as activation or ohmic losses. In order to reduce the overpotential, different methods have been examined such as increasing electrode surface area, solution conductivity and using catalysts (Oh and Logan, 2006). Using different catalysts has become one of the most beneficial way of achieving this target. The catalysts selected should decrease the overpotential and increase the selectivity and energy efficiency of the reaction. Over the past few decades, metal catalysts have been commonly used for electrochemical reduction of CO_2 and have received lots of attention in this area (Lim et al., 2014). Of all the metals, copper (Cu) seems to be the most widespread as it is well-known to catalyse CO_2 reduction reaction for hydrocarbons production (Kuhl et al., 2012, Loiudice et al., 2016). Zhao et al. reported the electrochemical reduction of CO_2 to formic acid using Pt as catalyst in cathode by MEC (Zhao et al., 2012). In addition, electrodeposited palladium (Pd) on Pt were used to convert CO_2 to formic acid (Kortlever et al., 2015). However, overpotential remained still high on metal electrodes. In addition, the chemicals produced

through electrochemical reduction of CO_2 are usually not diverse and limited to H_2 or C1 products (CO or formate). Furthermore, lack of robustness of these chemical catalysts at the surface of the electrodes is one of the challenges of using such catalysts, they are expensive and it will be costly to use this system in a larger scale. More importantly, this method is not environmentally sustainable. Photocatalytic reduction of CO_2 on TiO_2 and other metal oxides using solar energy is another method for CO_2 removal. However, as mentioned metals are expensive and not sustainable. In addition, it was reported that the efficiency of this technique is much lower than the natural photosynthesis (Habisreutinger et al., 2013). Non-metallic catalysts had been also used in CO_2 and CO conversion. Production of formate using formate dehydrogenase enzyme and also pyridine was investigated which are quite expensive and not economic for using in larger scale (Lim et al., 2014). Therefore, employing alternative catalysts are necessary. It was discussed that engineering bacterial pathways can be an efficient method for CO_2 sequestration (Hu et al., 2018). Bio-electrochemical system is potentially a sustainable method providing a promising approach to conversion of wastes to bio-energy and CO_2 to value-added products using bacterial catalysts at the cathodes. In comparison with metal catalysts, biocathodes are economic in cost of operation and construction, self-generated and robust (He and Angenent, 2006). Biotic cathodic reactions under anaerobic condition was primarily limited to H_2 production, which two electrons derived from anodic compartment was combined with 2 protons in cathodic compartment and generate one mole of H_2. Different bacteria such as sulphate reducing bacteria or even free bacterial enzymes were known responsible for H_2 production. However, MES has broaden this application of BES to reduction of CO_2 and production of value-added products. This approach has been derived from the outstanding capability of anaerobic autotrophic bacteria in reduction of CO_2 and synthesis of valuable chemicals discussed in Section 2.5.1. Anaerobic condition is not only preferable for such anaerobic autotrophic bacteria in BES, but also it is preferred from the electrochemical aspect, as oxygen reduction is thermodynamically more favourable than CO_2 reduction. Therefore, to direct cathodic reactions to production of valuable chemicals from CO_2, oxygen must be removed from the cathodic compartment. The rationale behind using such technique is catalysing the reduction of CO_2 using biocatalysts via the electrons derived from the anode and CO_2 in the catholyte, leading to production of both bio-energy and products. The significant advantages of this method are its low cost, sustainability and robustness as well as production of value-added products from gaseous waste of CO_2.

2.7.3 *Pure culture of acetogens as anaerobic biocathodes responsible for MES processes*

The term microbial electrosynthesis (MES) was used for the first time in 2010 by the study on pure culture of aceogen in BES (Nevin et al., 2010). In their study, Nevin et al. reported that pure culture of *Sporomusa ovata* was able to accept electron from the cathode and reduce CO_2 to acetate and small amounts of 2-oxobutyrate (Nevin et al., 2010). Another report published by the same authors investigated the ability of CO_2 reduction with wider range acetogens of *Sporomusa silvacetica, Sporomusa sphaeroides, Clostridium ljungdahlii, Clostridium aceticum* and *Moorella thermoacetica* through MES (Nevin et al., 2011). These studies proved the concept of CO_2 reduction as the cathodic reaction using the electrons derived from the cathode and production of value-added products through MES. Moreover, these studies showed the ability of different species of *Clostridium* to consume CO_2 and electrode as inorganic carbon and energy sources, respectively. *Clostridium* is the known type of acetogens able to produce different valuable chemicals from CO_2 through WL pathway. After these proof-of-concept studies, many studies on pure culture of different acetogens in BES confirmed the ability of acetogens in MES of acetate from CO_2 and cathodes. Different studies have been performed on pure culture of *Sporomusa* in BES. *Sporomusa* is one of the acetogens able to produce acetate as the major product through MES. Operational condition for growth of *Sporomusa* is similar to other acetogens, with optimal pH of 7.0. *Sporomusa* can grow on different organic substrates of sugars, organic acids and alcohols such as methanol and fomrate as well as H_2/CO_2 through WL pathway (Tremblay et al., 2015). Aryal et al. investigated the performance of different *Sporomusa* species in BES and their ability in reduction of CO_2 to acetate using the electrons derived from cathodes (Aryal et al., 2017a). In this study, it was reported that *Sporomusa aerivorans* didn't show any activity in BES, however, *Sporomusa Ovata* produced high rate of acetate (more than 6.5 mM day^{-1}) through MES processes (Aryal et al., 2017a). Different parameters to enhance the performance of *Sporomusa* in BES for production of acetate and ethanol form CO_2 was also investigated such as effect of tungstate in the medium (Ammam et al., 2016), or investigating different operational conditions or cathodic materials to enhance the biofilm formation and subsequently production (Zhang et al., 2013b, Aryal et al., 2017b).

Acetobacterium is another well-known acetogen recognised in production of valuable chemicals particularly acetate from CO_2 and H_2 (Demler and Weuster-Botz, 2011). Apart from H_2/CO_2 it can be grown with other organic substrates such as formate, methanol and lactate (Simankova and Kotsyurbenko, 2015). It was reported that *Acetobacterium woodii* is able to produce ethanol from CO_2 as well as utilize it as an electron donor (Buschhorn et al., 1989).

Compared to *Sporomusa*, lower and wider range of pH (5.2-8.0) is reported as the optimal condition for *Acetobacterium* (Balch et al., 1977, Simankova and Kotsyurbenko, 2015). In previous study on pure culture of *Acetobacterium woodii* in BES by Nevin et al no cathodic current and production was observed (Nevin et al., 2011). However, in studies on mixed culture of microorganisms *Acetobacterium woodii* was known responsible for MES processes which will be discussed in the next section. It was later discussed in the review by Lovley at el that *Acetobacterium woodii* is more likely not able to uptake electron directly from the cathode and requires source of H_2 (Lovley and Nevin, 2013).

2.7.4 *Acetogens responsible for MES processes in mixed culture anaerobic biocathode*

Diverse studies on MES using pure culture of acetogens showed the ability of these bacteria in production of organics utilising CO_2 and cathode as inorganic carbon and energy sources, respectively. To take MES to practical conditions, many studies were performed on BESs using mixed culture of microorganisms collected from environmental wastes. In most of these studies *Acetobacterium* was reported as one of the most dominant bacteria in anaerobic biocathode enriched during the experiment. This was despite the fact that no production was observed in BESs inoculated by *Acetobacterium woodii* in the study by Nevin et al as discussed in previous section. In their study no other source of energy was supplied, therefore it showed the lack of capability of *Acetobacterium woodii* to consume a cathode as an energy source (Nevin et al., 2011). It was suggested by Lovley et al that the dominance of *Acetobacterium* in biocathode responsible for MES processes can be due to consumption of H_2 produced biotically or abiotically in cathodic compartments (Lovley and Nevin, 2013), which will be discussed in the next section. This could also show the synergetic effect between different types of bacteria in mixed culture of microorganisms. Marshal et al studied biocahtode development in BES inoculated from brewery wastewater sludge and reported the *Acetobacterium* as the dominant bacteria at the cathode, in addition to archaea of methanogens (Marshall et al., 2012). In their study, methane, acetate and hydrogen were the products detected in cathodic compartments. In addition, further optimization in production through MES was reported by the same author in longer-term operation and by supressing methanogens activity (Marshall et al., 2013). Furthermore, a metabolic model of the dominant bacteria in biocathode in their study was also reported (Marshall et al., 2017). The effect of pH on the performance of *Acetobacterium*-dominated biocathode enriched from brewery wastewater was investigated. It was observed that at more acidic condition around 5, higher concentrations of hydrogen and acetate were

detected leading to enhance in BES performance (LaBelle et al., 2014b). Higher abundance of *Acetobacterium* was reported in biofilm of their study compared to that in solution. However, in the study reported by Patil et al in which acetate was the major product from bicarbonate, methanogens comprised the highest abundance of bacterial composition at the electrode, while *Sporomusa* and *Acetobacterium* were detected with relatively low abundance in suspension (< 10%) (Patil et al., 2015a). Author discussed that high concentration of H_2 in BES could be the reason of competition between acetogens and methanogens. Afterwards, Arend et al studied the bacterial community enriched through the continuous operational mode of MES processes (Arends et al., 2017). In their study, *Acetobacterium* was found as a dominant bacteria in supernatant, which was known as the reason of acetate production. In addition, *Desulfovibrio*, a known sulphate-reducing bacteria, was also found in the solution, similar to the study by LaBelle et al (LaBelle et al., 2014b). *Desulfovibrio* was found in the composition of H_2-producing anaerobic biocathode, in which showed the direct uptake of electron from cathode and production of H_2 (Croese et al., 2011, Aulenta et al., 2012). LaBelle et al suggested the syntrophic interaction of *Acetobacterium* and *Desulfovibrio* in which H_2 produced by *Desulfovibrio* from the cathode was consumed by *Acetobacterium* for the production of acetate from CO_2 (LaBelle et al., 2014b). Similar result was reported in a bioreactor (not BES) inoculated by *Acetobacterium* and *Desulfovibrio* (van Houten et al., 1995). Presence of CO_2 and H_2 in the reactor could supply acetate for *Desulfovibrio* produced by *Acetobacterium*, leading to production of H_2. It was observed in their study that change in pH ranging from 5.5 to 8 did not affect the abundance of microorganisms. It was reported in the previous study that H_2-dependent CO_2 reductase in *Acetobacterium woodii* could catalyse the production of formate and other energy carriers from H_2 and CO_2 (Schuchmann and Müller, 2013). In addition, under sulphate limiting condition, sulphate-reducing bacteria, which some of them also have CO_2 reductase, produces formate from H_2 and CO_2 (Schuchmann and Müller, 2013, da Silva et al., 2013). Formate is one of the well-known substrate for acetogens including *Acetobacterium*, *Clostridium* and *Sporomusa*. Therefore, this synergetic effect can increase the production and consequently efficiency of the system. Arend et al also found *Rummeliibacillus* in the bacterial community in solution correlated to production of butyrate. They also suggested that presence of *Rummeliibacillus* was more likely relevant to production of iso-propanol (Arends et al., 2017). Wenzel et al reported different dominant bacteria in biocathode in duplicates with similar operational conditions (Wenzel et al., 2018). In their study, *Clostridium* and *Serratia* dominated the bacterial community in one reactor, however *Burkholderia* and *Xanthobacter* were recognised as dominant bacteria in the other duplicate reactor. Three possible pathways for carbon fixation was suggested in their study, which WL was one of these pathways. Many other

reports studied MES processes using mixed culture of microorganisms but not all of them identified bacterial communities responsible for MES dominated biocathodes.

2.7.5 *Effect of cathodic potential on MES processes*

Despite the vast number of investigations on electron transfer in bioanode, the underlying mechanisms of CO_2 conversion using biocathode are still quite unclear, particularly those regarding the role of cathode and whether MES occurs at the electrode or in suspension. However, similar to bioanode and aerobic biocathode, two main mechanisms have been presumed for the extracellular electron transfer (EET) from a cathode to microorganisms through MES (Rabaey and Rozendal, 2010, Matysik and Borisov, 2014, Huang et al., 2011). The schematic of EET mechanisms is presented in Figure 2.7.

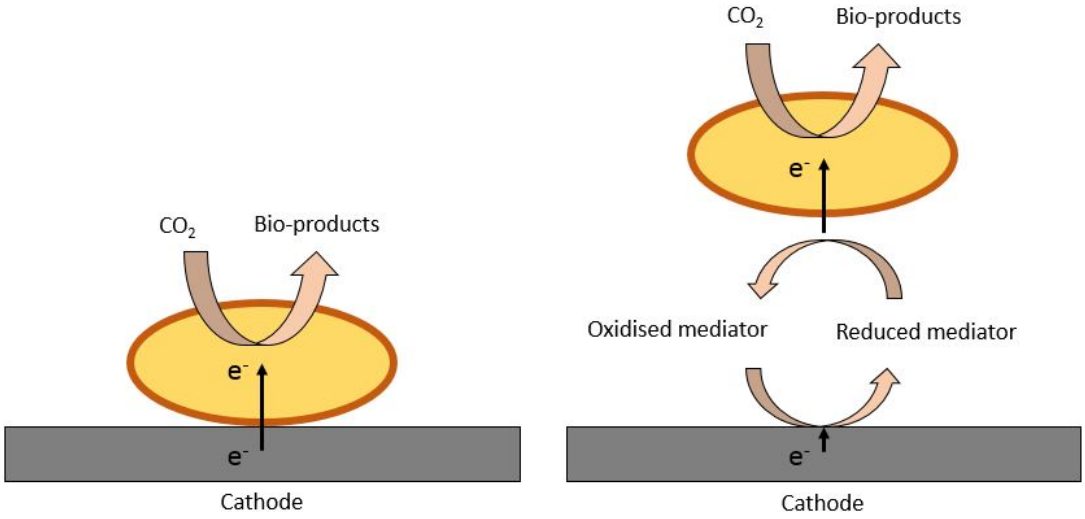


Figure 2.7. Schematic of electron transfer between anaerobic biocathode and cathode through MES processes.

It has been suggested that in the first mechanism, microorganisms are able to take electrons directly from an electrode and transfer them to the final electron acceptor; this mechanism is known as direct electron transfer (DET) (Tremblay et al., 2017, Lovley and Nevin, 2013). In this mechanism the direct contact of the bacterial cell membrane and the electrode is required. It has been postulated that electrons from cathode are received by the redox macromolecules in cells' outer membrane such as cytochromes for the bacterial metabolism and reduction of CO_2. The second mechanism is referred as indirect electron transfer (IET), usually through mediators.

These mediators can be self-excreted redox-active compounds that transfer the electrons between bacterial cells and electrodes (Karthikeyan et al., 2019). In this mechanism, the direct physical contact of bacterial cells and electrodes is not required as redox compounds (either cells self-excreted or external compounds) play the role of shuttles to carry out the IET mechanism. Additionally, IET can be performed using exogenous mediators and not self-excreted redox compounds. These additional mediators can be reduced/oxidised between bacterial cells and electrodes and facilitate the electron transfer. Hydrogen is a well-known mediator that can either be produced biotically (hydrogen produced by microorganisms) or abiotically (reduction of the protons in the electrolyte by the electrode) as shown in Equation 2.13, and supply the electrons required for bacterial community through MES (Huang et al., 2011).

$$2\ e^- + 2\ H^+ \rightarrow H_2 \qquad (2.14)$$

A polarised electrode provides the electrons required for the bacterial cells through MES. Therefore, the potential applied at the electrode is a key parameter affecting MES and the mechanism of electron transfer between bacteria and an electrode. The potential required to be applied at the cathode depends on the reduction reaction occurring at the surface of electrode. Thermodynamically, the production of acetate as the main product of MES through the WL pathway (Equation 2.13) from CO_2 requires -490 mV cathodic potential. However, this value is different in practical conditions due to the potential losses as associated with mass transfer or ohmic voltage losses (May et al., 2016). To study the electron transfer mechanism, choosing the suitable potential can be challenging due to the possibility of abiotic H_2 evolution reaction (HER) (Batlle-Vilanova et al., 2014). The theoretical redox potential of HER is -200 mV (pH: 7, 298.15 K, pH_2: 1 bar), however this can be different depending on the electrodes materials. It was observed that at the surface of carbon based electrodes -800 mV was the minimum potential that discard the possibility of HER (Deutzmann et al., 2015). Therefore, to study DET mechanism in previous studies the potentials more positive than -700 or -800 mV was poised at the cathodes to discard the possibility of abiotic H_2 evolution. The proof-of-concept MES studies investigated the possibility of DET mechanism in MES at the cathodic potential of -600 mV. At this low overpotential the possibility of hydrogen evolution is more likely ruled out, therefore any electrons consumption form the cathode is presumed as the direct electron uptake. This was suggested in the studies by Nevin et al. on pure culture of acetogens, where electrons

uptake and production of acetate was observed at a low overpotential (Nevin et al., 2010, Nevin et al., 2011). Although in these studies the columbic efficiency was more than 70%, the concentration of accumulated acetate in the catholyte was limited to less than almost 1.8 mmole (0.9 mM considering the volume of cathodic medium). At the potential of -700 mV, the production of acetate was studied using *Moorella thermoacetica* and *Moorella thermoautotrophica* as biocathodes at different temperature from 25 °C to 70 °C (Faraghiparapari and Zengler, 2017). In their study, best temperature was 60 °C, leading to acetate production rate and columbic efficiency of 6.9 ± 0.6 mM $m^{-2}day^{-1}$ and 79 ± 15% in BESs with *Moorella thermoacetica* and 11.6 ± 0.9 mM m^{-2} day^{-1} and 72 ± 4% in BESs with *Moorella thermoautotrophica*, respectively (Faraghiparapari and Zengler, 2017). Although DET mechanism has the advantage of low overpotential, the concentration of products are not very significant at the potentials more positive than -800 mV. Therefore, few methods were suggested to enhance the production through DET in MES. The recent study reported the effect of adding penicillin to MES with *Moorella thermoautotrophica* as biocathode on increasing the cells permeability and consequently enhance in MES performance by increasing in the formate and acetate concentrations at the cathodic potential of -600 mV (Chen et al., 2018a). In their study, it was hypothesized that increase in cells permeability could increase the redox activities of outmost cytochrome C and consequently the redox electron shuttles which facilitate the electron uptake and penetration of products through the cells (Chen et al., 2018a). Another study on MES using *Moorella thermoautotrophica* immobolized at the cathode poised at -600 mV reported the significant increase in acetate production and efficiency of the system by using carbon nanoparticles at the cathode of carbon cloth and increasing the operational temperature from 25 °C to 55 °C (Yu et al., 2017). Increasing the surface area and porosity of cathodes was another hypothesis to enhance MES processes by increasing the bacterial interaction with a cathode, studied by Nie et al. (Nie et al., 2013). In their study, they showed that at a cathodic potential of -600 mV, using a cathode of nickel nanowires anchored to graphite in MES increased the production by *Sporomusa* 2.3 fold compared to that by using non-modified graphite (Nie et al., 2013). Although using pure culture in MES has an advantage of selectivity of the products, it has few challenges such as the limits of providing a sterile experimental condition and low production. Using mixed culture of microorganisms in MES offers important benefits of synergetic effect of diverse bacterial community and the possibility of reactors operation without the limits of sterile conditions. Production of diverse products of acetate, propionate, butyrate, ethanol and butanol was reported in the BESs inoculated by mixed culture of microorganisms at the cathodic potential of -600 mV (Zaybak et al., 2013). In their study, inoculum was pre-enriched before transferring to BES. The pre-enrichment included

heterotrophic growth of inoculum for two weeks in incubator for two weeks, heterotrophic growth of inoculum for further weeks in BESs and autotrophic growth of inoculum in BESs. They hypothesized that this pre-enrichment could accelerate the start-up of the biocathode development and production. However, there is a risk of selection of fermenters over heterotrophic growth in such pre-treatment. Despite the advantage of the diverse products in their system, products concentration was not significant. The accumulated acetate, propionate and butyrate (the most concentrated products) were detected 1.90 ± 0.73 g L^{-1}, 2.09 ± 0.56 g L^{-1} and 2.25 ± 0.20 g L^{-1} respectively after almost 70 days of experiment (Zaybak et al., 2013).

Although in these studies DET was assumed as the mechanism of electron transfer (using cytochromes) due to the low overpotential, it has been presumed by many studies that self-excreted redox compounds are involved making the powerful combination alongside DET mechanism (Rosenbaum et al., 2011). Hydrogenase is one the well-known and important enzyme able to reduce and oxidise H_2. This enzyme has been found in vast range of anaerobic bacteria including methanogens and acetogens. Therefore, it is believed that microorganisms can catalyse the production of H_2 known as biotic H_2 production which is then oxidised by CO_2 reducing bacteria and used as an energy coupled with reduction of CO_2 (Rosenbaum et al., 2011). CO_2 reducers such as *Acetobacterium* oxidise H_2 and produce biological energy (ATP) by reducing NAD^+ to NADH (Xiu et al.). H_2-mediated mechanism using hydrogenase was observed in *Desulfovibrio* species (Rojas et al., 2018). Production of H_2 using membrane-bound (NiFe)-hydrogenase in *Thermococcus kodakarensis* and its effect on H_2 production was also studied (Kanai et al., 2015). Similar mediated electron transfer was also reported using other enzymes such as formate dehydrogenases recognised with its capability in production of formate which can be consumed by CO_2 reducers as an energy source. It was shown that formate dehydrogenase enzyme at the surface of the poised electrode was able to catalyse CO_2 reduction and produce formate (Reda et al., 2008, Srikanth et al., 2014). Many bacterial cells such as acetogens and sulphate-reducing bacteria produce formate dehydrogenase through their metabolism. Moreover, formate oxidises at the similar potential of H_2, therefore can be used as an energy source by bacteria through MES processes. Thus, production of energy carriers of H_2 and formate through the enzymatic reactions at the surface of electrode provides electrons required for reduction of CO_2, mimicking DET mechanism (Reda et al., 2008). Such mechanism was precisely reported in a successful study on electromethanogenesis (production of methane from CO_2 in BESs (Cheng et al., 2009b)) using *Methanococcus maripaludis* (Deutzmann et al., 2015).

The approach of DET mechanism has the key advantage of possibility of direct catalysis and low overpotential. However, one of the disadvantages of this mechanism is the long start-up of the biocathode and also low concentration of the products (Patil et al., 2015a). In addition, another disadvantage was reported as an internal and external diffusion. Internal diffusion limits the diffusion of substrate through the biofilm and external diffusion is related to the limits of the movement of the products within the biofilm, as the products of the microbes can provide the toxic condition for them (Rabaey et al., 2011). Applying more negative potential than -700 or -800 mV evidently provides more energy available for bacterial activity (Mohanakrishna et al., 2016). It has been postulated that more negative cathodic potential could enhance the biocathode start-up by overcoming the energy losses associated with the electrode overpotential, increasing the possibility of higher H_2 production and subsequently increase the concentration and diversity of the products. At a potential of -790 mV using mixed culture of microorganisms high concentration of products was reported (Marshall et al., 2012). Suppressing the methanogenic activity, acetate accumulated in the catholyte and acetate production rate reached a maximum of 175 mM (10.5 g L^{-1}) and 17.25 mM day^{-1} (1.04 g L^{-1} day^{-1}), respectively after 20 days (Marshall et al., 2013). Such high production was presumed as a result of high energy available for bacterial community provided by polarised cathodes. Furthermore, as H_2 was detected in their study, high production could be due to the effect of H_2 as a mediator generated at the cathodic potential of -790 mV on enhancing electrons transfer between the electrode and bacteria through IET mechanism (Marshall et al., 2013). It was reported in many studies that abiotic H_2 produced at the beginning of experiment in the BES accelerated the start-up of the MES processes and enhanced the production (Xiu et al.). Furthermore, in most of studies abiotic H_2 production was followed by biotic H_2 production confirmed by electrochemical analysis and consequently increase in production of organics. Electrochemical analysis such as cyclic voltammetry (CV) performed in these studies before and after the biocathode development showed significant shift in onset potential of HER in the voltammograms after biocathode development confirming biotic production of H_2 (Patil et al., 2015a, Matysik and Borisov, 2014). Therefore, higher production through MES at more negative potentials can be correlated with higher H_2 production. For instance, the cathodic potential of -1000 mV led to significant production of butyrate (maximum of 20.2 mM C) alongside acetate, ethanol and butanol. (LaBelle et al., 2014b). Production of methane using mixed culture of bacteria was also reported at the cathodic potential of -850 mV (Villano et al., 2010) and better performance at -1050 mV (Villano et al., 2011). Afterwards, due to higher and more diverse production through IET mechanism reported by applying cathodic potential lower than -700 mV, large number of studies selected this range of potentials to solve the limits of biocathode development and

production. Cathodic potentials of -900 mV (Zhen et al., 2015, Van Eerten-Jansen et al., 2013b), -1050 mV (Jourdin et al., 2014, Jourdin et al., 2015b, Jourdin et al., 2016), -1100 mV (Su et al., 2013) and even more negative potentials (Jiang et al., 2013, Patil et al., 2015a, Gildemyn et al., 2015) was used in recent studies to enhance the production through MES.

2.7.6 *Effect of inorganic carbon source on MES processes*

Type of inorganic carbon source is another crucial parameter affecting MES processes as it is a terminal electron acceptor. Availability of inorganic carbon source is important as many acetogens cannot grow under specific operation conditions, except inorganic carbon source is easily available (Drake et al., 2006). Inorganic carbon can be supplied in two forms for autotrophic growth and activity of biocathode: 1. providing gaseous CO_2 in solution or 2. adding $NaHCO_3$ in medium. Depending on the experimental and reactors designs, different inorganic carbon sources were selected in previous studies and it seemed that both types could provide available inorganic carbon for bacteria to consume through MES. Many studies purged CO_2 directly in the cathodic medium, which could provide sufficient inorganic carbon source required for bacterial activity. However, the pressure of the gas should be taken into consideration as high gas pressure can pressurize and break the membrane in case of two compartmnets design reactors. Recycling cathodic medium could be another operational method providing the constant CO_2 available for biocathode. In this design, cathodic medium can be recycled using a pump (adjusted at a desired flow rate) to the container outside of the reactor while the container is purged constantly by CO_2. GDE design could also enhance the mass transfer of CO_2 in catholyte (Bajracharya et al., 2016b, Srikanth et al., 2018b). Alternatively, many studies provided inorganic carbon source in form of bicarbonate constantly available in the solution, which $NaHCO_3$ was added to cathodic medium (Patil et al., 2015a).

Although both forms of CO_2 and bicarbonate could supply the available source of inorganic carbon for biocathode, there are few parameters related to these sources that make CO_2 more favourable source of inorganic carbon. One of these parameters is pH of the solution. Due to the equilibrium of $CO_2/HCO_3^-/CO_3^{2-}$ in the aqueous solution, dissolved CO_2 in solution provides the slight acidic pH (4.0-6.0), whereas pH is higher for the dissolved bicarbonate in solution (~8.0). This feature of these sources of inorganic carbon has a significant effect on MES processes, as pH of the growth medium is an important parameter in bacterial growth and activity. In the previous study in comparing CO_2 and $NaHCO_3$, catholytes' pH caused by using

different types of inorganic carbon seemed an important parameter affecting the MES processes (Mohanakrishna et al., 2016). It was reported in their study that supplying gaseous CO_2 controlled the solution pH around 7.0 leading to higher production of acetate, while $NaHCO_3$ in the catholyte provided alkaline pH contributed to low production (Mohanakrishna et al., 2016). This could be due two reasons. Firstly, lower pH makes the HER and acetate production reactions thermodynamically more favourable, according to Nernst equation. pH is the measure of protons activity. In case of DET mechanism, more proton in the catholyte steers the reaction demonstrated in Equation (2.13) to production of acetate. Moreover, as mentioned in the previous section H_2 plays an important role in MES processes through IET mechanism. Higher proton in the catholytes derives the reaction of Equation (2.14) to the production of H_2, and increase in H_2 is associated with increase in acetate production (Equation 2.13). The effect of pH on production of H_2 and acetate was studied by LaBelle et al. (LaBelle et al., 2014b). In their study it was reported that decreasing the pH of catholyte to 6.5 increased the production of H_2 (increase in H_2 partial pressure), leading to increase in the production of acetate through MES using *Acetobacterium* as a dominant bacterial community (LaBelle et al., 2014b). The correlation between pH, fermentative H_2 production and acetogenic VFAs (C2 and C4) in bioreactors was also reported previously (Lee and Rittmann, 2009, Demler and Weuster-Botz, 2011). In the study by LaBelle et al. it was also reported that acetate production was ceased at the pH of 5.0 despite the higher production of H_2 compared to that under pH 6.5 (LaBelle et al., 2014b). This could be relevant to the optimal pH for acetogens activity. It has been studied that wide range of anaerobic acetogens tend to maintain the constant pH inside their cells known as intracellular pH. Therefore, significant decrease in extracellular pH due to the acetogenic production of acidic products such as acetate and butyrate affects the growth and activity of acetogens (Menzel and Gottschalk, 1985). For instance, it was reported that *C. thermoaeetieum* cannot grow below pH 5.0 as the cells cannot maintain under the significant difference of intracellular pH (~5.8-6.0) and extracellular pH(<5.0) known as ΔpH (Menzel and Gottschalk, 1985). Thus, slightly acidic condition (pH between 5.0 and 6.0) can provide the proper condition for acetogenic activities. Some acetogens gain energy by the proton gradient between inside and outside of the cell caused by ΔpH (Batlle-Vilanova et al., 2016). For instance, It has been predicted that the proton gradient between inside and outside of *Clostridium Ljungdahlii* cells contributes to the ATP synthesis through H^+-translocating denylpyrophosphatase (ATPase) which is an enzyme catalysing the conversion of ADP to ATP (Tremblay et al., 2013). Another advantage of slightly acidic pH in the catholyte is the suppression of methanogenic activity. The intracellular pH of methanogens is reported around 6.7, therefore the extracellular pH less than 6.0 provides the proton gradients across the cytoplasmic layer, affecting the intracellular

production (Jarrell and Sprott, 1981, Tiwari and Ghangrekar, 2015). Although other methods such as heat treatment or using inhibitor chemicals are also known to control the activity of methanogens, acidic pH seems the effective method in MES to produce valuable chemicals from CO_2 as it enhances the acetogenic activity while supressing the methanogenic activity (Jadhav et al., 2018).

Apart from pH, it has been suggested that another advantage of using gas form of CO_2 for acetoegns is that it is more favourable for the protein synthesis of the some acetogens such as *Clostridium Kluyveri* than bicarbonate (Tomlinson and Barker, 1954, Grootscholten et al., 2013c). However, more investigation on the effect of CO_2 and bicarbonate on synthesis of acetogens cells is required. In addition, CO_2 generated from humans' activity which is believed as the main cause of climate change is emitted to atmosphere in gaseous form. Therefore, MES can be an effective method in converting gaseous CO_2 captured from the atmosphere and producing value-added products (Jiang et al., 2018).

2.7.7 *Role of biofilm at the cathode in MES processes*

Despite the vast number of investigations on MES, it is still not clear if MES processes occur in suspension or at the surface of electrode. In case of MES processes at the surface of a cathode, it is hypothesized that biofilm comprised of bacterial community responsible for MES forms at the electrode and uptakes electrons directly or indirectly from the cathode. Natural conductive biofilm composes of bacterial cells and extracellular polymeric substance (EPS) such as proteins or nucleic acids (e.g. DNA) (Bird et al., 2019). As described in previous sections, bacterial cells are able to produce small molecules such as hydrogenases to facilitate the electron transfer between the cells and electrodes. After electron transport networks bacterial cells attach to the surface of the electrodes and begin to produce EPS and make a conductive matrix allowing the bacterial cells to form biofilm (Kerr et al., 2003). Higher secretion of EPS is associated with formation of denser biofilms. The previous investigations on anodic biofilm of *Geobacter sulferreducens* showed the immediate decrease in the charge transfer resistance after formation of biofilm; however, over time that cells grow and biofilm get denser the number of dead cells increase in the inner layer of the biofilm leading to high diffusion resistance (Sun et al., 2016). Therefore, not only the density of the biofilm but also the presence of electroactive live cells in the biofilm is vital to increase the conductivity by enhancing the electron transfer to/from electrodes through the biofilm from/to electrodes (Angelaalincy et al., 2018). Such biofilms are able to grow, replicate and repair themselves as well as catalysing the desirable

reactions. These features of electroactive biofilms make them more promising, particularly through MES in cathodic compartments (Kiran and Patil, 2019). Similar to anodic biofilm formation, it has been suggested that cathodic biofilm formation from mixed culture of bacteria leads to greater performance in terms of electron uptake and production compared to that from pure culture (Zubchenko and Kuzminskiy, 2017). This can be due to the diversity of bacterial community in mixed culture and synergetic effects. Depending on dominant mechanism (DET or IET), the mechanism of electron transfer between an electrode and cells can be different. To boost electrons uptake, the attachment of bacteria at the surface of electrodes and formation of biofilm are important. Different parameters can affect the biofilm formation and composition such as electrodes materials, pH changes, gas in the solution, chemical changes such as change in the medium compounds and any other molecules produced by bacterial cells. Regarding electrodes materials, carbon based electrodes are commonly used because they are conductive, biologically compatible, porous and cheap. Higher surface area is important while selecting the electrodes. Many studies are performed to modify the carbon based electrodes to improve the porosity and surface area of the electrodes (Bird et al., 2019).

Thus far, many research studies and reviews have been focused on biofilm formation at the anodic electrodes. However, due to the challenges in biocathode development such as slow start-up or lack of biofilm formation at the surface of cathodes, less is known about the formation of biofilm at the cathodic electrodes through MES (Butler et al., 2010). Few methods have been proposed to improve the biofilm formation under anaerobic conditions (Jeremiasse et al., 2010, Jeremiasse et al., 2012). One of the methods to accelerate the biofilm development and subsequently start-up of the production was enriching and forming biofilm at an anodic electrode and using that as biocathode by switching the polarity in BES (Cheng et al., 2010, Rozendal et al., 2007). In the previous study by Pisciotta et al., after developing biofilm on the anode (carbon brush) in sedimentary MFC, they used this electrode as a cathode for MES at different poised potentials (-539, -639 and -739 mV), which they proposed the best production at the potentials more negative than -600 mV (Pisciotta et al., 2012). *Eubacterium limosum* (*Butyribacterium methylotrophicum*), *Desulfovibrio* sp. A2, *Rhodococcus opacus*, and *Gemmata obscuriglobus* were reported as dominant bacteria in their biofilm. Although this method suggested an effective way in biofilm formation, the composition of the biofilm may not be desirable. Before switching the polarity, many bacterial communities favourable for anodic reactions can be developed in biofilm which are not able to reduce CO_2 to desirable products after switching the polarity. Therefore, it can be difficult to control the selectivity of bacterial community and consequently the products using this method. Following that, another

method was proposed and published by Zaybak et al (Zaybak et al., 2013). In their study, they hypothesized that anaerobic facultative autotrophic bacteria can be selected and used as biocahtodes by first growing and enriching the mixed culture of microbes heterotrophically using glucose and later replacing glucose by bicarbonate by providing the autotrophic condition. Similar to the switching polarity method, undesired bacteria can be selected through heterotrophic growth such as fermenters decreasing system efficiency, which can be difficult to remove them form the biofilm matrix over the autotrophic condition.

Alternatively, many studies suggested the biofilm formation at cathodes by directly inoculating cathodic compartments and providing proper operational conditions for the growth and selection of desired bacteria. Studies on CO_2 conversion through MES reported biofilm formation at the surface of electrodes responsible for MES processes. One of these studies was published by Jourdin et al. showing the formation of biofilm at an unmodified cathode (Jourdin et al., 2018). Using an unmodified carbon felt as the cathode, scanning electron microscopy (SEM) images from the biofilm in their study showed the coverage of both carbon fibres and micro-porous gaps between the fibres through filamentous microorganisms (Figure 2.8). The cathodic potential was -1050 mV and both sources of inorganic carbon were used without particular order. The authors suggested that continuous feeding regime is the main reason for such a thick biofilm by providing constant fresh nutrients (Jourdin et al., 2018).

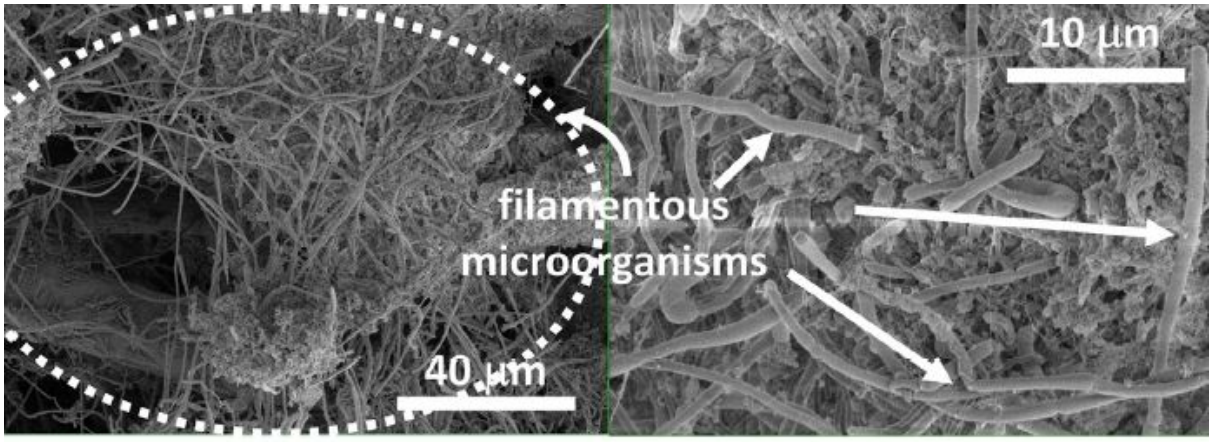


Figure 2.8. Scanning electron microscopy (SEM) images of the cathodic biofilm developed through MES at the carbon felt electrodes in a recent study by (Jourdin et al., 2018).

Similarly, Marshall et al. suggested that long-term operation of MES (180 days) and semi-batch operational mode contributed to biofilm development with *Acetobacterium* as a dominant bacteria at the cathode and consequently improve in acetate production from CO_2 (Marshall et

al., 2013). SEM images of the biofilm formed at the electrodes of their study are show in Figure 2.9.

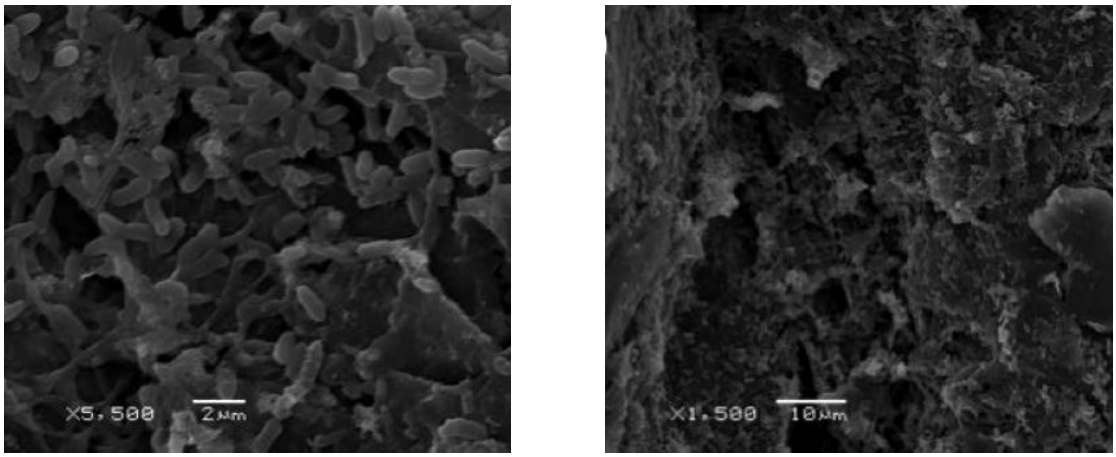


Figure 2.9. Scanning electron microscopy (SEM) images of the cathodic biofilm developed through MES at the graphite granules electrodes in a recent study by (Marshall et al., 2013).

Other studies reported the biofilm formation at the surface of the modified electrodes. Aryal et al. reported the attachment of few cells at the surface of carbon felt over MES, while thick biofilm of *Sporomusa ovata* was formed at the surface of the 3D-graphene functionalised carbon felt (Aryal et al., 2016). In their study, modifying the electrode increased the specific surface area 2 times more than non-modified electrode, which was associated with the increase in acetate production rate through MES by 6.8 fold. Similarly, Cui et al. reported the formation of multiple layers of biofilm of *Sporomusa ovata* at the surface of the metal-oxide modified carbon felt (Cui et al., 2017). Comparing the modified and unmodified carbon felt in their study, it was reported that the 5 times increase in the acetate production rate was achieved after using the modified carbon felt. These studies showed the correlation between the biofilm formation at the cathode and production through MES. This superior function in cathodes with denser biofilm through MES processes can be justified by the conductive matrix of the biofilm consisting of *c*-type cytochromes or active-redox compounds, which can enhance the transfer of the electrons (Bird et al., 2019). However, few studies on MES reported that no biofilm was formed at the surface of the electrode during the experiment and MES processes occurred in a bulk solution (Arends et al., 2017, Bajracharya et al., 2017b, Blanchet et al., 2015). Although continuous feeding regime was carried out and constant fresh medium was pumped inside the BESs in these studies, this operational mode could not promote the biofilm formation and authors reported that the bacterial community in suspension was responsible for CO_2

conversion to valuable chemicals. Due to the different results in previous reports regarding biofilm formation during MES, the parameters affecting biofilm development at cathodes and the role of biofilm in MES processes is still unclear.

2.8 Production of long chain organic acids and alcohols

2.8.1 *Production of long chain organic chemicals from CO_2*

As described in previous sections, acetogens are able to produce acetate from CO_2 known as acetogenesis mechanism through WL or Acetyl-CoA pathway. In general, the optimal pH for production of acetate through acetogenesis mechanism is reported between 5.0 and 7.0 (Balows et al., 2013). Indeed, butyrate, 2,3-butanediol, ethanol and butanol are also the natural end-products of some acetogens such as *Clostridium butyricum* and *Acetobacterium* (Schiel-Bengelsdorf and Dürre, 2012, Zigova and Šturdík, 2000, Bruant et al., 2010). However, environmental and operational conditions can also affect the diversity of the products and production of longer chain compounds by acetoegns. Accumulation of acetate or any other environmental reasons that decreases the medium pH (<5.0) is toxic to acetogens and inhibits their metabolism due to increase in difference between intracellular pH and extracellular pH and also $NADH/NAD^+$ ratio (Zigova and Šturdík, 2000). Under such condition, acetogens such as *Clostridium ljungdahlii* can convert their metabolism from acetogenesis to solventogenesis and re-assimilate the products to their corresponding alcohols such as ethanol or butanol (Vassilev et al., 2018). It has been known that low pH increases the stress level to bacterial community resulting in rejection of reducing equivalents by reducing organic products such as acetate and butyrate towards solvents such as ethanol and butanol instead of biomass (Richter et al., 2013, Martin et al., 2016). In thermodynamic point of view, conversion of organic acids to their corresponding alcohols requires proton translocation at specific sites of the pathways for gaining energy, which can be provided over the low pH condition (González-Cabaleiro et al., 2013). Solventogenesis is a defensive mechanism of acetogens under low pH. Presence of acetate and ethanol when *Clostridium* is presenting can steer the production to butyrate through reverse β-oxidation pathway and from butyrate to caproate known as chain elongation. Reverse β-oxidation pathway involves addition of Acetyl-CoA derived from ethanol to acetate and production of C4 and then C6 products (de Araújo Cavalcante et al., 2017). Production of butyrate can also occur through linear extension of the acetyl-CoA to butyryl-CoA and

eventually to butanol through solventogenesis (Arends et al., 2017). Production of acetone and further production of iso propanol was also reported (Arends et al., 2017). *Clostridium aceticum* is known in biosynthesis of acetone from acetate (Schiel-Bengelsdorf and Dürre, 2012).

β-oxidation pathway is a reversible metabolic pathway which can involve either oxidation or reduction reaction. Production of long chain organic acids through reverse β-oxidation requires specific conditions. One of the important condition is availability of energy-rich compounds. According to thermodynamic calculations, it has been predicted that production of butyrate from acetate and only H_2 is not feasible and other electron donor is required for this reaction (González-Cabaleiro et al., 2013). Electron donors supply energy and provide reducing equivalents such as NADH and Acetyl-CoA. Despite the previous reports on production of C4 and C6 products from H_2 as the sole electron donor in the system and acetate (Steinbusch et al., 2008, Grootscholten et al., 2013b), it has been proposed that ethanol was more likely generated initially from acetate, leading to production of butyrate and caproate from ethanol as an electron donor and acetate (González-Cabaleiro et al., 2013). Ethanol is one of the well-known electron donors in chain elongation reactions by making such reactions thermodynamically and kinetically favourable. It was reported that chain elongation of acetate using ethanol through reverse β-oxidation pathway consists of two steps: 1. Ethanol oxidation and 2. Reverse β-oxidation (Spirito et al., 2014). Table 2.1 shows the steps for the elongation of butyrate and caproate through acetate and ethanol according to previous studies on chain elongation (Angenent et al., 2016, Spirito et al., 2014). C2 elongation from every 5 molecules of ethanol is associated with conversion of 1 molecule of ethanol to acetate for generation of energy (ATP) and reducing equivalents (NADH) (Angenent et al., 2016).

Table 2.1. Reactions involved in elongation of butyrate and caproate from acetate and ethanol (ΔG°r values are at pH 7 and 25 °C, extracted from a review on chain elongation in anaerobic bioreactors (Spirito et al., 2014))

Chain elongation of acetate and ethanol to butyrate:		
Ethanol oxidation	Ethanol + H_2O → Acetate + H^+ + 2 H_2	($\Delta G°_r$ = 10.5 Kj/mole)
Reverse β-oxidation	Ethanol + Acetate → *n*-Butyrate + H_2O	($\Delta G°_r$ = -193.0 Kj/5 mole of products)

Overall	6 Ethanol + 4 Acetate → 5 *n*-Butyrate + H^+ + 2 H_2 + 4 H_2O ($\Delta G°_r$ = -182.5 Kj/5 mole of products)
Chain elongation of acetate and ethanol to caproate:	
Ethanol oxidation	Ethanol + H_2O → Acetate + H^+ + 2 H_2 ($\Delta G°_r$ = 10.5 Kj/mol)
Reverse β-oxidation	Ethanol + *n*-Butyrate → *n*-Caproate + H_2O ($\Delta G°_r$ = -194.0 Kj/5 mole of products)
Overall	6 Ethanol + 5 *n*-Butyrate → Acetate + 5 *n*-Caproate + H^+ + 2 H_2 + 4 H_2O ($\Delta G°_r$ = -183.5 Kj/5 mole of products)

Production of long chain products directed the attention of researchers to syngas fermentation. Syngas fermentation is a bioprocess in which syngas, a mixture of H_2, CO and CO_2, is consumed by microorganisms as a source of energy and inorganic carbon for the production of valuable chemicals (Zeng and Zhang, 2019). In the previous study on syngas fermentation by Steinbusch et al, chain elongation from acetate using H_2 and ethanol were compared showing the production of significant concentration of C6 and C8 organic acids (Steinbusch et al., 2011). In their study mixed culture was used in the fermentation reactors which was dominated by *Clostridium Kluyveri* (Steinbusch et al., 2011). *Clostridium Kluyveri* is a well-known acetogen which can grow on acetate and ethanol. This genome is unique in acetogens due to its valuable fermentation products of butyrate and caproate (Seedorf et al., 2008). Similar to many acetogens *Clostridium Kluyveri* cannot grow under the pH below 5.0. The recent study on alcohol production through acetogenesis and solventogeneisis, it was indicated that low pH around 4.7 – 4.8 led to solvents production of butanol and hexanol, however the pH around 4.5 was detrimental for the growth of bacterial community affecting the production of long chain organic acids (Ganigué et al., 2016). Production of more diverse products such as heptanoate and caprylate was also reported by addition of ethanol to municipal solid waste (Grootscholten et al., 2013a). It was suggested that the higher load of ethanol during chain elongation reactions led to higher production rate and selectivity of caproate (Grootscholten et al., 2013b). Thereafter, the effect of different ratio of ethanol:acetate on production was studied. Comparing different

ratio of ethanol:acetate (1:2, 1:1, 2:1 and 3:1), the highest caproate concentration of around 3033 mg L^{-1} was achieved at the ratio of 3:1 (Liu et al., 2016). In their study, it was also observed that when ethanol was depleted, H_2 played a key role in chain elongation as an indirect electron donor by production of acetate from acetogenesis process, leading to ethanol production through solventogenesis mechanism and using ethanol as an electron donor for chain elongation (Liu et al., 2016), in agreement with previous thermodynamic calculations (González-Cabaleiro et al., 2013). Following that, Spirito et al investigated the effect of 11 different ethanol:acetate ratios on production from syngas fermentation (Spirito et al., 2018). In this study they developed a thermodynamic model predicting that higher ratio of ethanol:acetate leading to more specificity in *n*-caprylate production (Spirito et al., 2018). Although it seemed high concentration of ethanol improved the chain elongation, Lonkar et al. reported that high concentration of ethanol in medium can be toxic for microorganisms resulting in inhibition of production (Lonkar et al., 2016). Investigating different concentration of ethanol in their study, it was reported that ethanol concentration higher than 40 g L^{-1} inhibited chain elongation (Lonkar et al., 2016). Therefore, the concentration of ethanol as an electron donor in the solution should be taken into consideration.

2.8.2 *Effect of different electron donors on production of long chain organic chemicals from CO_2*

Apart from ethanol, other energy-rich compounds were also reported as electron donors for chain elongation of acetate. Lactate is another known electron donor for production of C4 and C6 products through two steps, similar to ethanol. For the first step, lactate is converted to pyruvate resulting in formation of NADH, and energy (ATP) is generated through conversion of pyruvate to Acetyl-CoA (Spirito et al., 2014). Production of acetate, propionate, valerate and caproate was reported from lactate by *Rhodospirillum rubrm* and acetogens of *Clostridium kluyveri* and *Clostridium scatologenes* enriched from mixed culture of organisms (Elsden et al., 1956). Zhu et al. reported the production of caproate from lactate in a reactor microbiome, with *Clostridium IV* developed as a dominant bacteria (Zhu et al., 2015). Similar observation was also reported by Sträuber et al (Sträuber et al., 2016). Moreover, Kucek et al investigated the effect of medium pH on the selectivity of the products either butyrate or caproate using the continuous operational mode (Kucek et al., 2016). Chain elongation through acetate and methanol was examined using a mixed culture microbiome which initially was enriched using ethanol. Adding methanol as an electron donor led to production of butyrate (191 mM C) as a

dominant product and lower concertation of caproate (3 mM C) over the batch experiment, which caproate was almost negligible after changing the operational mode to continuous (Chen et al., 2016). Addition of propanol to the mixed culture microbiome including office paper and chicken manure resulted to production of acetate, propionate, valerate and caproate (Lonkar et al., 2016). In their study, it was observed that propanol concentration higher than 20 g L^{-1} inhibited chain elongation. In addition to propionate, valerate and caproate, butyrate production was also reported by Weimer et al through the growth of *Clostridium kluyveri* using propanol as an electron donor (Weimer and Stevenson, 2012). Chain elongation from amino acids and pyruvate was also reported by isolating *Eubacterium pyruvativorans* from sheep rumen (Wallace et al., 2003, Wallace et al., 2004). Despite the successful studies using different electron donors, ethanol is still the preferred electron donor for chain elongation. This is due to the fact that ethanol can present in the feedstock, or can be produced through the fermentation processes. It can also be produced off site and then added to the reactors for the chain elongation (Roghair et al., 2018).

Acetogens have diverse metabolic abilities, able to oxidise different components in addition to the compounds mentioned such as formate, ethylene, glycerol, sugars and etc. for the production of acetate and longer chain of VFAs and alcohols (Angenent et al., 2016, Drake et al., 2006). Many acetogens are reported to be able to grow on formate. *Moorella thermoacetica* can grow on formate or H_2/fromate and produce acetate (Drake et al., 2008). *Clostridium Ljungdahli*, *Clostridium coccoides*, *Clostridium aceticum*, *Acetitomaculum ruminis*, *Acetoanaerobium romashkovii*, *Acetrobacterium carbolinicum* and many more are reported as the acetogens able to consume formate and produce acetate as a sole electron donor (Gottschalk and BRAUN, 1981, Ljungdhal, 1986, Drake and Daniel, 2004, Ragsdale and Pierce, 2008, Ragsdale, 2008, Drake et al., 2006). As explained in section 2.5.1, WL pathway consists of methyl and carbonyl branches. The first step of methyl branch is formate synthesis from CO_2. Therefore, it is hypothesized that adding formate to the growth medium can improve the kinetic parameters, cells growth and subsequently production. This is due to the assumption that the energy that is saved by the use of formate can be consumed to increase the ATP production. Previously, the effect of supplying formate for *Clostridium ljungdahlii* and *Clostridium carboxidivorans* grown on syngas on their growth and production was investigated (Ramió Pujol et al., 2014). It was reported in their study that different medium pH could also affect the production in presence of formate. Although acetate was still the dominant product in presence of formate from both species, other products of ethanol and butyrate were also detected, with more alcohols at pH 5.0. Ethanol was detected in the medium of *Clostridium ljungdahlii* with

the maximum concentration of 230 mg C L^{-1} at pH 5.0, and butyrate was the other product in the medium of *Clostridium carboxidivorans* with the maximum of 14.9 mg C L^{-1} at pH 6.0 (Ramió Pujol et al., 2014). Formate was also reported as an inducer for *Clostridium butylicum* in production of formate, with the best performance at pH 4.8 (Ballongue et al., 1985). Apart from the positive impact of formate on production, it can be produced through fermentation bioprocess and can be present in feedstock. Moreover, it is reported that formate can be produced in a large quantity using electrochemical methods from CO_2 (Lu et al., 2014, Yang et al., 2017). Therefore, it can be an appropriate growth-support compound or electron donor for production of long chain organics.

2.8.3 *Production of long chain organic chemicals from CO_2 through electro-fermentation*

After proof-of-concept studies on MES and function of cathode as an electron donor for reduction of CO_2 (Nevin et al., 2010), the term ‘’electro-fermentation (EF)’’ was first reported in a comprehensive review about BESs and their potential applications (Rabaey and Rozendal, 2010). In this review, (cathodic) EF was referred to the electrochemical processes for the production of diverse chemicals (longer chain than acetate) through fermentation processes controlled by electric current of electrodes. As mentioned in the last section, acetate is the backbone of chain elongation, and similarly through EF. Therefore, the use of MES and EF together, considered as an integrated process of MES, has an advantage of reduction of CO_2 to acetate through MES and production of higher value products than acetate through EF, over the second stage (Jiang and Zeng, 2018b). Over MES processes, a cathode serves as a sole energy source as described in Section 2.5.3. This energy can be derived for bacterial activities through either DET or IET mechanisms. However, the role of electrodes in EF processes is still unclear. In the previous study on *Clostridium pasteurianum*, it was reported that butanol production through EF consumed 0.2% of the cathodic electrons, while 99.8% of the electrons were supplied from glucose substrate (Choi et al., 2014, Moscoviz et al., 2016). In comparison with the key role of cathodes in providing energy for MES processes, EF processes seem to require more energy sources than a sole electrode. In the recent review on EF processes, it was suggested that electrodes may not act as a sole energy source for EF, but they affect the bacterial metabolic pathways (Moscoviz et al., 2016). According to their review, electrodes potential known as extracellular oxidation-reduction potential (ORP) represents the activity of electrons in catholytes which affects the ratio of NAD^+/NADH of the cells. In addition, the ratio of NAD^+/NADH acts as intracellular ORP affecting synthesis of the enzymes and gene expression

and consequently the metabolic activity. Thus, it is assumed that the redox potential applied at electrodes influences the EF pathways and subsequently the spectrum of the final products (Jiang et al., 2018).

As described, compared to fermentation processes in bioreactors, BESs have the advantage of providing available and constant energy source. Previously, many successful studies reported the production of long chain VFAs and alcohols in BESs, including MES and EF processes without the need of supplying electron donors (Bajracharya et al., 2017a, Agler et al., 2011). It was observed in these studies that electron donors were produced during the experiment, leading to chain elongation from acetate. More specifically, under methanogenic activities suppression condition, CO_2 is reduced to mainly acetate (acetogenesis) using an electrode as a sole electron donor. Accumulation of acetate decreases the pH, switching the bacterial metabolism from acetogenesis to solventogenesis resulting in production of alcohols such as ethanol. Accumulation of acetate and ethanol in the solution leads to production of butyrate and eventually butanol. Depending on the acetogens species, increase in the accumulated acetate and butyrate steers the production to C6 and C8 products. Recently, research has targeted production of long chain VFAs and alcohols due to their value as industrial feedstock. Alcohols such as butanol can be used for car engines as biofuels. Production of ethanol using mixed culture of microorganisms was investigated using different mediators such as methyl viologen at the cathode potential of -750 mV (Steinbusch et al., 2009). Thereafter, significant production of butyrate (maximum concentration of 20.2 mM C) for the first time in BES with cathodic potential of -1000 mV from CO_2 was reported by Ganigue et al (Ganigué et al., 2015). In this study, acetate and ethanol were detected in parallel with butyrate, and the authors discussed that butyrate was more likely produced by chain elongation from acetate using ethanol as an electron donor. Long-term operation using continuous operational mode optimized the system by production of butyrate (highest concentration of 590 g L^{-1}) as a main product and also trace amount of propionate and caproate (Raes et al., 2017). It was also reported that long-term operation of BES led to accumulation of acetate up to 7 to 10 g L^{-1} at the cathodic potential of -900 to -1000 mV, leading to production of ethanol and butyrate (less than 0.4 g L^{-1}) (Bajracharya et al., 2017c). It was reported in their study that ethanol and butyrate production began when accumulated acetate concentration reached more than 1.5 g L^{-1} and pH was lower than 6.0 (Bajracharya et al., 2017c). Production of longer chain products of caproate and caprylate in BES was reported by Earten-Jansen et al (Van Eerten-Jansen et al., 2013a). In their report, BESs poised at -1100 mV were inoculated from the continuously anaerobic bioreactor dominated by *Clostridium kluyveri* from the mixed culture, therefore production of butyrate

and caproate as dominant products and smaller quantity of caprylate was observed immediately after starting the operation. However, CO_2 was not supplied and acetate was present in the medium (100 mM in cathodic medium); therefore, the study just focused on EF processes in BES (Van Eerten-Jansen et al., 2013a).

Over the batch mode, one of the issue can be extraction of products from catholyte. As explained, pH of solution is an important parameter affecting bacterial metabolism and production. To prevent the accumulation of acidic products and adjusting the pH of catholyte, different BESs configuration and operational mode were investigated. Three-chamber reactors including anodic, cathodic and extraction chambers or one anodic and two cathodic chambers were used to extract the products or adjust the pH (Gildemyn et al., 2015, Vassilev et al., 2019). In addition, continuous operational mode seemed a promising solution as not only the products are extracted but fresh growth medium and CO_2 are constantly available for bacterial community through MES processes. However, selection of hydraulic retention time (HRT) is an important factor in continuous mode, as fast flow rate can wash out the bacterial community from a cathodic compartment in case of absence of biofilm and presence of planktonic bacteria responsible for production. Previous study by Bajracharya et al compared the production in BESs from CO_2 in batch and continuous modes (Bajracharya et al., 2017b). Higher acetate production rate (149 mg L^{-1} day^{-1}) was achieved in their study over batch experiments compared to that in continuous operational mode (maximum acetate production rate of 100 mg L^{-1} day^{-1}). However, acetate production was not stable over the long-term continuous mode in their study. The authors discussed that this could be due to the enrichment of bacterial community responsible for MES processes in suspension over the biofilm attached to the electrode, which was more likely led to washing out the bacteria from the system. Investigating different HRTs of 3.3, 5 and 6.7 days in BESs with the fixed cathodic current of -5 mA (reported as equivalent potential of around -1200 mV), Arend et al discovered that at faster flow rate of HRT = 3.3 days, C1 and C2 products dominated the products, while at higher HRT, pH of the solution was less than 5.5 resulting in production of more diverse products of butyrate and iso propanol (Arends et al., 2017). It was observed in their study that planktonic bacterial cells were responsible for MES and EF processes and no biofilm formation was reported. Afterwards, Jourdin et al. reported a successful study on production of C2, C4 and C6 products in BESs operated in continuous mode with applied potential of -1050 mV (Jourdin et al., 2018). Maximum production rate of 9.8 ± 0.65 g L^{-1} day^{-1} acetate, 3.2 ± 0.1 g L^{-1} day^{-1} butyrate, and 0.95 ± 0.05 g day^{-1} L^{-1} caproate was reported in their study. The authors suggested that continuous removal of products and providing fresh nutrients led to formation of dense biofilm

correlated to such high production. Soon after, Vassilev et al reported production of acetate, iso butyrate, butyrate, caproate and their corresponding alcohols in BESs with cathodic applied potential of -1000 mV operated in batch mode (Vassilev et al., 2018). It was predicted through metagenomics analysis that acetogenesis, solventogenesis and β-oxidation were the mechanisms involved in chain elongation. It was discussed in their study that the low pH (≤ 5.0) caused by accumulation of acidic products over the batch operational mode led to solventogenesis metabolism and alcohol production (Vassilev et al., 2018). Among the studies on MES processes, only few of them reported the production of C6 products, which could be related to bacterial community (Wenzel et al., 2018). In general, in studies reported the production of C6 products, *Clostridium kluyveri* was observed as the dominant bacteria enriched from mixed culture of microorganisms, which is a well-known acetogen able to produce C6 products, as explained in Section 2.6.1. Therefore, the diversity of the products is strongly related to bacterial community.

2.9 Summary

As discussed in this review, wide range of studies have been performed on different applications of BES. Large number of studies focused on bioanode development and their function in electricity generation in MFCs. *Geobacter* has been known as one of the important electro-active bacterial community, able to degrade organics and use an anode as an electron acceptor. To enrich *Geobacter*, using acetate as a substrate seemed to select *Geobacter* from mixed culture of microorganisms. Despite the vast number of fundamental studies on MFCs, electricity generation in MFCs is still limited to lab scale. One of the challenges limiting the commercialization of MFCs are cathodic reactions limiting the electrons uptake derived from anodic electrodes. In case of ORR as a cathodic reaction in MFCs, there is a need for efficient, cost-effective and sustainable catalyst substituting chemical catalysts. Although many studies have been focused on aerobic biocatalysts formed from mixed culture of microorganisms, the slow development of biocathode is one of the major bottleneck of such catalysts. Therefore, there is a need to study the quick start-up aerobic biocatalysts comparable with aerobic biocathode. Indeed, reactors' designs play important roles in increasing the efficiency and decreasing the costs of operation. There is an opportunity to develop a highly active rapid start-up aerobic biocatalysts in optimised design MFCs.

Conversion of CO_2 and production of value-added products such as organic acids and alcohols through MES using autotrophic bacteria has been offered the promising approach towards BES applications. Organic products of acetogens from CO_2 through WL pathway, the most energy efficient non-photosynthetic fixation pathway, is more of interest than methane from methanogens. Among the parameters affecting MES processes, cathodic potential corresponds to energy for bacterial activities and inorganic carbon source seem key factors. However, so far the effect of these parameters on production in MES has not been studied systematically. In addition, the dominated mechanism of electron transfer between the biofilm and cathode is still unclear. Few studies reported the development of cathodic biofilm during MES processes responsible for production suggesting possible DET mechanism; however, other studies reported that bacterial community in planktonic cells were responsible for production, suggesting IET mechanism. It is still poorly understood about the mechanism of electron transfer between bacterial community and cathode, whether biofilm forms at the cathode during MES processes, morphology and composition of biofilm, its relation to production through MES and whether MES occurs at biofilm or in planktonic cells. In order to commercialize MES in future, more fundamental information are required in this area.

BES also provides the approach of production of longer chain organic chemicals than acetate using the electric current provided known as EF processes. It has been suggested that to steer the production in BES towards longer chain products, other electron donors than polarised cathode are required. Ethanol is one of the most well-known electron donors in chain elongation from acetate to butyrate and caproate. In addition, low pH caused by accumulation of such products leads the production towards corresponding alcohols known as solventogenesis. Although extra electron donors are required for chain elongation, it was suggested in previous review papers that cathodes affect the spectrum of products by affecting the intracellular ORP through the reduced/oxidised NAD ($NADH/NAD^+$). The involvement of cathode in production of long chain organic chemicals is still not clear. While many studies reported the production of longer chain organic acids and alcohols than acetate and ethanol in BES such as C4 and C6 products, products are still limited to short chain products in many other studies. Previous studies suggested this is more likely related to bacterial community and operational conditions provided. Due to higher value of long chain products, more investigations to provide better understanding of EF alongside MES processes, as well as more studies on different strategies for production of long chain products are required.

Chapter 3. Materials and Methods

This chapter gathers the materials, experimental designs and setups used in this thesis including aerobic and anaerobic biocathode experiments. In addition, the analysis and methods used to interpret the results are described in detail.

3.1 Aerobic biocathode experiment

3.1.1 *BESs operational design*

Dual chamber raectors with gas diffusion electrodes (GDEs) as cathodes were designed and fabricated from Perspex in this study. Reactors were constructed from one 9 × 8.5 × 3 cm (W × H × D) end plate used as an anode chamber, one 9 × 8.5 × 3 (W × H × D) middle plate as a cathode chamber and one 9 × 8.5 × 2 (W × H × D) gas diffusion end plate. Anodic and cathodic plates had a central void of 5 × 6 × 2 (W × H × D) for the electrolytes. Cathodic and gas diffusion plates had an additional 12.6 cm^2 central void to allow oxygen reach the gas diffusion electrode. All the plates had 4 M5 bolt holes in the corners to compress the plates after assembly. 2 ports were drilled and plugged on top of the plates. In anodic plates, one port was used for a reference electrode and the other one was for liquid sampling which was covered by stopper during the experiment. In cathodic plates, one port was used for a reference electrode while the other one was open during the experiment and used for liquid sampling. One inlet port was also plugged and connected to three-way valve at the bottom corners of anodic and cathodic plates in case of continuous feeding regime which were closed during the fed-batch operational mode. In addition, sampling ports on top of the plates were used as outlets during the continuous operational mode.

Anodic and cathodic compartments were separated by anion exchange membrane (AEM, Fumasep FFA-3-PK-130, Fumatech, Germany). Each reactor was assembled with the anodic

electrode connected to a titanium wire enclosed with a silicone gasket and a membrane. The other side of the membrane was a gasket and a cathodic plate which all were compressed using a gas diffusion cathode, gasket and a gas diffusion plate. The reactor operated for aerobic biocathode experiment and the setup are shown in Figure 3.1.

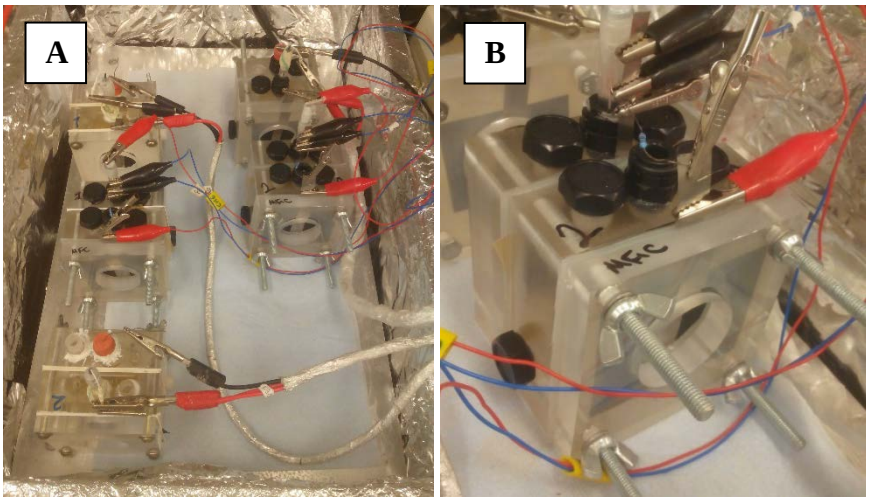


Figure 3.1. The image of the setup for aerobic biocathode experiment (A) and a GDE reactor operated for this experiment (B).

After assembly, all the reactors were operated and kept in 150 × 20 × 30 cm (W × H × D) covered in a polystyrene box equipped with heating mat to fix the temperature at 30 ℃ during the experiment.

3.1.2 *Electrodes*

In reactors during a half-cell experiment, 12 cm^2 rounded carbon paper with gas diffusion layer (GDL) of PTFE purchased from Quintech (H2315 I2 C6, Göppingen, Germany) was used as the working electrode. Before the reactors operation, carbon paper was pre-treated to increase hydrophilicity by immersing the electrode in a mixture containing 20 mL of concentrated H_2SO_4 (98%) and 2 g of $KMnO_4$ at room temperature for 2 minutes, washing properly by deionised water and finally heating at 150 °C for 2 hours (Qiu et al., 2015). The method used for pre-treating the carbon paper is reported in the literature as a modified Hummer's method

to increase roughness and surface area. The method allowed the introduction of hydrophilic groups such as hydroxyl, carbonyl and carboxylic groups at the surface of the carbon paper which was the main reason for following this method. The carbon paper purchased from Quintech undergoes a hydrophobic treatment during the production which is necessary for GDL products but also detrimental to interactions with bacteria/biofilms. Therefore, this reported method was mainly followed to increase the hydrophilicity of the carbon paper.

10 cm^2 rectangular platinum mesh and Silver / Silver chloride (Ag/AgCl, RE-5B, BASi, USA) were used as counter and reference electrodes, respectively. Counter electrode was connected to titanium wire passing through the gasket to reach the external circuit. Reference electrodes were +0.209 V vs. Standard Hydrogen Electrode (SHE). All the potentials throughout the thesis will be reported vs. Ag/AgCl reference electrode. To protect the reference electrodes from contamination by bacteria, 3 mole L^{-1} NaCl agar salt bridges were constructed in 5 ml pipette tips. Salt bridges consisted of 2.0 gr agar and 8.7 NaCl were dissolved in 50 ml DI water in a beaker. Mixture was heated to make a viscous gel which 2 ml of the gel was drawn into pipette tips and allowed to cool. The rest 3 ml of each pipette was filled with 3 mole L^{-1} NaCl and a reference electrode was inserted into the NaCl solution. The top part of each RE was sealed to the pipetted tip using parafilm sheets and glue.

When reactors were operated as microbial fuel cells (MFCs), they were equipped with 12 cm^2 platinised carbon paper (Pt-C) cathode, rectangular graphite felt (VWR, Cat. No. 43200.RR, Alfa Aesar) anode with the projected surface area of 6 cm^2 and thickness of 5 mm and a reference electrode in the anodic compartment. To prepare Pt-C, carbon paper (Quintech H2315 I2 C6, Göppingen, Germany) was coated with 0.5 mg cm^{-2} Pt. Titanium sheet with the thickness of 0.5 mm was used as current collectors for both anode and cathode electrodes. For anode electrodes, titanium sheet was drilled and screwed using plastic screws and bolts to the graphite felt and used as the current collector. The sheet was passed through the gasket to connect the anode to the external circuit. When required, the Pt-C cathodes were switched with the working electrodes from a half-cell experiment. For gas diffusion cathodic electrodes, titanium sheet was sandwiched between the gas diffusion plate and cathode to connect the cathodes to the external circuit.

Preparation of Pt-C was followed from the previous protocol including preparing a solution from ethanol as a solvent, Nafion solution (Nafion perfluorinated resin solution) as a binder and Pt and carbon powder (Platinum, nominally 20% on carbon black, HiSPEC 3000), and brushing a carbon paper with the solution (Middaugh et al., 2006). 0.5 ml of the solution was

brushed 6 times on the carbon side of carbon paper and electrodes were dried after each addition. Electrodes were dried by air at room temperature for 24 hours.

3.1.3 *Inoculum*

When reactors were operated as MFCs, inoculum used for bioanode was collected from the effluent from a glucose/glutamic acid-fed parent MFC operated over a year in the laboratory. MFCs were inoculated initially by activated sludge in wastewater treatment plant in Newcastle, UK. Parent MFCs were coupled with GDE design cathode and anodic and cathodic electrodes were connected to 200 Ω resistor, while the cells potentials were monitored using Pico Technology ADC-24 data logger (St Neots, U.K.). Effluent were collected when the cell potential was almost zero to assure that all glucose/glutamic acid was consumed.

Inoculum for cathodic compartments to enrich aerobic biocathode was collected from the Woodend Low level mine water discharge (a 'level' is a near-horizontal entrance into a mine), which flows into the Gate Gill (the local stream). The coordinates of its location are 54°37'33''N 3°02'48'' W, approximately 1 km to the north east of the village of Threlkeld in the northern Lake District. Figure A1 in Appendix A shows the image of the location where samples were collected. The sediment had an initial orange-brown colour and a pH of 3.6. Before using the inoculum in the experiment, samples were centrifuged to remove the precipitated iron in the samples and supernatant was used as an inoculum for development of aerobic biocathode.

3.1.4 *Preparation of electrolyte medium and medium replacement*

During half-cell experiment for acidophilic aerobic biocathode, anodic medium consisted of 0.4 g L^{-1} $(NH_4)_2SO_4$, 0.4 g L^{-1} KH_2PO_4, and 0.4 g L^{-1} $MgSO_4$ with the pH adjusted at almost 2.0.

For operation of MFCs during aerobic biocathode experiment, anolyte consisted of 50 mM phosphate buffer (PBS), 1 g L^{-1} of sodium acetate, 25 ml L^{-1} of macronutrients solution, 1 ml L^{-1} of micronutrients solution, and 0.5 ml L^{-1} of vitamins solution (composition of the solutions in Table A1 in Appendix A). Anolyte was purged by N_2 for 15 minutes after preparation to

remove the dissolved oxygen in the solution. When Pt-C was used as a cathode, catholyte was only 50 mM PBS.

When MFCs were operated in fed-batch mode, 60% of anodic medium was replaced by fresh medium every 4 days after completion of one batch cycle (after the current generated would drop). After switching the operation mode from fed-batch to continuous, 30 mL $hour^{-1}$ flow rate was used for refresh anolyte using a dosing pump (Jebao Dosing Pump Dp-4).

When cathodic compartment was inoculated by acidophilic aerobic biocathode cathodic medium was consisted of 1.0 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.4 g L^{-1} $(NH_4)_2SO_4$, 0.4 g L^{-1} KH_2PO_4, and 0.4 g L^{-1} $MgSO_4$ (Fontmorin and Sillanpaa, 2015). pH of medium was adjusted at 2.0 using 1 M H_2SO_4. 20% of the cathode medium was inoculated with the inoculum after the enrichment step. During continuous operational mode, 30 mL $hour^{-1}$ flow rate was used for refresh catholyte using a dosing pump (Jebao Dosing Pump Dp-4).

3.2 Anaerobic biocathode experiment

3.2.1 *BESs operational design*

BESs used in this study were cubic dual chamber Perspex reactors constructed from two 9 × 8.5 × 3 cm (W × H × D) end plates used as anode and cathode chambers. Anodic and cathodic plates had a central void of 5 × 6 × 2 (W ×H × D) for the electrolytes. All the plates had 4 M5 bolt holes in the corners to assemble the plates in addition to two top ports. The ports on top were plugged and used for headspace and a reference electrode in cathodic compartments. The ports for reference electrodes were designed and drilled close to the cathodes (< 2mm distance between cathodes and reference electrodes after assembly). In anodic compartments one port on top was used as a headspace and the other one was covered and sealed by butyl stopper. Additionally, one port was plugged to a three-way valve on the bottom corner of each anodic and cathodic plates to use as an inlet and also liquid sampling. Headspaces were 70 ml glass cylinders constructed with one port plugged and used for liquid outlet in case of continuous operational mode. Each headspace was sealed with a septum used for gas sampling.

Cation exchange membrane (CEM, Fumasep FFA-3-PK-130, Fumatech, Germany) was used to separate anodic and cathodic chambers. Each reactor was assembled with a cathodic electrode connected to titanium sheet as a current collector, sandwiched between a butyl

anaerobic gasket (Butyl rubber sheet, Rubberco, UK) and a membrane. The other side of the membrane, a gasket and an anodic electrode connected to a titanium wire were compressed by an anodic plate. The BES operated for anaerobic biocathode experiment is shown in Figure 3.2. The image of the setup is shown in Figure A2 in Appendix A.

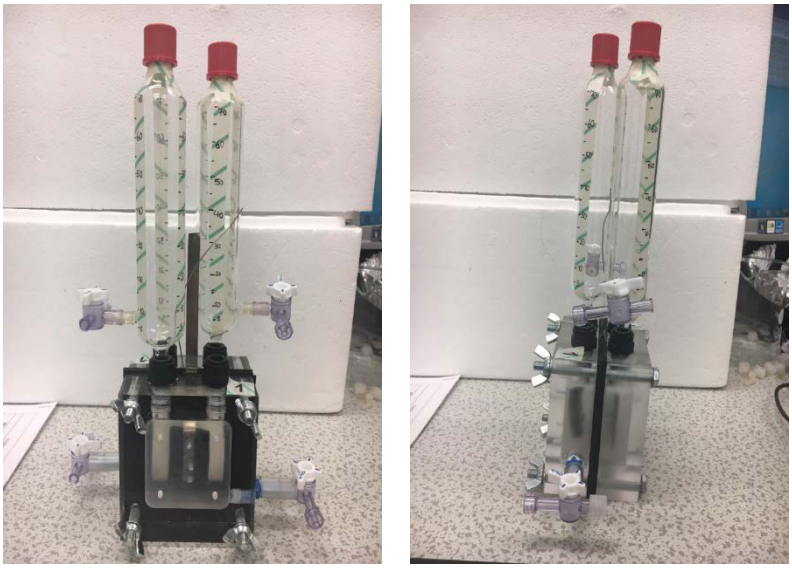


Figure 3.2. The image of the BES operated for anaerobic biocathode experiment

After assembly, all the BESs were operated and kept in 150 × 20 × 30 cm (W × H × D) covered polystyrene box equipped with heating mat to fix the temperature at 35℃ during the experiment.

3.2.2 *Electrodes*

Rectangular graphite felt electrodes (VWR, Cat. No. 43200.RR, Alfa Aesar) with the projected surface area of 4.9 cm^2 and thickness of 5 mm were used as working electrodes (cathode) through half-cell experiments. For system optimizations as will be mentioned in chapter 6, the same electrodes with higher projected surface area of 15.0 cm^2 were used as cathodes. Counter electrodes (anodes) were 10 cm^2 rectangular platinum mesh and reference electrodes were Silver / Silver chloride (Ag/AgCl, RE-5B, BASi, USA). Similar to aerobic biocathode experiment, reference electrodes were kept in salt bridges prepared before. Titanium sheet with the thickness of 0.5 mm was drilled and screwed using plastic screws and bolts to the graphite felt and used as the current collector. The sheet was passed through the anaerobic gasket to connect the cathode to the external circuit.

3.2.3 *Inoculum*

Inoculum for this study was collected from activated sludge in wastewater treatment plant in Newcastle, UK, stored overnight in the fridge at 4 ̊C and used for the enrichment step the next day.

3.2.4 *Preparation of electrolyte medium and medium replacement*

The medium used as the cathodic medium composed of K_2HPO_4 (0.35 g L^{-1}), KH_2PO_4 (0.25 g L^{-1}), NH_4Cl (0.25 g L^{-1}), KCl (0.5 g L^{-1}), $CaCl_2.2H_2O$ (0.15 g L^{-1}), $MgCL_2.6H_2O$ (0.6 g L^{-1}), NaCl (1.2 g L^{-1}), yeast extract (0.01 g L^{-1}), Trace metal solution (1 ml L^{-1}), Vitamin solution (2.5 ml L^{-1}), Tungstate-selenium solution (0.1 ml L^{-1}) (composition of the solutions in Table A2 in Appendix A) (Patil et al., 2015a). If required, sodium 2-bromoethanesulfonate (5 mM) was added as methane inhibitor in the medium.

When gaseous CO_2 was used as the sole inorganic carbon source, CO_2/N_2 (80/20) was purged in the catholyte for 20 minutes to provide the dissolved inorganic carbon source and also remove the dissolved oxygen in the solution, which provided 350.8 ± 20.2 mg L^{-1} inorganic carbon in the catholyte measured by TOC analyser. CO_2 was provided every 2-3 days in cathodic compartments by recirculating the catholytes in 250 ml duran bottle using peristaltic pump (520S, Watson-marlow) with hydraulic retention time (HRT) of 4 ml min^{-1}, which bottle was purged by CO_2/N_2 (90/10).When inorganic carbon source was provided in form of bicarbonate, 2.5 g L^{-1} $NaHCO_3$ was added to the catholyte, provided 357 mg L^{-1} inorganic carbon in the solution. To remove the dissolved oxygen in the catholyte prepared by $NaHCO_3$, N_2/CO_2 was purged in the catholyte for 20 minutes (90/10) before transferring to the cathodic compartment. To provide the fresh medium in the cathode compartment and remove the products in the solution, 40% of the catholyte was refreshed every 12-14 days. This period of time for changing the medium was chosen due to the consumption of inorganic carbon in the solution detected by TOC analyser. Whenever TOC showed inorganic carbon with the concentration less than 30 mg L^{-1}, catholyte were purged by CO_2 or $NaHCO_3$ was added to the catholyte. After purging the electrolytes, pH of the solutions were adjusted to the desired value.

During continuous operational mode, dosing pump (Jebao Dosing Pump Dp-4) was connected to 0.5 L Duran bottle of fresh medium continuously purged by CO_2/N_2 (90/10). Fresh medium was flushed continuously from the inlet port to cathodic compartment removing catholyte through the outlet port. Outlet ports were connected to anaerobic tubing to the closed effluent 0.5 L Duran bottle (different from 0.5 L fresh medium Duran bottle) to prevent the diffusion of oxygen through the ports.

The anode chamber was filled with the same growth medium used for the cathode compartment except inorganic carbon source, inoculum, trace mineral, vitamin and Tungstate-selenium solutions. Anolyte was continuously purged by N_2 using peristaltic pump (520S, Watson-marlow) at 0.5 ml $hour^{-1}$ to remove the oxygen produced in this compartment and prevent the crossover of oxygen to cathodic compartments through the membrane. Figure 3.3 shows the schematic of the experimental design in this section.

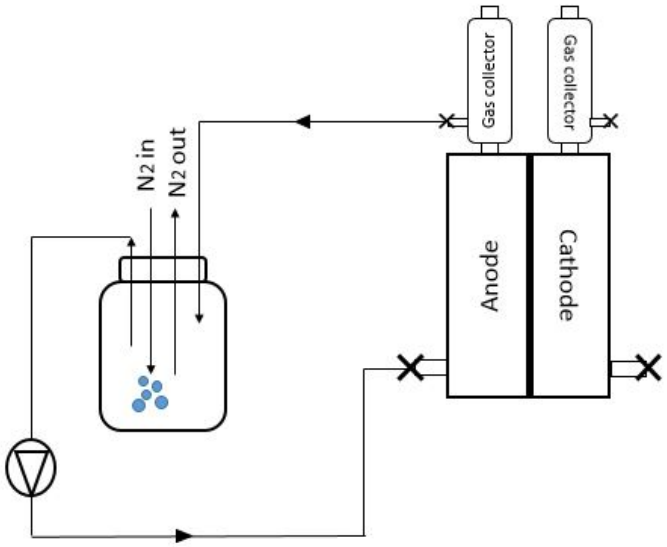


Figure 3.3. Schematic of the BES prepared for anaerobic biocathode experiment

3.3 Electrochemical methods

Electrochemical analysis was performed during the experiments to control the system and after the experiments to determine the electrochemical properties of the biocatalysts developed. The methods used to carry out these analysis are explained in this section.

3.3.1 *Potentials in MFCs*

MFCs in aerobic biocathode experiment were operated by connecting anode and cathode electrodes using external resistors of 200 Ω. Cell, anode and cathode potentials were recorded using a Pico Technology ADC-24 data logger (St Neots, U.K.).

3.3.2 *Chronoamperometry (CA)*

When the fixed potential at the surface of the electrodes was required during half-cell experiments, chronoamperometry (CA) was performed using a potentiostat (Whistonbrook, UK). Through CI, a constant potential is applied at the electrode and the current generated over time is recorded. Reactors were connected to the potentiostat and operated in 3 electrodes configuration. Depending on the experiment, either anode or cathode electrodes were considered as working or counter electrodes. The potentiostat poised the desired potential (vs. Ag/AgCl RE) at the surface of the working electrode and the current generated was monitored using the machine's software at the computer with the interval time of 5 minutes.

3.3.3 *Cyclic voltammetry (CV)*

Cyclic voltammetry (CV) is an electrochemical technique in which potential of the electrode is changed between two points back and forth at the stable scan rate and the current generated is recorded. CV was performed during the experiment using Autolab PGSTAT203 potentiostat and NOVA electrochemistry software (Metrohm,Switzerland) to evaluate the kinetic analysis and electrochemical properties of anode or cathode biofilms. The information required for the analysis such as upper and lower potentials and scan rate was given to the software, machine scanned the potentials between upper and lower potentials and current generated was recorded. Two scans were applied and the voltammograms of the second scan are reported throughout the thesis. A scan rate of 2 mV s^{-1} was selected with potential steps of 0.5 mV.

3.3.4 *Polarisation and power density curve*

Polarisation and power density curve was used to evaluate the performance of the MFCs in terms of power production. Polarisation and power density curve curve provides the information regarding the peak power of MFCs produced at different external resistors. After reaching the stable cell voltage in MFCs, polarisation and power density curve were recorded. The values of the external resistances used are: open circuit potential (OCP), 100 kΩ, 52.2 kΩ, 25.6 kΩ, 10.0 kΩ, 5.1 kΩ, 2.5 kΩ, 997 Ω, 505 Ω, 301 Ω, 103 Ω, 51 Ω, 12 Ω). The cell voltages and anode potentials for each resistance were recorded using a Pico Technology ADC-24 data logger (St Neots, U.K.). The Ohm law (V = R I) was used to calculate the current values for each voltage and resistance. OCP was measured manually using multi meter after 1 hour disconnecting the reactors from the potentiostats or data loggers.

3.3.5 *Electrochemical impedance spectroscopy (EIS)*

Electrochemical impedance spectroscopy (EIS) was performed from the anaerobic biocathodes to measure the charge transfer resistance caused by biofilm at the surface of cathodes and compare it with that in plain electrode (without biofilm). EIS analysis was carried out using Autolab PGSTAT203 with NOVA software (Metrohm, Switzerland) at 3 electrodes configuration to only assess the working electrode. To do that, the desired potential was applied at the working electrode (cathodes in this thesis) versus reference electrode and 50 frequencies were measured over the frequency ranging from 10000 to 0.1 Hz. After the analysis, Nyquist charts (the real impedance (Z') against the imaginary impedance (Z'')) were plotted. As shown in Figure 3.4, Nyquist chart provides the information regarding the resistance of the system such as ohmic resistance (R_{Ω}) and charge transfer resistance (R_{ct}). These data could be extracted and interpreted by fitting the equivalent circuits. In this study, R_{ct} were extracted from EIS analysis to determine the resistance of the system resulted from development of anaerobic cathodic biofilm at the electrodes and compare that with the system before biofilm development.

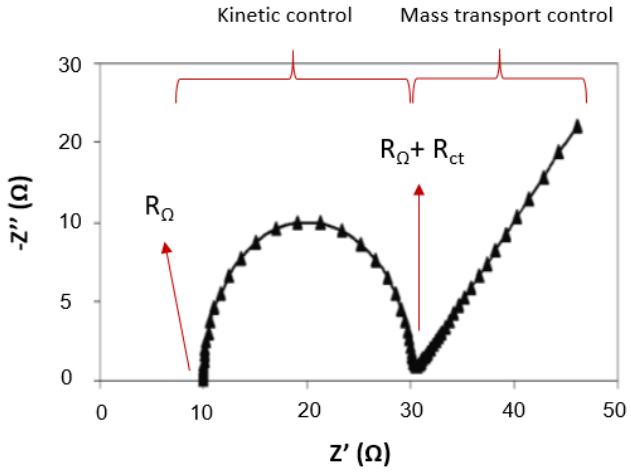


Figure 3.4. Example of Nyquist plot

3.4 Bio-imaging and biological analysis

Bio-imaging analysis was performed from the biofilms developed at the surface of the cathodes to investigate the physical properties of the biocatalysts developed during the anaerobic biocathode experiment, as well as bacterial cells growth through cell count analysis. This section will explain the methods used to perform these analyses.

3.4.1 *Sampling*

For the collection of liquid samples from the compartments, the bottom corner ports at the sides of compartments were used. In order to collect the samples, a 60 ml gas bag (0.6L Tedlar SCV Gas Sampling Bag w/ Thermogreen LB-2 Septa, Sigma Aldrich) was filled with N_2 and connected to the headspace of each reactor using a needle to pressurize the liquid solution, 3-way valve was opened and 4-5 ml sample was collected using a 5 ml syringe. Liquid samples collected for the cell count analysis were fixed in 50% ethanol and stored in 15 ml centrifuge tubes in a freezer (4˚C).

In case of biological analysis from the biofilm at the cathodic electrodes, 1 cm × 1 cm (projected surface area) of the electrodes were cut after terminating and disassembling the reactors. Regarding bio-imaging, cut electrodes were immediately prepared for the analysis; however,

they were kept in 5 ml vials, fixed in 50% ethanol in 5 ml vials in a 4 ̊C freezer for cell count analysis.

3.4.2 *Bacterial cell counts*

Flow cytometry with Accuri C6 flow cytometer (BD Biosciences) was used to count the number of the cells in the cathodic solutions and electrodes. Flow cytometry is the technique for detection and analysing the characteristics of cells through fluidic, optic and electronic systems. Particles are passed through the laser beams in a stream in a fluidic system, their light signals are directed to the related detectors through an optic system and the signal signals are converted to electronic signals in an electronic system (Biosciences, 2000). In order to count the number of the cells at the electrode after disassembling the cells, the electrodes were crushed using the sterile stainless steel rod. Crushing the biocathode allowed the biofilm to remove from the spongy structure of graphite felt, giving the homogenous liquid sample. Samples were prepared for flow cytometry by adding 100 µL surfactant (5% Tween 80 and 10 mM sodium pyrophosphate) to 900 µL sample and shaking it gently, diluting the samples ×200 in filtered sterile Ethylenediaminetetraacetic acid (EDTA) solution, staining the samples by 10 µL SYTO 9 (FilmTracer LIVE/DEAD Biofilm Viability Kit, Invitrogen) solution (diluted ×50 in Dimethyl sulfoxide) and incubating the stained samples in the dark at 60 °C for an hour. In flow cytometry, analysis is performed based on the gating. Desired gates and regions are typically placed through forward scatter (FSC). FSC represents the size of the bacterial cells, providing the proper method for detection of particles. Gating was performed through FSC using stained filtered sterile water to ensure removing the background noise caused by machine or stain. The image of the gating trough the software is presented in Figure A3 in Appendix A. Number of the cells of the prepared samples in the selected gate were measured by counting the number of particles in a set volume and reported by considering the dilution. The number of the cells at the electrodes was calculated with respect to the projected surface area. All the data of cell count in this study are reported in logarithmic scale.

3.4.3 *Confocal microscopy*

A confocal microscopy of Zeiss AxioObserver (LSM800/SDI) with reversed lens was used for investigation of the coverage, better understanding of the distribution and volume of dead and live cells in the cathodic biofilm recorded by ZEN software. Confocal microscopy is a bio-imaging technique from different depth within the sample. It has the optical system responsible for the collection of the light from a thin region of the sample called as focal plane. For the analysis, the desired depth can be set, the light from the single focal plane reaches the detector by filtering out the light form the rest fields of view (Kuehn et al., 1998). Z-scan can be made by taking serial focal plane images resulting to 3D image of the sample, particularly biofilm (Xavier et al., 2003, Popescu, 2016). To protect the live cells, electrode samples for confocal microscopy were prepared directly after disassembling the reactors. This was due to the fact that membrane of bacterial cells could lose their intact structure over freezing and defrosting steps. Samples were stained using the dead-live staining kit (FilmTracer LIVE/DEAD Biofilm Viability Kit, Invitrogen) based on the protocol provided for the kit. The kit contained two different dyes: SYTO 9 and Propidium iodide (PI). These dyes can be bound to DNA which enhances their fluorescence. Dead-live cells analysis of these dyes relies on the permeability of them in cells membrane. SYTO 9 is a permeable dye binding to all the DNAs in the sample. However, PI is not cell permeable and cannot enter in live cells with intact membrane and consequently binds the dead cells and extraneous DNA. When both dyes are used the emission spectrum of SYTO 9 and absorption spectrum of PI overlap, therefore PI suppresses the emission of SYTO 9. As a result, in presence of PI, SYTO 9 shows only live cells. Confocal microscope was set with 3 different channels, first channel visualized SYTO 9 shown as green for live cells, second channel visualized PI shown as red for dead cells and third channel visualized reflectance as blue channel for reflection of the graphite fibres as they are not optically transparent. Detailed information about the channels in confocal microscopy and its setting is summarized in Table 3.1.

Table 3.1. Setting of the confocal microscopy

Channel	**Colour**	**Mode**	**Excitation**	**Emission**	**Targeted objects**
1	Green	Fluorescence	482 nm	500 nm	Live cells
2	Red	Fluorescence	490 nm	635 nm	Dead cells
3	Blue	Reflectance	478 – 498 nm	478 – 498 nm	Carbon fibres

The staining protocol consisted of preparing the working solution (3 µl SYTO 9 and 3 µl PI per 1 ml filtered sterile water) and adding 200 µl working solution onto the biofilm sample. Samples after staining were placed on the coverslips and then on the microscope stage. As the microscope was reversed lens, lens was placed from bottom side of the cover slip. Magnification of ×100 and for more detailed images ×400 was used for the imaging. 3-5 different fields were chosen randomly for each sample and all the quantitative analysis are reported in average of number of fields and also reactor replicates. The z-scan from each field was conducted with the depth between 70 and 200 µm, based on the visibility of the graphite fibres and biofilm in each field of view. The percentage of live and dead cells biovolume in the cathodic biofilm were extracted from the stacks of the images and measured using the Huygens software by applying the best threshold. Threshold of each channel for each image was investigated using ZEN blue software to exclude the background noises. The percentage of dead and live biovolume visible at the cathodic electrode extracted using Huygens software was also calculated with respect to the image size at each field of view. To ensure that no noise affected the results and evaluate the accuracy of the analysis plain electrode was rinsed with ethanol and used as negative control.

3.4.4 *Scanning electron microscopy (SEM)*

A Tescan Vega 3LMU scanning electron microscope (SEM), which is a tungsten thermionic emission system with both high and low vacuum operations, fitted with Bruker XFlash 6 | 30 detector for energy-dispersive X-ray spectroscopy (EDS) analysis was used to take images from the surface of the cathodes, with and without biofilms. Samples were prepared directly after disassembling the reactors by fixing the cut cathodes in 2 % Glutaraldehyde for 24 hours, rinsing in the original medium matrix and dehydrating in a graded series of Ethanol. Dehydration step included rinsing electrodes for 30 minutes in 25 % ethanol, 30 minutes in 50 % ethanol, 30 minutes in 75 % ethanol and two times 60 minutes in 100 % ethanol. Final critical point dehydration was performed using carbon dioxide in a critical point dryer and finally electrodes were coated with gold in a SEM coating unit. Samples were imaged using a related software at different random fields of the electrodes surface, with different magnification adjusted manually, while other parameters such as scan speed or brightness were adjusted automatically by the machine. In addition, plain electrode rinsed in ethanol was used as negative control for SEM images.

3.5 Community analysis

Bacterial composition in the biofilms formed at the surface of the cathodes and planktonic cells in the catholytes of anaerobic biocathode experiment was identified through community analysis. This section describes the procedure of the community analysis performed.

3.5.1 *Sampling*

Similar method used for bio-imaging sampling was applied to collect liquid and cathodic electrode samples for community analysis. After sample collection, 4-5 ml liquid samples were stored in 5 ml vials in a freezer (4 ℃), while 1 cm × 1 cm (projected surface area) of the electrodes were cut, kept in 5 ml vials and stored in a freezer (4 ℃). Samples were defrosted in room temperature 2 hours before the analysis.

3.5.2 *DNA extraction*

To verify the identity of the bacteria in biocathode developed in BESs, Deoxyribonucleic acid (DNA) of the samples were extracted using DNeasy PowerWater Kit (14900-100-NF, QIAGEN, Germany). DNA extraction of bacterial community in catholytes was performed by collecting 2 ml of the liquid catholyte samples. After that, samples were filtered using 0.2 µm nylon membrane filters through filter holders (Swinnex, China) and the filters were then transferred to the bead kits. Regarding electrode samples, cut electrode samples were directly transferred to the bead kits. Extraction of DNAs (100 µl) were followed based on the protocols provided with the kit and the details are not presented here. DNA extracted samples were then stored at -18 ℃ freezer.

3.5.3 *PCR amplification*

Polymerase chain reaction (PCR) using Techne TC512 gradient thermocycler (Bibby Scientific, UK) was conducted to amplify the V4 – V5 region of 16S rRNA gene fragments. Sterile, filtered, DNA free water was used for the preparation. Thin-walled PCR tubes were used for PCR, each one filled by 2 µl DNA extracted sample, 0.5 µl barcoded V4 forward primer, 0.5 µl non-

barcoded V5 reverse primer and 22 µl PCR master mix containing Taq polymerase, PCR reagents and blue agarose loading dye(MegaMix-Blue; Cambio, UK). After preparation of all the samples in PCR tubes including a PCR tube of sterile DNA free water, they were loaded in the machine. The program set at the machine for the PCR amplification is shown in Table 3.2.

Table 3.2. Automated thermocycler program set for PCR amplification

Step	**Temperature (°C)**	**Holding time (min)**
Heated lid	95	-
Initial denaturation	95	4.00
35 cycles		
Denaturation	95	0.50
Annealing	55	0.75
Elongation	72	1.00
Final elongation	72	10.0
Final hold	4	∞

3.5.4 *Electrophoresis*

To confirm that PCR amplification was successful gel electrophoresis was performed. Gel was prepared using 1 gr agarose and 100 ml pH 8.5 TAE buffer which contained 40 mmole dm^{-3} Tris, 20 mmole dm^{-3} acetic acid and 1 mmole dm^{-3} pH 8.0 EDTA, and heated until the mixture was dissolved. 4 µl Ethidium bromide was added to the mixture of gel solution and dissolved. Gel solution was poured in the gel cast with the well combs and left to cool down. Once set, the well combs were removed from the cast and the gel was transferred to the electrophoresis tank containing pH 8.5 TAE buffer. First and final wells were filled by 5 µl molecular weight marker (Hyperladder 50 bp, Bioline, UK) and the rest were filled with 10 µl amplified DNA extracted samples. 130 V voltage was applied at the gel constantly for 35 minutes using PowerPac™ 300 power supply (Bio-Rad, UK). After that, gel was transferred to UV transilluminator to record the images of the gels using gel documentation system (BioSpectrum Imaging System; UVP, USA). Gels were imaged using Biochemi HR camera installed on the system and recorded by VisionWorks LS software. The bands appeared in the images of samples were compared with those appeared in the molecular weight ladder to ensure their presence between 400 and 500 bp bands.

3.5.5 *Quantification of the DNA*

The concentration of DNA in the extracted samples and the amplified DNA samples were quantified using Qubit dsDNA HS assay (Thermo Fisher Scientific, USA). Samples for Qubit were prepared following the protocol provided for Qubit dsDNA HS assay by addition of 5 µl sample to 195 µl working solutions. After calibrating the fluorometer with the reagents with the known concentration of DNA, DNA mass concentration (ng μl^{-1}) of the samples were measured by the fluorometer.

3.5.6 *Illumina 16S rRNA gene sequencing*

The communities of samples in this study were sequenced by Illumina MiSeq. For this reason the DNA extracted samples and their information including DNA concentrations and the size of the fragments from the electrophoresis images were submitted to Northumbria University, UK for Illumina MiSeq sequencing by evaluating the V4 region of 16S rRNA. 16S rRNA was chosen as it is the most well studied region with available sequences. Furthermore, 16S genes present in many species, therefore wide range of bacteria and archaea can be targeted by 16S rRNA (Caporaso et al., 2011). The Illumina MiSeq sequencer could produce 2 × 250 bp paired-end reads with V2 chemistry. The protocol including several steps for sequencing by Northumbria University was followed from Kozich et al (Kozich et al., 2013). The outputs of Illumina MiSeq sequencing were FASTQ files.

3.5.7 *Dada 2*

FASTQ files were analysed using the pipeline of ‘’Dada 2’’ package in R statistical platform. The steps of the package were followed from the Dada 2 tutorial in R (https://benjjneb.github.io/dada2/tutorial.html). Over the first step the 20 bases of primers were removed and the files were trimmed by keeping the first 240 and 190 bases from first (forward) and second (reverse) reads, respectively. Quality profiles are demonstrated in Figure A4 in Appendix A. After merging the first and second reads the final length was 213 bp. Next step was constructing a table of amplicon sequence variant (ASV). The remaining steps were

included of removing chimeras, tracking reads through the pipeline, assigning taxonomy and evaluating the accuracy. 2 databases of Silva and Ribosomal Database Project (RDP) were examined for the samples to choose the most compatible database for the assignment. The representative sequences in each ASV files were assigned using the database with the bootstrap confidence of 80. After taxonomy assignment, the relative abundance of each taxon was determined from kingdom to genus levels plotted in bar charts using ‘’phyloseq’’ package in R statistical platform (McMurdie and Holmes, 2013). Further analysis such as Bray-Curtis dissimilarity index was carried out to compare the difference between the samples. Bray-Curtis calculations in genus level were performed using ‘’vegan’’ package in R statistical platform (Oksanen et al., 2010). Following that, phylogenetic tree for selected sequences was plotted using ‘’ape ’’ package after sequences alignment using ‘’msa’’ package in R statistical platform (Paradis et al., 2004, Paradis et al., 2019, Bodenhofer et al., 2015).

3.5.8 *Controls for community analysis*

To evaluate the accuracy of the community analysis, mock bacterial community with specific composition of bacteria (ZymoBIOMICS® Microbial Community Standard) was used as the positive control. Positive control was composed of 8 bacteria: 4% Pseudomonas aeruginosa, 10% E. coli, 10% Salmonella enterica, 10% Enterococcus faecalis, 14% Listeria monocytogenes, 15% Staphylococcus aureus, 17% Bacillus subtilis, 18% lactobacillus fermentum. The genus level of the positive control is illustrated in Figure A5 in Appendix A.
Three negative controls with no sample were used for detecting the contamination during the DNA extraction processes. When the similarity of the samples and negative controls was less than 10 %, samples were considered as non-contaminated. However, higher similarity showed the contamination of the samples, therefore were removed from the analysis. Figure 3.5 illustrates the similarity of the sequences in the samples of this thesis and negative controls.

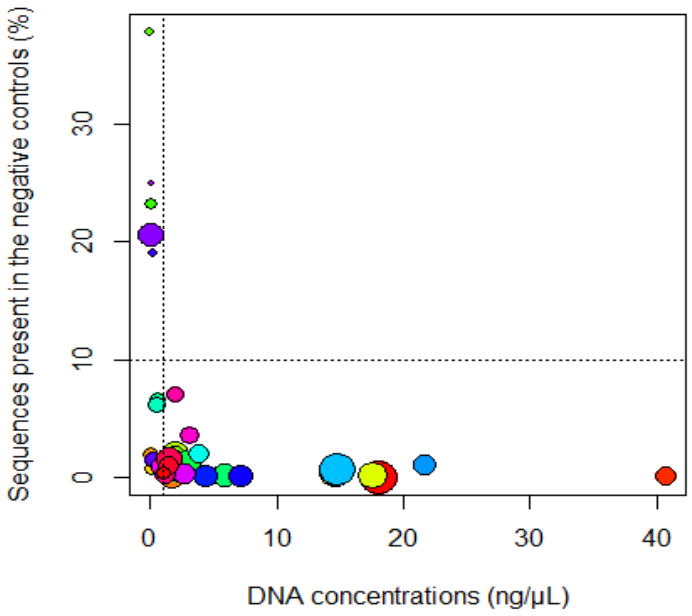


Figure 3.5. Similarity of the sequences in the samples and negative controls

3.5.9 *Adjusting gene copy number*

The data of relative taxon abundance from Dada 2 included the abundance of the sequences per taxon without considering the number of 16S rRNA gene copies of each taxon. Therefore, to calculate the accurate relative abundance of different organisms in the bacterial communities, the abundance data was corrected by the number of gene copies. This was performed by identifying the numbers of gene copies using the ribosomal RNA operon database (rrnDB). To keep the total abundance as 100 %, each sequence was weighted separately using the reciprocal mean gene copy number by the 'rrnDB Estimate' classifier tool (Stoddard et al., 2014).

3.6 Analytical methods

Analytical analysis was performed during aerobic and anaerobic biocathode experiments to gain more information about production and consumption of chemicals and general conditions in the reactors. The analysis performed are listed in this section.

3.6.1 *Sampling*

During aerobic biocathode experiment, liquid samples were collected using 5 ml syringe and a needle through a sampling port on top of the compartments.

For anaerobic biocathode experiments, however, it was essential to keep the cathodic chamber anaerobic. Therefore, the bottom corner ports at the sides of compartments were used for the collection of liquid samples from the compartments. In order to collect the samples, a 60 ml gas bag (0.6L Tedlar SCV Gas Sampling Bag w/Thermogreen LB-2 Septa, Sigma Aldrich) was filled with N_2 and connected to the headspace of each reactor using a needle to pressurize the liquid solution, 3-way valve was opened and 4-5 ml sample was collected using a 5 ml syringe. For analytical analysis, samples were filtered using 0.2 µm filter (PES membrane syringe filter, VWR, UK) in 5 ml Eppendorf tube and stored in a freezer (4˚C).

The headspaces on top of the Plexiglas reactors were designed to collect the gas products. For the collection of gas samples from the headspaces for further analysis, 0.5 ml gastight syringe (Hamilton syringe, Sigma-Aldrich) was used to collect gas samples through the septa on the headspaces of the reactors. To prevent pressurizing the headspace, a 60 ml gas bag was filled with N_2 and connected to the headspace of each reactor using a needle. By this, gas sample was replaced by N_2 after collection.

3.6.2 *Fe^{2+} and Fe^{3+} measurement*

Concentrations of iron species, Fe^{2+} and Fe^{3+}, were measured using spectrophotometer (6705 UV-Vis Jenway) following the previous protocol reported (Viollier et al., 2000). Three reagents were prepared in filtered sterile deionized water: A) Ferrozine solution:10 mM ferrozine (PDT disulfonate monosodium salt hydrate) in 100 mM ammonium acetate, B) Reducing agent: 1.4 M hydroxylamine hydrochloride, and C) Buffer: 10 M ammonium acetate with the pH adjusted at 9.5 using ammonium hydroxide. The protocol included of two steps. Over the first step 100 µl reagent A was added to 1ml sample and its absorbance was recorded. This could give the concentration of Fe^{2+}. Second step included addition of 150 µl reagent B to 800 µl mixture of step 1. Reagent B was a reducing agent converting Fe^{3+} to Fe^{2+}. After 10 minutes, when reduction was Fe^{3+} was completed, reagent C was added to the mixture and the absorbance of the mixture was recorded again. Therefore, subtraction of step 1 and step 2 could calculate the

concentration of Fe^{3+} in the solution. Calibration curves were prepared using different concentrations of Fe^{2+} (0 – 6 g L^{-1}) using $FeSO_4.7H_2O$ to calculate the concentrations of Fe^{2+} and Fe^{3+}. Before preparing the calibration curve, three different concentrations of Fe^{2+} was recorded in the spectrophotometer to choose the best wavelength that showed the maximum absorbance, which was 640 nm. Therefore, this wavelength was used to record the absorbance of standards and samples.

3.6.3 *pH measurement*

pH of the liquid samples were measured after each sampling. 1 ml of samples were transferred to 2 ml vials immediately after collection from the reactors and pH was measured using HI 9025 pH meter (Hanna Instruments, USA) with a pH probe (11542543; Fisher-Scientific, UK) calibrated between 2.0 and 9.0. pH of each condition was calculated in average of the duplicates.

3.6.4 *Total organic carbon (TOC) measurement*

A TOC 5050A Total Organic Carbon analyser (Shimadzu, Japan) was used to measure total inorganic and organic carbon in the solution. 4 ml 1/5 diluted samples were prepared in the machine's vials and loaded at ASI-5000 autosampler of the machine. Concentration (ppm) of total carbon (TC) was measured with an infrared detector by samples combustion at 800 ̊C and inorganic carbon (IC) was measured by acidifying the samples. Total organic carbon (TOC) was measured using the equation (3.1):

$$TOC = TC - IC \tag{3.1}$$

Over each set of analysis, TOC analyser was calibrated using three different standards with the known concentration of IC and TOC (using sodium bicarbonate and acetate).

3.6.5 *Gas concentration determination*

To detect the gas produced in the reactors during the experiment, 0.5 ml gas samples were injected in gas chromatography (GC-2010, Shimadzu Tracera, Japan) immediately after collection from the reactors. GC was equipped with Barrier Discharge Ionization (BID) detector which enhanced the accuracy of the detection. The column used for the gas detection was ShinCarbon ST micropacked column 80/100 (Restek) using Helium as a carrier gas with the pressure of 100 kPa. Machine was calibrated before analysing the samples. Gas compounds (H_2, N_2, O_2, CH_4 and CO_2) were detected according to their retention times.

3.6.6 *Acetone and alcohols concentration determination*

Concentrations of acetone and alcohols (methanol, ethanol, iso propanol, butanol and hexanol) were measured using gas chromatography (GC-2010, Shimadzu Tracera, Japan) equipped with BID detector and autosampler. The Zebron ZB-WAXplus capillary column (Phenomenex) was used for detection of acetone and alcohols. The machine was calibrated before the analysis using 7 standards of known concentrations of compounds. To prepare the samples for GC, 1 ml of each filtered liquid sample was acidified using 20 µl concentrated HCl in 2 ml tubes to reach the pH below 2, sonicated for 10 minutes and analysed by the machine.

3.6.7 *Volatile fatty acids (VFAs) concentration determination*

Concentrations of VFAs (formate, acetate, propionate, iso butyrate, butyrate, iso valerate, valerate and hexanoate) were measured using ion chromatography (Eco IC, Metrohm, Switzerland) equipped with the METROHM 6.1005.200 column and autosampler. The machine was calibrated before the analysis using 7 standards of known concentrations of compounds. To prepare the samples for IC, 1 ml of each filtered liquid sample was added to 1 ml filtered DI water. The mixture was acidified using 20 µl concentrated HCl to reach the pH below 2.0, sonicated for 40 minutes to remove the possible carbonate in the solutions in form of CO_2 to air and then analysed by the machine.

3.7 Evaluation of performance

3.7.1 *Production rate*

Production rate of the products in anaerobic biocathode experiment was quantified to investigate the performance of the BESs, which was calculated using equation (3.2):

$$Production\ rate = \frac{C2-C1}{t2-t1} \tag{3.2}$$

Where C1 is the concentration of the compound presented in the solution (ppm) at t1 (day) and C2 is the concentration of the same compound produced during the time of experiment (ppm) at t2 (day). Over the fed-batch operational mode, production rate was calculated over 3-5 days of sampling and during the continuous operational mode it was calculated over the completion of each hydraulic retention time.

3.7.2 *Columbic efficiency*

As in most of the reactors gas form of CO_2 was used as the source of inorganic carbon, there was a risk of losing inorganic carbon in preparation of liquid samples for inorganic carbon concentration measurement by TOC. Therefore, instead of mass balance considering the consumption of inorganic carbon source by autotrophic bacteria, columbic efficiency was calculated to evaluate the performance of anaerobic biocathode in consuming electrons from cathodes. Columbic efficiency (CE%) was calculated according to equation (3.3):

$$\mathrm{CE\%} = \frac{(C2-C1)\ V\ F\ n}{M \int_{t1}^{t2} I\ dt} \tag{3.3}$$

Where C1 is the concentration of the compound presented in the solution (g L^{-1}) at t1 (day), C2 is the concentration of the same compound produced during the time of experiment (g L^{-1}) at t2 (day), V is the volume of the electrolyte (L), F is faradic constant (96480 C $mole^{-1}$), n is number of moles, M is the molecular weight of the compound (g $mole^{-1}$) and I is the cathodic current consumed between the time of t1 and t2 (A).

Chapter 4. Development of a Gas Diffusion Electrode (GDE) Aerobic Biocathode Using Acidophilic Iron Oxidisers; A Rapid and Reproducible Method to Develop Highly Active Biocathodes for Oxygen Reduction Reaction

The main aim of this chapter was to develop active aerobic biocatalysts for the ORR in cathodic compartments of microbial fuel cells (MFC) a low cost alternative to precious metal chemical catalysts such as platinum. To achieve this aim the objectives of the study were:

- Enrichment of acidophilic iron oxidising bacteria (IOB) from the mixed culture of microorganism
- Development of an aerobic IOB biocathode with Fe^{2+} as a mediator in a bio-electrochemical system (BES) incorporating gas diffusion electrode (GDE) as the cathode
- Comparing the performance, with respect to power production, of an aerobic IOB biocathode and a conventional Pt-based cathode in microbial fuel cells (MFCs)

4.1 Introduction

MFC technologies remain limited to laboratory scale in part due to the low power outputs generated or the limitations associated with their scale up such as economic limitations (Du et al., 2007, Rahimnejad et al., 2015). The cathodic reaction is one of the main factors limiting MFC performance with respect to power generation (Rabaey et al., 2008, Rismani-Yazdi et al., 2008). The preferred cathodic reaction in conventional MFCs is ORR due to the virtually inexhaustible availability and the high standard potential of oxygen (E^0 = 1.229 V vs Standard Hydrogen Electrode). However, the kinetics of the oxygen reduction reaction (ORR) are slow,

associated with high overpotentials, and efficient catalysts are rare, thus the ORR is an important bottleneck for the development of MFCs.

In the past few years, several studies have focused on aerobic biocathodes for the ORR in the cathodic compartment of BES using mixed cultures of microorganisms enriched from natural environments and a number of cathodic communities have been identified as a promising alternatives to chemical catalysts (Huang et al., 2011). In a previous study of an aerobic biocathode developed from an activated sludge inoculum, it was shown that a particular group of *Gammaproteobacteria* was likely responsible for the ORR with direct electron uptake from the polarised cathode (Clauwaert et al., 2007b, Du et al., 2014, Jang et al., 2005, Rabaey et al., 2008). In spite of the ability of aerobic biocathodes to consume electrons directly from the cathode, development of the biofilms associated with the ORR is a long process (usually taking at least 50 days of enrichment). The utilisation of a chemical mediator can help overcome this limitation as some bacteria can reduce oxygen by using mediators as electron donors. Previous studies have shown that the acidophilic iron oxidising bacterium (IOB) *Acidithiobacillus ferrooxidans* is able to catalyse the ORR in the cathode compartment of an MFC at low pH (Ter Heijne et al., 2006, Carbajosa et al., 2010). It was reported that IOB are able to derive energy directly from the cathode (Carbajosa et al., 2010), however the presence of ferrous ions (Fe^{2+}) as a mediator significantly improves the kinetics at the cathode and facillitates faster biocathode development. *Acidithiobacillus ferrooxidans* is an autotroph that uses CO_2 as a carbon source, derives energy from the oxidation of Fe^{2+} and uses oxygen as an electron acceptor at low pH (typically around 3.0). Fe^{2+} is regenerated at the cathode by reduction of Fe^{3+} produced from the biological oxidation reaction (Lovley, 1991). Oxidising ferrous iron and reducing oxygen by the iron oxidisers under acidic conditions yields almost 30 kJ/mole energy for their growth. Using the Calvin cycle to fix CO_2, it has been estimated that *Acidithiobacillus ferrooxidans* oxidises 71 moles of Fe^{2+} to fix one mole of CO_2 (Hedrich et al., 2011). Although at neutral pH, neutrophilic iron oxidisers are able to oxidise Fe^{2+}, the reduction of oxygen – and thus the energy gain for the bacteria - is more favourable at pH 2.0 than at pH 7.0 (Ferguson and Ingledew, 2008). A successful scaled-up MFC with an acetate oxidizing anodic biofilm and a biocathode with acidophilic iron oxidisers at pH 2.0 exhibited high power density of 2.0 $W.m^{-2}$ (Ter Heijne et al., 2011a). However, in their study, expensive materials were used such as a bipolar membrane to overcome the pH shifts in the anode and cathode compartments as well as costly Pt- and Ir-coated electrodes.

The performance of aerobic biocathodes can be limited by oxygen mass transfer to the biofilm (Milner and Yu, 2018), thus to provide sufficient dissolved oxygen in the catholyte, forced is

required and increasing flow rate of air leads to an increase in the dissolved oxygen concentration and consequently to an increase in cathode and cell potentials (Milner and Yu, 2018). However, forced aeration is energy intensive and not economically viable for large scale application. Passive aeration using gas diffusion electrodes (GDEs) is an appealing alternative to forced aeration and GDE-based aerobic biocathodes have been shown to produce power output comparable to Pt based cathodes in BES (Xia et al., 2013). Despite the ability of GDE to overcome oxygen mass transfer limitation, few studies have focused on using GDE for development of aerobic biocathodes. To address this gap, we have developed a MFC using an aerobic biocathode with Fe^{2+} as a mediator in a dual chamber reactor equipped with a GDE. Acidophilic IOB were enriched from aquatic sediment as a cathodic biocatalyst. Reactors were started in batch mode and later changed to continuous operation to overcome pH shifts which impacted the performance of the MFC. The performance of the MFC was compared with a similar system equipped with a GDE coated with Pt catalyst.

4.2 Methods and experimental designs

4.2.1 *Pre-enrichment step*

To provide the proper condition for the growth and enrichment of acidophilic IOB from the inoculum after collection (as described in Section 3.1.3), 20 g L^{-1} $FeSO_4 \cdot 7H_2O$ was added to 100 mL of the environmental samples and incubated at 30°C with shaking at 170 rpm. 1 mL-samples were taken from the solution every 1-3 days to measure the pH and concentration of the Fe^{2+}/ Fe^{3+} ions using the ferrozine method (Viollier et al., 2000). When the pH of the solution dropped to 2.0, the colour of the solution was dark red-orange, 20% of the samples were transferred to a fresh cathodic medium (Section 3.1.4) for acidophilic IOB. After two more transfers, IOB were successfully enriched based on the colour, pH and the concentration of the Fe^{2+}/ Fe^{3+} ions (Ter Heijne et al., 2007, Hedrich et al., 2011, Malki et al., 2008), and 20% of the solution was transferred to the cathode compartment of a BES. In addition, control solution was prepared by autoclaving the inoculum after collection in a separate serum bottle. To prevent the contamination of the control solution, samples from this control solution were collected under sterile condition in fume hood. Colour, pH and iron concentrations in control solutions were analysed and compared with the inoculum to evaluate the bacterial activity.

4.2.2 *BESs operation*

BESs were operated, inoculated and started as mentioned in Section 3.1.1. Half-cell experiments were performed to investigate the development of an acidophilic iron-oxidising biocathode and investigate its performance in terms of cathodic current consumption. BES were operated in a 3 electrode configuration with carbon paper GDEs as working electrodes at an applied potential of 200 mV for 47 days.

Experiment in full MFCs with external resistors of 200 Ω were set up as follows. MFCs were first operated with Pt-C at the cathode as described in Section 3.1.2 in order to develop bioanodes. As mentioned in Section 3.1.3, anodic compartments were inoculated using the effluent from a glucose/glutamic acid-fed parent MFC operated over a year in the laboratory initially inoculated from activated sludge. To assure that no glucose/glutamic acid presented in the effluent, inocululm was collected from the outlet of parent MFCs when the cell potential was almost zero. After development of the bioanodes, Pt-C cathodes were replaced with an iron-oxidising biocathode developed in half-cells as described above and the same medium as used in half-cells was used for the catholyte. The details of the BES operation in this study are summarized in Table 4.1. pH of anodic and cathodic solutions were measured regularly to assure their stability. Anodic and cathodic medium were refreshed as described in Section 3.1.4, respectively during fed-bacth mode. To switch the operational mode to continuous, dosing pump was used to flush fresh medium in anodic and cathodic compartments as described in Section 3.1.4.

Table 4.1. BES operation strategy in Chapter 4

	Half-cell (47 days)	**Bioanode development in MFC (15 days)**	**MFC (12 days)**
Cathode	Carbon paper	Pt-C	Carbon paper with the developed biocathode from the half-cell
Anode	Pt mesh	Graphite felt	Graphite felt with the bioanode after the bioanode development

Catholyte	1 g L^{-1} $FeSO_4 \cdot 7H_2O$ 0.4 g L^{-1} $(NH_4)_2SO_4$ 0.4 g L^{-1} KH_2PO_4 0.4 g L^{-1} $MgSO_4$ at pH 2 (adjusted by H_2SO_4) 20% inoculum (IOB from enrichment step)	50 mM PBS	1 g L^{-1} $FeSO_4 \cdot 7H_2O$ 0.4 g L^{-1} $(NH_4)_2SO_4$ 0.4 g L^{-1} KH_2PO_4 0.4 g L^{-1} $MgSO_4$ at pH 2 (adjusted by H_2SO_4) 20% inoculum (IOB from enrichment step)
Anolyte	0.4 g L^{-1} $(NH_4)_2SO_4$ 0.4 g L^{-1} KH_2PO_4 0.4 g L^{-1} $MgSO_4$ at pH 2 (adjusted by H_2SO_4)	50 mM PBS 1 g L^{-1} Acetate 25 ml L^{-1} macronutrients solution 1 ml L^{-1} micronutrients solution 0.5 ml L^{-1} vitamins solution 20% inoculum (effluent of parent cell)	50 mM PBS 1 g L^{-1} Acetate 25 ml L^{-1} macronutrients solution 1 ml L^{-1} micronutrients solution 0.5 ml L^{-1} vitamins solution 20% inoculum (effluent of parent cell)

Cyclic voltammetry (CV) was performed during the experiment as described in Section 3.3.3 at a scan rate of 2 mV s^{-1} between the potentials of -1.0 and +1.0 V. After reaching the stable cell voltage in the MFC, polarisation and power density curves were recorded as explain in Section 3.3.4. The values of the external resistances used are: open circuit, 100 kΩ, 52.2 kΩ, 25.6 kΩ, 10.0 kΩ, 5.1 kΩ, 2.5 kΩ, 997 Ω, 505 Ω, 301 Ω, 103 Ω, 51 Ω, 12 Ω). The cell voltages and anode potentials for each resistance were recorded using a data logger. Ohm's law was used to calculate the current values for each voltage and resistance.

4.3 Results and discussion

4.3.1 *Enrichment of acidophilic iron-oxidising bacteria from the mixed culture of microorganisms before transferring to BES*

The enrichment of IOB was followed visually, with pH and iron speciation measurements. Environmental samples from the English Lake District (United Kingdom) were initially brown

and turned to red-orange after 5 days of enrichment. The change in the colour of the inoculated solutions was accompanied by a significant drop of pH (Figure 4.1). The pH of both inoculated and control solutions decreased from 3.5 to 2.7 on day 6, and to just under 2.0 on day 7, while the pH value of the control solution stayed stable circa 2.9. These observations can be associated with the faster and more advanced oxidation of Fe^{2+} into Fe^{3+} due to the presence and growth of IOB in the inoculated samples (Fontmorin and Sillanpaa, 2015).

In aerobic conditions it is known that the oxidation of Fe^{2+} can occur chemically. The rate of this reaction (Equation 4.1) depends on the environment conditions such as concentration of protons and dissolved oxygen (Stumm and Morgan, 1996):

$$-\frac{d[Fe^{2+}]}{dt} = k[O_2]\frac{[Fe^{2+}]}{[H^+]^2} \quad (4.1)$$

Therefore, at pH 2.0, the spontaneous oxidation of ferrous ions by molecular oxygen is very limited. In addition, at such pH, the redox potential of the oxygen/water couple is about 0.3 V more positive than at pH 7, which makes the oxidation of iron and the utilisation of oxygen as electron acceptor energetically favourable for IOB (Hedrich et al., 2011).

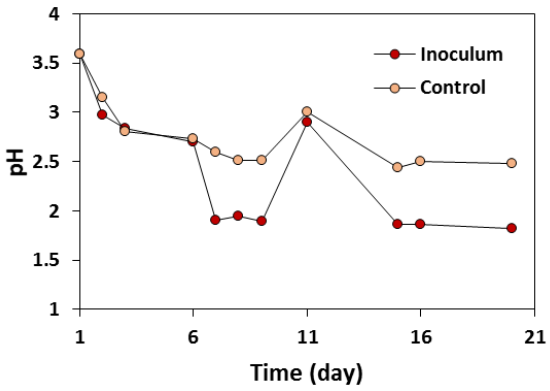


Figure 4.1. pH of the inoculated and control solutions during the enrichment step

At day 11 (see Figure 4.1), 20% of the inoculum was transferred to fresh medium containing 20 g L^{-1} $FeSO_4 \cdot 7H_2O$. A higher rate of the pH drop in the solution with inoculum compared to the control solution after the transfer and change in the colour of the inoculated solution confirmed the biological conversion of Fe^{2+} to Fe^{3+} (Ter Heijne et al., 2007). The colour of the control solution was orange-brown during the whole 20 days of the enrichment step. Fe^{2+} concentration was also measured during the enrichment step using ferrozine method (Viollier et al., 2000) in both inoculum and control solutions for 14 days. Before adding $FeSO_4 \cdot 7H_2O$, concentration of Fe^{2+} and Fe^{3+} were negligible in both solutions. After addition of $FeSO_4 \cdot 7H_2O$,

concentration of Fe^{2+} was around 4.4 g L^{-1} during 14 days in control solution, while concentration of Fe^{3+} was almost negligible. However, in inoculum solutions, concentration of Fe^{2+} began to decrease after day 4, reached the concentration of 0.2 on day on day 7. This was associated with increase in concentration of Fe^{3+} after day 4, reached the concentration of 3.6 and 4.6 g L^{-1} on days 7 and 11, respectively. Decrease in the concentration of the Fe^{2+} in the inoculated solution and stable concentration of Fe^{2+} in the control solution indicated the biological conversion of Fe^{2+} in the inoculated solution. This was also evident from the colour of the solutions after the enrichment period. As observed in Figure 4.2, colour of control solution remained brown, however the inoculum solution was orange at the end of enrichment indicating bacterial activity.

Figure 4.2. (a) Control solution and (b) inoculum solution after the enrichment period

The difference in colour, pH and Fe^{2+} concentration between the inoculated and control solutions indicated the successful enrichment of IOB in the inoculated solution which was used as the inoculum for the half-cells and MFCs experiments.

4.3.2 *Acidophilic iron-oxidising bacteria as a biocatalyst in the cathodic compartment of half-cell BES with a GDE cathode*

Half-cells equipped with GDEs were operated at 0.2 V and inoculated with the enriched environmental sample. Very low current density was measured over 17 days of the experiment in the control cell, therefore it was terminated after this period. As shown in Figure 4.3, electron uptake was observed in the half-cells inoculated from the beginning of the experiment: cathodic current densities of 0.3 mA cm^{-2} were measured just after inoculation and about 0.5 mA cm^{-2} after day 5 and for more than 45 days of operation (Figure 4.3). The presence of Fe^{2+}/Fe^{3+} as a mediator with iron-oxidizing bacteria as biocatalyst allowed the long start-up usually associated with the development of biocathodes catalysing the ORR, to be avoided. It has been reported in previous studies that iron-oxidizing bacteria such as *Acidithiobacillus ferrooxidans* can accept electrons directly from a cathode without the need for Fe^{2+} as a mediator (Carbajosa et al., 2010). However, in their study, current generation occurred after 22 days which corresponded to the time necessary for a biofilm to grow directly at the surface of the electrode. In addition, maximum current densities were measured at a potential of 0.05 V, potential corresponding to the catalysis of the ORR by the biofilm (Carbajosa et al., 2010).

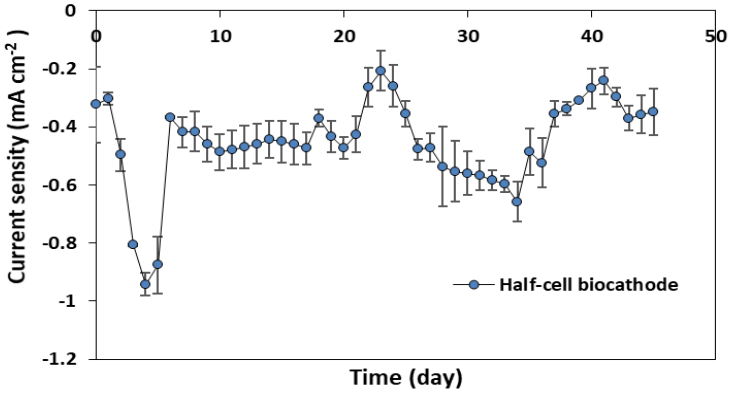
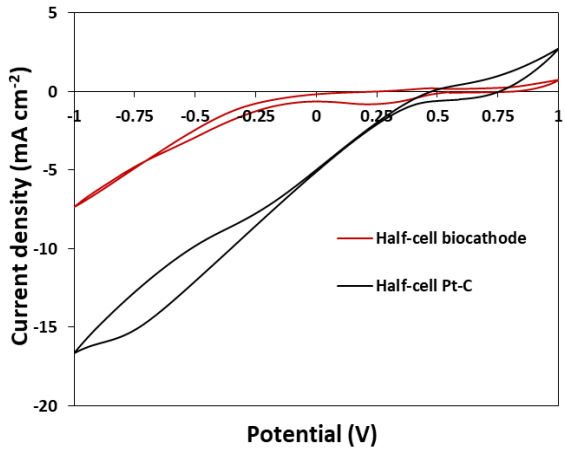


Figure 4.3. Chronoamperometry of half-cell poised at 0.2 V and inoculated by enriched IOB during the 47 day experiment

In order to confirm the mechanism of electron transfer between the cathode and bacterial community, CVs were recorded after 47 days of operation, and compared with those recorded with MFC with a Pt-C GDE (Figure 4.4). The voltammogram recorded on the Pt-C GDE (Figure 4.4 (a)) shows a clear catalysis of the ORR with an onset potential around 0.4 V. Carbajorsa et al reported that in absence of mediator, IOBs are able to accept electron directly from a cathode to catalyse the reduction of oxygen (Carbajosa et al., 2010). In the voltammograms recorded in their study after development of biocathode, clear oxidation and

reduction waves were observed indicating the bacterial electrochemical activity. In addition, authors compared the reduction peaks of voltammograms in presence and absence of oxygen which showed the evident ORR occurred in presence of oxygen and its significant decrease in the absence of oxygen. In our system using IOB and iron as mediator, however, there was no significant difference between the reduction peak of oxygen (at 0.4 V) when oxygen was provided with that in absence of oxygen. This could suggest that a pseudo-reversible signal of $E^{0'}$ about 0.4 V was attributed to the Fe^{2+}/Fe^{3+} redox couple at the surface of the cathode and not oxygen. Although oxygen was being used as a terminal electron acceptor by IOB, the mechanism of electron transfer between the cathode and bacterial community was more likely related to Fe^{2+}/Fe^{3+} redox couple rather than ORR.

(a)



(b)

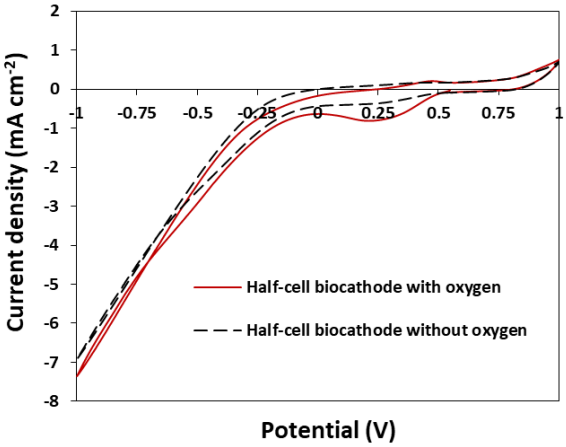


Figure 4.4. (a) Voltammograms recorded in half-cell reactors equipped with GDEs with Pt-C (black) and IOB biocathode as catalysts (red); (b) Voltammograms recorded in half-cells reactors equipped with GDEs and IOB biocathode as catalyst after 47 days of operation, in presence (red) and absence (dashed black) of oxygen. CVs recorded at a scan rate of 2 mV s^{-1}.

The proposed mechanism occurring between the electrode and bacterial community is more likely indirect as suggested in Figure 4.5. This shows the important role of cathode in regeneration of Fe^{2+}. Iron is the most abundant compound which can be found in environment such as low pH sediments of rivers. Combination of electrochemical and biological reactions of Fe^{2+}/Fe^{3+} redox couple can offer an efficient and quick start-up cathodic reaction as an alternative to the cathodic reactions using costly chemical catalyst. Therefore, it was important to investigate the performance of MFC coupled with acidophilic iron-oxidising biocathode in terms of power production and compare it with that in MFCs equipped with typical costly catalyst such as Pt. From CV results, it can be expected that the cathode potential of the MFCs equipped with IOB as biocatalyst and Fe^{2+} as mediator will be close to the reduction potential of Fe^{3+} into Fe^{2+} observed in Figure 4.4, i.e. about 0.3 V.

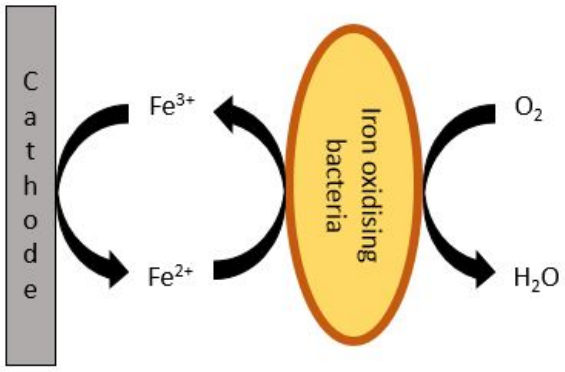


Figure 4.5. Schematic of the suggested reaction occurring in the cathodic compartment.

4.3.3 *The effect of an acidophilic iron-oxidising biocathode on the performance of MFC in fed-batch operational mode*

Following the half-cells experiments, MFCs were set up with GDE cathodes equipped with either Pt-C or IOB. Bioanodes from the effluent of parent MFCs effluent were first developed using Pt-C as cathodes. Parent MFCs were run and generated electricity over one year in the laboratory. Therefore, due to the enrichment of electro-active bacteria in parent MFCs, electricity production occurred rapidly after only a day following inoculation (Figure 4.6 (a)), reaching a cell potential of 0.3 V after 3 days in both duplicate MFCs. Acetate was fed as a sole carbon source for bioanode to select *Geobacter* from the bacterial community in the biofilm. After the 7^{th} batch cycle when the maximum cells potential reached the stable value (~ 0.35 V),

CVs were recorded to confirm the development of bioanode (Figure A1, Appendix A). To perform CVs without acetate in the solution, CVs were carried out when cell potential reached almost zero at the end of 7th batch cycle. Oxidation and reduction peaks appeared at potentials of -0.1, -0.30, -0.37 and -0.47 V (Figure A1, Appendix A) indicated the development of bioanode, similar to previous studies on the bioanode formation (Fricke et al., 2008, Richter et al., 2009). These studies reported the comprehensive electrochemical investigations on bioanodes colonized by *G. sulfurreducens* demonstrating similar voltammograms shapes as what observed in this chapter. Fricke et al reported redox peaks at -0.515, +0.059, -0.376 and -0.295 V which were attributed to electro-active pili or outer membrane cytochromes such as OmcB, OmcE and OmcS with the redox potential reported at -0.378 V (Fricke et al., 2008). This was also later confirmed by Richter et al, demonstrating the similar shapes and redox potentials from the biofilm of *G. sulfurreducens* and different outer membrane cytochromes (Richter et al., 2009). This suggested the successful development of bioanode in our system, more likely comprised of *G. sulfurreducens*. In addition, polarisation and power density curves of the cell showed maximum power production of 0.134 ± 0.010 mW cm^{-2} and open circuit potential (OCP) of 0.733 ± 0.009 V (Figure 4.6 (b)).

(a) (b)

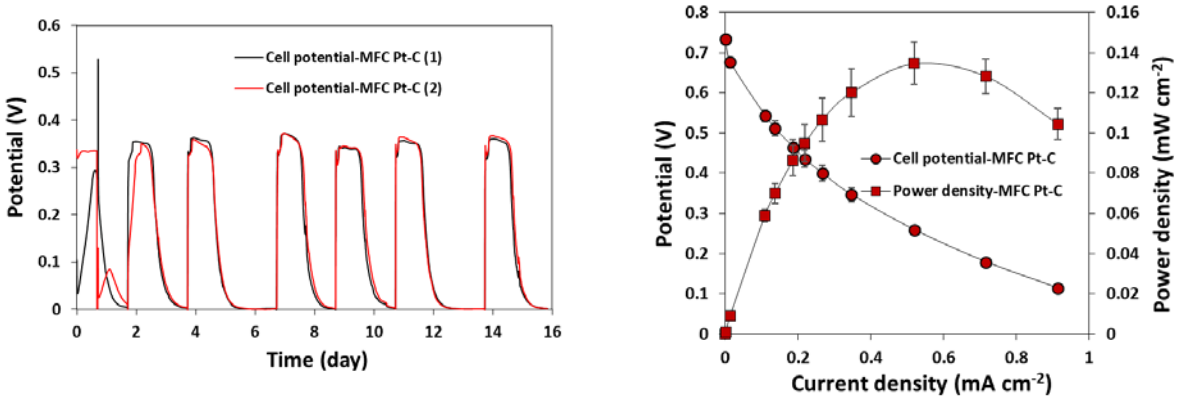


Figure 4.6. (a) Cell potential of MFC with the cathode of Pt-C over the development of the bioanode using the resistance of 200 Ω and (b) polarisation and power density curves of MFC after the bioanode development.

After the development of bioanodes, the platinised GDEs were replaced with GDEs with IOB as a biocatalyst. As discussed, the cathodic potential in MFCs equipped with iron-oxidisng

biocathiode was expected to be close to the potential of reduction of Fe^{2+}/Fe^{3+} (about 0.3 V) rather than ORR potential. Cell, anode and cathode potentials of the duplicate MFCs after replacing the platinised cathodes with the IOB biocathodes are reported in Figure 4.7. After switching cathodes, the cell potential in one of the duplicates decreased to 0.25 V after one hour and to 0.04 V after one day, before reaching almost zero at the end of day 2. The drop in cell potential was associated with the significant drop in the anode potential (Figure 4.7). After day 2, the medium of both anode and cathode compartments was replaced. Following the refreshment of medium, the cell potential reached 0.13 V and remained stable for almost 3 days before dropping back to zero on day 5 (Figure 4.7). In the other duplicate cell potential dropped to zero from day 1 until day 5. This drop in potential was accompanied by significant changes in the pH of the anolyte and catholyte.

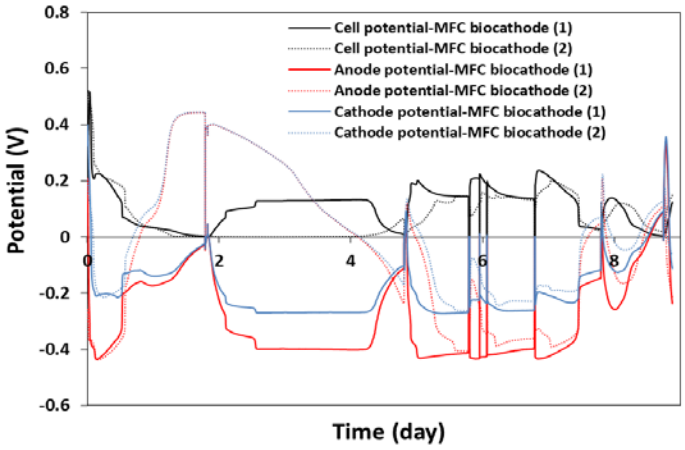


Figure 4.7. Cell, anode and cathode potentials of MFC equipped with GDE and IOB biocathode and operated in fed-batch mode.

Despite using 50 mM phosphate buffer at pH 7.0 in the anode compartment, the pH dropped to 4.0 after 2 days due to the high difference between the pH of the anolyte (pH 7.0) and catholyte (pH 2.0) separated by an AEM. Although daily refreshment of the anolyte and catholyte media helped to achieve a cell potential of 0.2 V, such a potential could only be sustained for a few hours as the pH of the anolyte rapidly decreased to 5.0. Similarly, a significant change was observed in the pH of the cathodic solution. Over the first 5 days after switching the cathodes, the pH of catholyte increased to about 5.0. The pH could be maintained at around 3.0 with regular medium replacement; however, it could not be stabilised at the initial and optimal pH of 2.0. Polarisation and power density curves of the MFCs equipped with Pt catalyst to those

equipped with IOBs as biocatalyst and iron as mediator demonstrated that the IOB biocathode did not perform as well as the platinised carbon cathode (Figure 4.8). To a large extent this was due to the inability to control the pH which had a major impact on the performance of the cells as the maximum power density achieved was 0.134 ± 0.010 mW cm^{-2} for MFCs operated in batch mode and equipped with Pt compared to 0.041 ± 0.011 mW cm^{-2} in MFCs with IOB biocathodes. The drop in pH was also associated with higher internal resistance in the MFC, as suggested by the increase of the slope of the polarisation curve (Figure 4.8).

pH of both anolyte and catholyte has a great impact on MFCs performance. It was shown that the optimal value of anolyte pH is between 7.0 and 8.0 and that a pH outside this range decreases the MFC performance (He et al., 2008). Previous studies reported that acidic anolyte (pH 5.0) affected the microbial activity of the biofilm which then caused lower current generation (Gil et al., 2003, Ren et al., 2007). Similarly, in the presence of IOBs and iron as catalyst and mediator, pH higher than 2.0 in the cathodic compartment results in iron precipitation which affects the performance of the biocathode (Ter Heijne et al., 2006). In addition, and as described before, pH of 2.0 provides optimal conditions for acidophilic IOB to produce ATP from the oxidation of Fe^{2+} and using molecular oxygen as terminal electron acceptor (Hedrich et al., 2011). Therefore, the pH of both the catholyte and anolyte need to be controlled to maintain the MFCs performance.

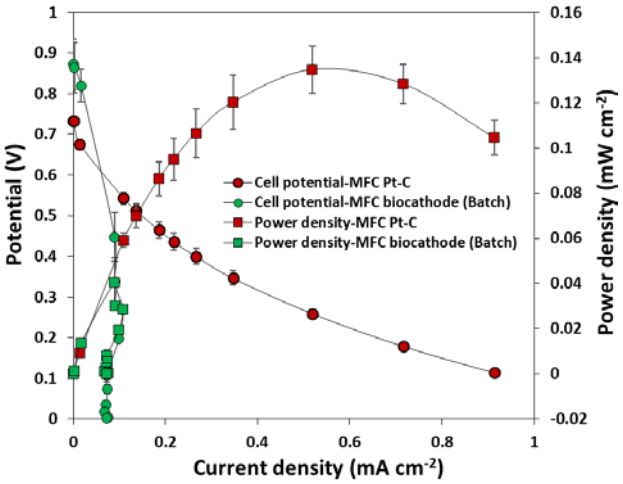


Figure 4.8. Polarisation and power curves density of MFCs with Pt-C and IOB catalysts operated in fed-batch mode.

4.3.4 *The effect of an acidophilic iron-oxidising biocathode on the performance of MFC in continuous operational mode*

In order to control the pH in both anode and cathode compartments and to avoid using expensive bipolar membranes, the cell operation was converted from fed-batch to continuous mode using a dosing pump for both anolyte and catholyte with similar hydraulic retention times (HRTs) of 2 hours. Such a low HRT helped stabilise pH in both compartments. Over the whole experiment in continuous mode, the pH of the anode and cathode media remained stable around 6.8 and 2.0, respectively. Five hours after switching from fed-batch to continuous mode, the anode potential in one of the duplicate MFCs (presented as (1) in Figure 4.7) dropped to -0.4 V whereas the cathode potential increased to -0.1 V, resulting in a cell voltage of 0.3 V (Figure 4.9). The anode potential stabilised at -0.5 V and the cathode potential increased slowly, reaching -0.2 V after 65 hours, for a corresponding cell potential of approximately 0.3 V, reaching the maximum of 0.4 V after 65 hours. However, other duplicate MFC (presented as (2) in Figure 4.7) could not recover from the effect of low pH resulted from the fed-batch mode. Although pH of anolyte and catholyte was adjusted at round 6.8 and 2.0, respectively in this MFC during continuous operational mode, cell potential did not increase and was zero during the experiment (Figure B2, Appendix B).

In order to confirm that the oxygen mass transfer was not limiting the bio-electrochemical process, the catholyte reservoir tank was purged with air using an aquarium air pump for 8 hours (between 40 and 48 hours). No significant change in cathode potential nor in cell voltage was observed (Figure 4.9), showing that passive diffusion of oxygen through the GDE is not a limitation to the performance of the MFCs. Therefore, such a design allows expensive aeration systems, which are not economically viable especially at larger scale, to be avoided.

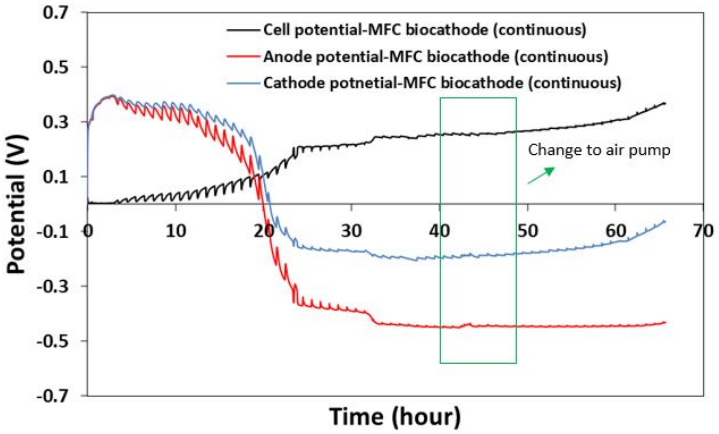


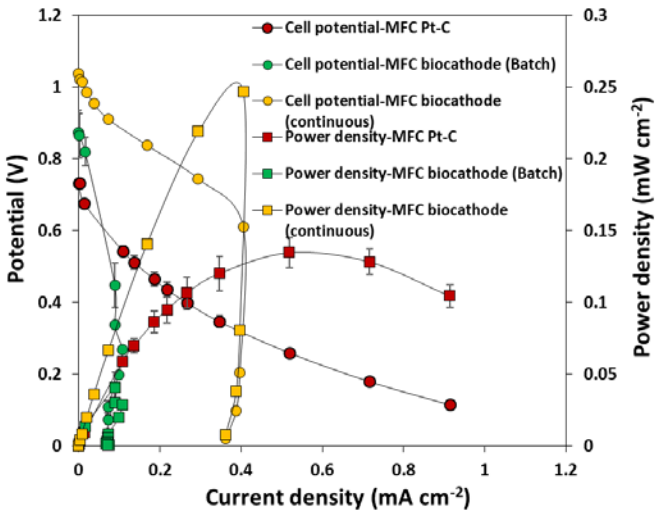
Figure 4.9. Cell, anode and cathode potentials of MFC with IOB biocathode operated in continuous mode.

Previously, GDE designed MFC was used more for abiotic cathodic reactions such as catalysis of the ORR than biotic cathodic reactions. It has been reported that air-cathode MFC with a GDE modified with activated carbon produced comparable power to an MFC operated with platinum as the cathode after one month of operation (Zhang et al., 2014). To date, few studies focused on the utilisation of GDEs for aerobic or anaerobic biocathode formation and operation. GDEs have generally been used to increase the diffusion of CO_2 through a gas diffusion layer to provide sufficient CO_2 for the development of anaerobic CO_2-reducing biocathodes (Bajracharya et al., 2016b). It was reported in that study that, with a GDE, mass transfer of carbon dioxide was higher than with direct purging of the catholyte with CO_2, leading to improvement of the BES performance in terms of CO_2 reduction and production. Studies focussing on MFCs equipped with GDEs for aerobic biocathodes are still scarce compared to systems with aeration, but the possibility of grwoing aerobic biofilms on such electrodes has been demonstrated in a number of studies (Wang et al., 2013, Xia et al., 2013, Montpart et al., 2018).(Rimboud et al., 2015). Therefore, the combination of GDE with IOBs as a biocatalyst and Fe^{2+} as mediator stands can be a good alternative to classical Pt-based catalysts in aerated system.

A maximum power density of 0.251 mW cm^{-2} was produced during continuous mode using IOB compared to 0.041± 0.011 mW cm^{-2} in fed-batch mode using IOB and 0.134 ± 0.010 mW cm^{-2} using platinised carbon paper as cathode (Figure 4.10 (a)). By stabilising the pH of both the anolyte and catholyte, continuous mode operation led to significant improvement in MFC output, then outperforming the MFC equipped with Pt catalyst (Figure 4.10). It can however be observed that there is a “doubling back” of the power curve for current densities exceeding 0.37

mA cm^{-2} which is also known as “power overshoot” (Kim et al., 2017). This phenomenon can be explained with the potential profiles of the anode and cathode recorded during the polarisation and presented in Figure 4.10 (b). As can be seen, the anode potential is relatively stable between -0.45 and -0.4 V until the external resistance is changed to 300 ohms. At this resistance, the cathode potential reaches approximately 0.25 V which is the potential at which Fe^{3+} starts to be electrochemically reduced into Fe^{2+} (as shown in CVs presented in Figures 4.4). The current required by the cathode thus increases and exceeds what the bioanode can supply. At this stage, it can be assumed that the bioanode becomes limiting, thus explaining the drop of both the anode potential and the cell voltage. In MFCs using oxygen as terminal electron acceptor, the power output is usually limited by the performance of the cathode, whether the ORR is catalysed by Pt or biocatalysts (Milner et al., 2016). Nevertheless, in the presence of IOBs and Fe^{2+} as mediator, and according to the potential profiles presented in Figure 4.10 (b), the bioanode appears to be limiting. This phenomenon was explained in a previous study showing that the power overshoot can occur when anodic kinetics are slower than cathodic kinetics, thus causing electron depletion (Kim et al., 2017). In our specific case, this could be explained by the development of a non-mature biofilm due to an enrichment process which has been too short, at lower external resistances (Hong et al., 2011), or by a biocathode outperforming the bioanode.

(a)



(b)

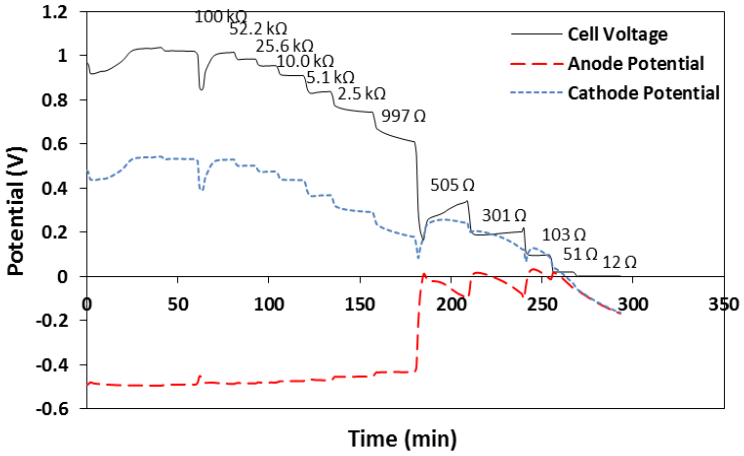


Figure 4.10. (a) Polarisation curve and power density of MFC equipped with different cathodes and operated in batch or continuous mode; (b) Potential profiles of the cell, anode and cathode during the polarisation of the MFC equipped with IOB biocathode and operated in continuous mode.

4.4 Conclusion

In this chapter, we demonstrated the feasibility of using iron-oxidising bacteria (IOB) as a biocatalyst and Fe^{2+} as a mediator as an alternative to a Pt catalyst in microbial fuel cells (MFCs) equipped with gas diffusion electrodes (GDEs). IOB were enriched from a natural environmental sample and first used as an inoculum in half-cell reactors. Stable current densities of around 0.5 mA cm^{-2} were produced over a period of 45 days. Due to the presence of Fe^{2+} as a mediator, no lag time was observed before such current densities were reached, thus avoiding the long start-up phase usually associated with aerobic biocathodes. Although the contribution of direct electron transfer between the cathode and the bacteria cannot be totally ruled out, cyclic voltammetry strongly suggested mediated electron transfer (by Fe^{2+}) as the main mechanism of electron transfer to oxygen. When MFCs were operated in batch mode, the performances of the cells containing IOB as a biocatalyst were comparable to those equipped with a platinized cathode. However, MFC performance could not be sustained due to the drop in pH in the anodic compartment and the increase of pH in the cathodic compartment. Operating MFCs in continuous mode helped stabilise pH in both compartments. Maximum power densities of 0.251 mW cm^{-2} were then reached, compared to 0.134 ± 0.010 mW cm^{-2} for MFCs equipped with a Pt catalyst. Finally, the passive diffusion of oxygen through the GDEs did not appear to be limiting factor to the performance of the MFCs, showing that the system studied

represent a good alternative to both chemical catalysts and non-mediated aerobic biocathodes without the need for costly aeration systems. In future studies, the impact of Fe concentration on the performance of the system will be studied, as it is known that IOB can accept electrons directly from polarised electrodes.

Chapter 5. Production of Valuable Organic Chemicals from CO_2 Using Microbial Electrosynthesis (MES)

This chapter focuses on the application of BES in conversion of CO_2 to valuable chemicals using anaerobic biocathodes. This research investigates the role of the cathode as an energy source in microbial electrosynthesis (MES) processes and biofilm formation driven by MES processes.

The specific objectives of this research were:

- Development of an active anaerobic biocathode for synthesis of organic compounds from CO_2
- Investigation of the effect of applied potentials and inorganic carbon sources on the development of an anaerobic biocathode
- Investigation of different operational parameters such as pH affecting anaerobic biocathode development
- Clarifying the electron transfer mechanism between the cathode and the bacteria
- Investigation of the role of a cathodic biofilm in MES processes
- Investigation whether synthesis of organic compounds occurs at the electrode or if it is carried out by planktonic cells.

5.1 Introduction

Microbial electrosynthesis (MES) has provided a cathode-driven approach to the application of bio-electrochemical system (BES) (Rabaey and Rozendal, 2010). In MES carbon dioxide reduction to valuable substances of volatile fatty acids (VFA) such as acetate can be driven at the cathode using electrons derived from renewable energy sources for example (Patil et al., 2015b). It is known that in BES, VFA particularly acetate can be produced by acetogens using

a cathode as an energy source and CO_2 as an inorganic carbon source through the Wood-Ljundhal (WL) pathway. The potential applied at the cathode and additional electron donors or carbon sources added to the system can affect the product profile generated from CO_2 in MES. According to Gibbs free energy of the system ($\Delta G^0 = -zF\ \Delta E^0$, ΔG^0: the change of standard Gibbs free energy of the system, kj mol^{-1}, z: number of electrons transferred from 1 mole of reactant/product, F: Faraday's constant 96485 C mol^{-1} and ΔE^0: standard redox potential), cathodic applied potential is linked to the energy supplied to microorganisms. Moreover, the form in which inorganic carbon is provided (e.g. HCO_3^- or CO_2) may also affect the progress of MES. In the recent review on microbial reactions, it was discussed that the phase of reactants or products affect ΔG^0 value. For instance, in CO_2 reduction reaction using hydrogenotrohic methanogens, ΔG^0 vary based on inorganic carbon source selected (gaseous CO_2 or HCO_3^-) (Amend and LaRowe, 2019). Several successful research studies demonstrating CO_2 or HCO_3^- conversion to different organic products such as acetate and butyrate in MES have been reported (LaBelle and May, 2017, Jourdin et al., 2016, Jourdin et al., 2015b, Marshall et al., 2012, Marshall et al., 2013). However, the mechanisms underlying microbial electrosynthesis remain unclear. Despite the recent focus on MES, major limitations exist such as slow development of CO_2-reducing biofilms on cathodes. These difficulties relate to the low growth rate and yield of bacteria that grow on the electrode surface, using the electrons provided from the electrode as a reductant and energy source and CO_2 as the carbon source, leading to the slow start-up of the biofilm formation and production processes (Jiang and Zeng, 2018a). Nevertheless, the role of the electrode in the system and whether MES occurs at the electrode surface or is catalysed by planktonic cells in suspension has not been fully established. It has been suggested in some studies, that the planktonic bacterial community in the catholyte is responsible for MES, as no biofilm was formed at the electrode (Arends et al., 2017). However, Jourdin et al. reported a development of mature biofilm at the cathode during MES processes responsible for CO_2 conversion (Jourdin et al., 2018). Hence, the ultimate aim of the work reported in this chapter was to clarify the impact of different potentials applied at the cathode, as an energy source for bacteria and the form of inorganic carbon provided (HCO_3^- or CO_2) on bacterial growth, development of an active CO_2-reducing biocathode, biofilm formation at the cathode and its performance in terms of cathodic current consumption and organic production, and its physical, biological and electrochemical properties.

5.2 Material and methods

5.2.1 Pre-treatment step

In this work, activated sludge was collected from a wastewater treatment plant, as an inoculum source for microcosms, and stored for 24 hours at 4 °C before heating for 20 minutes at 70 ± 2 °C. 40 ml of heated inoculum was then transferred to 4 replicate 120 ml serum bottles. Activated sludge was used as an inoculum due to its diversity of bacterial communities (Xia et al., 2018). Pre-treatment step before transferring to BESs was carried out to suppress methanogens and fermenters and select the acetogenic bacteria from the diverse bacterial community in activated sludge. 40 ml cathodic medium described in Section 3.2.4 with 3 g/L sodium bicarbonate was added to each serum bottle and the solution was purged for 10 minutes with N_2/CO_2 (90/10) to remove dissolved oxygen and then bottles were sealed by butyl rubber septa. Finally, 40 ml headspace of each serum bottle was purged by H_2 for 5 minutes and inverted (to ensure headspace gases were preserved) in a shaking incubator set at 35 °C and 80 rpm under anaerobic condition. The pH of each solution was between 6.5 and 7.0 after purging the solution and headspace.

The microcosms were run in batch cycles defined as the period between preparing the serum bottles until the medium was changed (approx. 5 to 7 days). After each batch cycle, 50% of the solution was replaced with fresh and degassed cathodic medium, and headspaces were purged, similar to the first cycle. Overall, three batch cycles were performed and the resultant fourth cultures were used to inoculate the BESs.

Two different controls were prepared for the pre-treatment procedure:

1) To ensure that the bacterial community was responsible for the production of any chemicals, 2 replicate serum bottles were prepared by autoclaving cathodic medium which was then degassed and operated under sterile condition.

2) To ensure that H_2 was used as an energy source, 2 replicate serum bottles were prepared in the same way as the 4 inoculated serum bottles, except with N_2 instead of H_2 purged into the headspace.

Concentration of H_2 was calculated using the ideal gas law;

$$PV=nRT \quad (5.1)$$

where P is pressure (atm), V is volume of gas (ml), n is moles of gas and R is the gas constant 82.057 ml atm K^{-1} mol^{-1}. P was assumed to be 1 atm because the serum bottles were

prepared at atmospheric pressure, V was the volume of headspace (40 ml) and T was the set point temperature of the incubator (308 ^{o}K; 35 ^{o}C)).

Gas and liquid samples were collected at the beginning and end of each batch cycle and analysed by GC and IC, respectively to measure products concentration as described in Sections 3.6.5, 3.6.6 and 3.6.7. Total organic carbon in the catholytes were measured at the end of each batch cycle as explained in Section 3.6.4 to measure the organics in the solution. As acetate was the major product during the enrichment, mass balance was calculated based on consumption of H_2 in the headspace and concentration of acetate produced (equivalent to H_2 consumption) in each batch as shown in Equation (2.13). All results from the enrichment were reported as an average of the replicate serum bottles.

5.2.2 Evaluating abiotic hydrogen production at different cathodic potentials

Before starting the biotic experiment in BESs, the possibility of abiotic evolution of H_2 at different potentials was investigated. To achieve that, 4 different reactors in duplicates (8 reactors in total) were fabricated as described in Section 3.2.1 and started similarly to ensure the equal experimental conditions in all the reactors. To provide sterile conditions for the abiotic control experiment, all the reactors were washed with ethanol, rinsed with deionised water and imposed them under UV light for 20 minutes. The reactors were started with autoclaved anodic and cathodic media. Each duplicates were poised at different potentials of -0.7, -0.8, -0.9 and -1.0 V for 13 days with potentiostat as described in Section 3.3.2 and the cathodic current generated was recorded. To determine the abiotic production of hydrogen at each applied potential, gas samples was collected from the headspaces every 3 - 4 days and analysed using GC. Liquid samples were also analysed to detect the organic acids in the catholytes.

5.2.3 Evaluating the effect of electrode potential on MES with bicarbonate

Selection of the best condition in terms of energy and inorganic carbon sources for start-up of biocathode development and production using the pre-treated inoculum was investigated over 100 days test experiment. After 13 days of abiotic experiment described in the previous section, cathodes of the reactors were replaced with the identical fresh cathodes and 50% of the catholytes were replaced by the cultures from the treated microcosms. All the headspaces were

purged with nitrogen to remove oxygen. Over the first 50 days, sodium bicarbonate was used as a sole inorganic carbon source in cathodic compartments. Bicarbonate was provided by dissolving $NaHOC_3$ in cathodic medium as described in Section 3.2.4. 30% of medium was refreshed every 10 - 20 days. Cathodes used for both abiotic and biotic experiments were washed in acetone beforehand and then washed several times in sterile deionised water to remove the acetone. A duplicate BES was operated with the similar experimental condition but under open circuit potential (OCP) condition as a control to specify the effect of cathodic poised potential on MES.

Gas and liquid samples were collected and analysed regularly to detect the gaseous and liquid products and measure the pH of liquid samples. Total inorganic and organic carbon in the catholytes were measured irregularly (5 times over experiment) to establish the consumption of inorganic carbon and production of organics. In order to verify the cells growth in cathodic compartments, liquid samples were collected every 16 to 20 days, for cell count analysis using a flow cytometer reported log-transformed as explained in Section 3.4.2.

To identify the electrochemical activity of any biocathode formed during the first 50 days of experiment, CVs were recorded from all the BESs on day 1, before inoculating the system and on day 50 as described in Section 3.3.3. To provide similar conditions in the BESs for the CV analysis and to prevent the solution pH affecting results, 40% of all media were refreshed and the pH of all catholytes were adjusted to ~ 6.5 using 1 M NaOH or 1 M HCl before performing CV.

5.2.4 Evaluating the effect of electrode potential on MES with CO_2

From day 50 to 100 of the test experiment, inorganic carbon source was changed from bicarbonate to CO_2. Therefore, 60% of each catholyte was replaced with fresh cathodic medium without sodium bicarbonate and instead, CO_2 was purged as a sole inorganic carbon source. Every 2 or 3 days, CO_2 was provided in cathodic compartments by recirculating the catholytes in 250 ml duran bottle using peristaltic pump (hydraulic retention time of 4 ml min^{-1}), which bottle was purged by CO_2/N_2 (90/10). 30 % of medium was also refreshed every 10 – 20 days. Following the first 50 days, gas and liquid samples were collected to detect the products, pH, total inorganic carbon, total organic carbon and number of the cells in the catholytes.

On day 100, 40% of catholytes were refreshed and their pH were adjusted to ~ 6.5 using 1 M NaOH or 1 M HCl before performing CV. CVs were recorded from all the BESs on day 100 and compared with the voltammograms of day 1 and day 50.

The experimental design of 100 days of biotic experiment and control are summarized in Table 5.1.

Table 5.1. Experimental design used for 100 days of test experiment.

Reactor	**BESs -0.7 V**	**BESs -0.8 V**	**BESs -0.9 V**	**BESs -1.0 V**	**OCP**
Potential (V)	-0.7	-1.0	-0.8	-1.0	OCP
Inorganic carbon	0-50 days $NaHCO_3$ 5-100 days CO_2	0-50 days $NaHCO_3$ 5-100 days CO_2	0-50 days $NaHCO_3$ 5-100 days CO_2	0-50 days $NaHCO_3$ 5-100 days CO_2	0-50 days $NaHCO_3$ 5-100 days CO_2
Cathodic inoculum	Inoculum from 4^{th} cycle of treatment	Inoculum from 4^{th} cycle of treatment	Inoculum from 4^{th} cycle of treatment	Inoculum from 4^{th} cycle of treatment	Inoculum from 4^{th} cycle of treatment

5.2.5 Evaluating the effect of cathode potential on biofilm formation and MES

One of the important aim of this research was to study whether cathodic biofilm forms during cathodic reactions and its role on MES. To achieve that, 3 different fresh BESs were operated over the second phase of the experiment. From the 100 days of test experiment, the catholyte of the BESs that showed the highest cells growth and production through MES was used for inoculating 50% of the new 3 BESs. To investigate the effect of poised potential on the biofilm formation at the cathode and MES processes as an energy source, two potentials of -0.8 and -1.0 V were applied at the cathodes of two different duplicate BESs supplied by dissolved CO_2. CO_2 was provided similar to the method mentioned in Section 3.2.4. In addition, biotic control experiment was carried out using the similar BESs of the biotic experiment but under OCP condition. This could evaluate the formation of biofilm when no energy source was provided. When cathodic medium was refreshed, pH of the catholytes were adjusted at 6.8-7.0 by purging

CO_2/N_2 (90/10) in the catholytes. However, pH was not adjusted during the experiment to compare the natural effect of different conditions provided. The experiment was executed for 104 days.

The experimental design used for the biotic and control experiments are summarized in Table 5.2.

Table 5.2. Experimental design used for 104 days of the experiment investigating the effect of cathodic potential on biofilm formation and MES processes.

Reactor	**BESs 1**	**BESs 3**	**OCP**
Potential (V)	-1.0	-0.8	OCP
Inorganic carbon	CO_2	CO_2	CO_2
Cathodic inoculum	Inoculum enriched in BESs -1.0 V	Inoculum enriched in BESs -1.0 V	Inoculum enriched in BESs -1.0 V

During 104 days of experiment, gas and liquid samples were collected every 2 to 5 days and analysed to detect the gaseous and liquid products. Total inorganic and organic carbon in the catholytes were measured irregularly. pH of the catholytes was also measured at each liquid sampling. At the end of this experiment (104 days) after adjusting the pH of the catholytes to ~ 6.5 using 1 M NaOH or 1 M HCl, CV was performed from all the BESs to compare the electrochemical properties of the biocatalysts developed under different conditions during the 104 days of the experiment. Additionally, before terminating the experiment, more electrochemical analysis of CV and EIS were performed from BESs 1 as explained in Section 3.3.5 to gain further insight toward the role of biofilm formed at these BESs.

5.2.6 Evaluating the effect of inorganic carbon source on biofilm formation and MES

In parallel to the investigation of the effect of poised potential, the effect of different inorganic carbon source types on biofilm formation and MES processes was also examined. To achieve

that, a duplicate set of BES reactors were operated by cathodic applied potential of -1.0 V with bicarbonate addition to the catholyte instead of purging with CO_2 during 104 days of experiment. When cathodic medium was refreshed, pH of the catholytes were adjusted at 6.8-7 by purging CO_2/N_2 (10/90) to catholytes. However, pH was not adjusted during the experiment. The performance of the BESs in terms of production was compared with that observed in BESs poised at -1.0 V and supplied by CO_2 (BESs 1).

Gas and liquid samples were collected every 2 to 5 days and analysed to detect the gaseous and liquid products. pH of the catholytes was also measured at each liquid sampling. Total inorganic and organic carbon in the catholytes were measured irregularly. At the end of the experiment (104 days) after adjusting the pH of the catholytes to ~ 6.5 using 1 M NaOH or 1 M HCl, CV was performed from these BESs to compare the electrochemical properties of the biocatalysts developed when bicarbonate was provided with that recorded from BESs 1.

The experimental design used for the biotic and control experiments are summarized in Table 5.3.

Table 5.3. Experimental design used for 104 days of the experiment investigating the effect of inorganic carbon source on biofilm formation and MES processes.

Reactor	**BESs 1**	**BESs 2**
Potential (V)	-1.0	-1.0
Inorganic carbon	CO_2	$NaHCO_3$
Cathodic inoculum	Inoculum enriched in BESs -1.0 V	Inoculum enriched in BESs -1.0 V

5.2.7 *Evaluating physical properties of the cathodic biofilms*

After terminating and disassembling BESs 1, BESs 2, BESs 3 and OCP on the last day of experiment (day 104), electrodes were collected and cut for the further analysis. SEM images

from the electrodes were performed as described in Section 3.4.4 to gain a visual representation of physical properties of biofilms developed at different conditions.

In addition, confocal microscopy was carried out from the electrodes as described in Section 3.5.3 to gain quantitative information from the biofilms developed. Results from confocal microscopy were processed using Huygens software as described in section 3.4.3, to extract the distribution, coverage and bio-volume of live and dead cells of the biofilms formed at the electrodes at each condition. The live and dead bio-volume of cathodic electrode was corresponded to the coverage of the cathode by the biofilm and was calculated as the percentage of live or dead cells per each image size, by dividing the visible bio-volume of the cells considering the depth (z-scan) of the image by size of the image. Live and dead bio-volume of the cathodic biofilm was calculated by the percentage of live or dead cells of the total volume of the biofilm.

5.2.8 *Identifying bacterial composition in cathodic biofilms and planktonic cells*

It was important to compare the bacterial composition in cathodic biofilm and planktonic cells in the catholytes to calrify one of the important question of this research study, whether MES occurs at the electrode or in suspension. Combining with the electrochemical anaylsis mentioned, this could also clarify the mechanism of electron transfer dominating in MES processes between the cathode and bacterial cells. Therefore, cell counts were carried out using a flow cytometer to count the number of the cells on the cathodes and catholytes reported log-transformed. Cell densities for the biofilm at the cathodes were calculated per cm^2 projected surface area of the cathodes, while for catholytes they were measured in 1 ml solution. Furthermore, 16S rRNA gene-based community analysis was performed as described in Section 3.5 to reveal the identity of the bacteria in the biofilm formed at the surface of the electrodes and planktonic cells in the catholytes. For that, DNA was extracted from the cathode and catholyte samples of BESs 1, BESs 2 and BESs 3 that significant number of bacterial cells were observed, which was also amplified using Polymerase chain reaction (PCR). For DNA extraction, 2 samples were collected from each reactor. As each condition was operated in duplicate, therefore 4 samples were collected for DNA extraction from each condition. The extracted DNA from all the samples were quantified using Qubit and calculated in average of 4 samples per condition. The communities of samples from electrodes and solutions were sequenced using Illumina MiSeq and processed with ‘’Dada 2’’ package in R statistical platform as explained in Section 3.5.7. Before taxonomy assignment, diversity of the samples

was carried out using Shannon index analysis from data (ASV table). Shannon index is a diversity index describing the diversity of the samples using this equation:

$$\text{Shannon index} = -\sum p\_ASV . \ln(p\_ASV) \quad (5.2)$$

Where p_ASV is calculated by dividing the number of one particular ASV in each sample by the total ASV found in the sample. For assignment of the sequences two different databases of SILVA and Ribosomal Database Project (RDP) with bootstrap 80 were compared by plotting abundance of assignment of the sequences from the levels of kingdom to genus. The database that presented the sequences of the samples was selected. Further analysis such as Bray-Curtis dissimilarity index was carried out to compare the difference between the samples as described in Section 3.5.10.

5.2.9 *Summary of the analyses*

The summary of the biotic experiments executed in this chapter is demonstrated in Figure 5.1. The analyses carried out from the BESs throughout the experiments are also summarized in Table 5.4.

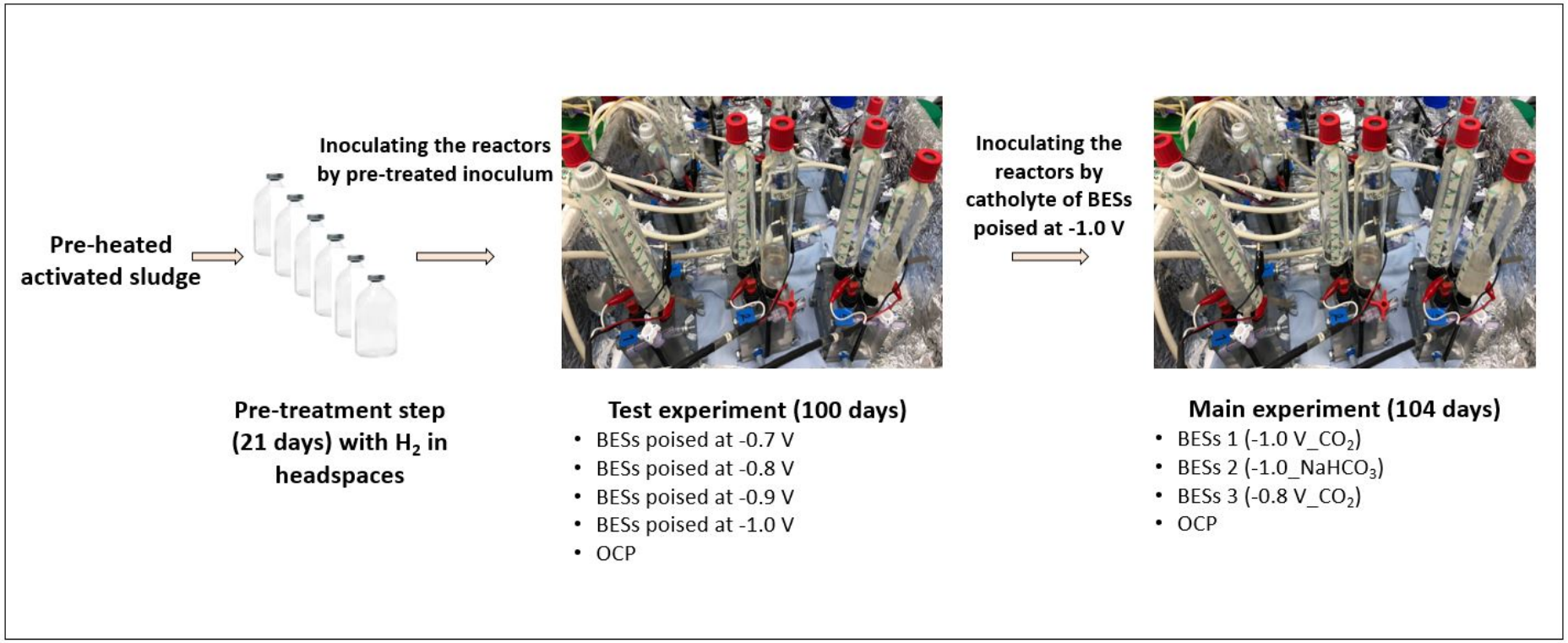


Figure 5.1. Summary of the biotic experimental design in this chapter.

Table 5.4. Electrochemical, analytical and biological analyses carried out from the BESs during the experiments in this chapter.

Analysis / BESs	CA	CV	EIS	Products detection	pH	Cell count of cathodic biofilm	Cell count of catholyte	Confocal microscopy	SEM	16S of cathodic biofilm	16S of catholyte
-0.7 V	✓	✓	×	✓	✓	×	✓	×	×	×	×
-0.8 V	✓	✓	×	✓	✓	×	✓	×	×	×	×
-0.9 V	✓	✓	×	✓	✓	×	✓	×	×	×	×
-1.0 V	✓	✓	×	✓	✓	×	✓	×	×	×	×
OCP	×	×	×	✓	✓	×	✓	×	×	×	×
BESs 1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
BESs 2	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓
BESs 3	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓
OCP	×	×	×	✓	✓	✓	✓	✓	✓	×	×

5.3 Results and discussion

5.3.1 *Pre-treatment step*

Selection and enrichment of acetogens from mixed culture of microorganisms can be challenging, as they require the same conditions (namely inorganic carbon and energy) as methanogens and thus, these two types of microorganisms usually coexist (Balows et al., 2013). Therefore, scavenging the methanogens is essential, as they are the known competitive for acetogens, affecting the MES processes and decreasing the efficiency of the system. To ensure that methanogens were supressed before transferring to BESs and acetogens were selected from the mixed bacterial community, pre-treatment step was carried out before transferring the activated sludge to the BESs. This was performed using the modified procedure adapted from previous study as described in Section 5.2.1 (Mohanakrishna et al., 2015). The reason for modification was that in the cited procedure, inoculum was heated at 90 °C for an hour, grown heterotrophically with glucose and finally grown autotrophically with only sodium bicarbonate and hydrogen. However, heterotrophic growth of inoculum was suspected to promote the enrichment of competitors particularly fermenters which will be difficult to control them after transferring to BESs. In addition, heating at 90 °C for an hour could kill many species of acetogens in the inoculum as likely not all of them are resistant to high temperature (Drake et al., 2006). During the 21 days of pre-treatment step in the microcosm bottles, acetate was the dominant product and other products of propionate, butyrate and iso valerate were also detected in trace quantities (Figure 5.2). Acetate concentrations increased over each batch cycle, after refreshing the medium, and reached the maximum concentration of 680.1 ± 70.2 ppm (22.6 ± 2.3 mmole C L^{-1}) with the maximum of 901.3 ppm (30.0 mmole C L^{-1}) in one of the bottles at the end of third batch cycle. In addition, no methane was detected in the headspaces during the pre-treatment step.

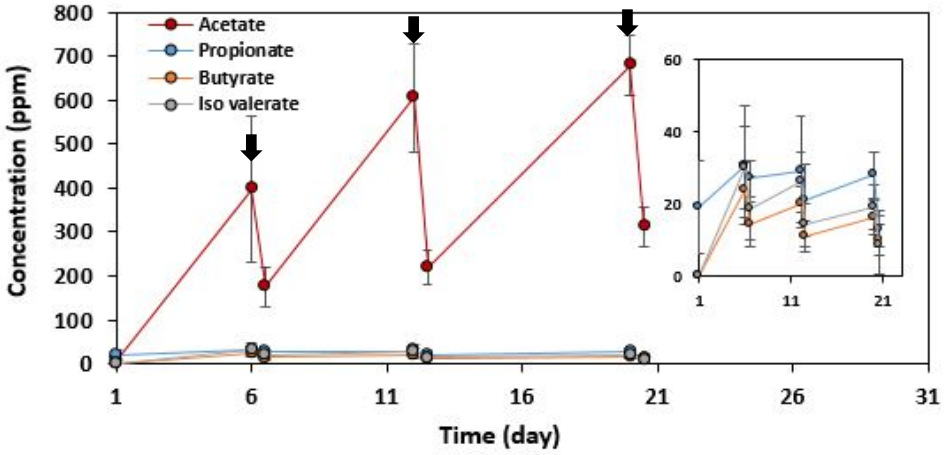


Figure 5.2. Organic acids detected in the serum bottles over the 21 days of pre-treatment step after heating treatment of activated sludge. Insert figure is the zoom in of small section of production. Black arrows show the medium refreshing throughout the pre-treatment step.

The acetate produced during the pre-treatment step in the medium was compared to the acetate expected to be produced (Figure 5.3) due to the consumption of H_2 calculated according to its stoichiometric equation (Equation 2.11). For the first batch cycle, not all the H_2 was consumed, as around 41.0 ± 6.0 % H_2 was detected in the headspaces. However, consumption increased over the batch cycles, when no H_2 was detected in the headspaces at the end of further batch cycles, showing the consumption of all the H_2 in the bottles by microorganisms. At the end of first batch cycle, higher concentration of acetate was detected than that calculated considering consumption of H_2. This was more likely due to the presence of organics in the initial activated sludge and therefore fermentation processes interfered the pre-treatment step. This was also confirmed by the TOC results which was much higher than the TOC represented the products. However, regular medium refreshment during the experiment could wash out the organics inside the solution. This was associated with the complete consumption of H_2 in the headspaces in the last batch cycle, indicating the enrichment of autotrophs over heterotrophs.

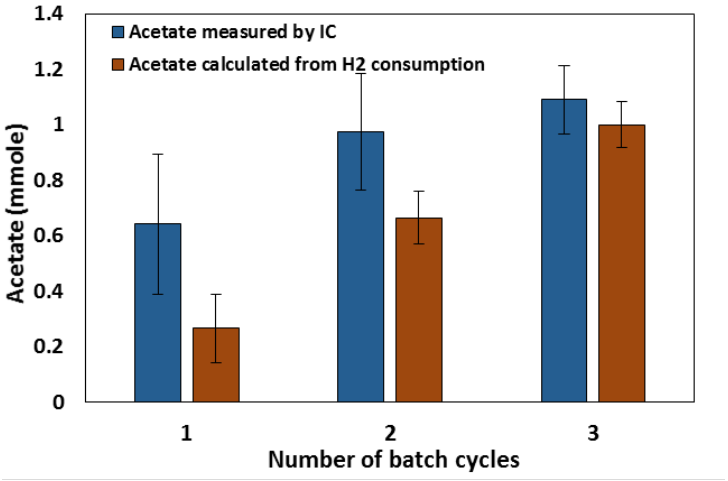


Figure 5.3. Comparing the acetate produced during the pre-treatment step in the medium and the acetate calculated to be produced according to the consumption of H_2.

The control experiment for the pre-treatment step was performed by operating similar serum bottles but without H_2 in the headspaces. The reason of performing the control experiment was to verify whether the organics production was related to the fermentation or acetogenic processes (Figure 5.4). Similar to the observations from pre-treatment step, acetate was produced over the first batch cycle due to the fermentation processes of organic matters in activated sludge. However, after regular refreshing the medium, no production was observed in the serum bottles. Therefore, acetate production when H_2 was provided could be attributed to acetogenic processes using inorganic carbon and H_2. In addition, no organic acids or gas products were detected in abiotic control experiment carried out in parallel to pre-treatment step, confirming that bacterial community was responsible for the production in inoculated serum bottles.

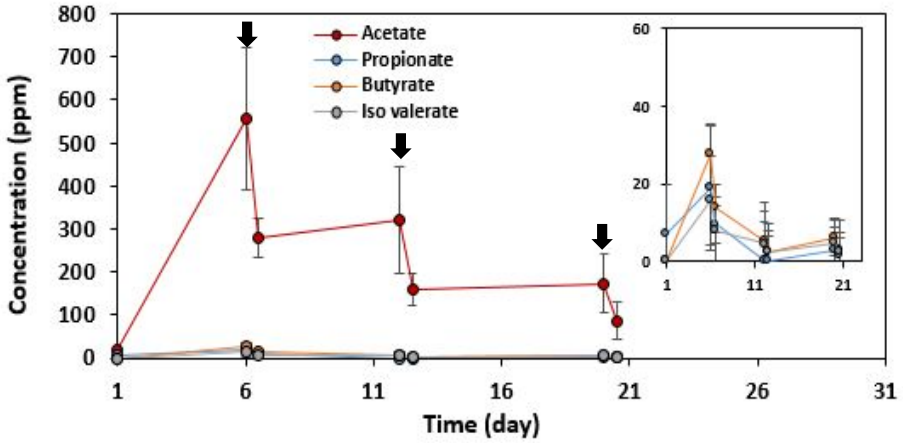


Figure 5.4. Organic acids detected in the serum bottles without H_2 in the headspaces over the 21 days of control experiment after heating treatment of the activated sludge. Insert figure is the zoom in of small section of production. Black arrows show the medium refreshing throughout the pre-treatment step.

No methane was detected in the headspaces of serum bottles during the pre-treatment step. Given the results from pre-treatment step supporting the suppression of methanogenic activity and improve in acetogenic activity, the pre-treated community from the fourth cycle was used as an inoculum for the next step in BESs.

5.3.2 *Investigation of abiotic hydrogen evolution at different applied potentials*

Before inoculating the BESs, a control abiotic experiment was carried out for 13 days to evaluate the possible abiotic cathodic current consumption and H_2 production at different applied potentials. Cathodic current consumption over the abiotic experiment is presented in Figure C1 in Appendix C. The highest cathodic current was recorded at the duplicate reactors poised at -1.0 V, approximately between -0.01 and -0.03 mA cm^{-2} during the experiment. At the applied potential of -0.9 V, abiotic cathodic current was almost around -0.01 mA cm^{-2}, whereas this value was much lower at the potentials of -0.7 and -0.8 V, which were $\leq$ 0.004 mA cm^{-2}. Detection of 1.8 % and 0.19 % H_2 in the headspaces of reactors poised at -1.0 and -0.9 V, respectively (Table 5.5) elucidated the higher cathodic current consumption at these conditions, while no H_2 was detected in the headspaces of reactors poised at -0.7 and -0.8 V. It is also established that carbon is not a suitable catalyst for abiotic H_2 production as high overpotential

is required for the production of H_2 at a carbon based surface (Dong et al., 2015a). In addition, no organic products were detected in the catholytes of the reactors during the abiotic experiment.

Table 5.5. Abiotic H_2 produced over the 13 days of abiotic control experiment in the reactors poised at four different cathodic potentials.

Potential (V)	-0.7	-0.8	-0.9	-1.0
Average H_2 produced (%) during 13 days of abiotic experiment	-	-	0.19 ± 0.0	1.8 ± 0.1

5.3.3 *Determination of best applied potential and inorganic carbon source for bacterial growth and acetate production through MES*

After the abiotic control experiment, the biotic experiment was initiated in the fresh reactors and inoculated with the inoculum treated through the pre-treatment step. Figure 5.5 demonstrates the cathodic current density at each batch cycle of BESs poised at four different potentials from -0.7 to -1.0 V over the whole 100 days of the biotic test experiment. The main target of 100-day test was selection of the best operational conditions for MES. Similar to abiotic experiment, the current consumption trend throughout the experiment was: (-1.0 V) > (-0.9 V) > (-0.8 V) > (-0.7 V) (current consumption increased with greater applied potential). Over the first 50 days of the experiment that bicarbonate was used as an inorganic carbon source in the catholytes, the BESs poised at -0.9, -0.8 and -0.7 mV showed the similar current density to that in abiotic experiment. However, comparing the abiotic and BESs cathodic current density in the reactors poised at -1.0 V, an increase in the current density was observed after inoculation, indicating the involvement of bacterial community in electrons uptake. Maximum cathodic current density in the BESs poised at -1.0 V was approximately around -0.06 mA cm^{-2} during the first 50 days of experiment. After changing bicarbonate to CO_2 as the sole inorganic carbon in the catholyte, current uptake increased significantly in the BESs poised at -1.0 V, reaching the maximum current density of almost -0.2 mA cm^{-2}. A small increase in the electron uptake was also observed in the BESs poised at -0.9 V, however it did not change in BESs poised at -0.7 and -0.8 V and was less than -0.004 mA cm^{-2} during the whole 100 days of the experiment, similar to that in the abiotic experiment.

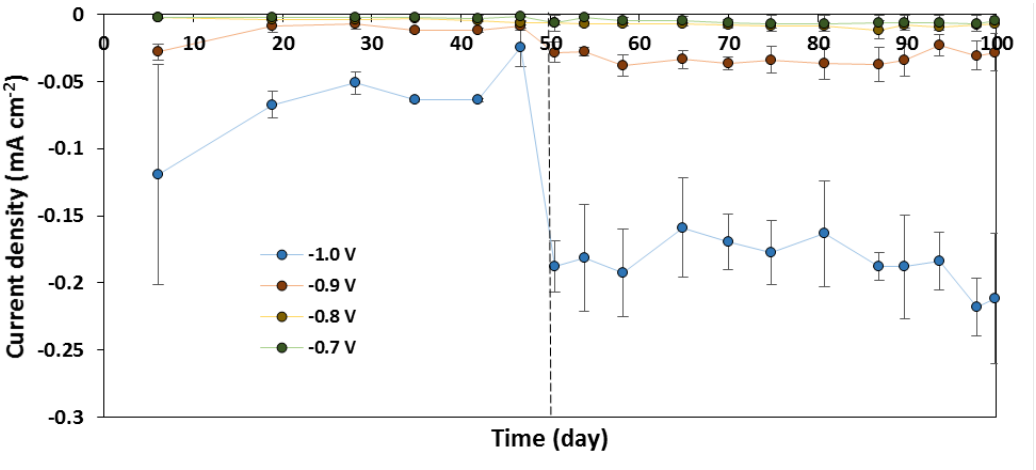


Figure 5.5. Cathodic current density during the biotic experiment at the BESs poised at -1.0, -0.9, -0.8 and -0.7 V in average of duplicates

Over the first 50 days of the biotic experiment that bicarbonate was used in the medium, each batch cycle was around 11 to 19 days due to the low rate of bicarbonate consumption measured by TOC. Concentration of inorganic carbon was around 357 ppm at the beginning of each batch cycle and did not decrease to less than 150 ppm at the end of the cycle. This low consumption rate led to a low production rate of acetate which was the sole product during the 100 days of test experiment. Figure 5.6 demonstrates the concentration of acetate in cathodic compartments of BESs during the experiment.

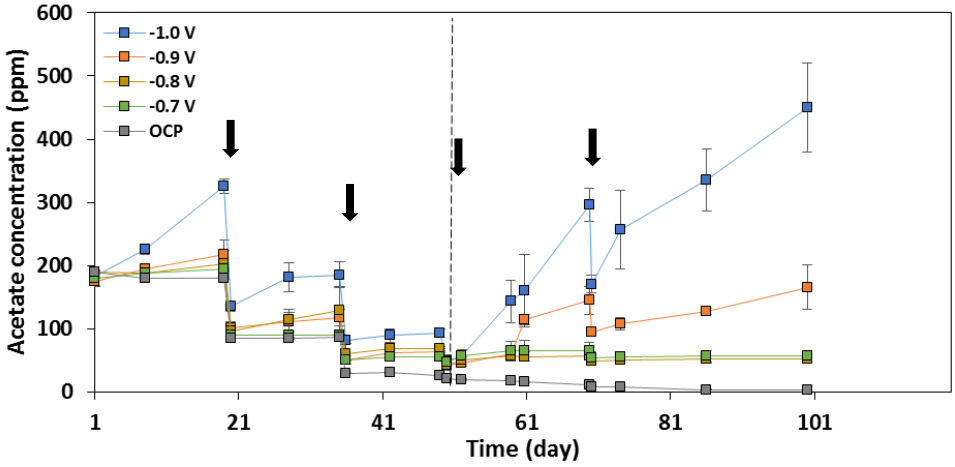


Figure 5.6. Concentration of acetate produced through MES in the BESs poised at -1.0, -0.9, -0.8 and -0.7 V.

Due to more considerable acetate production rate in BESs poised at -1.0 and -0.9 V, acetate production rate was calculated for these reactors (Figures C2 (a) and (b), Appendix C). As shown in Figure 5.6, the most significant increase in acetate concentration was observed in BESs -1.0 V, which was highest over the first batch from 182.2 ± 3.0 ppm (6.1 ± 0.1 mmole C L^{-1}) at day 1 to 325.3 ± 11.1 ppm (10.8 ± 0.0 mmole C L^{-1}) at day 19 (production rate of around 7.5 ppm day^{-1} (0.25 mmole C L^{-1} day^{-1}). However, this trend decreased over the next two batch cycles. The acetate production rate declined to 3.1 and 0.8 ppm day^{-1} over the second and third batch cycles, respectively. This reduction was associated with increase in catholyte pH. Although the medium pH was adjusted between 6.7 and 7.0 after purging N_2/CO_2 (80/20) at the beginning of each batch cycle, it was increasing to around 8.0 after almost two days. This could be due to the higher rate of protons reduction for H_2 evolution than production through MES by bacterial community. Negligible consumption of bicarbonate by bacteria and therefore, low production of acidic products caused the accumulation of bicarbonate in the solution and increase in pH over the batch cycles. In BESs poised at -0.8 and -0.7 V, pH was also increasing to almost 7.8 – 8.0 at the end of each batch cycle, with negligible production of acetate detected.

After switching the inorganic carbon source type from bicarbonate to dissolved CO_2, pH of the catholytes decreased and remained almost stable around 6.0 due to the equilibrium of CO_2 in solution. This was also associated with increase in acetate production at the BESs applied at -1.0 and -0.9 V. Acetate concentration, the sole product in the catholyte, reached 295.4 ± 26.2 ppm (9.8 ± 0.8 mmole C L^{-1}) and 144.5 ± 21.9 ppm (4.8 ± 0.7 mmole C L^{-1}) at day 70 in BESs -1.0 V and BESs -0.9 V, respectively. This also increased after changing the medium and reached the maximum concentration of 450.0 ± 70.7 ppm (15.0 ± 2.3 mmole C L^{-1}) at BESs -1.0 V and 165.0 ± 35.3 ppm (5.5 ± 1.2 mmole C L^{-1}) at BESs -0.9 V. Highest acetate production rate was achieved at BESs -1.0 V which was 22.1 ± 8.0 ppm day^{-1} (0.7 ± 0.0 mmole C L^{-1} day^{-1}) on day 74. It is worth noting that almost no hydrogen was detected in the headspaces of any BESs over the 100 days of test experiment, which could show its consumption through MES to produce acetate. More significant acetate production in BESs -1.0 V and BESs -0.9 V while supplying CO_2 than that when providing bicarbonate could be due to either favourability of low pH for bacterial activities provided by bubbling CO_2 or greater feasibility of consumption of dissolved CO_2 by bacteria. Despite the production of acetate at BESs -1.0 and -0.9 V, acetate production was almost negligible at the BESs of -0.7 and -0.8 V over the 100 days of experiment, regardless of inorganic carbon source types. While replacing bicarbonate in the catholyte by CO_2 improved acetate production in BESs -1.0 and -0.9 V, no improvement was observed in BESs -0.7 and -0.8 V after changing the type of inorganic carbon source. This indicated that

applied potentials of -0.7 and -0.8 V at the cathodes did not provide sufficient energy for reduction of CO_2 through MES processes. Inferior production in BESs poised at -0.7 and -0.8 V was more likely related to the lack of H_2 production observed in Section 5.3.2. In addition, no production was observed at the OCP control experiment, indicating the key role of cathode in providing the energy source for bacterial activity in MES processes.

To investigate the impact of cathodic applied potentials and inorganic carbon source types on providing the appropriate conditions for bacterial activities and growth, cell count was performed during the 100 days of experiment (Figure 5.7). As can be observed, number of the cells in the first sample was 10^7 cells ml^{-1}, which decreased in all the BESs over the first 50 days of experiment when bicarbonate was fed in the catholyte. This decrease was more significant in BESs poised at -0.7 and -0.8 V, indicating that not only type of inorganic carbon source in the system but also the cathodic potential was not enough to support the growth of bacterial cells. With CO_2 as the inorganic carbon source, cell numbers in BESs poised at -1.0 and -0.9 V began to increase which reached to more than 10^7 cells ml^{-1} in the BESs poised at -1.0 V. Over the 100 days of experiment, the highest number of cells was around $1.6 \times 10^7 \pm 1.0 \times 10^6$ cells ml^{-1} measured in BESs -1.0 V after changing bicarbonate to CO_2. This indicated that -1.0 V and CO_2 provided the best condition in terms of energy and inorganic carbon sources, respectively for bacterial metabolisms and growth.

The cell numbers in the BESs -0.8 and -0.7 V were less than 6 cells ml^{-1}, one magnitude lower. Lack of enough energy for the bacterial activities and MES reactions at these potentials could be concluded for the reactors at -0.8 V and -0.7 V. Therefore, bacterial cells were not able to uptake enough electrons from the electrode leading to low cathodic current consumption, production of acetate and biomass. As a result, bacterial community were more likely washed out from the cathodic compartment by each medium change. Similarly, no cell growth was observed in the OCP condition, regardless of the inorganic carbon sources, indicating the key role of cathode potential as an energy source for bacteria for MES.

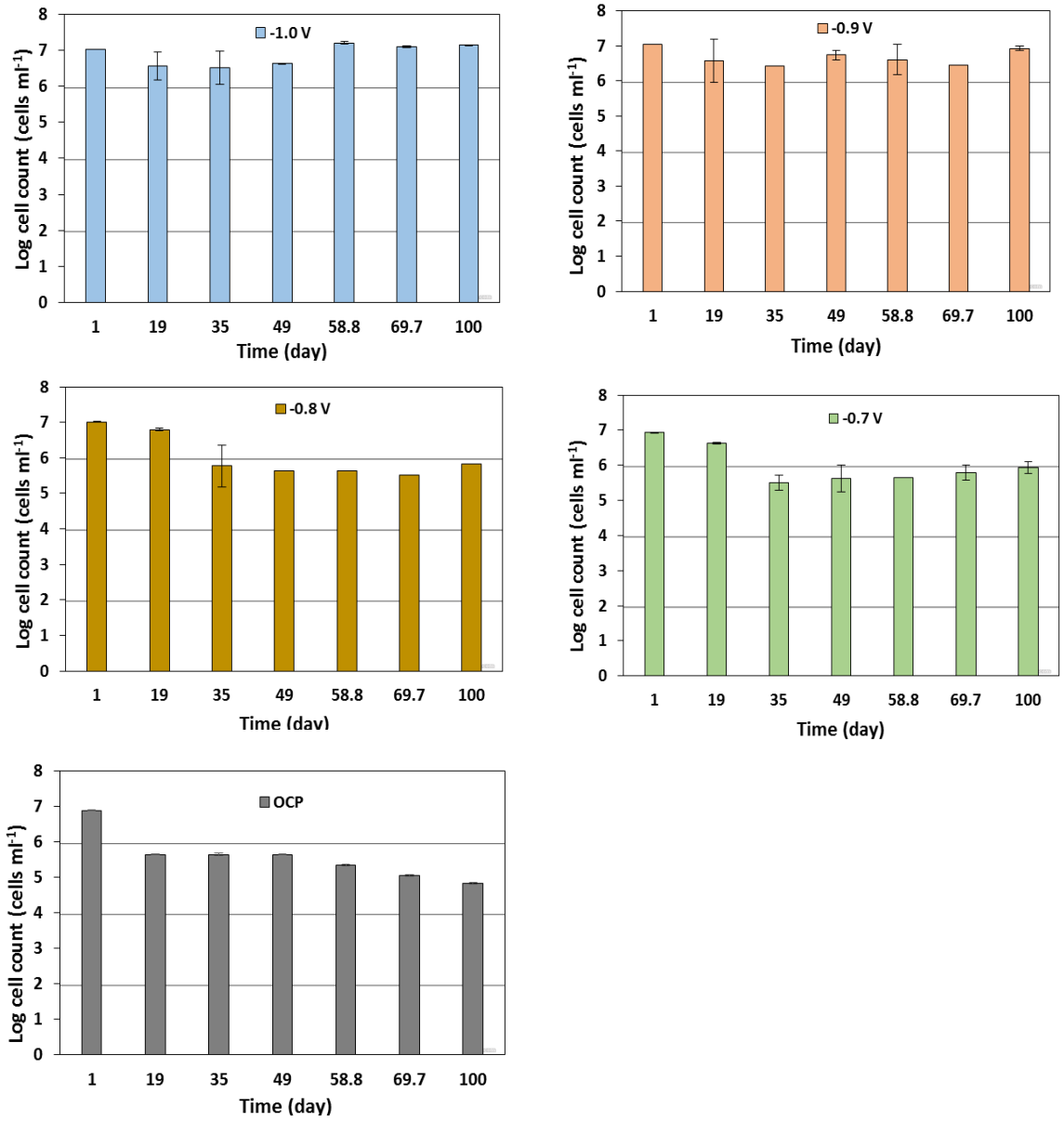


Figure 5.7. Number of the bacterial cells in logarithmic scale during the first round of experiment in the catholytes of the BESs poised at -1.0, -0.9, -0.8, -0.7 V and OCP.

5.3.4 *Electrochemical properties of the biocathodes developed through MES*

To gain better understanding of the electrochemical properties of the biocathode developed in the BESs during 100 days of experiment, CV was performed from all the BESs after 50 days of using bicarbonate and later on day 100, 50 days after using CO_2. pH of the catholytes was adjusted manually to around 6.5 in all the BESs before performing CV to provide the similar condition in all the reactors. Figure 5.8 demonstrates the voltammograms from the biocathode developed in the cathodic compartments with that monitored from the plain electrode before

inoculating the system. As demonstrated in Figure 5.8, no noticeable change was observed in the voltammograms of BESs -0.7 V during the 100 days of experiment, indicating no active redox components were enriched during the experiment. Voltammograms of BESs -0.8 V revealed the small shift in onset potential of H_2 evolution from approximately -1.0 to -0.97 V. More significant changes in CV figures were observed in BESs -0.9 V and, particularly BESs -1.0 V. In BESs -0.9 V, voltammograms of day 50 were very similar to that of plain electrode; however, H_2 evolution potential shifted from around -1.0 to -0.9 V at the end of experiment, showing that replacing bicarbonate by CO_2 improved the system in terms of biotic H_2 production. The most significant change in voltammogrmas appeared in the BESs -1.0 V. After first 50 days of the experiment, significant redox reaction appeared in the voltammogram of the BESs poised at -1.0 V between -0.18 and -0.6 V. However, after 50 days of changing the inorganic carbon type to CO_2, when BESs reached the best condition in terms of production, redox reactions in the voltammograms changed to 2 small oxidation peaks at -0.18 and -0.65 V, and onset potential of H_2 evolution shifted from -1.0 to -0.86 V. Significant difference in shapes of CVs when bicarbonate and CO_2 was used was not very clear. Therefore, more investigations were carried out from the CV analysis and the oxidation peaks appeared in the voltammograms recorded from the developed biocathode, reported at the end of this chapter in Section 5.3.8.

Alongside the evident shift in onset potential of H_2 evolution, the considerable increase in the cathodic current was observed from the potential scanned from -1.0 to 1.2 V. CVs present that the current achieved in BESs -1.0 V at the potential of -1.2 V, the lower vertex potential, increased from -0.77 mA cm^{-2} at the plain electrode to -1.8 mA cm^{-2} on day 50, and to -2.62 mA cm^{-2} on day 100, showing 2.33 and 3.40 times increase in the cathodic currents, respectively compared to that in the plain electrode. This increase in the cathodic current was also observed in the BESs -0.9 V to -2.65 mA cm^{-2} and BESs -0.8 V to -1.8 mA cm^{-2} only when using CO_2 as an inorganic carbon source. This increase indicates the involvement of biocatalysts in electron transfer from the cathode, which can be due to either involvement of bacterial enzymes in production of H_2 as suggested before (Deutzmann et al., 2015) or higher rate of removal of H_2 at the surface of the electrode by microorganisms.

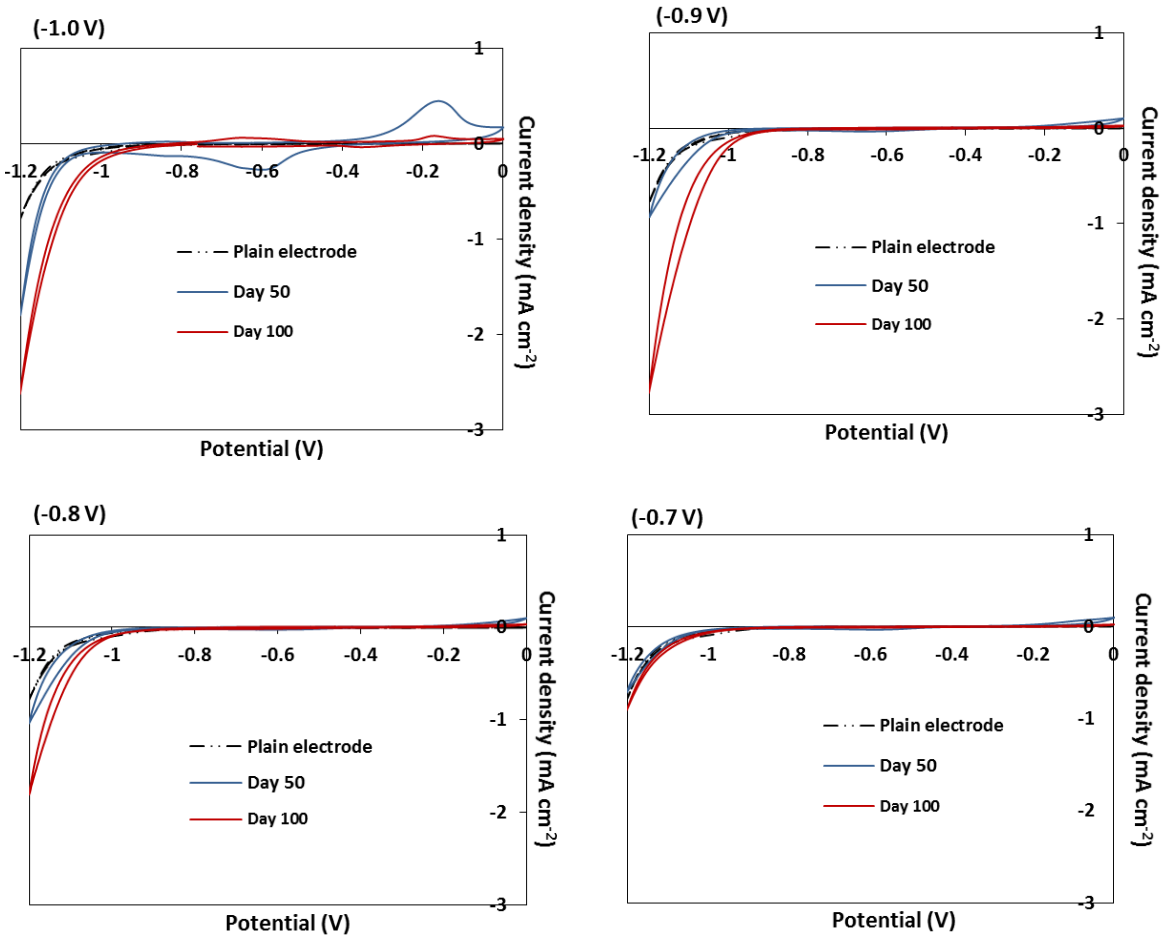


Figure 5.8. Cyclic voltammograms recorded from the cathodes in the BESs poised at -1.0, -0.9, -0.8 and -0.7 V, before, 50 days and 100 days after the biotic experiment.

The results extracted from CVs were in agreement with the results of cells growth and production. It was observed that cathode potential and inorganic carbon source had direct effects on the biocathode development and MES reactions. In addition, it seemed that operational low pH provided by supplying CO_2 played a potential role in enhancing the bacterial growth and MES processes. Therefore, the observations from 100 days of the test experiment assisted to design the further systematic experiment to answer the main research questions of this chapter related to biofilm formation during MES processes.

5.3.5 *Effect of cathodic potential on MES*

The main target of this section was to gain better understanding of electron transfer between the electrode and microorganisms on the cathode, whether biofilm forms at the cathode during

MES processes and its responsibility, investigating the imperative parameters affecting the formation of biofilm and whether MES occurs in the suspension or at the electrode. Hence, the effect of different applied potentials of -0.8 and -1.0 V on MES and biofilm development were investigated using CO_2 (concluded from the test experiment). Additionally, the effect of different inorganic carbon source types of CO_2 and HCO_3^- on MES and biofilm development were investigated at the best cathodic applied potential of -1.0 V (concluded from the test experiment).

Three different reactors in duplicate and a biotic control experiment under OCP condition were started and inoculated by the catholyte of the reactors poised at -1.0 V from the previous experiment. Current consumption in all the BESs of this study, more significantly in BESs 1 (-1.0 V_CO_2), started immediately after inoculating the cells. Figure 5.9 shows the maximum current density in each batch cycle. Cathodic current consumption at BESs 1 began at -0.9 ± 0.2 mA cm^{-2} and stayed almost stable over the first 20 days. After day 20, electron uptake began to increase and reached the maximum of -1.8 ± 0.3 mA cm^{-2} on day 60. However, current uptake was much lower in BESs 2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2) during the experiment. The maximum cathodic current was -0.2 ± 0.0 mA cm^{-2} in BESs 2 and -0.03 ± 0.0 mA cm^{-2} in BESs 3.

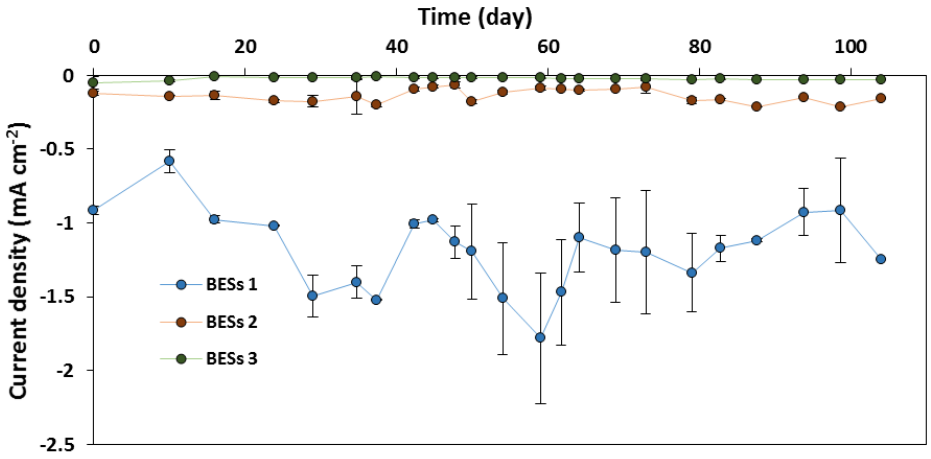
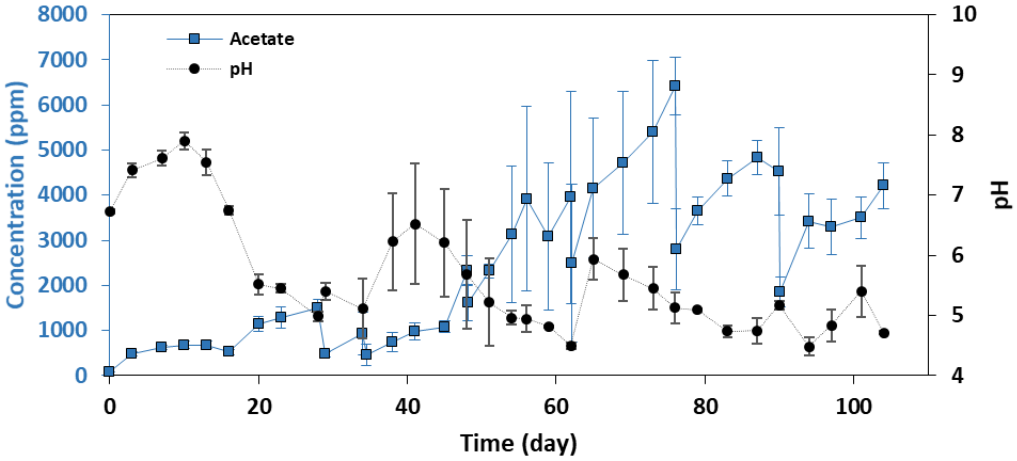


Figure 5.9. Cathodic current consumption of BESs 1 (-1.0 V_CO_2), BESs 2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2) during the experiment.

Due to the higher electron uptake from the cathodes in BESs 1 compared to other BESs, it was expected to observe higher production through MES. Figure 5.10 presents acetate production and catholytes pH in BESs 1 and BESs 3 throughout the experiment. As illustrated in Figure

5.10 (a) acetate was the only product in BESs 1 over the first 20 days of the experiment, with the maximum concentration of 1145.2 ± 158.1 ppm (38.2 ± 5.3 mmole C L^{-1}) and maximum production rate of 155.3 ± 23.9 ppm day^{-1} (5.2 ± 0.8 mmole C L^{-1} day^{-1}) (Figure C3 in Appendix C) both reached at day 20. Although pH of the catholyte was adjusted at 6.5 after purging CO_2, it increased to 7.0 – 7.5 during the first 20 days of the experiment, which could be due to proton reduction and hydrogen production at the cathode. However, the absence of hydrogen in the headspace would suggest its consumption by CO_2 reducers through indirect electron transfer (IET) mechanism. After day 20, a slight increase in the concentration of acetate was observed in BESs 1, before increasing more significantly after day 40. The increase of acetate concentration was associated with drop in the solution pH due to increase of acidic products in the catholyte. A significant surge in production of acetate was observed in BESs 1 after day 45, reaching the highest concentration of 6500.4 ± 633.6 ppm (216.7 ± 21.1 mmole C L^{-1}) at day 74 (and highest production rate of 660.7 ± 101.1 ppm day^{-1} (22.1 ± 3.4 mmole C L^{-1} day^{-1}) at day 54. This surge in acetate production 45 days after the BESs operation could be consistent with the development of biofilm at the cathode, as cathodic biofilms require longer time to enrich and develop compared to anodic biofilms (Milner et al., 2016, Jourdin et al., 2015a, Zaybak et al., 2013).

(a)



(b)

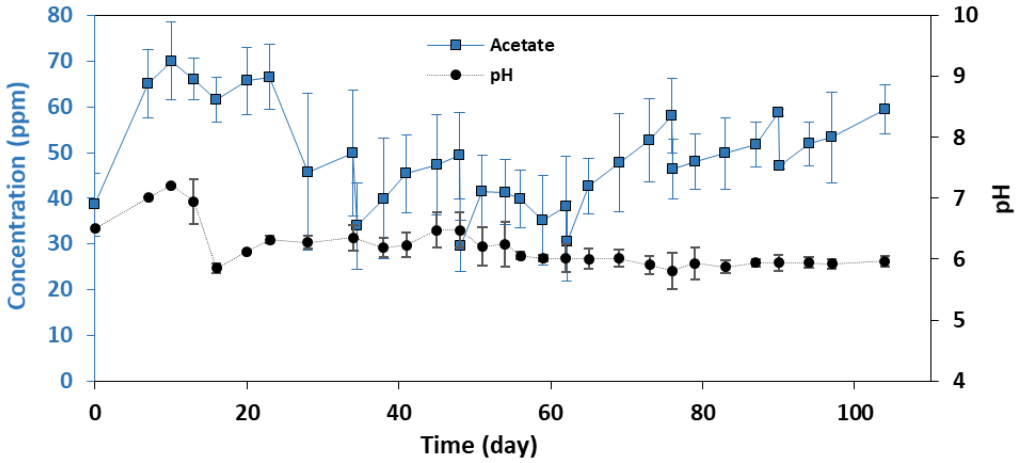


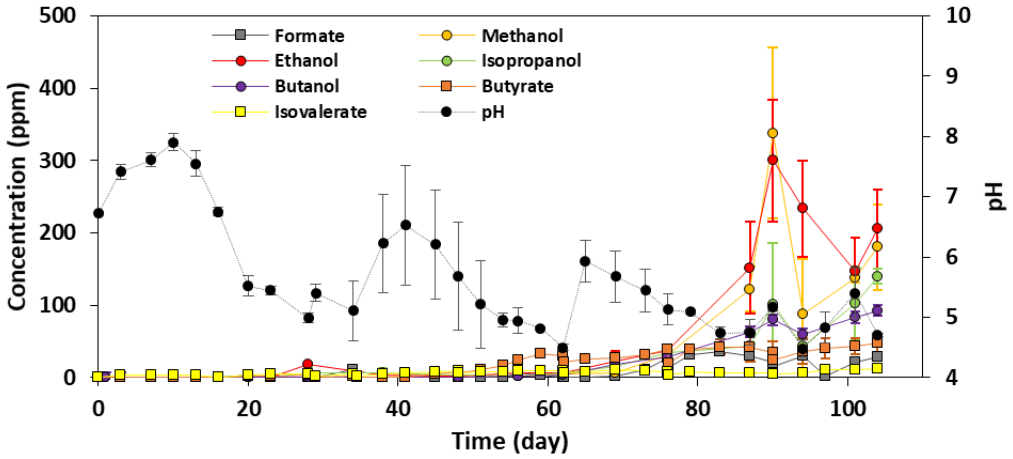
Figure 5.10. Concentration of acetate produced in (a) BESs 1 (-1.0 V_CO_2) and (b) BESs 3 (-0.8 V_CO_2) during 104 days of the experiment through microbial electrosynthesis.

To gain the better understanding regarding the effect of applied potential on MES processes, two potentials of -0.8 V and -1.0 V were compared. Figure 5.10 (b) illustrates pH and acetate concentration in BESs 3. During the experiment, acetate concentration was not notable in BESs 3. Maximum concentration of acetate was achieved on day 10 (70.6 ± 8.2 ppm, 2.6 ± 0.3 mmole C L^{-1}), with the maximum production rate on day 65 (5.2 ± 1.1 ppm day^{-1}, 0.2 ± 0.0 mmole C L^{-1} day^{-1}), as shown in Figure C4 in Appendix C. Comparing BESs 1 and BESs 3, acetate production was almost negligible in BESs 3, although pH of the catholyte was around 6.0 in both systems. This fact indicated that -0.8 V was not enough for H_2 production or direct electron uptake by bacteria and consume them for the reduction of CO_2. In BESs of biotic control experiment under OCP condition, concentration of initial acetate in the inoculum decreased linearly after changing the medium during the experiment, reaching a negligible value after 20 days as no production was observed. This fact strongly confirmed the role of cathode as a source of energy for bacterial community in MES processes.

Although acetate was the dominant product over 104 days of the experiment, other products were detected after day 58 in BESs 1 as shown in Figure 5.11 (a). The production of more diverse products such as butyrate and iso valerate after day 58 was associated with the decrease in the concentration of acetate and its production rate, suggesting chain elongation through acetate. Respective butyrate and isovalerate concentrations of 47.2 ± 10.1 (2.2 ± 0.4 mmole C L^{-1}) ppm and 12.3 ± 4.0 ppm (0.6 ± 0.0 mmole C L^{-1}) were detected at day 104. It is understood that the WL pathway plays a key role in the production of acetate in MES by fixing CO_2 (Tremblay and Zhang, 2015). Production of VFAs from CO_2 by acetogens through this pathway,

also known as acetogenesis, can be carried out under anaerobic condition, by synthesizing acetyl-CoA from CO_2 reduction and conserving energy (Ragsdale and Pierce, 2008). The production of butyrate can occur either via linear extension of acetyl-CoA to butyril-CoA, or via reverse β-oxidation known as microbial chain elongation (Arends et al., 2017).

(a)



(b)

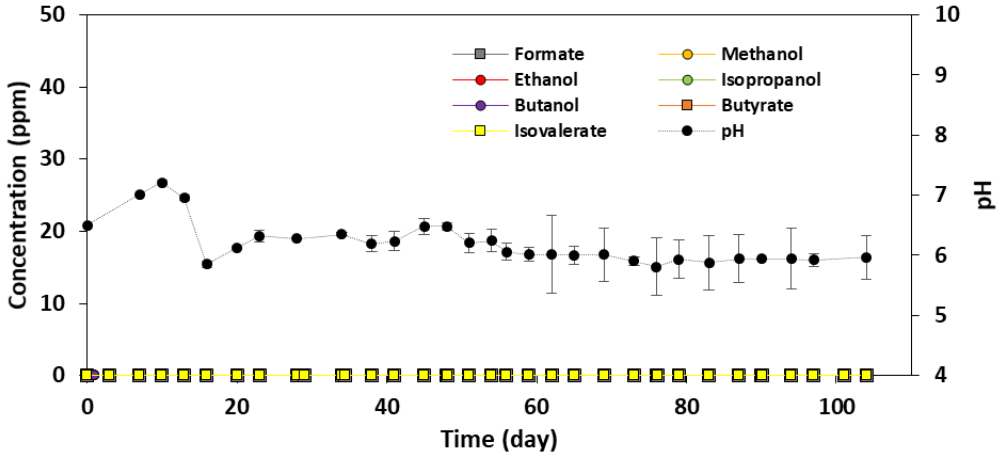


Figure 5.11. Concentration of organic acids and alcohols produced in (a) BESs 1 (-1.0 V_CO_2) and (b) BESs 3 (-0.8 V_CO_2) alongside acetate during 104 days of the experiment through microbial electrosynthesis.

Alongside of production of VFAs, alcohols production was observed in BESs 1 after almost 60 days of the experiment. Ethanol, iso-propanol and butanol were also produced after day 60, with maximum concentrations of 300.2 ± 84.4 ppm (13.0 ± 2.8 mmole C L^{-1}) at day 90, 103.0 ± 50.0 ppm (5.1 ± 1.6 mmole C L^{-1}) at day 101 and 92.3 ± 2.0 ppm (4.9 ± 0.0 mmole C L^{-1}) at day 104, respectively. The production of alcohols in the catholyte is consistent with the drop of pH measured due to the accumulation of acidic products during the same period. It was indeed

reported that decrease in pH leads to a toxic environment for microorganisms, thus switching metabolism from acetogenesis to solventogenesis as a defensive mechanism, leading to production of solvents such as alcohols from acetate (Zigova and Šturdík, 2000). During solventogenesis also called as ”acid crash” in acetogens such as bacteria belonging to class of *Clostridia*, products are re-assimilated to their corresponding alcohols such as ethanol or butanol (Vassilev et al., 2018, Ramió-Pujol et al., 2015). From a thermodynamic point of view, conversion of organic acids to their corresponding alcohols requires proton translocation at specific sites of the pathways for gaining energy, which can be provided over the low pH condition (González-Cabaleiro et al., 2013). Ethanol and butanol are recognised as the main products of the solventogenesis metabolism due to low pH condition (Lee et al., 2010). Besides, notable concentration of isopropanol was detected due to possible solventogenesis, while negligible concentration of acetone was detected. This was in agreement with a previous study published by Arends et al. (Arends et al., 2017). In their study, Arends et al. suggested that the production of acetone can occur during an acetone-butanol-ethanol (ABE) fermentation, and acetone can be further reduced to isopropanol during an isopropanol-butanol-ethanol (IBE) fermentation. It can therefore be assumed that although acetone may have been produced, it was further reduced to isopropanol, thus explaining that isopropanol was only detected in trace amounts (Arends et al., 2017). The diversity of products in our study could be due to the diversity of bacterial community enriched from mixed culture of microorganisms. Notably, ethanol concentration was not stable during the experiment indicating its consumption. It was previously reported that H_2 does not provide sufficient energy for chain elongation and other electron donors such as ethanol or lactate are required as intermediates through the reactions (González-Cabaleiro et al., 2013). It has been suggested that production of long chain VFAs through acetate using H_2 as an electron donor is not thermodynamically favourable. However, this was shown feasible when ethanol was present as an electron donor (González-Cabaleiro et al., 2013). The observation from the present research is also in agreement with a previous study on acetate production in MES which reported that ethanol and butyrate production began significantly when the accumulated acetate concentration reached more than 1500 ppm and the pH of the catholyte was slightly acidic (Bajracharya et al., 2017c). In BESs 3, however, no other organic acids than acetate or alcohols were detected as observed in Figure 5.11 (b). The potential applied at the cathode derives the energy required for MES processes considered as an important operational factor affecting MES. In theory, reduction of CO_2 to acetate (Equation 2.11) requires cathodic potential of -490 mV (25 °C, pH 7). However, the significant difference between BESs 1 and BESs 3 showed that more negative cathodic potential could overcome the potential losses in BESs and promote production from CO_2 reduction.

Due to the high production in BESs 1 during the experiment, it could be concluded that a bacterial community responsible for the cathodic CO_2 reduction were enriched. As acetate was the major product throughout the experiment, most electrons of the cathode were consumed for the MES of acetate from CO_2. Therefore, columbic efficiency of the system was calculated considering the production of acetate for almost every 20 days of experiment demonstrated in Figure 5.12. Columbic efficiency over the first 20 days of experiment was 51.5 ± 19.4 %. It was even lower from days 20 to 41 (30.4 ± 4.7 %). However, after day 41 that acetate production was more significant, columbic efficiency increased to around 69 % between days 40 and 63 and remained almost stable until the end of experiment. The rest of electrons could be consumed for the production of H_2, which may be trapped in the liquid or leaked from the reactors, or production of other products (butyrate, isovalerate and alcohols); however, their concentrations were almost negligible compared to acetate concentration. Biomass production can be a further possibility for the consumption of electrons from the cathodes. The average coulombic efficiency of 63% for the last 60 days of experiment for the production of acetate showed the successful consumption of electrons derived from cathodes through MES processes.

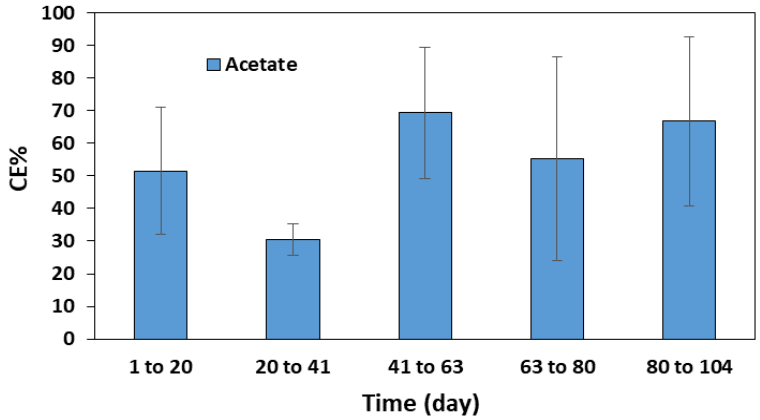


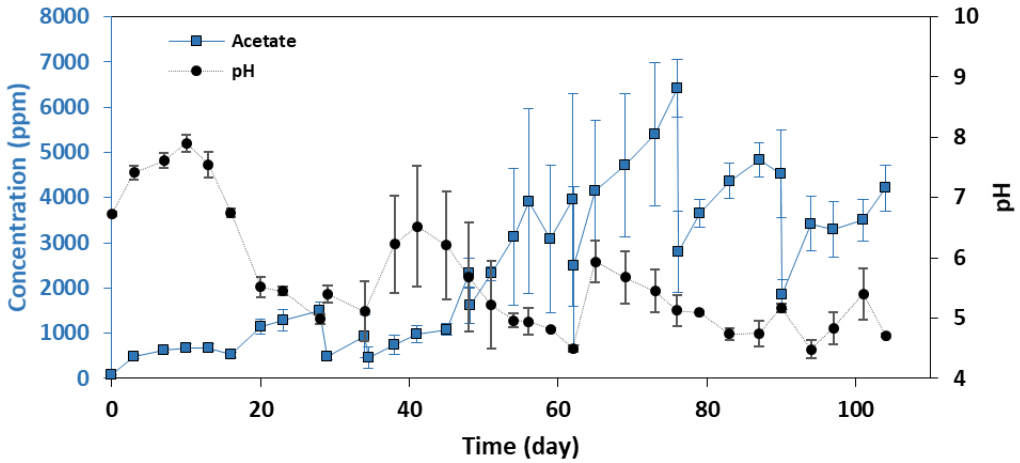
Figure 5.12. Columbic efficiency of acetate production in BESs 1 (-1.0 V_CO_2) during 104 days of the experiment.

5.3.6 *Effect of inorganic carbon source on MES*

In order to compare the impact of different types of inorganic carbon source on production through MES, the performances of BESs 1 and BES 2 were compared. In previous studies on MES, CO_2, bicarbonate or both forms of inorganic carbon were used in cathodic medium

depending on experimental designs (Bajracharya et al., 2015, Gildemyn et al., 2015, Bajracharya et al., 2016b). Bicarbonate is a liquid form of CO_2 dissolved in solution. One of the main differences between these two forms is pH of solution with CO_2 or bicarbonate, due to their equilibrium in the liquid phase. Thus far, the effect of inorganic carbon source on the performance of BES has not been studied systematically. In this direction, the ability of bacteria in utilization of these two inorganic carbon sources at the same applied potential was studied in this work.

(a)



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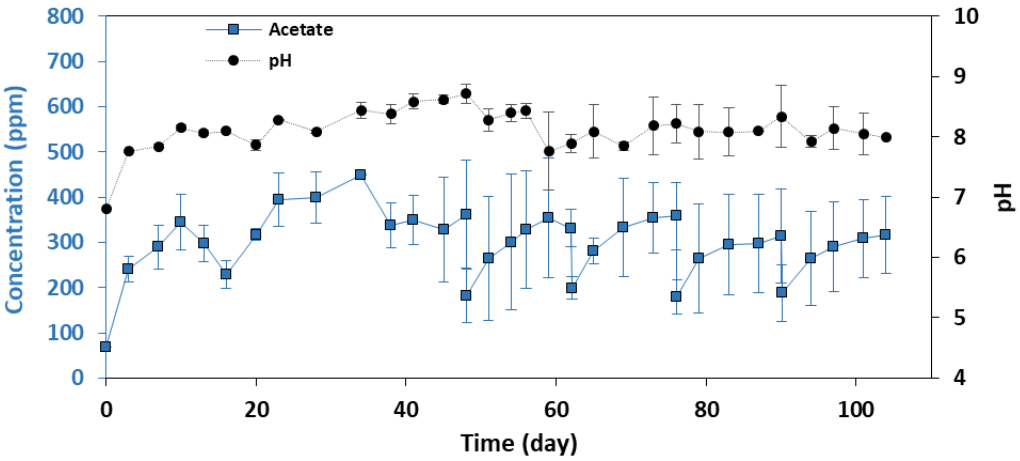
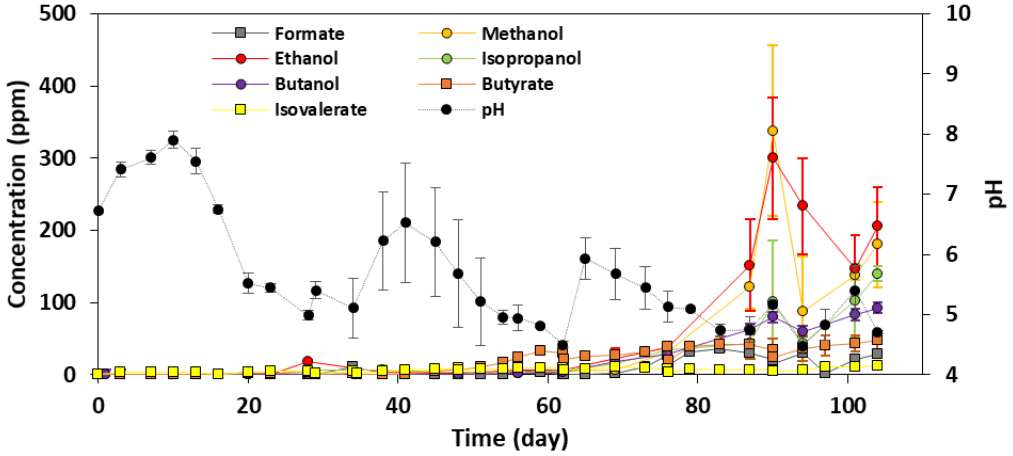


Figure 5.13. Concentration of acetate produced in (a) BESs 1 (-1.0 V_CO_2) and (b) BESs 2 (-1.0 V_$NaHCO_3$) during 104 days of the experiment through microbial electrosynthesis.

(a)



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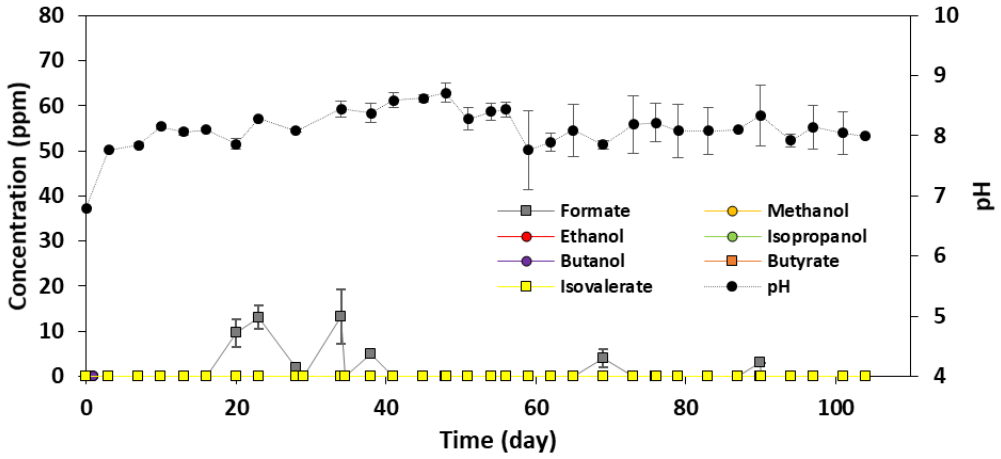


Figure 5.14. Concentration of organic acids and alcohols produced in (a) BESs 1 (-1.0 V_CO_2) and (b) BESs 2 (-1.0 V_$NaHCO_3$) alongside acetate during 104 days of the experiment through microbial electrosynthesis.

As can be observed in Figure 5.13 (b), acetate concentration in BESs 2 was much lower than that in BESs 1 over 104 days of the experiment, with maximum acetate concentration of 450.9 ± 1.3 ppm (15.0 ± 0.0 mmole C L^{-1}) on day 34 and maximum acetate production rate of 58.2 ± 9.4 ppm day^{-1} (1.9 ± 0.3 mmole C L^{-1} day^{-1}) on day 103 (Figure C5 in Appendix C). The superior performance of BESs 1 compared to BESs 2 could be attributed to pH of the catholytes. Although pH of the catholytes were adjusted to around 6.8-7.0 for every medium change, they were rapidly increasing in BESs 2 to between 7.5 and 8.5 after just a day following the medium change. In contrast to the significant pH drop in BESs 1 (due to accumulation of acetate in the solutions), pH remained almost stable between 7.5 and 8.5 in the catholytes of BESs 2, as no significant acetate production was observed when bicarbonate was the sole inorganic carbon in

the catholyte. Small percentage of H_2 (~ 5 %) in the headspaces of these reactors showed that H_2 was not consumed through MES by bacterial community, justifying the higher pH in BESs 2 due to protons reduction. Low concentration of acetate and high pH in the catholytes of BESs 2 led to lack of production of longer chain of organic acids and alcohols as observed in Figure 5.14 (b). Comparing the production at two different types of inorganic carbon provided, results show that production through MES could be correlated to pH of the catholytes indicating the availability of protons in the solutions. Lower pH and higher possibility of hydrogen production in the BESs fed by gaseous CO_2 could be presumed as the important parameter resulting significant production in these BESs. Also, it is known that optimal pH for acetogens is slightly acidic between 5.0 and 6.0 (Drake et al., 2006, Batlle-Vilanova et al., 2016). Additionally, during 104 days of the experiment TOC was measured periodically to examine the organics equivalent to the products. It was observed that concentration of total inorganic carbon in BESs 1 was decreasing over each batch cycle (3-5 days); however, decrease in total inorganic carbon in BESs 2 was negligible. This raised the question concerning the feasibility of consumption of different types of inorganic carbon by CO_2 reducers. In a study on acetogenic pure culture of *Clustridium Kluyveri*, CO_2 was known as a vital nutrient on its protein synthesis (Tomlinson and Barker, 1954). It was suggested that using CO_2 as an inorganic carbon source may lead to faster proceed of the protein synthesis than using HCO_3^- (Grootscholten et al., 2013c). Using HCO_3^- in their study, it was shown that at higher pH than 7.0, almost all the inorganic carbon source was presented as bicarbonate, showing the low bicarbonate consumption and subsequently low production. Whereas, production enhanced at the pH around 6.0, as around 50% of added bicarbonate presented as CO_2 (Grootscholten et al., 2013c). Similar trend was observed in this study.

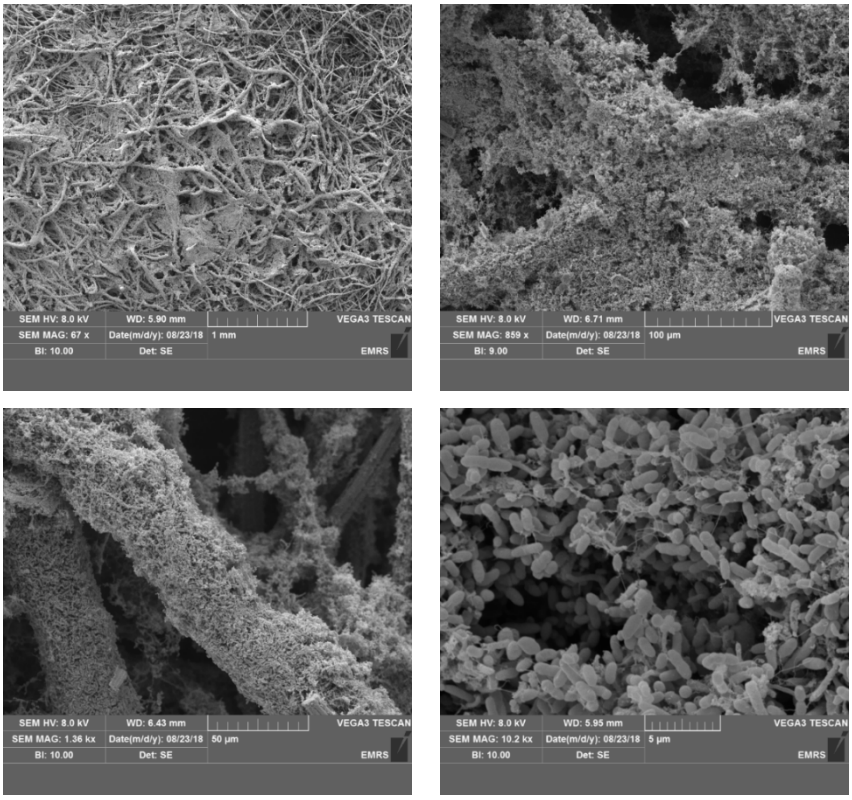
Despite the advantages associated with the utilisation of a mixed microbial community such as diversity of products, robustness and synergetic effects, it is also usually concomitant with the competition between acetogens and hyrogenotrophic/acetocalstic methanogens (Molenaar et al., 2017, Jadhav et al., 2018). For the production of VFAs and alcohols from CO_2 through MES, acetogens are the preferred community, especially in comparison to the production of methane via methanogens. Several methods have been suggested to inhibit the activity of methanogens such as utilisation of chemicals, temperature control, inoculum heat pre-treatment and low pH operation (Kadier et al., 2017). In this experiment, although the activated sludge used as inoculum was pre-heated and no methane was detected during 21 days of pre-treatment and 100 days of test experiment steps, 2-4% methane concentration was detected in the headspaces of BESs 1 and 5% in BESs 2 over the first 30 days of experiment. Therefore, sodium 2-

bromoethanesulfonate (5 mM) was added as methane inhibitor in the medium of all the BESs on day 30. No methane was detected in the headspaces of BESs from day 30 until the end of experiment. In addition, after the significant drop of pH from 6.5 to around 5.0 in BESs 1, sodium 2-bromoethanesulfonate was removed from the medium in these reactors from day 48 until the end of experiment. No methane production in such condition in BESs 1 confirmed the low pH condition as a proper method in suppression of methanogens.

5.3.7 *Effect of cathodic potential and inorganic carbon source on physical properties of biofilms*

In order to visualize and compare the biofilm formed at the electrodes over three different treatments, SEM was performed from the projected surface of the electrodes at different BESs (Figure 5.15). As demonstrated in Figure 5.15 (a), complete coverage of the graphite felt by biofilm was observed at the electrodes of BESs 1. This coverage could even be observed with naked eyes (Figure C6 in Appendix C). At some parts of the electrodes, the biofilm connected the small gaps between the graphite fibres, which referred as “filamentous biofilm” in the study by Jourdin et al. (Jourdin et al., 2018). A similar electrode coverage by biofilm was observed in their study with the cathodic potential of -1.05 V (Jourdin et al., 2018). In their study, both CO_2 and $NaHCO_3$ were used as inorganic carbon sources without specific feeding pattern, therefore the correlation between the nature of the carbon source and the presence of a biofilm could not be elucidated. According to the authors, the long-term and continuous mode of operation, in addition to microbial inoculum source played important roles in biofilm development. In the SEM images from the electrodes of BESs 2 that bicarbonate was fed, only coverage of individual graphite fibres by biofilm was observed. From the images of BESs 2 shown in Figure 5.15 (b), it can be evidently seen that not only the biofilm formed around the graphite fibres was not as dense as that in BESs 1, but also the gaps between the fibres were not covered by biofilm. In the SEM images of the electrodes of BESs 3 at different fields (Figure 5.15 (c)) almost no biofilm was observed at the graphite fibres, with few bacterial cells at different parts of the electrode, similar to those from the electrode under OCP condition (Figure 5.15 (d)) and plain electrode (Figure 5.15 (e)).

(a)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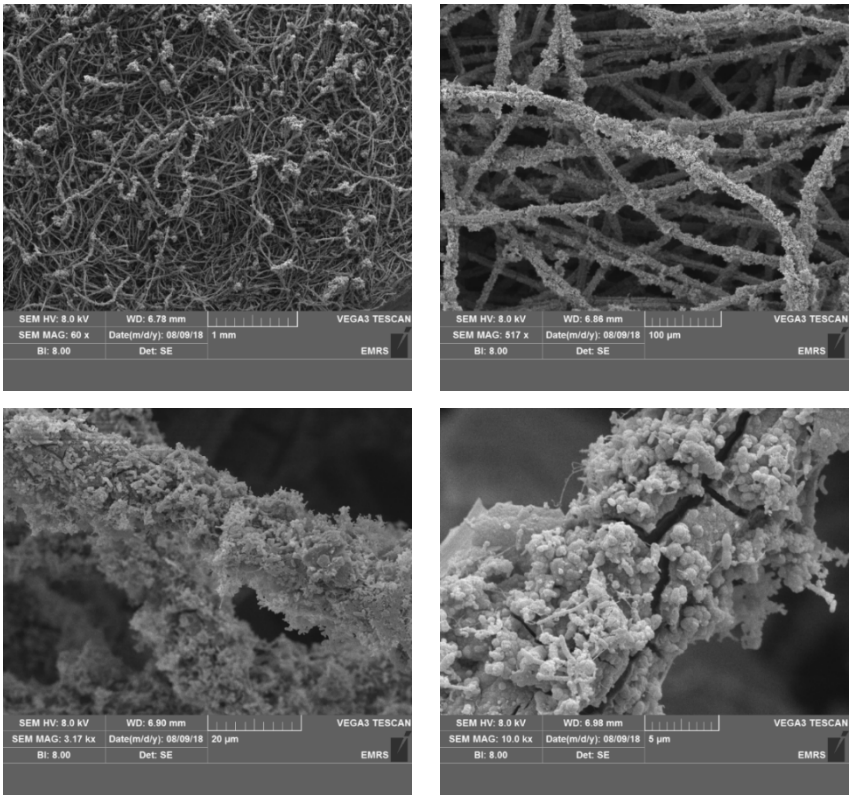
(b)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WD: 6.98 mm
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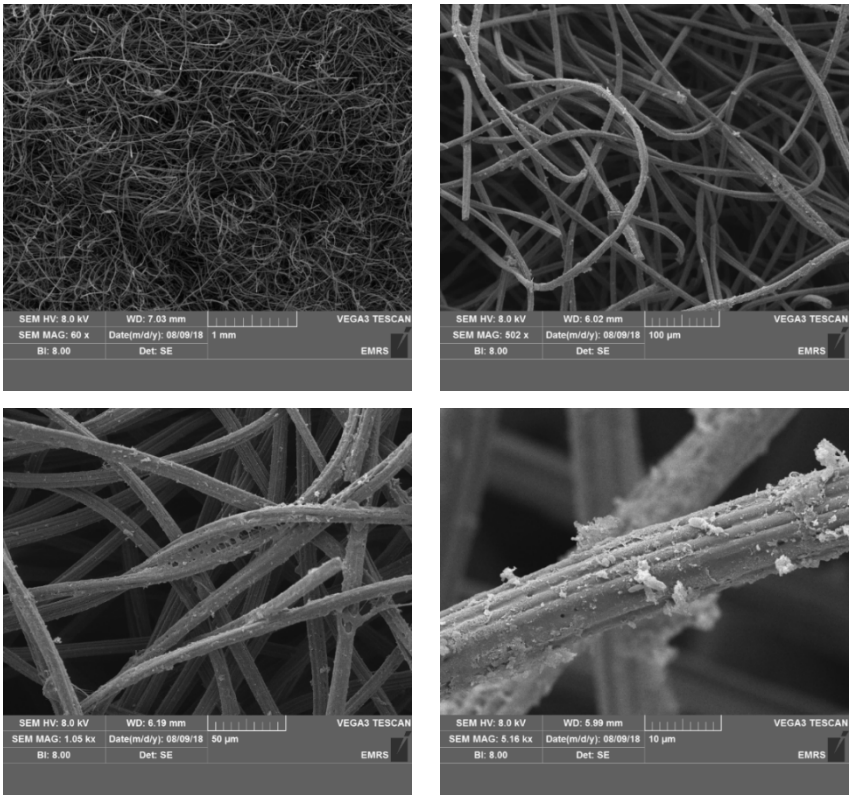
(c)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EMRS
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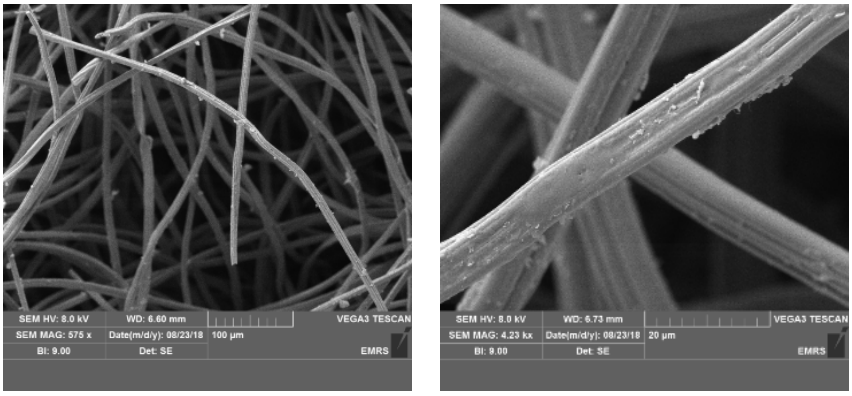
EMRS

(d)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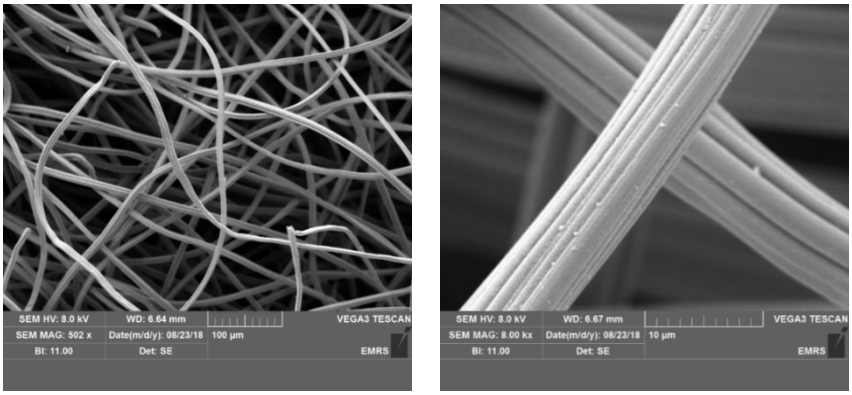
Det: SE
EMRS

(e)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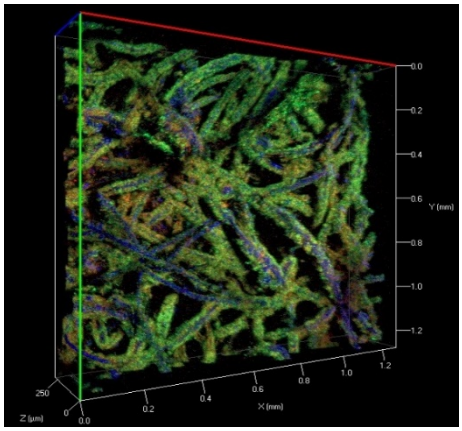
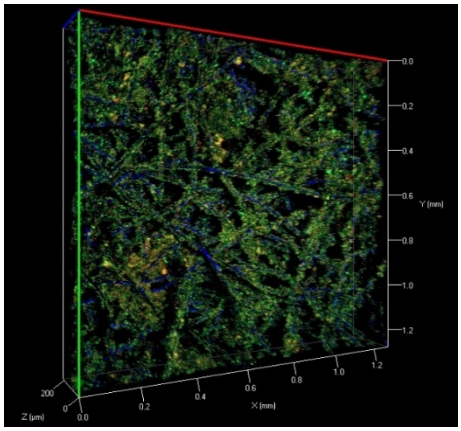
10 µm
BI: 11.00
Det: SE
EMRS

Figure 5.15. SEM images from the electrodes surface after 104 days of experiment in (a) BESs 1 (-1.0 V_CO_2), (b) BESs 2 (-1.0 V_$NaHCO_3$), (c) BESs 3 (-0.8 V_CO_2), (d) biotic control under OCP condition, and (e) plain electrode.

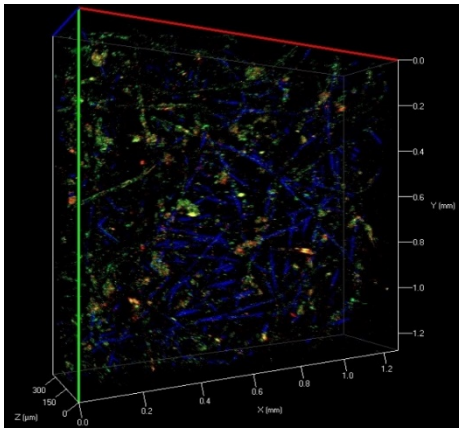
To obtain more detailed information about physical properties of the biofilms, confocal microscopy was performed from the electrode samples at each condition. Figure 5.16 demonstrates the 3D images of a plain graphite felt and the electrodes from all the conditions through confocal microscopy to study the correlation of cathodic biofilm formation at the surface of graphite felt and production through MES. Quantitative information was extracted from the 3D images of confocal microscopy and used subsequently to compare the biofilm coverage, dead and live cells at the cathodic electrodes. Percentage of live and dead cells at the cathodic biofilm is also demonstrated in Figure C7 in Appendix C. The highest coverage of graphite fibres was observed at the electrode of BESs 1. 18.9 ± 8.1 % and 2.0 ± 1.0 % of the cathode in BESs 1 was covered by live and dead cells respectively (total biofilm coverage of 19.1 ± 8.3 %) , while this coverage was much lower at the electrodes of BESs 2 with the 6.2 ± 2.3 % of live cells and 2.8 ± 1.4 % of dead cells (total biofilm coverage of 8.5 ± 3.9 %). This showed that better condition for bacterial growth and metabolism was provided when inorganic carbon source was provided in form of dissolved CO_2 in the solution. Comparing the 3D images and the quantitative information extracted from electrodes poised at -1.0 V and -0.8 V both fed by CO_2 (shown in Figures 5.16 (a), (c) and (f)), it can be seen that the percentage of biofilm coverage at the electrode poised at -0.8 V was similar to that under OCP condition (Figure 5.16 (d)) . In terms of bio-volume of cells at the electrodes of BESs 3, 1.1 ± 0.7 %, 0.7 ± 0.5 % and 0.3 ± 0.2 % of the cathodes were covered by the total biofilm, live cells and dead cells, respectively, which was much higher at the electrodes of BESs 1.

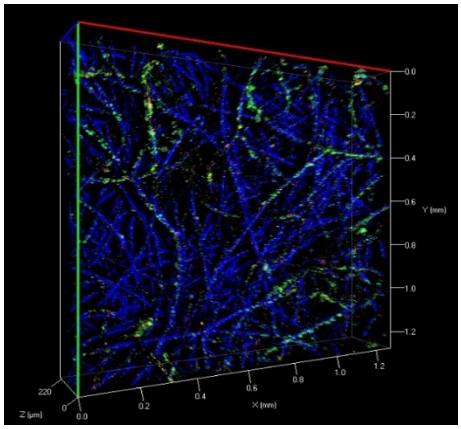
(a) (b)



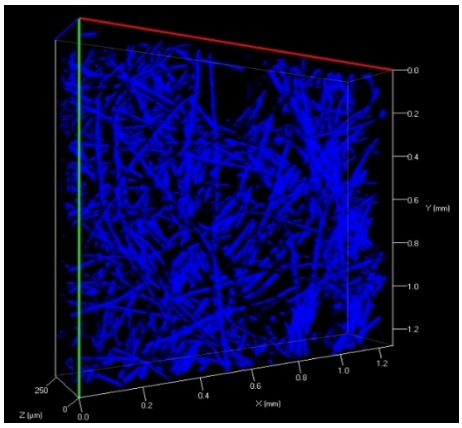


(c) (d)





(e) (f)



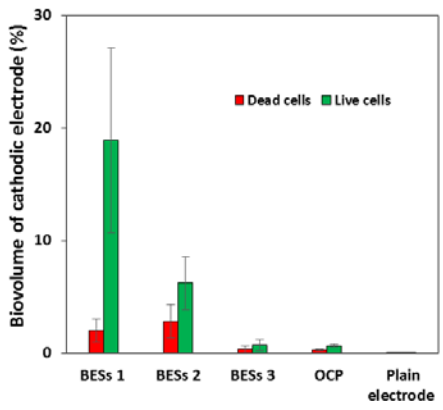


Figure 5.16. 3D images of the confocal microscopy from the electrodes surface after 104 days of experiment in (a) BESs 1 (-1.0 V_CO_2), (b) BESs 2 (-1.0 V_$NaHCO_3$), (c) BESs 3 (-0.8 V_CO_2),(d) biotic control experiment under OCP condition,(e) plain electrode, and (f) quantitative analysis of the 3D electrodes extracted from the images using Huygens software (Green, red and blue colours in the images indicate live cells in biofilm, dead cells in biofilm and graphite fibres, respectively).

Bio-imaging results pointed two important facts in this study. Firstly, it strongly showed that higher production in BESs was correlated with the density of the biofilm. Although not many studies focused on the characterisation of cathodic biofilm, direct correlation of thickness of anodic biofilm of *G. sulfurreducens* and electron production from acetate was reported previously (Reguera et al., 2006). In another study, the biofilm of *Sporomusa ovata* formed at the surface of the plain graphite felt and at the surface of modified hierarchical electrode were compared. It was shown that the thicker biofilm formed at the modified cathode contributed to 5 times higher acetate production rate (Cui et al., 2017). Similarly in this study, the thickest biofilm formed at the electrodes of BES 1 with the highest percentage of live cells was associated with the highest and most diverse production compared to other conditions, indicating the correlation between biofilm density and production through MES. In addition, regardless of inorganic carbon source, complete graphite fibres coverage in BES 1 and BES 2 showed the biofilm formation at the cathodes applied at -1.0 V, while no biofilm was observed at the cathodes applied at -0.8 or OCP. This strongly elucidated the effect of applied potentials on the cathodic biofilm formation and its physical properties.

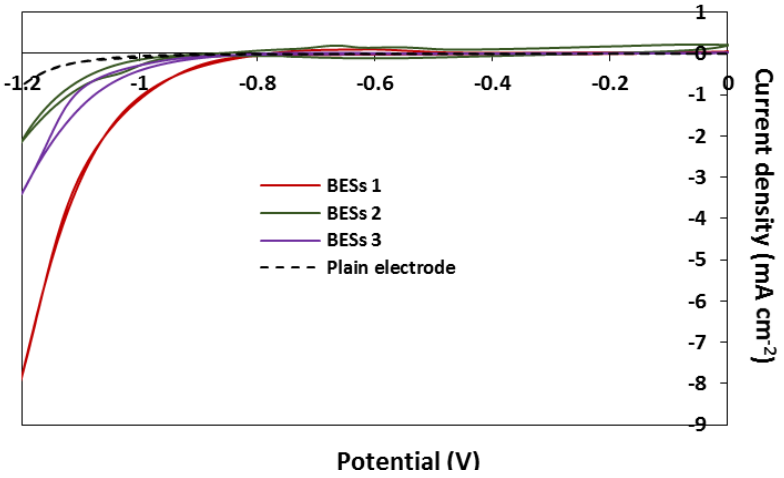
5.3.8 *Effect of cathodic potential and inorganic carbon source on electrochemical properties of biofilms*

Given the evidence from the bio-imaging analysis in this study supporting the formation of mature biofilm at the surface of the electrode of BESs 1, it arraised the question concerning the electrochemical characterisation of biofilm and the role of biofilm in transfer of the electrons and reduction of CO_2. Therefore, CVs were recorded after 104 days, at the end of experiment and compared with the voltammogram recorded from the plain electrode at the beginning of experiment. CVs were performed after changing the media and adjusting pH around 6.5. According to Figure 5.17, a significant shift in the onset potential of the hydrogen evolution reaction (HER) and consequently increase in draw currents at the potentials less than -1.0 V could be observed in all the BESs compared to that observed at plain graphite felt electrode. More specifically, the onset HER potential was around -1.0 V at the plain electrode, while it decreased to around -0.9 V in BESs 2 and BESs 3 and -0.8 V in BESs 1. Therefore, it was expected to observe H_2 production in BESs 1; however, no H_2 was detected in the headspaces of BESs 1. Although the absence of H_2 in the headspaces of BESs 1 could be due to the trapping of H_2 in the solution or the leakage of small molecule of H_2, according to the high production in these reactors H_2 is more likely consumed as an energy source through IET mechanism by

the bacterial community responsible for the production measured. Similarly, no H_2 was detected in the headspaces of BESs 3 throughout the experiment, while small percentage of H_2 was detected in the headspaces of BESs 2 irregularly. The shift in HER potential was much lower in BESs 2 and BESs 3, which can correlate the low chance of H_2 evolution to low production in these BESs. In addition, the correlation between the thickness of the biofilm in BESs 1 observed in the bio-imaging analysis and the significant shift in HER onset potential strongly suggests the implication of the biofilm developed in the production of H_2. This observation also confirms the key role of H_2 as an intermediate between the electrode and bacterial community responsible for production and IET as a dominant mechanism of electron transport through MES in this study. At sufficiently negative potentials poised at a cathode, the abiotic formation of H_2 or formate at the surface of the graphite electrode is thermodynamically favourable, but it is kinetically very slow (Rabaey and Rozendal, 2010). However, the involvement of related extracellular enzymes or coenzymes such as hydrogeneses, fomrate dehydrogenase or cytochromes are known to catalyse the formation of small compounds of H_2 or formate by interacting with the surface of a polarised cathode (Rosenbaum et al., 2011). Duetzmann et al. investigated the electron uptake from the electrode by *M. maripaludis* at the poised potential of -800 mV (Deutzmann et al., 2015). It was reported that free enzymes such as hydrogenases or formate dehydrogenases were released over the culturing and catalysed the evolution of H_2 or formate as an intermediate. Intermediates were rapidly consumed by bacteria, mimicking the direct electron transfer (DET) mechanism, for microbial catabolism and production of methane (Deutzmann et al., 2015). This could more likely be the reason in the shift of HER in all the BESs.

Although by far the contribution of DET between the cathode and bacterial community could not be totally ruled out, it seemed evident that biofilm formed at the cathodes poised at -1.0 V was related to production of H_2 molecules, confirming H_2 mediated electron transfer as a dominant mechanism between the bacteria and cathode in MES processes. Moreover, the negligible abiotic H_2 production at the plain electrode poised at -1.0 V described in abiotic control experiment (Section 5.2.2), could be the motive force for the rapid start-up of production through MES in BESs 1.

(a)



(b)

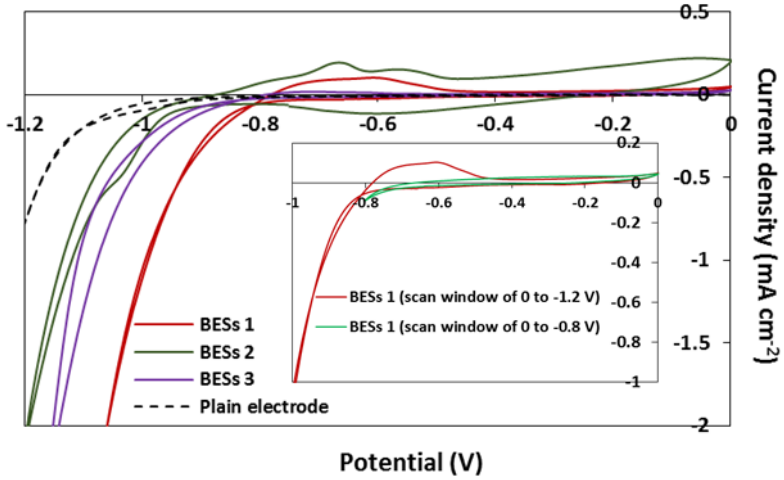


Figure 5.17. (a) Cyclic voltammograms recorded from the plain electrode and cathodes in BESs 1 (-1.0 V_CO_2), BESs 2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2) after 104 days of experiment at scan rate of 2 mV s^{-1}, (b) Zoom in of small section of the figure (a). Insert in figure (b) is cyclic voltammograms recorded from BESs 1 (-1.0 V_CO_2) at two different potential windows.

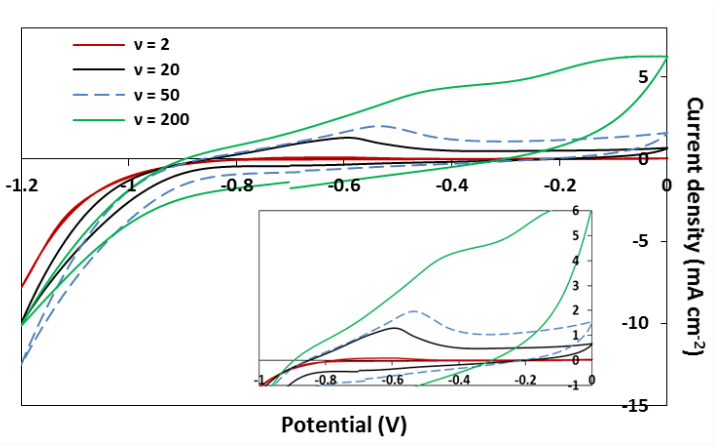
Figure 5.17 (b) also highlights specific redox features on the volatommograms recorded from BESs. It was hypothesized that the oxidation peak appeared in the voltammogram of BESs 1 at the end of experiment was related to H_2 production. In order to confirm the link between the oxidation peak and the biotic production of H_2, the potential window of CV was changed from ‘’0 to -1.2 V’’ to ‘’0 to -0.8 V’’, just before the onset potential of HER observed in BESs 1. As can be observed in the insert of Figure 5.17 (b), the oxidation peak disappeared after removing

the possibility of proton reduction and H_2 production. This confirmed the fact that oxidation peak appeared in the voltammogram of BESs 1 was related to H_2 oxidation.

Comparing the voltammograms of BESs 1 and BESs 2 in Figure 5.17 (b), more redox peaks was observed at the voltammogram of BESs 2 at -0.58, -0.6 and -0.68 V. Similar peaks were observed in the voltammograms recorded from an acetate producing BES in a previous study after the development of biocathode using bicarbonate (Patil et al., 2015a). In their study, cathode current was controlled in a galvanostatic condition which controlled the cathode potential at around -1350 mV. The authors suggested that the redox peaks appeared in the CVs were less likely involved in the MES as more negative potential was applied at the cathode during the experiment. In this experiment, higher capacitance was observed in the voltammogram of BESs 2 compared to that in other BESs. The resistance caused by the biofilm formed at the cathode was assumed to affect the voltammogram. Higher number of dead cells at the electrode or non-electroactive biofilm observed in confocal microscopy from cathodic biofilm of BESs 2 could deteriorate the electron uptake from the electrode and transfer to the CO_2 reducers, causing the higher resistance at the electrode (Kim et al., 2011, Kang et al., 2012). However, more analysis is required concerning the capacitance and the redox peaks in BESs fed by bicarbonate.

Additionally, it was observed that oxidation peak appeared in the voltammogram of BESs 1 related to H_2 oxidation was growing by increasing the scan rate. To check whether this oxidation peak appeared over time in the CVs between the potentials -0.5 and -0.7 V were surface absorbed or diffusive, CVs were performed at 4 different scan rates from 1 to 200 mV s^{-1} presented in Figure 5.18 (a), and the oxidation peaks were plotted versus $v^{1/2}$ (Figure 5.18 (b)). As can be observed, the plot of current peak against $v^{1/2}$ shows the linear trend. This linearity implies that the redox reaction is likely diffusive, and not limited to the surface of the electrode.

(a)



(b)

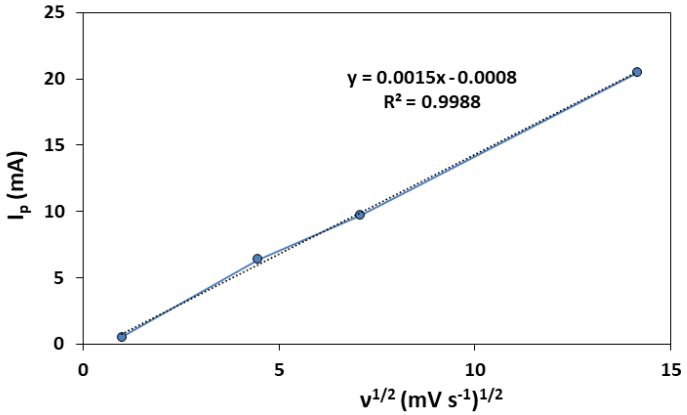


Figure 5.18. (a) Cyclic voltammograms recorded from cathode in the BESs 1 (-1.0 V_CO_2) after 100 and 104 days of the experiment at the scan rate of 2, 20, 50 and 200 mV s^{-1}, Insert figure is zoom in of small section of the figure (a), (b) the oxidation peak heights plotted versus $v^{1/2}$.

Although cathodic biofilm catalyses CO_2 reduction reaction, one of the concerns regarding biofilm growth is the limitations caused by a dense biofilm such as charge transfer resistance (Rabaey et al., 2011). In case of anodic biofilm, it was reported that the dense biofilm led to increase in electron transfer resistance between the anode and bacterial community (Sun et al., 2016). Therefore, as the densest biofilm was observed at the cathodes of BESs 1, EIS analysis was performed at these reactors on day 104 and compared with that recorded from the plain electrode. EIS was carried out with 3 electrodes configuration at the applied potential of -1.0 V. This applied potential was selected as it was poised during the 104 days of experiment at the surface of the cathodes in BESs 1. However, other potentials were also investigated through EIS analysis which are demonstrated in Figure C8 in Appendix C. Comparing the Nyquist plots illustrated in Figure 5.19, it can be observed that charge transfer resistance decreased significantly, more than 10 times, through the biofilm at the electrode in comparison with the plain electrode. This strongly confirms the fact that the dense biofilm formed in BESs 1 was conductive facilitating the electron transfer between the cathode and CO_2 reducers compared to the plain electrode. Considering the evidences supporting the biotic production of H_2 from CV results, it could be assumed from the electrochemical analysis that improve in the charge transfer through the biofilm is due to the involvement of bacterial community in the electron uptake to produce H_2. H_2 was then consumed by acetogens for the reduction of CO_2 and synthesis of organics particularly acetate by CO_2 reducers leading to superior production in BESs 1 compared to BESs 2 and BESs 3.

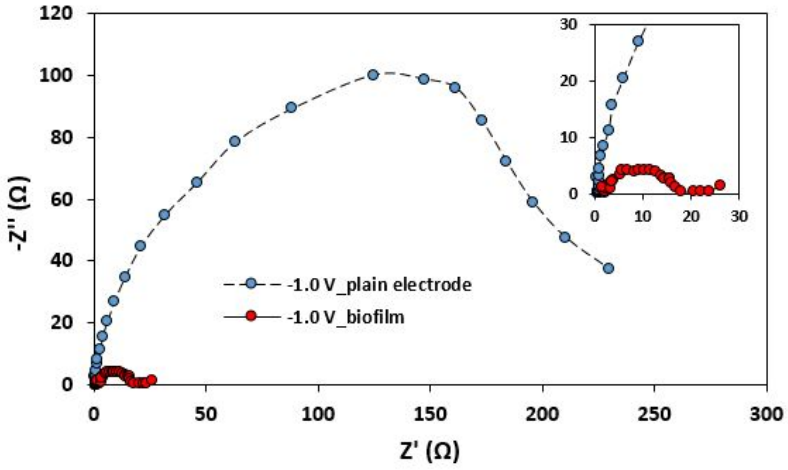


Figure 5.19. Nyquist plots at the potential of -1.0 V poised at plain electrode and the electrode of BESs 1 (-1.0 V_CO_2) at the end of experiment. Insert figure is the zoom in of small section.

5.3.9 *Effect of cathodic potential and inorganic carbon source on cells growth in biofilms and planktonic cells*

One of the questions of this thesis was to determine the difference between the bacterial community in the biofilm formed at the cathodes and planktonic cells in catholytes and whether bacteria tend to grow at the surface of the electrode or in suspension. Solving this question could also give the better understanding about MES reactions and whether MES occurs in the catholytes or at the cathodes. Therefore, to compare the cells growth in the biofilm at the cathodes with planktonic cells in the solution, cell count analysis was performed from all the BESs and OCP control using flow-cytometer. Figure 5.20 compares the cells densities in logarithmic scale at the cathodes and catholytes in all the BESs. In all the conditions, higher cells density was observed at the electrodes than in solutions. The highest bacterial cell density measured was $1.4 \times 10^9 \pm 4.7 \times 10^8$ cells cm^{-2} at the cathodic biofilm of BESs 1, whereas it was of $8.0 \times 10^8 \pm 4.6 \times 10^8$ and $6.3 \times 10^7 \pm 1.3 \times 10^7$ cells cm^{-2} in BESs 2 and BESs 3, respectively. Similar trend was observed in cells desnity in the catholytes but with lower values ($4.6 \times 10^7 \pm 2.7 \times 10^7$, $2.4 \times 10^7 \pm 1.3 \times 10^7$ and $9.0 \times 10^6 \pm 2.0 \times 10^6$ cells ml^{-1} in BESs 1, BESs 2 and BESs 3 respectively). The cell density in OCP condition, however, was much lower compared to those in BESs with polarised cathodes. The cell densities were $2.0 \times 10^5 \pm 3.6 \times 10^4$ cells cm^{-2} at the cathode and $1.0 \times 10^5 \pm 3.6 \times 10^4$ cells ml^{-2} at the catholyte in OCP control, pointing out the key role of cathode on bacterial growth by supplying energy.

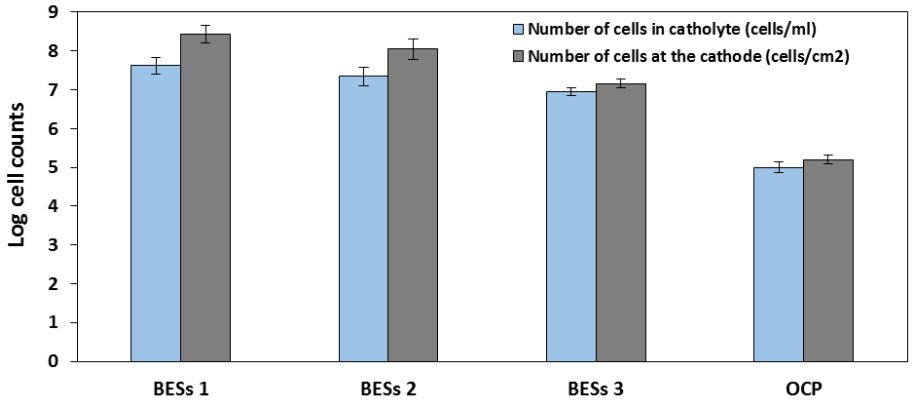


Figure 5.20. Number of the bacterial cells in logarithmic scale in 1 ml of catholytes and at 1 cm^2 projected surface area of the cathodes in BESs 1, BESs 2, BESs 3 and biotic control experiment under OCP condition.

5.3.10 *DNA extraction and selection of best database for community analysis*

Table 5.6 shows the concentration of DNA extracted from the samples of biofilm and planktonic cells. The highest DNA concentration (22.7 ± 12.0 ng μl^{-1}) was extracted from the biofilm at the cathodes of BESs 1, while lowest was measured in the biofilm at the cathodes of BESs 3 (1.6 ± 0.7 ng μl^{-1}). The quantity of extracted DNA in all the samples increased after PCR showing the generation of copies from the extracted DNA.

Table 5.6. DNA extraction quantification

Sample	DNA (ng μl^{-1})
BESs 1 (electrode)	22.7 ± 12.0
BESs 1 (solution)	2.0 ± 0.3
BESs 2 (electrode)	8.7 ± 4.6
BESs 2 (solution)	11.3 ± 8.5
BESs 3 (electrode)	1.6 ± 0.7
BESs 3 (solution)	1.8 ± 0.9

According to the equation of Shannon index (Equation 5.1), lower value of Shannon index indicates the higher diversity in the samples. Table 5.7 compares the Shannon index in the samples. Due to this table, higher community diversity was observed in the BESs poised at -1.0 V (BESs 1 and BESs 2); however, bacterial diversity was less than other conditions in BESs

3. Moreover, comparing the Shannon index of electrode and solution in each condition, diversity of samples at the electrodes of BESs 1 and BESs 2 was slightly higher than that in solutions, however, opposite trend was recorded in BESs 3.

Table 5.7. Shannon diversity index

Sample	Shannon index
BESs 1 (electrode)	2.81 ± 0.14
BESs 1 (solution)	2.92 ± 0.42
BESs 2 (electrode)	2.93 ± 0.28
BESs 2 (solution)	3.75 ± 0.29
BESs 3 (electrode)	4.03 ± 0.32
BESs 3 (solution)	3.82 ± 1.05

The representative sequences in each ASV files were assigned using the database with the bootstrap confidence of 80. 16S rRNA is a proper standard for identification of taxa. Selection of 16S database is important as the up-to-date taxonomic information is required in 16S database for evaluating the identity of taxa (Park and Won, 2018). There are few public 16S databases available such as Silva, Greengens and RDP. Silva is one of the most comprehensive 16S database containing taxonomic information for the kingdom of Bacteria, Archaea and Eukarya and has been used in many studies (Quast et al., 2012, Pruesse et al., 2007). In this study, first, Silva database was selected for the taxonomy assignment. Figure 5.21 illustrates the abundance of assignment of the sequences from the levels of kingdom to genus plotted using Silva database. It can be observed in this figure that not all the sequences were involved in the assignment, particularly at family and genus levels. Therefore, processing using Silva database could not represent all the sequences in the samples.

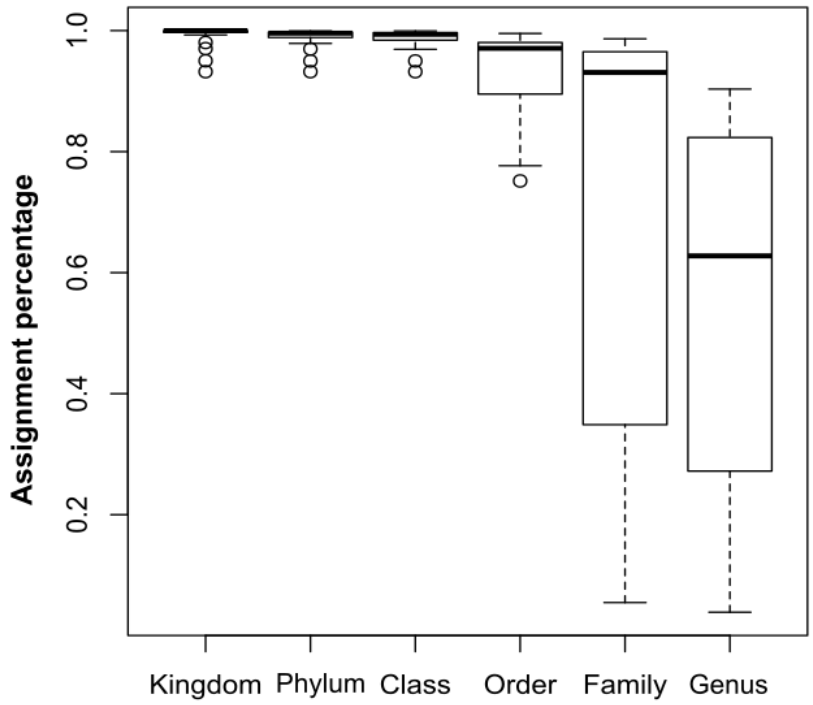


Figure 5.21. Abundance of assignment at 6 levels of kingdom, phylum, class, order, family and genus using Silva database with bootstrap 80.

Therefore, RDP was used for the taxonomic assignment of the samples. RDP is a known 16S database containing the taxonomic information of Bacteria, Archaea and Fungi (Eukarya) (Cole et al., 2013). As demonstrated in Figure 5.22, all the levels from kingdom to genus levels can represent the sequences in the samples. One of the differences between different databases is the alternative names used for taxa (Balvočiūtė and Huson, 2017). However, it seemed that in this study the issue originated from one unique sequence. The bar charts plotted using both Silva and RDP showed the significant difference in the abundance of bacteria particularly in genus level. Figure C9 in Appendix C compares the genus levels plotted using Silva and RDP as databases and it was observed that one of the genus (*Pullulanibacillus*) was not identified by Silva which comprised the significant percentage of bacterial community of the samples. Due to this limit observed in Silva database, RDP was used to assign the taxa throughout the thesis.

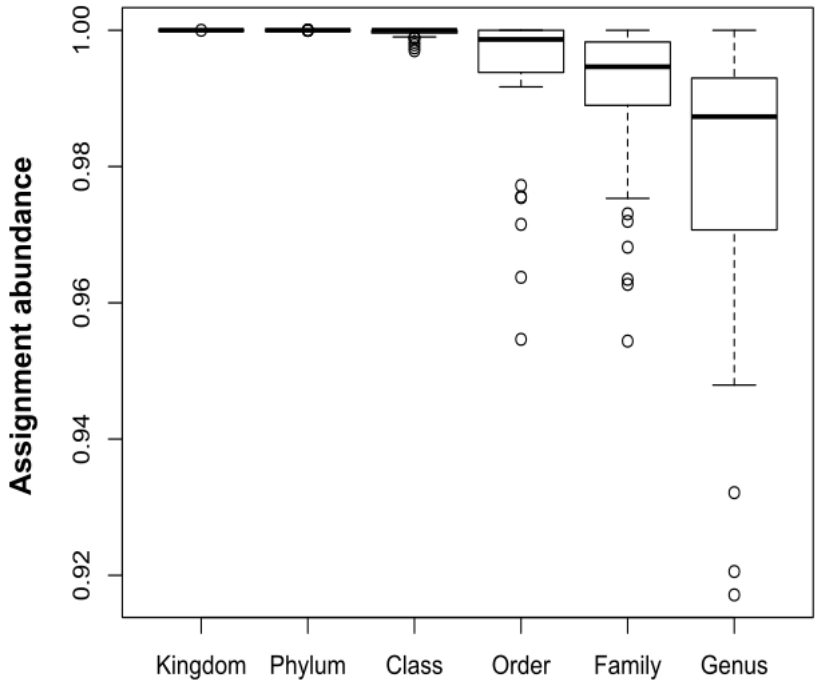
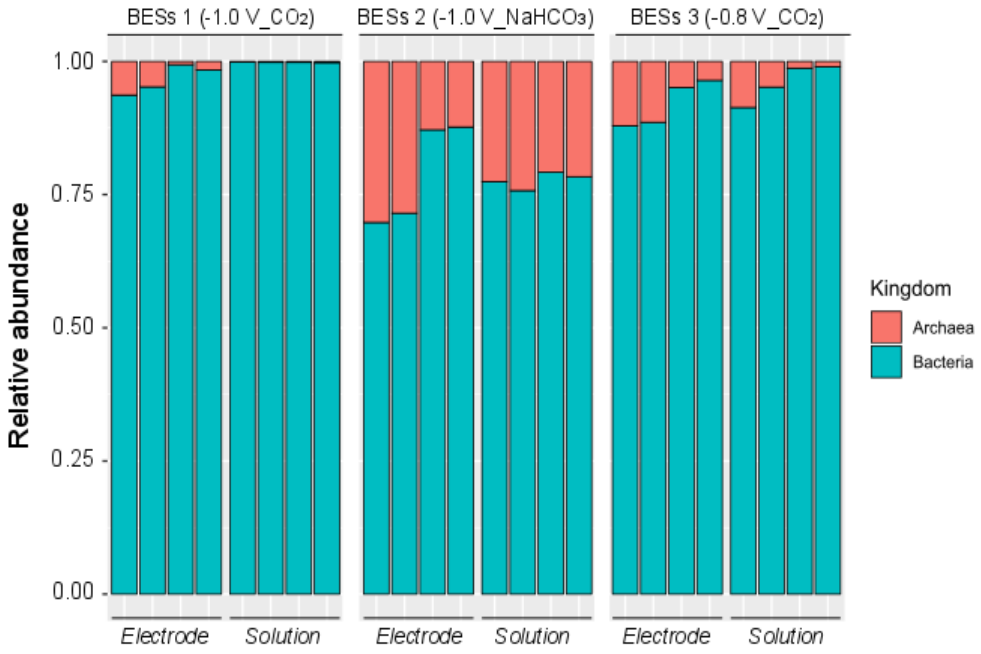


Figure 5.22. Abundance of assignment at 6 levels of kingdom, phylum, class, order, family and genus using RDP database with bootstrap 80.

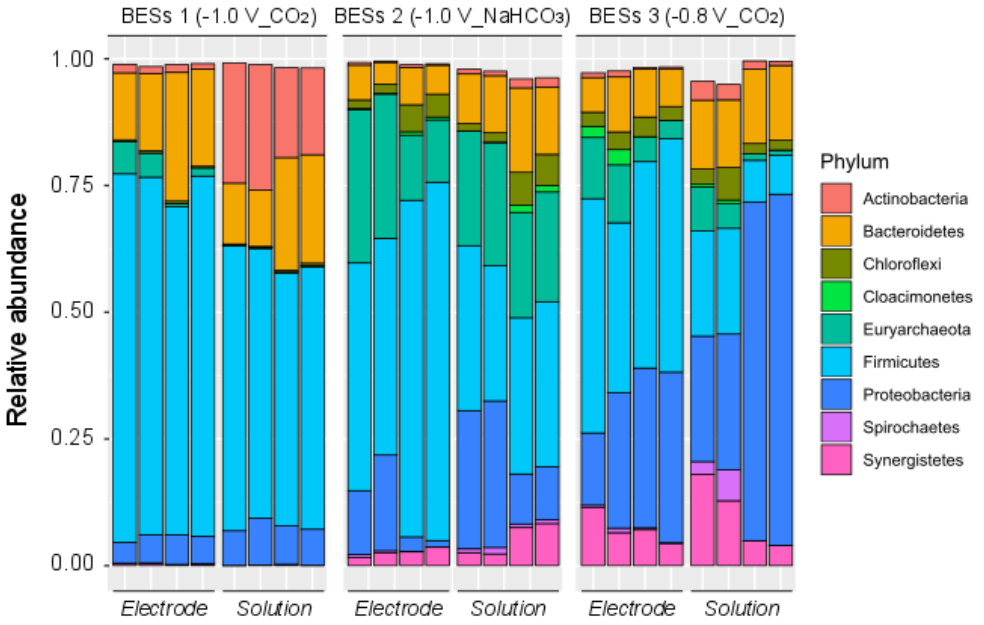
5.3.11 *Effect of cathodic potential and inorganic carbon source on composition of biofilms and planktonic cells*

After taxonomy assignment, the relative abundance of each taxon was determined from kingdom to genus levels as illustrated in bar charts in Figures 5.23 and 5.24. The abundance of weighted cell density were also plotted using the number of the cells in the electrodes and solutions by correcting the number of 16S rRNA gene copies of each genus through the classifier tool of ‘’rrnDB Estimate’’ as described in Section 3.5.9 (Stoddard et al., 2014). The unit of log cell density shown in Figure 5.24 (c) are presented as ‘’cells per ml’’ for the solution samples and ‘’cells per cm^2’’ for electrode samples.

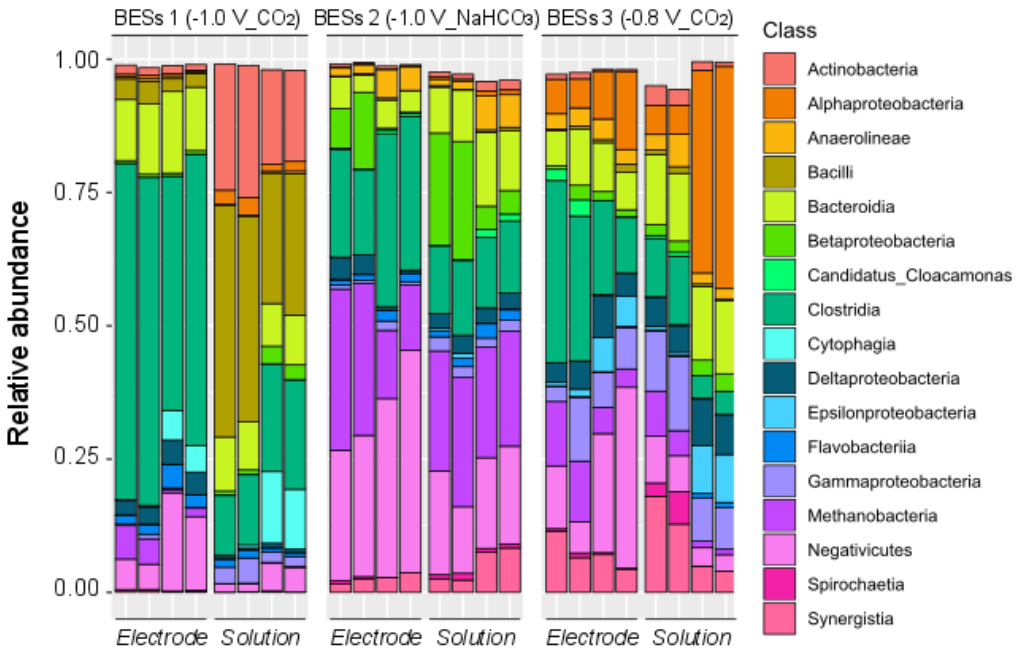
(a) Kingdom level



(b) Phylum level



(c) Class level



(d) Order level

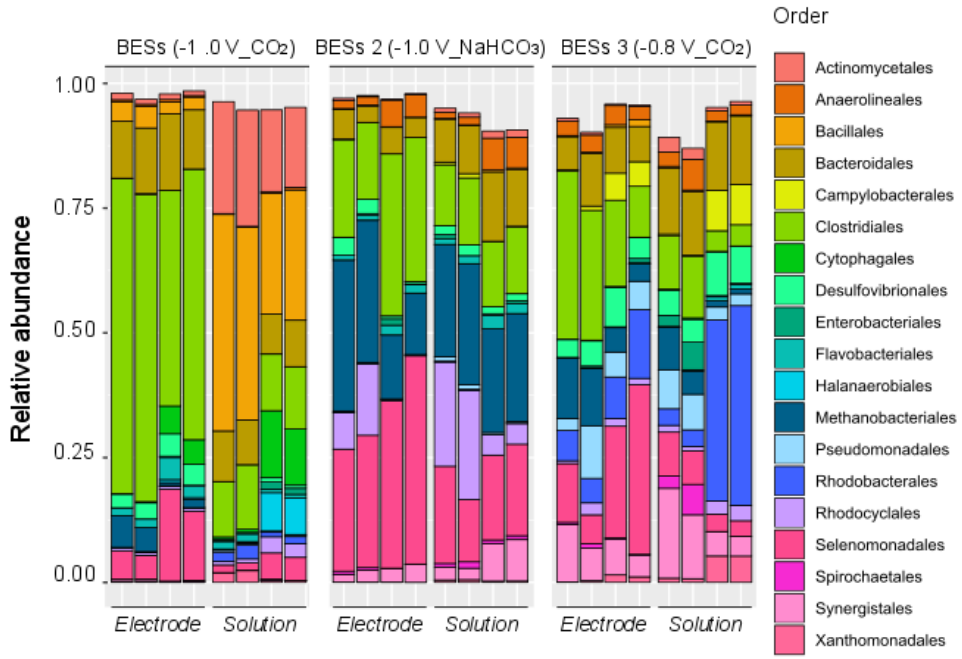
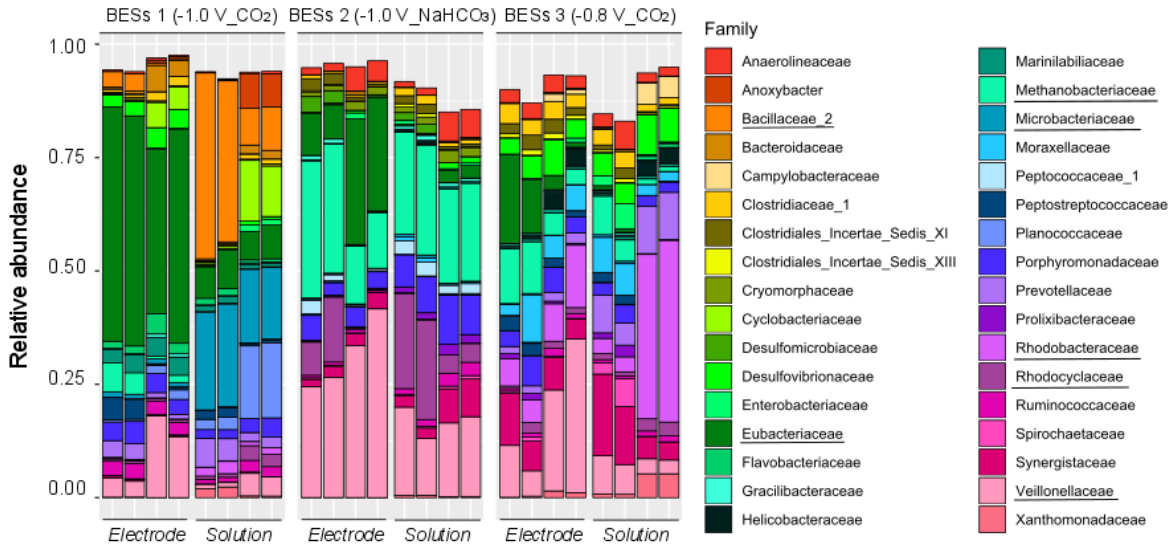
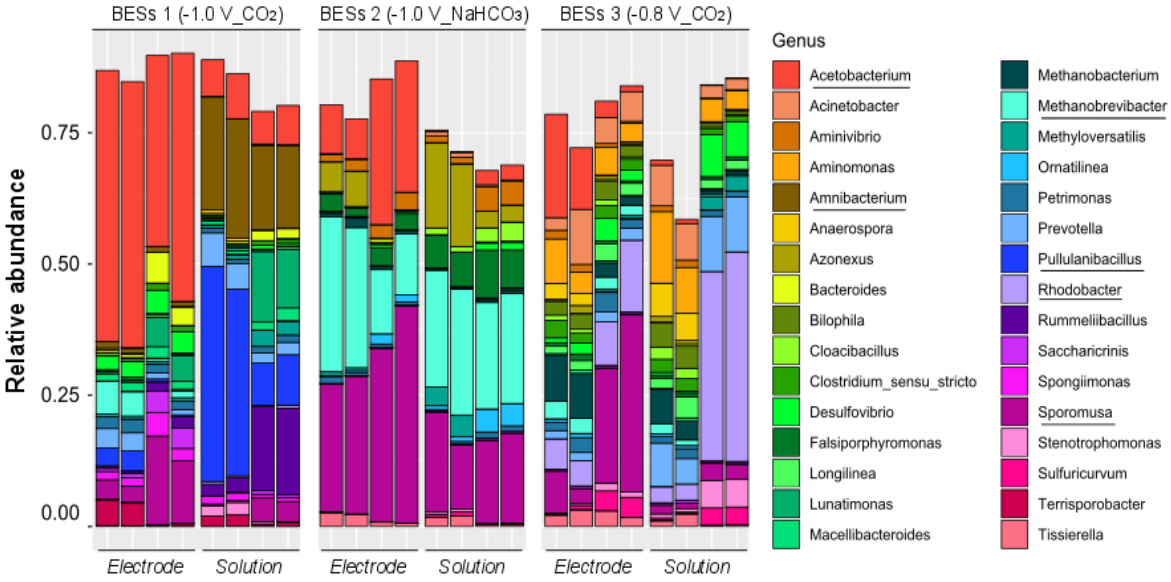


Figure 5.23. Relative abundance of bacterial community in (a) kingdom (b) Phylum (c) class and (d) order levels obtained from Illumina sequencing of 16S rRNA genes from electrodes and solutions in BESs 1 (-1.0 V_CO_2), BESs2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2).

(a) Family level



(b) Genus level



(c) Weighted Genus level

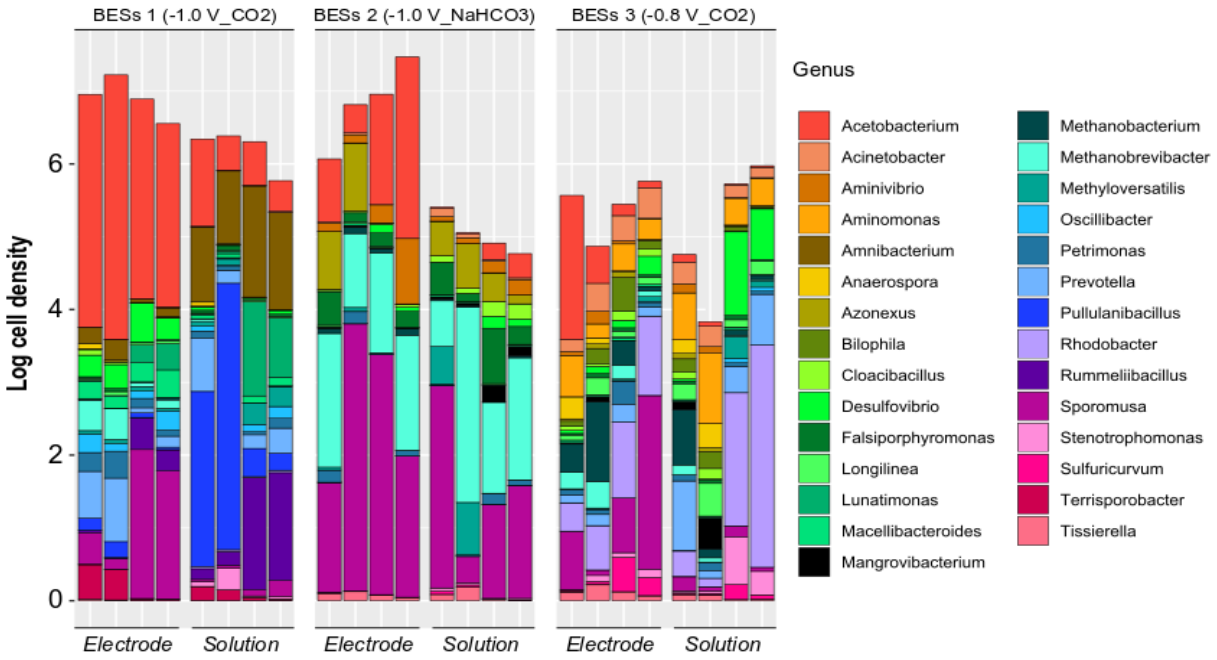


Figure 5.24. Relative abundance of bacterial community in (a) family and (b) genus levels and (c) weighted cell density (cell ml^{-1} for solution samples and cell cm^{-2} for electrode samples) obtained from Illumina sequencing of 16S rRNA genes from electrodes and solutions in BESs 1 (-1.0 V_CO_2), BESs2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2).

The difference (=distance) between the samples was evaluated using Bray-Curtis dissimilarity index. The similarity of the samples could be determined by constructing the distance matrix according to the bacterial composition. To partition the distance matrix, clusters were developed based on observations. Clear clustering was observed in replicate samples as well as 4 different clusters including BESs 1 (-1.0 V_CO_2) solution, BESs 1 (-1.0 V_CO_2) electrode, BESs2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2). The reliability of clustering in 4 groups was confirmed by multivariate analysis of variance (MANOVA) executed from all the taxa (p-value = 0.001, R^2 = 0.65). This showed the bacterial composition difference between three different treatments. Moreover, different bacterial communities were determined at the electrodes of BESs 1 compared to that in solutions; however, this was similar at the electrodes and solutions of BESs 2 and BESs 3. This indicated that in BESs 1, the cathode provided different conditions than catholyte in BESs 1, however conditions of electrodes and solutions were similar in BESs 2 and BESs 3. Data set can be visualised in non-metric multi-dimensional scaling (NMDS) illustrated in Figure 5.25 (stress level = 0.08795, R^2 = 0.96). Stress level indicates the level of disagreement between the original data set and NMDS, and R^2 defines the linear relation

between Bray-Curtis dissimilarity distance and ordination distance on NMDS plot which were extracted from Figure C10 in Appendix C. In addition, 20 genus with the highest abundance in all the samples were added to NMDS plot. The size of each genus is proportional to its abundance. The location of species could be interpreted with their distance to the clusters. Species closer to the clusters have greater chance to be more abundant in those clusters. The order of statistical analysis in this section is summarised in Figure 5.26.

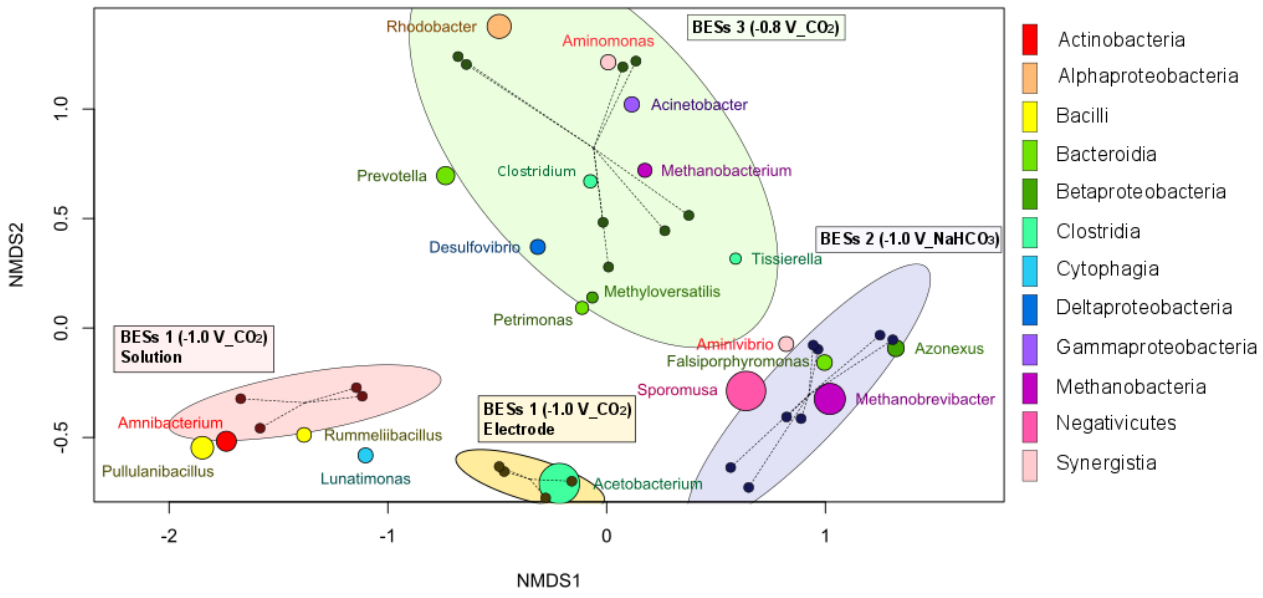


Figure 5.25. Non-metric multidimensional scaling (NMDS) of a Bray-Curtis distance matrix with 4 clusters of BESs 1 (-1.0 V_CO_2) Solution, BESs 1 (-1.0 V_CO_2) Electrode, BESs2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2) and 20 genus with the highest abundance in communities of all the samples. The diameter of each cluster was the 90% confidence interval from the centre of the samples (stress = 0.08795).

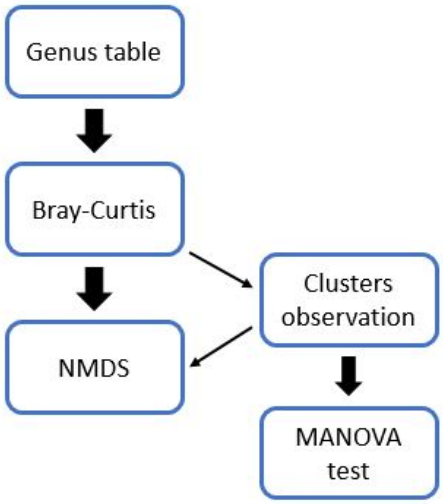


Figure 5.26. The statistical steps performed from genus levels of samples.

5.3.12 *Acetogenic bacteria responsible for MES*

Figure 5.27 shows the abundance of 6 genus which had the total relative abundance greater than 0.2 in all the samples. The difference between the abundance of these genus was interpreted using analysis of variance (ANOVA) test. One of the major reasons for the difference between 4 clusters of BESs 1 (-1.0 V_CO_2) solution, BESs 1 (-1.0 V_CO_2) electrode, BESs2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2) was the presence of *Acetobacterium* in the samples which was located close to the cluster of BESs 1 (-1.0 V_CO_2) electrode as observed in Figure 5.25. This indicated the enrichment of *Acetobacterium* extensively at the surface of the cathodes in BESs 1. Comparing the abundance of *Acetobacterium* in all the BESs, p-value of 5.7×10^{-5} was calculated through ANOVA indicating the dissimilarity of the abundance of this genus in different conditions.

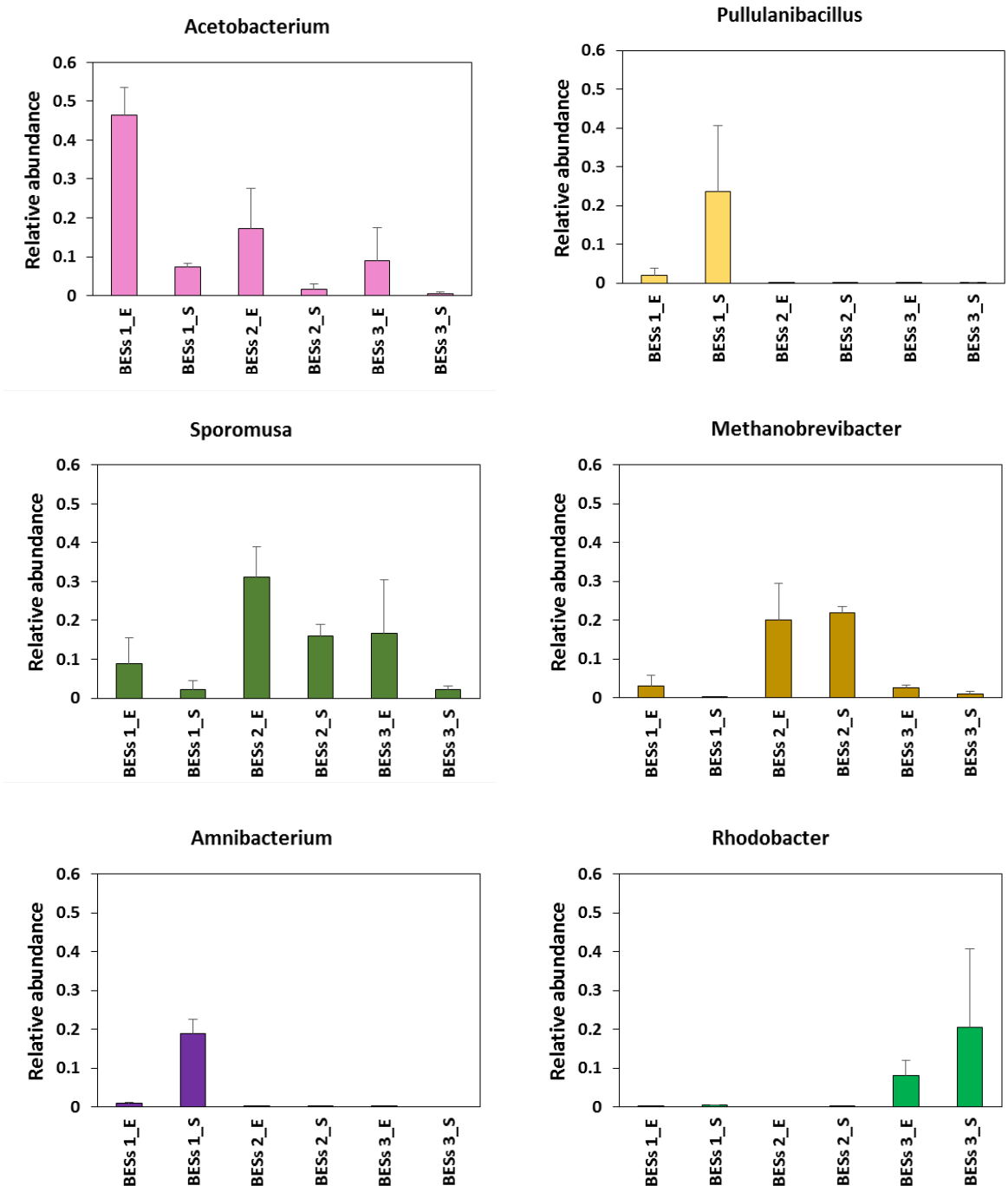


Figure 5.27. Average relative abundance of 6 genus with the total abundance greater than 0.2 in all the community samples, at the electrodes and solutions of BESs 1 (-1.0 V_CO_2), BESs2 (-1.0 V_$NaHCO_3$) and BESs 3 (-0.8 V_CO_2).

The highest abundance of bacterial community at the cathodic biofilm of BESs 1 was comprised by *Acetobacterium. Acetobacterium* are the oval-shaped, short rod and strictly anaerobic baceteria belonged to the phylum of *Firmicutes*, class of *Clostridia*, order of *Clostridiales* and family of *Eubacteriaceae*. Known as acetogenic bacteria, they consume H_2 and CO_2 as an

energy source and inorganic carbon source, respectively and produce acetate autotrophically as the major product through WL pathway (Simankova and Kotsyurbenko, 2015, Drake et al., 2006). Compared with other acetogens such as *Clostridium*, they have different morphological feature that they do not make endospores. However, they can consume other chemicals for acetogenic activities such as sugars and alcohols (Balch et al., 1977). Optimal condition for *Acetobacterium* is temperature around 30 °C and pH of 6.0-8.0. It has been discovered that ATP synthesis through WL pathway occurs through chemiosmotic mechanism by the presence of sodium-proton across the cytoplasmic membrane (Bertsch and Müller, 2015). This feature explains the flexibility of *Acetobacterium* to well adapt to different operational conditions such as low pH of BESs 1 in this chapter (Simankova and Kotsyurbenko, 2015). *Acetobacterium* was found with the highest abundance of 46.4 ± 7.01 % at the electrodes of BESs 1. This can justify the major production of acetate in this condition.

However, the abundance of *Acetobacterium* was much lower in the solution of BESs 1 (7.2 ± 0.9 %). The percentage of *Acetobacterium* abundance at the electrodes of BESs 2 (17.3 ± 10.3 %) was much lower than that in BESs 1 and even less in BESs 3 (8.9 ± 7.0 %), however in all the treatments similar significant difference was observed between the abundance at the electrodes and solutions. 1.5 ± 0.2 % and 0.4 ± 0.3 % of the planktonic cells in the catholytes of BESs 2 and BESs 3 were comprised of *Acetobacterium*, respectively. Depending on the initial inoculum source and operational conditions provided, different types of acetogens such as *Clostridium* can be enriched from the mixed culture of microorganisms through MES processes (LaBelle et al., 2014b, LaBelle and May, 2017, Giddings et al., 2015, Patil et al., 2015a). In this chapter, it was discovered that inorganic carbon source is one of the key parameter in enriching the acetogens responsible for acetate production through MES. As discussed, one hypothesis for favourability of CO_2 rather than bicarbonate for bacterial community could be due to the pH difference provided in the catholytes. Low pH can enhance H_2 production at the electrodes by reducing protons available around the cathodes. This might be also the reason that higher abundance of *Acetobacterium* was observed in the biofilm rather than in the solution. In other words, it can be concluded that *Acetobacterium* was grown where the highest concentration of H_2 was produced which was at the cathodes. Following that hypothesis, electrons were taken from the cathodes through IET mechanism by bacteria. In addition, over extensive studies on mechanism of electron transfer between anodes and *Geobacter*, it has been confirmed that cytochrome of bacterial cells played an important role in transferring the electrons. However, in case of biocathodes, it was reported that *Acetobacterium* does not have cytochromes (Kracke et al., 2015). Furthermore, the proof-of-concept study by Nevin et al reported the lack of direct

electron uptake from the cathode by *Acetobacterium woodii* , showing non-electroactive activity of this genus (Nevin et al., 2011). This could confirm H_2 consumption by *Acetobacterium* as an energy source instead of direct electrons uptake form the cathodes.

Although these findings can highlight the assumption of IET mechanism at the cathodes, the DET mechanism cannot be completely ruled out, particularly when plenty of pili wires were observed in the SEM images (Figure 5.15) of the biofilm at the cathodes of BESs 1. Pili is made of protein or membrane of the cells which can be highly conductive and play an important role in transferring electrons from bioanode to the anode (Reguera et al., 2005, Pham et al., 2009, Šefčovičová et al., 2011). The pili revealed in the SEM images of biofilm can be non-electroactive compounds as part of the biofilm matrix only connecting the cells together. However, there can be also a possibility that these pili were conductive facilitating the transfer of the electrons within the cells and between the cathode and cells through the synergetic effect of different bacterial community in the biofilm.

Besides pH, higher enrichment of *Acetobacterium* in BESs 1 compared with BESs 2 could confirm the feasibility of dissolved CO_2 reduction by bacteria rather than bicarbonate. Little is reported about the difference between the CO_2 and HCO_3^- on acetogens activities, however, similar observation was reported by Grootscholten et al (Grootscholten et al., 2013b). In their study, it was suggested that bacterial protein formation may proceed faster with CO_2 rather than HCO_3^- (Grootscholten et al., 2013b). The assumption in their study originated from the previous study on *Clostridium* showing that CO_2 was required for the synthesis of protein for bacterial cells (Tomlinson and Barker, 1954).

Another acetogen found in the samples with high abundance was from the class of *Negativicutes*, genus of *Sporomusa*. P-value of 0.47 showed that *Sporomusa* was more likely found in all the conditions, however, Figure 5.25 demonstrates the higher abundance of *Sporomusa* in BESs 2 particularly at the electrodes compared to other conditions. This could be due to the pH difference in different treatments which was the highest (~ 8.0) in BESs 2. The effect of pH on enrichment of two most abundant acetogens in the system was analysed. Figure 5.28 demonstrates the correlation between the average pH of the cathodic medium and relative abundance of *Acetobacterium* and *Sporomusa*. Although pH at the surface of electrodes could be slightly different from the solution pH, the solutions pH was considered for both electrodes and solutions, as it was not possible to measure the pH at the surface of electrodes during the experiment. It can be observed that increase in the pH in the BESs was associated with increase in abundancy of *Sporomusa*. This indicated the effect of pH as an important parameter on *Sporomusa* enrichment in the reactors. However, not clear relationship was observed in the pH

and enrichment of *Acetobacterium*. Although previous studies on pure culture of *Sporoumsa* showed their enrichment to wide range of pH for their growth (5.7-8.0), it seemed in this study that *Sporomusa* could not grow under acidic conditions (below 5.8).

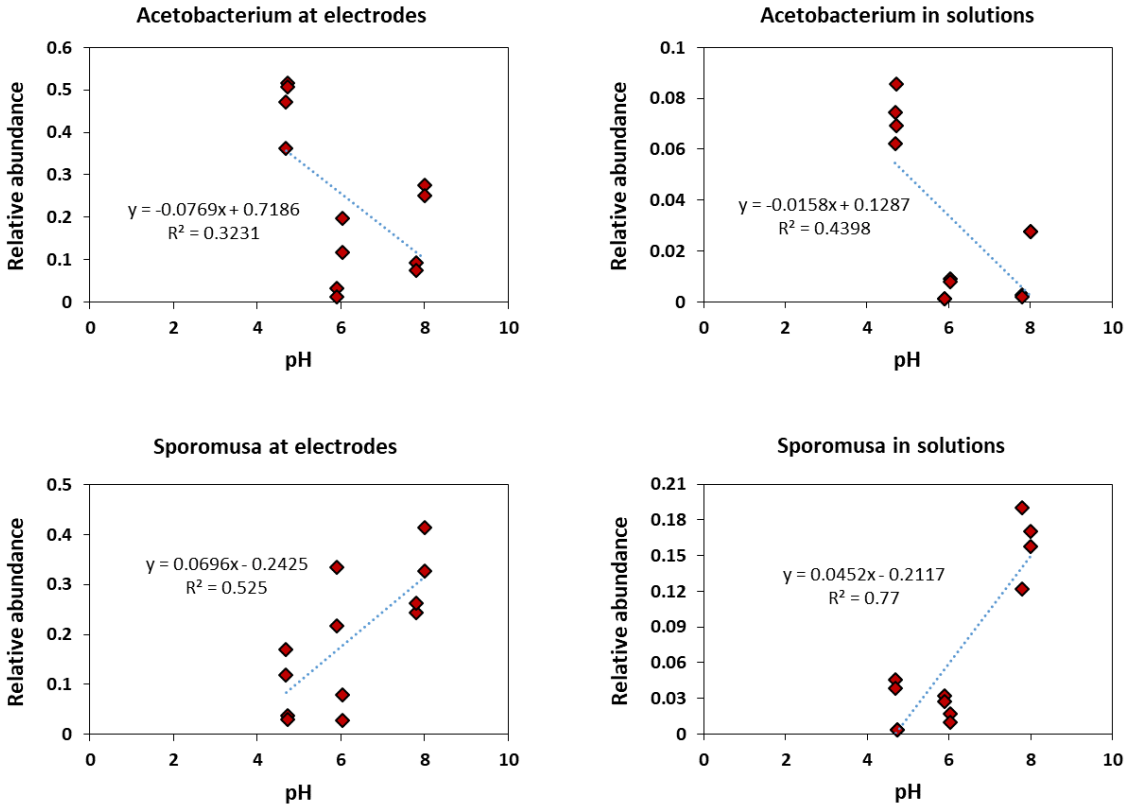


Figure 5.28. Correlation between the abundance of *Acetobacterium* and *Sporomusa* at the electrodes and solutions and pH.

Acetobacterium and *Sporomusa* were the most abundant acetogens in the solutions. To clarify which genus was more involved in synthesis of acetate from CO_2, Figure 5.29 was plotted which indicates the correlation between the acetate concentration produced in the BESs and the relative abundance of *Acetobacterium* and *Sporomusa*. Concentration of acetate are extracted from the last day of experiment (day 104). According to Figure 5.29, there was a clear linear correlation between the abundance of *Acetobacterium* in bacterial community of both electrodes and solutions and acetate production; however, this was different with the abundancy of *Sporomusa*. Although both genus are known in production of organics particularly acetate, it seemed that *Acetobacterium* was more responsible for synthesis of acetate from CO_2 in this experiment.

Considering *Acetobacterium* as the most responsible bacterial community in acetate production through MES, which its enrichment was not affected by pH, this can eliminate the hypothesis that CO_2 provided the better condition due to the lower pH rather than that by supplying bicarbonate. Hence, it can highlight this hypothesis that CO_2 is more feasible and favourable for bacterial activities compared to bicarbonate. However, more investigations is required in this context.

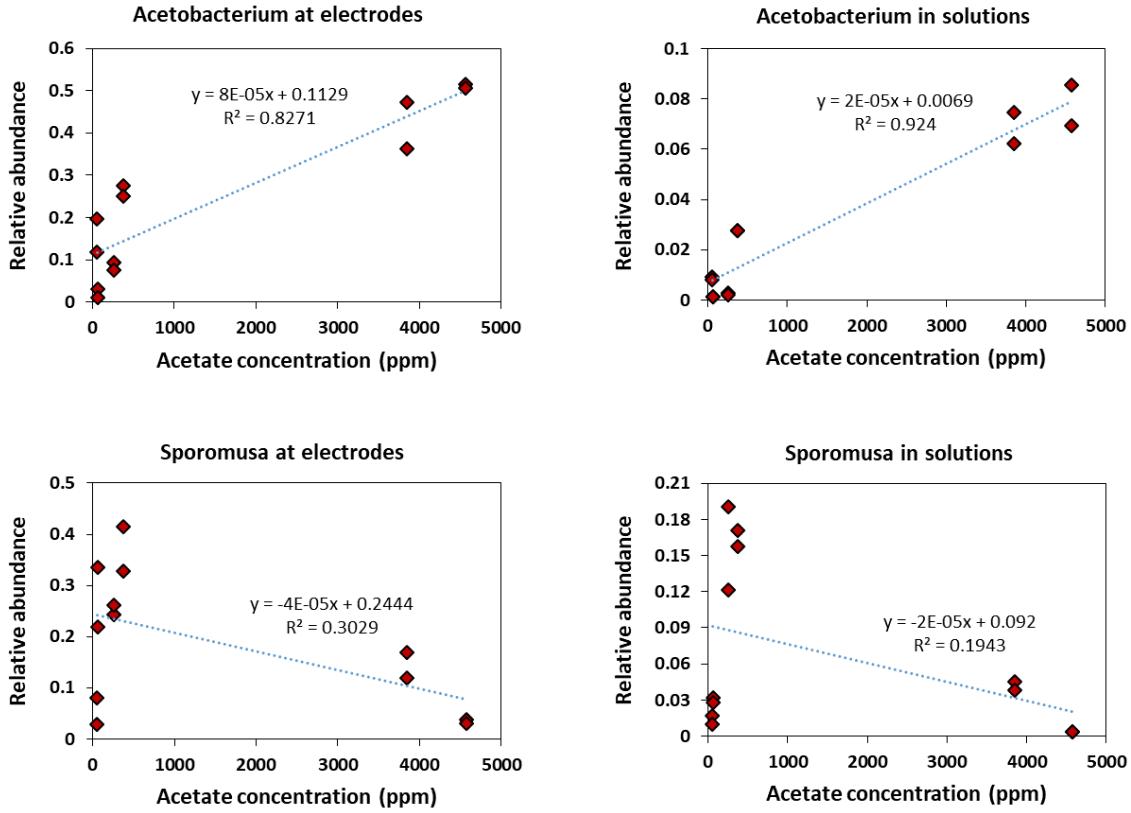


Figure 5.29. Correlation between the abundance of *Acetobacterium* and *Sporomusa* at the electrodes and solutions and acetate production.

5.3.13 *Dominated bacterial community from family of Bacillaceae_2 developed during MES*

Pullulanibacilus (p-value = 0.099) is the genus belonged to the phyla of *Firmicutes*, class of *Bacilli* and family of *Bacillaceae_2* which was only found with the significant abundance (~ 23%) in the planktonic cells of BESs 1 solutions and less than that (~ 2%) at BESs 1 electrodes. BLAST (Basic Local Alignment Search Tool) searches of the NCBI (National Center for Biotechnology Information) from this sequence revealed that *Pullulanibacilus* was reported as an uncultured bacterium in the previous study using Silva as a database. Figure 5.30 shows the

phylogenetic tree from 16S rRNA gene sequences presented in the biofilm and suspension of BESs 1 and highly similar sequences recovered from NCBI BLAST search. The sequences of the present study and from the NCBI BLAST search are coded according to their ASV numbers and genbank accession numbers, respectively. The similarity of *Pullulanibacilus* and the uncultured bacterium from the previous study can be evidently observed in Figure 5.30. In the study focused on the production of acetate from hydrogen and CO_2 at the hollow-fibre membrane reactor, *Pullulanibacilus* was found as planktonic cells in the solution in addition to *Clostridium* in which the authors correlated the acetate production to *Clostridium* species found in the solution (Zhang et al., 2013a). In addition, similar to operational condition in BESs 1, solution in their study had highly acidic condition (pH ~ 4.5). Presence of *Pullulanibacilus* in the catholytes could be related to either presence of organic products through MES processes particularly in in the solution of BESs 1 and utilizing the organics or its involvement in production of organic products. Although not much is reported about *Pullulanibacillus*, it was reported as the spore-forming bacteria of *Firmicutes* in the previous review on classes of *Bacilli* and *Clostridia* (Galperin, 2013). Few temperature resilient species of *Pullulanibacillus* were found in the environment with the optimum temperature of 37 °C and acidic pH of around 4 (Pereira et al., 2013).

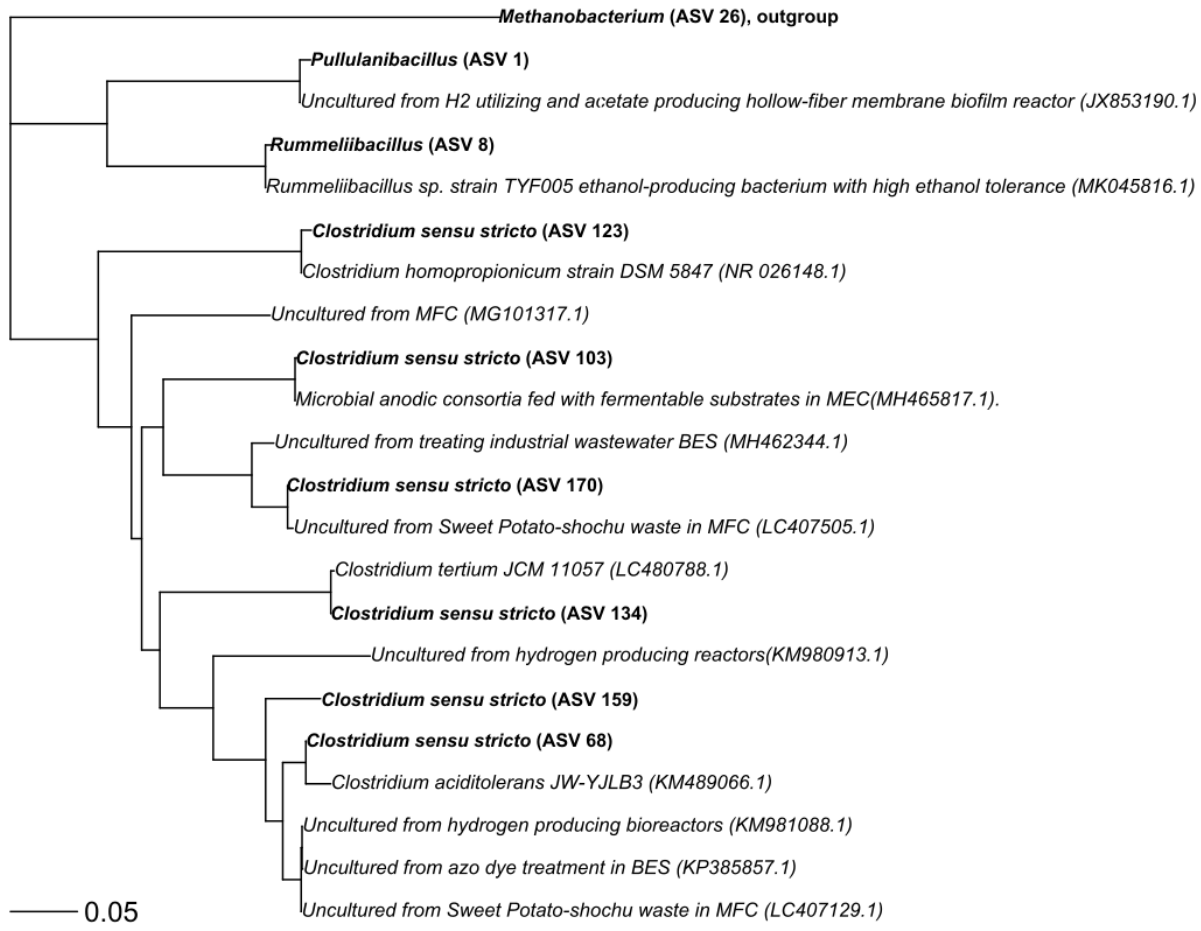


Figure 5.30. Phylogenetic tree of 16S rRNA gene sequences extracted from community analysis of electrode and solution samples from BESs 1 (-1.0 V_CO_2) presented in bold (coded by their ASV numbers) compared to highly similar sequences recovered from NCBI Nucleotide database collection (coded by their genbank accession numbers). The scale bar shows the number of nucleotide position changes. *Methanobacterium* was used as an outgroup from Archaea.

Another genus from the family of *Bacillaceae_2* identified in the samples was *Rummeliibacillus* (p-value = 0.09), which was found more abundantly in the solutions of BESs 1 also confirmed by NMDS plot (Figure 5.25). The abundance percentage of this genus in the electrodes and solutions was 9.3 ± 7.9 % and 1.2 ± 0.8 %, respectively with the significant abundance of approximately 16 % in the solution of one of the replicate reactors, while it wasn't found in other conditions. This genus was also found in the catholyte of BES reported by Arends et al (Arends et al., 2017). Redundancy analysis in their study showed the strong correlation between the presence of *Rummeliibacillus* and production of butyrate confirmed by plotting the correlation between the butyrate concentration and single operational taxonomic unit (OTU) abundance. They also reported that *Rummeliibacillus* was related to the production of iso-

propanol (Arends et al., 2017). Similarly, in this study butyrate and iso-propanol production was observed only in BESs 1 which could be closely associated with the enrichment of this genus in the catholytes. In addition, according to Figure 5.30 *Rummeliibacillus* was also reported as the ethanol producing bacteria in an unpublished work.

5.3.14 *Clostridium developed during MES*

Small production of other products than acetate such as butyrate and alcohols could be also related to the *Clostridium* found in both biofilm and suspension of BESs 1. Although operational condition in BESs 1 seemed the most favourable for *Acetobacterium*, different species of *Clostridium* were found in these reactors, which can be due to the acidic conditions. It has been reported that different species of *Clostridium sensu stricto* were isolated from acidic conditions (Kuhner et al., 2000, Wiegel et al., 2006, Matthies et al., 2001). Moreover, Clostridium can ferment wide range of organics in addition to inorganic carbon which both were presented in BESs 1. Different sequences all named as *Clostridium sensu stricto* belonging to the class of *Clostridia* and order of *Clostridiales*, was found in BESs 1 but with low abundant population (~ less than 1 %). Figure 5.30 illustrates these sequences from the present study coded by their ASV numbers. The sequences from NCBI BLAST search highly similar to the *Clostridium sensu stricto* sequences in this study were found in bio-electrochemical or acetogenic bacterium reactors in previous studies. *Clostridium sensu stricto* (ASV 68) was highly similar to *Clostridium aciditolernas* found in the C4, C6 and C8 producing reactors from syngas (Ganigué et al., 2016). In their study, both alcohol and organic forms of long chain products were detected from syngas as substrate in a bioreactor. *Clostridium aciditolernas* is an acid tolerant bacterium with broad substrate spectra able to fix CO_2 through WL pathway and produce organic acids and alcohols. It has been reported that the fermentation products of this bacterium are typically acetate, butyrate and ethanol (Lee et al., 2007). *Clostridium aciditolernas* was also found in BESs producing C1-C5 organic acids and alcohols from CO_2 with higher abundance in suspension (46%) than in biofilm (16%) in a previous study (Wenzel et al., 2018). Besides, similar sequences to *Clostridium sensu stricto* (ASV 68) was previously found in the similar conditions provided in BESs 1 such as bio-electrochemical and H_2 producing reactors. High similarity was observed between the sequences of *Clostridium sensu stricto* (ASV 123) and *Clostridium sensu stricto* (ASV 134) with *Clostridium propionicum* and *Clostridium tertium*, respectively. Previous studies on *Clostridium tertium* reported acetate, butyrate and H_2 as the fermentation products (Kim et al., 2013). Comprehensive study on the

phylogeny of genus *Clostridium* including *Clostridium propionicum* showed that *Clostridium* are extremely diverse, able to produce wide range of products depending on the substrates (Collins et al., 1994). Therefore, if *Clostridium sensu stricto* and *Rummeliibacillus* were responsible for the production of butyrate and alcohols in BESs 1, minor concentration of these products compared to acetate could be justified by the low abundance of these bacterial communities in the system.

5.3.15 *Methanogens developed during MES*

One of the other differences between the samples was the presence of *archaea* in BESs 2 more than other conditions. Cathodic biofilm and planktonic cells of BESs 2 were dominated by the class of *Methanobacteria* corresponded to the genus of *Methanobrevibacter* (p-value = 0.71). *Methanobrevibacter* is a known hydrogenotrophic methanogen found with almost similar abundance of approximately 20% at both electrodes and solutions in BESs 2 (Miller et al., 1982). However, much lower percentage of cathodic biofilm (2.5 – 3 %) in BESs 1 and BESs 3 was comprised by *Methanobrevibacter*. As the pH of the catholytes in both BESs 1 and BESs 3 was less than 6.5 over the experiment, it can be concluded that low operational pH was the main reason of the low abundance of *Methanobrevibacter* in these reactors, while higher percentage of *Methanobrevibacter* was enriched in BESs 2 with the alkaline operational pH. Low operational pH was known as one of the methods to suppress the methanogenic activities (Jadhav et al., 2018). In addition, higher enrichment of methanogens in BESs 2 could be related to the difference in standard Gibbs free energy of the system when different phases of reactants are provided and the higher favourability of the reaction when bicarbonate is used (Amend and LaRowe, 2019). Another methanogen found in BESs 2 was *Methanobacterium* (~ 0.5 %) from the class of *Methanobacteria*. Similar to *Methanobrevibacter*, *Methanobacterium* is an anaerobic hydrogenotrophic methanogens which consumes CO_2/H_2 and produces methane (Zehnder and Wuhrmann, 1977). Despite the enrichment of methanogens in BESs 2, no methane was detected in the headspaces of these reactors. The presence of hydrogenotrophic methanogens in the samples while no methane production was observed can be due to few reasons such as suppression of methanogens by chemical inhibitor, trapping of methane in the solution, or dead cells of these *archaea* (dead cells observed from confocal microscopy).

5.3.16 *Fermenters developed during MES*

Amnibacterium was another genus with p-value of 0.06 dominated approximately 18.9 ± 3.6 % of the planktonic cells in the solution of BESs 1; however, its abundance in other conditions is almost negligible. *Amnibacterium* is from the phylum of *Actinobacteria* and the family of *Microbacteriaceae* which is an anaerobic soil organism. The fact that it was found with high abundance in the solution of BESs 1 could be due to the presence of acetate in the catholytes produced in BESs 1 with the highest concentration compared to other conditions leading to enrichment of heterotrophic bacterial community such as *Amnibacterium* (Kim and Lee, 2011).

Rhodobacter was found only in BESs 3 (p-value = 0.01), more abundantly in the solution (~ 20%). Classified in the phylum of *Proteobacteria* and the family of *Rhodobacteraceae*, *Rhodobacter* is known as a facultative photosynthetic bacterium capable of respiration in both anaerobic and aerobic conditions (Ermler et al., 1994, Wenzel et al., 2018, Pujalte et al., 2014). *Aceniobacter* was another soil organism from the phylum of *Proteobacteria* (and order of *Pseudomonodales*) living in anaerobic conditions found more significant in both electrodes and solutions (abundance of 2-10%) of BESs 3. *Amnivibrio* with the small abundnnce around 1.5 – 2% was found in both conditions of BESs 2 and BESs 3; however, it was not enriched in BESs 1. *Amnivibrio* is an anaerobic soil organism from the family of *Synergistaceae*. *Amnivibrio* is known as an amino-acid degrading bacteria which first was isolated from the propionate oxidising culture enriched from soil in Japan (Honda et al., 2013). Similarly, *Aminomonas* was found only in BESs 3 with relative abundance of 3-13%. *Aminomonas* is also anaerobic amino-acid degrading bacteria belonged to the family of *Synergistaceae* (Baena et al., 1999). Other organic consuming bacteria such as *Terrisporobacter*, *Tissierlla*, *Macellibacterodes*, *Bacteroides* and *Prevotella* were found in all the BESs particularly in BESs 3 with quite small relative abundancy (0.5 – 3 %) which are reported as anaerobic fermenters (Deng et al., 2015, Harms et al., 1998, Jabari et al., 2012). In addition, although no sulphate was provided in the medium few sulphate-reducing bacteria with small percentage (less than 5%) such as *Desulfovibrio* or *Sulfuricurvum* were found in the solutions and electrodes of BESs.

In general, it was observed that conditions provided in BESs 1 was more favourable for the enrichment of acetogenic bacterial community from mixed culture of microorganisms responsible in production of organics from CO_2 leading to the superior function of these reactors. However, bacterial community enriched in BESs 2 and BESs 3 was more comprised of methanogens and fermenters, respectively.

5.4 Conclusion

Enrichment of anaerobic biocathode able to reduce CO_2 and produce valuable chemicals from mixed culture of microorganisms is challenging. In this chapter, the parameters affecting this enrichment in BES were studied. The effect of different cathodic potentials corresponded to the energy available for bacterial community and different forms of inorganic carbon source on the development of biocathode and production was investigated. The greatest cell growth was observed in BESs poised at -1.0 V and fed by the gaseous form of CO_2. Consequently, the best production was also obtained in these reactors in which maximum acetate concentration of 6500.4 ± 633.6 ppm (216.7 ± 21.1 mmole C L^{-1}) acetate and other C4, C5 organic acids and alcohols were detected. While methanogenic activities are suppressed, small abiotic hydrogen production at the carbon-based electrodes with the cathodic potential of -1.0 V could be the motive force for the selection of acetogens from mixed culture of microorganisms leading to their growth, enrichment and activity.

It was clearly observed that cathodic potentials affected the formation of biofilm at the surface of the electrodes. The clear correlation was observed between cathodic potentials and physical properties of biofilm such as thickness of biofilm, its distribution at the cathode and live bacterial cells in the biofilm by comparing reactors under OCP conditions, BESs with potentials of -0.8 V and -1.0 V fed by CO_2, leading to higher production in BESs poised at -1.0 V.

Inorganic carbon source seemed to have a direct effect on the composition of bacterial community. Approximately 50 % of the biofilm at the cathodes of BESs poised at -1.0 V and supplied by CO_2 was comprised of *Acetobacterium*, which was postulated as the main bacteria responsible for acetate production through MES. It also seemed that consumption of inorganic carbon by acetogens was more feasible in form of CO_2 than bicarbonate. However, when bicarbonate was provided methanogens were the dominant bacterial community enriched during MES processes.

Although regardless of inorganic carbon source biofilm formation was observed only at the cathodes of BESs applied at -1.0 V, community analysis indicated the significant effect of inorganic carbon source on composition of bacterial community in the biofilms, proposing the enrichment of bacteria responsible for MES by providing gaseous CO_2 rather than bicarbonate.

Electrochemical analysis discovered that dense biofilm formed at the cathodes of BESs poised at -1.0 V and supplied by CO_2 was highly conductive. However, community analysis showed

that almost half of the bacterial community was comprised of *Acetobacterium*, known as non-electroactive bacteria. Significant shift in onset potential of H_2 evolution to around -0.8 V in these BESs could reveal that the conductivity of the biofilm was related to the biotic H_2 production. Biotic H_2 production could be more likely through free redox enzymes of bacterial community such as hydrogenases, which was reported that could be released during routine culturing (Deutzmann et al., 2015), playing a key role in biotic H_2 production. Therefore, it is fair to conclude that *Acetobacterium* grew and developed a biofilm where H_2 was more available which was at the surface of the electrodes. This strongly shows the role of cathode in BES as a source of H_2 rather than providing the electrons for the direct electron transfer to acetogens.

In addition to energy and inorganic carbon sources, pH of the solution seemed to play a key role not only in electrochemical property of the system but also enrichment of desired bacteria. Acidic pH seemed the best method in suppressing the activity of methanogens in BESs, without the need for costly inhibitor chemicals. Furthermore, pre-heating the inoculum before inoculating the BESs performed in this chapter could not suppress the methanogens completely, as methane was still detected in BESs after inoculation. As low pH conditions showed the successful methanogens suppression, pre-heating the inoculum can be removed for the future studies.

Chapter 6. Strategies for Production of Long Chain Organic Chemicals through Optimized Microbial electrosynthesis (MES) and Parameters Affecting the Optimization

After development of a biocathode able to reduce CO_2 and produce organics through microbial electrosynthesis (MES) and comprehensive study on biofilm formed during MES discussed in Chapter 5, this chapter focuses on optimizing the system efficiency and steering the production towards longer chain products than acetate. Therefore, two different strategies were investigated: 1) continuous operational mode and 2) supplying electron donors in addition to a cathode.

Therefore the main objectives were:

- Investigating the effect of continuous feeding regime on operational condition and production in bio-electrochemical system (BES) with acetogenic biocathode
- Investigating the effect of different electron donors on production in BES with acetogenic biocathode
- Clarifying the role of cathode in production of short and long chain organic acids and alcohols

6.1 Introduction

MES shows promising application for converting CO_2, a destructive waste from human activities, to valuable chemicals particularly acetate and ethanol, by providing constant energy source to a bacterial community via polarised electrode (May et al., 2016). However, research in MES has been directed to production of longer chain organic acids and alcohols such as caproate and hexanol rather than acetate and ethanol in BESs due to their commercial value (Vassilev et al., 2018). Production of these high value biofuels with the significant industrial and agricultural applications is possible by controlling the fermentation pathways using the

electrons derived from an electrode (Moscoviz et al., 2016). Wood-Ljungdahl (WL) pathway is an established pathway through acetogenesis mechanisms by reducing CO_2 using H_2 as an energy source through synthesizing acetyl-CoA and producing acetate as the major product. Production of long chain products in MES typically occurs from acetate as the base unit of the reaction known as chain elongation through reverse β-oxidation pathway or linear extension of acetyl-CoA to butyril-CoA. To direct the production towards alcohols, it has been discussed that increasing the stress level such as low pH to bacterial community leads the bacterial culture to reject the reducing equivalents by reducing acetate and butyrate towards their corresponding solvents such as ethanol and butanol instead of biomass (Richter et al., 2013, Martin et al., 2016, Ramió-Pujol et al., 2015). Therefore, bacterial metabolism will switch from acetogensis (production of organic acids) to solventogenesis (production of solvents such as alcohols) (Molitor et al., 2016). In this context, depending on the operational conditions and bacterial community, various chemicals and biofuels can be produced through MES and electro-fermentation (EF, chain elongation in BES) processes. In addition, it has been postulated that chain elongation requires other sources of electrons in addition to the electric current served by electrodes as electric current does not provide sufficient energy source for long chain organics production in BES (Jiang et al., 2018). Ethanol, methanol, lactate and formate are the known examples of electron donors for the production of short and long chain carboxylates and alcohols (Moscoviz et al., 2016). Although few studies showed a high production rate of caproate in BESs, this was limited to short chain carboxylates in other studies due to the lack of sufficient electron donors in the solution. Production rate of ethanol can be low, limiting the energy source for the chain elongation through acetate. In a previous study, adding mediators to bioreactors for the production of ethanol from acetate was suggested (Steinbusch et al., 2009), however, addition of electron donors in BES has not been studied. Although electron acceptors seemed to be more involved in chain elongation, previous comprehensive reviews on MES and EF processes emphasized the role of polarised cathodes in the nature of the products (Jiang et al., 2018, Moscoviz et al., 2016).

In Chapter 5, it was observed that acetogenic biofilm was formed at the surface of the cathodes dominated by *Acetobacterium* and acetate was the major product in the BESs poised at -1.0 V with dissolved CO_2 as an inorganic carbon source over fed-batch mode. Due to the high concentration of acetate in the solution, pH of the catholytes at the end of most batch cycles dropped to around 4.8-5.0 which is toxic for these bacteria (Angenent et al., 2016, Xu et al., 2018). Furthermore, some products such as formate and ethanol had unstable concentration during the experiment showing their consumption through production of longer chain products

than acetate. Small abundance of *Clostridium* was found in the biofilm and planktonic cells that were more likely involved in production of long chain organics. In this direction, it was hypothesized that providing constant fresh medium, adjusting catholytes' pH and supplying electron donors in addition to cathodes would improve acetate production (required for chain elongation) through *Acetobacterium*, growth of *Clostridium* leading to chain elongation in BESs and subsequently efficiency of the system. Therefore, two different strategies were considered in new experiments. To adjust the pH of catholytes by extracting the products, effect of continuous flow mode on adjusting pH by applying different hydraulic retention times (HRTs) and consequently production was studied. Moreover, addition of 2 different electron donors of ethanol and formate on production was investigated. Ethanol is a well-known electron donor involved in chain elongation from acetate in acetogens (Grootscholten et al., 2013b). In addition, formate has the similar redox potential as H_2 which can be formed through enzymatic activities (for instance formate dehydrogenese) using the electrons supplied from cathodes and be consumed as an energy source for production (Deutzmann et al., 2015). Over the first step of methyl and carbonyl branches in WL pathway, formate is synthesised through formate dehydrogenase which is reduced later for the conversion of inorganic carbon to valuable chemicals. Therefore, it was hypothesized that providing ethanol can steer the production towards longer chain organics and providing formate can optimize acetate production kinetically, which can further divert the reactions to chain elongation. In addition, function of cathode in chain elongation through acetate was studied by further analysis with addition of electron donors to reactors with non-polarised cathodes.

6.2 Material and methods

6.2.1 *Evaluating the effect of continuous operational mode on production through MES*

To investigate the effect of continuous flow mode on production through MES, 2 different reactors in duplicates (4 reactors in total) were operated as described in Section 3.1.2. Reactors were inoculated by 50% of the catholytes of BESs 1 (-1.0 V_CO_2) from Chapter 5. Reactors were poised at -1.0 V and gaseous CO_2 was provided. BESs were started in a fed-batch mode for 14 days to develop biocathode by allowing bacterial community to adapt to fresh reactors. Thereafter, operational mode was switched to a continuous feeding regime using a dosing pump. The dosing pump was connected to 0.5 L Duran bottle of fresh medium continuously purged by CO_2/N_2 (90/10). Fresh medium was flushed continuously from the inlet port to cathodic

compartment removing catholyte through the outlet port. Outlet ports were connected to anaerobic tubing to the closed effluent 0.5 L Duran bottle (different from 0.5 L fresh medium Duran bottle) to prevent the diffusion of oxygen through the ports. No chemical methane inhibitor was added to the cathodic medium. The total volume of the cathodic compartment was 80 ml and the flow rate was adjusted based on the desired HRT. The effect of two different HRTs of 3 and 7 days were investigated on production in BESs. From day 14 to day 56 (42 days) a HRT of 3 days was applied and it changed to 7 days for the following 42 days.

Liquid and gas samples were collected over the fed-batch mode and at the end of each retention time after switching to continuous mode, from the BESs to detect the products using IC and GC, as described in Sections 3.6.5, 3.6.6 and 3.6.7 and measure the pH of liquid samples. Total inorganic and organic carbon in the catholytes were measured irregularly as described in Section 3.6.4. After 102 days of continuous flow experiment and adjusting the pH to ~ 6.5 using 1 M NaOH or 1 M HCl, CV was performed as described in Section 3.4.3 to compare the electrochemical properties of the biofilm formed at the cathode with the biofilm enriched under fed-batch mode (Chapter 5).

6.2.2 *Evaluating the effect of addition of electron donors on production through MES*

The effect of adding two different electron donors of formate and ethanol with polarised cathodes on production through MES was investigated. 3 different reactors in duplicates (6 reactors in total) were operated in a fed-batch mode similar to reactors described in Section 3.1.2, but with larger cathodes (projected surface area of 15 cm^2) to provide enough cathodic energy source for bacterial community. Reactors were inoculated by 50% of the catholytes of BESs 1 (-1.0 V_CO_2) from Chapter 5, poised at -1.0 V and gaseous CO_2 was purged in the catholytes every 2-3 days through recirculation as described in Section 3.3.3. 40% of cathodic medium was refreshed every 10 - 15 days. No chemical methane inhibitor was added to the cathodic medium. BESs were started in a fed-batch mode for 35 days to develop biocathode by allowing bacterial community to adapt to fresh reactors. On day 35, formate and ethanol were added separately to the BESs with the final concentrations of half the molarity of acetate presented in the catholyte. Ethanol and formate were added to the cathodic compartments during the recirculation of catholytes while providing CO_2. Electron donors were added to the BESs for 45 days from day 35 to day 79 comprising 13 batch cycles. Each batch cycle lasted approximately 3-4 days, between each addition of electron donors. pH of the solution was

adjusted to ~ 5.8 at the beginning of each batch cycle. Concentration of electron donors in the catholytes were topped up to reach half the molarity of acetate at the end of each cycle, to achieve the similar ratio of ethanol/acetate and formate/acetate (1/2) at the beginning of each batch cycle. However, as no ethanol was consumed between days 44 and 51 in reactors with ethanol, 40% of medium was changed by fresh medium without adding ethanol on day 51. On day 58 of the experiment, ethanol (half the molarity of acetate in the catholyte) was added to the solution and no ethanol was added after that to the catholytes until the end of the experiment due to the low rate of ethanol consumption.

Liquid and gas samples were collected every 3-4 days to detect the products using IC and GC, and measure the pH of liquid samples. Total inorganic and organic carbon in the catholytes were measured irregularly.

The experimental design used for the experiments explained in this section is summarized in Table 6.1.

Table 6.1. Experimental design used for the experiment (79 days) to investigate the effect of addition of electron donors on production through MES.

Reactor	**BESs_formate**	**BESs_ethanol**	**BESs_control**
Potential (V)	-1.0	-1.0	-1.0
Inorganic carbon	CO_2	CO_2	CO_2
Cathodic inoculum	Inoculum enriched in BESs 1 (-1.0 V_CO_2)	Inoculum enriched in BESs 1 (-1.0 V_CO_2)	Inoculum enriched in BESs 1 (-1.0 V_CO_2)
Electron donor	Formate	Ethanol	-

6.2.3 *Identifying bacterial composition in planktonic cells*

Biological analysis were carried out from the catholytes of BESs in this chapter at the end of the experiments to investigate the effect of different strategies on the bacterial cells growth and their composition. Cell count and bacterial community analysis was performed from the catholytes of BESs after continuous operational mode and addition of electron donors and were

compared to that in the initial inoculum, which was BESs 1 (-1.0 V_CO_2) from Chapter 5. Cell counts were carried out using a flow cytometer as described in Section 3.5.2 to investigate the bacterial cells growth by counting the number of cells in the catholytes reported log-transformed. 16S rRNA gene-based community analysis was performed as described in Section 3.5 to reveal the identity of planktonic cells in the catholytes. For that, DNA was extracted from the catholyte samples of the BESs, which was also amplified using Polymerase chain reaction (PCR). For DNA extraction, 2 samples were collected from each reactor. As each condition was operated in duplicate, therefore 4 samples were collected for DNA extraction from each condition. The communities of samples from the catholytes were sequenced using Illumina MiSeq and processed with "Dada 2" package in R statistical platform as explained in Sections 3.5.6 and 3.5.7. Before taxonomy assignment, diversity of the samples was carried out using Shannon index analysis from data (ASV table). Sequences were assigned using Ribosomal Database Project (RDP) as a database with bootstrap 80.

6.2.4 *Investigating function of cathode in production of acetate and longer chain organics in BES*

One of the important questions in MES and EF processes is the role of cathode in nature of the products in BES. Therefore, only electron donors were provided for the developed biocathode. After 45 days adding electron donors to BESs, reactors were disconnected from potentiostat. 50% of cathodic medium were refreshed to provide the fresh nutrients for biocathodes. Electron donors were added for another 30 days to BESs_formate and BESs_ethanol with non-polarised cathodes, under open ciruit potential (OCP) condition, while no electron donor was added to BESs_control during this period. Production in the reactors were investigated when energy for bacterial activity was supplied only through electron donors without the effect of polarised cathodes in BESs_formate and BESs_ethanol, while no energy source was provided in BESs_control. Similar to the experiments of BESs with polarised cathodes, dissolved CO_2 was provided in the reactors during OCP experiment.

Formate and ethanol with half the molarity of acetate present in the catholytes were added to the reactors two times during the experiment; on day 1 and day 15 and products were detected during the experiment. Liquid and gas samples were collected every 3-4 days to detect the products using IC and GC, and to measure the pH of liquid samples.

6.2.5 *Summary of the analyses*

The analyses carried out from the BESs throughout the experiments in this chapter are summarized in Table 6.2.

Table 6.2. Electrochemical, analytical and biological analyses carried out from the BESs during the experiments in this chapter.

Analysis / **BESs**	CA	CV	Products detection	Cell count of catholyte	16S of catholyte	OCP control experiment
BESs at continuous operational mode	✓	✓	✓	✓	✓	×
BESs_formate	✓	×	✓	✓	✓	✓
BESs_ethanol	✓	×	✓	✓	✓	✓
BESs_control	✓	×	✓	✓	✓	✓

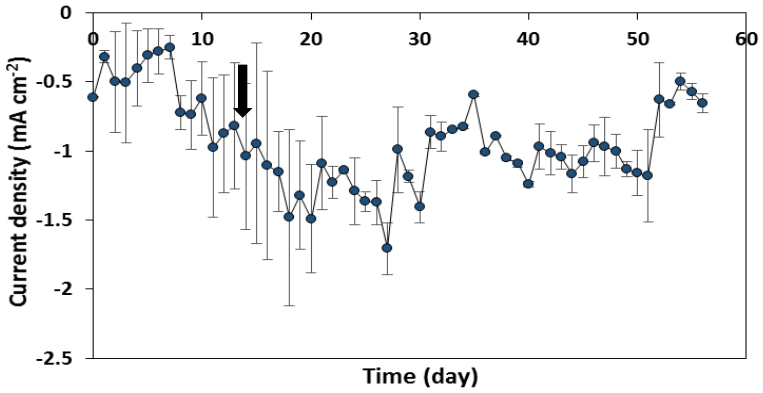
6.3 Results and discussion

6.3.1 *Effect of continuous operational mode on production through MES (HRT: 3 days)*

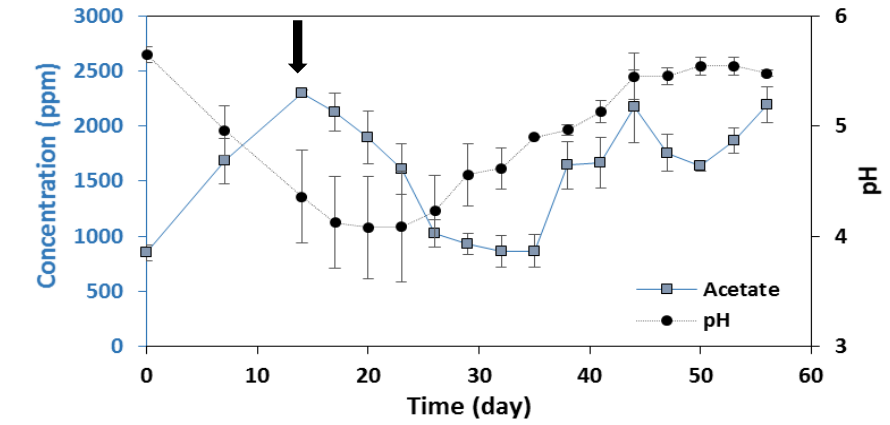
The effect of continuous feeding regime on production in MES was investigated. Regular refreshment of medium could not only maintain the pH of the catholytes close to the desired value and recover the products leading to enhancement in the reaction equilibrium, but also supply CO_2 and fresh nutrients constantly available for microorganisms (LaBelle et al., 2014a). Two fresh BESs were operated in fed-batch mode for 14 days and the operational mode was switched to continuous feeding regime with HRT: 3 days from day 14. HRT of 3 days was selected as due to Chapter 5, after approximately 3 days catholytes pH dropped to almost 4.8-5. Inoculating from the active and developed biocathode led to the rapid start-up of the BESs in cathodic electron uptake and production of acetate. As can be observed in Figure 6.1 (a), cathodic current consumption started from -0.6 ± 0.0 mA cm^{-2} immidiately after starting the BESs, which gradually increased to around -1.0 ± 0.5 mA cm^{-2} at day 14, before switching the operational mode to continuous. During the experiment of continuous feeding regime, current

consumption varied between -1.0 and -1.5 mA cm^{-2} until day 56, the last day of applying HRT of 3 days.

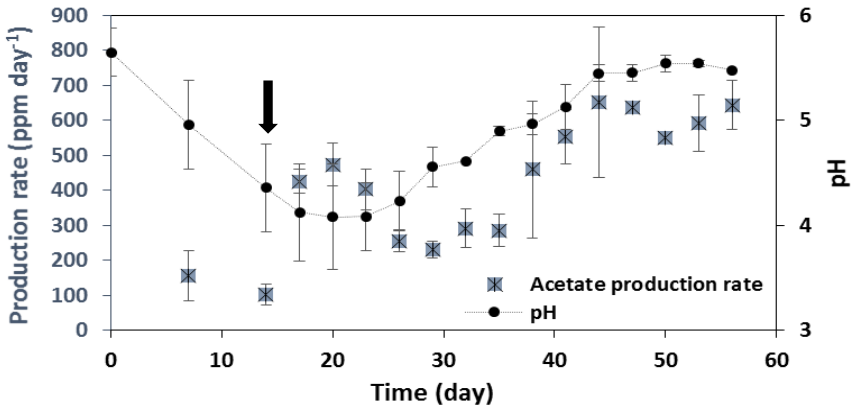
(a)



(b)



(c)



(d)

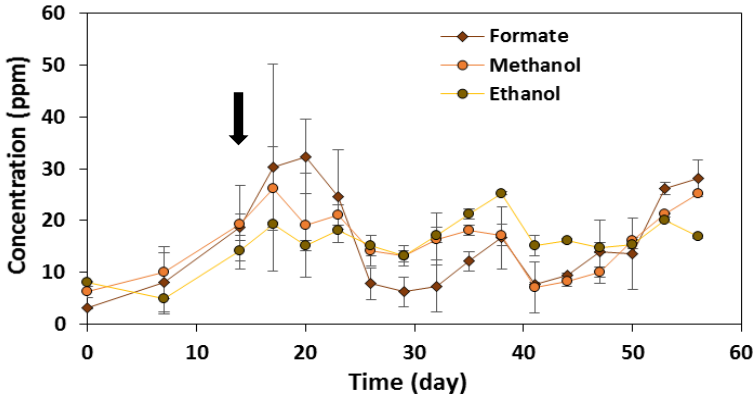


Figure 6.1. (a) Cathodic current consumption, (b) acetate concentration (c) acetate production rate and (d) other products than acetate detected in the BESs poised at -1.0 V and fed by CO_2 before and after changing to continuous operational mode with HRT of 3 days. Black arrow shows switching the operational mode from fed-batch to continuous.

Notable concentration of acetate was produced during the first 14 days fed-batch mode and reached the concentration of 2297.3 ± 29.5 ppm (76.5 ± 0.9 mmole C L^{-1}) at day 14, with acetate production rate of 155.2 ± 71.0 and 102.1 ± 30.2 ppm day^{-1} at day 7 and 14, respectively (Figure 6.1 (b)). Production of acetate was associated with the significant drop of catholytes pH from 5.6 ± 0.0 at day 1 to 4.4 ± 0.4 at day 14. Figure 6.2 demonstrates columbic efficiency of the system through acetate production during the experiment as the major product of the BESs. Similar to BESs 1 from previous chapter under fed-batch operational mode, columbic efficiency in average of the 14 days experiment was 58.4 ± 23.0 %. After switching the operational mode to continuous with HRT of 3 days, acetate concentration in the solution began to decrease due to the removal of acetate accumulated in the catholytes. Columbic efficiency of the system between days 14 and 35 was 55.2 ± 14.8 % similar to that in fed-batch mode over the first 14 days of experiment.

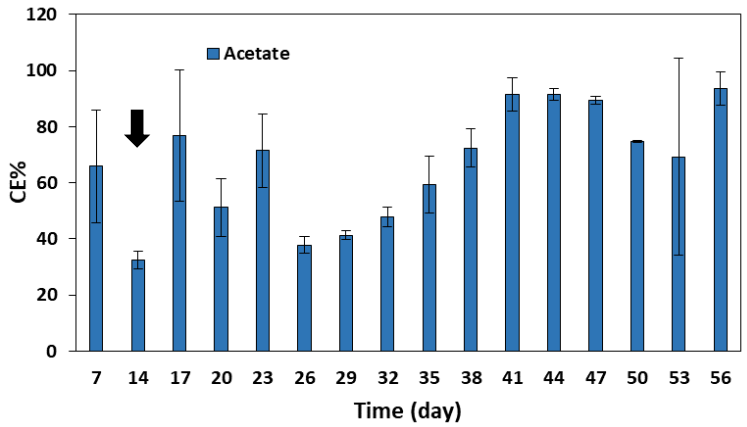


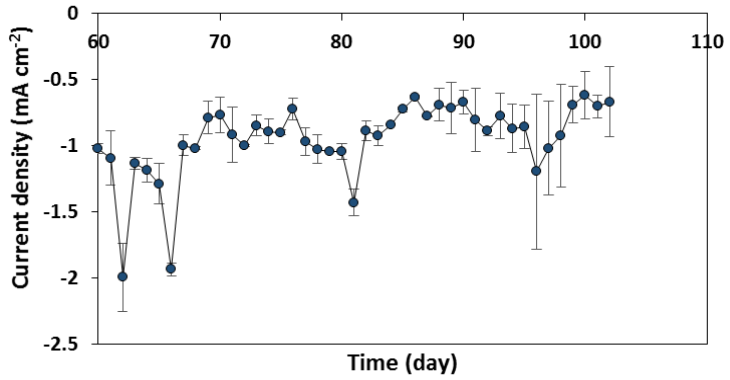
Figure 6.2. Columbic efficiency of BESs during the experiment of continuous operational mode (HRT: 3 days). Black arrow shows switching the operational mode from fed-batch to continuous.

As can be observed in Figure 6.1 (c) acetate production rate began to increase, reaching the maximum production rate of 651.8 ± 214.2 ppm day^{-1} (21.7 ± 7.14 mmole C L^{-1} day^{-1}), with maximum 803.3 ppm day^{-1} (26.8 mmole C L^{-1}) in one of the duplicates, 30 days after switching the operation to continuous feeding regime. In addition, columbic efficiency reached the average value of 83.2 ± 10.5 % between days 35 and 56, which was the highest value during the experiments (maximum of 90 % in one of the duplicates). This strongly indicated superior performance of BESs operated on continuous operational mode compared to fed-batch operation in terms of acetate production and systems efficiency by constant removal of acetate from the catholyte and pH adjusted around 5.5. Over the 56 days of experiment, acetate was the dominant product detected in the solution and no H_2 was detected in the headspaces. Trace concentration (less than 40 ppm) of formate, methanol and ethanol were detected irregularly in the catholytes during the experiment as shown in Figure 6.1 (d), but no other longer chain of organic acids or alcohols were detected. Number of the cells in the catholyes measured in the last day of fed-batch mode (day 14) and last day of continuous mode (day 56) with HRT of 3 days was almost stable, indicating the persistent cells growth. The number of cells was around $5.5 \times 10^7 \pm 4.5 \times 10^7$ on day 14 and $1.1 \times 10^7 \pm 4.6 \times 10^6$ on day 56. Slight decrease in the number of the cells could be due to the removal of biomass accumulated in suspension. Higher columbic efficiency achieved in BESs at continuous mode (HRT: 3 days) than BESs at fed-batch mode indicated that higher inorganic carbon was converted to products rather than biomass when fresh medium was flushed constantly in the cathodic compartment. At the end of the experiment, a visible biofilm could be observed at the surface of the cathode.

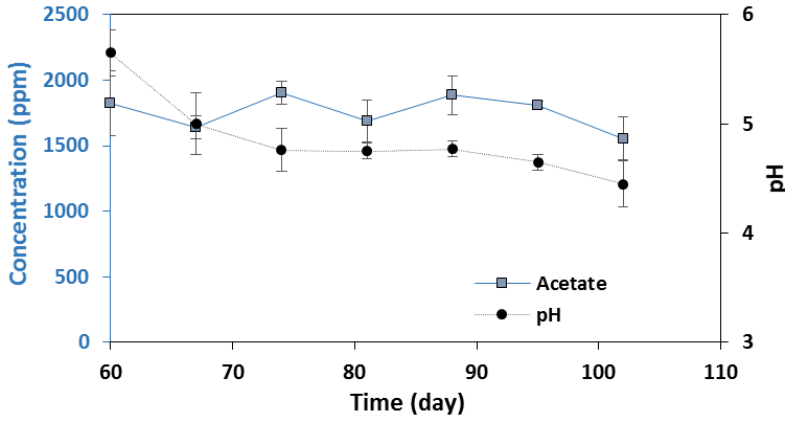
6.3.2 *Effect of continuous operational mode on production through MES (HRT: 7 days)*

After 42 days operation of continuous flow with HRT 3 days, retention time was changed to longer period of 7 days. HRT of 7 days was selected to investigate the acidic pH provided by accumulation of the products on production while fresh medium was constantly flushed to the catholytes. The cathodic current, products concentrations and pH are demonstrated in Figure 6.3.

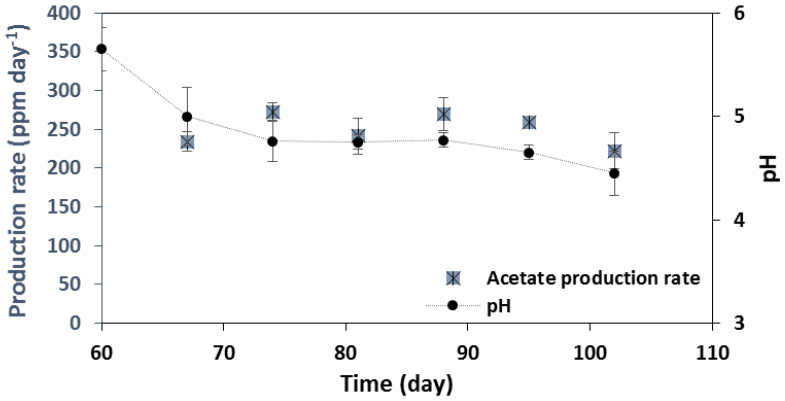
(a)



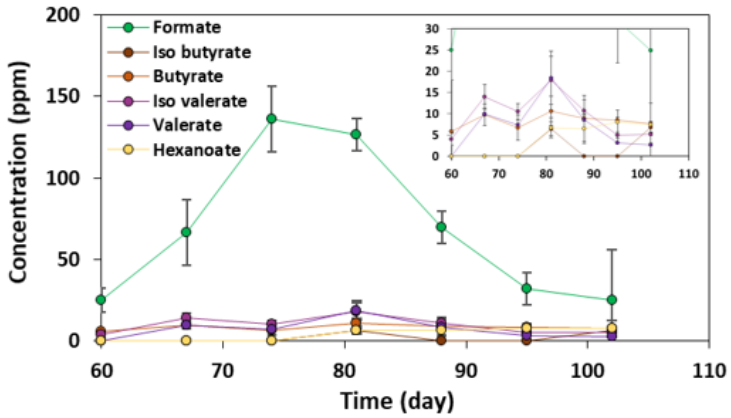
(b)



(c)



(d)



(e)

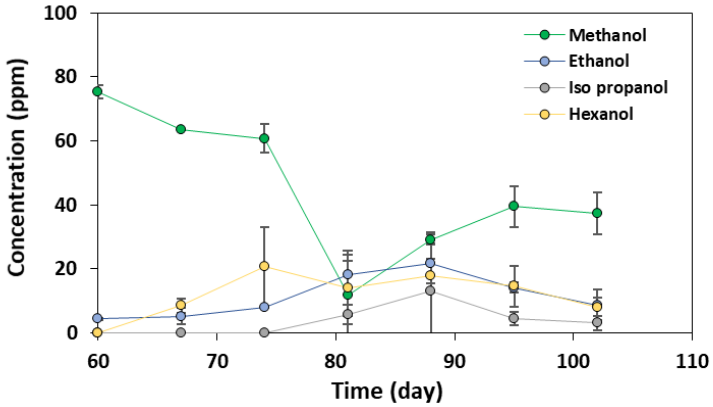


Figure 6.3. (a) Cathodic current consumption, (b) acetate concentration, (c) acetate production rate, (d) organic acids (insert: zoom in), and (e) alcohols detected in the BESs poised at -1.0 V and fed by CO_2 at continuous operational condition with HRT of 7 days.

Applying longer retention time led to pH drop to around 4.3-4.7 during the experiment. Current density at HRT: 7 days was around -1.0 mA cm^{-2} with decrease from day 80. The concentration

of acetate detected in the solution was lower than that in BESs at HRT: 3 days but more stable. The highest acetate concentration was achieved on day 74 of experiment 1904.1 ± 86.2 ppm (63.5 ± 2.8 mmole C L^{-1}) with acetate production rate of 272.0 ± 12.3 ppm (9.1 ± 0.4 mmole C L^{-1}). Lower acetate production rate during HRT: 7 days compared to that of HRT: 3 days could be due to accumulation of products and consistent acidic condition provided during HRT of 7 days (less than 5), which was reported that could provide toxic conditions for acetogenic activity (Demler and Weuster‐Botz, 2011, Ge et al., 2015a, Kucek et al., 2016). Formate was detected with higher concentration than that observed in HRT: 3 days, at around 140 ppm 15 days after switching the retention time to 7 days, however its concentration was not stable.

The pH drop was associated with production of diverse products of butyrate, iso butyrate, valerate, iso valerate, hexanoate and hexanol, which were not detected in the reactors at HRT of 3 days; however, the concentration of these products was less than 25 ppm during the experiment. This was the first time that C6 products were detected in this study, although with trace concentration, which could be related to the composition of bacterial community responsible for their production. Initial inoculum used for continuous flow experiment was dominated by *Acetobacterium* as described in Chapter 5, which is known in production of acetate as a major product from CO_2. Production of longer chain organic acids than acetate could be related to other bacterial community found in initial inoculum such as *Clostridium*, which their trace concentration could be due to low abundance (< 1%) of these bacterial community in the inoculum. Production of longer chain alcohols was more likely related to the low pH provided. Similar observation was reported in the previous report (Ganigué et al., 2016). The authors discussed that pH lower than 5.0 ceased the production of long chain VFAs and promote the production of alcohols through solventogenesis mechanism. After 42 days of continuous operational mode with HRT: 7 days, number of the cells in the catholytes were measured. In contrast to cell count after HRT: 3 days, number of the cells in the catholytes increased to of $3.9 \times 10^7 \pm 1.8 \times 10^6$. Comparing BESs on continuous operational mode of HRT: 7 days with BESs on fed-batch mode from Chapter 5 (BESs 1), number of the cells was almost similar in both conditions, which indicated the accumulation of biomass in suspension. However, columbic efficiency of the system in acetate production improved during continuous feeding regime compared to that at fed-batch mode. Due to the low concentration of other products than acetate and lack of detection of hydrogen in BESs, columbic efficiency was calculated considering the acetate production as illustrated in Figure 6.4. Similar to HRT: 3 days, columbic efficiency was significant, with the highest value of approximately 81% over the last 21 days of continuous feeding regime experiment with HRT of 7 days. Slight decrease

in columbic efficiency at HRT: 7 days than that calculated in HRT: 3 days for acetate production was more likely due to trace production of long chain products detected.

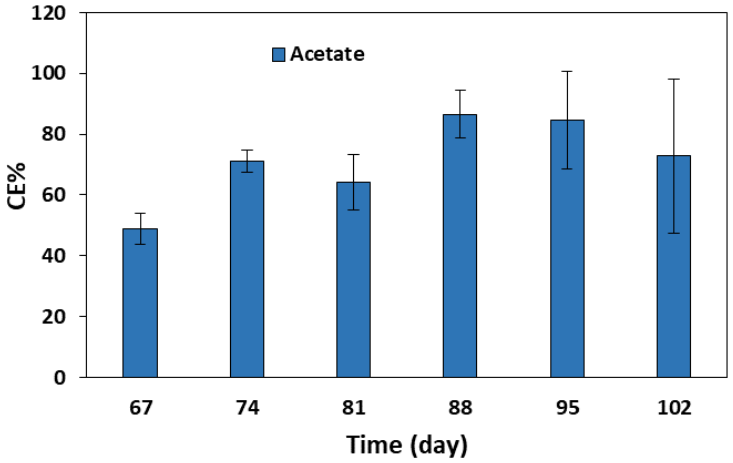


Figure 6.4. Columbic efficiency of BESs during the experiment of continuous operational mode (HRT: 7 days).

Results from Chapter 5 showed that slightly acidic medium at the pH between 5.5 and 5.8 provided an optimal condition for acetogenic growth and subsequently MES processes. Similar result was also reported from previous studies (Abubackar et al., 2011, Batlle-Vilanova et al., 2016). In this study, switching the operational mode from batch to continuous feeding regime showed the promising strategy for improvement in acetate production and efficiency of the system. Highest acetate production rate (803.3 ppm day^{-1}) of the experiments in this thesis was achieved over the continuous operational mode at HRT of 3 days when pH was adjusted at around 5.5. In addition, achieving the columbic efficiency more than 80 % showed the significant improve in the efficiency of the system over the continuous operational mode with both values of HRT (3 and 7 days) in comparison with that (maximum ~ 69 %) over fed-batch operation observed in the previous chapter. WL pathway is the only CO_2 fixation pathways that is contributed to energy conservation (Schuchmann and Müller, 2014). Indeed, cells growth and production of acetate from CO_2 is coupled with ATP formation. Therefore, higher biomass turnover rate can lead to enhance in the performance of BESs in terms of higher electron recovery and acetate production (Schuchmann and Müller, 2014). Comparing two HRTs of 3 and 7 days, it could be concluded that shorter retention time implied higher removal rate of biomass and products leading to higher energy demands for bacteria and thus higher growth

rate. Similar trend was observed in the previous study on different feeding regime in BES (Arends et al., 2017). Three different HRTs of 3.3, 5 and 6.7 days were investigated in their study which acetate was the major product in all conditions, but the highest acetate production rate (~ 0.9 g $m^{-2}_{cathode}$ day^{-1}) was reported at lowest HRT of 3.3 days when pH was adjusted around 5.5 leading to less diversity in products. However, longer chain products of butyrate and isopropanol were detected at longer retention time due to lower pH provided from accumulation of acidic products. As H_2 was the second significant product in their study, columbic efficiency considering acetate production reported (~ 63%) was lower than that in this study. This could be due to higher surface area of the cathode (500 cm^2 L^{-1} ration of cathode projected surface to cathodic volume) and more negative cathodic potential (-5 A $m^{-2}_{projected\ surface\ area}$ fixed, equivalent to approximately 1200 mV applied) in their study, leading to higher production rate of H_2 than its consumption through MES. However, no H_2 was detected in the headspaces of the reactors in this chapter over 102 days of continuous operational mode contributing to higher columbic efficiency for acetate production.

Apart from adjusting pH and providing fresh nutrients for bacterial community, constant removal of products and providing soluble CO_2 was postulated as another key reason for optimizing the system. Removal of acetate from the catholytes, the major product during continuous flow experiment, and providing soluble CO_2 could divert the acetate production reaction from CO_2 (Equation 2.11) to higher production of acetate to achieve the equilibrium contributing to highest acetate production rate in this chapter. Similar trend was observed in the recent study, in which continuous operational mode enhanced the production of organics from soluble CO_2 and chain elongation (production of C4 and C6 products) significantly (Jourdin et al., 2019). Similarly, it was reported that continuous feeding regime improved the production of C4 products in BES (Raes et al., 2017). Although continuous feeding regime strategy reported in this section was implied to steer the production towards longer chain products, acetate was still the major product more likely due to bacterial community dominated by *Acetobacterium* enriched from initial inoculum. This could also be related to the shorter term of experiment (102 days) compared to previous studies which were operated more than 200 days (Jourdin et al., 2018, Jourdin et al., 2019). (Srikanth et al., 2018a) also reported production of long chain products up to C4 during 320 days experiment.

It is worth mentioning that during the continuous feeding regime, biofilm visible at the surface of cathodes got also much denser than that during fed-batch mode which could be seen by naked eyes. Figure 6.5 shows the image of the dense slightly white biofilm developed at the surface of the electrodes after 102 days of continuous operational mode. Previous studies on pure

culture of acetogens reported the white colour of biofilm of *Acetobacterium* (Balows et al., 2013). As the BESs were inoculated from the catholyte of previous experiment with significant abundance of *Acetobacterium* and due to the high production of acetate during continuous feeding regime experiment the biofilm was more likely comprised of *Acetobacterium*. Formation of dense biofilm at the cathode could be as a result of constant supplying of fresh nutrients for bacterial community. The positive effect of minerals and vitamins on activity and production through acetogens was reported before (Ammam et al., 2016).

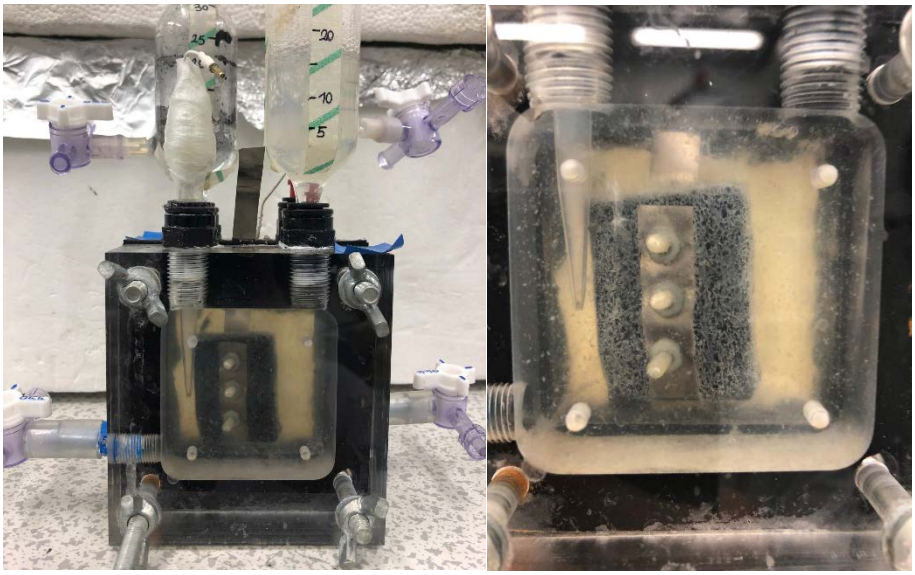


Figure 6.5. Images of the biofilm at the surface of the cathodes (back side) after 102 days of continuous feeding regime experiment.

6.3.3 *Effect of continuous operational mode on electrochemical properties of biofilms*

Formation of denser visible biofilm at the surface of cathode after 102 days at continuous operational mode compared to that after 104 days of fed-batch mode experiment, arraised the question regarding the difference in electrochemical property of the biofilms developed in different operational mode conditions. Figure 6.6 represents the CVs recorded from plain electrode, biofilm after 104 days of fed-batch mode from Chapter 5 (BESs 1) and after 102 days of continuous mode in this chapter. pH in the reactors were adjusted at around 6.5 manually before performing CV. The most significant change between the voltammograms was increase in the draw current between the potentials of -1.0 and -1.2 V. Insert figure shows the shift in onset potential of H_2 evolution from -0.8 V at the end of fed-batch experiment to around -0.6 V

at the end of continuous experiment. This strongly confirms the positive effect of continuous feeding regime on biotic production of H_2, even though no H_2 was detected in the headspaces of the BESs showing its consumption through MES processes. This justifies the more significant production rate of acetate during continuous operational mode compared to fed-batch mode.

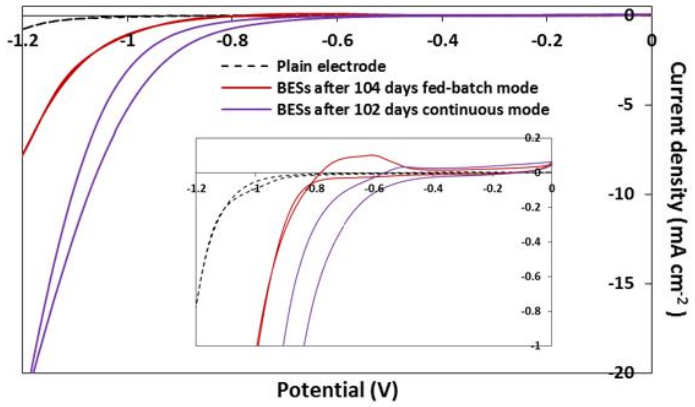


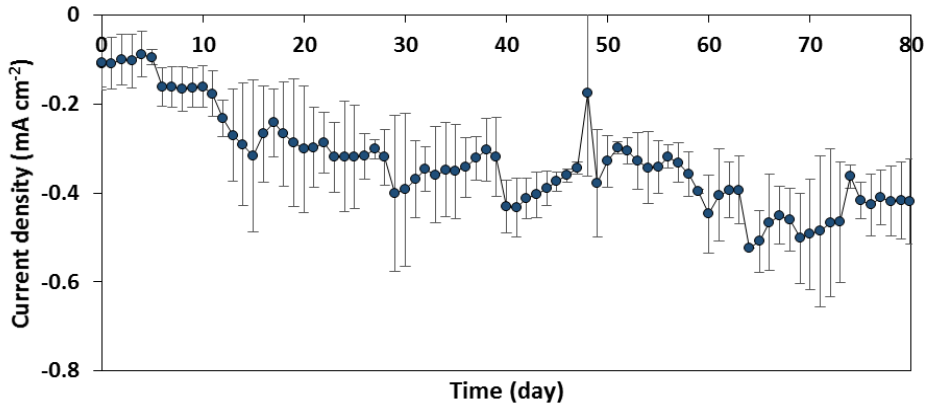
Figure 6.6. Cyclic voltammograms recorded from the plain electrode, cathode with biofilm formed after 104 days fed-batch mode (previous chapter) and cathode with biofilm formed after 102 days continuous operational. Insert figure is the zoom in of small section of the voltammograms.

6.3.4 *Effect of increase in cathode surface area on production through MES*

Six new reactors (3 duplicates) as described in Section 6.2.2 were operated and inoculated with catholytes of BESs 1 from Chapter 5, with the pH adjusted at 5.8 at the beginning of each batch cycle. In the experiments performed by far for CO_2 conversion in BESs, the ratio of cathode projected surface area to catholyte volume were 61 cm^2 L^{-1}. However, in this part of the study reactors were designed with larger ratio of cathode surface to catholyte volume of ~ 187 cm^2 L^{-1} to provide sufficient energy for bacterial activity. This was due to the observations from previous experiments in Chapter 5 that indicated polarised cathode played an important role in supplying energy for MES processes and biofilm formation. Thus, graphite felt with larger projected surface area was used in this part of the experiment to assure the availability of electrons required for MES and EF processes, either in form of H_2 as a mediator, which showed the dominant mechanism of electron transfer between microorganisms and cathode (Chapter 5)

or through direct electron transfer. Two out of three duplicate reactors (BESs_formate and BESs_ethanol) were operated to investigate the effect of addition of formate and ethanol separately on production, whereas no electron donor was added to the other duplicate (BESs_control). Figure 6.7 shows the cathodic current consumption and production in BESs_control. As biocathode was already enriched through the previous experiment, electron uptake from the electrode and production began immediately after starting the reactors. Cathodic current started from approximately -0.1 mA cm^{-2} and increased over time, remained almost stable around -0.5 mA cm^{-2} from day 40 onward. Similar to what observed in previous experiments in this thesis, acetate was the major product throughout the experiment, with the maximum concentration of 4729.2 ± 164.3 ppm (157.6 ± 5.4 mmole C L^{-1}) on day 79. Production rate of acetate was around 300 ppm day^{-1} (10 mmole C L^{-1} day^{-1}) over the first 10 days, fluctuated throughout the experiment and reached the value of 219.8 ± 30.1 ppm day^{-1} (7.3 ± 1.0 mmole C L^{-1} day^{-1}) at the end of experiment (day 79). Slow production of butyrate was observed over the first 50 days of the experiment and its concentration reached the maximum of 51.5 + 4.9 ppm (2.4 + 0.2 mmole C L^{-1}) on day 79. Methanol, ethanol and trace concertation of iso propanol were the only alcohols detected during this 79 days. Concentration of methanol, ethanol and formate were not stable and remained less than approximately 50.0 ppm as they were more likely consumed through MES processes. Trace concentration of iso valerate was also detected in the catholyte, which did not reach to more than 9 ppm. In contrast to the previous experiments in this thesis operated with the smaller ratio of cathode surface to catholyte volume (61 cm^2 L^{-1}), H_2 was detected during the experiment in the headspaces of BESs_control. Assembling cathode with larger projected surface area led to lower cathodic current density but H_2 detection throughout the experiment. Highest percentage of H_2 was detected over the first 20 days of the experiment (16.5 ± 4.9 %), which was the start-up period of the reactor, indicating the low rate of H_2 consumption and consequently low rate of acetate production. However, H_2 percentage decreased to (9.0 ± 2.8 %) after day 20, and reached the lowest value of 4.5 ± 0.7 % over the last 10 days of the experiment, when the highest acetate and butyrate concentration was observed indicating the consumption of H_2 during production. Slight decrease in the percentage of H_2 detected over time in the system indicated the constant growth and enrichment of H_2 consuming acetogens in the cathodic compartment. This strongly confirmed the fact proved in Chapter 5 regarding the role of cathode in providing electrons more likely in form of H_2 as a mediator for cathodic reactions.

(a)


Current density (mA cm-2)
Time (day)
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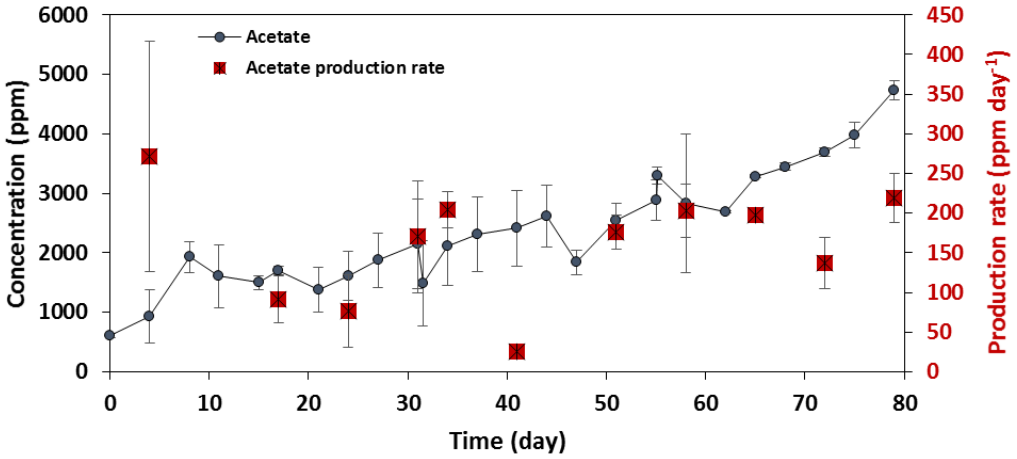
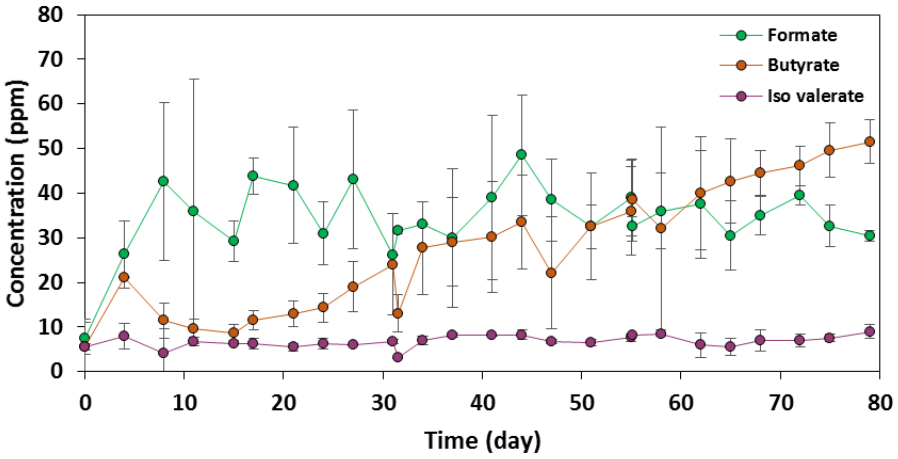
-0.4
-0.6
-0.8
0
10
20
30
40
50
60
70
80

(b)


Acetate
Acetate production rate
Concentration (ppm)
Production rate (ppm day-1)
Time (day)
6000
5000
4000
3000
2000
1000
0
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
80

(c)


Formate
Butyrate
Iso valerate
Concentration (ppm)
Time (day)
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70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80

(d)

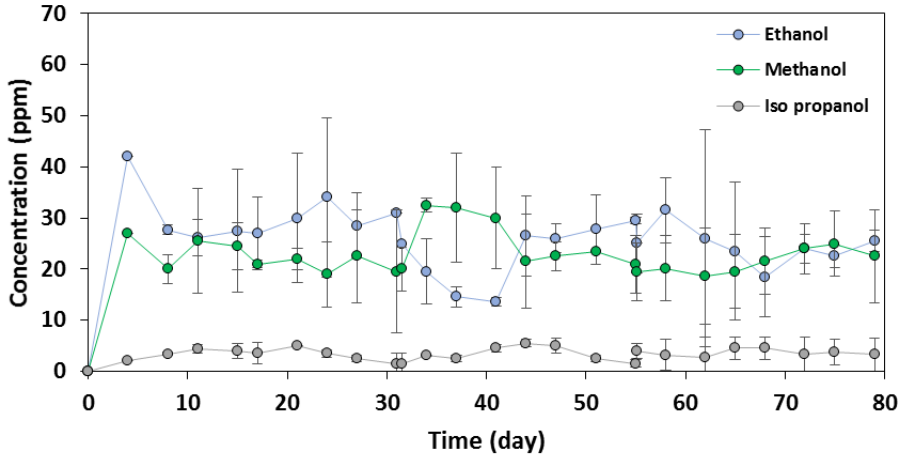


Figure 6.7. (a) Cathodic current consumption, (b) acetate concentration and acetate production rate, (c) organic acids and (d) alcohols detected in BESs_control during the 79 days of experiment.

6.3.5 *Effect of providing formate in BES on production through MES*

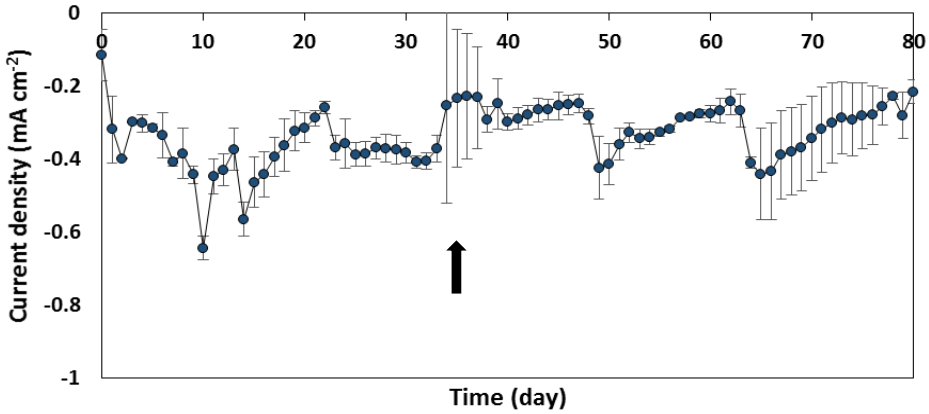
BESs_formate were operated in fed-batch mode for 34 days without addition of formate. Similar to BESs_control, cathodic current consumption began after starting the experiment and remained around -0.5 mA cm^{-2} over the first 34 days (Figure 6.8 (a)). Similarly, as shown in Figures 6.8 (a), (b) and (c) gradual increase was observed in the concentration of acetate and butyrate during this period and reached the maximum concentration of 2305.9 ± 278.8 ppm (76.8 ± 17.9 mmole C L^{-1}) and 37.9 ± 7.9 ppm (1.7 ± 0.3 mmole C L^{-1}) on day 19, respectively. Trace amount of iso valerate, methanol and ethanol (less than 8 ppm) and unstable concentration of formate (remained less than 40.0 ppm) was also detected during the first 34 days. The production in BESs_fromate showed the similar trend as that observed in BESs_control during the first 34 days of experiment. Concentrations of acetate and formate were measured on day 34 and formate was added to the medium to reach the final concentration of half the molarity of acetate presented in the catholyte. Two days after adding formate to the BESs, current collector in one of the duplicates split from the cathode, therefore BES had to be disassembled under anaerobic condition and the electrode was re-connected to the current collector. Hence, large error bars observed in Figures 6.8 (b) and (c) for acetate and formate concentrations are due to the fact that formate was not consumed in this BES during the first batch. Although cathodic current and formate consumption in this reactor recovered after the second batch cycle, no depletion in formate concertation was observed at the end of few batch

cycles (8^{th} batch cycle (day 58), 9^{th} batch cycle (day 62) and 13^{th} batch cycle (day 75)), while it was consumed gradually over the following cycles. In the other duplicate, however, trace concentration of formate was detected in the solution after 3-4 days, indicating the consumption of formate during each batch cycle. Formate consumption observed during the experiment could be relevant to its function as an additional source of carbon or energy apart from CO_2 or H_2. Moreover, formate would be synthesized via formate dehydrogenase in the first step of WL pathway. Presence of additional formate could provide the supplemental energy which could minimize the energy required for production of acetate from CO_2 (Ramió Pujol et al., 2014).

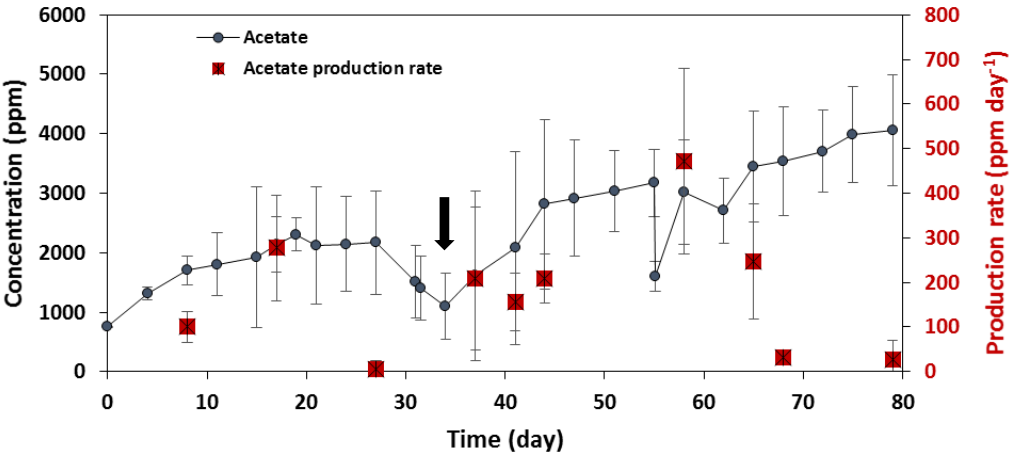
Providing formate in cathodic medium contributed with few notable changes in production. The first significant change was increase in the concentration of acetate in the catholyte over the first days of adding formate. Between days 34 and 44 acetate production rate was approximately ~208 ppm day^{-1} (6.9 mmole C L^{-1} day^{-1}). Changing 50% of medium on day 55 led to increase in the rate of acetate production, reaching the value of 472.4 ± 208.3 ppm day^{-1} (15.7 ± 6.9 mmole C L^{-1} day^{-1}). However, this rate was much lower, around ~30 ppm $days^{-1}$ (~1 mmole C L^{-1} day^{-1}) between days 44 and 55, and days 58 and 79 more likely due to its consumption through chain elongation observed. The concentration of iso valerate did not change during the formate addition. However, considerable change was observed in the concentration of butyrate in the solutions after day 65, increasing from 19.1 ± 4.4 ppm (0.9 ± 0.2 mmole C L^{-1}) on day 65 to 54.9 ± 22.4 (2.5 ± 0.0 mmole C L^{-1}) on day 68 and reached the highest concentration of 142.7 ± 22.2 ppm (6.5 ± 1.0 mmole C L^{-1}) on the last day of experiment (day 79), 158.8 ppm (7.3 1.0 mmole C L^{-1}) in one of the duplicates. This was the highest concentration of butyrate by far detected in BESs of this experiment, which showed the role of formate involved in its production. The butyrate production rate in average of 5 last batch cycles was 8.5 ± 3.7 ppm day^{-1} (0.4 ± 0.1 mmole C L^{-1} day^{-1}). The other notable change was increase in diversity and concentration of alcohols. Irregular production of methanol and ethanol was observed in 79 days of experiment in BESs_control which remained less than 40.0 ppm. However, in BESs_formate they began to increase after addition of formate while their consumption was also observed during the experiment, reaching the maximum concentration of 114.7 ± 88.1 ppm (3.6 ± 2.7 mmole C L^{-1}) methanol on day 51 and 172.2 ± 83.6 ppm (7.5 ± 3.6 mmole C L^{-1}) ethanol on day 65. Besides, longer chain of alcohols of butanol and trace value of hexanol were detected in the catholytes. Maximum concentration of hexanol was detected on day 65 around 27.4 ± 7.9 ppm (1.6 ± 0.4 mmole C L^{-1}). Additionally, alongside the significant increase in the butyrate concentration, butanol began to increase as well reached the maximum value of 126.9 ± 5.1 ppm (6.8 ± 0.3 mmole C L^{-1}) on day 79. Similar to BESs_control

trace concentration of acetone and iso propanol (less than 14 ppm) were also detected which seemed that addition of formate did not affect the production of these compounds. Although the pH of the catholytes were almost stable around 5.8-6.0 at the beginning of each batch cycle, it decreased to around 4.8 after 3-4 days, which could also cause considerable production of alcohols, in addition to the possible effect of presence of formate on alcohols production.

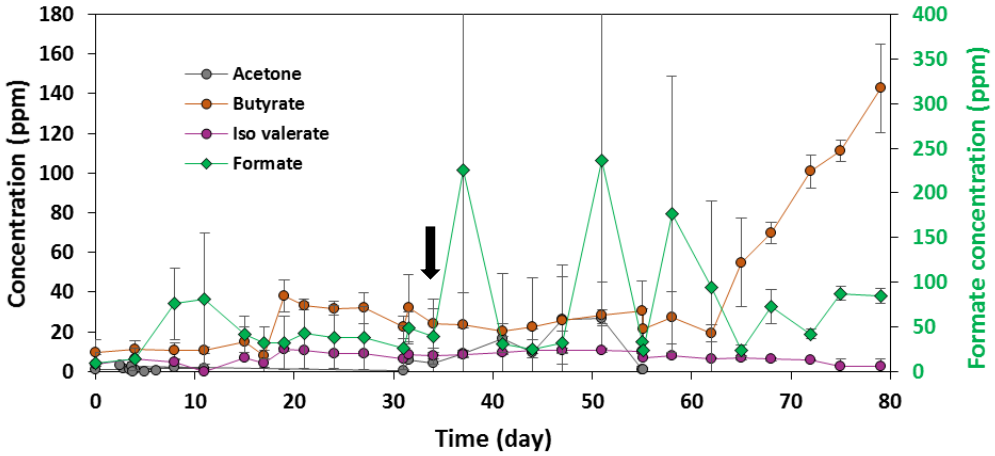
(a)



(b)



(c)



(d)

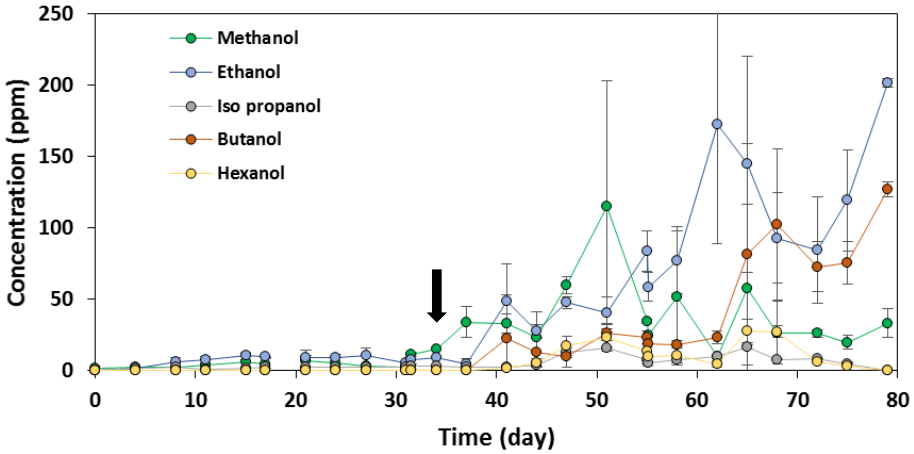


Figure 6.8. (a) Cathodic current consumption, (b) acetate concentration and acetate production rate, (c) acetone and organic acids and, (d) alcohols detected in BESs_formate during the 79 days of experiment. Black arrow shows the beginning of formate addition to cathodic medium.

Comparing the products in BESs_control and BESs_formate, it can be observed that much higher rate of acetate production was measured occasionally in BESs_fromate. When acetate production rate was decreasing, it was simultaneous with detection of longer chain products specifically butyrate and butanol, indicating consumption of acetate through chain elongation. These observations could postulate that considerable proportion of formate was more likely consumed for the production of acetate as a major product and also methanol and ethanol. In addition, high concentration of acetate in the solution led to chain elongation for significant production of butyrate and butanol. Production of trace concentration of hexanol could show the effect of formate in steering the production toward longer chain by increasing the concentration of acetate. One possibility could be involvement of formate in acetate production

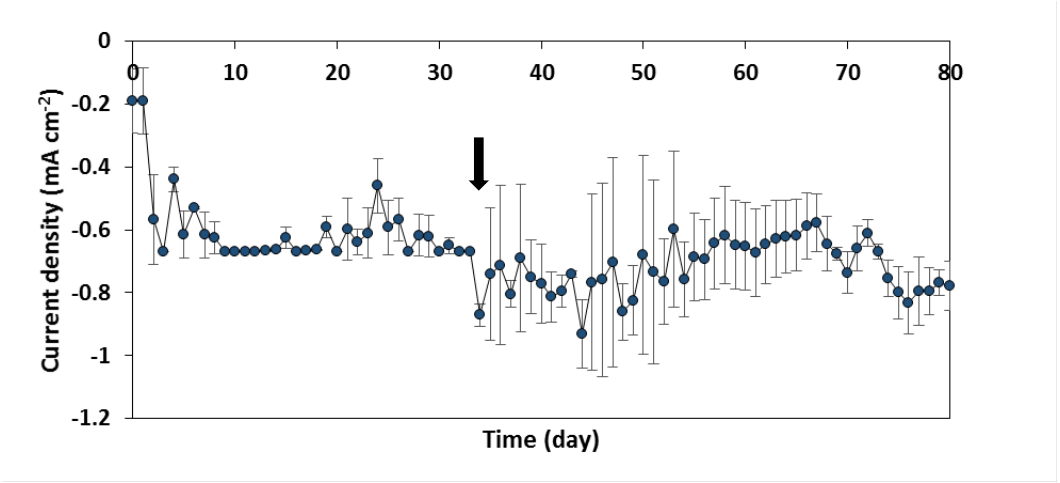
leading to increase in the acetate accumulated in the catholyte. This then resulted in production of C4 and trace C6 products using electrons derived from cathode and/or formate. Another possibility could be involvement of formate only in production of acetate and ethanol, and chain elongation through acetate using ethanol as an electron donor. In both cases, the role of formate as an electron donor was confirmed by detection of large percentage of H_2 in the headspaces. Similar to BESs_control, H_2 was detected in the headspaces during the first 34 days of the experiment, which decreased to approximately 5% between days 24 and 34 by growth in acetogens. However, by adding formate in the medium percentage of H_2 began to increase, which was detected around 26.6 ± 8.9 % throughout the next 45 days of experiment. Due to the complete consumption of formate observed in each batch cycle, this could indicate that formate provided a proper source of energy competing with H_2 for bacterial activities led to limiting the H_2 consumption.

6.3.6 *Effect of providing ethanol in BES on production through MES*

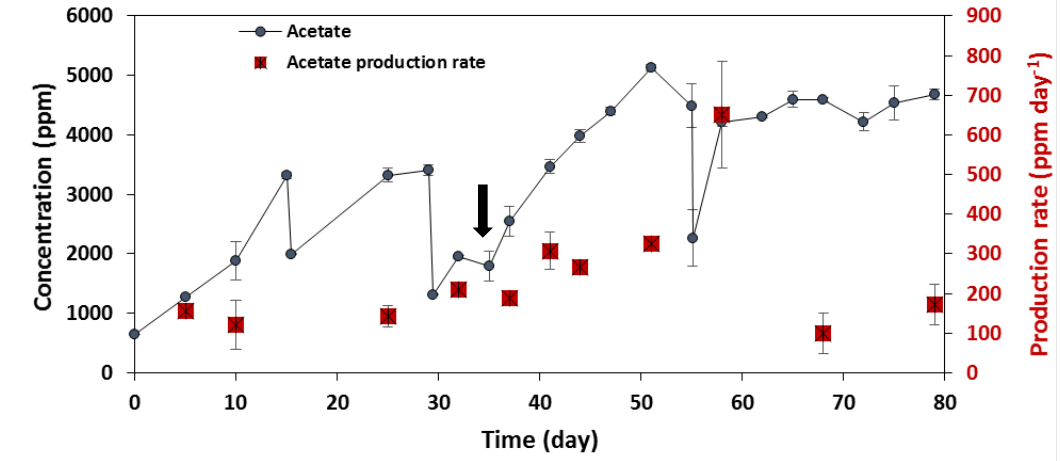
Similar to BESs_formate, concertation of acetate in BESs_ethanol increased over the first 34 days when no ethanol was provided and reached the maximum of 3325.1 ± 119.0 ppm (110.8 ± 3.9 mmole C L^{-1}) on day 29. The average of acetate production rate during this period was 163.8 + 35.7 ppm day^{-1} (5.46 ± 1.2 mmole C L^{-1} day^{-1}). Butyrate (maximum concentration of 29.0 ± 7.0 ppm (1.3 ± 0.3 mmole C L^{-1})) and unstable concentration of formate (maximum of ~ 80.0 ppm (1.8 mmole C L^{-1})), trace amount of iso valerate, methanol and ethanol were also detected in the catholyte during this period (Figures 6.9 (a), (b) and (c)), comparable with the production observed in BESs_control. Ethanol concentration was not stable and remained less than 20.0 ppm during the first 34 days. After day 34, supplying ethanol on day 34 to the BESs was associated with two significant changes in production: 1. Increase in acetate concentration 2. More diversity in products detected throughout the experiment. In addition to all the products detected in catholytes of BESs_formate, trace concentration of iso butyrate (maximum concentration of 8.2 ppm on day 79) and hexanoate (maximum concentration of 10.0 ppm on day 79) were also found in catholytes of BESs_ethanol between days 69 and 79. Moreover, higher concentration of hexanol than that in BESs_formate was detected with the maximum concentration of 39.8 ± 11.1 ppm (2.3 ± 0.6 mmole C L^{-1}) on day 79. Acetone concentration also increased after supplying ethanol in the medium with the maximum concentration of 53.4 ± 46.3 ppm (2.7 ± 2.3 mmole C L^{-1}) on day 62, however it seemed that it was consumed during the experiment as it was detected occasionally. Increase in the concentration of butyrate and

butanol was also observed, particularly over the last 17 days of ethanol adding experiment (from day 62 to 79), however their production rate and final concentration were not as significant as those observed in BESs_formate. Butyrate and butanol concentrations increased from 36.9 ± 4.3 ppm (1.7 ± 0.2 mmole C L^{-1}) and 35.9 ± 15.4 ppm (1.9 ± 0.8 mmole C L^{-1}) on day 62 to 63.15 ± 3.7 ppm (2.9 ± 0.1 mmole C L^{-1}) and 62.4 ± 28.9 ppm (3.4 ± 1.5 mmole C L^{-1}) on day 79, respectively. Moreover, similar to BESs_formate only trace concentration of iso propanol (less than 15.0 ppm) was detected during the whole experiment.

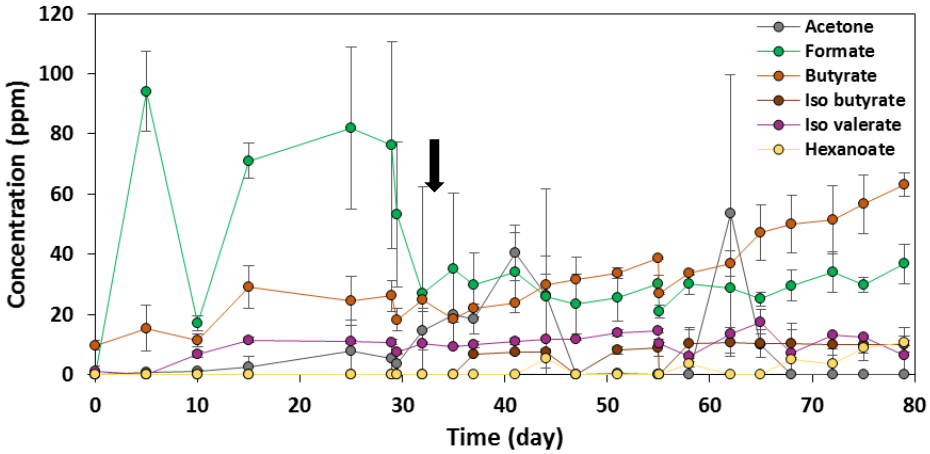
(a)



(b)



(c)



(d)

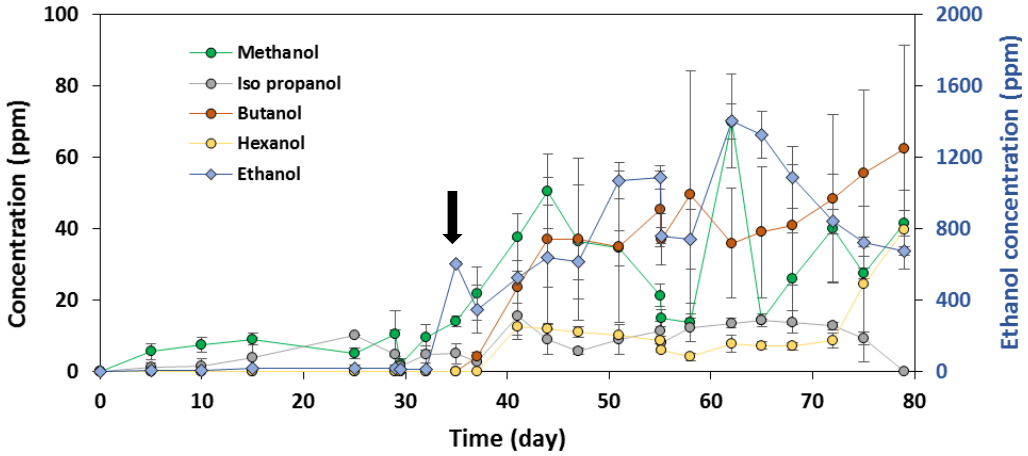


Figure 6.9. (a) Cathodic current consumption, (b) acetate concentration and acetate production rate, (c) acetone and organic acids, and (d) alcohols detected in BESs_ethanol during the 79 days of experiment. Black arrow shows the beginning of ethanol addition to cathodic medium.

Despite the complete consumption of formate perceived in BESs_formate in most of the batch cycles, ethanol added to the medium was not totally consumed at the end of any batch cycle in BESs_ethanol. Between days 34 and 44, while consumption of ethanol in the solutions was observed, concentration of all the products began to increase. During this period, significant products detected were methanol 50.4 ± 3.9 ppm (1.5 ± 0.1 mmole C L^{-1}), acetate 3984.5 ± 102.9 ppm (132.8 ± 3.4 mmole C L^{-1}), butyrate 29.8 ± 3.5 ppm (1.4 ± 0.2 mmole C L^{-1}) and butanol 72.1 ± 45.4 ppm (3.9 + 2.4 mmole C L^{-1}) on day 44. In addition, trace concentration (less than 15.0 ppm) of acetone, iso propanol, isobutyrate, iso valerate, hexanoate and hexanol were found in the catholytes of these reactors.

From day 44 to day 51 no ethanol in the solution was consumed and also the only production observed in the solution was for acetate from 3467.2 ± 116.3 ppm (115.6 ± 3.8 mmole C L^{-1}) on day 44 to 4399.3.3 ± 80.8 ppm (143.0 ± 2.5 mmole C L^{-1}) on day 51 from CO_2 reduction. As no significant production of other products than acetate was observed in this period, there was a probability of toxicity caused by ethanol in the solution. Therefore, 60% of the solutions in BESs_ethanol were changed by the fresh medium without ethanol on day 51 and only CO_2 was fed to the catholytes until day 58. After day 58, ethanol with the final concentration equal to half of the molarity of acetate was added to the medium, and due to the slow consumption rate of ethanol in the BESs, no ethanol was added to the system until the end of experiment (day 79). As illustrated in Figures 6.9 (c) and (d), the concentration of products began to increase again after adding ethanol on day 58, and in case of butyrate, butanol, hexanoate and hexanol reached the maximum concentration on day 79.

In addition, the similar trend of H_2 production observed in BESs_Formate were detected in BESs_ethanol. H_2 was less than 10% before adding ethanol, however the average of H_2 over 45 days of experiment that ethanol was supplied was 23.6 ± 9.6 %. In BESs_fromate it was assumed that large percentage of H_2 detected in the headspaces while formate was provided in the cathodic medium was due to the consumption of formate as an available and favourable source of energy in addition to H_2 in MES processes. The same hypothesis can also be presumed for BESs_ethanol, but it is also probable that H_2 was detected in BESs_ethanol due to the toxicity of ethanol for bacteria and affecting the MES and EF processes, even though the ratio of ethanol to acetate was adjusted at 1 to 2. Lack of production apart from acetate and no consumption of ethanol in the solutions between days 44 and 51 could be due to this toxicity, which the toxic nature of ethanol to acetogens was also reported in the previous study (Lonkar et al., 2016). However, in their study the concentration of ethanol around 40.0 g L^{-1} inhibited the microorganisms' activity, while in this study the ethanol concentration did not reach higher than 2.0 g L^{-1}. In previous studies on chain elongation of acetate and ethanol in bio-reactors to high value products of caproate and caprylate, It was shown that the higher ratio of ethanol to acetate lead to enhance in the production (Spirito et al., 2018). Studying different ratio of ethanol to acetate, It was also reported that increase in the production of long chain VFAs and alcohols occurred when the concentration of ethanol was higher than acetate in the solution, more efficient at the ratio of 3:1 (ethanol:acetate) (Liu et al., 2016). However, in this study concentration of ethanol was half the molarity of acetate in the solution. Comparing the number of cells in the catholytes on days 0 and 79, it was observed that it declined slightly from 7.6 ± 0.2 to 7.1 ± 0.1 in both BESs_ethanol and BESs_formate. However, it is worth noting that

during the ethanol supplying experiment biofilm began to develop at the surface of the electrode covered the bottom part of the electrodes of both duplicates which could be seen visually with naked eyes (Figure D1 in Appendix D).

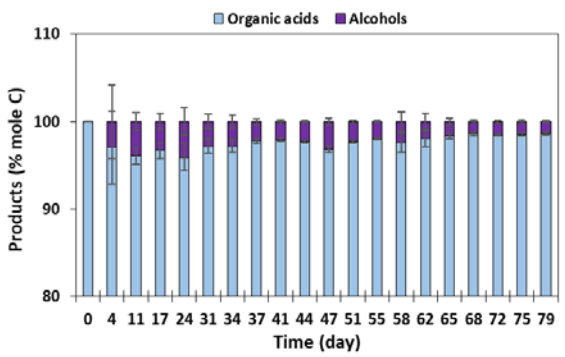
6.3.7 *Effect of adding electron donors in BES on the diversity of products*

Figure 6.10 (a) demonstrates the effect of different electron donors of formate and ethanol on the abundance of the products, by comparing the amount of carbon converted to organic acids and alcohol in BESs_control, BESs_formate and BESs_acetate. Figures 6.10. (b) and (c) also depict more details regarding the molarity of the carbons in the products formed during the experiment. Although there was a possibility of production of formate in BESs_fromate and ethanol in BESs_ethanol, the concentrations of formate and ethanol were not included in the calculations of products after day 34 illustrated in the figures, to exclude the concentration of these compounds added manually to the BESs. In addition, the concentration of formate and ethanol produced over the first 34 days was quite negligible compared to the concentration of these compounds added manually to the catholytes from day 34 to 79. More than 98% of the total carbon in all the BESs was fixed as organic acids in all the BESs during the first 34 days of the experiment. After adding electron donors to the reactors, the percentage of alcohols in the total products increased in both conditions, however more significantly in BESs_formate. At the end of experiment (day 79), the maximum percentage of alcohols of 10.7 ± 2.0 % represented the total products in the catholytes of BESs_formate, while this was lower in BESs_ethanol, with maximum value of 5.8 ± 3.4% on day 79. Figures 6.10 (b) and (c) indicate the increase in diversity and number of the mole of carbon. In BESs_formate more alcohol production was observed over time, with a gradual increase from the beginning of formate spiking until the end of experiment, with the dominant products of ethanol and butanol. In the last batch cycle that the highest molarity of carbon was fixed/produced, 8.76 ± 0.13 mmole C L^{-1} C2 and 6.86 ± 0.28 mmole C L^{-1} C4 in alcohol products and 135.53 ± 31.08 mmole C L^{-1} C2 and 6.56 ± 1.02 mmole C L^{-1} C4 in organic acids were the most significant products detected in the catholytes. In BESs_ethanol, however, more carbon was fixed in form of C2 in organic acids, as the highest molarity of C2 was achieved on day 51 with concentration of 170.91 ± 1.97 mmole C L^{-1}. Various products in form of C1, C5 and C6 were detected in parallel of C2 production particularly in organic acids products, however with small quantities. In the last batch cycle these products were as low as 0.82 ± 0.14 13 mmole C L^{-1} of C1, 0.32 ± 0.21 13 mmole C L^{-1} of C5 and 0.52 ± 0.13 mmole C L^{-1} of C6. Alternatively, the most significant

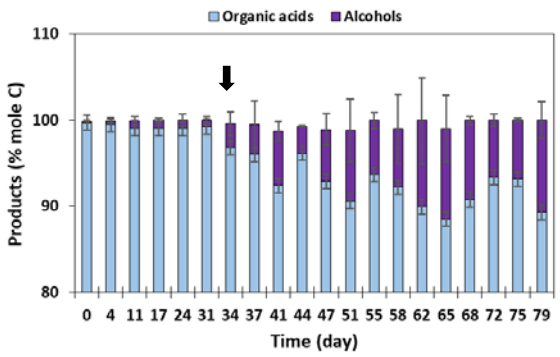
products in the last batch cycle were 3.37 ± 1.56 mmole C L^{-1} C4 and 2.33 ± 0.65 mmole C L^{-1} C6 in alcohol products and 156.02 ± 0.32 mmole C L^{-1} C2 and 3.07 ± 0.32 mmole C L^{-1} C4 in organic acid products. Carbon was also fixed in very small quantity of C1, C5 and C6 in organic acid products with the concentrations of 0.82 ± 0.14, 0.32 ± 0.21 and 0.52 ± 0.13 mmole C L^{-1}, respectively.

(a)

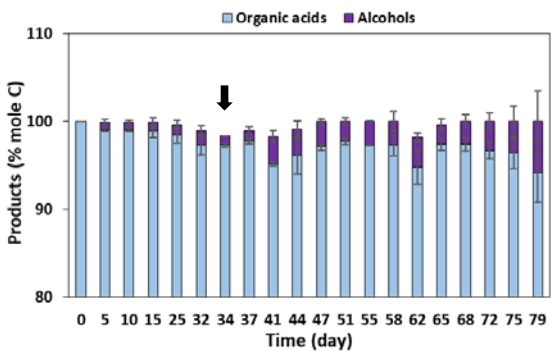
BESs_control



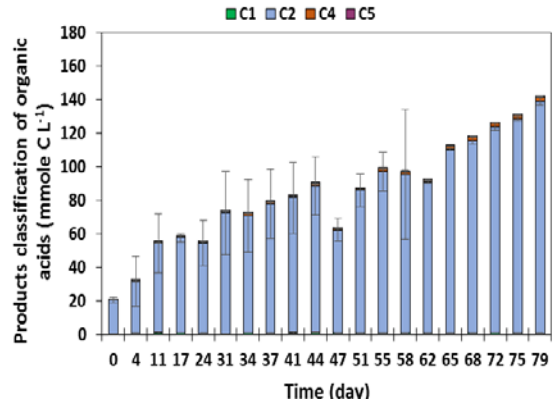
BESs_formate

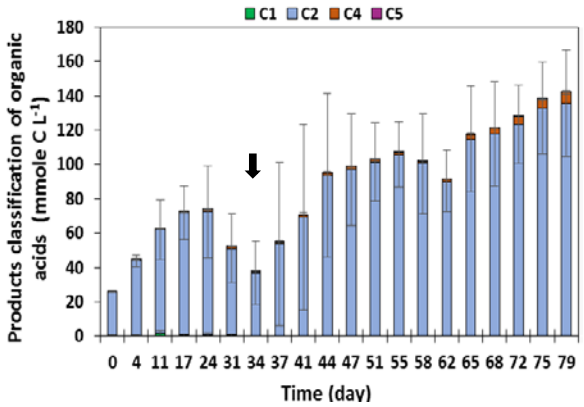


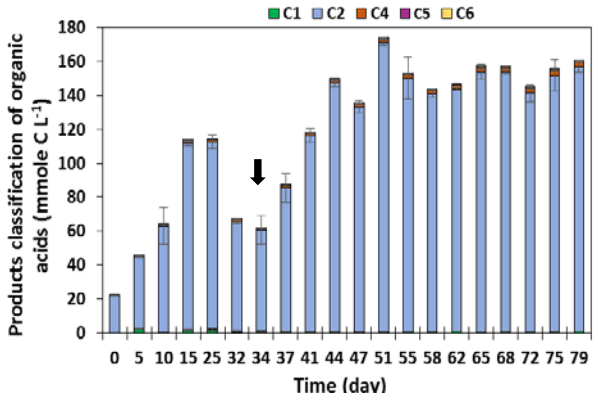
BESs_ethanol



(b)







(c)

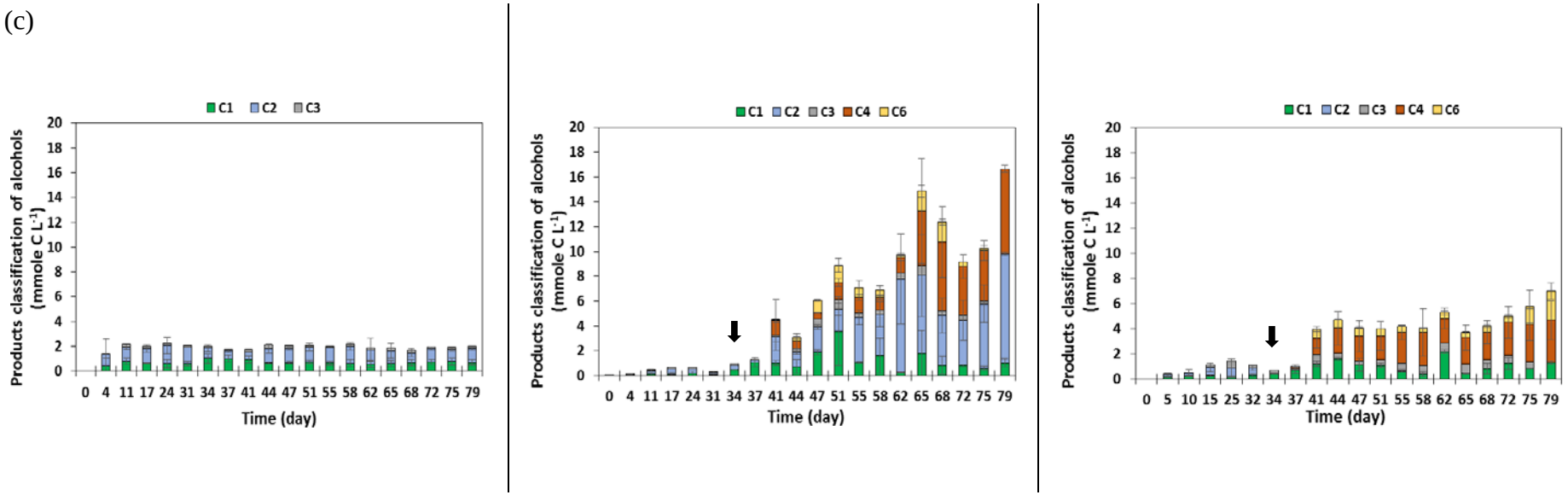


Figure 6.10. Percentage of alcohols and organic acids in products and classification of the products in forms of C1 to C6 in alcohols and organic acids in (a) BESs_control, (b) BESs_fromate, and (c) BESs_ethanol. Black arrows show when electron donors experiments began.

In spite of higher alcohol formation in BESs_formate, lower percentage of alcohol products was hexanol in these reactors compared to BESs_ethanol. The maximum percentage of 16.8 + 10.9 % and 31.9 + 17.9 % of the alcohols formed as hexanol in BESs_formate on day 75 and BESs_ethanol on day 51 of the experiment, respectively. Additionally, small percentage of alcohols (less than 18.0 %) were presented as iso propanol in both reactors. Simultaneously, trace concentration of acetone was occasionally detected in both conditions. Significant production of iso propanol was observed in the previous study by Arends et al, in which it was described that acetone can be produced during acetone-butanol-ethanol fermentation and consumed for the production of iso propanol over iso propanol-butanol-ethanol fermentation (Arends et al., 2017). Although more diversity in products was depicted after adding electron donors, it is clearly demonstrated in Figures 6.10 (a) and (b) that the highest percentage of the products was C2 in organic acids during the whole experiment in both BESs. As described before, high production of acetate, even after providing different electron donors, could be related to presence of *Acetobacterium* dominated the bacterial community enriched from initial inoculum. Reducing CO_2 as a terminal electron acceptor, acetogens are able to produce different products through acetyl-CoA pathway depending on their cultivation. However, it has been reported that different genera of acetogens such as *Acetobacterium* are able to produce only acetate as a sole product even by consuming different types of electron donors (Drake et al., 2006). Similarly, previous study on actogens such as *Clostridium* species showed among acetate, ethanol, butyrate and butanol from syngas fermentation, acetate was the major end-product (Bruant et al., 2010). Large number of studies on BES also reported acetate as the main product, while much less quantity of by-products of formate, propionate, butyrate, methanol, ethanol and butanol were detected alongside acetate (Marshall et al., 2013, LaBelle et al., 2014b, Zhang et al., 2013b, Nie et al., 2013, Bajracharya et al., 2017c).

6.3.8 *Cell density and community analysis of the bacterial cells in suspensions of BESs in different strategies*

As BESs and the biofilm at the surface of electrodes were needed for the further analysis, only liquid samples from the catholytes were analysed for cell count and community analysis. Liquid samples were collected from the reactors at the end of experiments. In case of BESs in continuous operational mode, samples were collected after 102 days on continuous mode. For BESs-formate and BESs-ethanol this was after 79 days of experiment. Figure 6.11 illustrates the number of cells (in logarithmic scales) in catholytes of BESs in batch mode, continuous mode, BESs_formate and BESs_ethanol analysed by flow cytometer. BESs_batch are the samples of initial inoculum from BESs 1 (-1.0 V_CO_2) described in Chapter 5, after 104 days of experiment on batch mode. Bacterial community in the samples from BESs in this Chapter was compared with that in initial inoculum to clarify the changes in the composition of bacterial community in the catholytes. Number of the cells in the catholytes in all the BESs were quite similar, however slight decrease was observed from the initial inoculum. Cell count was $4.6 \times 10^7 \pm 2.7 \times 10^7$ in BESs on batch mode; however, it decreased slightly to $2.9 \times 10^7 \pm 2.0 \times 10^6$ after 102 days of continuous mode. In addition, cell count value was $1.2 \times 10^7 \pm 5.7 \times 10^5$ and $1.2 \times 10^7 \pm 2.9 \times 10^6$ after 79 days of electron adding experiment in solutions of BESs_formate and BESs_ethanol.

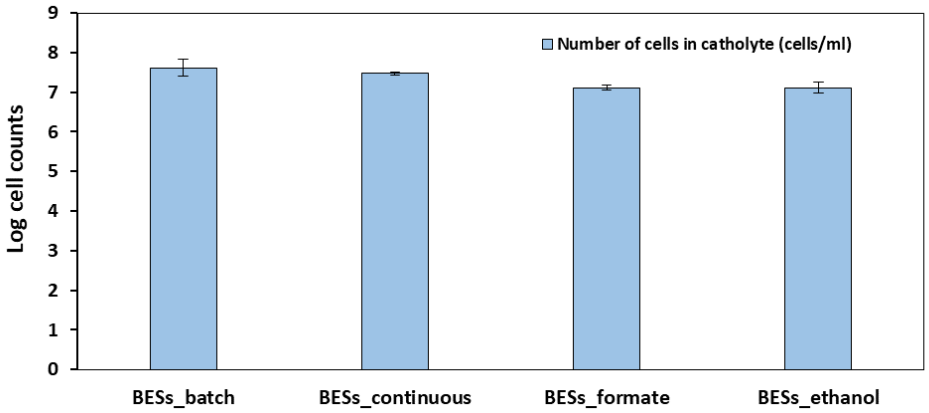


Figure 6.11. Number of the bacterial cells in logarithmic scale in 1 ml of catholytes BESs in batch mode, BESs in continuous mode, BESs_formate and BESs_ethanol.

DNA was extracted from the samples from solutions of BESs in continuous mode, BESs_formate and BESs_ethanol and further diversity and community analysis were compared

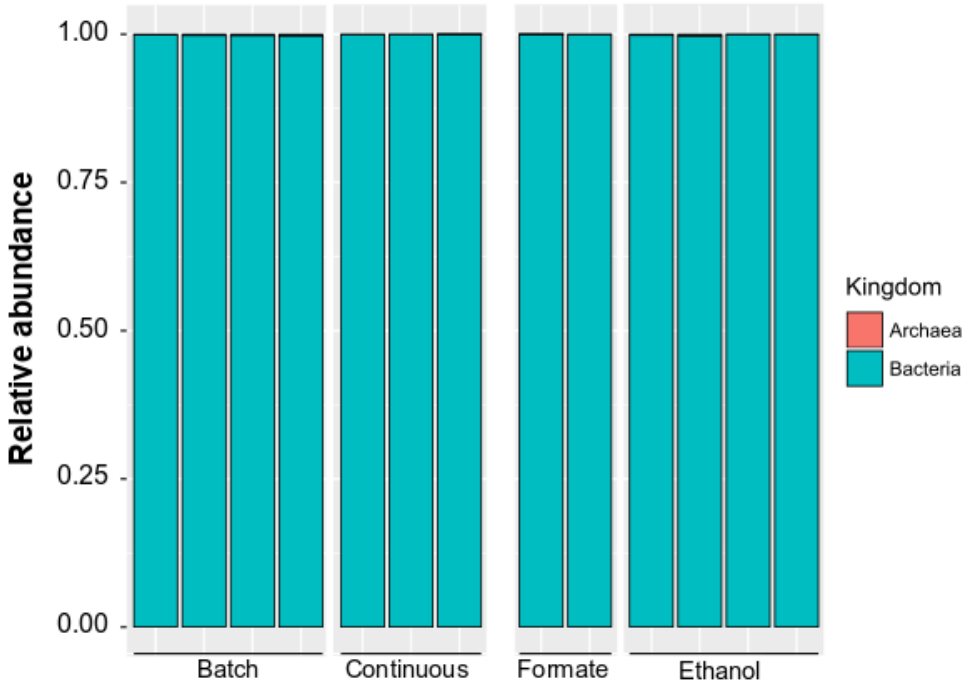
with those from solution samples in BESs in batch mode. Before taxonomy assignment, Shannon diversity index was determined using Equation (5.2) from the ASV table of samples. As shown in Table 6.3, it can be observed that the diversity of bacterial community in the solutions was not different after batch and continuous operational modes as Shannon index was almost similar. However, Shannon index was slightly lower in BESs-formate and BESs-ethanol compared to BESs on batch and continuous modes indicating the slight increase in the diversity of the bacterial community by providing electron donors more than cathodes in BESs.

Table 6.3. Shannon diversity index

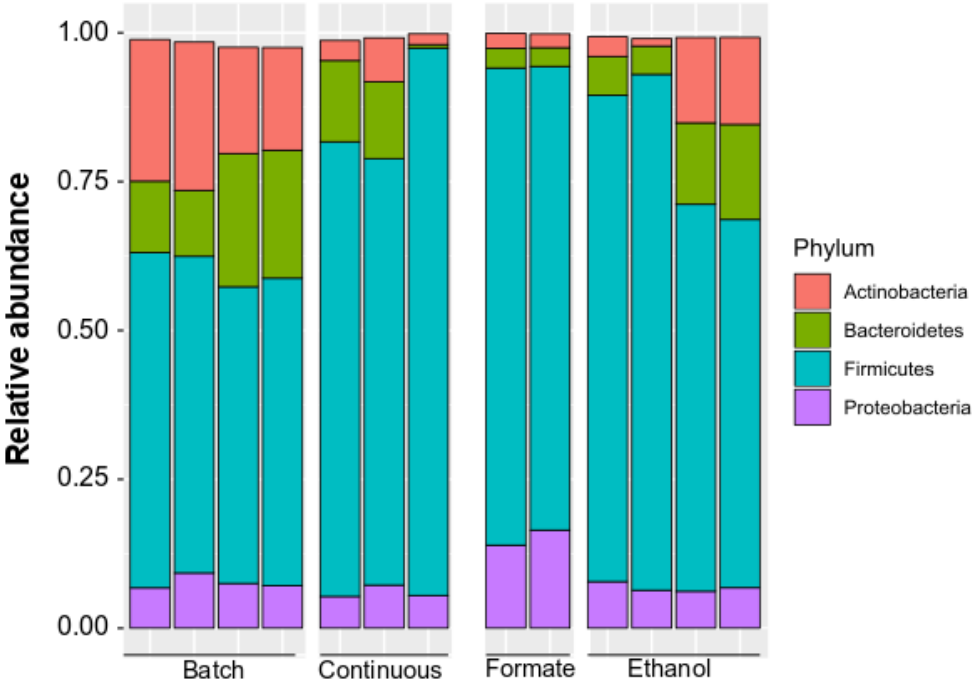
Sample	Shannon index
BESs_batch	2.91 ± 0.42
BESs_continuous	2.89 ± 1.77
BESs_formate	1.98 ± 0.03
BESs_ethanol	1.97 ± 0.38

The representative sequences in each ASV files were assigned using RDP database with the bootstrap confidence of 80. RDP database was selected due to the reasons discussed in the Chapter 5. After taxonomy assignment, the relative abundance of each taxon was determined from kingdom to genus levels (Figures 6.12 and 6.13). The bar charts were plotted using ‘’phyloseq’’ package in R statistical platform (McMurdie and Holmes, 2013).

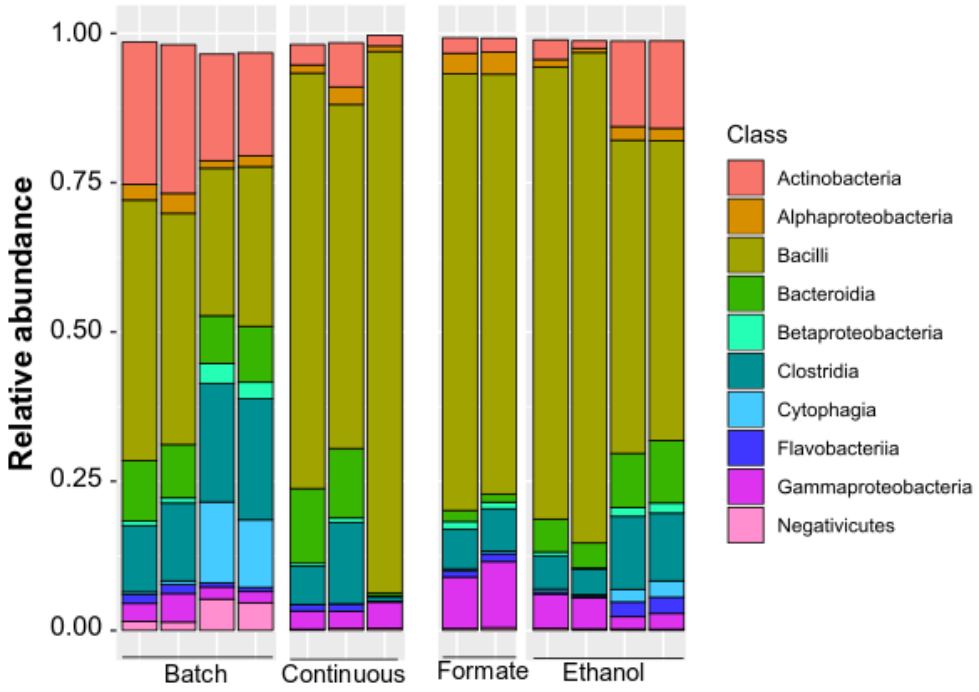
(a) Kingdom level



(b) Phylum level



(c) Class level



(d) Order level

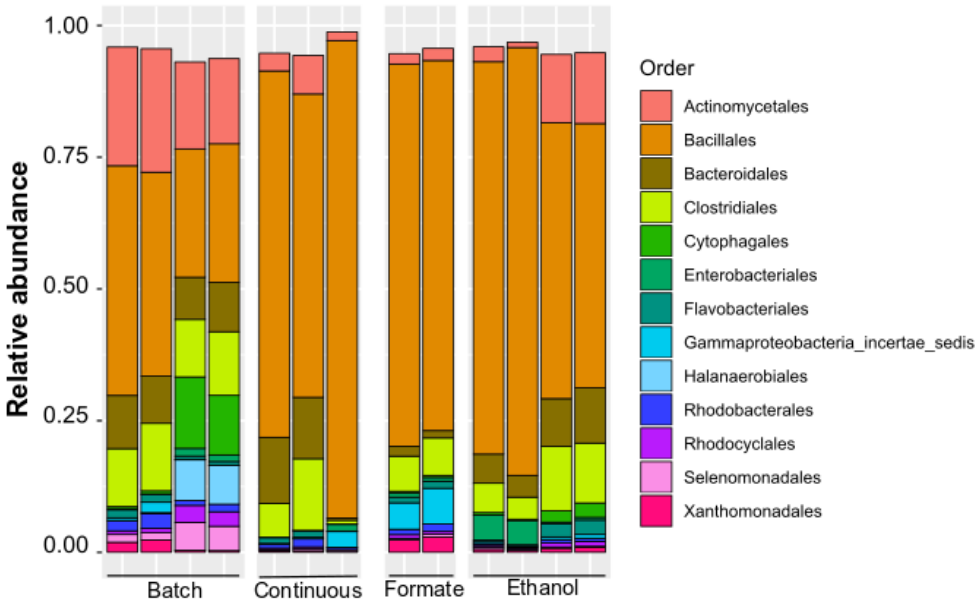
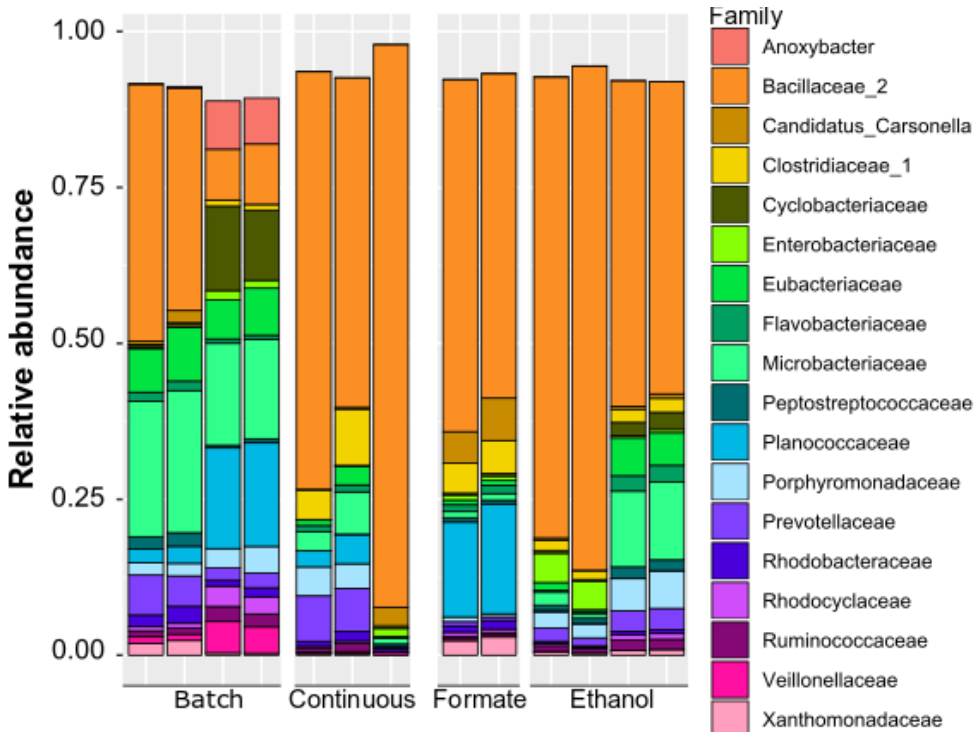
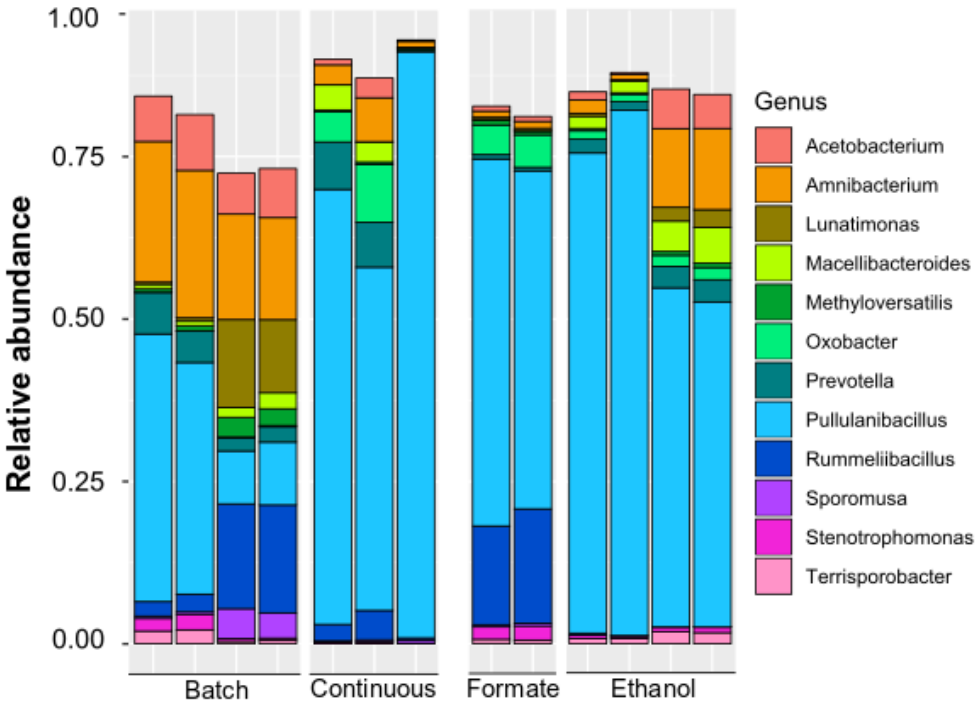


Figure 6.12. Relative abundance of bacterial community in (a) kingdom (b) Phylum (c) class and (d) order levels obtained from Illumina sequencing of 16S rRNA genes from solutions of BESs on batch operational mode , BESs on continuous operational mode, BESs_formate and BESs_ethanol named Batch, Continuous, Formate and Ethanol, respectively in the figures.

(a) Family level



(b) Genus level



(c) Weighted Genus level

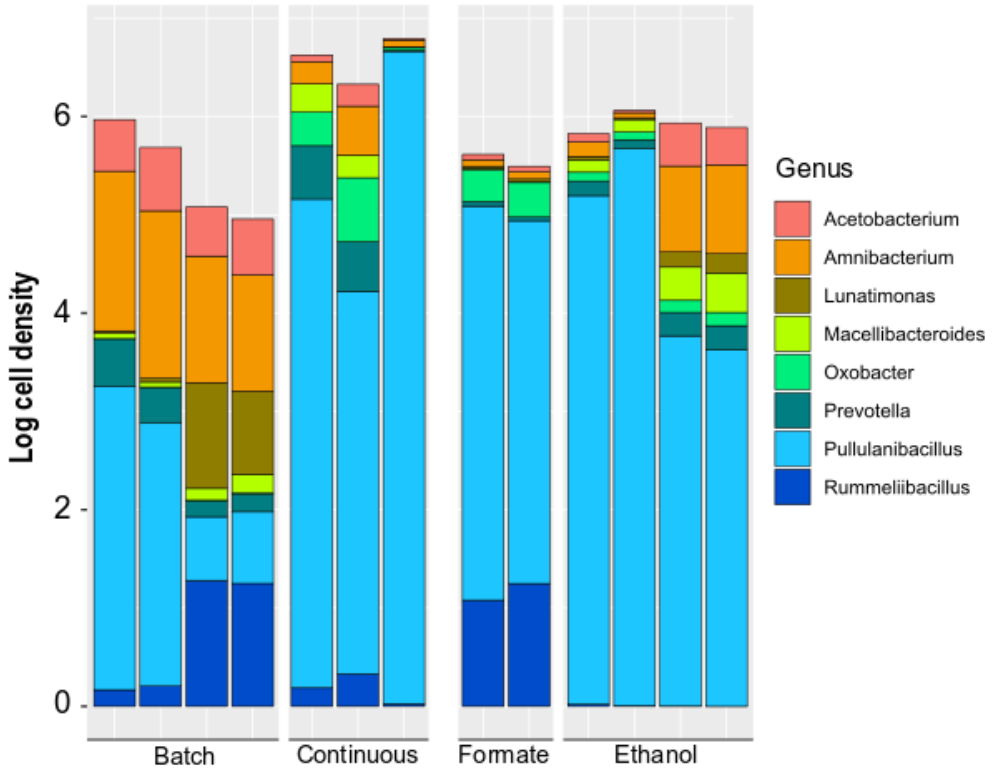


Figure 6.13. Relative abundance of bacterial community in (a) family, (b) genus, and (c) weighted cell density (cell ml^{-1}) obtained from Illumina sequencing of 16S rRNA genes from solutions of BESs on batch operational mode, BESs on continuous operational mode, BESs_formate and BESs_ethanol named Batch, Continuous, Formate and Ethanol, respectively in the figures.

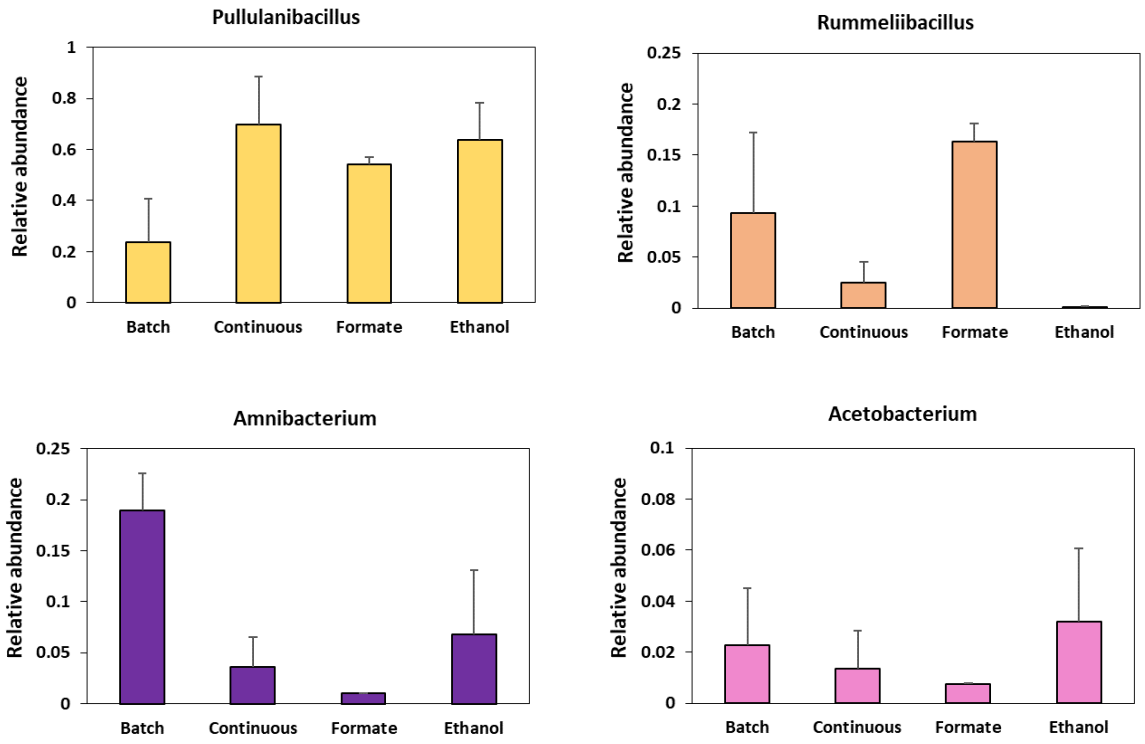
Figure 6.14 demonstrates the abundance of 5 genus including most dominant bacteria and two acetogenic bacteria of *Acetobacterium* and *Sporomusa* in the catholytes of the BESs on batch operational mode with those in the BESs on continuous operational mode, BESs_formate and BESs_ethanol. The most significant change was increase in the abundance of *Pullulanibacillus* in the solutions. Detected as the most abundant genus in all the BESs, it comprised 69.8 ± 1.3 %, 54.0 ± 0.7 % and 63.5 ± 3.2 % of solutions community of the BESs on continuous feeding regime, BESs_formate and BESs_ethanol respectively, while it was only 23.5 ± 2.2 % in initial inoculum from BESs on batch mode. As mentioned in Chapter 5, *Pullulanibacillus* is from the family of *Bacillaceae_2* which is the spore-forming bacteria from *Firmicutes* with the optimum temperature of 37 °C and acidic pH of around 4.5 (Pereira et al., 2013, Galperin, 2013, Zhang et al., 2013a). Not much is known about *Pullulanibacillus*, however highly similar sequence was found in the acetate producing bioreactors from CO_2 and H_2 with pH around 4.5 (according

to tree plot of Figure 5.30) similar to operational conditions in the BESs of this chapter (Zhang et al., 2013a). Whether *Pullulanibacillus* was involved in the production over MES processes or consumption of organics due to the high concentration of organics in the BESs is still unclear. Low percentage of *Rummeliibacillus* found only in the solutions of BESs 1 was hypothesized to be correlated to presence of butyrate and iso propanol as discussed by Arends et al (Arends et al., 2017). However, it seemed more related to the presence of butyrate in this study as it was found more abundant in BESs_formate which butyrate and butanol were the most significant products in this condition. Although similar to *Pullulanibacillus* little is known about the metabolism of *Rummeliibacillus*, it was reported as the butanol-tolerating bacteria (Kanno et al., 2013). In this study, *Rummeliibacillus* included almost 20% of the bacterial community in the solutions of BESs_formate, while it was negligible in that in BESs_ethanol which less butanol was detected in these BESs. It is worth noting that similar to Chapter 5, small abundance but diverse *Clostridium* genus was found in the catholytes. *Clostridium sensu stricto* (ASV 68), which showed the high similarity to *Clostridium aciditolerans* (Figure 5.30), was found in all the samples, with highest abundance in the catholytes of BESs_formate. Compared to fed-batch mode from Chapter 5, only the abundance of this genus from *Clostridium* increased. Approximately 1 – 5 % of the planktonic cells in BESs_ethanol and 2 - 16% of the planktonic cells in BESs_formate were comprised of *Clostridium sensu stricto* (ASV 68), with the highest abundance in one of the duplicates of BESs_formate (16 %). This could be related to C4 production in these reactors (Lee et al., 2007). As discussed in Section 5.3.14 of Chapter 5, *Clostridium aciditolerans* was found in a bioreactor producing long chain organics through syngas fermentation (Ganigué et al., 2016). It was also reported as a bacteria with broad range of fermentation products such as acetate and butyrate (Lee et al., 2007), which could be related to increase in C4 production in this study after adding electron donors. Minor production of C6 products detected could also be due to small abundance of diverse *Clostridium sensu stricto* found in the suspension of the reactors. In previous studies on chain elongation through acetate, *Clostridium Kluyveri* was reported as the responsible bacteria for production of C6 products (Steinbusch et al., 2011, Coma et al., 2016, Diender et al., 2016, Roghair et al., 2018). However, this bacterium was not found in the samples of this study.

As discussed in Chapter 5 *Acetobacterium* was recognised as the responsible bacteria in production of acetate through MES in BESs 1. Although it was the dominant bacteria in the biofilm (~50 % of the bacterial community in biofilm), lower abundance of *Acetobacterium* (~7 %) was still detected in the solutions. Concluded from Chapter 5, optimal condition for *Acetobacterium* growth and activities could be provided by soluble CO_2, cathodic potential of

-1.0 V and slightly acidic pH (~ 5.8). According to this observation, it was assumed that inoculating all the reactors of this chapter by catholytes of BESs 1 and imposing the conditions favourable for *Acetobacterium* growth could lead to formation of biofilm at the cathodes dominated by *Acetobacterium* responsible for acetogenic activities, while lower abundance could be found in the catholytes. Figure 6.14 shows that *Acetobacterium* was found in the solutions of all the BESs operated in this chapter, however, the highest abundance (~4 %) was found in the catholytes of BESs_ethanol. In addition, it was observed that *Sporomusa* did not enrich in the BESs with acidic pH. As the catholytes pH in BESs on continuous operational mode, BESs_formate and BESs_ethanol was acidic, the experimental conditions were not favourable for the growth and enrichment of *Sporomusa* leading to decrease in the abundance of *Sporomusa* in the catholytes similar to the observation from Chapter 5. Moreover, as discussed in the previous chapter *Amnibacterium* was less likely involved in the MES processes, which decrease in its relative abundance in the catholytes of the BESs on continuous mode, BESs_formate and BESs_ethanol could confirm its wash out from the reactors over medium refreshments.

In addition, no *archaea* was found in the catholytes of the BESs in this chapter while no chemical methane inhibitor was added to the cathodic medium. This confirmed the successful suppression of methanogens in the BESs under acidic conditions.



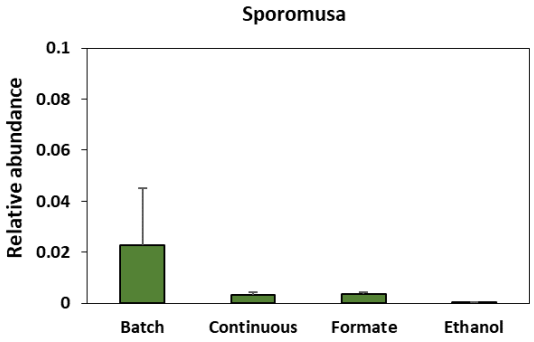


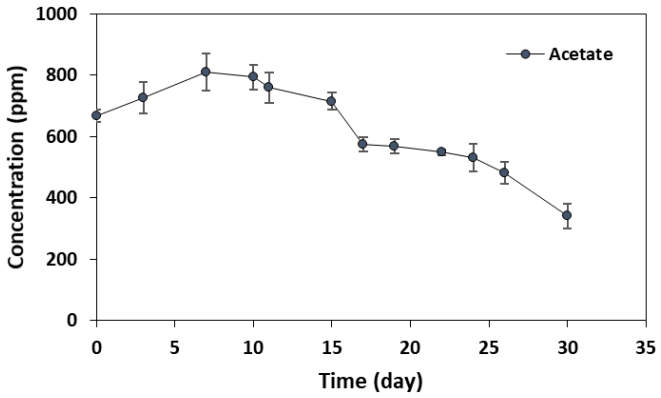
Figure 6.14. Average relative abundance of 5 genus in all the community samples in the catholytes of BESs on batch operational mode, BESs on continuous operational mode, BESs_formate and BESs_ethanol named Batch, Continuous, Formate and Ethanol, respectively in the figures.

6.3.9 *Function of cathode in MES and EF processes*

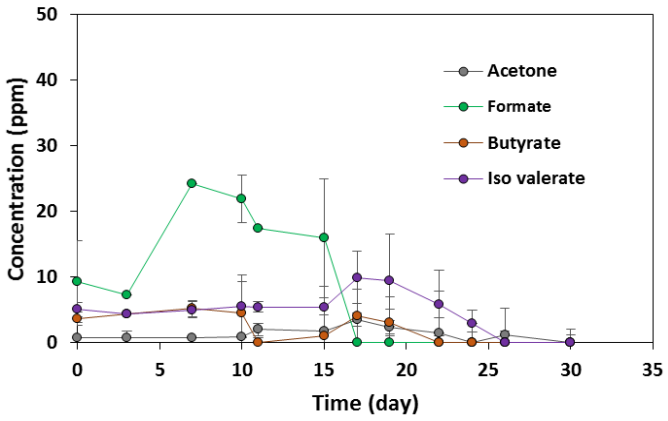
One of the important questions reagrding CO_2 reduction in BESs is the role of cathode through MES and EF processes. From Chapter 5, it was observed that cathodic current affected the formation of biofilm at the cathodes by providing the energy required for CO_2 reduction to acetate through MES processes. However, it is still not clear if a cathode is involved in production of longer chain products than acetate. To achieve that, OCP control experiment was designed by removing the involvement of cathode for production. OCP control experiments were carried out after electron donors adding experiments, using non-polarised cathodes. Comparing different applied potentials of cathodes showed their important role in providing energy source for MES processes. This was also confirmed through OCP control experiment in BESs_control by providing CO_2 in BESs_control without energy source of cathode. Slight increase in acetate concentration (less than 200.0 ppm) demonstrated in Figure 6.15 (a) was observed over the first 6 days, however no acetate was produced from day 6 to day 30 of OCP control experiment and only consumption of acetate was observed. Despite the enrichment of acetogens dominated bacterial community in the biofilm, fermenters were also detected in cathodic compartments (Figures 5.24 and 6.13), which were more likely responsible for the consumption of acetate. The lack of acetate production under OCP condition strongly indicates the key role of cathode in supplying energy for CO_2 reduction through WL pathway, through either IET or DET mechanism, in MES processes. Trace concentrations (less than 30.0 ppm) of other organic acids and alcohols such as formate, butyrate, iso valerate, ethanol, methanol

and iso propanol, , were also detected during the 30 days of OCP experiment as observed in Figures 6.15 (b) and (c), however they were consumed and reached almost negligible concentrations at the end of experiment. These observations could confute the mechanism of chain elongation in BES proposed by (Raes et al., 2017, Jourdin et al., 2018), that CO_2 is converted to acetate using a cathode as an energy source and C4 and C6 products are formed from the CO_2 provided and acetate. It confirms the alternative approach in chain elongation that C4 and C6 products are formed from the CO_2 provided and acetate using another source of energy than cathode (Jourdin et al., 2019).

(a)



(b)



(c)

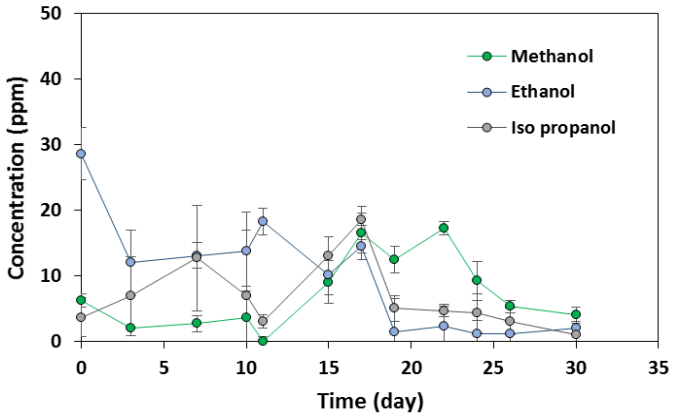
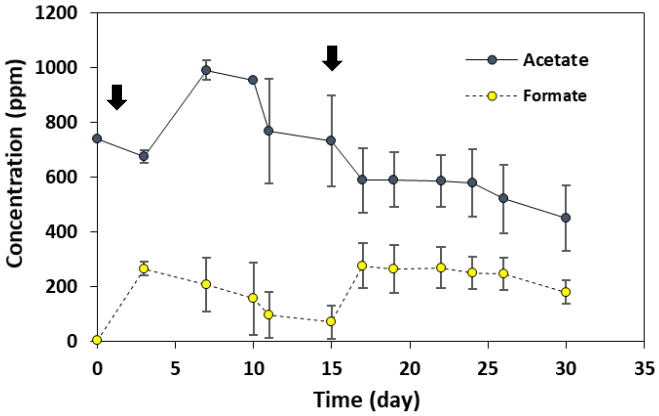


Figure. 6.15. (a) Acetate concentration, (b) acetone and organic acids, and (c) alcohols detected in BESs_control during OCP control experiment.

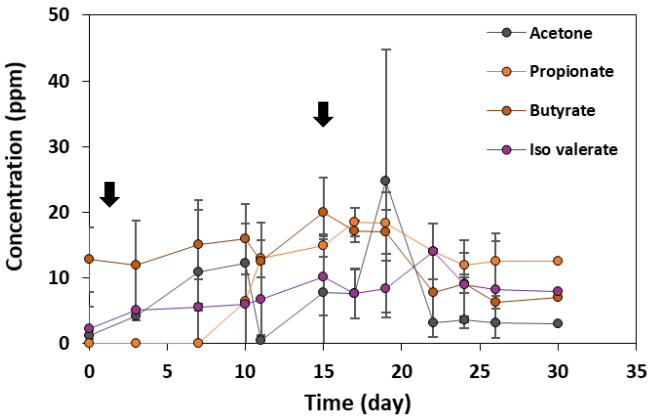
In BESs_formate, formate was added to acetogenic biofilm under OCP condition. We hypothesized that formate could be an alternative to cathode as an energy source leading to production of acetate and longer chain of products, even without polarised cathodes. However, as illustrated in Figures 6.16 (a), (b) and (c), the results were different to what observed during 79 days experiment with polarised cathodes. Over the first 7 days formate was consumed associated with slight increase in acetate concentration. Acetate concentration increased from 676.3 ± 23.1 ppm (22.5 ± 0.7 mmole C L^{-1}) on day 3 to 991.0 ± 35.4 ppm (33.0 ± 1.2 mmole C L^{-1}) on day 7, however, it began to decrease after that until day 15 associated with consumption of formate and negligible production of acetone, propionate, butyrate and iso valerate (almost less than 20.0 ppm). Although formate was reported as a carbon and energy source for acetogens in bioreactors (Ramió Pujol et al., 2014), it seemed that it could not be consumed in OCP experiment of this study when polarised cathodes were not provided. Despite the rapid consumption of formate over 3-4 days in experiment with polarised cathode, formate was not completely consumed during first 15 days of OCP control experiment. Slow consumption of formate was more considerable over the second 15 days. On day 15, formate was topped up to half the molarity of acetate. During the second 15 days, almost no formate was consumed and consequently no significant increase in the concentration of acetate or other products was observed. Rapid consumption of formate during BES experiment and slow formate consumption during OCP experiment can suggest the role of polarised cathode in enhancing the kinetic of cathodic reactions, highlighting one of the important advantage of BES over bioreactors. Moreover, throughout 30 days of OCP control experiment methanol, ethanol and iso propanol were the only alcohols detected irregularly which their concentrations did not

reach higher than 45.0 ppm. It is interesting to note that no butanol and hexanol or significant concentration of butyrate were detected in OCP experiment in contrast to what observed in BESs_formate with polarised cathodes. It may show that cathode is not only involved in MES processes but also can influence the production of longer chain products through acetate in EF processes. Studies on EF processes showed that electrode is not the only source of energy and other sources are required to produce long chain products (Jiang and Zeng, 2018b). However, cathodic potential represents the activity of electrons in the solution which affects the ratio of NAD^+/NADH within bacterial cells. In addition, over metabolic activities, genes expression and enzymes synthesis is controlled by NAD^+/NADH. Therefore, it is sensible to assume that cathodes potential affects the fermentation pathways and therefore the range of products (Moscoviz et al., 2016, Jiang et al., 2018).

(a)



(b)



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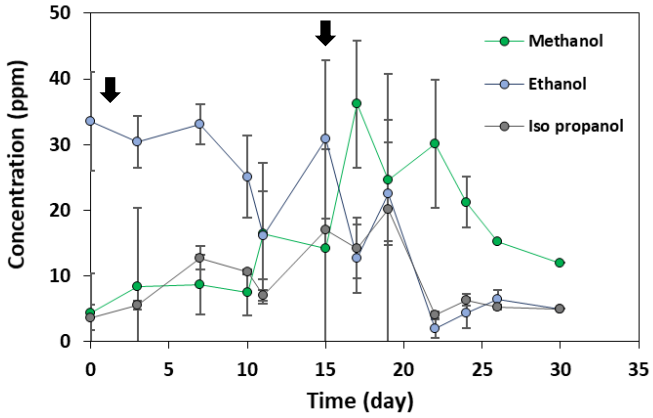


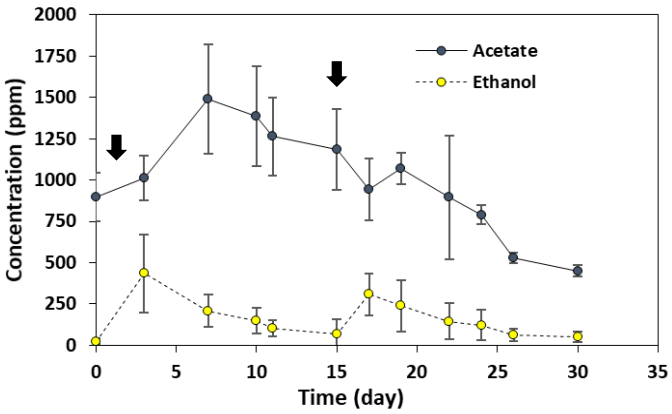
Figure. 6.16. (a) Acetate concentration, (b) acetone and organic acids, and (c) alcohols detected in BESs_formate during OCP control experiment. Black arrow shows when formate was added to the cathodic medium.

Result of OCP control experiment in BESs_ethanol demonstrated in Figures 6.17 (a), (b) and (c) was very different from what observed in BESs_control and BESs_formate. First, concentration of acetate began to increase immediately after addition of ethanol on days 1 and 15, increased from 896.7 ± 146.3 ppm (30.0 ± 4.8 mmole C L^{-1}) on day 1 to 1489.3 ± 331.0 ppm (49.6 ± 11.0 mmole C L^{-1}) on day 7 and from 943.9 ± 187.9 ppm (31.5 ± 6.3 mmole C L^{-1}) on day 17 to 1067.8 ± 95.8 ppm (35.6 ± 3.2 mmole C L^{-1}) on day 19. However, in following days after addition of ethanol, acetate concentration began to decrease indicating its consumption, either through fermentation or chain elongation. Production of longer chain products was evidently observed in OCP experiment of BESs_ethanol by high production of C4 products indicating chain elongation through acetate. Over the first 15 days of OCP experiment, butyrate production was almost negligible. However, after day 17 it began to increase significantly, reaching the maximum value of 416.3 ± 50.0 ppm (19.1 ± 2.3 mmole C L^{-1}) on day 30 associated with slight increase in the concentration of butanol to 33.2 ± 2.0 ppm on day 30. This was the highest concentration of butyrate detected in this thesis. This showed that butyrate production in BESs poised at -1.0 V supplied by ethanol was less likely related to polarised electrode. Similar trend was observed in the previous study (Batlle-Vilanova et al., 2017). Although no OCP experiment was performed in their study, it was shown that providing sufficient H_2 from the cathode and CO_2 along with low pH led to reduction of acetate to ethanol and further production of butyrate from acetate and ethanol, which seemed that cathode was less likely involved in chain elongation from acetate. However, the effect of time on bacterial growth and production cannot be neglected. Higher butyrate production rate in the reactors

during OCP experiment compared to BES experiment could be related to time of the experiments. As OCP experiment was performed after the BES experiments, it is possible that bacterial community was developed over the longer period of time during OCP control experiment leading to higher concentration of butyrate produced during OCP experiment.

Furthermore, no C6 products was detected in OCP experiments in BESs_ethanol. Although the concentration of hexanol detected in BESs_ethanol during the BES experiment was almost negligible, the lack of detection of that in OCP experiments could suggest that polarised cathode was involved in the chain elongation of C6 products through acetate in BES.

(a)



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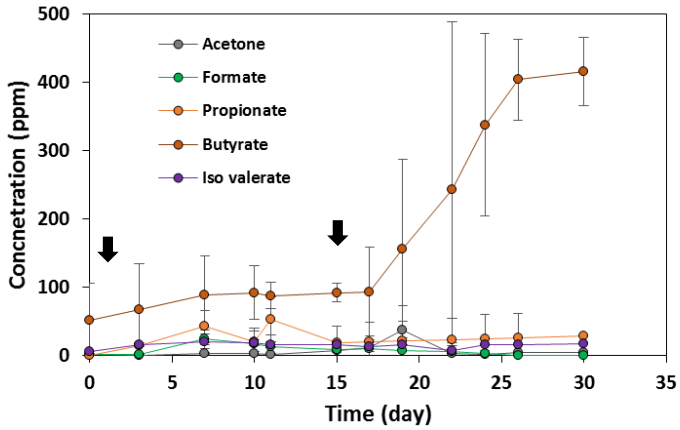

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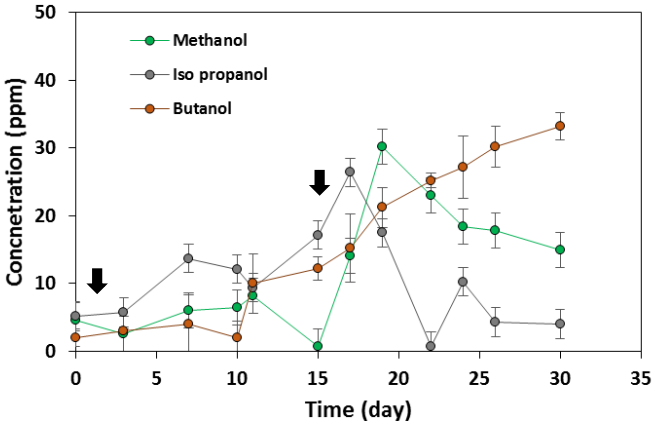


Figure. 6.17. (a) Acetate concentration, (b) acetone and organic acids, and (c) alcohols detected in BESs_ethanol during OCP control experiment. Black arrow shows when ethanol was added to the cathodic medium.

6.4 Conclusion

In this chapter, production through MES could be successfully optimised using two different strategies. Constant flushing of fresh nutrients and soluble CO_2 and removal of products through continuous operational mode led to significant improve in acetate production rate and columbic efficiency, compared to that in fed-batch mode. In addition, continuous feeding regime contributed to more electro-active biofilm catalysing HER leading to superior performance of BES in terms of production and efficiency. Two different HRTs of 3 and 7 days applied for flushing the fresh medium showed slight different results. In both HRTs, acetate was the major product from CO_2 with almost similar columbic efficiency (maximum 90% at HRT: 3 days and 81% at HRT: 7 days), while highest acetate production rate was achieved over HRT of 3 days (803 ppm day^{-1}). This could be related to pH of the solution provided at different HRTs which was around 5.0-5.5 for HRT 3 days and 4.7-5.0 for HRT 7 days. Lower pH and higher acetate accumulated in BESs at HRT 7 days resulted in production of more diverse products (C4, C5 and C6 products), however their concentration ($\leq$ 20 ppm) were almost negligible compared to the concentration of acetate.

The main target of addition of electron donors to BES with active CO_2 reducing biocathode with and without polarised cathodes was clarifying the role of cathode and different electron donors as energy sources in acetate and longer chain organics production in BES. Providing extra electron donors in addition to cathodes in BESs resulted to production of more diverse products. Providing formate in BESs was associated with significant increase in production of

butyrate as the second major organic acid product (after acetate) and also ethanol and butanol, reaching the maximum concentration of 142.7 ± 22.2, 172.2 ± 83.6 and 126.9 ± 5.1 ppm, respectively. Significant change in the BESs supplied by ethanol was production of more diverse C4, C5 and C6 products but with negligible concentrations compared to acetate concentration. Addition of formate and ethanol to the polarised cathodes BESs led to increase in the concentration of organic acid of acetate, however after switching to OCP condition, only BESs with ethanol continued to produce acetate.

Figure 6.18 summarizes the observations through electron donors' addition experiments to reactors with and without polarised cathodes. It was clearly observed that acetate was produced from CO_2 using the cathode as an energy source, as acetate production was ceased during OCP experiment. Although formate was reported to be consumed as the sole carbon and energy source by acetogens of *Clostridia* (Ramió Pujol et al., 2014), it was not consumed in this experiment with non-polarised cathode. Polarised cathode could possibly enhance the kinetic of the reaction leading to formate consumption and production of acetate in BESs. In addition, lack of production of longer chain products when formate was supplied in OCP experiment could indicate that formate was less likely involved in chain elongation in BESs. However, it seemed that ethanol played an important role in C4 production. Previous study on the feasibility of reactions in chain elongation suggested that production of butyrate from acetate was not thermodynamically feasible by only H_2, however this reaction was favourable using ethanol as an energy source (González-Cabaleiro et al., 2013). The authors suggested that in the system that ethanol is not supplied, production of butyrate from acetate occurs more likely from ethanol as an electron donor produced as a by-product throughout the experiment by different bacterial community. This was in agreement with the findings in this chapter, which higher butyrate and butanol production was observed when ethanol was supplied to the reactors, with and without polarised cathodes. It was also suggested that production of hexanoate required ethanol as an energy source and production of C6 products from C4 is not feasible with only H_2 (González-Cabaleiro et al., 2013). However, this was different from the observations in this chapter. Detection of C6 products in BESs with polarised cathodes supplied by ethanol and lack of C6 production in the OCP experiment when ethanol was supplied showed that cathode was involved in production of C6 products. A comprehensive review on chain elongation in BES also discussed the important effect of polarised cathodes on the ratio of NAD^+/NADH within bacterial cells and consequently the range of products (Moscoviz et al., 2016). However, due to the low concentration of C6 products detected when cathodic potential (-1.0 V) was applied at the cathodes, more investigations are required in this area.

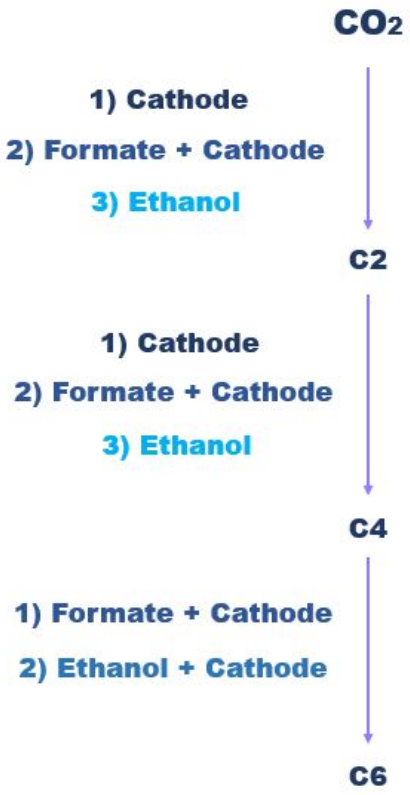


Figure 6.18. Electron donors involved in C2, C4 and C6 production from CO_2 through MES and EF processes according to the findings of this chapter.

Chapter 7. Conclusions and Future Work

7.1 Summary

Bio-electrochemical systems (BES) is a potential sustainable technology. As outlined in Chapter 1, the main aim of this thesis was a comprehensive study on aerobic and anaerobic biocathode to gain fundamental understanding related their development and their effect on BES performance. In case of energy harvesting, the aim was developing a biocatalyst from mixed culture of microorganisms in a cathodic compartment comparable with chemical catalysts for catalysing oxygen reduction reaction (ORR) and consequently enhancing energy generation. In case of CO_2 reduction through microbial electrosynthesis (MES), enrichment of autotrophic bacteria of acetogens from mixed culture of microorganisms is challenging. The aim for MES processes was first enriching and developing a biocathode able to consume CO_2 and produce valuable organics and investigating the parameters affecting MES processes. Thereafter, biofilm formed at cathodes during MES was comprehensively studied. In addition, different strategies were performed to optimize the system in terms of production.

The key findings including the challenges and experimental limitations are summarised below:

- Iron oxidising bacteria (IOB) was enriched from the natural environmental sample and used as an active biocathode catalysing ORR. Presence of Fe^{2+} as a mediator led to a short lag time, thus avoiding the long start-up phase usually associated with aerobic biocathodes. Cyclic voltammetry (CV) analysis strongly suggested mediated electron transfer (by Fe^{2+}) between the cathode and bacterial community; however, direct electron transfer (DET) cannot be totally ruled out.
- Microbial fuel cell (MFC) comprising of bioanode and IOB biocathode was successfully operated; however, the low pH required for IOB (acidophilic, pH ~ 2.0) in the cathodic compartment was detrimental for bioanode due to the significant pH difference between

anodic and cathodic compartments. 2 days after operation of MFC equipped with IOB biocathode, anodic pH dropped from 7.0 to 4.0 due to the high difference between the pH of the anolyte (pH 7.0) and catholyte (pH 2.0) separated by anion exchange membrane (AEM), leading to significant decrease in power production. Operating MFCs in continuous mode helped adjusting pH in both compartments. MFCs produced maximum power density of 0.251 mW cm^{-2}, compared to 0.134 mW cm^{-2} for MFCs equipped with Pt catalyst (0.5 mg cm^{-2} Pt on carbon paper) indicating advantages of fully microorganism catalysed system, and importance of operational parameters in such systems.

- The passive diffusion of oxygen through a Gas diffusion electrodes (GDEs) showed the proper method for overcoming the limiting factor of oxygen mass transfer and the costs associated with using air pump, by providing the constant available oxygen available for ORR. IOB biocathode operated in GDE design studied in this thesis showed a promising alternative to both chemical catalysts and non-mediated aerobic biocathodes without the need for long-term start-up and costly aeration systems.
- Regarding anaerobic biocathode, one of the factors affecting the enrichment of acetogens from mixed culture before transferring to BESs was presence of undesirable bacterial community such as methanogens and fermenters. Fermenters could be washed out from the inoculum by providing CO_2 as a sole carbon source leading to removal of heterotrophs and growth of autotrophic bacteria. However, removal of hydrogenotrophic methanogens was challenging as they coexist with acetogens, consuming CO_2 and H_2 and producing methane. Among different methods used before and after transferring the inoculum in BESs including pre-heating the inoculum, using chemical methane inhibitor and low pH, low pH provided by accumulation of acidic products (from acetogens) showed the most efficient and cost-effective result in suppressing the activity of methanogens successfully.
- During the whole experiments, acetate was the major product of MES processes. During the fed-batch mode experiment without system optimising, highest concentration of acetate accumulated was 6500.4 ± 633.6 ppm (216.7 ± 21.1 mmole C L^{-1}) in BESs poised at -1.0 V and inorganic carbon source provided as gaseous CO_2 with the highest acetate production rate of 660.7 ± 101.1 ppm day^{-1} (22.1 ± 3.4 mmole C L^{-1} day^{-1}) and negligible concentration of C4 and C5 products and alcohols. However, maximum acetate concentration detected in BESs poised at -1.0 V and fed by HCO_3^- and BESs poised at -0.8 V and fed by CO_2 was much lower, 450.9 ± 1.3 ppm (15.0 ± 0.0 mmole C L^{-1}) and 70.6 ± 8.2 ppm (2.6 ± 0.3 mmole C L^{-1}) respectively. CV analysis performed

from the reactors with different conditions showed the most significant shift in onset potential of H_2 evolution in BESs poised at -1.0 V and fed by gaseous CO_2 from around -1.0 V to -0.8 V. Highest production of H_2 could be one of the reasons for the superior acetate production in such condition. This strongly confirms the biotic H_2 production, more likely through the enzymes of the bacterial cells such as hydrogenase (Deutzmann et al., 2015). Enzymatic H_2 produced was then consumed by acetogens for production of acetate as no H_2 was detected in the headspaces of these reactors, confirming the H_2-mediated electron transfer mechanism between the biofilm and electrode.

- One of the important findings in this thesis was the key role of biofilm in MES processes. It was observed that MES processes occur at the cathodic biofilm rather than planktonic cells. This was due to the difference in bacterial community enriched in biofilm and planktonic cells in BESs poised at -1.0 V and fed by gaseous CO_2. In contrast to bacterial community in suspensions, the most abundant bacteria in cathodic biofilm was *Acetobacterium* (~ 50 %) responsible for acetate production through MES, while *Pullulanibacillus* (~ 20 %) was the most dominant bacteria in suspension. Direct correlation was evidently observed between the density of cathodic biofilm, abundance of acetogens in the biofilm, number of live cells in the biofilm and production from CO_2. In addition, electrochemical impedance spectroscopy (EIS) analysis showed significant decrease in charge transfer resistance at the electrode after formation of dense biofilm. Although *Acetobacterium* was reported as non-electroactive bacteria (Nevin et al., 2011, Kracke et al., 2015), the high conductivity of the biofilm was assumed to be due to the active enzymatic H_2 production, facilitating the electrons transfer. In contrast to BESs poised at -1.0 V and fed by gaseous CO_2, non-metric multidimensional scaling (NMDS) figure showed no difference between the bacterial cells in the biofilm and planktonic cells of BESs poised at -1.0 V and fed by HCO_3^- and BESs poised at -0.8 V and fed by gaseous CO_2.
- The important effect of cathodic potential and type of inorganic carbon source observed on production through MES seemed to be reflected from their effect on physical properties and bacterial composition of biofilm, respectively. The difference between the biofilms formed at the cathodes of two different potentials of -1.0 and -0.8 V (both using inorganic carbon source of gaseous CO_2) showed that much denser biofilm was formed at the potential of -1.0 V, while no biofilm was observed through confocal microscopy and SEM analysis at the cathodes poised at -0.8 V similar to them in open circuit potential (OCP) condition. Minor abiotic H_2 production at the potential of -1.0 V could give the initial energy to bacterial community leading to start-up of the processes,

growth and development of the bacterial biofilm. Inorganic carbon source showed the significant impact on the composition of biofilms. Comparing community analysis of biofilms formed in BESs fed by CO_2 and bicarbonate at the similar potential (-1.0 V) showed different bacterial community developed in the biofilm. Almost 50 % of the biofilm in BESs that gaseous CO_2 was provided as inorganic carbon source was comprised by *Acetobacterium*, the responsible bacteria for production from CO_2. However, the abundance of *Acetobacterium* in the biofilm of BESs fed by bicarbonate was almost 20 %. In addition, highest abundance of methanogen (~ 20 %) was enriched at the cathodes of BESs fed by bicarbonate. Enrichment of methanogens was more likely related to the pH of the catholyte provided in BESs with bicarbonate as inorganic carbon source. Bicarbonate was not consumed significantly contributed to its accumulation in the catholyte, lack of acidic products production and increase in catholytes pH (~ 8.0) due to abiotic reduction of protons, providing more favourable condition for methanogens than acidic condition. Additionally, *Sporomusa*, a known acetogen, was also detected in the biofilm of BESs fed by bicarbonate, however no relationship was observed between the abundance of *Sporomusa* and acetate production.

- The evident effect of inorganic carbon source of CO_2 on enrichment of *Acetobacterium* from mixed culture of microorganisms can be due to two reasons: 1. Lower pH provided by CO_2 in the catholytes 2. More favourability of CO_2 for acetogens activity rather than bicarbonate. Apart from the effect of pH on the selection and enrichment of acetogens, pH corresponds to the protons in solutions. Higher protons in the catholyte is associated with higher proton reduction and H_2 production at the polarised cathode. Higher molecules of H_2 at the cathodes was more likely the reason of higher abundance of *Acetobacterium* in the biofilm in BESs poised at -1.0 V and fed by CO_2 compared to BESs poised at -1.0 V and fed by HCO_3^-. In this thesis no strong linear relationship was obsereved between pH and *Acetobacterium*, highlighting the second reason for higher *Acetobacterium* enrichment when CO_2 was provided. This could strogly suggest that inorganic carbon source provided in form of CO_2 was more favourable and feasible to consume by cathodic bacterial community responsible for MES rather than bicarbonate.
- The role of cathode is rather producing H_2 for CO_2 reduction than providing electrons directly for MES. The constant availability of H_2 in BES is one of the important advantages of this system compared to natural environments and bioreactors with limited availability of H_2. In bioreactors, H_2 requires to be provided which includes the costs and limitations of purification, transportation, distribution and storage of H_2 (Balat, 2008). During 104 days fed-batch mode experiment in the BESs poised at -1.0 and fed

by CO_2 in Chapter 5, the energy consumed by potentiostat to apply the constant potential at the cathode was 8.98 KWh considering the average cathodic current consumed during the experiment. The power cost for applying potential during these 104 days was £ 1.29 for each BES calculated according to the average national electricity price (14.37 p/KWh). The electricity can also be supplied by renewable sources of energy. Therefore, it could be concluded that BES offers a unique application of CO_2 reduction and organic compounds production using a low cost energy.

- For increasing the efficiency of BES, two strategies seemed to be efficient: 1. Providing low pH in the catholyte by constant flushing of CO_2 and fresh medium and removal of products over continuous operational mode 2. Providing more electron donors in the catholyte in addition to polarised cathodes.
- Continuous operational mode enhanced the acetate production rate and efficiency of the system significantly compared to fed-batch mode. As *Acetobacterium* was the dominated bacterial community in the cathodic biofilm, acetate was still the major product after switching the operational modes to continuous. Choosing HRT is a critical factor during the continuous operational mode. High flow rate can wash out the bacterial community from the reactors and low flow rate could lead to accumulation of products in the catholytes. Highest production rate of acetate 651.8 ± 214.2 ppm day^{-1} (21.7 ± 7.14 mmole C L^{-1} day^{-1}) in this thesis was achieved during the continuous feeding regime with HRT of 3 days, when the pH of catholyte was adjusted around 5.5. Catholyte pH was lower (4.3-4.7) when HRT of 7 days was applied due to the accumulation of acetate in the solution. Lack of constant removal of acetate from the catholyte was associated with decrease in acetate production rate to 272.0 ± 12.3 ppm (9.1 ± 0.4 mmole C L^{-1}) and increase in the biomass production. Low pH in this condition led to production of longer chain products than acetate such as C4, C5 and C6 organic acids but their small concentrations were due to the low abundance of *Clostridium* in the biocathode.
- During the fed-batch mode the average columbic efficiency for acetate production during the experiment was around 61 %; however, it enhanced significantly to more than 80 % during continuous operational mode. The highest columbic efficiency for acetate production in this thesis was achieved when BES was operated in continuos feeding regime with HRT of 3 days, around 90 %, while this was slightly lower at HRT of 7 days, around 81%. The superior function of BESs in continuous feeding regime was due to flushing of fresh nutrients and providing CO_2 constantly in the cathodic compartments. In addition, constant removal of products could direct the reactions to

acetate production more than that when acetate was accumulated in the catholytes during fed-batch mode.

- Supplying electron donors in addition to polarised cathode could steer the production of longer chain organic acids than acetate or chain elongation. Although higher alcohols were detected in the catholytes when electron donors were provided in the BESs compared to BESs without electron donors, more than 95 % of the products were still detected in form of organic acids. This showed that despite the acidic condition provided solventogenesis was not the dominated mechanism in the system. Between two electron donors investigated in this thesis, formate promoted the production of C4 products in presence of polarised cathode more significantly than other products compared to polarised cathode as a sole energy source. Highest concentration of butyrate and butanol in the BESs supplied by formate was 142.7 ± 22.2 ppm (6.5 ± 1.0 mmole C L^{-1}) and 126.9 ± 5.1 ppm (6.8 ± 0.3 mmole C L^{-1}), respectively. However, formate without polarised cathode did not seem to be consumed for bacterial activity. As formate was reported a known carbon and energy source for acetogens in bioreactors (Ramió Pujol et al., 2014), it can be concluded that BES enhanced the formate consumption reaction kinetically. Ethanol, however, increased the diversity of the products in BESs in both conditions, with and without polarised cathodes. This could show that ethanol was the sufficient electron donor for both MES of acetate and chain elongation through acetate, in contrast to what observed when formate was supplied. Highest concentration of butyrate (416.3 ± 50.0 ppm) was achieved in this thesis when ethanol was supplied in the reactors with non-polarised cathodes.
- In addition to acetate and C4 products during continuous flow and electron adding experiments, trace amount of C6 products were detected in BESs. C6 products were found in form of organic acid and alcohol in samples of BESs supplied by ethanol and formate in presence of polarised cathodes. Production of C6 products were more likely related to *Clostridium* bacteria found in the biocathode, in which the negligible quantity of these products could be justified by the low abundance of *Clostridium*. Involvement of *Clostridium* for chain elongation was also confirmed by increase in abundance of *Clostridium sensu stricto* with highest similarilty to *Clostridium aciditolerans* in catholytes. However, during OCP control experiment where cathodes were not polarised, no C6 products were detected. It seemed that polarised cathode affect the nature of the products and played an important role in C6 chemicals production. However, this cannot be completely concluded as the concentration of C6 products was not significant in the BESs with polarised cathodes.

7.2 Recommendations for future work

The outcomes of this thesis provide the optimistic approach towards different applications of BES in energy harvesting, CO_2 reduction and bio-conversion. More future studies on different experiment and configuration designs can make these applications more practical. One of the examples can be operation of larger scale MFC coupled with bioanode and IOB biocathode, following the successful operation of these reactors in lab scale in this thesis. Although, GDE design and cheap biocatalyst could remove the extra cost for aeration of system, there was a need to a constant continuous operation in both compartments of MFCs. Life-cycle assessment can evaluate the costs of such systems required for scale-up.

For MES processes, it was shown in this thesis that pre-heating the inoculum before inoculating the BESs or using chemical inhibitor of sodium 2-bromoethanesulfonate could not totally suppress the methanogens, as methane was still detected in BESs after inoculation. As low pH conditions showed the successful methanogens suppression, pre-heating the inoculum can be removed for the future studies. In order to accelerate the start-up of MES in future studies, BES can be inoculated by mixed culture of batceria directly after collection (without pre-treatment step) by providing 3 key parameters: 1) pH around 5.5-5.8, 2) cathodic potential of -1.0 V, and 3) constant available inorganic carbon source of CO_2.

Gaseous CO_2 was the preferable source of inorganic carbon during MES processes than bicarbonate, while the method for its capture and availability in cathodic compartment needs to be taken into consideration. Gas diffusion design is one of the proper method in solving gas mass transfer limits in solutions as confirmed in aerobic GDE design in Chapter 4. In case of MES processes using anaerobic biocathode, adding a gas chamber next to a cathodic compartment with GDE design not only provides the constant CO_2 for bacterial activity but also offers the simple way in providing CO_2 for bacterial community in the biofilm rather than purging CO_2 in the catholyte. As a consequence of this PhD thesis, the new set-up has been designed and operated with adding the gas chamber to the BES design in a new project. The target of this project is promoting the production towards long chain organic acids and alcohols. The concept of this project is demonstrated in Figure 7.1.

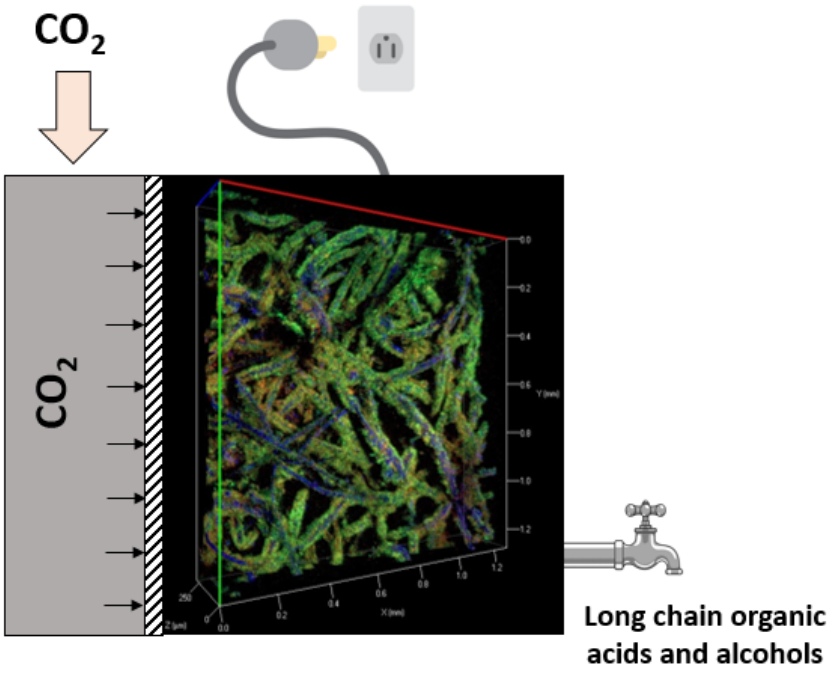


Figure 7.1. The concept of optimising MES processes and chain elongation by adding constant CO_2 available through gas chamber in GDE design BES

In addition, extraction of products needs to be taken into account. Products particularly acetate are soluble in water and are difficult to be extracted from the cathodic compartment. Three chamber BES were designed and operated before to extract acetate and store on the third chamber; however, this system needed another membrane which could increase the cost of the system (Gildemyn et al., 2015, Vassilev et al., 2019). Directing the production to longer chain chemicals has this advantage that they are less soluble in water and can be extracted from the solution more conveniently. Long chain organics have also more commercial value than short chain products. Therefore, more studies are required to extract the products from the catholytes. To steer the production in MES processes towards longer chain products, more diverse electron donors needs to be investigated such as lactate or methanol. It is not economic to add electron donors to BES particularly in larger scales. Therefore, it is recommended to study the methods (using different catalysts and biocatalysts) and conditions to enhance the production of these electron donors in the system in parallel to MES, which can be consumed by bacteria responsible for chain elongation.

The role of cathode in MES processes was clarified in this thesis; however, this was not completely clear through chain elongation particularly in C6 production. To clarify that, it is recommended to analyse bacterial community in the biofilm at the cathodes after electron

adding experiment with polarised cathodes and compare them with those enriched after electron adding experiment without polarised cathodes. This comparison can justify the difference of the products particularly C6 products detected in BESs before and after OCP control experiment. The focus of this thesis was development of CO_2 reducing biocathode, therefore the BESs were operated in half-cell to remove the effect of anodic compartment on the cathodic reactions. Counter electrode used for this thesis was Pt mesh to provide the sufficient electrons for CO_2 reduction. Although Pt is a proper catalyst for water hydrolysis, it is an expensive metal (~ £ 16.7 per gram) particularly not economic for larger scale reactors. Different metals or cheaper electrodes can be used as counter electrodes. In addition, CO_2 reducing biocathode can be coupled with active bioanode in BES and its performance can be investigated.

Considering the power required to apply the constant potential at the surface of a cathode calculated in the last section, it is fair to say that BES is cost effective in terms of electricity consumption. However, comparing to bioreactors, BES has more complicated designs including electrodes and membrane. Therefore, there is a need of a complete life-cycle assessment considering the materials costs and value of the products.

Chapter 8. Appendices

Appendix A. Material and methods data

List of Figures

List of Tables

Figure A1. Image of the river where the sediment was collected as an inoculum for aerobic biocathode.

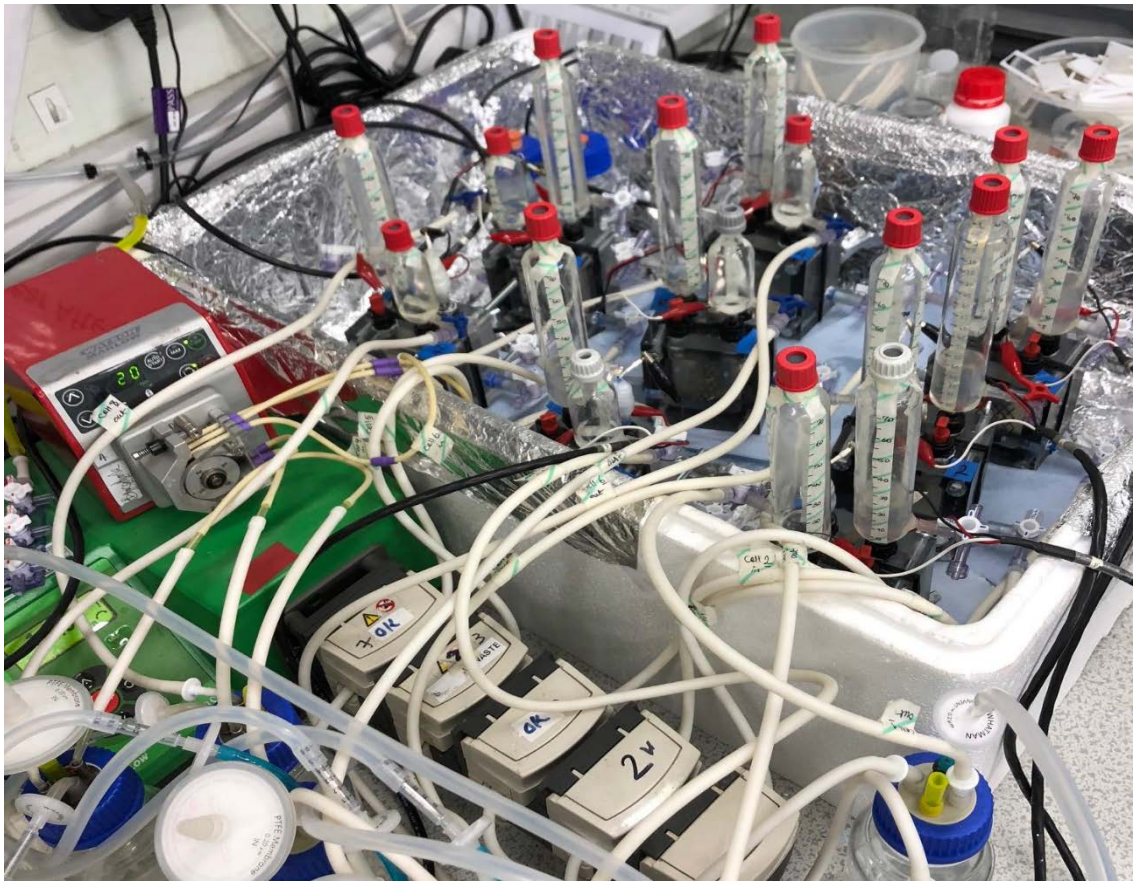


Figure A2. Image of the setup of the anaerobic biocathode experiment.

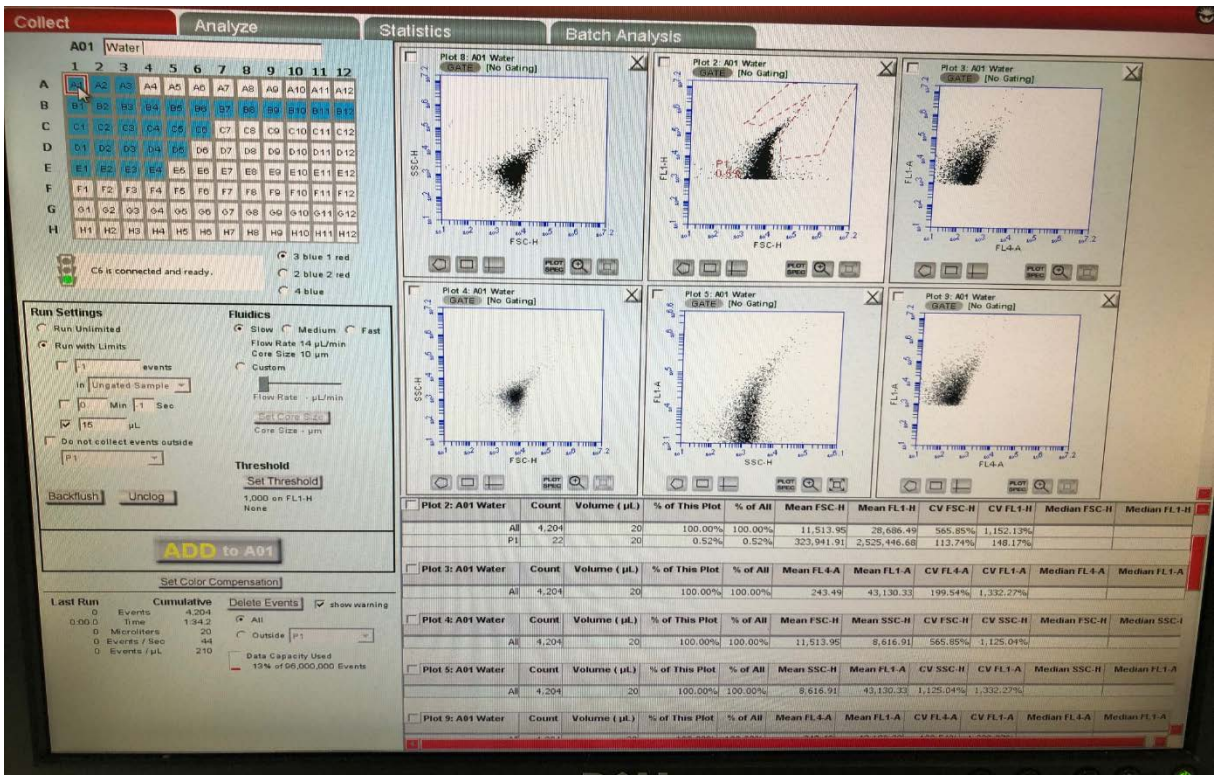
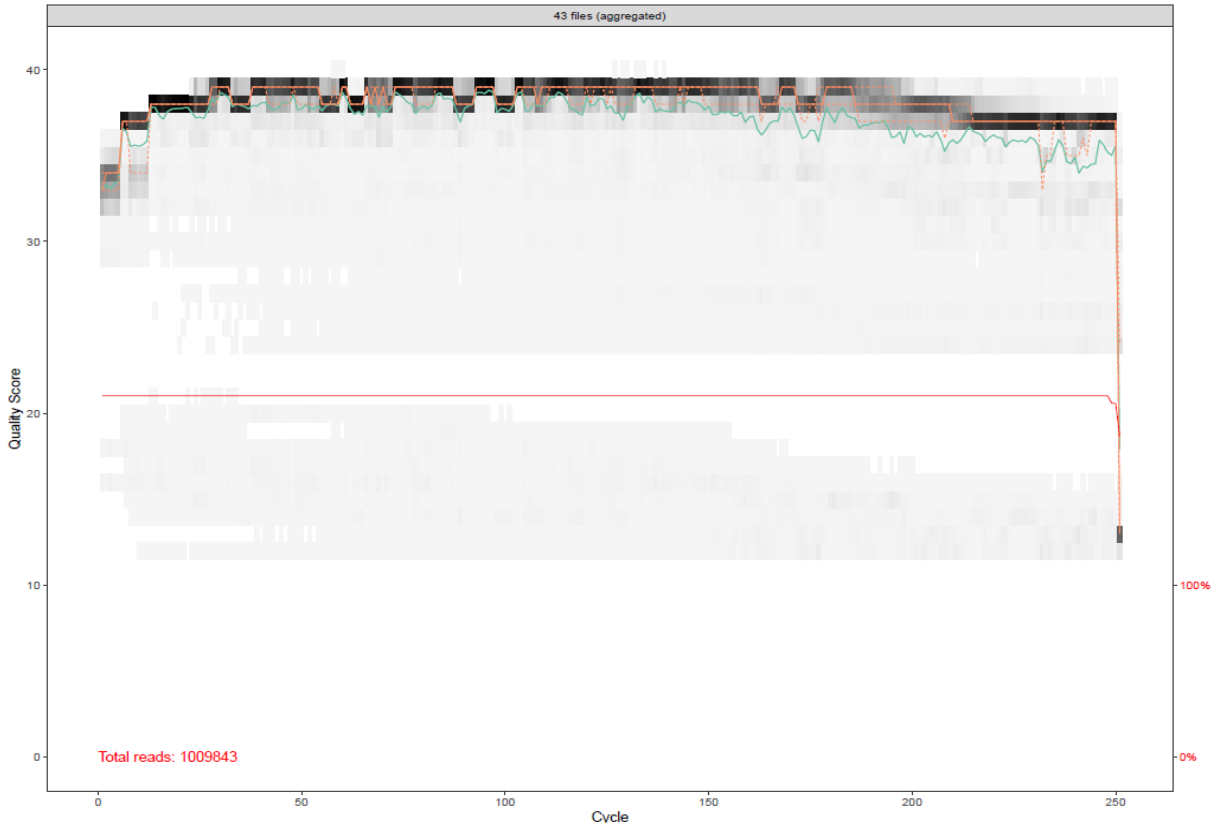
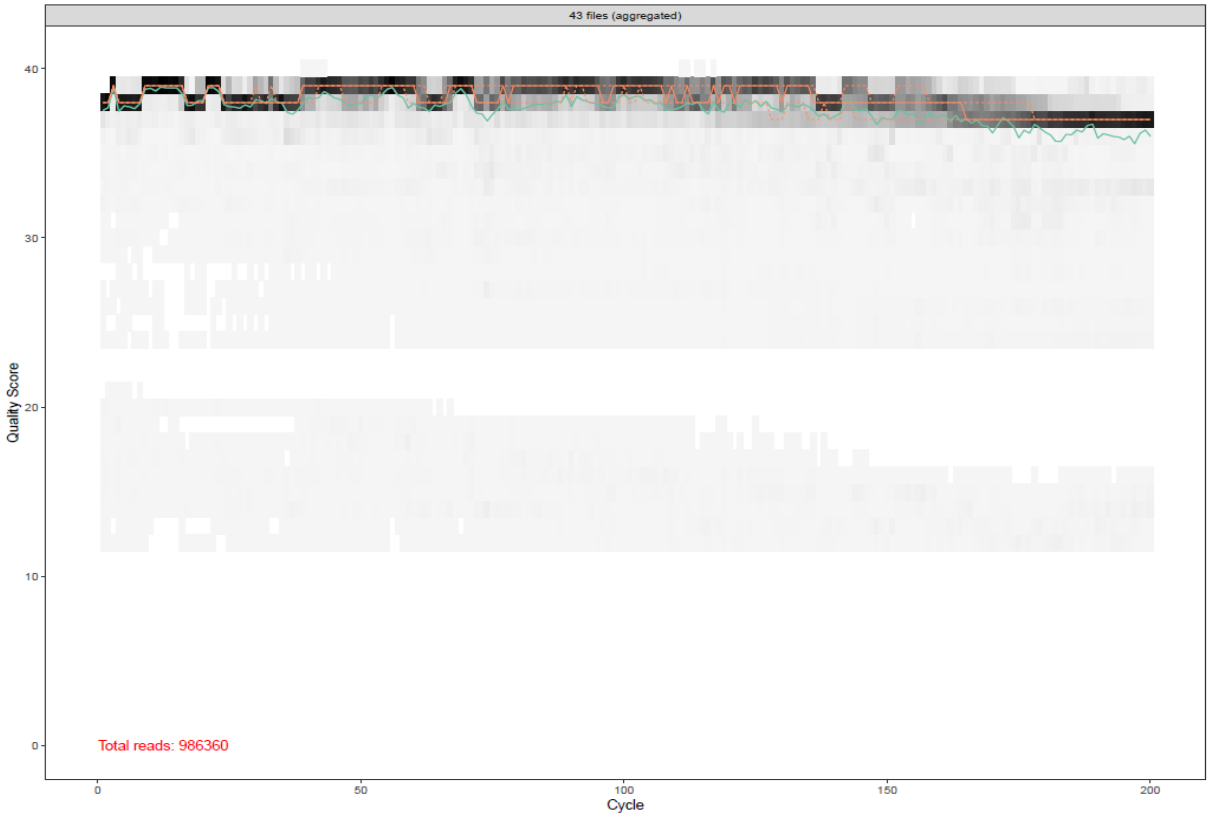


Figure A3. Image of the software over gating through FSC using stained filtered sterile water.

(a)



(b)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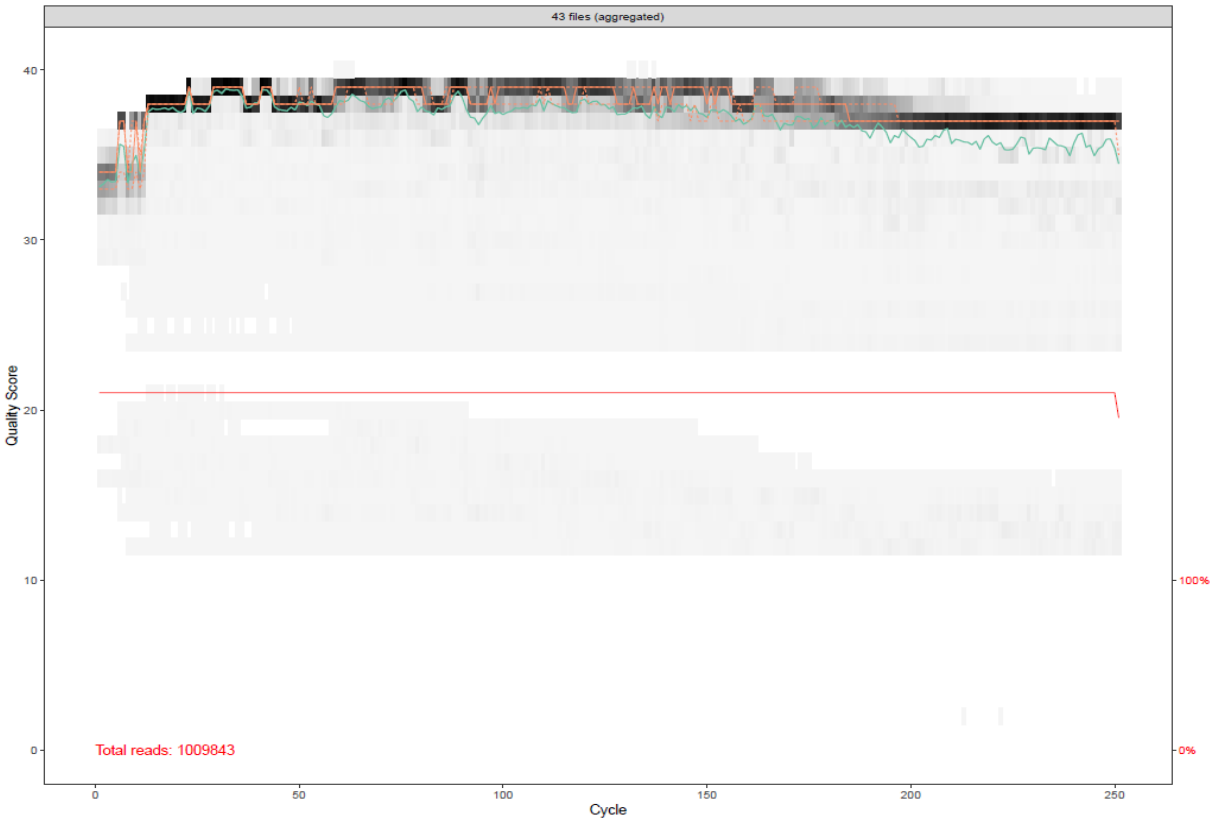
(c)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Cycle
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(d)

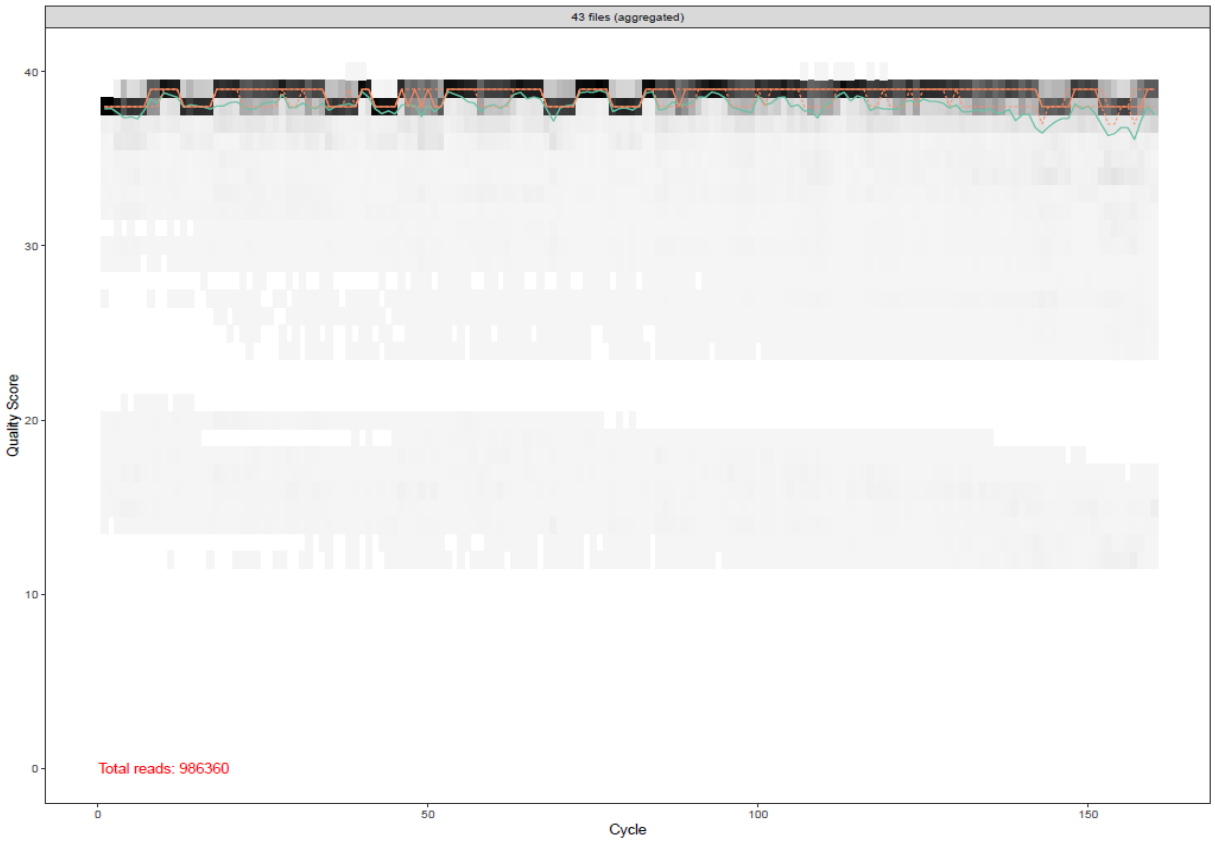


Figure A4. Quality profile of the files (a) first (forward) reads, (b) first (forward) after trimming, (c) second (reverse) reads, and (d) second (reverse) reads after trimming.

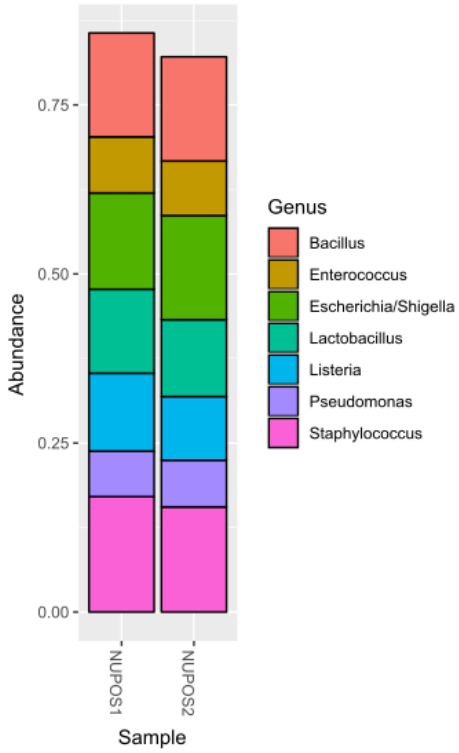


Figure A5. Relative abundance of bacterial community in positive control in genus level.

Table A1. Composition of solutions used in anode medium of MFCs in aerobic biocathode experiment

Composition of PBS (mg L^{-1})	
HK_2PO_4	5024
H_2KPO_4	2877
Composition of macronutrients (mg L^{-1})	
NH_4Cl	280
$CaCl_2$ _ $2H_2O$	5.7
$MgSO_4$ _ $7H_2O$	100
Composition of micronutrients (mg L^{-1})	
$FeCl_2$ _ $4H_2O$	4
$CoCl_2$ _ $6H_2O$	2
$MnCl_2$ _ $4H_2O$	1
$ZnCl_2$	0.1
H_3BO_3	0.1
$CuCl_2$ _ $2H_2O$	0.08
$(NH_4)6Mo_7O_{24}$ _ $5H_2O$	0.14
$NiCl_2$ _ $6H_2O$	2
Na_2SeO_3 _ $5H_2O$	0.32
Composition of vitamins (mg L^{-1})	
Pyridoxine	1
Nicotinic acid	0.5
Riboflavin	0.25
Thiamine	0.25
Biotin	0.2
Folic acid	0.2
Vitamin B12	0.01

Table A2. Composition of solutions used in cathode medium of BESs in anaerobic biocahtode experiment.

Composition of trace metal solution (g L^{-1})	
Nitrilotriacetic acid (dissolve with KOH; pH 6.5)	1.5
$Mg_2Cl_2.6H_2O$	3.0
$MnCl_2.2H_2O$	0.5
NaCl	1.0
$FeCl_2$	0.1
$CoCl_2$	0.1
$CaCl_2.2H_2O$	0.1
$ZnCl_2$	0.1
$CuCl_2$	0.1
$AlCl_3.6H_2O$	0.1
H_3BO_3	0.1
$Na_2MoO_4.2H_2O$	0.1
Composition of trace vitamin solution (g L^{-1})	
Sodium ascorbate	10
Biotin	2.0
Folic acid	2.0
Pyridoxine hydrochoride	10
Thiamine hydrocloride	5.0
Riboflavin	5.0
Nicotinic acid	5.0
DL-calcium pantothenate	5.0
Vitamin B12	0.1
p-aminobenzoic acid	0.5
Lipoic (thioctic) acid	5.0
Pantothenic acid	5.0
Composition of tungstate-selenium solution	
0.1mM Na_2WO_4 and 0.1mM Na_2SeO_3 in 20 mM NaOH	

Appendix B. Aerobic biocathode development data

List of Figures

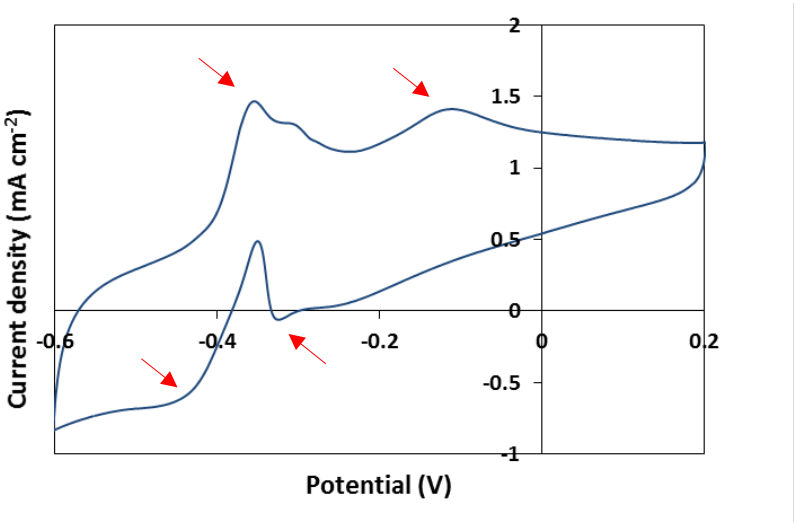


Figure B1. Voltammograms recorded in MFCs equipped with Pt-C as cathodes after 15 days of development of biocathode. Red arrows show the redox peaks attributed to development of Geobacter in bioanode. CVs recorded at a scan rate of 2 mV s^{-1}.

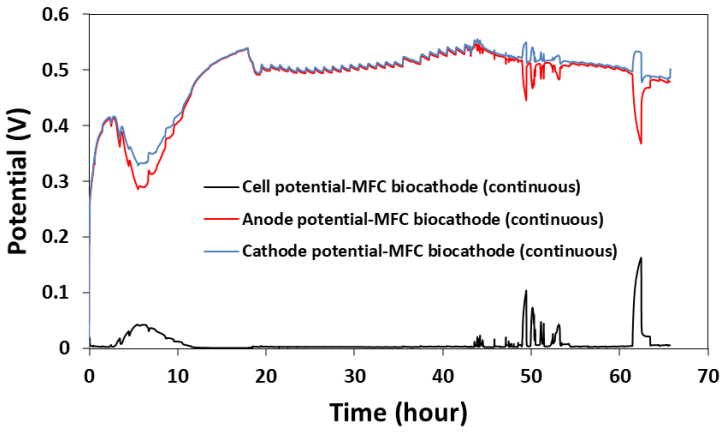


Figure B2. Cell, anode and cathode potentials of MFC (2) equipped with IOB biocathode and operated in continuous operational mode.

Appendix C. Anaerobic biocathode development data

List of Figures

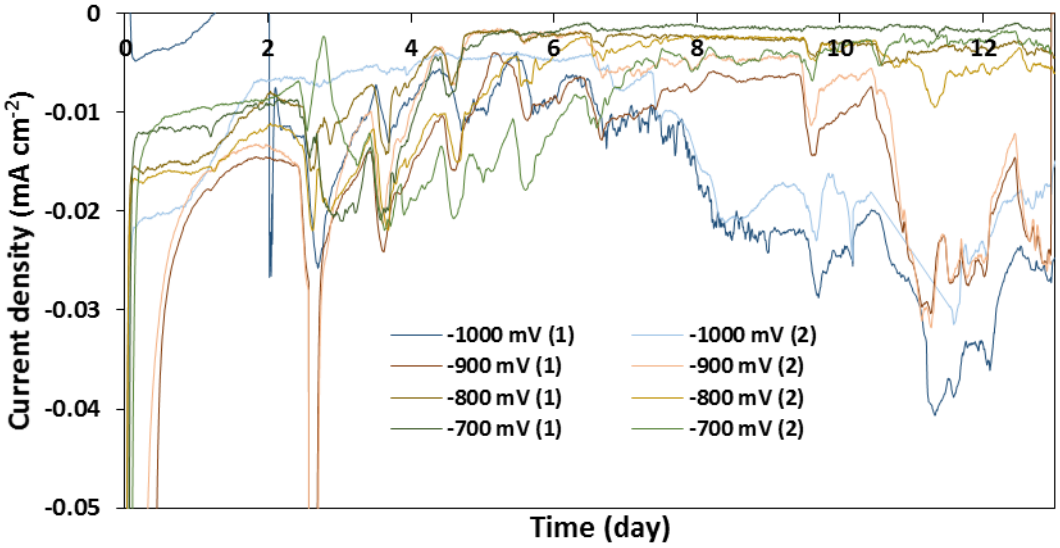


Figure C1. Cathodic current density during the 13 days of abiotic control experiment at the reactors poised at 4 different potentials of -700, -800, -900 and -1000 mV.

(a) (b)

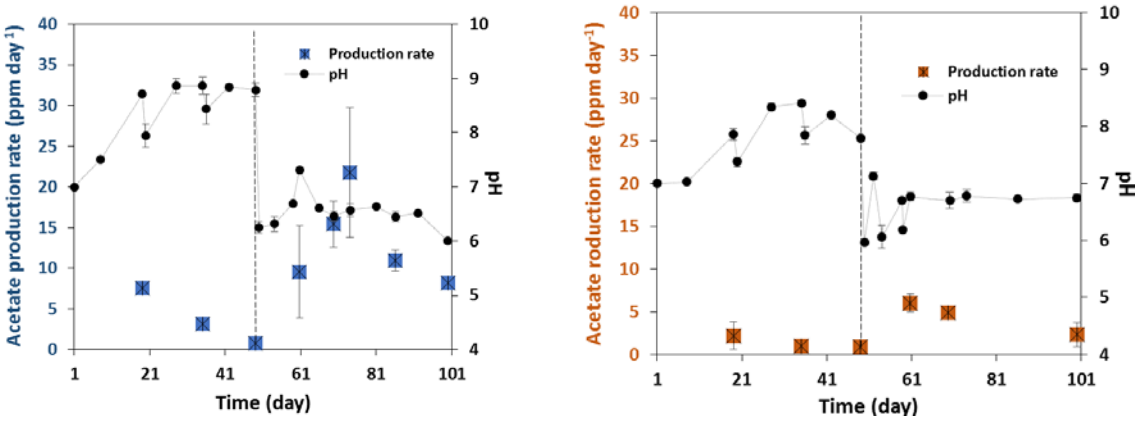


Figure C2. Production rate of acetate through MES in the BESs poised at (a) -1.0 and (b) -0.9 V.

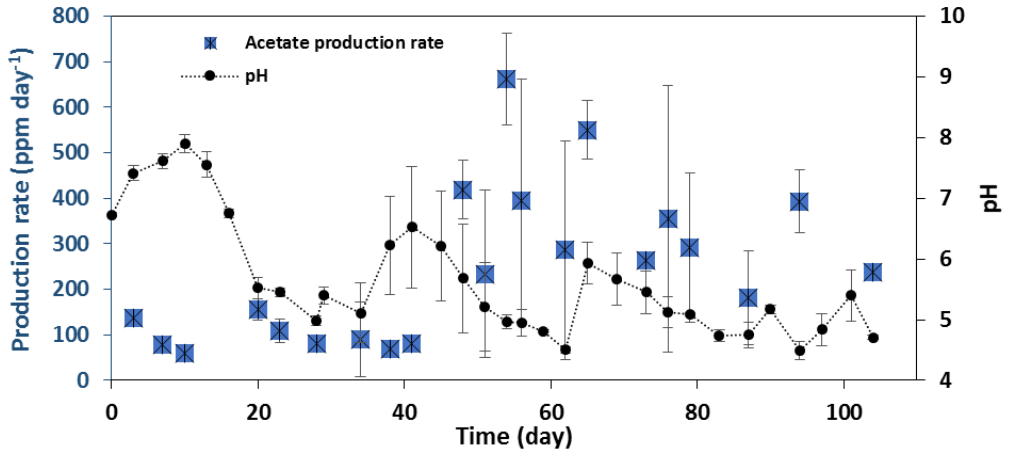


Figure C3. Acetate production rate during the experiment in BESs 1 (-1.0 V_CO_2).

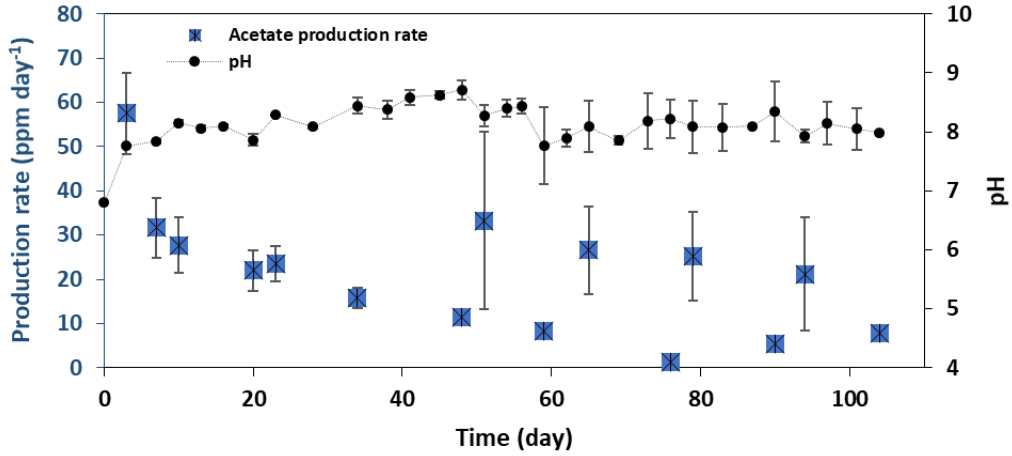


Figure C4. Acetate production rate during the experiment in BESs 2 (-1.0 V_$NaHCO_3$).

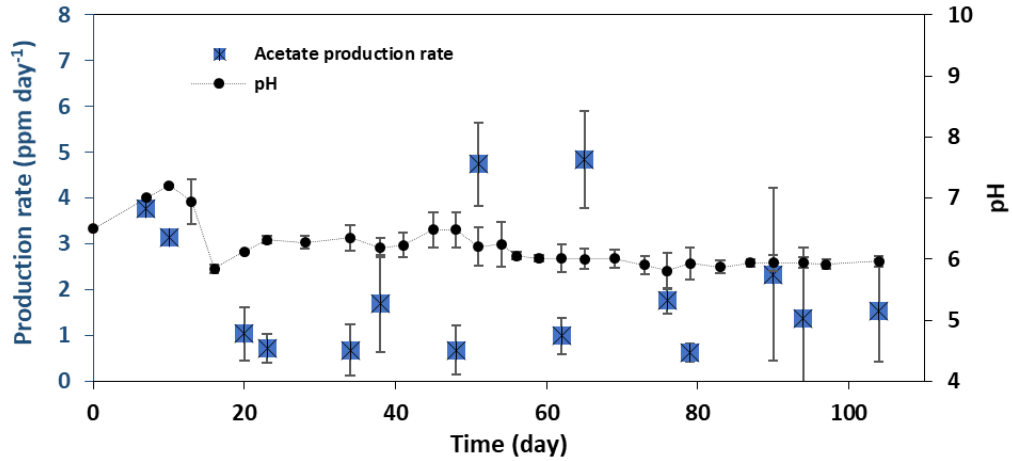


Figure C5. Acetate production rate during the experiment in BESs 3 (-0.8 V_CO_2).

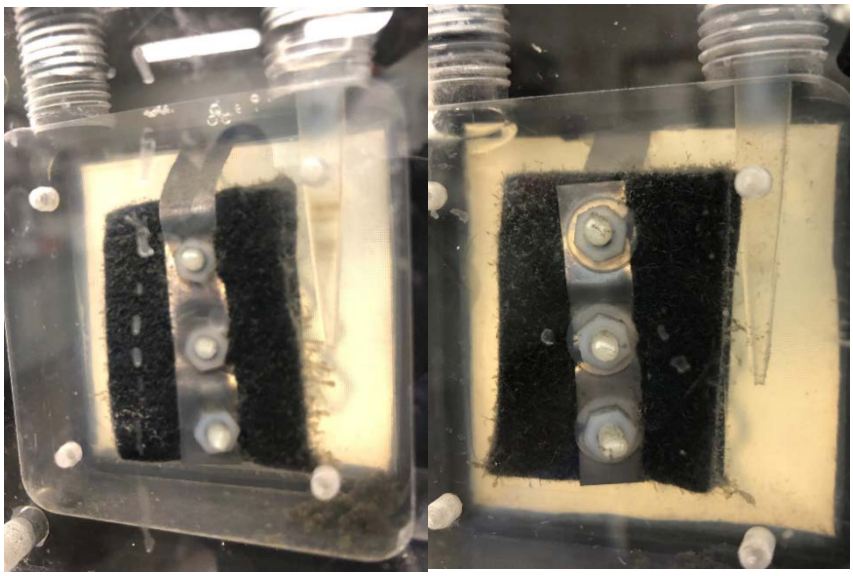


Figure C6. Image of the electrode of BESs 1 (-1.0 V_CO_2) before terminating the reactors.

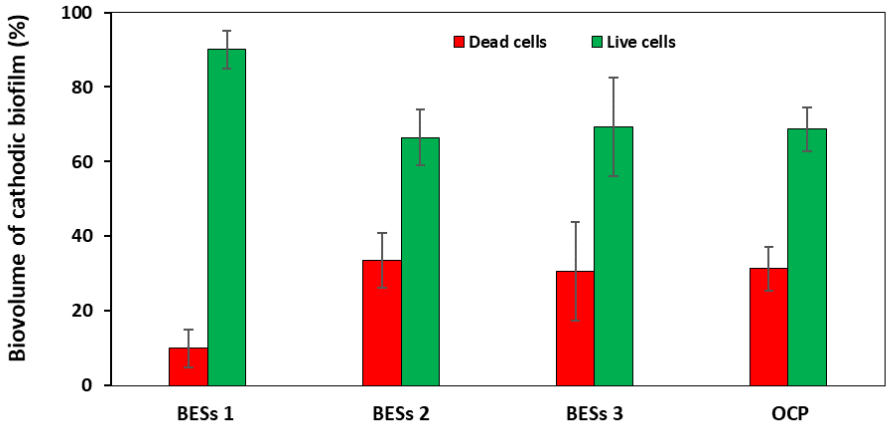


Figure C7. Percentage of live and dead cells in the cathodic biofilm of BESs 1 (-1.0 V_CO_2), BESs 2 (-1.0 V_$NaHCO_3$), BESs 3 (-0.8 V_CO_2) and OCP.

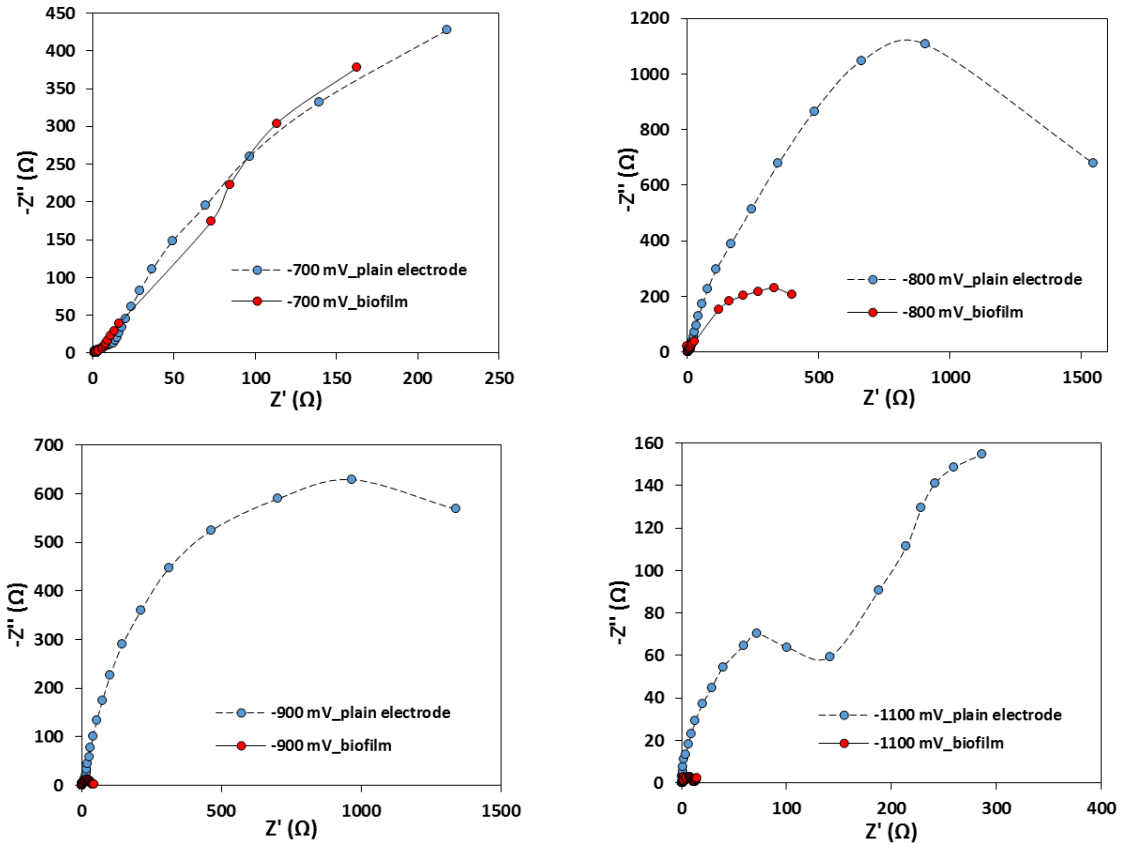
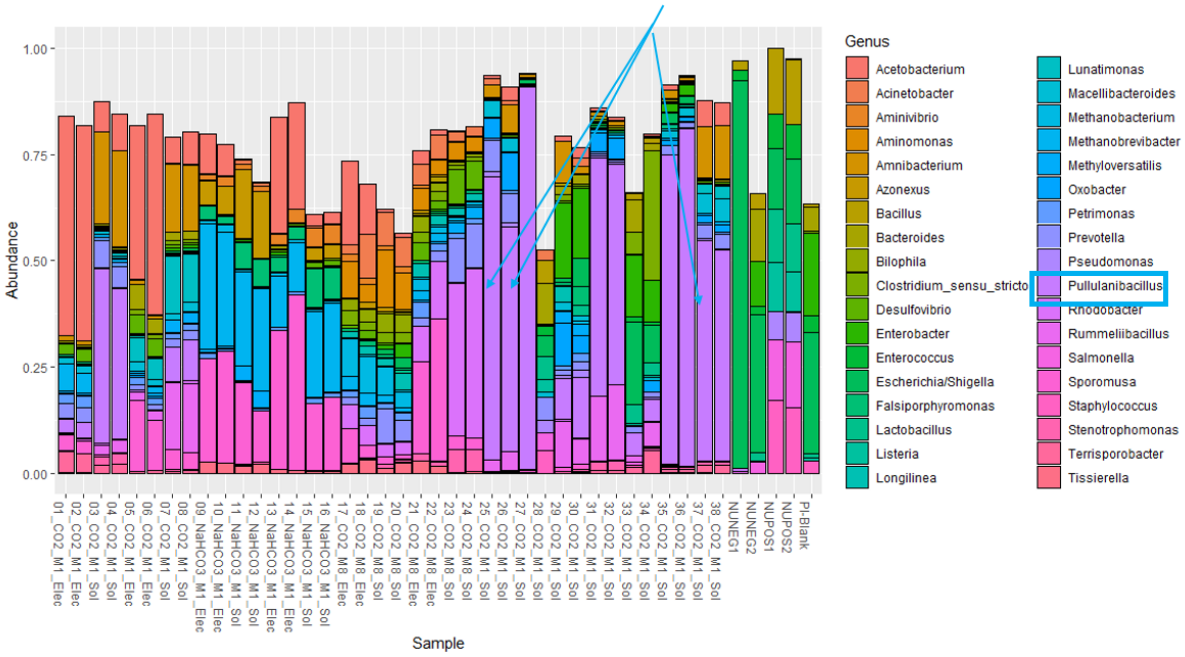


Figure C8. Comparing the EIS analysis recorded from the electrode in BESs 1 (-1.0V_CO_2), and plain electrode at the potentials of -0.7, -0.8, -0.9 and -1.1 V applied at the electrode.

(a)



(b)

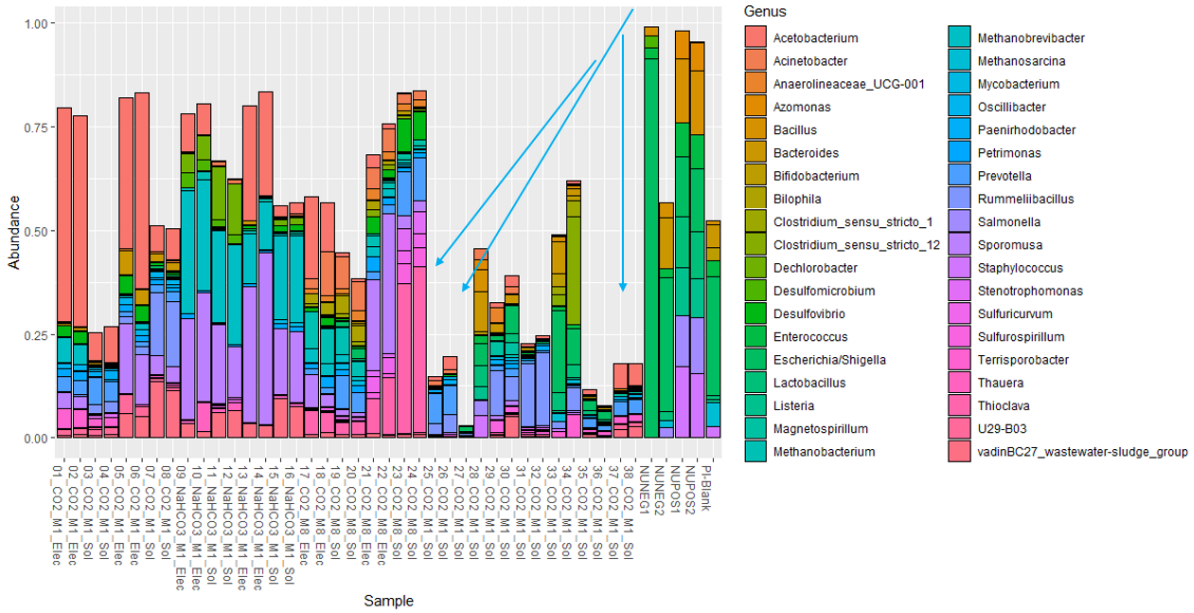


Figure C9. Relative abundance of bacterial community in genus level obtained from Illumina sequencing of 16S rRNA genes from all the sample using (a) RDP and (b) Silva databases with bootstrap 80. The blue arrows show the presence and lack of presence of *Pullunubacillus*.

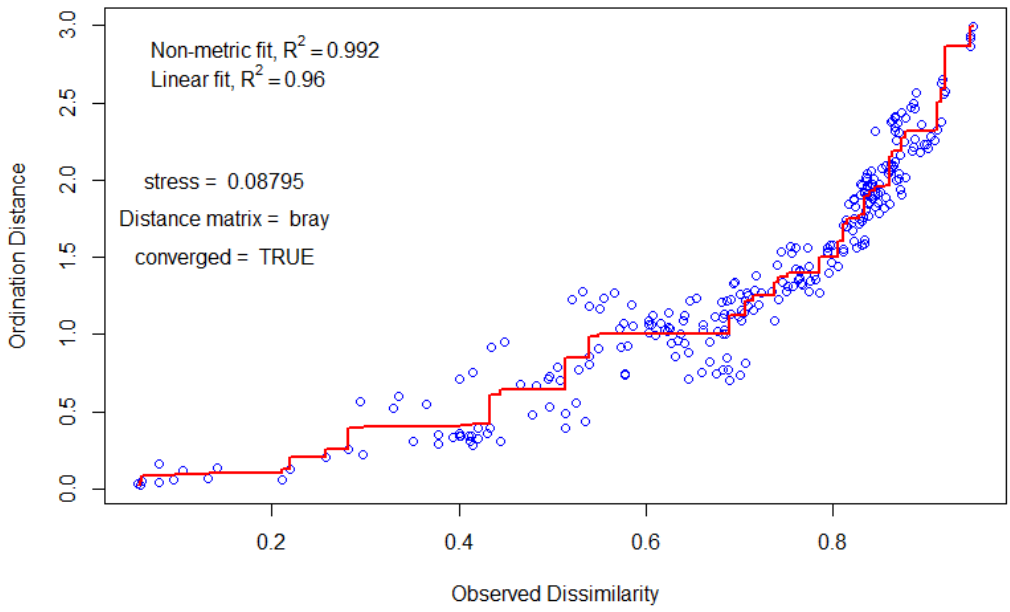


Figure C10. The level of agreement between the original data set and NMDS figure.

Appendix D. Chain elongation in BES data

List of Figures

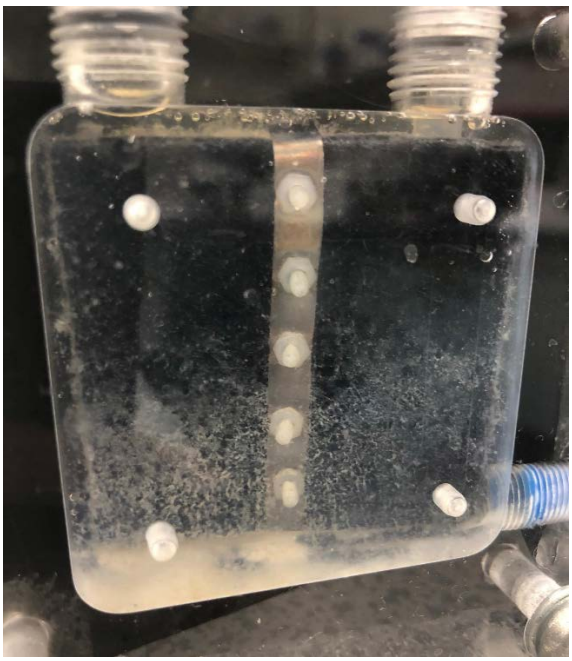


Figure D1. Image of the cathode (back side) of BESs_ethanol after 79 days of experiment.

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