THE UNIVERSITY of ADELAIDE

Understanding Biochemical Interactions and Optimization of Electrochemical Activities in Microbial Fuel Cell System for Dichlorophenol Degradation and Electricity Generation using Microbial Consortia

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To my dearest

Amazing parents ***Che Yun*** *&* ***Hassan***

Lovely wife ***Nurul Afiqah***

Beloved daughter ***Nur Aisyah*** *&* *son* ***Muhammad Thaqif***

Declaration

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Preface

This doctoral thesis is prepared in "Publication" format according to the "Specifications for Thesis (2017)" of the University of Adelaide. It includes publications that have been published, submitted for publication, or prepared in publication format:

1) H. Hassan, B. Jin, S. Dai, Application of microbial fuel cell in toxic contaminants biodegradation. (Prepared in publication style)
2) H. Hassan, B. Jin, S. Dai, T. Ma, C. Saint, Chemical impact of catholytes on *Bacillus subtilis*-catalysed microbial fuel cell performance for degrading 2,4-dichlorophenol, **Chemical Engineering Journal** 301 (2016) 103-114.
3) H. Hassan, B. Jin, E. Donner, S. Vasileiadis, C. Saint, S. Dai, Bioelectrochemical analysis of microbial fuel cell systems inoculated by *Bacillus*- and *Arcobacter*-dominated petrochemical and domestic microbial consortia. (Submitted and under review for Applied Energy Journal)
4) H. Hassan, B. Jin, S. Dai, Biochemical kinetics and pathways of 2,4-dichlorophenol biodegradation by domestic and industrial microbial consortia in microbial fuel cell system. (Prepared in publication style / ready to submit)
5) H. Hassan, B. Jin, S. Dai, K.H. Low, G. Tristanto, Application of polynomial models for optimizing microbial fuel cell system for electricity generation and dichlorophenol degradation. (Prepared in publication style)

Some relevant components of the project work have been orally presented in international conferences with the following proceeding papers:

1) H. Hassan, L.S. Illingheim, B. Jin, S. Dai, Degradation of 2,4-dichlorophenol by *Bacillus subtilis* with concurrent electricity generation in microbial fuel cell, **Procedia Engineering** 148 (2016) 370-377. (Presented at 4th International Conference on Process Engineering and Advanced Materials (**ICPEAM 2016**), 15-17 August 2016, Kuala Lumpur, Malaysia)

2) H. Hassan, B. Jin, S. Dai, C. Ngau, Formation of industrial mixed culture biofilm in chlorophenol cultivated medium of microbial fuel cell, **AIP Conference Proceedings**. (Presented at 4th International Conference on Fundamental and Applied Sciences (**ICFAS 2016**), 15-17 August 2016, Kuala Lumpur, Malaysia)

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Abstract

Toxic pollutants such as phenols and dyes in industrial wastewater have raised increasing environmental and human health concerns in many industrialized countries around the world. There is an ongoing need to develop sustainable and cost-effective technologies to remove these pollutants. Bioelectrochemical technology such as microbial fuel cell (MFC) system has been approved as promising and sustainable treatment process for removing of these organic toxic pollutants, while generating electricity by exoelectrogenic bacteria.

The exceptional MFC ability to degrade chlorophenol as one of the recalcitrant pollutants in industrial wastewater has been explored in this study. Three functional bacteria: pure culture *Bacillus subtilis*, and two mixed microbial consortia derived from domestic and industrial petrochemical wastewaters have been utilized for electricity generation and 2,4-dichlorophenol (2,4-DCP) degradation in a double chamber MFC system. The selection of microorganisms is based on their ability to degrade phenols and its derivatives so as to discover good exoelectrogenic bacteria to drive MFC system for degradation of 2,4-DCP and production of electricity. The industrial petrochemical wastewater as a highly phenolic-contaminated wastewater is expected to provide its mixed consortium better acclimatization in 2,4-DCP-mediated MFC environment as compared to typical mixed consortium from domestic wastewater.

Bacillus subtilis has been approved to show its great ability to generate maximum current density of 64 mA/m^2 in persulfate-based catholyte MFC system while degrading up to 60% of 2,4-DCP. Chemical properties of catholytes, for instance, oxidizing and buffering abilities, could improve the MFC performance through well controlled pH and electron transfer mechanism. The experimental results revealed that low-cost and low-toxicity catholytes, such as potassium persulfate, M9 and phosphate buffer could amplify the electricity generation with simultaneous 2,4-DCP degradation in double chamber MFC system.

Like *B. subtilis*, mixed consortia from both domestic and industrial wastewaters have demonstrated high performance in electricity generation and 2,4-DCP degradation using Pt/Ti electrode in MFC systems. The important bacteria in domestic mixed consortium

for 2,4-DCP degradation have been identified as *Arcobacter* and *Cloacibacterium* which showed positive response towards the toxic pollutants in anodic MFC. Industrial mixed consortium in which *Bacillus* dominated the cultures, performed well in generating 156 mA/m^2 current density, with 41% phenolic degradation as compared to domestic consortium with 123 mA/m^2 and 62% phenolic degradation. This study proved that *Bacillus* sp. from petrochemical wastewater could have high adaptation in chlorophenol containing medium through its high current generation profiles in MFC despite of its relatively low 2,4-DCP degradation capability.

The performance of both mixed consortia was further investigated for its growth kinetics and 2,4-DCP biodegradation pathways in double chamber MFC systems. Domestic consortia biofilm was found to have higher phenol degradation ability with a high growth constant of 27 mg/L and specific biodegradation rate of 0.32 mg/L/h. Although industrial consortia yielded lower apparent kinetic parameters, its growth profile at low 2,4-DCP concentration implied its excellent bacterial acclimatization with higher voltage outputs. The toxicology analysis of the final metabolites of 3-oxoadipate and acetate produced by industrial and domestic consortia suggests that both consortia could degrade 2,4-DCP into very much less hazardous and simpler compounds in the MFC system.

Finally, an optimization study on MFC performance by *B. subtilis* was investigated using statistical central composite design (CCD) of response surface methodology (RSM). The optimized parameters, including anode pH, cathode pH and inoculum size, were found to have good interaction to generate optimal current density of 106 mA/m^2 with over 70% 2,4-DCP degradation rate through the developed quadratic models. Analysis of variance revealed that the optimum current density could be achieved at anode pH 7.5, cathode pH 6.3-6.6 and 21 – 28% inoculum size. Only inoculum size-cathode pH interaction appeared to be significant for phenolic degradation response where the optimum predicted phenolic degradation could be attained at cathode pH 7.6 and 29.6 % inoculum size.

This project therefore has contributed several valuable fundamental and biosystem outcomes in terms of systematic biochemistry, microbiology electrochemistry and optimization in MFC system. Pure culture *Bacillus subtilis*, *Arcobacter*- and *Bacillus*-

dominated domestic and industrial microbial consortia have been discovered their special capabilities in generating electricity and 2,4-DCP degradation, thus could be promising bacterial cultures in forthcoming MFC studies.

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List of Abbreviations

2,4-DCP	2,4-dichlorophenol
ΔpH	pH change
ACN	Acetonitrile
AD	Anaerobic digestion
ADG	Australian Dangerous Goods Code
ANOVA	Analysis of variance
AWQC	Ambient Water Quality Criteria
BES	Bioelectrochemical system
BOD	Biochemical / biological oxygen demand
CCD	Central composite design
CCV	Closed circuit voltage
CDW	Cell dry weight
CE	Coulombic efficiency
CEM	Cation exchange membrane
COD	Chemical oxygen demand
CP	Chlorophenol
CV	Cyclic voltammetry
DMC	Domestic microbial consortium
dMFC	Domestic microbial consortium-Microbial fuel cell
DNA	Deoxyribonucleic acid
EET	Extracellular electron transfer
EIS	Electrochemical impedance spectroscopy
FD	Factorial design
FMN	Flavin mononucleotide
HPLC	High performance liquid chromatography
IET	Intracellular electron transfer
IMC	Industrial microbial consortium

iMFC	Industrial microbial consortium-Microbial fuel cell
IRR	Internal rate of return
LC-MS	Liquid chromatography mass spectrometry
LSV	Linear sweep voltammetry
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
NCBI	National Center for Biotechnology Information
NPW	Net present worth
OCV	Open circuit voltage
OD	Optical density
OTU	Operational taxonomic unit
PB	Phosphate buffer
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
PEM	Proton exchange membrane
PP	Potassium persulfate
RDP	Ribosomal database project
RPM	Rotation per minute
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
SEM	Scanning electron microscope
SHE	Standard hydrogen electrode
SRA	Sequence read archive
SSU	Small ribosomal subunit
TOF	Time-of-Flight
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultraviolet
WHS	Work health and safety

CHAPTER 1

~ INTRODUCTION ~

Chapter 1: Introduction

1.1 Project Background

Microbial fuel cell (MFC) has gained intensive research interests as a sustainable technology in wastewater treatment and renewable energy generation since decades ago. The functional microorganisms known as exoelectrogenic bacteria that could produce electrical current from chemical utilization and degradation make the MFC an exceptional tool for exploiting its bioelectrochemical reaction capabilities for wastewater treatment. The electrons transfer from bacteria to anode surface could be performed through direct, mediator and/or nanowires transfer mechanisms which result in current generation. The reduction of O_2 to H_2O in cathode which accepts electrons and flow of protons through ion-exchange membranes complete the electrical circuit in MFC system.

Latest studies on MFC cover a broad range of research subjects, including electrode materials [1 – 3], MFC designs [1], biocathodes [4 – 6], exoelectrogenic microorganisms and substrates [7, 8]. Recent progress of MFC studies on wastewater treatment focuses on organic loading reduction, nitrification/denitrification and toxic pollutant removal [9]. Therefore, MFC appears to be one of the best options in sustainable wastewater treatment to replace current conventional wastewater treatment which are generally chemical- and energy-intensive techniques [9].

Hazardous pollutants such as phenol compounds and derivatives which are found abundantly from industrial effluent could expose serious threat especially to aquatic organisms and human beings. Phenols and their derivatives for instance chlorophenols (CPs) have aromatic ring structures containing at least one chlorine atom (-Cl) and one hydroxyl (-OH) group at the benzene rings. These aromatic ring structures have been recognized based on their chemical characteristics, for example, monochlorophenols, polychlorophenols, chloronitrophenols, chloroaminophenols, and chloromethylphenols [10]. The major sources of the phenol contaminants are industrial wastes, pesticides, herbicides, and complex chlorinated hydrocarbons [11]. CPs and their derivatives are highly toxic to living beings due to their carcinogenic, mutagenic and cytotoxic properties [12]. The World Health Organization and the International Agency for Research on Cancers have characterized several poly-CPs as potential human carcinogens [12].

Microbial treatment or biodegradation of these toxic phenolic compounds leading to complete mineralization in aerobic or anaerobic cultivations have shown to be economical and versatile, and have been widely studied in the last two decades [13]. This biodegradation technique exploits the ability of bacteria including pure and/or single strains, mixed bacterial consortia or yeast genera to convert this toxic organic pollutant to water, carbon dioxide and biomass, by avoiding undesirable by-products or secondary pollutants [14]. For this instance, the integration of biodegradation in MFC systems appears to be the most environmentally benign method for toxic chlorophenol removal with simultaneous generation of energy. Some recent studies have discovered several functional microorganisms to degrade poly-CPs in MFC [15 – 17]. However, there is lack of fundamental studies on biodegradation kinetics and pathways, and identification of positive response-bacteria towards CPs degradation in MFC. These missing scientific elements are significant to comprehend further the CPs biodegradation phenomena associated with bioelectrochemical activities and electron transfer mechanisms in MFC systems.

Therefore, this thesis project was aimed to develop a double chamber MFC system for generating electricity and chlorophenol degradation. The laboratory scale MFC system was bio-catalyzed with specific bacterial cultures namely pure *Bacillus subtilis*, and mixed consortia derived from domestic and industrial petrochemical wastewaters. The utilization of *B. subtilis* in MFC is due to its excellent role in degrading phenolic compounds [10] supported by its ability to generate electricity in MFC [18, 19]. The ability of mixed bacterial consortia to acclimatize in potential-gradient bioelectrochemical reactions is a pre-requisite to maintaining the electricity generation in MFC systems. Thus, the selection of mixed consortia from highly phenolic contaminated industrial petrochemical wastewater is believed to provide better adaptation of the bacteria in MFC systems while degrading chlorophenol in synthetic cultivation medium. The domestic mixed consortia as normally isolated and utilized microorganisms in MFC studies, were used to compare its performance with petrochemical industrial wastewater based consortium. This study particularly analysed the double chamber MFC performances in terms of catholytes roles in intervening the electron transfer mechanisms in MFC, identifying the positive response bacteria for 2,4-dichlorophenol degradation, kinetics and biodegradation pathways and

current generation associated with physicochemical interactions using polynomial design approach.

1.2 Project Objectives

This study is aimed to investigate the ability of three selected bacterial cultures including pure *Bacillus subtilis*, and mixed bacterial consortia derived from domestic and industrial petrochemical wastewaters for electricity generation and 2,4-dichlorophenol (2,4-DCP) degradation in double chamber MFC system. The main objectives of this study have been successfully demonstrated throughout this project and are highlighted as the followings:

1 – To investigate the chemical impact of catholytes on MFC performance in generating electricity and 2,4-DCP degradation by *B. subtilis* with respect to pH change profile and electron transfer mechanism.

2 – To explore the dynamic changes of bioelectrochemical activities and bacterial community towards 2,4-DCP degradation using domestic and industrial petrochemical mixed consortia in MFC systems.

3 – To evaluate the biodegradation kinetics and pathways of 2,4-DCP by domestic and industrial petrochemical mixed consortia in MFC systems.

4 – To optimize the current generation and 2,4-DCP degradation by *Bacillus subtilis*-catalysed MFC systems using polynomial design of response surface methodology.

1.3 Thesis Outline

This thesis is presented based on the thesis-by-publication format which consists of four main chapters as the core contents in this study. The thesis is introduced with project background in Introduction chapter which briefly explains the MFC main principle and operations, recent related MFC studies, and key environmental problems to be addressed and applied in the MFC investigations associated with the study gaps within the project scope. The project background is followed by the aims and main objectives that govern the overall project scope.

The second chapter of the thesis is Literature Review. This chapter reviews and discusses the recent research progresses and previous studies on MFC systems, including principles, concepts, and its key bioelectrochemical and systematic design components which are useful for the implementation of this study. The literature review is then followed by the first main technical content of this project (Chapter 3). This chapter reports the study on the ability of *Bacillus subtilis* to generate electricity and 2,4-DCP degradation through the impact of catholytes and electron transfer mechanisms on the overall MFC performance.

After having studied the behaviors of *B. subtilis* and catholytes in MFC, the roles of mixed bacterial consortia from domestic and industrial petrochemical wastewaters in MFC were analyzed as presented in Chapter 4. This chapter describes one of the most important discoveries of this project which is the identification of responsive and exoelectrogenic bacteria from the biofilm and suspension of anodic MFC towards 2,4-DCP degradation. The bioelectrochemical activities and the comparison study between MFC and anaerobic biodegradation system were also discussed in this chapter.

Then, the 2,4-DCP biodegradation kinetics and pathways using both mixed consortia were studied as the key research component of this study. The growth and degradation rate profiles were analysed using various kinetic models, which are the key data to propose the 2,4-DCP biodegradation pathways. The toxicology analysis of relatively simpler and less toxic metabolites was briefly discussed at the end of the Chapter 5.

The final technical content of the thesis reported in Chapter 6 is the study on optimization of *B. subtilis*-catalysed MFC system for electricity generation and 2,4-DCP degradation using polynomial design of response surface methodology (RSM) statistical software. The thesis is ended with the last chapter Conclusion, in which the core findings of this study were concisely reiterated followed by a number of recommendations for further improvements in future MFC studies.

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CHAPTER 2

~ LITERATURE REVIEW ~

Statement of Authorship

Title of Paper	Application of microbial fuel cell in toxic contaminants biodegradation
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Principal Author

Name of Principal Author (Candidate)	Huzairy Hassan
Contribution to the Paper	Manuscript writing, review and analysis.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 19/01/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Bo Jin
Contribution to the Paper	Supervising the development of the work, manuscript review and correction.
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2. Application of Microbial Fuel Cell in Toxic Contaminants Biodegradation

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Chapter 2: Application of Microbial Fuel Cell in Toxic Contaminants Biodegradation

Abstract

The rapid industrialization and population growth require a very efficient and sustainable toxic wastewater treatment through various economical and eco-friendly methods. Current toxic wastewater treatment technologies which entail high energy and cost-intensive seems to be non-sustainable to meet this water sanitation need. Microbial fuel cell (MFC) system appears to be a promising technology which provides a better option for toxic wastewater treatment while generating sustainable bio-energy through its bioelectrochemical reactions. This paper reviews the operation, mechanism and performance of MFC in generating electricity with focus on organic and inorganic toxic contaminants degradation. This review also discusses the degradation of refractory contaminants that heavily found in industrial wastewater including phenols, metals and dye, which are also received special attentions among scientific communities especially in the MFC studies. This paper is concluded by the overview of current MFC technical and economic challenges and several constructive recommendations for future MFC improvements.

Keywords: Microbial fuel cell; Toxic contaminants; Phenols; Biodegradation

2.1 Introduction

Microbial fuel cell (MFC) technology emerges as a potential engineering tool in sustainable bio-energy generation and wastewater treatment. The bioelectrochemical process of MFC which is carried out by unique exoelectrogenic bacteria has attracted intensive research interests among scientific community since decades ago. The widespread research topics in MFC studies include the use of immobilized bacteria on the electrode surfaces, electrode materials, MFC design, fuel cell operation, reliability and applications [1].

The first work using MFC principle was initiated in the early 20th century, where a chemical mediator was used to transfer electrons from the bacteria in the cell to the anode [2]. Then, a mediatorless MFC began to be developed in the 1970s where the bacteria itself can transfer the electrons directly to the anode through electrochemically active redox protein (cytochromes) on its outer membrane [2]. In early 21st century thus far, MFC becomes a topical field of study among investigators for searching the optimum designed and configuration, operational parameters, types of microbial cultures and electrode materials. Furthermore, the researchers have recently shift their MFC research direction to develop a commercial wastewater treatment using MFC principle [3].

MFC is also viewed as an important technology to treat water pollution while harvesting electricity. MFC seems to be a greener and cost-effective way to replace the conventional wastewater treatment methods which normally require high energy and chemical use for example chemical oxidation, distillation, adsorption, membrane separation, and chromatography. Previous studies have found that MFC could reduce significantly the organic loading termed as COD of the wastewater using varieties of bacteria and MFC designs [4, 5]. While others attempted to treat other waste compounds in the MFC for instance phenolic, carbon, glycerol, sulfide, and metals [6 – 11]. These studies showed that the MFC systems could reduce and degrade the waste compounds effectively at the range of 60 - 99 % waste reduction. With this achievement, MFC undoubtedly could be further improved especially in its application in the real wastewater environment.

Besides, we are concerned about the water pollution which is very challenging as the waste and wastewater could be produced enormously from time to time. One of the critical waste compounds that need to be paid significant attentions is phenolic compounds. Phenols and their derivatives have been plentifully found in wastewater especially from chemical, petrochemical, pharmaceutical, oil refineries, and pulp and paper industries [12]. These compounds possess dangerous and recalcitrant properties including acute toxicity, bioaccumulation due to its lipophilicity [13], emission of a strong odour, persistence in the environment, and suspected carcinogenicity and mutagenicity [14]. Other than phenols, metals and coloring dyes are also toxic and mutagenic compounds that require special attention as they are heavily found in industrial effluent. Therefore, it is very essential to treat these toxic compounds in the wastewater before they could destruct more aquatic lives as well as human being. In view of that, the MFC which is great at its biodegradation capability, seems to be a promising technology to degrade these type of wastes while gaining useful energy from its process.

This chapter provides an overview of the MFC operation, mechanism and performance in generating electricity and wastewater degradation. We focus on the MFC capabilities in the toxic wastes biodegradation, their kinetics and degradation pathways involved in the process. A special attention is given to the degradation of phenols, metals and dyes in MFC. In addition, the bacterial communities known as exoelectrogens involved in the bioelectrochemical process which are vital backbones in the MFC biodegradation and overall performance are also integrated in the subsections.

2.2 MFC Operations and Mechanisms

Microbial fuel cell is a device that uses microbes to convert the chemical energy stored in organic and inorganic compounds into electricity, providing a low-cost and low-maintenance reactor that produces a small amount of sludge as the by-product [15]. The electron production and transfer as a result of the oxidation and reduction reactions will subsequently generate electricity in the MFC system. MFC normally consists of two chambers: an anaerobic anode chamber and an aerobic cathode chamber separated by an ionic exchange membrane.

2.2.1 Anode MFC

The organic wastes used as substrates (for example glucose) are oxidized in anode MFC which produce electrons and protons. The bacteria in the form of biofilm pass the electrons to anode which then migrate through external circuit producing current. The protons will pass through the membrane to the cathode, where they combine with oxygen and electrons to form water [16]. The chemical reactions involved are described by the followings:

Anode (Glucose): $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$ (Rxn. 2.1)

Cathode: $24H^+ + 24e^- + 6O_2 \rightarrow 12H_2O$ (Rxn. 2.2)

The anode MFC or sometimes called bioanode utilizes microorganisms in the form of biofilm and/or suspension to perform substrate oxidation as well as contaminant biodegradation. The anodic biofilms plays major role especially in electron transfer mechanisms as they are directly attached on the electrode surface as illustrated in Figure 2.1. There are several mechanisms of electron transfer as a result of substrate oxidation by bacteria (Figure 2.2); 1) Direct electron transfer through outer membrane cytochromes, 2) electron transfer through mediators, and 3) electron transfer through nanowires [17].

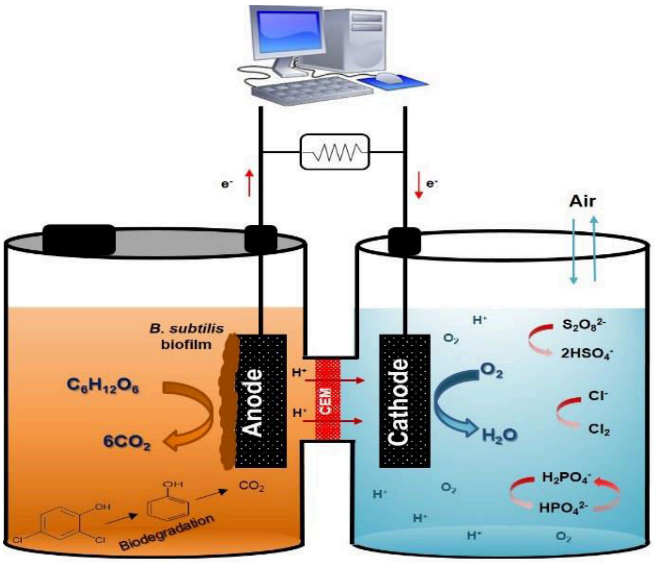

Figure 2.1 Schematic diagram of a double chamber microbial fuel cell system.

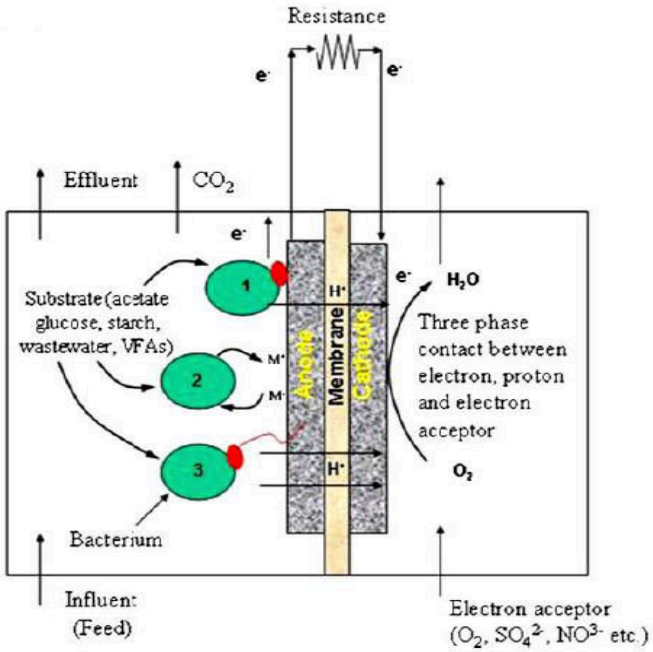

Figure 2.2 Simplified view of a two-chamber MFC with possible modes of electron transfer: (1) Direct electron transfer (via outer membrane cytochromes); (2) electron transfer through mediators; and (3) electron transfer through nanowires [17].

2.2.1.1 Bioanode Bacterial Cultures

The oxidation of substrates is normally coupled with the electrons transfer from bacterial biofilm to anode which generate electrical current. This type of bacteria is called exoelectrogenic, electrogenic or electrochemically active bacteria. Fermentative bacteria are needed to convert complex substrates into carboxylic acids including acetate, which can then be digested by electrogenic bacteria [18]. For example, the bacteria (*Bacillus subtilis*) will oxidize phenolic compounds and generate electrons, protons and carbon dioxide (Figure 2.1). Apart from that, there are also bacteria in suspension mixture which might be electrochemically or non-electrochemically active that contribute to the biodegradation of contaminants.

Various electrochemically active bacterial species have been discovered and isolated in the anodic biofilm which showed good performance in electron transfer as well as waste reduction in MFC. A well-known species in MFC is *Geobacter sulfurreducens*. *G. sulfurreducens* is a metal-reducing and electrogenic bacterium widely found in nature, able

to directly transfer electrons to the electrode [19]. This species has received a great attention since its complete genome was sequenced, providing insights into its physiological, biochemical and genetic properties [20]. Commault et al. [21] recently studied the capability of *Geobacter*-dominated biofilms in MFC to treat synthetic wastewater. The COD was removed up to 0.8 g/L by well-attached biofilms and achieved about 100 mW/m^2 power density. This species has also been found to maintain the electricity generation over 60 days in MFC with acetate as electron donor and obtained maximum power density of 7 mW/m^2 [22].

Other important bacterial species have also been reported to produce current in MFC for instance *Shewanella*, *Bacillus*, and *Pseudomonas*. *Shewanella* sp. HN-41 has been found to have strong ability to reduce Fe(III) with lactate or glucose as electron donor, and secreted flavin mononucleotide (FMN) and riboflavin in MFC [23]. As an electron shuttle, flavins can mediate extracellular electron transfer, and some microorganisms have been found to have the ability to use flavins for extracellular electron transfer [24]. *Shewanella*, as an exoelectrogen, can not only secrete flavins, but can also produce higher power output by utilizing exogenous flavins [23].

Previous studies also used pure *Bacillus* sp. to generate electricity in MFC [25, 7, 10]. Although the amount of electrical current produced by this bacterium is relatively lower than that of mixed bacterial consortia, the ability of *Bacillus subtilis* in phenolic degradation in MFC should be highly considered in the toxic waste treatment [10]. Some *Bacillus* genus for example *Lysinibacillus sphaericus* and *Bacillus safensis*, have been isolated to be dominant in industrial distillery wastewater and able to generate 400 mV of closed circuit potential in MFC [26]. Furthermore, *Bacillus* sp. have been found to form flagella structure on its biofilm development [27]. This flagella structure is important for the biofilm functionality including in the bacterial motility, genetic communication among the microbial community and strengthening the biofilm attachment to the electrode [27], thus improve the electron transfer in the MFC system.

Another excellent exoelectrogenic bacterium isolated in the MFC biofilm is *Pseudomonas* sp. This species has been largely researched owing to its excellent capabilities in defining metabolic diversity, colonizing wide range of niches and ease of culturing [28]. *Pseudomonas aeruginosa* is generally associated with the phenazines and

pyocyanin secretion which are important natural products for cystic fibrosis patients as well as for biological control in soil environment.

2.2.1.2 Anode Material

Other than bacterial cultures which could be the main backbone in the MFC performance, anode also plays a major role in enhancing the efficiency of electron transfer, thus current generation. Widespread anodic materials including carbonaceous, graphite, metal and metal oxides and composite material have been used in MFC [29]. The MFC performance resulted from these materials were varied which could also be contributed by other factors for instance the MFC designs, types of substrates and bacterial cultures, and physicochemical factors. The most widely used anode material is carbonaceous material due to its good biocompatibility, chemical stability and conductivity, and relatively inexpensive [1].

The modifications of the anode materials have also been an attractive method in order to improve the current generation in the MFC. Liu et al. [30] found that the electron transfer could be improved by 38% when using carbon cloth anodes modified with formic acid. Zhang et al. [31] recently increased MFC performance by 24.5% using manganese dioxide (MnO_2) electrodeposited carbon felt anode compared to bare carbon felt. This finding could be explained by the synergistic effect of the material properties (biocompatibility, high specific surface area and pseudocapacitive behavior) which could facilitate the electron transfers [1].

Besides, the surface area and morphology of the anodic materials are important factors that determine the biofilm formation and efficiency of the electron transfer in the MFC. Metallic anode materials including stainless-steel, titanium or gold, despite of excellent conductive properties, are not good choices as they have smooth surface which obstructs the attachment of bacterial biofilm. That is why the carbonaceous materials are more preferable in the anodic MFC. However, the use of metallic electrode materials is more preferable to be applied at the cathode MFC as they could improve catalytic activities for oxygen-reduction process in air-cathode MFC [32].

2.2.2 Cathode MFC

Cathode MFC is a chamber where the reduction of oxygen occurs as a result of H^+ ions from the substrate oxidation in anode MFC to complete the current circuit. In a double chamber MFC, cathode chamber normally contained with various chemicals as catholytes for example saline NaCl, phosphate buffer, potassium persulfate, ferrycyanide, and permanganate [10, 33, 34]. Whereas in single chamber MFC or also known as air-cathode MFC, the cathode typically consists of catalyst sandwiched together with proton exchange membrane (PEM). The cathode is exposed to the air for oxygen reduction into water as illustrated in Figure 2.3.

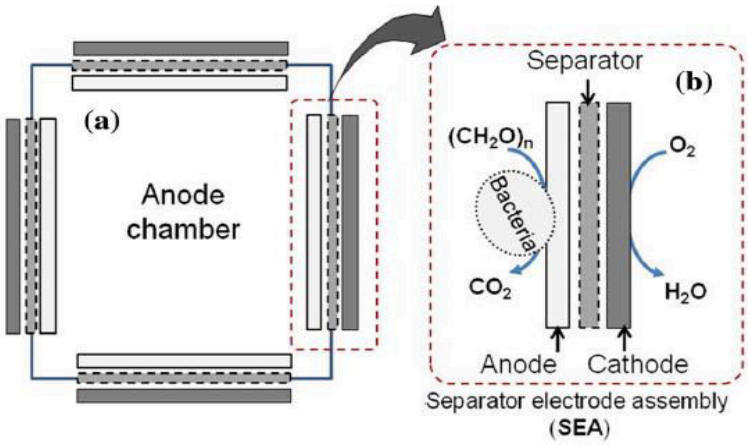

Figure 2.3 (a) Schematic diagram of the top-view of the single-chamber microbial fuel cell **(b)** and a detailed description of the separator-electrode assembly [35].

2.2.2.1 Catholytes in cathode MFC

The key role of an excellent catholyte in MFC operation is to provide good medium for electron and proton transfers from anode to cathode efficiently. Furthermore, the ability of a catholyte to have oxidizing and buffering properties is significant in order to enhance the performance of the MFC [10]. The oxidizing property of a catholyte promotes the reduction of oxygen into water. When the rate of oxygen reduction is increased, the flow of electrons is improved, thus, enhancing the current production in the MFC system. The buffering property of a catholyte can preserve the pH of the cathode from any changes due to anionic reduction process, and thus affects the MFC performance even at very little pH change [10,

34]. In addition, the principle of reduction process is applied in the chemical cathode to produce derived chemicals as well as to remove environmental pollutants (Figure 2.).

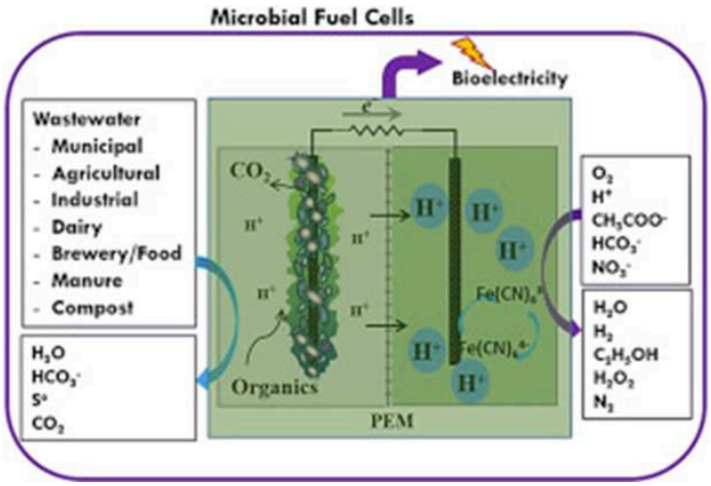

Figure 2.4 Microbial fuel cell for wastewater treatment with a chemical cathode: anode chamber can be fed with various wastewater sources while the cathode chamber can be used to produce useful chemicals or remove environmental pollutants [3].

2.2.2.2 Biocathode MFC

The reduction reaction occurred in cathode chamber of MFC could also be performed by microorganisms. Biocathode MFC has been studied in comparison to chemical cathode for evaluating the MFC performance and improved electron transfer. The use of hazardous chemicals such as ferricyanide or expensive platinum catalyst for electron accepting mechanism in the cathode seems impractical especially for large scale environmental application [3]. The application of biocathode as a driver for the electricity generation process may improve the functionality of the technology by providing advanced wastewater treatment while reducing the energy, environmental and cost footprint of the overall technology [36]. Hence, the recently emerged biocathode MFC system could replace the expensive abiotic cathodes for electricity generation and waste reduction [3].

Examples of waste reduction capabilities in biocathode MFC include COD removal, nitrification/denitrification, metal, sulfate and phenolic removal. *Cyanobacteria* has been well-known for performing nitrification and currently it showed effective nitrogen removal

in cathode MFC while generating electricity [37]. Both the enrichment of the heterotrophic denitrifying bacteria with the organic matter added in the cathode and the stimulated metabolic activities of the nitrifiers and denitrifiers in the electric field probably contributed to the observed enhanced nitrogen removal efficiencies in the MFC system [37].

The biocathode MFC is found to have comparable performance with abiotic cathode. The uncultured *Gammaproteobacteria* in aerobic cathode showed comparable performance in electricity generation to a platinized cathode [38]. It was found that bacteria catalyzed the oxygen reduction through electron transport chain. About 62 $\mu W/cm^2$ power has been harvested by this biocathode compared to Pt-cathode with 70 $\mu W/cm^2$ power output. This indicates that the aerobic biocathode can provide a promising self-sustained, free to use, stable alternative to chemical catalysts for oxygen-reduction reaction in MFCs [38].

Metal-reducing bacteria can be a potential tool in the treatment of heavy metal polluted wastewater. Metal ions present in wastewater could not be biodegraded into harmless end products and thus reduction mechanism by metal-reducing bacteria seems to be a safer method for this problem [39]. Besides, some of these heavy metal-containing groups have high redox potentials and therefore could be utilized as electron acceptors in order to reduce and precipitate [39]. Song et al. [40] demonstrated electricity production and hexavalent chromium (Cr(VI)) reduction in a biocathode MFC. The graphene biocathode was found to increase the electricity generation of Cr(VI)-reducing MFC.

The biocathode MFC could also recover cobalt (Co) at a rate of 0.08 mmol/L.h and yielded cobalt hydroxide of 0.24 mol/mol COD while generating 1.5 W/m^3 power output [41]. Manganese and iron have been used to transfer electrons from the electrode to oxygen by means of biological processes [42, 43]. Ter Heijne et al. [44] developed an oxygen cathode mediated by the couple Fe_{3p}/Fe_{2p} at very low pH with biological re-oxidation of ferrous ions with oxygen by a culture of *Acidithiobacillus ferrooxidans*. Compounds other than oxygen can also be used as terminal electron acceptors. Nitrate, sulfate, iron, manganese, uranium, selenate, arsenate, urinate, fumarate, and carbon dioxide are all possible candidates for MFC applications [45]. One recent study by Song et al. [40] is a good example to sum up the significance of modified biocathode materials in MFC for

degradation of chromium. Their findings showed that microbially-graphene-modified biocathode has significantly reduced Cr(VI) via bacterial respiration and improve electron transfer by providing more adsorption sites for chromium. Therefore, these findings could reveal a promising approach for treating and recovering useful metallic material through an environmental friendly manner in MFC systems.

2.3 Biodegradation of Toxic Organics and Inorganics in MFC

Several studies on the biodegradation of toxic organics and inorganics using MFC have been reported. The toxic contaminants include metals, phenols, coloring dyes, and pesticides. This topic is particularly significant since the toxic contaminants can endanger lot of flora and fauna species as well as human beings which in the long run adversely affect our ecosystem. The capability of bacteria to biodegrade the toxic compounds as their substrates in bioelectrochemical system is extremely useful to be further explored since this can be sustainable options of waste treatment compared to conventional chemical treatments. This section elaborates the operation and mechanisms of toxic contaminant biodegradation with the active microbial species that involved in the MFC biodegradation system.

2.3.1 Phenolic Degradation

Phenol and its derivatives are still being extensively studied especially on its treatment methods as they are recalcitrant, and have high toxicity and acute effect if exposed to humans and aquatic organisms. There are found most abundantly from industrial wastewater including chemical, pharmaceutical, oil refinery, petrochemical, paper and pulp industries [46]. Apart from conventional treatment methods for example chemical oxidation, adsorption, membrane separation or chromatography, a more sustainable method, i.e., biodegradation process is appreciably preferable to treat phenolic compounds in wastewater.

Numerous studies on phenolic biodegradation using conventional degradation processes have been reported. However, application of MFC for biodegrading phenols becomes more significant nowadays. More studies on phenolic biodegradation using MFC are needed in order to understand the mechanisms and biodegradation pathways by exoelectrogenic bacterial cultures. Table 2.1 summarizes the updated studies related to

phenolic removal in the MFC system. These reported studies mainly focused on promoting the phenolic removal efficiency using mixed bacterial cultures with simultaneous electricity generation. Most of the studies used the typical two-chambered MFC setup with varieties of electrode designs and materials as well as other physicochemical parameters.

2.3.1.1 Phenolic Biodegradation Mechanisms and Pathways

The phenolic biodegradation phenomena in the MFC may proceed through different degradation pathways in the sense that the bacterial cultures consume the substrate in a bio-electrochemical environment, or in other words, the electron transfer activities and substrate consumption occur simultaneously. Therefore, it is interesting to investigate the behavior of the microbial population which involve in the biodegradation process through the specific pathways with simultaneous ability of electron transfer. Phenolic compounds can be degraded to carbon dioxide, firstly by fermentation to structurally simpler and more easily biodegradable compounds, with subsequent respiration using different electron acceptors [54]. Fermentation is generally considered as rate limiting in the biodegradation of phenols [55], however, some studies may use it as sole electron donor in the MFC cultivation.

There are limited literatures discussing on phenolic degradation pathways in MFC system. Liping Huang and her team studied the pentachlorophenol (PCP) degradation in MFC by mixed bacterial cultures from domestic wastewater sludge and proposed the PCP degradation pathways occurred in MFC [52]. This finding may be the first to be reported in this topic. According to this study, PCP in particular was co-metabolized by the bacteria in MFC through reductive dehalogenation (or dechlorination) in the presence of easily degradable substrate (glucose or acetate). The PCP served as an electron acceptor and the easily degradable substrate was used to reduce the toxicity and growth inhibition of PCP on microorganisms. The easily degradable substrate can also act as inducing agent for biodegradative enzymes as well as an electron donor for bacterial growth [56]. Figure 2.5 shows the PCP degradation pathways where the intermediates were released in the bioanodes (Figure 2.5 (a)) and PCP mineralization occurred in the biocathodes (Figure 2.5 (b)).

Table 2.1 Studies on phenolic degradation using MFC systems.

Phenolic compound	Bacterial culture / substrates	MFC setup	Max. phenolic removal (%) / rates	Max. current / power density (mA/m^2 / mW/m^2)	References
4-Chlorophenol 2,4-Dichlorophenol	Anaerobic digester sludge	- Tubular two-chamber - Graphite fiber and felt as electrodes - 43 mL working anode volume - Aerobic and anaerobic biocathode	0.15 $mol/m^3/d$	4.2 W/m^3	[47]
Phenol Cresols Xylenols	Contaminated groundwater	- H-type dual chamber using 250 mL Schott bottles - Carbon cloth electrodes - Nafion 117 proton exchange membrane (1.5 cm diameter)	~ 54%	1.8 mW/m^2	[48]
Phenol	Anaerobic sludge	- Series of single chamber membrane free air cathode (14 mL) - Carbon cloth electrodes (7 cm^2) - MFC contains electrosorption unit (ACFs as anode, nickel foam as cathode)	2.47 mmol/g (MFC-sorption system)	783 mW/m^2	[49]
Pentachlorophenol	Domestic wastewater (effluent of primary sedimentation tank)	- Tubular two-chamber - Graphite felt and carbon fibers as electrodes - 85 mL working cathode volume - Cation exchange membrane (CEM; CMI-7000)	> 90%	5.2 W/m^3	[50 – 52]
p-Nitrophenol	PNP-degrading strains (soil river)	- Two-chamber with *in situ* biosensor of monitoring PNP (working volume 240 mL) - PEM, Nafion117 - Carbon felt electrodes	> 90%	13 mW/m^2	[53]
2,4-Dichlorophenol	*Bacillus subtilis*	- Two chamber 250 mL Schott bottles - Carbon cloth electrodes - Cation exchange membrane (CEM; CMI-7000) - Various catholytes	~ 60%	9.5 mW/m^2	[10]

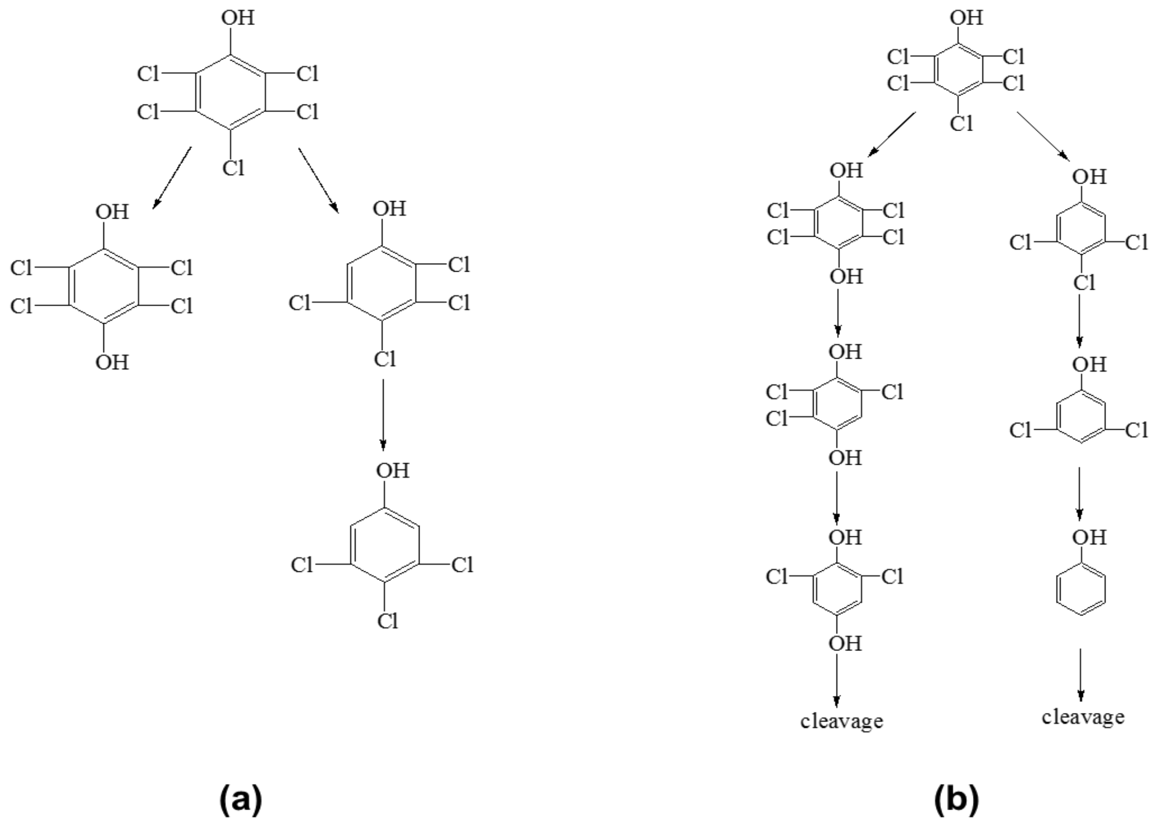

Figure 2.5 Proposed PCP degradation pathways in the **(a)** bioanodes and **(b)** biocathodes of MFC at a potential of 200 mV (vs. SHE) [52].

The most recent study on phenol degradation by bacterial community from contaminated groundwater in MFC system was reported by Hedbavna et al. in 2016 [48]. The biodegradation metabolites were identified as 4-hydroxybenzoic acid and 4-hydroxy-3-methylbenzoic acid by mass spectrometry. Similar metabolites were obtained through anaerobic degradation of chlorophenols and polychlorophenols in the conventional phenolic biodegradation study [57]. Hedbavna et al. [48] found that the phenolic compounds diffused from the anode chamber to the cathode chamber via the membrane along a concentration gradient. The lower than expected final phenol concentration in the cathode chamber can also be explained by its aqueous volatility characteristics [58]. As biodegradation of phenols proceeded, the concentration of metabolites in the anode increased to the maximum values.

In addition, the mechanism of phenolic biodegradation and mass transfer in MFC involve two important parameters including diffusion and electromigration induced by electrical current in the MFC system [48]. The electromigration flux, J_E and diffusion flux, J_D are given by the following equations:

$$J_E = D\,\frac{zFc}{RT}\frac{\Delta E}{\Delta L} \tag{2.1}$$

$$J_D = -D\frac{\Delta c}{\Delta L} \tag{2.2}$$

where D is the solute diffusion coefficient, z is the charge of the ion, F is the Faraday constant (96 485 C/mol), R is the universal gas constant (8.314 J/mol·K), c is the solute molar concentration, L is the distance, T is the temperature in Kelvin and ΔE is the voltage. According to the principle of bioelectrochemical system (BES) technology, the negatively charged metabolites or contaminants could be drawn towards the anode, keeping the biodegrading microorganism, contaminants, organic metabolites and electron acceptors in close proximity [48].

The values of pH of the anolytes and catholytes which are associated with the electron transfer and diffusion might play an important role in the substrate degradation in the MFC system. Wang et al. [59] suggested that a stable pH favours the fibre degradation and electricity generation in mixed-rumen MFC. However, the nature and extent of anolytes and catholytes affecting the MFC performance in terms of phenolic degradation are still incompletely understood. A possible explanation for this might be the molecular interactions between anolyte and catholyte which in turn yield varied MFC performance based on different catholytes [10].

Furthermore, the bacterial growth rate also showed a slightly direct relationship with phenolic degradation rate [10, 60]. Hassan et al. [10] suggested that the best degradation of 2,4-DCP occurred when the *B. subtilis* reached the highest growth level. The bacterial growth and phenolic degradation profiles also indicated a short lag phase which corresponds to the rapid bacterial acclimatization of 2,4-DCP by *B. subtilis*. Since there is still lack of data relating the phenolic degradation pathways with the electrochemical activities, future work is required to establish a mechanism of phenolic degradation interaction with respect to the condition of electron transfer in the MFC system.

2.3.1.2 Exoelectrogens in MFC Phenolic Degradation

The electrochemically active or exoelectrogenic bacteria that form the biofilm in MFC have been quite numerously studied including *Geobacter*, *Pseudomonas*, and *Shewanella* [21, 61, 62]. Apart from being inoculated or isolated mainly from domestic wastewater (anaerobic sludge), these bacterial cultures were also utilized in the MFC in pure culture form. Considering the phenolic compound in MFC, to our knowledge, there is no or limited number of reported literatures identifying and isolating the microbial strain which is electrochemically active while being specifically capable of degrading phenolic compound as either electron donor or acceptor. This shortage of data is applied to the microbial screening studies using mixed bacterial consortia from any waste water sources since there are attempts of using pure culture or phenolic-degrading strain to degrade this compound in the MFC [10, 53].

Nevertheless, this subsection demonstrates the discoveries on the microbial strains which possibly participate in the phenolic degradation while generating electricity in the MFC. The closest bacterial strain which has been recorded to have ability to degrade phenol [63] is *Variovorax paradoxus*. This bacteria was present in the MFC acclimatization of 4-chlorophenol and 2,4-dichlorophenol as discovered by Huang et al. [47]. This study also found that *Comamonas* sp. which was reported far earlier to degrade monocyclic aromatic hydrocarbons [64] was present during chlorophenol acclimatization in the MFC. The presence of phylogenetic relation to *Desulfobacterium aniline* in MFC biocathode acclimatization with pentachlorophenol was also an interesting microbiological finding to be explored [50]. This is due to its ability to anaerobically mineralize multiple aromatic compounds including aniline, phenol, benzoate, 2-hydroxybenzoate, 4-hydroxybenzoate, 4-hydroxyphenylacetate, 2-aminobenzoate, 2-fluorophenol and 2-fluorobenzoate [56]. Several potential phenolic degrading bacteria are summarized in Table 2.2 which shows the closest sequence of bacteria found in related phenolic MFC studies together with their isolation sources from reported literatures.

Table 2.2 Potential bacterial strain for phenolic degradation in MFC studies.

Phenolic compound (acclimatization in MFC)	Bacterial strains or species / conditions in MFC	Isolation sources (previous non-MFC studies)	References
4-Chlorophenol 2,4-Dichlorophenol 2,4,6-Trichlorophenol	*Variovorax paradoxus* (aerobic & anaerobic at biocathodes)	Degrading phenol and reducing nitrate.	[47]
	Comamonas sp. (aerobic & anaerobic at biocathodes)	Degrading monocyclic aromatic hydrocarbons and diclofenac / ibuprofen.	
Pentachlorophenol	*Desulfobacterium Aniline*	Can anaerobically mineralize aniline, phenol, benzoate, 2-hydroxybenzoate, etc.	[50]
	Rhodococcus erythropolis	Aerobically degrade chlorophenols.	
	Actinomycetes and *Streptacidiphilus*	Aerobically degrade mono-chlorophenols.	
	uncultured *Nitrospira* sp.	Exist in environments contaminated recalcitrant wastes (pharmaceuticals).	
Pentachlorophenol	*Alphaproteobacteria* (at 200 mV vs. SHE)	Uncultured *Sphingomonas* sp. as degrader of alkylphenols including PCP, 2,3,4,6-tetrachlorophenol and bisphenol A (GenBank).	[52]
		Alphaproteobacterium UBF11 as degrader of polycyclic aromatic hydrocarbon (GenBank)	
	Bacteroidetes (at -300 mV vs. SHE)	Uncultured *Bacteroidetes* sp. clone NEVC4 in Ethene-enriched consortium degrading vinyl chloride and chlorophenol (in anode and cathode) (GenBank).	
	Betaproteobacteria (at -300 mV vs. SHE)	*Comamonas* sp. p19 in degrading PCP, quinoline, and chlorinated aromatic hydrocarbons (in anode and cathode).	
		Comamonas testosteroni sp. PA1 as PCP-degrading and exoelectrogen.	
p-Nitrophenol	*Pseudomonas monteilii* LZU-3	Co-metabolically degrading chlorinated phenols in conventional biological processes.	[53]
Pentachlorophenol	*Gammaproteobacteria* (PCP-glucose)	Highly existing in polyaromatic hydrocarbon polluted sites.	[65]
	Alphaproteobacteria (PCP-acetate)	Degrading petroleum.	
	Mycobacterium (high relative abundance in 15 mg/L PCP)	PCP degradation in conventional aerobic process.	
	Pseudomonas	Co-metabolically degrading chlorinated phenols in conventional biological processes.	

From Table 2.2, in general, *Pseudomonas*, *Rhodococcus*, and *Actinomycetes* could be potential bacterial strains for degradation of phenolic compound while taking part in bioelectrochemical activities in MFC system. *Pseudomonas* in particular, has been known to have strong ability to degrade chlorophenol in the conventional biodegradation studies [56, 66]. Although the presence of the aforementioned bacteria showed a positive interaction of phenolic biodegradation in MFC, the electrotrophic activities and their role in chemolithotrophic dechlorination processes remain unknown [50]. Further investigation of the electrotrophic activities of bacteria with simultaneous phenolic reduction with pure cultures is still needed.

2.3.2 Metal Reduction / Removal

Metals are also among dangerous contaminants mostly found in the wastewaters from industrial activities. The discharge of this untreated wastewater into natural water bodies causes a serious threat to humans as well as other forms of life [40]. Therefore, various wastewater treatments have been developed to treat this toxic metal including chemical, electrochemical and biological reductions. The chemical and electrochemical reduction methods generally proceed in an acidic solution and involve legally required pH adjustments [40]. In addition, the use of reducing agents with high energy consumption make these methods expensive. Therefore, it can be stated that biological reduction using reducing microorganisms which yield lower operating cost is more preferable to be utilized. However, the shortcomings of biological reduction process are its low reduction efficiency at a neutral pH and long acclimation time for some reducing bacteria [67].

Recently, biocathode MFCs have been explored to reduce several hazardous metals using various reducing bacteria. One highly considered toxic metal is hexavalent chromium (Cr(VI)), since it is well-known to be carcinogen, mutagen and highly mobile in the environment [68]. Several studies have shown good reduction efficiency of Cr(VI) using biocathode-MFC while generating significant amount of current output [40, 69, 70]. The simultaneous reduction reaction involves electron release from anaerobic oxidation of glucose in the anode. While in the cathode, Cr(VI) receives the electrons and is reduced to Cr(III) as shown by Reaction 2.3 [40]:

$$Cr_2O_7^{2-} + 14H^+ + 6e^- \rightarrow 2Cr^{3+} + 7\,H_2O \qquad \text{(Rxn. 2.3)}$$

The biocathode acclimatization period was found to be shortened by 19 days and the Cr(VI) reduction rate was increased by a factor of 2.9 [70]. Microbial community analyses of acclimatized biocathodes revealed that *Gamma-proteobacteria* and *Bacteria* dominated the system, thus showing these bacterial species could potentially have positive response towards Cr(VI) reduction capability.

Furthermore, electrode materials are also important factor to improve the recovery of metal in biocathode. Huang et al. [71] demonstrated that graphite fibers were superior to graphite felt or graphite granules for Cr (VI) reduction in the biocathode and that increasing the cathode surface area could lead to higher cathodic reaction rates. However, very little effort has been spent on biocathode material fabrication for Cr (VI) reduction, especially using nanostructured materials. For Cr(VI) reduction, the properties of a good biocathode material will not only improve the electrical conductivity of the electrodes, but also provide the bacteria and Cr(VI) with more reaction sites [40].

Other dangerous metals have also been reduced using biocathode MFC including copper (Cu(II)) [72], cobalt, manganese, iron, uranium and arsenate (See subsection 2.2.2.2 Biocathode MFC). These metals together with nitrate, sulfate, fumarate and carbon dioxide are possible candidates for MFC reduction applications [45]. The MFC technology using cathodic reduction seems to be promising in treating the contaminants and recovering essential metals from the industrial wastewater. Its environmental-friendly operation makes it as a highly preferable option for the wastewater treatment. Future studies are still needed to improve the efficiency of metal recovery and reduction as well as its feasibility in the real wastewater applications.

2.3.3 Removal of Colouring Dyes

Other than phenol, pyridine and dichloroethane, colouring dyes also make the lists of hazardous compounds to be highly treated. Azo dye is a critical organic material in dye industry and is found to be toxic and mutagenic [73]. The azo dye degradation could be favoured in a distinctive anaerobic bioelectrochemical environment of the MFC anode, which processes were believed to be simultaneously occurred according to the theory of biological degradation of azo dyes and MFC operating principle [74]. However, the degradation of azo dyes in anaerobic environment yields aromatic amines which are also

biorefractory compounds and harmful to aquatic plants and animals [75]. Hence, the dye degradation in anode MFC has gained considerable attentions as many focus on the further degradation of dye products in MFC environment.

In the presence of alternative electron acceptors, MFC technology is feasible to augment *in-situ* biodegradation [76]. The degraded metabolites of dye could act as electron shuttles in enhancing colour removal in MFC system [77]. In addition, to improve MFC performance, Chen et al. [78] and Sun et al. [79] indicated that exogenous augmentation of redox mediators (or electron shuttles) was feasible to increase power generating and dye degrading capabilities of MFCs. For example, according to identification of dye metabolites of RG19 by *Enterobacter cancerogenus* BYm30-bearing MFCs, organic sulphides (e.g., 3-methyl-thiopropionic acid or 4-methylthiobutanol) were suspected electron shuttles for such autocatalysis [80]. Furthermore, phenyl methadiamine and 5-sulfoanthranilic acid in decolorized metabolites of reactive blue 160 (RBu160) by *Proteus hauseri* ZMd44 or *Klebsiella pneumonia* ZMd31-seeded MFCs were also capable to act as electron shuttles to enhance MFC performance [74]. Therefore, it can be stated that the optimal exogenous or endogenous accumulation of electron shuttles would be crucial to maximize electron transfer phenomena in MFCs. In particular, using MFC as mode of operation for reductive degradation of azo dye(s) seemed to be electrochemically favourable because of sufficient electron transfer for dye removal [77].

Like other studies on contaminants removal using MFC, anode materials in MFC for dye removal were also one of the topics of interest. The modification of graphite-felt anode surface through electrodeposition of humic acid and riboflavin demonstrated excellent electrocatalysis activity which improved power density by 66% and internal resistance decrement by 49% [81]. It is interesting to note that the formation of redox mediator crystals on the modified anode could accelerate electron transfer, which benefited both bioelectricity generation and decolourization. Huang et al. [81] also proved that the rates of decolourization were relatively low at the starting MFC cultivation (~2 hours) because the microorganism did not adapt itself to the azo dye molecule which was just added and had a certain toxicity. Further MFC operating time to 10 h showed steady decolourization process, microorganisms had gradually adapted to azo dye and displayed the degradation ability. On the next hours, the decolourization rate was tend to slow down, since the azo

dye concentration was lower than before, which meant the dye molecules number existed in the anolyte were more difficult to contact with microorganisms.

The crucial role of microorganisms as backbone in MFC performance is also applied in the dye degradation study in MFC. It was found that textile dyeing sludge based inoculum resulted in higher azo dye decolourization compared to municipal sludge [82]. The textile dyeing sludge although generated relatively lower power output compared to municipal sludge, achieved fast Congo red decolourization with first-order rate constant, 34% higher than that obtained by municipal sludge. Since the azo dye decolourization in the anode MFC was an electron consumption process, the electron transfer from co-substrate to azo dye was the determining factor for its decolourization rate [78]. The presence of specific functional bacteria in the anode for instance *Pseudomonas* sp. and *Aquamicrobium* sp. (from textile dyeing sludge) could be responsible for the faster decolourization of Congo red dye [82].

In addition, the degradation pathways analysis showed that there were similar metabolite products of azo dye decolourization produced by both inoculum sludge. The similar three products detected as Congo red metabolites were 2-Amino-1,4-naphthoquinone, 2,2'-diaminodiphenyl and benzidine. Similar degradation products suggested similar degradation routes for Congo red but did not indicate a similar anodic microbial community because different species were capable of fulfilling the same function and the loss of one species will be compensated by another species in the community, resulting in a similar degradation performance [83].

The aforementioned studies on azo dye degradation revealed that the anodic MFC plays important role in assisting and improving the simultaneous mechanisms of dye degradation and electron transfer by microorganisms. Since the metabolite products of azo dye are still refractory to aquatic species, future studies are required to achieve zero dye metabolite products in the anolyte after MFC decolourization process. This could be attained by careful selection and isolation of the microbial strains responsible for dye degradation as well as through kinetic improvement for its biodegradation pathways.

2.4 Practical and Economical Challenge in Wastewater Treatment using MFC

In order to promote the MFC system as an alternative for sustainable wastewater treatment, engineers and scientists need to highly consider various economic and environmental factors. Logan [84] proposed elementary economics for an MFC based wastewater treatment system. A city with 100,000 population would generate 16.4 billion litres of wastewater over a year with a potential to produce 2.3 MW of electricity (based on 300 mg/L BOD concentration) which can be harvested in MFCs. Accounting for other barriers such as reactor detention times and energy recovery limitations, about 0.5 MW of electricity can be easily captured using MFCs. The economics of such a system for power production were reported to be comparable with other conventional alternatives [3]. Other important achievement of MFC in wastewater treatment is the ability to provide clean water for irrigation purpose as recently highlighted by Abourached et al. [85]. The cost analysis demonstrated that the MFC has potential of producing reclaimed water for irrigation with simultaneous electricity generation; both of large economic benefit to farmers. The total MFC cost for HRT 1.5 hour is only 9% of the total conventional treatment cost. If only the aeration system of the conventional plant is replaced by MFC and all other treatment costs are assumed to be common to both MFC and conventional treatment plant, $8.37 million in capital and aeration energy costs would be saved [85].

MFC economic analysis has been compared with other related wastewater treatment systems i.e., activated sludge with and without anaerobic digestion (AD) system and microbial electrolysis cells (MECs-producing hydrogen) [86]. From the brief analysis, MFC is found to meet only 50% total costs demonstrating it is rather uneconomical compared to MEC. The cost distribution in MFC technology development partially contributed by a major portion of anode and cathode materials, followed by current collector materials and other essential parts. For this reason, inexpensive electrode materials must be used to improve power performance and economic benefits in MFC technology [86]. Among the electrode materials, semicoke and activated carbons are preferable which showed relatively more excellent performance in generating power density (20.1 W/m^3 and 24.3 W/m^3, respectively) compared to graphite and carbon cloth (14.1 W/m^3 and 17.1 W/m^3, respectively) [87].

In addition, the current economic analyses suggest that electricity generation alone from MFCs may not be adequate to turnout the wastewater systems to be energy-neutral or energy-positive or even cost-effective. For this reason, energy recovery strategies from wastewater should be diversified to produce methane, lipid-rich biomass and bioelectricity [3]. With this advanced scheme, the overall economics of the process could be significantly improved. The MFC-integrated system for instance MFC-AD and photobioreactor-yeast fermenter [88], may show a positive net present worth (NPW) value over the 20 year plant life at a 10% rate of return in investment (IRR). This integrated facility would generate bioethanol, bioelectricity (to be used in bioethanol production process), and produce valuable biomass while sequestering CO_2 emissions in the integrated process [3].

For their practical implementation, MFCs need to be scaled-up by several orders of magnitude from the laboratory scale (10^{-6} to 10^{-3} m^3) to a scale suitable for wastewater treatment (1 to 10^3 m^3). Such implementation of microbial fuel cells also requires an increase in efficiency and the use of new low cost materials and, to date, pilot-scale attempts have proved to be unsatisfactory [89]. Hence, modelling and simulation of MFCs have been seen to be useful tools for optimizing their performance. Optimization study is necessary to reduce technical constraints, costs of MFC materials and operation, and to increase the MFC performance. Many scientific efforts have been put into optimizing MFC devices since this technology was viewed as a promising way to generate clean energy [89]. However, modelling and mathematical optimization of MFCs have taken more time to gain the attention of researchers and only recently have works dealing with MFC modelling proliferated [90]. The phenomena that can be taken into account in a model cover a wide range of processes, such as mass transport through the cell, microbial growth, phases of matter and its boundary conditions, anode and cathode reaction kinetics or the electrochemical behaviour of the cell [89]. An increase in number of models is expected in the coming years and these should increase our understanding on this technology to improve its performance through cost-effective means.

2.5 Concluding Remarks

The technology of MFC is foreseen to be suitably developed and applied especially in wastewater treatment system in near future. Recent data suggest that MFC could be a practical and preferred option among sustainable bioenergy processes. Various scientific works have demonstrated that this technology offers potential prospects for wastewater treatment and power generation for instance in water desalination, COD removal or nitrification/denitrification. However, more studies are needed to investigate the biodegradation mechanisms of recalcitrant wastes, especially phenols, metals and coloring dyes compounds, while maintaining the electricity generation in MFC. This is because the phenol and its derivatives, for example, have become the major and priority pollutants throughout the industrialized nations worldwide, and their toxicity effect can adversely disrupt our environment.

The appropriate selections of microbial cultures and/or substrates, physicochemical parameters, internal resistances, MFC designs, and electrode materials are highly important during the design and development stage of the MFC system in order to maximize its power output and waste degradation capabilities. Technological improvements to reduce the consumption of energy for wastewater treatment, substrates utilization, and microbial energy harvesting along with a suitable mathematical model are required for MFC optimization. Other than that, the opportunities to integrate the MFC system with other existing wastewater treatment methods should be optimized in order to widen the prospects of MFC to be applied in the larger real world scale.

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CHAPTER 3

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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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3. Chemical Impact of Catholytes on *Bacillus subtilis*-catalysed Microbial Fuel Cell Performance for Degrading 2,4-Dichlorophenol

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Chapter 3: Chemical Impact of Catholytes on *Bacillus subtilis*-catalysed Microbial Fuel Cell Performance for Degrading 2,4-Dichlorophenol

Abstract

This study was to explore a *Bacillus subtilis*-catalysed microbial fuel cell (MFC) system for electricity generation and dichlorophenol degradation. Our research focused on understanding the chemical interactions of various catholytes having distinctive properties (oxidizing, buffering, and salinity) and their impacts on the electrochemical activities, bacterial growth and phenolic degradation in the MFC system. Our experimental results revealed that *B. subtilis* is a potential exoelectrogenic bacterium for producing current density of 64.0 mA/m^2 while degrading 2,4-dichlorophenol in the MFC. Chemical properties of the catholytes and the pH change profiles could have significant impact on the bio-electrochemical activities, therefore the performance of the MFC system. Potassium persulfate was found to be the most suitable catholyte for generating the maximal power density of 9.5 mW/m^2 with a peak current of 1.11 mA over a potential of 0.45 V, while degrading over 60 % 2,4-dichlorophenol. The *B. subtilis*-catalysed MFC could be a feasible technology for removal of hazardous phenol pollutants from industrial wastewater, while generating electricity.

Keywords: *Bacillus subtilis*; Microbial fuel cell; Catholytes property; 2,4-Dicholorophenol

3.1 Introduction

Microbial fuel cell (MFC) system is a unique sustainable biotechnology that is able to generate electricity through biodegradation of various organic compounds. MFC has received increasing research interests in broad fields; biochemistry, biochemical engineering and wastewater treatment. MFC has been recognised as a potential technology for the integration of electricity generation with wastewater treatment. MFC system relies on exoelectrogenic microorganisms to catalyse the electrochemical reactions occurring on electrode surfaces. To date, the applications of a number of pure or mixed cultures including *Clostridium* [1], *Pseudomonas* [2], *Shewanella* [3] and *Geobacter* [4] species have been reported.

Phenol and its derivatives are considered as refractory hazardous pollutants in wastewater. *Bacillus* species have been employed in many studies on the degradation of phenolic compounds. Pankaj [5] studied the decolourization of nitrophenol compound by *Bacillus subtilis* and reviewed the degradation of chlorophenols and their derivatives by various bacteria including *Bacillus* species [6]. *Bacillus cereus* isolated from a phenolic contaminated pond was found to be able to remove 2,4-dichlorophenol [7] and a strain of *Bacillus subtilis* was able to oxidize mono- and di-methoxyphenols using laccase bound enzymes [8]. These phenolic biodegradation studies were carried out in the conventional biodegradation experiments and not in the MFC systems. To the best of our knowledge, there was no reported work so far in which the pure culture *B. subtilis* is used to degrade recalcitrant toxic compounds (particularly 2,4-dichlorophenol) through MFC systems. With the ability of *B. subtilis* to generate electricity in the MFC [9, 10], we anticipate that this species could be a potential microorganism to catalyse MFC for electricity production while degrading organic hazardous waste including chlorophenols.

Many biochemical and systematic factors need to be considered in the development of MFC technology, including MFC design, microbial communities, electrodes and substrate chemistries [11]. Chemicals involved in the MFC system as catholytes, substrate, nutrients and reagent for pH control could have significant impact on the bio-electrochemical reactions and overall performance of the MFC system. Seeking suitable catholytes able to enhance the bio-electrochemical engineering performance of the MFC system is mainly empirical in previous studies. It should be noted that the diffusivity and transport

mechanism of electrons in catholyte solutions can amplify current production in MFC systems. Previous studies used relatively high concentration of ferricyanide and permanganate as catholytes for investigating their effects on MFC performance [12, 13]. However, these catholytes might be transformed into hazardous pollutants which impose negative impact on aquatic environments when discharged. Yongtae and Bruce [14] found the use of saline catholyte to replace phosphate buffer in MFC resulted in high current production. However, the concentration of saline catholyte needed to be relatively higher in order to offset the number of ions present in the catholyte solution that could produce greater electricity. High concentration of catholyte solution could also inhibit the performance of proton exchange membrane due to the concentration gradient imposed between the anode and cathode chambers. Therefore biochemical accessibility, electron transfer characteristics and costs of electrolytes need to be taken into consideration while studying MFCs.

Considering the significance of suitable microorganisms and catholytes used for MFC, this study aims at exploring *B. subtilis*-catalysed MFC system for simultaneous production of electricity and degradation of chlorophenol contaminant. Chlorophenol compounds are mostly disposed as organic wastes from palm oil, chemical processing and petrochemical industries. Our research focuses on studying how the chemicals of catholytes and chlorophenol as target hazardous pollutant could alter electrochemical mechanisms and affect the overall performance of the *B. subtilis*-catalysed MFC in terms of the electricity production and degradation of 2,4-dichlorophenol (2,4-DCP). Five catholytes, namely potassium persulfate (PP), M9 medium (M9), phosphate buffer solution (PB), saline NaCl (NaCl) and tap water (Water), were examined in this study. The MFC experiments were carried out using low-concentrated catholytes with absence of air-purging so as to explore a low-cost and environmental-friendly operation mode. These experiments were expected to yield higher current generation and phenolic degradation.

3.2 Materials and Methods

3.2.1 Microorganism, Culture Medium and Catholytes

Bacillus subtilis (AWQC 111) provided by the Australian Water Quality Centre, was used as a model bacterium in this study. The bacteria were grown in 100 mL of nutrient broth in a 250-mL shake flask for 20 h on an incubator shaker (175 rpm) at 37 ºC. The bacterial cells were used as the seed culture for the MFC system.

M9 medium was used as the growth medium for the *B. subtilis*, which was prepared according to Miller [15] with slight modification by adding yeast extract to supply vitamin and amino acid. The modified M9 medium was composed of 0.2% glucose and 1 g/L yeast extract in mineral salts (1 g/L NH_4Cl, 3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl, 1 mmol/L $MgSO_4$, and 0.1 mmol/L $CaCl_2$). The pH of M9 medium was adjusted to pH 7.0±0.2 with NaOH or HCl. The medium was autoclaved at 121 ºC for 15 min. The glucose solution was sterilized using 0.22 μm filter unit (Millipore membrane PVDF) and then added to the autoclaved medium. The culture and medium preparation was carried out in a biological safety cabinet (Class II, BH2000 Series) to avoid culture contamination.

The MFC experiments were carried out using five catholytes with different initial pH (based on the original pH following the catholyte solutions preparation) and their distinctive characteristics were listed in Table 3.1. The pH changes of both anolytes and catholytes in the MFC systems were monitored throughout MFC operations using a pH meter (Eutech Instruments).

3.2.2 MFC Reactor

A dual-chambered membrane MFC reactor was constructed from two glass bottles (250 mL) joined with a glass bridge, which consists a cationic exchange membrane (CMI-7000, gel polystyrene cross linked with divinylbenzene) with a 3-cm inner diameter. A 3.0 cm × 3.0 cm carbon cloth was used as the anode and cathode materials. Both electrodes were pierced with titanium wire projecting outside connecting to an external electrical circuit. The MFC glasses were autoclaved and the setup was carried out in the biological safety cabinet to avoid the glasses and culture contamination.

Table 3.1 Characteristics of catholytes used in the *B. subtilis*-catalysed MFC.

Catholytes (concentration)	pH*	Conductivity** (mS/cm)	Buffering property	Oxidizing property
Potassium persulfate, PP (50 mM)	3.0	11.79	-	√
M9	6.92	20.70	√	-
Phosphate buffer, PB (50 mM)	7.10	6.30	√	-
NaCl (50 mM)	5.83	11.15	-	-
Water	7.04	0.472	-	-

* Initial pH of catholyte measured before running the MFC experiment

** Conductivity of catholyte measured at 25 °C

After transferring inoculum to the anodic chamber, air purging was immediately supplied in the anodic chamber to facilitate bacterial growth. The anodic chamber turned turbid after 12 hours, indicating the bacterial growth. Then, air supply was disconnected at this stage and the joined parts of the anodic chamber were sealed with plasticine to block air penetration, thus creating an anoxic environment. The biofilm formation on the electrode surface was observed afterwards. The cathodic chamber was open for aeration. The MFC system was topped on a magnetic plate to ensure well mixing during operation and maintained in a room temperature. Figure 3.1 shows a schematic diagram of the MFC setup used in this study with anolyte medium and 2,4-dichlorophenol, and various catholytes at the cathodic chamber under investigation. Various electrochemical reactions taking place in the MFC systems will be evaluated in the succeeding sections.

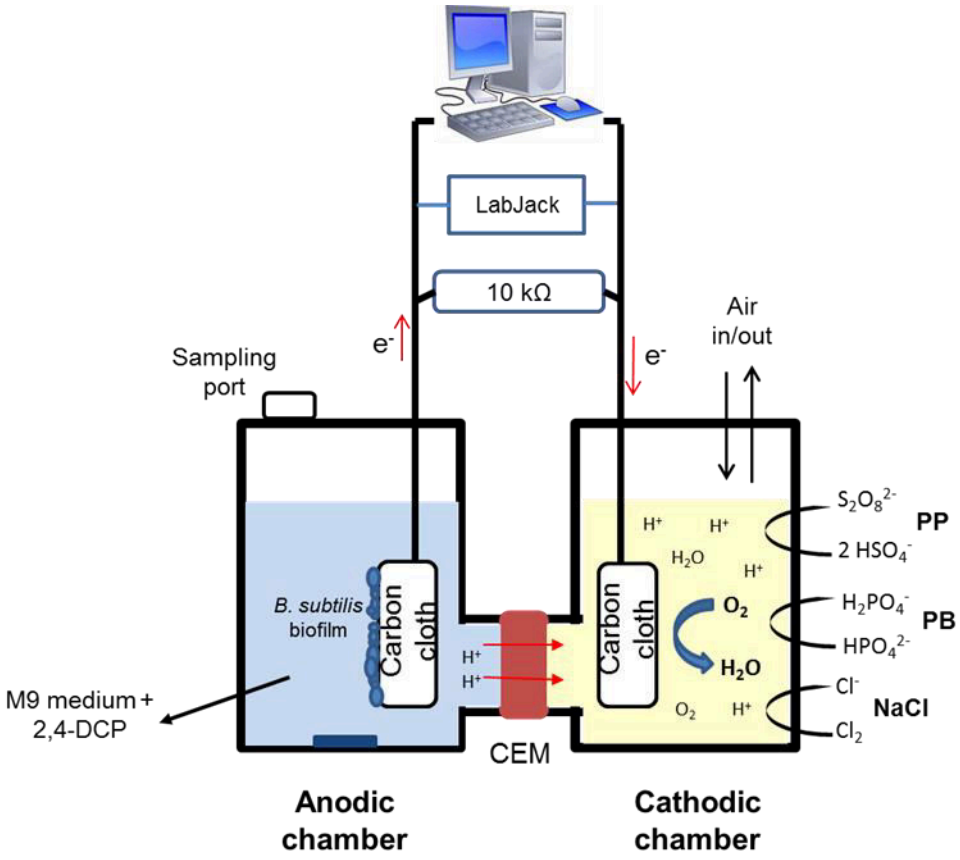

Figure 3.1 Schematic diagram of double chamber MFC setup with various catholytes.

3.2.3 Biochemical Analysis

3.2.3.1 Optical Density vs. Phenolic Degradation

The optical density (OD) of bacterial growth was periodically determined using a UV-Visible spectrophotometer (Shimadzu, UV-1601) at a wavelength of 660 nm. The model chlorophenol compound used in this study was 2,4-dichlorophenol (2,4-DCP) (Alfa Aesar), which is a chlorinated derivative of phenol with the molecular formula $C_6H_4Cl_2O$. 10 mg/L 2,4-DCP was fed into the anodic chamber under anoxic condition. The 2,4-DCP concentration was examined using the UV-Visible spectrophotometer at a wavelength of 750 nm as described by Wilfred and Ralph [16]. Phenolic degradation was calculated based on the difference in 2,4-DCP initial and final concentrations.

3.2.3.2 Coulombic Efficiency

To achieve a high power output MFC, it is expected to convert as much electrons stored in the substrate as possible to current [17]. The recovery of electrons is determined in terms of coulombic efficiency (CE) as described in Eq. 3.1,

$$CE = \frac{C_R}{C_{th}} \times 100\% \tag{3.1}$$

where C_R is the total coulombs through the external circuit in a complete circle by integrating current over time, and C_{th} is the theoretical amount of coulombs that can be calculated based on phenolic substrate removal.

3.2.3.3 Catholyte Ionic Change

The chemical changes of three selected catholytes namely potassium persulfate (PP), phosphate buffer (PB) and NaCl were analysed in terms of anionic reduction. The catholyte samples were taken before and after MFC operation. The concentrations of phosphate (PO_4^{3-}) and chloride (Cl^-) were measured using ion chromatography technique equipped with a HPIC pump (Waters, Model 515) and a conductivity detector (Waters, Model 430) with sample volume of 100 µL. The column used was anion column (Waters, IC-Pak A) with dimension of 4.6 × 50 mm, particle size of 10 µm and capacity of 30 ± 3 µeq/mL at a flow rate of 1.0 mL/min.

The concentration of persulfate ($S_2O_8^{2-}$) was measured using spectrophotometry technique equipped with UV-Vis spectrophotometer (Metertech, SP 8001). This spectrophotometry technique was based on procedures described by Liang et al. [18] with modified wavelength of 450 nm. The relevant data of ion chromatography and spectrophotometry techniques was provided in the **Supplementary Data**.

3.2.4 Electrochemical Measurement

3.2.4.1 Voltage-Current Data Acquisition

Voltage and current outputs were continuously recorded against time by a LabJack U6 recorder with an external load of 10 kΩ connected to a computer during 5 days operation of the MFC system. The current and anodic and cathodic potentials were measured with respect to operational time. To develop the polarization curve, different external loads were

applied for a complete batch cycle, with a variable resistance ranging from 10 Ω to 100 kΩ.

3.2.4.2 Cyclic Voltammetry and Linear Sweep Voltammetry

Data for cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were recorded using a CHI 650 D bipotentiostat (CH Instruments, Inc., USA). The CV was conducted at a scan rate of 0.1 V/s ranging from 0 to 0.5 V (vs Ag/AgCl), while the LSV was conducted at a scan rate of 0.1 V/s ranging from 0.1 to 3.0 V (vs Ag/AgCl). To analyze the chemical impact of 2,4-DCP on the electrochemical activity with respect to various catholytes used, the CVs and LSVs were performed before and after 2,4-DCP feeding into the anode solution.

The experiments were carried out in the MFC using three electrodes; working electrode, reference electrode (an Ag/AgCl electrode) and counter electrode. The working and counter electrodes were made of carbon cloths with the same size of 9.0 cm^2, pierced with titanium wires. All three electrodes were inserted into the MFC, avoiding any contact among these electrodes. The CVs and LSVs of the MFC with different catholytes were analysed during before and after 2,4-DCP feeding into the culture medium in order to examine the phenolic impact on the electrochemical activities of the *B. subtilis*-catalysed MFC system.

3.2.4.3 Electrochemical Impedance Spectroscopy

Data for electrochemical impedance spectroscopy (EIS) was obtained using the CHI 650 D bipotentiostat. The electrode materials were the same with CV and LSV as described above. To measure the anode impedance, the anode was connected to the working terminal, while the cathode and Ag/AgCl electrode were connected to the counter terminal and reference terminal, respectively. The working terminal was shifted to cathode and the counter terminal to anode when the cathode impedance was measured [19]. The EIS tests were carried out at a frequency range of 1.0 – 100 kHz with 0.005 V.

3.3 Results and Discussion

3.3.1 Electricity Generation

Electricity generation in term of current density (mA/m^2) was monitored in the *B. subtilis*-MFC reactors using five catholytes; PP, M9, PB, NaCl and Water. Figure 3.2 illustrates the electricity generation profiles in the MFC using five catholytes during 5 days operation. In general all catholytes-fed MFCs exhibited similar current generation profiles, implying similar biochemical activities occurred. During the first 20 hours, *B. subtilis* seemed to adapt themselves to the new MFC environment showing a slow growth, resulting in low current densities. The bacterial growth was further maintained by feeding the glucose after 10 h as indicated by the solid arrow in Fig. 3.2. Thereafter, the MFC anodic chamber was fed with 10 mg/L of 2,4-DCP as shown by the dotted arrow in Fig. 3.2. This operation was to study the 2,4-DCP degradation associated with the current generation as well as the bacterial growth in the MFC.

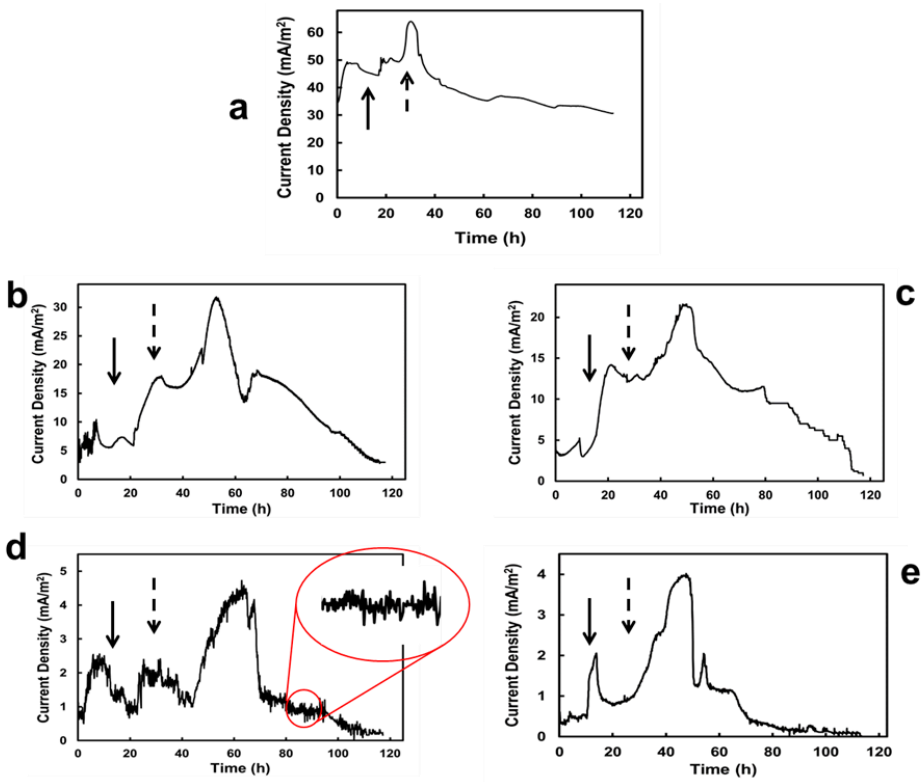

Figure 3.2 Current density evolution profiles for *B. subtilis* MFCs using the catholytes of **(a)** PP, **(b)** M9, **(c)** PB, **(d)** NaCl and **(e)** Water. The solid arrow shows the glucose feed while the dotted arrow shows 2,4-DCP feed.

The highest current density of 64.0 mA/m^2 was generated by PP-MFC, followed by M9-, PB-, NaCl- and Water-MFCs with 32.0, 22.0, 4.6 and 4.0 mA/m^2, respectively. The high current density generation in PP-MFC suggests that the oxidizing and conductivity properties (Table 3.1) of potassium persulfate was able to accelerate the reduction reaction and electron transfer, thus intensifying the current production in the MFC system. After approximately 40 – 60 hours of cultivation, PP-MFC (Fig. 3.2 (a)) showed trivial decline of bacterial growth as evidenced by steadier drop of current density compared to other catholytes-fed MFCs that exhibited significant current decrease. This result demonstrated that there was a substrate limitation after a long cultivation, which affected the microbial metabolism and hence the current output [9]. It is also expected that the decline of current density for all MFC systems was due to the descending growth rate of *B. subtilis* in recalcitrant medium condition of 2,4-DCP.

It is interesting to note that the fluctuation profiles in current generation were observed in the NaCl- and Water-MFC systems as compared to the other three MFC systems (see amplification of fluctuation trend in Fig. 3.2 (d)). NaCl-MFC showed larger fluctuation in current generation as compared to Water-MFC (Fig. 3.2 (d) and (e)). This is because the Na^+ and Cl^- ions in the NaCl solution could interfere the flow of H^+ to the cathode chamber, thus misbalancing the proton concentration in the MFC systems leading to current fluctuation. Furthermore, NaCl- and Water-MFCs showed a tremendous drop of current densities to almost below 1.0 mA/m^2 after 70 hours. It can be deduced that NaCl, despite of its relatively high conductivity, showed inferior stability in the MFC, leading to inconsistency in current generation. In addition, it can be noted that the minimal conductivity level of Water-MFC hindered the electron transfer between the MFC electrodes, thus resulting in its lowest current generation among all other catholytes-MFC systems.

It can be suggested that the PP, M9 and PB assisted the MFC to produce more stable electricity indicated by no current fluctuation in these systems. Moreover, PP-, M9- and PB-MFCs displayed insignificant current falls after 60 hours. This can be inferred by the buffering capability for minimising pH changes as that could affect the electron transfer between MFC electrodes, thus inhibiting the current generation (in case of M9 and PB) [20]. Use of PP catholyte was found to be able to increase cathodic potential and reduce

over potential during charge transfer process [21]. Moreover, the oxidizing property possessed by the PP could play an integral role in maintaining the redox reaction and electron transfer, thus stabilizing the current generation in the MFC.

3.3.2 Chemical Impact of Catholytes

3.3.2.1 pH Change Profile

The pH changes (ΔpH) in the anodic and cathodic chambers of the catholyte-MFCs were monitored during the operation and the results are shown in Fig. 3.3 (a). Since there was a cross-over of ions from anodic chamber to cathodic chamber through ion-exchange membrane, it is expected that the pH of anolytes and catholytes in the MFC were speckled throughout the cultivation period. In the anodic chamber, the microbes oxidized substrates such as glucose and some refractory organics (i.e., 2,4-DCP). For example, the oxidation of glucose generates electrons, protons and carbon dioxide (Reaction 3.1). In the cathodic chamber, on the other hand, a reduction process occurs with the presence of oxygen molecules to form water as indicated by Reaction 3.2.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad \text{(Rxn. 3.1)}$$

$$24H^+ + 24e^- + 6O_2 \rightarrow 12H_2O \quad \text{(Rxn. 3.2)}$$

Microbial reaction and phenolic degradation in the anodic chamber resulted negligible pH fluctuation (ΔpH < 0.09) in all MFC systems. Raghavulu et al. [12] reported that the anodic environment generally controls the kinetics of electron transfer and thus current generation from microorganism to the electrode. Wang et al. [13] found that stable pH favours the fibre degradation and electricity generation in mixed-rumen MFC. These results further support that the unvaried pH in anodic chambers of our MFC systems can sustain the current generation throughout operations.

Unlike the anodic chambers, the catholyte solutions in the cathodic chambers showed diverse ΔpH among the five MFCs. A very low ΔpH measured in both anode and cathode of M9-MFC contributed to relatively higher current density among the other systems. The negligible ΔpH in both chambers of M9-MFC may be elucidated by the ions that are balanced between those chambers. This is due to the transfer of ions rather than H^+ and OH^- [14]. Furthermore, using PB, there was no ΔpH found in the cathodic chamber besides

an extremely low ΔpH (~0.08) in its anodic chamber. It is clear to state that the negligible ΔpH in M9- and PB-MFCs was due to the buffering characteristic of these catholytes, which obstructed ΔpH thus improving the MFC performance in electricity generation.

Nevertheless, the pH changes in PP-, NaCl- and Water-MFC systems demonstrated contrary profiles as compared to the other two MFCs. The ΔpHs of the cathodic solution in the NaCl- and Water-MFC showed the highest ΔpH (0.55 and 0.51, respectively), followed by PP-MFC with 0.43 ΔpH. This effect proposed that the fluctuations and low current production in those MFCs were due to the incapability (in case of NaCl and Water) to maintain its initial pH throughout the operation. A similar performance was recorded by Yongtae and Bruce [14], who found that the pH of a 240-mM saline catholyte-MFC dropped dramatically to almost pH 5.5 during cultivation, and identified that pH < 6 might inhibit current generation. The relatively high ΔpH in PP-MFC was obviously due to the absence of buffering effect to maintain the pH of catholytes during reduction process.

The variance in pH change among these catholytes-fed MFCs was also due to the behaviours of the electron transfer and ionic diffusion through the ion-exchange membrane. That can be described by Nernst-Planck equation (Eq. 3.2), where J_i is the ionic flux, D_i the diffusion coefficient for the ion, z_i the charge, ∇c_i the concentration gradient of the ion in solution, k Boltzmann's constant, e_o the elementary charge, E the electric field strength, and T the absolute temperature. It calculates the ion flux under the influence of both ionic concentration gradient and the electric field.

$$J_i = -D_i \nabla c_i + \frac{D_i z_i e_o E c_i}{kT} \tag{3.2}$$

The diffusion coefficients of PP, M9 and PB catholytes were relatively higher than that of NaCl and water (which contains trace level of ions): SO_4^{2-} (~16.0×10^{-6} cm^2/s), PO_4^{3-} (~1.0×10^{-5} cm^2/s), OH^- (~5.0×10^{-9} cm^2/s) and Cl^- (~2.0×10^{-9} cm^2/s). The small diffusion coefficients in NaCl and water could be favourable for the ion transport to the anode chamber through the membrane and increased the solution pH. It was reported that the pH decrease in the anodic chamber and pH increase in the cathodic chamber were due to less charge transferred by protons compared to other ions [14, 20]. This further proved that the unbalanced protons present in the anode and cathode chambers for the NaCl- and Water-MFCs resists the electron transfer in the circuit, hence lowering the current generation in

these systems. Despite of the high diffusion coefficients (based on D_i of phosphate ions) of M9 and PB, the negligible ΔpH in M9- and PB-MFCs was due to the compensation of buffering effect in these solutions. This can result in stable and balanced protons and ions transfer between anodic and cathodic chambers, thus generating higher currents in MFC systems.

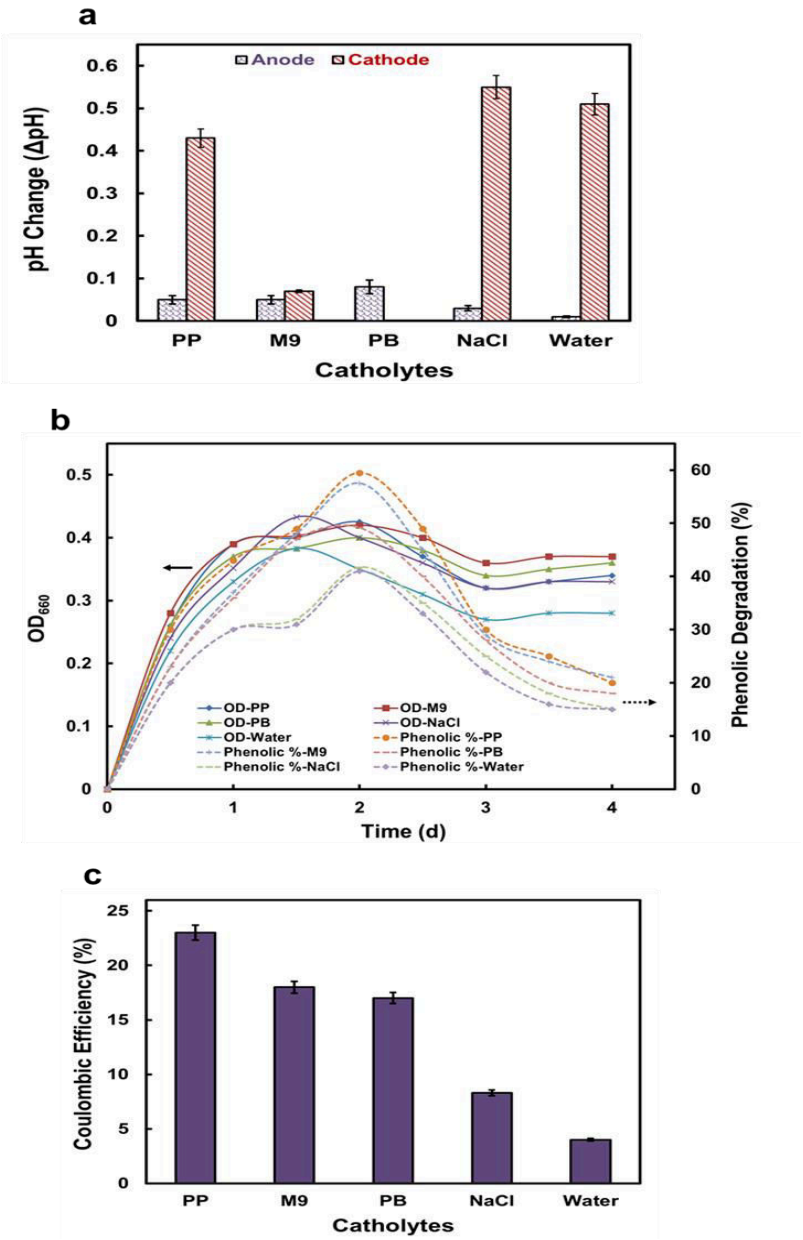

Figure 3.3 (a) pH Change (ΔpH) of the anodic and cathodic solutions of the MFCs with different catholytes **(b)** Phenolic degradation with respect to growth profile of *B. subtilis* in the MFC with various catholytes **(c)** Coulombic efficiency by *B. subtilis* using various catholytes.

3.3.2.2 Anionic Reduction in Catholytes

PP, PB and NaCl were selected to study the reduction of their anions throughout the MFC operations in order to understand the relationship between the pH and anionic concentration changes. Ion chromatography data showed that these three catholytes endured minor changes in terms of their anions concentrations (data was provided in the **Supplementary Data**). 5.6% reduction of Cl^- concentration was found in the NaCl catholyte, followed by PP with its persulfate ($S_2O_8^{2-}$) of 1.05% and lastly PB (PO_4^-) with no notable change in concentration at all. These anionic reduction were in accordance to the pH change of the catholytes. The ΔpH of PP and NaCl (0.43 and 0.55, respectively) resulted in 1.05 and 5.6% of anionic reduction. The ΔpH in PB catholyte was minor in accordance to the absence of anionic reduction in the system. Although these anionic concentration changes were very small, the reduction reaction in catholytes might play vital role in associating the effect of pH change to the MFC performance.

The reduction in $S_2O_8^{2-}$ concentration in the catholyte gave precedence to its strong oxidizing and conductivity properties with an oxidation potential of 2.12 V (Reaction 3.3). Its small concentration changes of 1.05% could be attributed to slow reaction kinetics at ordinary temperatures [22]. Under acidic conditions, persulfate anion can hydrolyse to form hydrogen peroxide (Reaction 3.4).

$$S_2O_8^{2-} + 2H^+ + 2e^- \rightarrow 2HSO_4^- \quad \text{(Rxn. 3.3)}$$

$$S_2O_8^{2-} + 2H_2O \rightarrow H_2O_2 + 2HSO_4^- \quad \text{(Rxn. 3.4)}$$

The retaining capability of persulfate anion and its oxidizing property in the MFC catholytes might also be contributed by the presence of various oxidants and radicals with higher oxidation potentials including hydrogen peroxide (1.77 V) (Reactions 3.5 – 3.7) [22, 23]. The strong oxidants and free radicals build up in the catholyte might offset the presence of persulfate anions, thus minimizing the anionic reduction reaction.

$$S_2O_8^{2-} + H_2O \rightarrow HSO_5^- + HSO_4^- \quad \text{(Rxn. 3.5)}$$

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{-\cdot} \quad \text{(Rxn. 3.6)}$$

$$SO_4^{-\cdot} + e^- \rightarrow SO_4^{2-} \quad \text{(Rxn. 3.7)}$$

For saline NaCl catholyte, the solubilisation of the saline in water produces sodium and chloride ions (Reactions 3.8 and 3.9). The chloride anions were further reduced to chlorine Cl_2 which might affect the change of pH of the catholytes.

$$NaCl + H_2O \rightarrow Na^+ + Cl^- + H_2O \quad \text{(Rxn. 3.8)}$$

$$2Cl^- \rightarrow Cl_2 + 2e^- \quad \text{(Rxn. 3.9)}$$

Phosphate buffer system consists of dihydrogen phosphate ions ($H_2PO_4^-$) as hydrogen-ion donor and hydrogen phosphate ions (HPO_4^{2-}) as hydrogen-ion acceptor. These two ions are in equilibrium with each other as indicated by Reaction 3.10.

$$H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+ \quad \text{(Rxn. 3.10)}$$

The presence of H^+ ions from anode affected insignificantly on the equilibrium state of phosphate anions since the value of molar concentration of H^+ ions is equal to the value of the equilibrium constant. The pH is equal to pKa namely 7.21 (or in the range of 6.9 – 7.4). That is the main reason of the ΔpH absence in the PB catholyte, resulting in no notable change in phosphate anion concentration.

3.3.3 Bacterial Growth vs. Phenolic Degradation and Coulombic Efficiency

The bacterial growth and 2,4-DCP degradation were monitored over four days of the MFC operation. Bacterial growth 2,4-DCP degradation profiles with respect to catholytes used are presented in Fig. 3.3 (b). The highest 2,4-DCP degradation of 60% was obtained by the PP-MFC, followed by M9-, PB-, NaCl- and Water-MFCs with 57.5%, 49.2%, 42.5% and 41.7%, respectively. The trend of phenolic degradation seems to follow the fashion of bacterial growth in the MFC systems. Day 1.5 - 2 denoted the highest growth with optical density (OD) of the range 0.4 – 0.45. This optimum cultivation time corresponded to the highest phenolic degradation as depicted in the figure. These results revealed that the best degradation of 2,4-DCP occurred when the *B. subtilis* reached the highest growth level. These results agree with the results by Silambarasan and Vangnai [24], where a 10 mg/L toxic 4-nitroaniline was completely removed by *Acinetobacter* at day 1 – 2 of cultivation when the cell density reached constant value of 0.45 OD. The growth and phenolic degradation profiles also indicated a short lag phase which corresponds to the rapid bacterial acclimatization of 2,4-DCP by *B. subtilis*. The phenolic degradation started to

decline almost sharply after day 2.5, however, the bacterial growth was steadily proceeded with small decreasing trend. These profiles suggest the efficient ability of *B. subtilis* in not only surviving in high toxicity of 2,4-DCP, but also reflect its prompt induction and response to 2,4-DCP when detoxification is required [24].

The OD vs. phenolic degradation profiles also suggest that there were relatively small difference of growth level and phenolic percentage degradation among the catholytes in which the PP resulted the highest and water catholyte achieved the lowest level for the two parameters. These findings raise intriguing questions regarding the nature and extent of catholytes affecting the MFC performance in terms of bacterial growth and phenolic degradation. A possible explanation for this might be the interaction between anolyte and catholyte which in turn assisted to yield varied MFC performance based on different catholyte. Nevertheless, future work is required to establish a mechanism of interaction especially between the phenolic or waste degradation with catholyte system in the MFC.

The coulombic efficiency (CE) of MFCs using five catholytes is depicted in Fig. 3.3 (c). CEs of 23.0%, 18.0% and 17.0% in PP-, M9- and PB-MFCs, respectively, were much higher than that of NaCl- and Water-MFCs with 8.3% and 4.0%, respectively. The degradation of substrate was expected to be highly favourable for the biochemical reactions in NaCl- and Water-MFCs compared to maintaining the electron transfers between anode and cathode chambers. Possible oxygen transference from the cathode to anode could result in decreasing CE output in MFC [14]. In contrast, the relatively higher CEs in the M9- and PB-MFCs might be attributed to the anoxic condition of methanogenesis suppression for the controlled-pH catholytes (buffering properties of PB and M9) [25]. Furthermore, the use of PP and M9 could increase the system conductivity, resulting in a decrease of internal resistance. This improved the CE and current density production [14].

3.3.4 Polarization Curve and Power Generation

Polarization curve is an essential parameter to evaluate the performance of the MFC system. In this study, polarization curves as depicted in Fig. 3.4 were developed in the period of stable current generation using external loads ranging from 10 Ω to 100 kΩ. The highest open circuit voltage (OCV) of 661 mV was obtained by PP-MFC (Fig. 3.4 (a)), corresponding to current density of 7.34 mA/m^2, followed by M9-, PB-, NaCl- and Water-

MFCs with 264 mV, 238 mV, 72.0 mV and 60.2 mV, respectively. The linear trends of the polarization curves showed that the ohmic losses occurred using all catholytes. The internal resistances calculated from the polarization curves were found to be 700 Ω, 1.1 kΩ, 1.15 kΩ, 5.6 kΩ and 5.5 kΩ for PP-, M9-, PB-, NaCl- and Water-MFCs, respectively. It is obvious that the internal resistances in NaCl- and Water-MFCs were much higher than that in PP-, M9- and PB-MFCs which led to lower electricity generation and phenolic degradation. Besides, the higher potential generated in PP-MFC may indicate that this MFC had lower mass transport limitation at its electrodes [9].

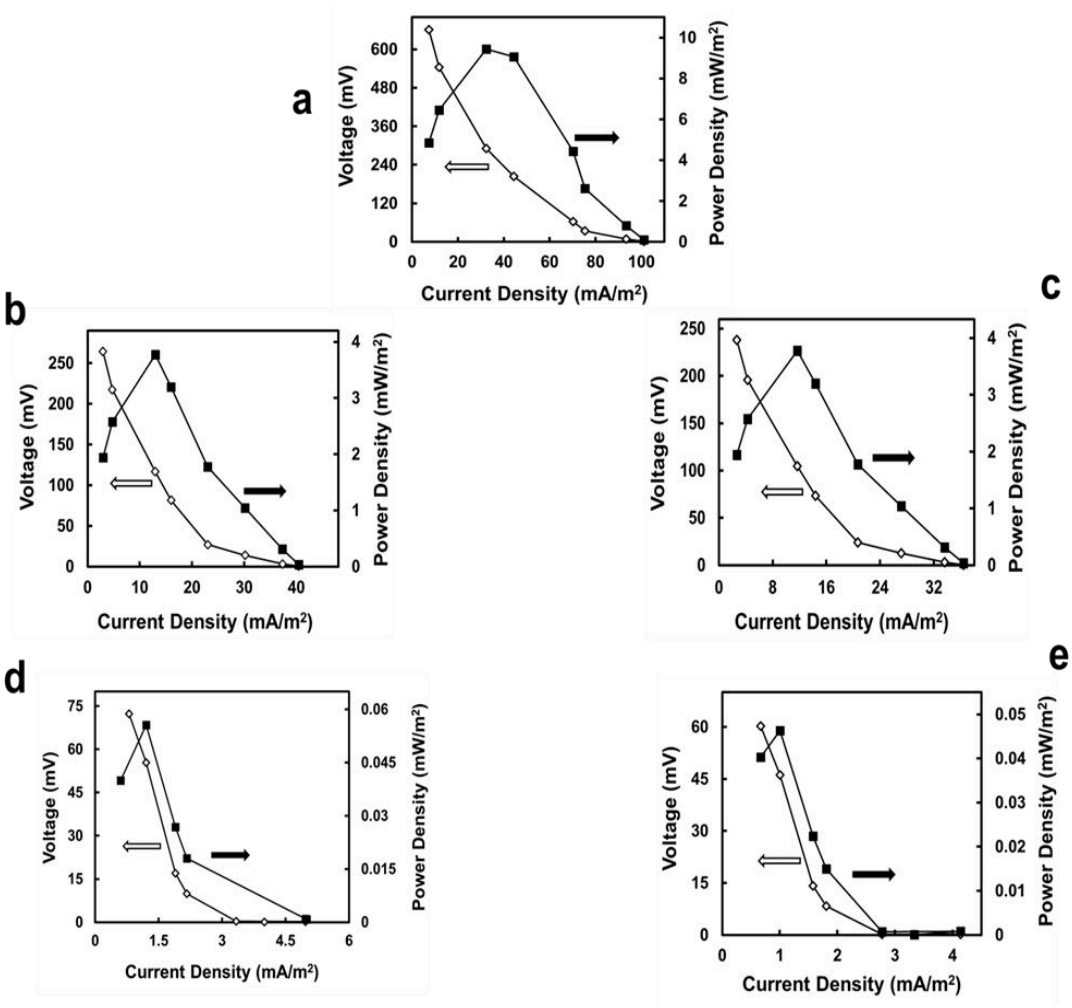

Figure 3.4 Polarization curves and power generation using the five catholytes **(a)** PP, **(b)** M9, **(c)** PB, **(d)** NaCl and **(e)** Water.

Figure 3.4 also shows similar power density profiles of the MFCs using PP, M9 and PB as the catholytes. The maximum power density of 9.4 mW/m^2 was obtained by the PP-MFC at 32.4 mA/m^2 current density, followed by M9-, PB-, NaCl- and Water-MFCs with 3.8, 3.4, 0.06 and 0.05 mW/m^2, respectively. The distinct advantage of PP-MFC over the other catholyte-fed MFCs is due to its oxidizing property and ability to produce more efficient electron transfer from the anode biofilm to the cathode, which yielded higher power density in the MFC system. The higher power densities obtained by the PP, M9 and PB catholytes may be attributed to the improved cathode dynamics with increasing electron acceptor concentration as compared to water catholyte [26].

NaCl and Water (Fig. 3.4 (d) and (e)) appeared to have insignificant effect on MFC performance in term of power recovery. The dramatic decline of power density using these two catholytes could be elucidated by several factors. Firstly, the use of NaCl or water as catholytes could lead to severe over-potential for oxygen reduction without catalysis of noble metals (e.g. platinum) [20, 27]. Secondly, biofilm formation during acclimating phase would also restrict the oxygen mass transfer, thus aggravating the over-potential in the MFC system [27]. Thirdly, the metabolic by-products excreted could impose adverse impacts on the power generation.

The overall results were consistent with the findings of Fan et al. [28], who proposed that the increase in the power density of the MFC could be attributed to the decrease in its internal resistance. The potential and power density obtained by Hongyan et al. [29] were also comparable with our results in which their 50 mM sodium bromate catholyte attained 640 mV and 0.7 W/m^3, respectively. Therefore, these data suggested that PP-, M9- and PB-MFC systems were favourable for solving diffusional transfer and electrochemical restrictions in MFC by *B. subtilis* compared to the other two catholyte systems.

3.3.5 Electrochemical Activity and Electron Transfer Mechanism

3.3.5.1 Cyclic Voltammetry

Cyclic voltammetry (CV) characterizes the electrochemical activity of the redox compounds and reveals the performance of a catalytic process. Figure 3.5 presents typical CVs of the five catholytes-fed MFCs. Generally, the CVs profiles show slight differences before and after 2,4-DCP feeding, except for the PP- and M9-MFCs (Fig. 3.5 (a) and (b)). The CVs of before 2,4-DCP feeding for PP- and M9-MFCs show oxidation peaks of (1.11 mA / 0.45 V), (1.12 mA / 0.47 V) and reduction peaks of (-0.43 mA / 0.36 V), (-0.37 mA / 0.04 V), respectively, which were higher than these after 2,4-DCP feeding [oxidation peaks: (0.53 mA / 0.36 V), (0.42 mA / 0.42 V); reduction peaks: (0.07 mA / 0.41 V), (-0.27 mA / 0.04 V)], respectively. These CVs profiles in Fig. 3.5 (a) and (b) suggest that the 2,4-DCP substrate marginally affected the performance of current generation in the MFC. It was also noted that the 2,4-DCP feeding in the anodic chamber slightly altered the pH of the medium for *B. subtilis* growth. When the pH was reduced from 7.0, the *B. subtilis* growth might be inhibited, leading to current decline. This also could be interpreted that a very small pH change could have significant effect on the MFC performance and thus *B. subtilis* growth. In addition, the increase in oxygen reduction peak current of after 2,4-DCP feeding proved that the existence of microbes with the function of transferring electrons donated by the electrode to oxygen, hence accelerating the reduction of oxygen [30, 31].

In contrast, after 2,4-DCP feeding for PB- and NaCl-MFCs showed a slight increase in the current generation, as shown in Fig. 3.5 (c) and (d). The oxidation cycle of PB-MFC occurred at 0.88 mA / 0.17 V after 2,4-DCP feeding, while a lower points of 0.66 mA / 0.17 V was found before 2,4-DCP feeding. This could be described by its buffering characteristic which compensates the electrons transfer mechanism, thus maintaining a high level of the current generation even after phenolic addition in its cultivation medium. Whereas, salt ions facilitated the electron transfer between anode and cathode chambers in NaCl-MFC, resulting in the small difference in current generation before and after phenolic feeding.

PP-, M9- and PB-MFCs showed vibrant oxidation and reduction peaks compared to NaCl- and Water-MFCs which demonstrated inferior peaks. These profiles proved the presence of mediators in PP-, M9- and PB-MFCs, which could be reversibly oxidized and reduced during CV tests that demonstrated the presence of active redox compounds responsible for the electrochemical activity in the broth solutions [32]. These CV data were also comparable to the results reported by Vanita et al. [33], where the MFC operated at pH 7 showed the highest current generation of about 1.0 mA over the potential of 0.5 V. It is remarkably noted that the 2,4-DCP produced insignificant impact on the *B. subtilis* growth, therefore on the biochemical activities for the current generation in the MFC system.

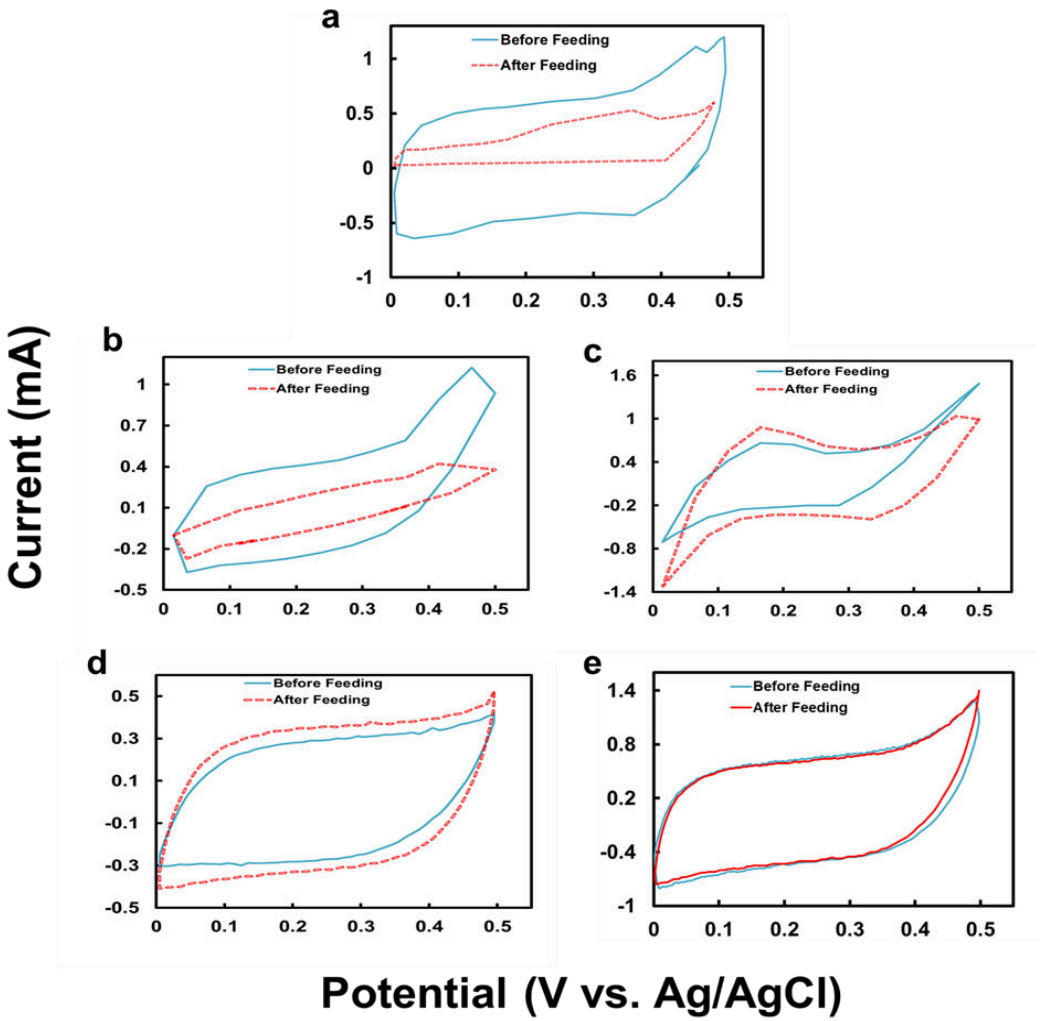

Figure 3.5 Cyclic voltammograms (CV) of the different catholytes **(a)** PP, **(b)** M9, **(c)** PB, **(d)** NaCl and **(e)** Water performed before and after phenolic compound feeding.

3.3.5.2 Linear Sweep Voltammetry

To gain insight into the electrochemical characteristics of the cathode in the MFC, linear sweep voltammetry (LSV) at a scan rate of 0.1 V/s was performed to select the optimal operation parameters, such as operational current and potential. The analogous profiles of LSVs in Fig. 3.6 indicate that all catholytes exhibited similar biochemical activities in the systems [34]. The difference in the MFC performance using different catholytes was caused by the varied chemical reactions of the cathodes, rather than the anode as suggested by Zejie et al. [31]. The oxidation reaction started approximately at a voltage range of 0 – 0.8 (V vs. Ag/AgCl) for all catholyte-fed MFC systems. Then, linear increment of currents developed over subsequent potential flow which showed improvement in the microbial activities at this particular time and become constant (or slightly decreasing) afterwards.

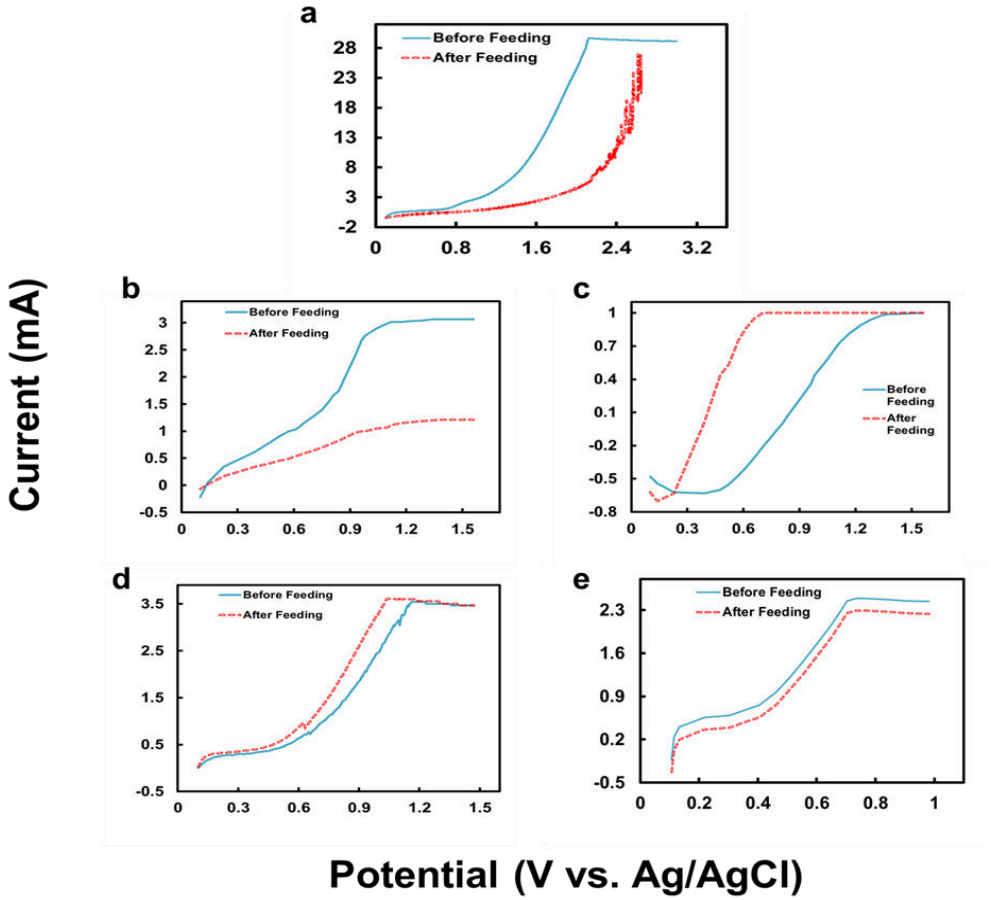

Figure 3.6 Linear sweep voltammograms (LSV) of the different catholytes **(a)** PP, **(b)** M9, **(c)** PB, **(d)** NaCl and **(e)** Water performed before and after phenolic compound feeding.

PP-, M9- and PB-MFCs in Fig. 3.6 (a), (b) and (c) reveal significantly different LSV curves between before and after 2,4-DCP feeding operations. PP-MFC demonstrated the highest current generation of 28.9 mA at 2.11 V before the feeding. The same results were found in M9-MFC (Fig. 3.6 (b)). However, PB-MFC exhibited contrary results with the same peak current of 1.0 mA, which were achieved at 1.34 V and 0.7 V, before and after the feeding, respectively. Increasing the applied voltage caused a little change in peak current for these experiments. The data of LSV for PB-MFC was slightly differed from its CV profile (Figure 3.5 (c)) might be due to some electrochemical interferences during scanning of linear region of potential sweep. However, both CV and LSV profiles achieved the same current peaks for before and after phenolic feeding. The similar profile can be observed from Fig. 3.6 (d), showing a peak current of 3.53 mA achieved at 1.03 V and 1.14 V in the NaCl-MFCs before and after the feeding, respectively. Water-MFC (Fig. 3.6 (e)), on the other hands, showed insignificant different performance before and after the feeding experiment where the current peak approximately occurred at 2.30 mA / 0.7 V and achieved a constant current over further voltage spectrums.

The current increment over potential sweep from its initial electrochemical kinetic equilibrium region is a function of the availability of organic substrates and the activity of electron transfer [35]. Our results revealed that any further increment in potential flow after the peaks could result in constant current for all catholytes fed MFCs. This phenomenon was due to the decrease in microbial activities associated with the microbial biofilm growth and the increasing diffusion for the substrate mass transport to the electrode surface [35]. The higher LSV current generation shown in Fig. 3.6 (a) could be attributed to the combined effect of the higher redox potential and higher electron transfer number of the substrate reduction reaction compared to those in Fig. 3.6 (c) and (e). A similar LSV profile was reported by Jun et al. [36], who used Fe- and K-based electrolytes and found no notable difference in electrolyte reactions as the electrolyte pH increased from 2.0 to 10.0.

The variance in current peaks with corresponding potential for all catholyte-MFC systems gives us a better understanding that the properties of catholytes are of significant factors in evaluating the performance of the MFC in electricity generation and phenolic degradation. Although the LSV curves for PP-, M9- and PB-MFCs could be rehabilitated by the 2,4-DCP feeding, their resilient oxidizing and buffering properties could otherwise

tolerate the changes in maintaining the current generation and phenolic degradation in the MFC system.

3.3.5.3 Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy (EIS) was used to analyze the internal resistances and electrochemical behavior of anode and cathode individually or the whole MFC system. Figure 3.7 shows the Nyquist plots of the MFC for anode and cathode before and after phenolic feeding. There are three resistances can be interpreted from the EIS curves: ohmic resistance (R_{ohm}), charge transfer resistance (R_{ct}) and diffusion resistance (R_d) [29]. In the high frequency region, the intercept of the curve and the real axis could be regarded as R_{ohm} [37]. In the low-frequency, the semicircle curves could be regarded as R_{ct}. Figure 3.7 shows diverse profiles of Nyquist plots between anode (Fig. 3.7 (a)) and cathode (Fig. 3.7 (b)). The R_{ohm} values in anode were slightly varied and became significantly differed in the low-frequency region (R_{ct}). Water and NaCl show the highest R_{ct} with 800 and 700 Ω, respectively, followed by PP (520 Ω), M9 (500 Ω) and PB (410 Ω). The better result for PB which had the lowest R_{ct} value could be due to its buffering capability in maintaining pH stabilization in the MFC system. The significant variance in R_{ct} of anode could also be attributed by the biofilm development. The thicker anode biofilm during the process of cultivation could decrease charge transfer efficiency which resulted in high R_{ct} values.

On the contrary, cathode impedance curves in Fig. 3.7 (b) showed similar trends which could be explained by analogous electrochemical activities occurred in the MFC catholyte systems. As a catholyte used in the MFC, water showed the highest R_{ct} of 512 Ω, while the lowest R_{ct} was yielded by PP and M9 with 400 and 383 Ω, respectively. These low values of R_{ct} were mainly due to excellent electrical conductivity of PP and M9 catholytes (Table 3.1), which increased the rate of electrochemical reactions in the MFC systems [21]. The lower overall impedance resistance values in cathodes as compared to anode gave us understanding that the electrochemical activities in cathode have higher influence on improving the MFC performance than that of anode. The internal resistances resulted in EIS analysis were in a good accordance with the ones obtained from polarization methods at which Water and NaCl catholytes yielded the highest internal resistances, whereas the PP, M9 and PB resulted in lower resistances.

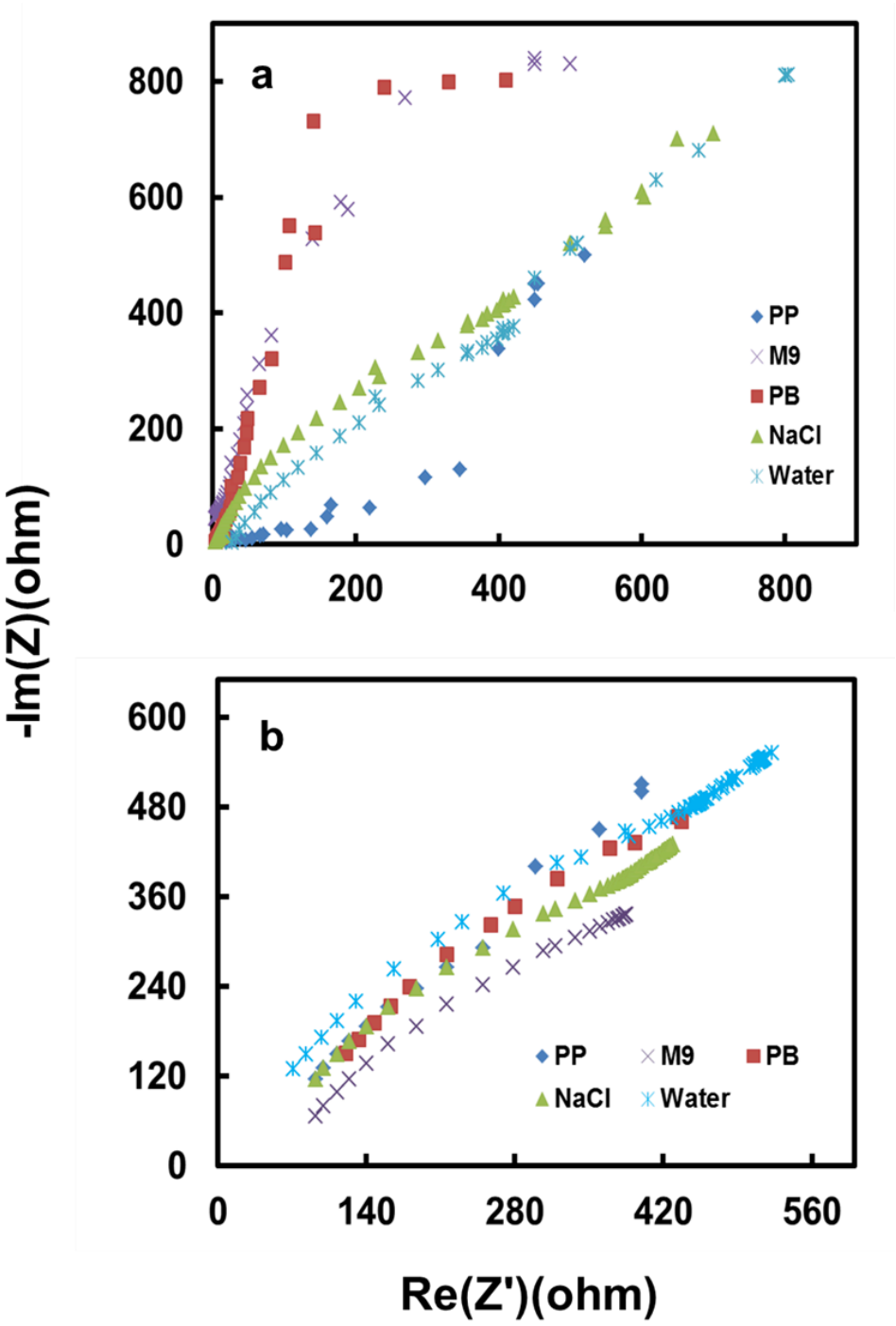

Figure 3.7 Fitted Nyquist impedance spectra of **(a)** anode and **(b)** cathode using various catholytes in the MFC system.

3.4 Conclusions and Future Works

In this study, we experimentally investigated double chamber MFC systems using pure culture *B. subtilis* with various catholytes for electric current production and 2,4-DCP degradation. *B. subtilis* was found to have capability to degrade 2,4-DCP while generating electric current in the MFC system. Chemical characteristics of the catholytes have significant impact on the bio-electrochemical activities, and therefore the performance of the MFC system. The current density profiles show that PP-MFC exhibited significantly the highest current generation (64.0 mA/m^2) and 2,4-DCP degradation, while NaCl and water appeared to be less promising catholytes for MFCs. The phenolic degradation and CE data supported the findings that PP, M9 and PB were the potential catholytes. The polarization curve data indicated that PP had high capability of solving diffusional and electrochemical restriction by *B. subtilis*. The analyses of electrochemical and electron transfer activities using cyclic voltammetry and linear sweep voltammetry validated our initial hypothesis that PP, M9 and PB were vital catholytes in enhancing MFC performance to generate current as compared to NaCl and Water catholytes. Therefore the results of this study proved that the low-concentrated, low-cost and low-toxicity catholytes could amplify the electricity generation with simultaneous 2,4-DCP degradation. The proposed system design is potentially very useful towards the development of low-cost and sustainable MFC applications.

In future works, the MFC performance could be studied using variants of the persulfate-based catholytes. Studies involving the utilization of mixed culture from wastewater could be carried out to explore the performance of the MFC in terms of phenolic degradation and current generation. Besides, future study is necessary to investigate the mechanism of interaction between catholytes and bacterial growth or waste degradation in the MFC system.

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Supplementary Data

Spectrophotometry analysis of persulfate anion reduction:

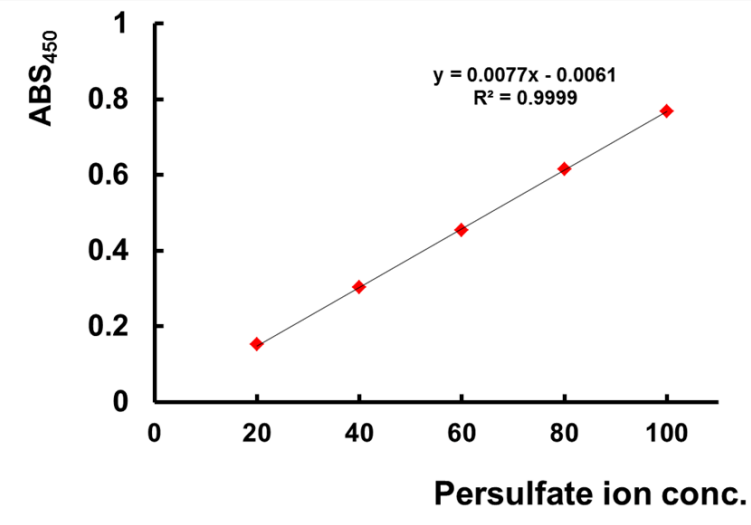
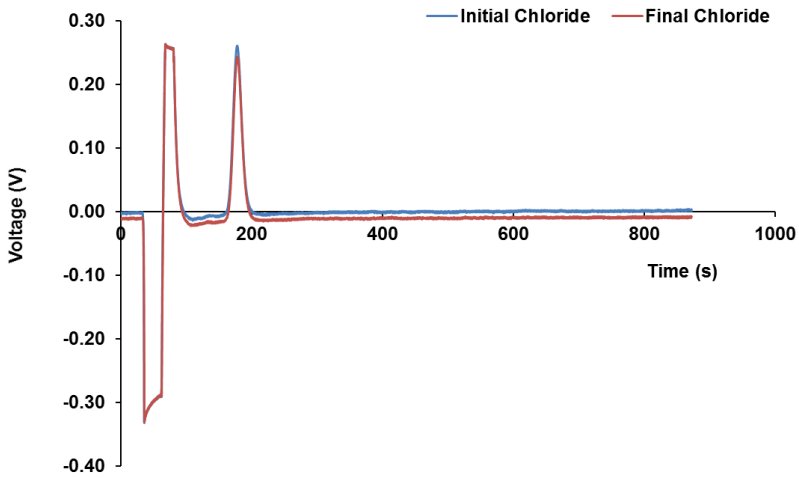


Figure S3.1 Standard data for persulfate ($S_2O_8^{2-}$) anion using spectrophotometry analysis with a wavelength of 450 nm.

Table S3.1 Reduction of persulfate anion concentration in potassium persulfate (PP) catholyte.

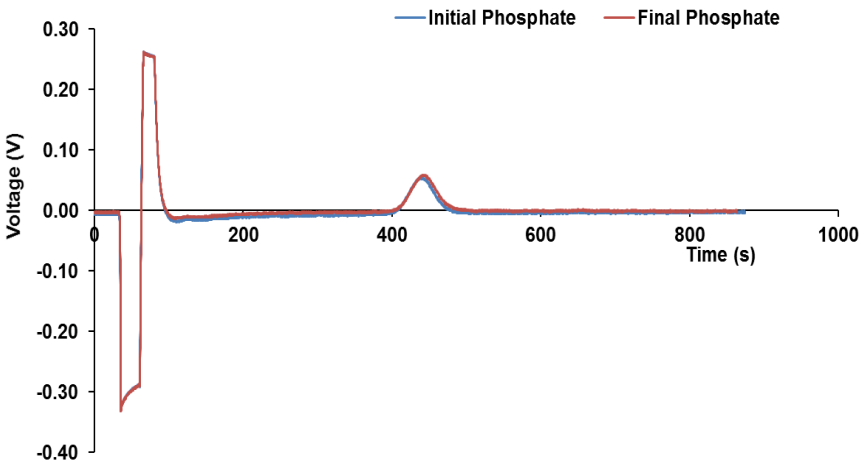
Initial concentration	34.32 mM	9278.5 mg/L
Final concentration	33.96 mM	9180.5 mg/L
% Reduction	**1.05**	

Ion chromatography analysis of phosphate and chloride anions reduction:



	Min.	Max.	Mean	Std. Dev.	Count	**Area**
Initial Chloride	-8.48E-04	0.266	0.081	0.091	525	**4.243 s**
Final Chloride	-7.72E-04	0.256	0.099	0.089	403	**4.005 s**

Figure S3.2 Ion chromatography peaks for initial and final chloride anions concentration.



	Min.	Max.	Mean	Std. Dev.	Count	**Area**
Initial Phosphate	-8.36E-04	0.061	0.025	0.02	980	**2.497 s**
Final Phosphate	4.68E-05	0.061	0.024	0.02	1064	**2.546 s**

Figure S3.3 Ion chromatography peaks for initial and final phosphate anions concentration.

Table S3.2: Reduction of peak area for phosphate (HPO_4^-) and chloride (Cl^-) anions in phosphate buffer (PB) and NaCl catholytes, respectively.

Anions	**Initial (peak area)**	**Final (peak area)**	**% Reduction**
Cl^- initial	4.243	4.005	**5.6 %**
HPO_4^-	2.497	2.546	**-2.04 %** (considered as no significant change by ion chromatography measurement)

CHAPTER 4

Statement of Authorship

Title of Paper	Bioelectrochemical analysis of microbial fuel cell systems inoculated by *Bacillus*- and *Arcobacter*-dominated petrochemical and domestic microbial consortia
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Huzairy Hassan
Contribution to the Paper	Designing and conducting the experiments, data analysis, and manuscript writing.
Overall percentage (%)	50%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 21/12/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Bo Jin
Contribution to the Paper	Supervising the development of the work, data interpretation, manuscript review and correction.
Signature	Date 19/01/2017

Name of Co-Author	Erica Donner
Contribution to the Paper	Assisting in DNA analysis, data interpretation, and advice on manuscript preparation.
Signature	Date 21/12/16

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Contribution to the Paper	Assisting in DNA sequencing preparation, bioinformatics and statistics. Assisting with data interpretation and manuscript preparation.		
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Contribution to the Paper	Assisting with DNA analysis and manuscript review		
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Contribution to the Paper	Helping in manuscript review and correction.		
Signature		Date	21-12-2016

4. Bioelectrochemical Analysis of Microbial Fuel Cell Systems inoculated by *Bacillus*- and *Arcobacter*-dominated Petrochemical and Domestic Microbial Consortia

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Chapter 4: Bioelectrochemical Analysis of Microbial Fuel Cell Systems inoculated by *Bacillus*- and *Arcobacter*-dominated Petrochemical and Domestic Microbial Consortia

Abstract

Microbial fuel cell (MFC) and its bio-system have been intensively investigated for energy generation using domestic or municipal bacterial consortia. Yet, lack of detailed studies on microbial and bioelectrochemical activities in the MFC system, which is operated for removal of toxic contaminant in wastewater, while generating electricity. Hence, this study evaluates the microbial community and electrochemical activities in laboratory scale MFC reactor using petrochemical industrial and domestic microbial consortia. The systematic investigation of the natural microbial consortia-inoculated MFC was aimed at degrading 2,4-dichlorophenol (2,4-DCP) and generating electricity. Cutting-edge microbial community analysis techniques were used for identifying bacterial species and bioelectrochemical activities involved in the MFC. Industrial microbial consortium (IMC) performed well in generating 156 mA/m^2 current density with 41% phenolic degradation as compared to domestic microbial consortium (DMC) with 123 mA/m^2 and 62% phenolic degradation. *Arcobacter* and *Cloacibacterium* in DMC were found to be important bacteria for 2,4-DCP degradation while *Bacillus* dominated IMC contributing to generate higher electricity. These results provide new insight in manipulating suitable bacteria for phenolic biodegradation while generating electrical energy outputs in MFC systems.

Keywords: Microbial consortia; Microbial fuel cell; 2,4-Dichlorophenol; *Arcobacter*; *Bacillus*; *Cloacibacterium*.

4.1 Introduction

Microbial fuel cell (MFC) has emerged as one of the potential tools for sustainable energy production and wastewater treatment. Although the low amount of electricity generation is still an ongoing technical issue in the MFC, the capability to produce the electricity needs to be improved in current research practices. Furthermore, the utilization of harmless microorganisms as exoelectrogenic bacteria makes the MFC more unique and safe to produce energy with simultaneous biodegradation of organic, especially toxic contaminants in wastewater.

As the increasing environmental risks of wastewater from growing industrial activities, MFC has been viewed as an important and sustainable technology for wastewater treatment. MFC offers a number of advantages including direct production of energy and value-added products, good effluent quality obtained through effective combination of biological and electrochemical processes, as well as good operating stability achieved through inherently real-time monitoring and control [1]. MFCs may produce up to 1.43 kWh/m^3 from a primary sludge or 1.8 kWh/m^3 from a treated effluent [2]. MFCs consume only 0.024 kW or 0.076 kWh/kg-COD in average which is about ten times lesser than activated sludge-based aerobic processes (~0.3 kW or 0.6 kWh/kg-COD) [3]. This suggests that MFCs operate at very efficient energy consumption level compared to conventional wastewater treatment while recovering energy through its exoelectrogenic based bioelectrochemical process. Other than that, much smaller amount of sludge could be produced (0.07-0.16 gVSS/gCOD cell yield) in MFC as compared to activated sludge process (0.35-0.45 gVSS/gCOD) [4] at which the sludge management is a considerable problem in an industrial wastewater treatment process.

The latest research findings demonstrate promising systematic outcomes in electricity generation and wastewater reduction using MFC. For instance, noble materials (Acrylonitrile Butadiene Styrene and Nanocure) were used as electrodes in MFC to enhance the electricity generation (~4.5 mW/m^2) and COD removal (~53%) [5]. An up-flow MFC system was designed to achieve maximum power generation (372 mW/m^2) with a very high cell density retain [6]. Enhanced power generation (1063 mW/m^2) was reported in a lab scale MFC system using flame-oxidized hematite anode catalyzed by identified anodic dominant *Geobacter* sp. [7]. However, the relatively expensive materials and high-

profile configuration make these MFC designs debatable especially in its large scale process for practical applications.

The aforementioned studies typically evaluated the MFC performance for electricity generation and organic loading reduction in terms of chemical oxygen demand (COD). To the best of our knowledge, there are still limited reported studies which systematically investigated MFC systems for removal of specific chemicals, especially toxic pollutants in wastewater such as heavy metals, phenolic or other hazardous compounds. Phenolic compounds become one of the major toxic contaminants in wastewater from many chemical, pharmaceutical, textile and oil refinery industries [8]. With their recalcitrant characteristics including acute toxicity, and suspected carcinogenicity and mutagenicity [9-10], phenolic compounds therefore have been drawn serious attentions in various researches.

This study was to analyse mixed bacterial consortia derived from industrial and domestic wastewaters in a double chambered MFC system, which was systematically assessed in terms of its electrochemical capability for biodegradation of 2,4-dichlorophenol (2,4-DCP), while generating electricity. The industrial wastewater was collected from petrochemical refinery plant in which the phenolic contaminated level was expected to be high. As a commonly used microbial consortium for MFC studies, domestic wastewater from a sewage treatment plant was comparatively analysed. Special attention was given to determine changes in microbial communities in MFC using both consortia in response to the addition of 2,4-dichlorophenol. The microbial consortia were analysed using recently reported technique of the Illumina HiSeq analysis of 16 rRNA amplicons [11]. The traits of selected operational taxonomic units (OTUs) on their phylogenetic affiliations have been explored using the cutting-edge step-by-step DNA analysis including LotuS, FLASH, USEARCH/UPARSE, UCHIME, Bayesian classifier, SILVA and UPGMA. This study provides mainstays in microbial behaviour exploration for systematically selecting the suitable microorganisms profoundly involved in phenolic degradation as well as MFC electron transfer activities.

4.2 Materials and Methods

4.2.1 Mixed Bacterial Consortia and Growth Media

4.2.1.1 Mixed Bacterial Consortia

Domestic microbial consortium (DMC) was derived from domestic wastewater collected from Glenelg Wastewater Treatment Plant, South Australia. Industrial microbial consortium (IMC) was derived from petrochemical wastewater collected from Australia Mobil Oil Plant, South Australia. Both samples were collected from clarifier tanks in the form of mixed sludge. DMC sample was directly used as inoculum for the MFC experiment. IMC sample was first separated using separatory funnel and the remaining sludge was used as inoculum. 30% (v/v) of inoculum was used for both microbial consortia. The phenolic compound in both domestic and industrial wastewaters was determined according to Garcia et al. [12]. Wastewater quality data for both wastewaters are provided in Table S4.1 **(Supplementary Data)**.

4.2.1.2 Growth Media

The growth medium for both microbial consortia was made of artificial wastewater consisting of 2.0% glucose, 0.386 g/L $(NH_4)_2SO_4$, 0.149 g/L K_2SO_4, 3.31 g/L NaH_2PO_4, 10.31 g/L Na_2HPO_4, 1 g/L NaCl, 0.2 g/L $MgSO_4$ and 12.5 mg/L vitamin. The pH was adjusted to 7.0 ±0.1 with NaOH or HCl. The medium was sterilized at 121 ºC for 15 min. The glucose solution was sterilized using 0.22 µm filter unit (Polyvinylidene difluoride (PVDF) millipore membrane). 2,4-dichlorophenol (2,4-DCP) with 10 mg/L was fed into the growth medium after 12 hours of MFC cultivation and denoted as 'phenolic feeding' operation. The 'non-phenolic feeding' MFC was operated with glucose feeding only at the same abovementioned time. 50 mM phosphate buffer was used as MFC catholyte in this study.

4.2.2 Microbial Community Analysis and Characterization

4.2.2.1 DNA Analysis of Microbial Community

The biofilms on anode electrode surface and suspended culture in anolyte were sampled from phenolic and non-phenolic feeding MFCs for microbial community analysis. The bacterial genomic DNA was extracted from 0.5 ml cell suspension or 0.5 g dried biofilm of these samples using FastDNA^{TM} SPIN Kit and FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). The bacterial V3-4 hypervariable region of the bacterial small ribosomal subunit coding gene (SSU) was amplified via polymerase chain reaction (PCR) performed on the DNA extracts. The products of all samples were then multiplexed as described in Vasileiadis et al. [11] and were sequenced with the Illumina HiSeq 2500 platform for 300 cycles using the paired-end reads module by Fasteris SA (Geneva, Switzerland). The received sequences were analysed with the LotuS v1.44 software suit [13]. Analysis steps included: read de-multiplexing to their samples of origin and quality control with the LotuS native simple de-multiplexer (smd) v1.26 using the default parameters with the exception of a minimum good quality sequence length of 170 bp; merging of read pairs with FLASH v1.2.8 [14]; clustering of reads into 97% identity operational taxonomic units (OTUs) with the USEARCH/UPARSE v8.0.1623 algorithm [15]; removal of chimeric OTUs with UCHIME v4.2 [16] and the ribosomal database project (RDP) gold database version; OTU representative sequence classification using the RDP naïve Bayesian classifier v2.11 [17] and the SILVA v123 database [18]. The output matrices were analysed using the R v3.2.3 software [19] for: addressing sample relations via hierarchical clustering and the unweighted pair group method with arithmetic mean (UPGMA); assessing treatment group related OTU differential abundances with Fisher's exact tests as implemented by the Edge R v3.12.0 package using the Holm correction for multiple hypothesis testing [20].

The final per-sample sequence numbers were sufficient for screening the vast majority of the existing diversity as indicated by the Good's coverage to estimate the values which were translated into the 97.2-100% of the environmental bacterial SSU diversity being uncovered given our methods [21].

4.2.2.2 Characterization of Biofilm Morphology

Morphology of the DMC and IMC biofilms formed onto the anode surface was characterised by a scanning electron microscope (SEM) (Philips XL30). The carbon cloth-anode attached biofilms were aseptically removed from the MFC anodic chamber and washed with phosphate buffer saline solution. Then the biofilm sample was dried at 80 °C for 24 h. The sample was coated using platinum for high resolution SEM imaging.

4.2.3 MFC Reactor Setup

A double chambered MFC reactor was constructed from two glass bottles (250 mL) joined together with a glass bridge, consisting a 30-mm inner diameter cationic exchange membrane (CEM, CMI-7000, gel polystyrene cross linked with divinylbenzene). Anode material was made up of 40 × 30-mm carbon cloth, while the cathode material was made of 40 × 30 × 2-mm platinised titanium (Pt/Ti) catalysts plate. Both electrodes were wired with titanium projecting outside connecting to an external electrical circuit, and placed as closed as possible to the membrane bridge.

The anodic chamber was tightly closed from air penetration to provide anoxic condition for culture growth and the cathodic chamber was open for aeration. Both DMC- and IMC-MFC systems were operated on a magnetic plate to ensure well mixing during operation in room temperature. After one day of cultivation, the medium in anodic chamber turned turbid, indicating the bacterial growth. The biofilm was gradually formed on the electrode surface. The MFC experiments were conducted in duplicate. An abiotic MFC without microbial inoculation was operated as a control experiment under the same conditions.

4.2.4 Data Acquisition, Polarization Curve, and Cyclic and Linear Sweep Voltammetry

The electrode circuit voltage was continuously monitored and recorded by a computer program. The anode and cathode were directly connected to a LabJack U6 recorder with an external load of 1 kΩ circuit connection. The MFC system was then operated over 4 days under a single constant resistance. The anodic and cathodic voltage and current were measured during the operational period. To develop the polarization curves, different external loads were applied for a complete batch cycle in a variable resistance range of 10 - 100 kΩ.

Cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were recorded using a CHI 650 D bipotentiostat (CH Instruments, Inc., USA). The CVs were conducted at a scan rate of 0.1 V/s ranging from 0 to 0.5 V (vs. Ag/AgCl), while the LSVs were conducted at a scan rate of 0.1 V/s ranging from 0.1 to 2.5 V (vs. Ag/AgCl). The experiments were carried out in a conventional three-electrode arrangement consisting of a working electrode, reference electrode (an Ag/AgCl electrode) and a counter electrode. All three electrodes were inserted into the MFC, avoiding any contact among these electrodes.

4.2.5 Evaluation of Chlorophenol Degradation

2,4-dichlorophenol (2,4-DCP) (Alfa Aesar) was used as a model phenolic compound. 2,4-DCP is a chlorinated derivative of phenol with the molecular formula $C_6H_4Cl_2O$. 2,4-DCP with 10 mg/L was fed into the MFC anodic chamber after 12 hours of cultivation. A 5-mL sample was collected from anodic chamber every 24 hours over 4 days. The sample was then centrifuged using Megafuge (1.0 R Heraeus) at 4000 rpm for 10 minutes at 4 °C. The supernatant was used for further quantitative and qualitative analyses using spectrophotometry and high performance liquid chromatography (HPLC), respectively. Two sets of control experiment using both DMC and IMC were conducted in an anaerobic chamber in order to compare the 2,4-DCP degradation in MFC with these conventional biodegradation trials. The inoculum size, medium solution and phenolic feeding time were the same as operated in MFC experiments.

The phenolic degradation was analysed using spectrophotometer (UV-1601; Shimadzu) according to Folin Ciocalteau's phenol reagent method as described by Garcia et al. [12]. The phenolic degradation (%) was calculated by the difference between initial (C_0) and final concentration (C) of 2,4-DCP. 2,4-DCP degradation metabolites were determined using the HPLC (VARIAN, ProStar) equipped with a C_{18} capillary column (Microsorp-MV 100-5, 150 X 4.6 mm). The column was stored in 10% methanol prior to use. The ultraviolet detector (VARIAN, ProStar) was set at 227 nm. The mobile phase was prepared by dissolving methanol in milli-Q water with 50:50 v/v ratio with 2% acetic acid pre-set at pH 3. All metabolite samples were tested in duplicates and compared against pre-run standard solutions (analytical / chromatography Sigma-Aldrich standards) chosen in 95 - 99% conformity with Arora and Bae [22]. The phenolic compounds that are naturally present in the consortia samples would not interfere with the fed 2,4-DCP as the HPLC

could specifically detect and analyse only the target compound of 2,4-DCP with pre-determined chromatogram conditions.

4.3 Results and Discussion

4.3.1 Microbial Community Analysis

Classification of sequences into taxa using the Ribosomal Database Project (RDP) classifier for 80% bootstrap cutoff resulted in matrices that were used for assessing the taxon based sample relations. Inclusion of taxonomical levels in the analysis was based on their relative abundances. Starting from the genus level and in the case of unclassified or taxa participating with less than 1% in at least one sample the higher taxonomical level was used. The sequences analyzed in this study are publicly available in the sequence read archive (SRA) of NCBI under the sequence accession numbers **SRR4302501-16**.

Using these cutting-edge techniques as described in subsection 4.2.2, microbial community in the growth media, suspension and biofilms in the MFC systems was analyzed with respect to consortium sources and chlorophenol feeding operation.

4.3.1.1 Industrial Microbial Consortium

The industrial mixed consortium (IMC) samples were identified according to the composition of their communities in dominant operational taxonomic units (OTUs) with the OTU approximating the species concept. Based on the OTU identification as shown in Fig. S4.1 **(Supplementary Data)**, the IMC yielded about 83.6% relative participation identified from *Bacillus* genus and others from the *Bacillales* order (16.2%) and rare OTUs, which have less than 0.1% relative participation. It is obvious that IMC showed a pattern of singularity in its microbial classification in which *Bacillus* dominated the microbial community in MFC systems. The other DNA data analysis are not discussed in this paper, and are available in the **Supplementary Data**, unless mentioned otherwise.

Bacillus categorized as an obligate aerobe or facultative anaerobe [23] is known for its ability to form a tough, protective and metabolically quiescent endospores [24]. These resilient characteristics allow it to be resistant to chemical stresses [24-25], for example, in the petrochemical contaminated environment. Our data suggest that the *Bacillus* exhibited excellent current generation in the MFC with or without 2,4-DCP feeding. Hence, it may

be concluded that this *Bacillus* genus show high resistance and adaptability to the toxicity of 2,4-DCP in the MFC.

Other than that, *Bacillus subtilis* has been reported as a good current generator in MFC system [26]. Samsudeen et al. [27] isolated abundance of *Bacillus* genus for example *Lysinibacillus sphaericus* and *Bacillus safensis* from distillery wastewater, and found to generate 400 mV of closed circuit potential in the MFC. Other industrial wastewater sources such as food, alcohol, dairy and chemical wastewaters have also been used as media to generate power in MFC. However, detailed bacterial population analysis seems missing in many reported studies [28, 29]. To the best of our knowledge, there were still limited studies on analysing petrochemical based microbial consortia for generating electricity in the MFC. Therefore, the microbial community from IMC is very significant.

4.3.1.2 Domestic Microbial Consortium

The domestic microbial consortium (DMC) samples were restructured based on their taxon composition using UPGMA algorithm (in the mixture of phylum, class, family and genus classifications). Results of microbial community analysis for DMC are illustrated in Fig. 4.1. There was a wide existence of microbial diversity in the DMC as compared to IMC (supported also by colony appearances on nutrient agar as shown in Fig. S4.2). Generally, the predominant populations in DMC belong to *Arcobacter*, *Aeromonas*, and *Pseudomonas*, followed by the smaller bacterial groups affiliated by *Acinetobacter*, *Cloacibacterium*, *Shewanella*, and others from *Bacteria* domain. It is found that low abundance of *Trichococcus*, *Clostridiales*, *Actinomycetales* and *Parabacteroides* were also involved in the process. The microbial population in DMC generally show common bacterial consortium associated largely with phyla *Proteobacteria*, and others with *Firmicutes*, and *Bacteroidetes*.

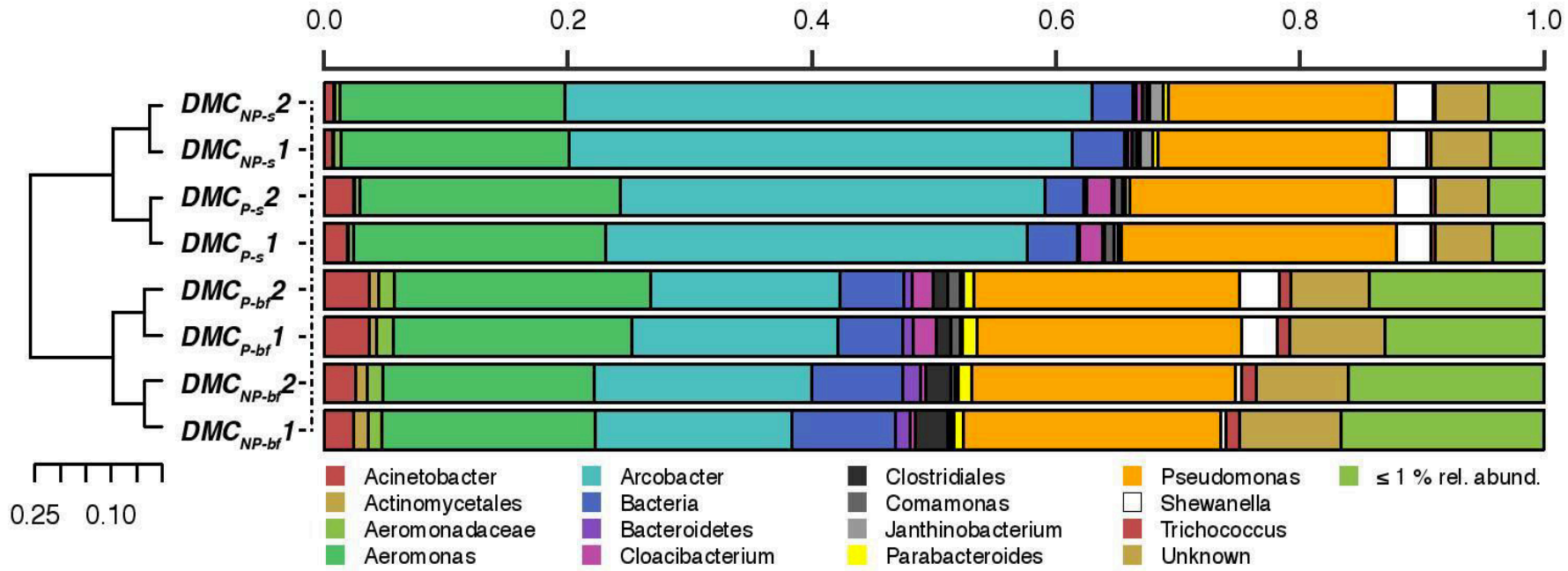

Figure 4.1 Stacked bar plot and hierarchical clustering (UPGMA algorithm) for DMC results only according to the Bray-Curtis dissimilarities of samples based on their taxon composition. Taxa participating equal or more than 1 % in at least one sample are shown here.

(Note: NP: non-phenolic feeding, s: suspension liquid, P: phenolic feeding, bf: biofilm, Number 1 & 2: duplication)

The widespread presence of facultative anaerobic and aerobic bacteria recommends that anodic chamber of the MFC was not strictly anaerobic due to the crossover of oxygen from the cathodic chamber through CEM. It is reported that anaerobic enriched MFC showed good tolerance to oxygen in the anodic chamber [30]. For example, *Pseudomonas* and *Aeromonas* are classified as facultative anaerobic and aerobic [31-32]. A reasonably anew found bacterium, *Arcobacter* is classified as aerotolerant *Campylobacters* and was reported to grow under microaerobic and anaerobic conditions in the MFC biofilm [33-34]. However, some typical exoelectrogenic bacteria and obligate anaerobes, for example *Geobacter* were not found in this DMC. This may be because the air exposure or diffusion from cathode slightly hindered the anaerobic exoelectrogens growth [35], or they might be outcompeted by more competent bacteria in the system.

4.3.1.3 Biofilm vs. Suspension in DMC-MFC

Figure 4.2 (A) shows the comparison results of existing bacteria in suspension liquid and biofilm of the anodic chamber in DMC-MFC. With respect to the relative abundance, the biofilm formation at the anode was extensively associated with the 'rare' taxa (comprising less than 1% of the total bacterial communities) followed by *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Clostridia*, *Parabacteroides* and *Trichococcus* (based on the star sign which indicates the comparison is significant). These data revealed that these type of bacteria were relatively involved in generating electric current through biofilm attached on the anode surface. Previous studies found that *Aeromonas* is an excellent electrochemically active strain in the MFC biofilms [32, 36]. *Pseudomonas* is also a unique bacterium which is a good exoelectrogen in MFC as reported by Raghavulu et al. [37]. *Pseudomonas* spp., especially *Pseudomonas aeruginosa* has been broadly researched due to its excellent capabilities in defining metabolic diversity and is able to colonize a wide range of niches [38]. The nature of *Pseudomonas* has unipolar motility and flagellar characteristics in helping its biofilm development [39], and thus could enhance its role for current generation in the MFC system.

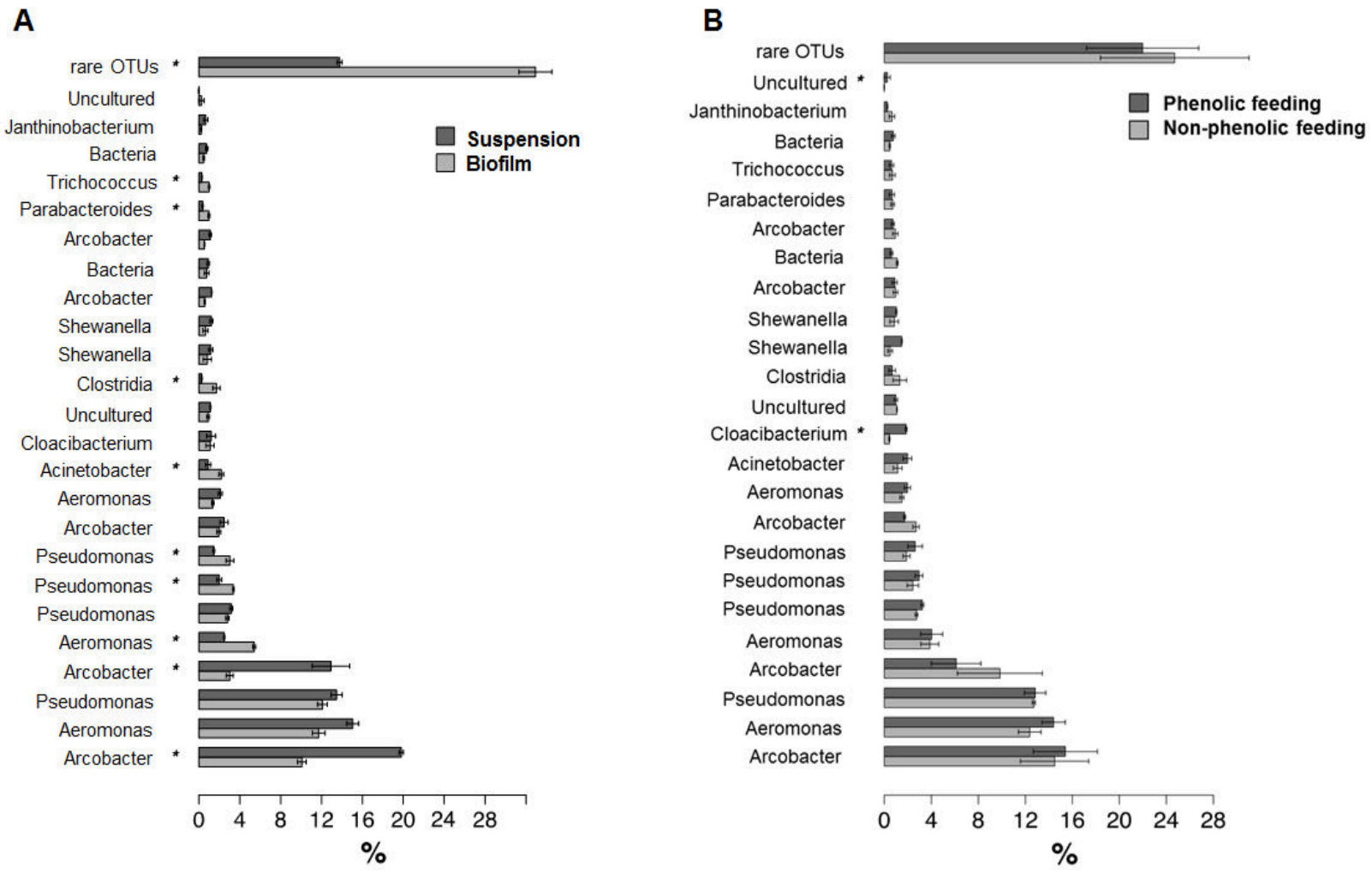

Figure 4.2 Bar plot with standard error bars showing the Fisher's exact test output for differentially abundant OTUs identification between **(A)** suspension and biofilm and **(B)** phenolic and non-phenolic feedings. Stars show differentially abundant OTUs for adjusted P values ≤ 0.05 using the Holm correction method. Only DMC samples were used in this analysis.

Arcobacter species, on the other hands, was found to offer more substantial impact on the MFC performance through suspension liquid rather than biofilm (Fig. 4.2 (A)). *Arcobacter* bacteria, in particular *Arcobacter butzleri* from the electrode biofilm were reported in a few of recent studies [33, 40]. This suggests that the electron transfer process by *Arcobacter* in the MFC is associated with both suspension liquid (by mediators or pili) and biofilm (by direct transfer).

Arcobacter butzleri was isolated from the electrode of an acetate-fed MFC and is found to be the first exoelectrogenic *Epsilonproteobacterium* demonstrated to act as an exoelectrogen [33]. It has been speculated that the *Arcobacter* may produce exopolysaccharides which could help the microorganism to colonize around the MFC electrodes [40] through its suspension liquid. Other recent notable findings by Medrano et al. [41] revealed that the *A. butzleri* can express a protein called flagellin and resemble filamentous pili for biofilm formation, and has involved novel cytochromes in its electron transport chain. These previous findings may support our result in which the *Arcobacter* was found to be useful in (i) anode-cell communication related to suspension liquid; (ii) electrogenicity in its biofilm formation, and (iii) improving current generation in the MFC through its electron transfer mechanisms.

4.3.1.4 Impact of chlorophenol on DMC-MFC performance

It could be hypothesized that additional 2,4-DCP may impact on the bacterial activities, microbial community, and consequently the bio-electrochemical activities in MFC. Microbial community was analysed in the DMC-MFC with and without 2,4-DCP feeding. Figure 4.2 (B) shows that there are insignificant microbial community changes with respect to the phenolic addition except for the *Cloacibacterium*. The high abundance of *Cloacibacterium* in the phenolic medium can be positively related to the 2,4-DCP degradation efficiency in the MFC. Very few studies on this Gram-negative, non-motile and facultative anaerobic bacteria were reported since it was discovered in 2006 [42]. Hence, it is worthwhile to note that our study may be the first to report *Cloacibacterium* in the phenolic based MFC. Further study on the molecular level of *Cloacibacterium* seems necessary to improve the phenolic degradation while generating electricity in the MFC. Previous study found that *Mycobacterium* and *Desulfobacterium* are abundantly associated with the pentachlorophenol degradation in MFC [43-44]. Some evidences suggested that

many of the aerobic chlorophenol-degrading strains were found belonging to the genera *Mycobacterium* and *Sphingomonas* [45].

Figure 4.2 (B) also indicates that more *Shewanella* and *Acinetobacter* sp. were found in the 2,4-DCP fed MFC. *Acinetobacter* sp. was reported in previous studies on chlorophenols degradation [46]. *Shewanella* was also reported to degrade phenol in an anaerobic degradation process [47], and was found as a good electroactive species in the MFC [48]. Nevertheless, this paper may be the first to report the significant existence of *Acinetobacter* and *Shewanella* sp. in the phenolic contaminated environment of the anodic MFC.

To sum up, results from microbial community analysis demonstrate that phenolic toxicity through the 2,4-DCP addition may have limited impact on the variation in microbial populations especially on *Cloacibacterium*, and those dominants *Arcobacter*, *Aeromonas* and *Pseudomonas* in the DMC-MFC. *Pseudomonas* along with other dominants of *Proteobacteria* and *Bacteroidetes* for example were found to have strong ability to degrade chlorophenol in a biodegradation study [49]. Therefore, considering the relative abundance analysis and other physicochemical factors, the *Cloacibacterium* and other dominants in DMC could play a significant role in degrading 2,4-DCP in our MFC system.

4.3.2 Morphologies of Biofilms

Scanning electron microscopy (SEM) images in Fig. 4.3 reveal unique biofilm morphologies and structures in DMC- and IMC-MFC systems. It is clearly observed that the microbial biofilms for both mixed consortia (Fig. 4.3 (B) and (C)) were formed and attached on the carbon cloth electrode as compared to the clean carbon cloth in Fig. 4.3 (A). The bacteria on the DMC biofilm (Fig. 4.3 B) were more morphologically diverse compared to IMC (Fig. 4.3 C). The DMC biofilm appeared to be more packed and caused the anode surface to be unrefined. On the contrary, IMC biofilm demonstrated better structure showing the anode surface undisrupted. The well-structured and uniform biofilm formation lead to low charge resistance as suggested by Yang et al. [50], thus increasing the efficiency of electron transfer and current generation in the IMC-MFC.

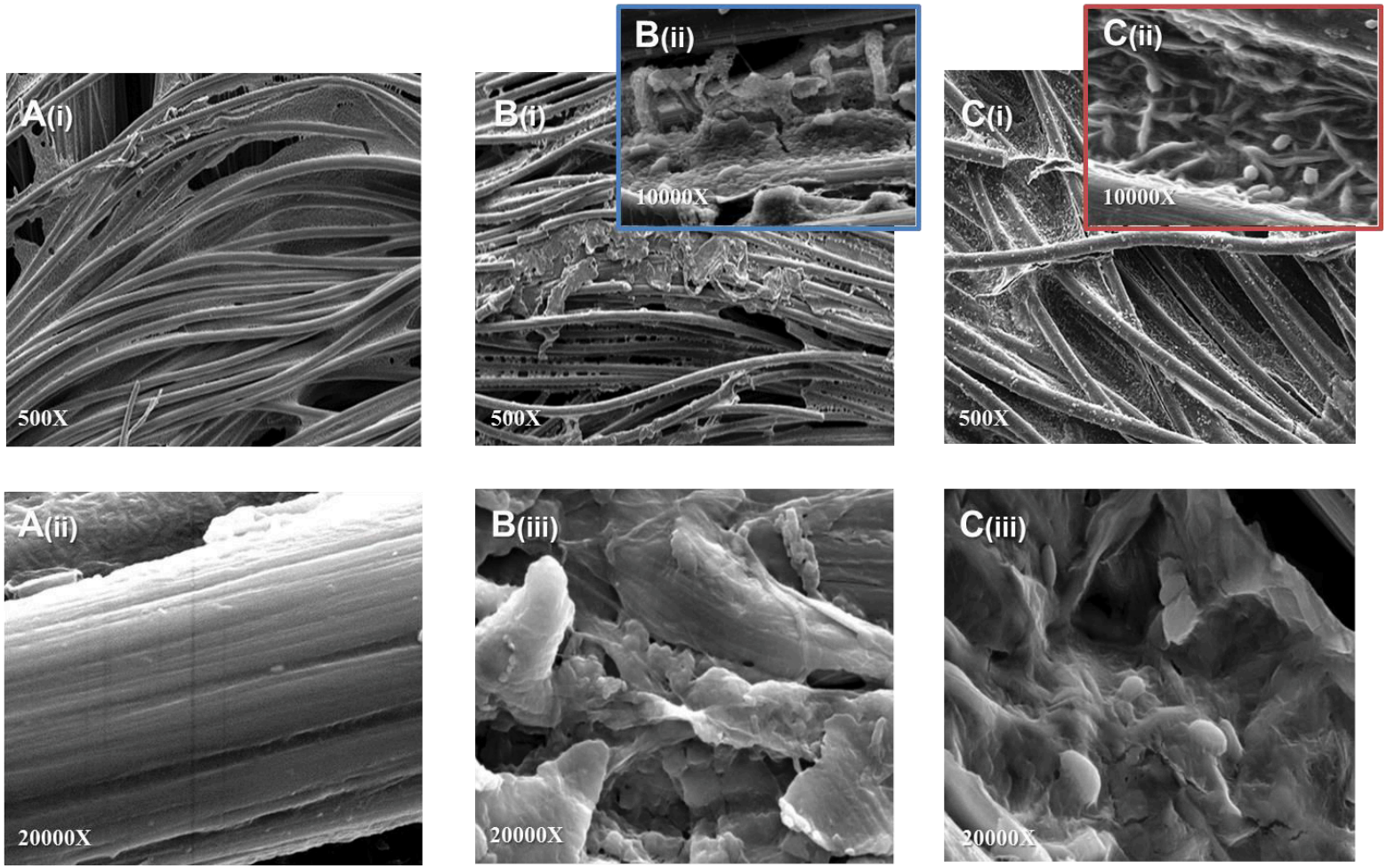

Figure 4.3 XL30 SEM images of **(A)** clean carbon cloth, **(B)** DMC biofilms and **(C)** IMC biofilms attached on the carbon cloth surfaces.

Figure 4.3 (C (i) and (ii)) also shows the formation of flagella-like structures with single rod-shaped bacteria. This bacterium was apparently proved to be *Bacillus* as evidenced by the microbial community analysis in the previous section. The flagella formed by *Bacillus* sp. were very much associated with the biofilm development and acts as an important structure for the bacterial motility and genetic communication among the microbial population in the biofilms [51-52]. Furthermore, the flagella structure allows the bacteria to have motility in the biofilm formation [52], resulting in forming more biofilms on the anode surface, hence improving the direct electron transfer in MFC. Some coccal-shaped bacteria were also spotted on the IMC biofilms which might suggest the presence of *Staphylococcus* (under *Bacillales* order) that appear round or cocci in nature.

The DNA analysis for DMC reveals that the microorganisms in the anode appeared to have a great bacterial diversity, resulting in forming intense multilayer and thicker biofilms. The thicker biofilms could reduce the mass and electron transfers, resulting in decreasing overall performance and electricity generation efficiency of the MFC [53]. Thus, this structure might negatively influence the electrochemical activities of the DMC in the MFC systems.

4.3.3 Electrical Energy Generation and Electrochemical Activities

4.3.3.1 Current Density

Electric output in terms of current density by DMC and IMC inoculated MFC (noted as DMC-MFC and IMC-MFC), was monitored for approximately 120 hours against abiotic MFC as the control experiment (Fig. 4.4 (A)). IMC-MFC yielded higher current output than DMC-MFC for both phenolic and non-phenolic feeding MFC systems. The different current outputs might result from the biochemical reactions catalysed by the microbial consortia of DMC and IMC, and the chemical composition changes, especially when 2,4-DCP was fed in MFC. IMC was derived from wastewater source that composed relatively higher phenolic substances (20-30 mg/L) and COD (600-1000 mg/L) (Table S4.1) than that of DMC. This gives us understanding that the microbial consortia in industrial wastewater have greater capability to adapt themselves in chlorophenol contaminated growth media, thus assisting to generate better current output. The current generation performance by IMC- and DMC-MFCs was comparable with previous studies which

mostly used domestic wastewater based microbial cultures in double chamber bio-electrochemical systems [54].

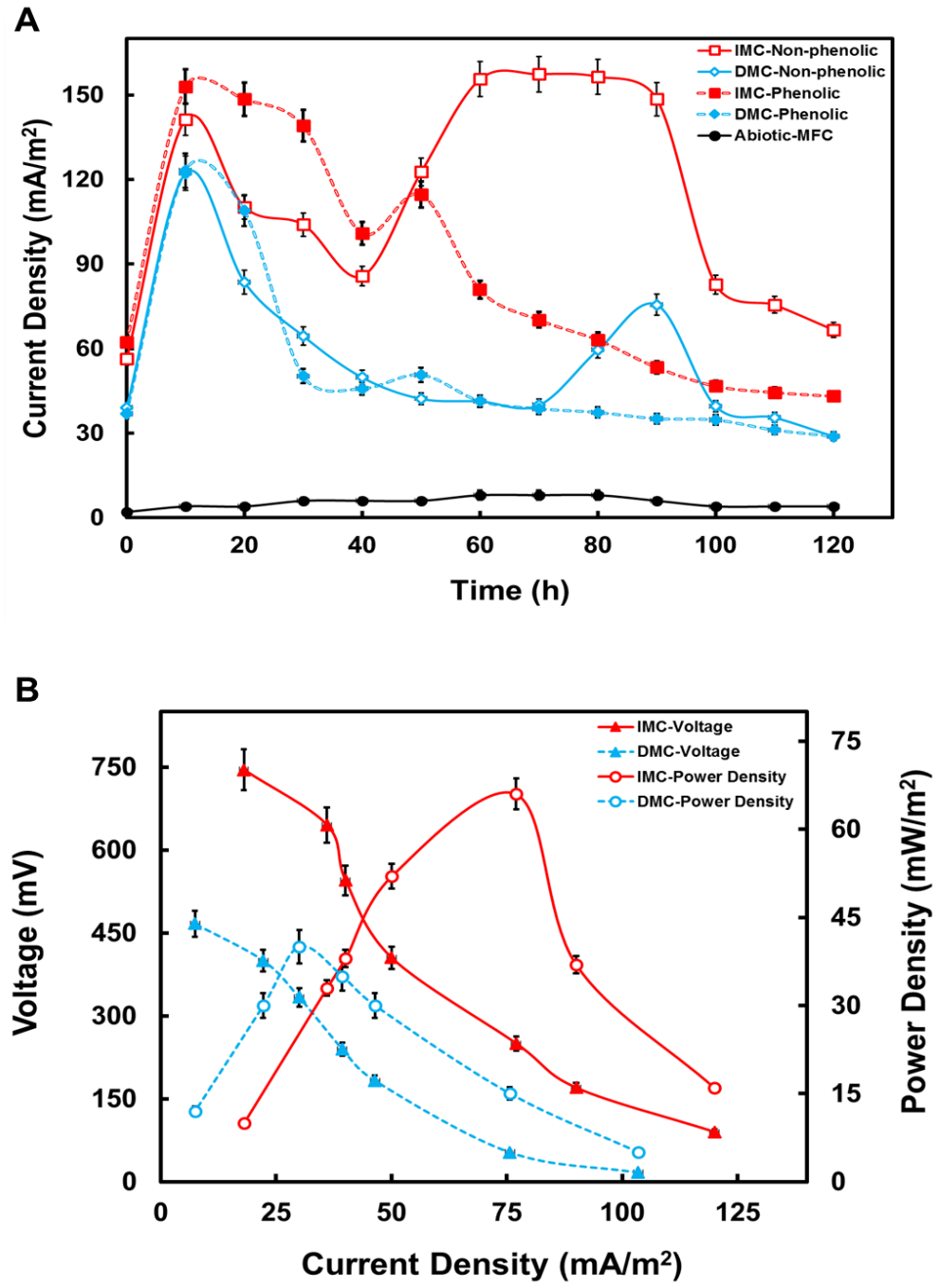

Figure 4.4 (A) Current generation profiles by DMC- and IMC-MFC with non-phenolic and phenolic feeding against abiotic MFC **(B)** Polarization and power curves by DMC- and IMC-MFC (phenolic feeding only).

The current density profiles displayed by all MFC systems appeared to be slightly distinguishable. The current density rose quickly in the first 15 hours. This could be attributed by high level of bacterial activity at this adaptation and nutrient-rich phase of MFC. The current declined quite dramatically afterward in both DMC-MFC and IMC-MFC which might be due to nutrient lacking over prolonged MFC operation. The current density re-escalated after about 40 hours in IMC–MFC (non-phenolic) and remained stable at approximately 156 mA/m^2 over 60 hours. Without additional nutrient to MFC, the current output over a longer operation period could be explained by the endurance of the bacterial consortia in the nutrient limited MFC. It is reported that substrates such as glucose (as in non-phenolic feeding MFC) could stabilize anode microbial activities which maintained the electricity output at this current density level [55]. It is also interesting to note a high current output was measured after 80 hours in the non-phenolic fed DMC-MFC over a much smaller time range, suggesting that there was a lesser sensitivity of glucose-fed compared to that of IMC-MFC.

The current generation profiles demonstrated by both IMC and DMC in phenolic feeding MFC systems appear to correspond well to the bacterial activity inhibition by 2,4-DCP. The current remained constant at a higher current density in IMC-MFC for longer time even after phenolic feeding. This result further reveals that microbial consortia in IMC have high phenol adaption ability to grow in the high phenolic loading media. The DMC-MFC, however, performed a current decline after the 2,4-DPC feeding. This shows that 2,4-DCP at this concentration had more appreciable effect on the DMC microbial growth, thus negatively influence on the overall MFC performance.

4.3.3.2 Polarization Curve and Power Density

The performance of the MFCs with Pt/Ti cathodes is illustrated in Fig. 4.4 (B). The highest closed circuit voltage (CCV) of 745 mV was obtained by IMC-MFC at a corresponding current density of 18.0 mA/m^2, while only 467 mV at 7.4 mA/m^2 were measured in DMC-MFC. The CCV of both DMC- and IMC-MFCs declined over higher current density with their internal resistances of 1 kΩ and 700 Ω, respectively. It is known that the MFC internal resistance is an important factor to determine the efficiency of power recovery from organic matters. A high maximum power density is based on a low internal resistance [56].

Therefore, it can be deduced that the IMC is able to increase the power generation efficiency in MFC with its lower internal resistance than DMC.

Figure 4.4 (B) also shows a typical power density profile of MFC. The power density profiles demonstrated that both IMC and DMC performed similar bio-electrochemical activities with different power strength in the MFCs. The different power densities can be attributed to the different microbial communities enriched in the MFCs during the start-up cultivation periods [30]. The data support the highest power density (66.0 mW/m^2) generated by IMC-MFC with the corresponding current density of 77.0 mA/m^2 as compared to DMC-MFC. The lower power density obtained by DMC-MFC could be due to the existing complex and competitive environment by the electrochemically and non-electrochemically active bacteria in the biofilms where the electron transfer and substrate consumption occurred simultaneously. The highly packed microbial structures of DMC could also hinder the mass transfer close to the anode surface and reduce the proton transfer efficiency to the membrane, thus increasing the internal resistance [6]. The figure displays the tremendous drop of power density by both mixed consortia over extended current density, which was possibly owing to the tolerance level in the toxicity environment containing 2,4-DCP in the medium.

4.3.3.3 Cyclic and Linear Sweep Voltammetries

The extracellular electron transfer mechanism depends strongly on the redox activities in the MFC system which can be achieved by conductivity within biofilm and electrodes. Since the biofilm conductivity itself is not enough to transfer electrons fluently to/from the electrodes [54], this study used conductive nanostructured Pt/Ti cathode catalysts to assist the electron transfer by both microbial cultures.

The redox activities in the DMC-MFC and IMC-MFC were analyzed through measuring the cyclic voltammetry (CV) at a scan rate of 0.1 V/s (Fig. 4.5 (A)). The CV results show that DMC-MFC achieved lower redox activities by generating lower oxidation current at approximately 1.16 mA, 0.5 V (vs. Ag/AgCl) compared to that of IMC-MFC (2.35 mA, 0.5 V (vs. Ag/AgCl)). The size of CV cycle in the IMC-MFC demonstrated higher electrochemical activities in the MFC [57]. However, there were no apparent oxidation-reduction peaks in the IMC-MFC at which similar CV profiles obtained by

Yamashita et al. [7], indicating the importance of biofilms responsible for the electrochemically catalytic activities [58]. In addition, the cathode catalyst normally offset the presence of mediators in the MFC which is associated with clear and outward redox peaks in CV tests. That is why there is lack of oxidation-reduction peaks of CV tests in both DMC- and IMC-MFC, even both of them generated significant and comparably higher voltages. This phenomenon could be supported by a study conducted by Wang et al. [59] in which the Pt-cathode catalyst showed no catalytic activity in the absence of oxygen during CV test in a double chamber MFC.

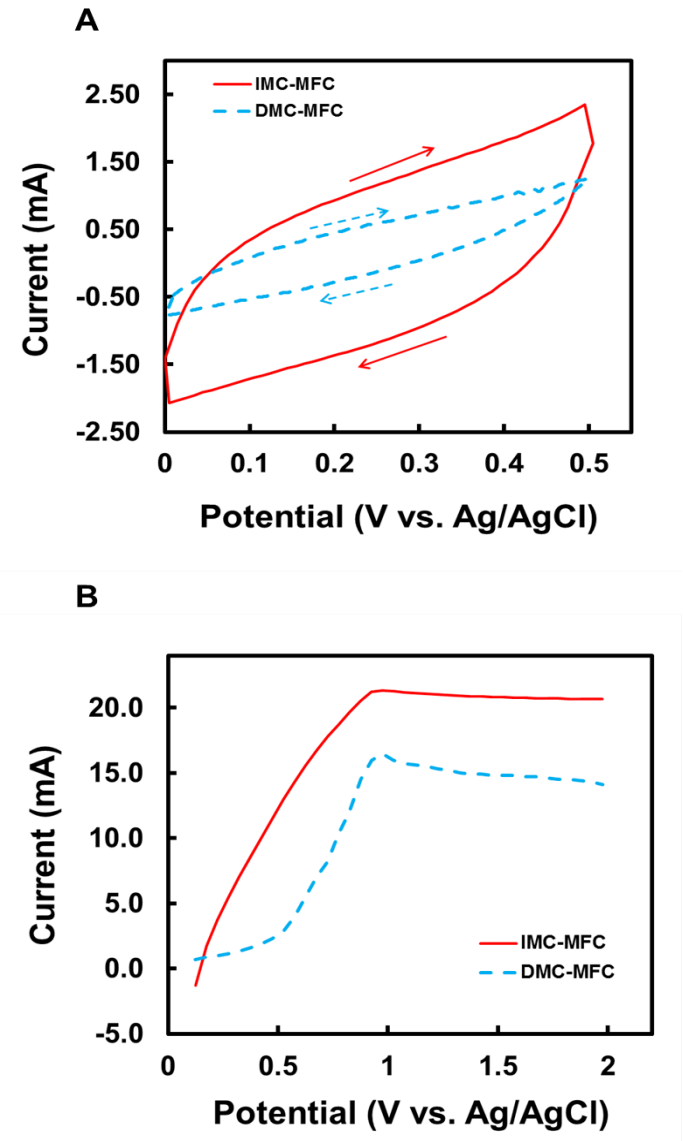

Figure 4.5 (A) Cyclic voltammetry (CV) and **(B)** linear sweep voltammetry (LSV) curves for DMC- and IMC-MFC (phenolic feeding only).

To obtain more insight into the electrochemical activities of the cathode in the MFC, linear sweep voltammetry (LSV) at a scan rate of 0.1 V/s was performed. LSV profile is displayed in Fig. 4.5 (B). The analogous LSV profiles show that both mixed consortia performed similar electrochemically catalytic activities in the MFC systems. Nevertheless, the DMC showed a fairly gradual current increase at a potential range of 0 – 0.5 V to reach the peak. This result is consistent to convince that the thick and unrefined biofilm in DMC-MFC resulted in low current generation rate and long reaction time required to reach a peak value as suggested by Choi and Choi [53]. The cathodic catalytic data of LSV also indicates that the biological oxidation by DMC-MFC occurred at a lower current-potential value of approximately 2.90 mA, 0.53 V, while a higher value was achieved by IMC-MFC (5.40 mA, 0.28 V). This again proves that the IMC was more capable in utilizing the organic compounds at the starting period and efficiently transferring the electrons in the MFC system. Furthermore, the peak current in the IMC-MFC at 21.0 mA with 0.93 V was slightly higher than that in DMC-MFC (16.0 mA, 0.94 V). This is because the internal resistance in the IMC-MFC was lower thus allowing it to reach higher peak of current generation.

4.3.4 Chlorophenol Degradation

The 2,4-DCP degradation performance of both DMC- and IMC-MFC was assessed in comparison with biodegradation ability of DMC and IMC in anaerobic tests (denoted as only DMC and IMC). Degradation profiles for the four bench scale experiments are shown in Fig. 4.6. Although the anaerobic bioprocess is generally considered rate limiting in the biodegradation of phenols [60], the positive selection of microbial consortia especially in MFC systems could improve the phenolic degradation. DMC-MFC showed the highest 2,4-DCP degradation with 62% followed by IMC-MFC (41%), DMC (30%) and IMC (20%) which unanimously occurred after 108 hours. This results strongly support that the MFC system efficiently improve the degradation of 2,4-DCP. The effective 2,4-DCP biodegradation by DMC might be due to synergistic support of bacterial dominants especially *Cloacibacterium* (refer to subsection 4.3.1.4.) which have positive responses towards the compound. Furthermore, the presence of diverse microbial species in the DMC could in-turn contribute to the higher phenolic degradation capabilities as compared to homogenous microbial community in the IMC. The phenolic degradation capability of our

DMC-MFC is more promising compared with these reported studies in which contaminated groundwater was used to remove phenols in dual chamber MFC with lower degradation rate [60].

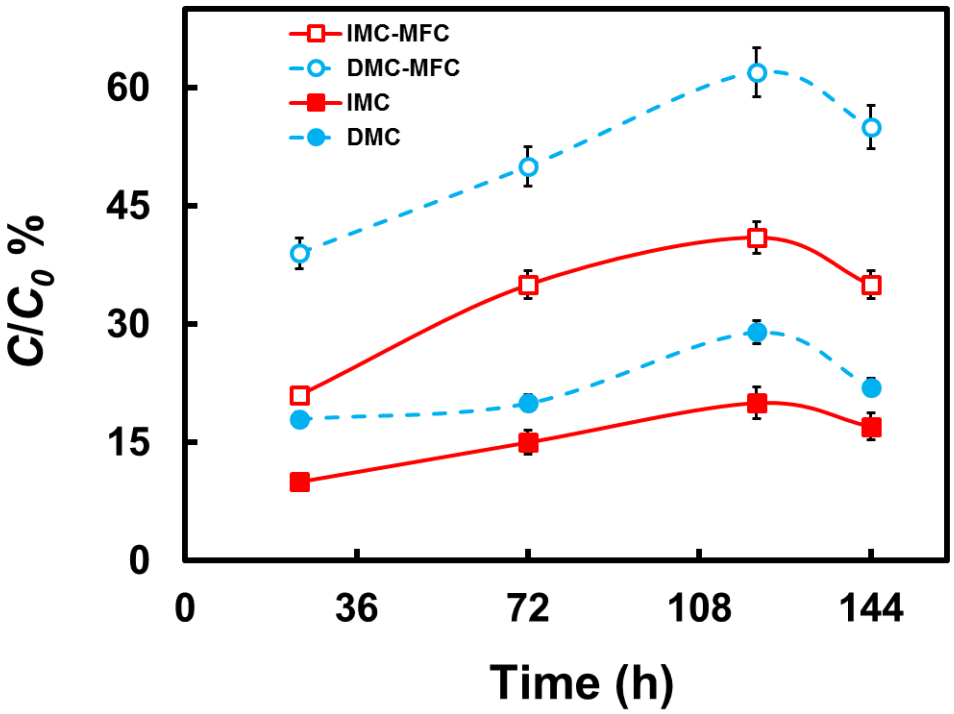

Figure 4.6 Degradation profiles of 2,4-DCP by IMC- and DMC-MFC against conventional anaerobic system (denoted as IMC and DMC).

Our results revealed that the electrochemical reactions in MFC can enhance the oxidation mechanisms which are beneficial for the phenol biodegradation. It was reported that the consortial biofilms have positive influence towards the target organic substances such as phenol compounds and are increasingly viable nearer the electrode [61], which may improve substrate capture and degradation. In the conventional biodegradation system, on the other hand, the electron transfer is improvised to make organic loading to be elevated, thus slowing down the phenolic degradation rate by the bacteria as demonstrated by the biodegradation trials inoculated with DMC and IMC in Fig. 4.6.

The metabolites of 2,4-DCP degradation were also examined using HPLC as shown in Fig. 4.7. It is interesting to note that there was slight difference in metabolites produced by the MFC systems against conventional biodegradation trials. 3,5-dichlorocatechol was detected in both DMC- and IMC-MFC systems. DMC-MFC shows a remarkably high degradation ability, resulting in a complete 3,5-dichlorocatechol removal during the final degradation experiment. Benzoic acid was detected in DMC-MFC but not in IMC-MFC. 4-chlorophenol and phenol were detected in all trials and were significantly removed by all four microbial systems.

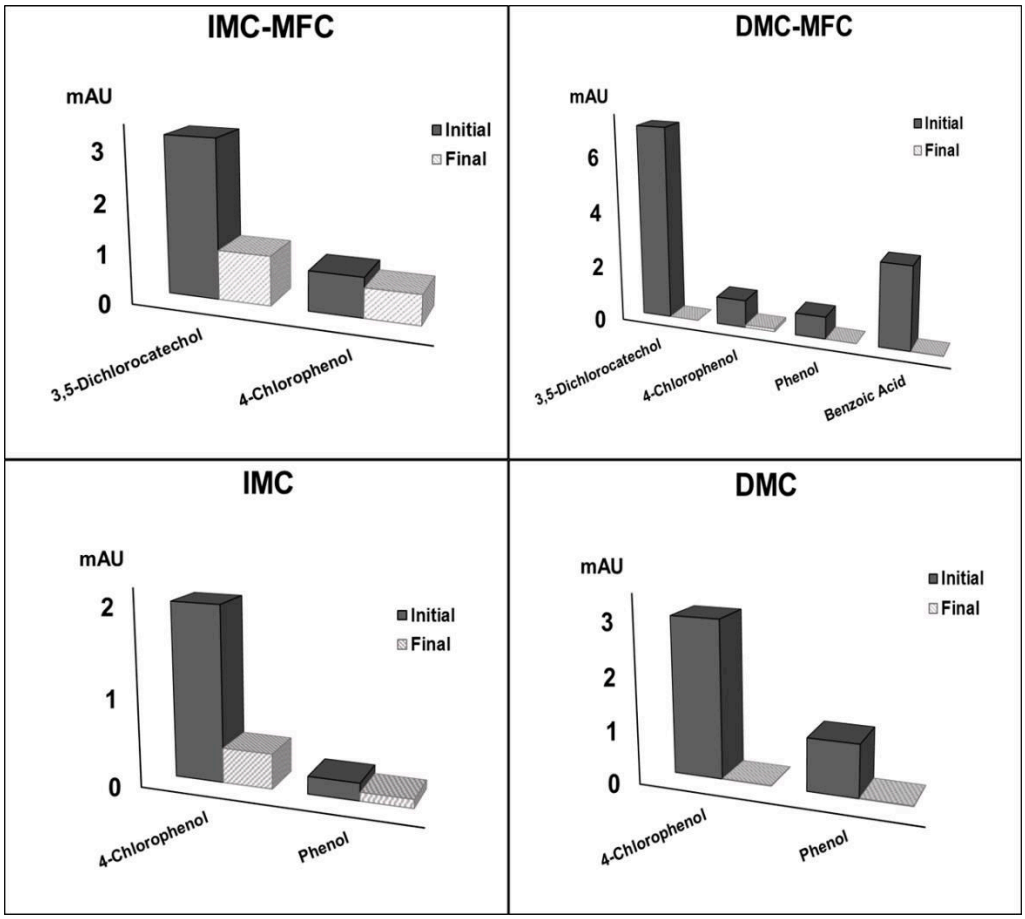

Figure 4.7 Metabolites of 2,4-DCP degradation by IMC- and DMC-MFC against conventional anaerobic system (denoted as IMC and DMC).

Among these degradation experiments, DMC in both MFC and anaerobic systems again showed better performance by almost completely removing all the metabolites during the final day of operation. On the other hands, the IMC in spite of its excellent performance in generating electricity, could degrade lesser amount of 2,4-DCP in the MFC. The metabolites produced by IMC in both MFC and anaerobic test could still be observed at the end of MFC operation.

The metabolites produced by all systems agree with the results reported by Arora and Bae [22], suggesting that our MFC system was not strictly anaerobic with the presence of 3,5-dichlorocatechol metabolite. The anaerobic trials using DMC and IMC were able to degrade the 2,4-DCP in an anaerobic pathways through reductive dehalogenation process where only 4-chlorophenol and phenol were detected as metabolites [22]. From the experimental and analytical data, it could be deduced that the *Cloacibacterium* with other dominants in DMC play a significant role in 2,4-DCP degradation in MFC compared to *Bacillus* in IMC-MFC, although it performed a higher current generation rate than that of DMC.

The performance of the MFC in the current study was compared with other related studies as summarized in Table 4.1 for phenolic based MFC operation and Table 4.2 for electric output comparison. This result was found comparable with previous studies which could be preferable due to its low-profile and simple design and operation. Furthermore, the comprehension of the microorganisms which have potential electrogenicity with positive response towards chlorophenol degradation can be simply derived from our study.

Table 4.1 Comparison of DMC- and IMC-MFCs performance with previous related studies using phenolic substrate in MFC.

Phenolic compounds / concentration	Substrates / inoculum	Bacterial community identification on phenolic	MFC setup	Length of degradation experiment	Maximum current density / voltage	Maximum phenolic degradation / rate	References
Chlorophenols / 0.08 mM	Anaerobic digester sludge	- *Variovorax paradoxus* - *Comamonas* sp.	- Tubular two-chamber - Graphite fiber and felt electrodes - 43-mL working anode volume - Aerobic and anaerobic biocathode	36 hours	300 mV	0.15 mol/m^3/d (~ 75%)	[62]
Phenol / 0.213 mM	Contaminated groundwater	Not discussed	- H-type dual chamber using - 250 mL Schott bottles - Carbon cloth electrodes - Nafion 117 PEM	> 50 days	90 mV	54 %	[60]
Pentachlorophenol / 0.056 mM	Domestic wastewater (primary sedimentation tank)	- *Gammaproteobacteria* - *Alphaproteobacteria* - *Mycobacterium* - *Pseudomonas*	- Two chamber - Graphite felt electrodes - 100-mL working volume - CEM (CMI-7000)	96 hours	~7 A/m^3	0.16 mg/L/h	[43]
2,4-Dichlorophenol / 0.061 mM	Domestic wastewater (sludge from clarifier tank)	- *Cloacibacterium* - *Arcobacter*	- Double chamber - Carbon cloth anode - 200-mL working volume - CEM (CMI-7000)	132 hours	123 mA/m^2	62%	This study
	Petrochemical wastewater	*Bacillus* sp.			156 mA/m^2	41%	

Table 4.2 Comparison of DMC- and IMC-MFCs performance in terms of energy output and COD removal efficiency with related MFC studies.

Substrates / inoculum	MFC design	Power generation	Coulombic efficiency (CE)	COD or substrate removal	References
Activated sewage sludge / human urine	- single-chamber air-breathing MFCs - carbon fibre veil sheets anode and cathode - replenish substrates with continuous operation - CEM (CMI-7000) - 6.25 mL working volume	4.5 mW/m^2	N/A	53%	[5]
Cultures from compost	- Vertical up-flow two-chamber MFCs with fed-batch operation - Plain graphite electrodes - CEM (CMI-7000) - Working volume of anode (500 mL); cathode (300 mL)	372 mW/m^2	21%	N/A	[6]
Rice straw hydrolysate	- Two chamber - Graphite felt electrodes - 100-mL working volume - CEM (CMI-7000)	293 mW/m^2	17.9%	72%	[63]
Activated sludge	- Flame oxidation air-cathode MFC - Stainless steel anodes and carbon cloth electrode with Pt catalyst - 125 mL reactor volume	1063 mW/m^2	21%	N/A	[7]
Domestic wastewater (sludge from clarifier tank)	- Double chamber - Carbon cloth anode - 200-mL working volume - CEM (CMI-7000)	66 mW/m^2	N/A	62%	This study
Petrochemical wastewater		37 mW/m^2		41%	

4.4 Conclusions

Domestic and petrochemical industrial microbial consortia have been evaluated in terms of microbial community and bioelectrochemical activities associated with degradation of 2,4-dichlorophenol in double chamber MFC systems. Industrial microbial consortium (IMC) were found to synergistically improve the MFC electrical energy outputs with relatively lower performance in phenolic biodegradation. Conversely, the domestic microbial consortium (DMC) with *Arcobacter* dominant performed more effective phenolic biodegradation with relatively lower energy output. *Cloacibacterium* appeared to be significant towards 2,4-DCP biodegradation in the DMC-MFC compared to *Bacillus* in its IMC-MFC system. This study offered valuable microbiological and electrochemical data for biodegradation of phenolic contaminants while producing electricity in the MFC. Future bacterial genetic study is essential to analyse the capability of *Cloacibacterium* in degrading 2,4-DCP and its role in electron transfer mechanisms for improving the MFC performance.

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Supplementary Data

Table S4.1 Domestic and industrial wastewater composition.

Parameters	Domestic (DMC)	Industrial (IMC)
pH	7.50 ± 0.2	6.70 ± 0.2
TSS (mg/L)	80 – 100	400 – 600
COD (mg/L)	100 - 200	600 – 1000
Total Phenolic (mg/L)	5 – 10	20 – 30

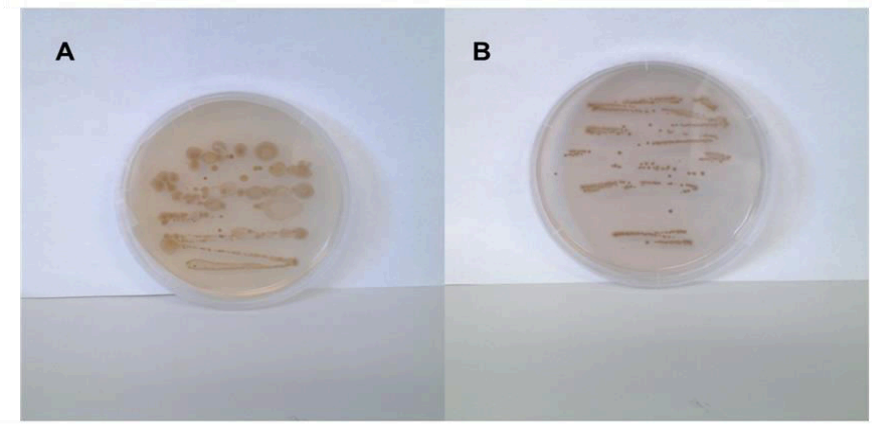

Figure S4.2 (A) DMC **(B)** IMC on agar plates.

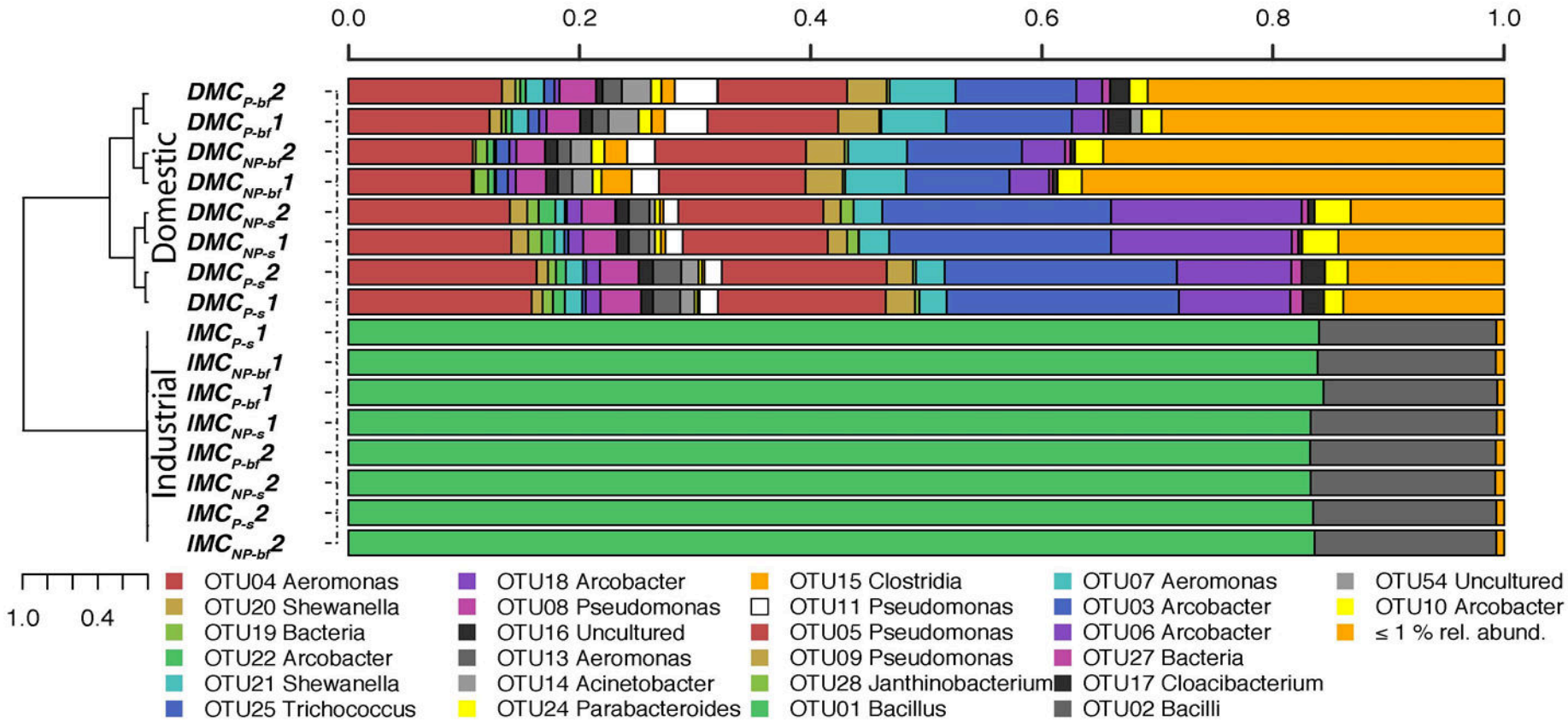

Figure S4.1 Stacked bar plot and hierarchical clustering (UPGMA algorithm) results between samples in 97% identity operational taxonomic unit (OTU) composition. OTUs with less than 1.0 % relative participation are considered as rare OTUs.

Table S4.2 Sequence distribution among samples and alpha diversity indices.

Sample	seq# used	Good's Coverage	Gini-Simpson 1-λ	Simpson 1/λ	Fisher's α	Effective number of OTUs at q*	Observed S	Chao 1	Chao 1 se	ACE	ACE se
H1_B097	39260	0.997	0.956	22.5	173.4	226.4	941.0	993.7	13.0	1000.2	15.0
H2_B098	24336	0.994	0.952	21.0	158.3	197.0	798.0	879.4	18.0	887.6	14.0
H3_B099	29987	0.996	0.950	20.0	153.2	177.7	809.0	880.0	16.4	886.8	13.9
H4_B100	7256	0.972	0.949	19.5	141.5	147.6	560.0	738.5	32.8	784.6	15.1
H5_B101	39326	0.997	0.898	9.8	93.3	68.0	564.0	628.8	16.1	636.4	11.6
H6_B102	14919	0.990	0.894	9.4	78.2	55.8	411.0	574.8	35.2	574.2	12.7
H7_B103	17678	0.992	0.899	9.9	78.8	62.0	427.0	548.9	27.9	553.0	11.8
H8_B104	20130	0.993	0.898	9.8	78.8	60.7	437.0	554.0	26.0	574.6	12.2
H9_B105	93837	1.000	0.273	1.4	2.4	1.9	25.0	25.3	0.9	26.0	2.5
H10_B106	146112	1.000	0.276	1.4	2.2	1.9	24.0	27.0	4.6	25.2	2.4
H11_B107	97142	1.000	0.265	1.4	2.1	1.9	23.0	23.0	0.2	23.3	2.4
H12_B108	93420	1.000	0.282	1.4	2.5	1.9	26.0	26.0	0.1	26.6	2.6
H13_B109	112682	1.000	0.281	1.4	1.9	1.9	21.0	21.0	0.0	21.0	2.3
H14_B110	84454	1.000	0.281	1.4	3.5	2.0	35.0	39.2	4.3	40.3	3.2
H15_B111	56899	1.000	0.271	1.4	1.6	1.8	17.0	18.0	2.3	17.9	2.1
H16_B112	156503	1.000	0.278	1.4	1.7	1.9	19.0	19.0	0.0	19.0	2.0

Note: The sequences analyzed in this study are publicly available in the sequence read archive (SRA) of NCBI under the sequence accession numbers **SRR4302501-16**.

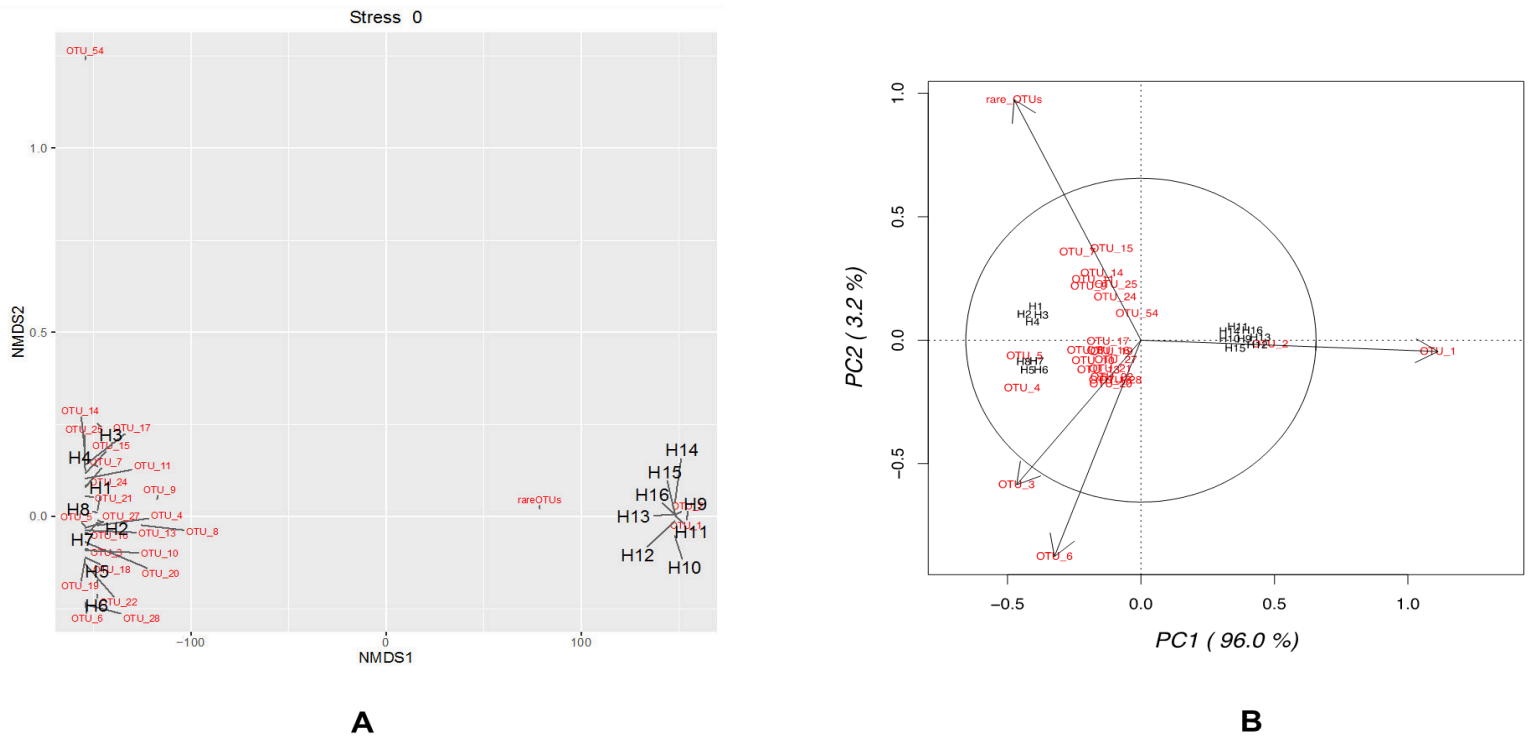

Figure S4.3 (A) Non-metric multi-dimensional scaling (nMDS - using the Bray-Curtis dissimilarities) biplot of OTU and sample scores with a stress value of nearly 0 (an upper limit of 200 iterations until solution was set). Name-tag de-convolution was carried out for avoiding text overlap and is indicated by the grey lines; **(B)** Principal components analysis (PCA) biplot on the Hellinger transformed OTU values as previously suggested for reducing PCA biases in ecological data (Legendre and Gallagher, 2001). OTUs with the highest impact on the observed sample scores are indicated by the arrows and are residing outside the circle (Kindt and Coe, 2005). Partial name-tag de-convolution was carried out here as well.

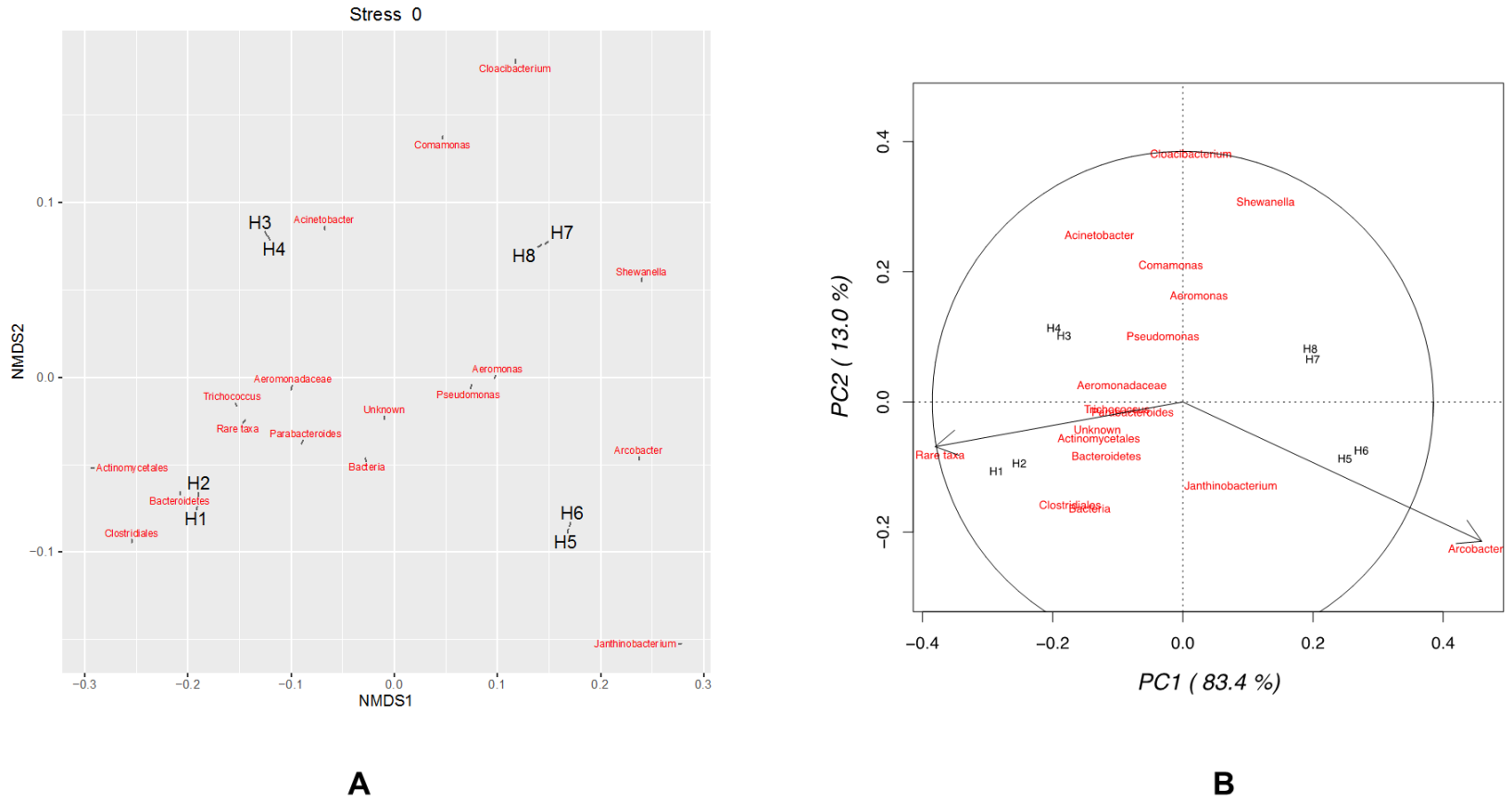

Figure S4.4 (A) nMDS biplot of taxa and sample scores with a stress value of nearly 0 performed using the same parameters as in Fig. S4.3 (A); **(B)** PCA based on taxon compositions of samples performed according to the parameters and transformations described in Fig. A4.3 (B).

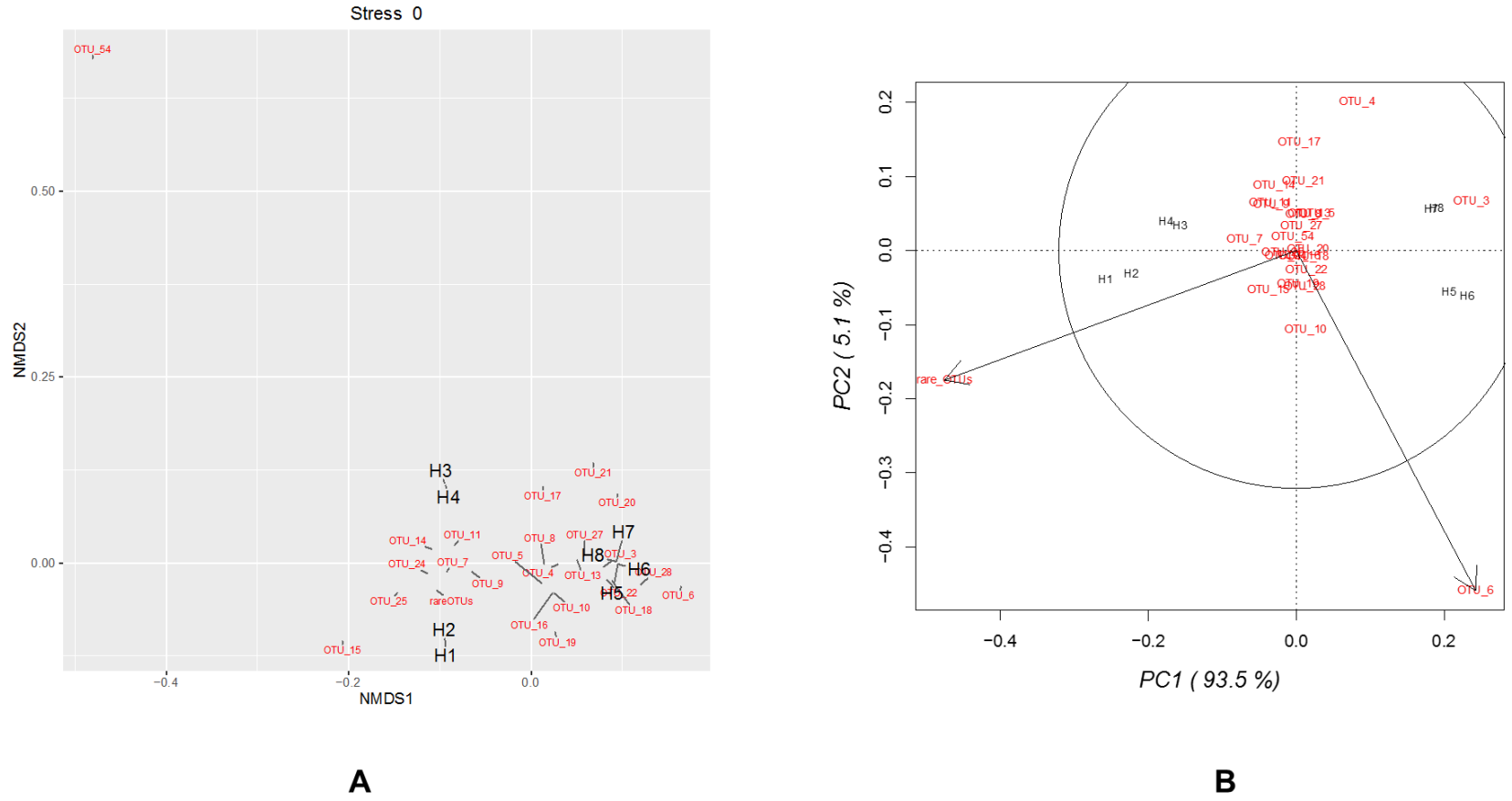

Figure S4.5 (A) nMDS performed and plotted as described in Fig. S4.3 (A) only for samples DMC; **(B)** PCA performed as described in Fig. S4.3 (B) only for sample DMC.

Table S4.3 Classifications based on OTUs for all samples of DMC and IMC.

OTU	Domain	Phylum	Class	Order	Family	Genus
OTU_1	*Bacteria*	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae 1*	*Bacillus*
OTU_2	*Bacteria*	*Firmicutes*	*Bacilli*	*Bacillales*	*?*	*?*
OTU_3	*Bacteria*	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*
OTU_4	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*
OTU_5	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
OTU_6	*Bacteria*	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*
OTU_7	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*
OTU_8	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
OTU_9	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
OTU_10	*Bacteria*	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*
OTU_11	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
OTU_12	*Bacteria*	*Firmicutes*	*Bacilli*	*Bacillales*	*?*	*?*
OTU_13	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*
OTU_14	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
OTU_15	*Bacteria*	*Firmicutes*	*Clostridia*	*Clostridiales*	*?*	*?*
OTU_16	*?*	*?*	*?*	*?*	*?*	*?*
OTU_17	*Bacteria*	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Cloacibacterium*
OTU_18	*Bacteria*	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*
OTU_19	*Bacteria*	*?*	*?*	*?*	*?*	*?*
OTU_20	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Shewanellaceae*	*Shewanella*
OTU_21	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Shewanellaceae*	*Shewanella*
OTU_22	*Bacteria*	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*
OTU_23	*?*	*?*	*?*	*?*	*?*	*?*
OTU_24	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Parabacteroides*
OTU_25	*Bacteria*	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Trichococcus*
OTU_26	*Bacteria*	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*
OTU_27	*Bacteria*	*?*	*?*	*?*	*?*	*?*
OTU_28	*Bacteria*	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Janthinobacterium*
OTU_29	*Bacteria*	*?*	*?*	*?*	*?*	*?*
OTU_30	*Bacteria*	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*?*	*?*
OTU_31	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*?*
OTU_32	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Alteromonadales*	*Shewanellaceae*	*Shewanella*
OTU_33	*Bacteria*	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*
OTU_34	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Enterobacter*
OTU_35	*Bacteria*	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Thauera*
OTU_36	*Bacteria*	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Chryseobacterium*
OTU_37	*Bacteria*	*Bacteroidetes*	*Sphingobacteriia*	*Sphingobacteriales*	*?*	*?*
OTU_38	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*?*
OTU_39	*Bacteria*	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Simplicispira*
OTU_40	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*?*
OTU_41	*Bacteria*	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae 1*	*Bacillus*
OTU_42	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Raoultella*
OTU_43	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Salmonella*
OTU_44	*Bacteria*	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*
OTU_45	*Bacteria*	*Bacteroidetes*	*?*	*?*	*?*	*?*
OTU_46	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
OTU_47	*Bacteria*	*?*	*?*	*?*	*?*	*?*
OTU_48	*Bacteria*	*Chloroflexi*	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	*?*
OTU_49	*Bacteria*	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*
OTU_50	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
OTU_51	*?*	*?*	*?*	*?*	*?*	*?*
OTU_52	*Bacteria*	*Candidatus Saccharibacteria*	*Saccharibacteria_genera_incertae_sedis*	*Saccharibacteria_genera_incertae_sedis*	*Saccharibacteria_genera_incertae_sedis*	*Saccharibacteria_genera_incertae_sedis*
OTU_53	*?*	*?*	*?*	*?*	*?*	*?*
OTU_54	*?*	*?*	*?*	*?*	*?*	*?*

CHAPTER 5

Statement of Authorship

Title of Paper	Biochemical kinetics and pathways of 2,4-dichlorophenol biodegradation by domestic and industrial microbial consortia in microbial fuel cell system
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Huzairy Hassan
Contribution to the Paper	Designing and conducting the experiments, data analysis, and manuscript writing.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 19/01/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Bo Jin
Contribution to the Paper	Supervising the development of the work, data interpretation, manuscript review and correction.
Signature	Date 19/01/2017

Name of Co-Author	Sheng Dai
Contribution to the Paper	Helping in experimental design and manuscript review.
Signature	Date 19/01/2017

Please cut and paste additional co-author panels here as required.

5. Biochemical Kinetics and Pathways of 2,4-Dichlorophenol Biodegradation by Domestic and Industrial Microbial Consortia in Microbial Fuel Cell System

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Chapter 5: Biochemical Kinetics and Pathways of 2,4-Dichlorophenol Biodegradation by Domestic and Industrial Microbial Consortia in Microbial Fuel Cell System

Abstract

Chlorophenol compounds are the significant industrial pollutants which have raised considerable environmental concerns and have attracted ongoing research interests in seeking cost-effective treatment processes. Microbial fuel cell (MFC) could be a promising sustainable technology for degradation of chlorophenols, while generating electricity. However, there is lack of detailed studies on biodegradation kinetics and pathways in MFC system. This study was to get better understanding of the bioelectrochemical reaction kinetics, to propose biodegradation pathways, and to determine degradation products and their toxicology from MFC-driven degradation of 2,4-dichlorophenol (2,4-DCP). The biodegradation kinetics were studied in MFC systems using two bacterial consortia derived from industrial petrochemical and domestic wastewater, noted as iMFC and dMFC system, respectively. The experimental data were evaluated by Haldane, Michaelis-Menten and Hanes-Woolf models to determine bacterial growth and degradation kinetic parameters. Over 60% 2,4-DCP degradation was achieved by dMFC compared to iMFC (40%). The dMFC showed better kinetic results with growth constant of 27 mg/L and specific biodegradation rate of 0.32 mg/L/h. The iMFC performed excellent microbial growth profile at low 2,4-DCP concentration and higher electrical outputs. The final metabolites of 3-oxoadipate and acetate produced by iMFC and dMFC, respectively, signified their capabilities to degrade 2,4-DCP into much less hazardous compounds. This result has provided new insight for future study on biodegradation kinetics and relationship with bioelectrochemical activities employed in MFC system.

Keywords: Kinetics; Microbial fuel cell; 2,4-Dichlorophenol; Biodegradation pathways.

5.1 Introduction

Biodegradation process is one of the most preferable methods for removing hazardous pollutants in wastewater owing to its environmental-friendly and simple operations using various safe microorganisms. Over the past several decades, researchers intensify their efforts to employ biodegradation principles for removing recalcitrant pollutants including toxic organic and inorganic compounds, and complex chemicals. Until recent years, the research directions of biodegradation study have been shifted to using advanced technologies such as microbial fuel cell (MFC) system. However, there is still lack of detailed studies on elucidating the biodegradation kinetics in the MFC system. For instance, chlorophenols could be demineralized and biodegraded in the MFC in a few case studies [1 – 3]. These studies are important in supporting the roles of biodegradation in the MFC. However, further fundamental information is needed to clarify the specific microbial community and biodegradation kinetics which could contribute to the overall performance of biodegradation in MFC systems for industrial applications.

Biodegradation process is mainly carried out by various bacterial cultures especially for removing complex and hazardous compounds such as chlorophenols. The main factor driving this investigation is due to recalcitrant biochemical properties of chlorophenols including acute toxicity, bioaccumulation as their lipophilicity [4], strong odour, and suspected carcinogenicity and mutagenicity [5]. Chlorophenols enter the environment primarily through the effluents of various industries, including petrochemical, oil refining, tannery, pulp and paper mills [6]. Enormous studies have been published to explore wild microorganisms, microbial consortia and biosystems for degradation of phenol and other toxic organic contaminants in wastewater. Biodegradation of organic pollutants using bioelectrochemical systems such as MFC has drawn attention in recent years. Martínková et al. [7] reported the biodegradation of phenolic compounds by *Basidiomycota* using oxidase enzymes. Moussavi et al. [5] studied the biodegradation of 2-chlorophenol in a granular anoxic baffled reactor. Kong et al. [8] and Wen et al. [9] evaluated the dechlorination of 4-chlorophenol in bioelectrochemical systems.

Pure cultures *Pseudomonas* [10, 11] and *Bacillus* [12], as well as mixed bacteria consortia derived from wastewaters [13] were used in a few of biodegradation studies on removing phenols. The maximum specific growth rates, μ_{max} was reported to be in a range

of 0.036 – 0.385 h^{-1} depending on strains and culture conditions [14]. Phenol is normally recognized as an inhibitory substrate. Thus, Haldane equation can be used to describe the relationship between the growth and substrate-inhibition paradigms.

However, the biodegradation kinetics and mechanisms of chlorophenols in the MFC systems may be different from those in the conventional biodegradation system. The biodegradation in the MFC may proceed through different pathways in the sense that the bacterial cultures consume the substrate through complex bioelectrochemical reactions. In other words, the electron transfer activities and substrate consumption occur simultaneously. Therefore, it is interesting to investigate the microbial population and bioelectrochemical kinetics which are involved in the biodegradation process through the specific pathways with simultaneous ability to transfer electrons to the electrodes in MFC. Till now, there seem no reported studies showing biodegradation kinetics and pathways associated with phenol degradation using MFC system.

This study utilized two microbial consortia derived from industrial and domestic wastewaters to degrade 2,4-dichlorophenol (2,4-DCP) in a double chambered MFC system. The industrial wastewater was collected from petrochemical refinery plant in which the phenolic contamination level was expected to be high. The Pt/Ti cathode catalyst was used to investigate the dynamic behaviour of microbial communities, and biodegradation kinetics and pathways for better understanding of the overall bioelectrochemical reactions in the MFC system. The proposed pathways showed useful information for understanding the microbial abilities in degrading the complex and recalcitrant 2,4-DCP into much less toxic compounds. This study also provides mainstays in determining the kinetics and rate of degradation of toxic pollutants by selected microbial strains in MFC systems.

5.2 Materials and Methods

5.2.1 Chemicals and Media

The cultivation medium for bacterial growth consisted of 5.0% glucose in mineral salts (pH 7.0 ± 0.2) [0.386 g/L $(NH_4)_2SO_4$, 0.149 g/L K_2SO_4, 3.31 g/L NaH_2PO_4, 10.31 g/L Na_2HPO_4, 1 g/L NaCl, 0.2 g/L $MgSO_4$ and 12.5 mg/L vitamin]. The medium was sterilized at 121 ºC for 15 min. The glucose solution was sterilized using 0.22 μm filter unit (Millipore membrane PVDF) and then added to the autoclaved medium. 2,4-dichlorophenol (2,4-DCP) (99% purity; Alfa Aesar) with various concentrations (5 – 50 mg/L) was fed into the growth medium in order to study the chlorophenol biodegradation and kinetics in the MFC system. To investigate the capability of the bacterial culture in biodegrading the chlorophenol, 2,4-DCP were fed to the MFC anode cultivation in one-off basis. Catholyte used in this study was 50 mM phosphate buffer at pH 7.0 ± 0.2.

5.2.2 Microbial Inoculum and DNA Identification

5.2.2.1 Microbial Culture and Inoculum Preparation

Two microbial consortia were utilized in this study namely, domestic and industrial bacterial consortia. Domestic bacterial consortium was derived from domestic wastewater sample collected from Glenelg Wastewater Treatment Plant, South Australia. Industrial bacterial consortium was derived from industrial petrochemical wastewater sample collected from Australia Mobil Oil Plant, Birkenhead Terminal, South Australia. Both wastewater samples were collected from clarifier tanks in the form of mixed sludge. Domestic sample was directly used as inoculum, while the oil layer from industrial sample was first separated using separatory funnel and the remaining sludge was used as inoculum for the enrichment procedure.

The two consortia (50 mL) were enriched with the abovementioned medium fed with 2,4-DCP over 2 days in MFC systems. The biofilms developed on the MFC anode surface were inoculated using streak-plate method onto the pre-prepared nutrient agar in sterile petri dish consisting of sufficient proportions of peptone, yeast extract, sodium chloride and agar. The inoculated agar was incubated at 37 ºC for 24 hours. The subculture were repeated three times under the same conditions. These subcultures from biofilms of

microbial consortia were used to produce seed culture for further MFC biodegradation experiment.

Two to three streaked bacterial loops from the sub-cultured nutrient agar were inoculated and grown in a 250 mL of nutrient broth in a 500-mL shake flask for 48 hours on an incubator shaker (175 rpm) at 37 ºC. 10-mL sample was taken every three hours; 5 mL from this sample volume was checked for the optical density (OD) using UV-Vis Spectrophotometer (Shimadzu, UV-1601) at 660 nm wavelength. The other 5 mL sample was used to determine cell dry weight (CDW, mg/L) of the bacterial cultures. The bacteria sample was filtered using pre-weighed filter paper (Whatman 110 mm) with the application of vacuum pump. The bacteria left on the filter paper were dried at 80 ºC for 24 hours. The dried filter paper was incubated in a desiccator for 24 hours prior to weighing. The CDW was determined using Eq. 5.1:

$$CDW = \frac{W_f - W_i}{V} \tag{5.1}$$

where W_f and W_i were the final and initial weight of filter paper (g) and V is the volume of sample (5 mL). The OD and CDW experiments were carried out in triplicate for the two bacterial cultures. The highest growth rate was determined from the OD and CDW data and become the basic start-up period for further kinetic experiments using MFC.

5.2.2.2 Microbial Community Identification

The microbial community of both microbial consortia that were extracted from the MFC-enriched biofilms on the anode electrode surface were analysed. The extraction of genomic DNA, amplification using polymerase chain reaction (PCR), multiplexed, sequencing and bioinformatics data analysis were carried out as previously described [15]. The final per-sample sequence numbers were sufficient for screening the vast majority of the existing diversity as indicated by the Good's coverage estimate [16] values which translate into the 97.2-100% of the environmental bacterial SSU diversity being uncovered given our methods. The results of the microbial community for both consortia are shown in Table 5.1.

5.2.3 MFC Reactor Setup and Voltage Acquisition

A double chambered MFC reactor was constructed from two glass bottles (250 mL) joined together with a glass bridge, consisting a 30-mm inner diameter cationic exchange membrane (CEM, CMI-7000, gel polystyrene cross linked with divinylbenzene). Anode material was made up of 40 × 30-mm carbon cloth, while the cathode material was made of 40 × 30 × 2-mm platinised titanium (Pt/Ti) catalysts plate. Both electrodes were wired with titanium projecting outside connecting to an external electrical circuit.

The anodic chamber was tightly closed from air penetration as to provide anaerobic condition for bacterial growth and the cathodic chamber was open for aeration. The MFC system was operated in a room temperature, topped on a magnetic plate to ensure well mixing during operation. The period of MFC operation was prolonged over 4 days. After one day of cultivation, the anodic chamber turned turbid, indicating the bacterial growth. The biofilm formation on the electrode surface was observed.

The electrode circuit voltage was continuously monitored by a computer. The anode and cathode were directly connected to a LabJack U6 recorder under open circuit voltage (OCV) connection. The MFC experiments were performed in duplicate.

5.2.4 Analysis of Bacterial Growth and 2,4-Dichlorophenol Biodegradation Kinetics

Effect of initial concentrations of 2,4-DCP (5 - 50 mg/L) on bacterial cell growth and biodegradation kinetics was investigated for both bacterial consortia in MFC systems. 5, 25, and 50 mg/L of 2,4-DCP were defined as low, medium and high concentration, respectively, for the following Result and Discussion section. At a designed time interval, a sample was collected to measure cell growth in term of cell density (CD) using optical density (OD) measurement at 660 nm by a spectrophotometer (Shimadzu, UV-1601). The sample was then centrifuged at 4000 rpm for 10 minutes to obtain cell-free solution, each of which was used for the analysis of residual concentration of 2,4-DCP using the spectrophotometer (750 nm) as described by Garcia et al. [17], and for the degradation metabolites (as described below).

5.2.5 Analysis of 2,4-Dichlorophenol Degradation Metabolites

An advanced version of HPLC technique in corporation of mass spectrometry or simply called LC-MS technique was used to analyse further the metabolite compounds released from the degradation of 2,4-DCP. LC-MS with mass spec detector uses the principal of reverse phase chromatography, where the metabolite binds to the column by hydrophobic interactions in the presence of a hydrophilic solvent (for instance water) and is eluted off by a more hydrophobic solvent (methanol or acetonitrile). As the metabolites appear from the end of the column they enter the mass detector, where the solvent is removed and the metabolites are ionised.

In this study, the LC (1260 Infinity, Agitech) and the MS (6230 TOF, Agilent Technologies) were operated with EC-C_{18}, 2.7 μm column (Poroshell 120) in order to examine the metabolites of 2,4-DCP degradation using both mixed cultures. Two types of solvents used in the LC-MS analysis which were water-methanol (95%) and water-acetonitrile (ACN) to obtain a refined and confirmed data of the excreted metabolites.

5.3 Results and Discussion

5.3.1 Microbial Growth, Biodegradation and Electrical Output Analysis

5.3.1.1 Bacterial Community Analysis

The identification of domestic and industrial microbial community (Table 5.1) would hypothesize that the bacterial growth and 2,4-DCP biodegradation reactions could be diversified. The industrial microbial consortium (IMC) biofilms were found to have non-assorted microbial community in which *Bacillus* sp. highly dominated the consortium with up to 84%. The *Bacillales* (under the same order of *Bacillus*) comprised about 16% of total bacterial community probably consisting of *Bacillus* and *Staphylococcus* genus. It can be stated that these non-diversified bacteria contributed to the electrogenicity and substrate degradation in the MFC system.

Table 5.1 Summary results of operational taxonomic units (OTUs) identifications based on UPGMA algorithm for sub-cultured enriched 2,4-DCP-dependent biofilms of domestic and industrial microbial consortia.

Microbial community	OTUs composition (Genus / Order) (Relative abundance, %)
Industrial microbial consortia	*Bacillus* (84%) *Bacillales* (16%) * Rare OTUs (< 1%)
Domestic microbial consortia	*Pseudomonas* (21.5%) *Aeromonas* (19.6%) *Arcobacter* (14.6%) * Rare OTUs (30.4%) *Shewanella*, *Trichococcus*, *Acinetobacter*, *Clostridia*, *Cloacibacterium*, etc. (1 - 3%)

* Rare OTUs are group of OTUs having genus/order with less than 0.1% relative participation of overall microbial community.

Unlike IMC, the domestic microbial consortium (DMC) showed more diverse microbial pattern in which *Pseudomonas*, *Aeromonas* and *Arcobacter* were determined as the dominants in chlorophenol-based MFC cultivations. Rare OTUs were those bacterial groups classified to contribute less than 0.1% relative participation and become the highest proportions of overall microbial community. Other small bacteria compositions for instance *Shewanella*, *Trichococcus*, *Acinetobacter*, *Clostridia*, and *Cloacibacterium* were also found in the DMC.

It is interesting to note that *Bacillus* and *Pseudomonas* are greatly discovered as good toxic pollutants and phenols degraders [10, 12, 18, 19]. Our data are also supported by previous MFC studies where *Pseudomonas* and *Variovorax paradoxus* were identified in chlorophenol-cultivated MFC system [2, 3]. Their presence in the consortia was expected to favour chlorophenol degradation and microbial growth in 2,4-DCP-fed MFC systems.

5.3.1.2 Microbial Growth and 2,4-Dichlorophenol Degradation

Figure 5.1 presents the kinetic profiles of bacterial growth and 2,4-DCP biodegradation in MFC using both consortia. 2,4-DCP at a concentration range of 5 - 50 mg/L demonstrated different impact on the bacterial growth (solid lines) and biodegradation rate (dotted lines). iMFC showed excellent bacterial growth at a low 2,4-DCP concentration (5 mg/L) as compared to dMFC which demonstrated almost insignificant growth increment (Fig. 5.1 (A)). Following short lag phase, an exponential growth phase of iMFC occurred at 20 – 60 hours of cultivation and then succeeded by prolonged stationary phase. This result suggested that iMFC had a rapid bacterial acclimatization in the media containing 2,4-DCP. The better bacterial growth shown by iMFC could also be attributed by its inoculum source from petrochemical industrial wastewater that is expected to have higher adaptability towards phenolic compounds.

The prompt 2,4-DCP biodegradation was noticed in the first 60 hours before both consortia started to slow down or cease the biodegradation. In this instance, dMFC showed a higher 2,4-DCP biodegradation with 60% than iMFC (40%). The increase in 2,4-DCP degradation rate indicated a low inhibitory of the phenolic compound at low concentration [20]. Furthermore, the slow bacterial growth in dMFC could be the manifestation of its strive in response to 2,4-DCP toxicity while carrying out the biodegradation process.

Different bacterial growth pattern by both microbial consortia was found when subjected to higher 2,4-DCP concentrations (Fig. 5.1 (B) and (C)). Using 25 mg/L 2,4-DCP, iMFC reached longer and lower level of log phase than dMFC. Unlike iMFC, dMFC confronted with a significant decrease of growth following 60 hours of log phase. This could be due to the low remaining energy and substrate left. This profile is believed to reflect its efficient ability to not only survive in a highly toxic medium but also degrade 2,4-DCP at a high concentration, suggesting its quick induction and response to 2,4-DCP when detoxification process is required [21]. Similar finding was observed where *Bacillus cereus* strain AKG2 required 60 hours to degrade phenol at a high concentration with high biodegradation rate as reported by Banerjee and Ghoshal [12].

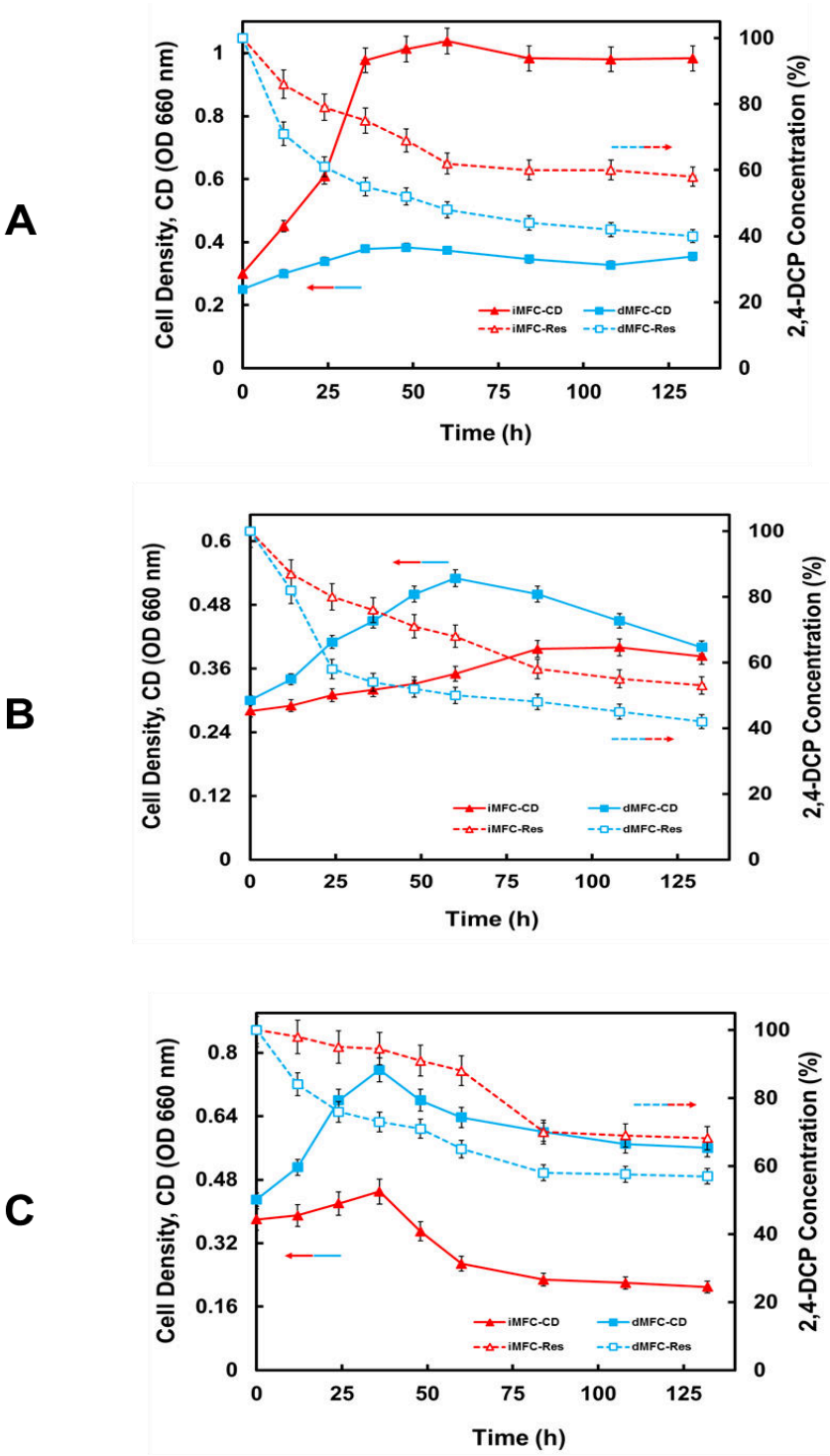

Figure 5.1 Biodegradation of 2,4-DCP by iMFC and dMFC and bacterial growth with 2,4-DCP concentrations: **(A)** 5 mg/L, **(B)** 25 mg/L, and **(C)** 50 mg/L. During MFC cultivation period, 2,4-DCP residual (Res, %) (dotted line) and 2,4-DCP-dependent bacterial growth in terms of cell density (CD, $OD_{660\ nm}$) (solid line) were plotted. The data were mean ± percentage from duplicate experiments.

At a high concentration of 2,4-DCP (50 mg/L), dMFC showed a good bacterial growth at early cultivation period with a small decline after 36 hours. On the contrary, iMFC could not maintain high growth after 36 hours when it showed a tremendous drop of cell density below 0.25 OD. High substrate concentrations or/and the presence of inhibitory substances in the medium are known to inhibit microbial growth [22]. This principle could be applied to iMFC where 2,4-DCP acted as inhibitory substrate in the MFC system. This finding also corresponded to its low ability to degrade only 32% of 2,4-DCP at very high concentration compared to that of dMFC.

It could be deduced that the 2,4-DCP influenced significantly on the growth of both microbial consortia in MFC while contributing less effectively to the biodegradation rate. Although the biodegradation in MFC might proceed in more complex condition due to the engagement of bioelectrochemical activities by bacteria with the presence of potential gradient, these microbial growth and biodegradation profiles were moderately supported by previous findings from conventional biodegradation experiments [21, 23]. The transformation of various intra or extra cellular metabolites that lag behind the 2,4-DCP utilization rate might have contributed to the observed growth of both microbial consortia toward the end of the MFC biodegradation period [20].

5.3.1.3 Voltage Generation

Figure 5.2 shows the profiles of open-circuit voltage (OCV) generation corresponding to various 2,4-DCP concentrations by both microbial consortia. Relatively similar trends could be observed for voltage output curves in which iMFC prioritized dMFC with higher voltage output. A significantly higher OCV attained by iMFC compared to dMFC in low 2,4-DCP concentration (Fig. 5.2 (A)) could be correspondingly associated with their abilities to grow in the MFC with low 2,4-DCP (see the growth profile). The OCV generation appeared to be constant (or slowing down) after approximately 75 hours of cultivation at which the biodegradation rates and growth tended to be constant. This suggests that the bioelectrochemical activities are associated with the bacterial growth and closely linked to 2,4-DCP biodegradation.

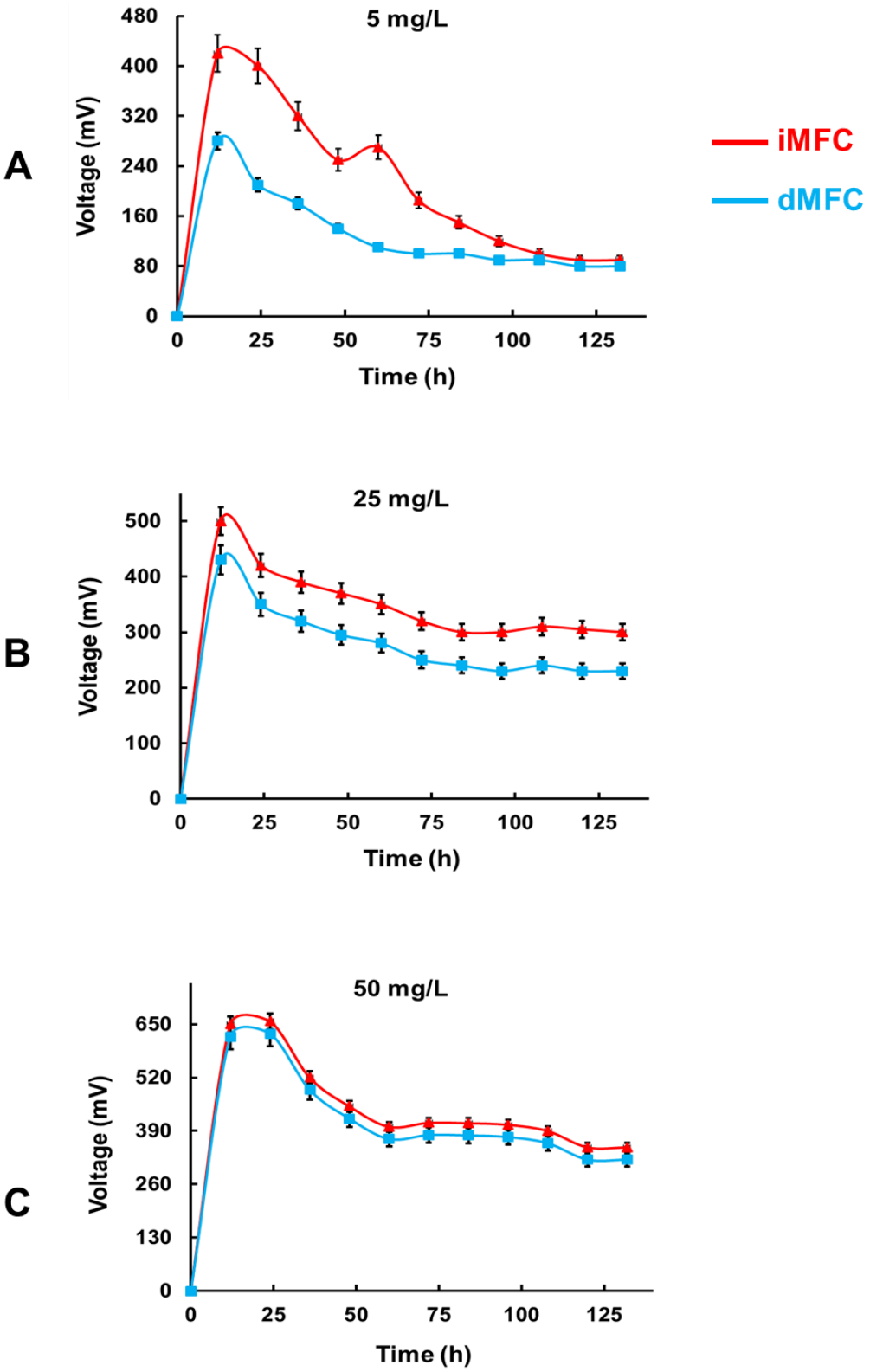

Figure 5.2 Open circuit voltage (OCV) generation profiles by iMFC and dMFC with various 2,4-DCP concentrations: **(A)** 5 mg/L, **(B)** 25 mg/L, and **(C)** 50 mg/L.

The growth association phenomena might not be pertinent to the MFC voltage generation profiles when the initial 2,4-DCP concentrations were increased (Fig. 5.2 (B) and (C)). However, the OCV generations by dMFC in a moderate to high level of 2,4-DCP were almost as high as that of iMFC. In other words, the dMFC performance to generate electricity was enhanced with the increase of initial 2,4-DCP concentration. It could be suggested that higher 2,4-DCP concentration could trigger the electron transfer in the dMFC systems, thus, leading to higher OCV generation. For instance, the increase in OCV with the increasing of 2,4-DCP concentrations i.e., from 280 - 420 mV (5 mg/L) to ~650 mV (50 mg/L) could be due to the dependence of the reaction activation losses on 2,4-DCP concentration as suggested by Huang et al. [1]. Furthermore, the retaining capability of voltage generation as well as the microbial growth in the medium with a broad range of 2,4-DCP concentrations over prolonged cultivation period reflected the efficient role of biofilms in the MFC systems.

5.3.2 Bacterial Growth Kinetics and Biodegradation Pathways

5.3.2.1 Growth Kinetic Analysis

Kinetic analysis for microbial growth and target substrate degradation is a vital tool to obtain an understanding of bacterial growth activities, degradation reactions and metabolites in a system. Growth kinetic profiles of both microbial consortia were analysed in terms of specific growth rate (μ, h^{-1}) with various initial 2,4-DCP concentrations (S) using a non-linear regression of Haldane inhibition model (Eq. 5.2). Haldane model is used to analyse the microbial growth when toxic substrate (normally analysed for phenolic compounds) with potential inhibitory effect is considered [21]. Then, specific kinetic parameters including maximum specific growth rate (μ_{max}), a half-saturation growth constant (K_s) and inhibition constant (K_i) can be determined from the plotted graph (Fig. 5.3 (A)).

$$\mu = \frac{\mu_{max} S}{S + \left(\frac{S^2}{K_i}\right) + K_s} \tag{5.2}$$

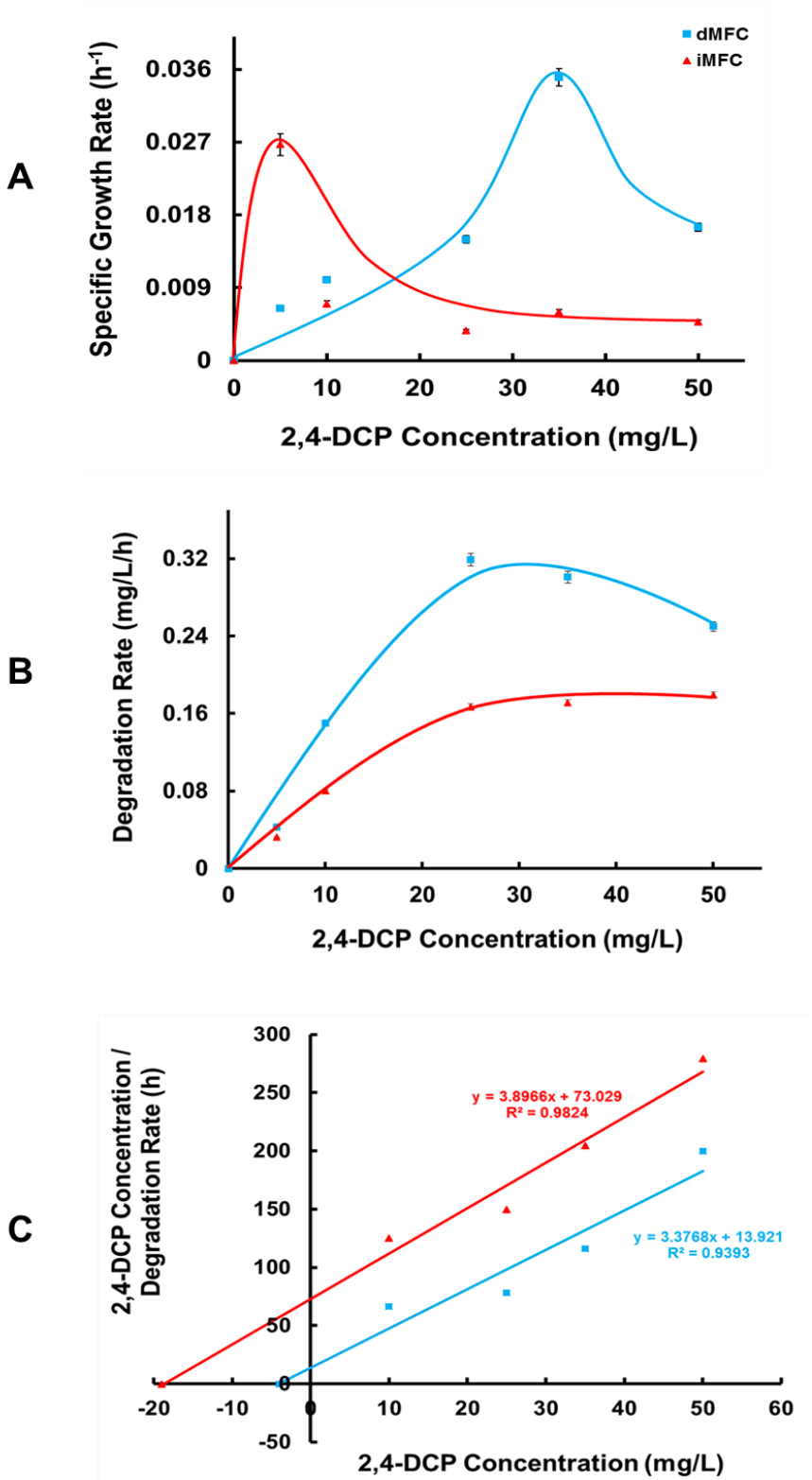

Figure 5.3 (A) 2,4-DCP-dependent specific growth kinetics of iMFC and dMFC fitted with Haldane inhibition model; 2,4-DCP biodegradation kinetics analysis of iMFC and dMFC using: **(B)** Michaelis-Menten plot, and **(C)** Hanes-Woolf model.

The μ_{max} for iMFC and dMFC was determined to be 0.028 and 0.037 h^{-1}, respectively (Table 5.2 (A)). These values were relatively lower than those previously reported [14]. This could be due to the type of microbial culture in which pure culture might achieve higher specific growth rate compared to mixed bacterial culture or consortia as in our case. Although the μ_{max} of dMFC is higher, the peak was greatly shifted to the right which might suggest its slow acclimatization rate at low 2,4-DCP concentration. The estimated K_s values were determined to be 1.5 mg/L and 27 mg/L for iMFC and dMFC, respectively, which indicate that the dMFC had higher preference towards this toxic pollutant. This result also corresponds to the high biodegradation rate achieved by dMFC. Most of the K_s values found in the literature lie between 1 and 110 mg/L [14]. The K_s in our iMFC and dMFC were determined within the low range. This might be attributed by the bioelectrochemical activities and microbial consortia employed in the MFC system, which compensate the low attainable K_s as well as μ_{max} values.

Table 5.2 Kinetic parameters for **(A)** bacterial growth and **(B)** biodegradation rate profiles of 2,4-DCP by iMFC and dMFC.

Kinetics parameters	**Kinetic models**			
(A) Bacterial growth	Haldane model			
	iMFC		dMFC	
μ_{max} (h^{-1})	0.028		0.037	
K_s (mg/L)	1.5		27	
K_i (mg/L)	33		28	
(B) Biodegradation	Michaelis-Menten model		Hanes-Woolf model	
	iMFC	dMFC	iMFC	dMFC
V_{max} (mg/L/h)	0.178	0.32		
K_s (mg/L)	10.2	11.0		
V_{max}/K_s (h^{-1})	0.017	0.03		
R^2	0.990	0.985	0.982	0.939
1 / V_{max} (L·h/mg)			3.897	3.377
K_s/V_{max} (h)			73.03	13.92
- K_s (mg/L)			-19.0	-4.0

The K_i values determined from the iMFC and dMFC had small difference (33 mg/L and 28 mg/L for iMFC and dMFC, respectively). These results denoted that the partial inhibition of the cell growth occurred at a high 2,4-DCP concentration. Furthermore, these results suggest that both consortia might tolerate 2,4-DCP at high concentrations, while maintaining satisfactory specific growth rate [21]. Despite of low organic and biomass loading in the anolyte cultivation medium of MFC, the biodegradation kinetics of both microbial consortia were comparable with previous studies in conventional anaerobic biodegradation systems [12 – 14].

5.3.2.2 Biodegradation Kinetic Analysis

Further analysis on 2,4-DCP biodegradation kinetics at different initial concentrations was examined by fitting experimental data with a non-linear Michaelis-Menten model (Eq. 5.3) [24], and a linear Hanes-Woolf plot model (Eq. 5.4) as follows:

$$\frac{ds}{dt} = -V_{max}\frac{S}{S+K_S} \tag{5.3}$$

$$\frac{S}{v} = \frac{1}{V_{max}}[S] + \frac{K_S}{V_{max}} \tag{5.4}$$

where V_{max} is the maximum biodegradation rate obtained from a slope value of a linear plot as 1/ V_{max}, and K_s is a half saturation concentration determined with intact bacterial cells as described previously and obtained from an x-axis intercept of a linear plot as $-K_s$ [25, 26]. The kinetic paradigms shown by both microbial consortia fitted well with Michaelis-Menten and Hanes-Woolf models as depicted in Fig. 5.3 (B) and (C) respectively. The apparent kinetic parameters extracted from those models were tabulated in Table 5.2 (B).

In general, dMFC achieved a higher maximum biodegradation rate of V_{max} (0.32 mg/L/h) and V_{max} /K_s (0.03 h^{-1}). This proved that dMFC was highly capable to degrade 2,4-DCP compared to iMFC. The ratio of V_{max} /K_s (known as specific substrate affinity) could be considered as a useful index for the whole-cell reaction efficiency and acts as better alternative of analysing kinetics considering nutrient uptake efficiency and substrate assimilation ability [27], especially when utilizing mixed microbial consortia. The higher value signified the bacteria could well uptake and assimilate the target pollutant (2,4-DCP). In addition, experimental data from iMFC and dMFC fitted very well to Michaelis-Menten model, representing the biodegradation kinetic of the consortia in MFC systems. Hanes-

Woolf's kinetic parameter, on the other hand, showed that iMFC and dMFC were dynamically comparable where iMFC demonstrated better fitting with higher R^2 value. Other apparent kinetic values also suggest that kinetic data from iMFC was better fitted with Hanes-Woolf model considering the period based linear profile.

5.3.2.3 Degradation Pathways

To better understand the 2,4-DCP biodegradation reactions and the intermediates as well as final metabolites of iMFC and dMFC, biodegradation pathways were analyzed based on the detected metabolites using LC-MS analysis as illustrated in Fig. 5.4 (dMFC) and Fig. 5.5 (iMFC). The dMFC was proposed to biodegrade 2,4-DCP anaerobically and aerobically (Fig. 5.4). dMFC biodegraded the 2,4-DCP into phenol through anaerobic routes (shown by solid arrows). Phenol is produced through reductive dehalogenation reaction [28] and further degraded into benzoic acid through carbonation of p-hydroxybenzoic acid (not detected by LC-MS). The final detected metabolite by the dMFC through anaerobic route is acetate where it will be further transformed into CH_4 and CO_2 as the end by-products. The LC-MS data for the final detected metabolite of acetate is provided in the **Supplementary Data**.

While for the aerobic routes (shown by dotted arrows), firstly, the hydroquinone was oxygenated into 1,2,4-benzenetriol by phenol-2-monooxygenase enzyme [29] and subsequently cleaved into maleylacetate by dioxygenase enzyme [21, 30]. 3,5-Dichlorocatechol was also produced through aerobic route by the reaction of 2,4-DCP hydroxylase enzyme and further ring-cleaved into maleylacetate. The metabolites through aerobic route were expected to finally pass into tricarboxylic acid (TCA) cycle.

A more complex biodegradation pathway was shown by iMFC as compared to dMFC (Fig. 5.5). Like dMFC, iMFC was also proposed to biodegrade 2,4-DCP through anaerobic and aerobic routes. Following anaerobic route, the iMFC reduced 2,4-DCP into phenol through two metabolites i.e., 2-chlorophenol and 4-chlorophenol by reductive dehalogenation reaction. The phenol was successively reacted with CO_2 to produce p-hydroxybenzoic acid and further reduced to benzoic acid. Then, the benzoic acid was decarboxylized into benzene and further aromatic ring-cleaved into 2-chloromaleylacetate.

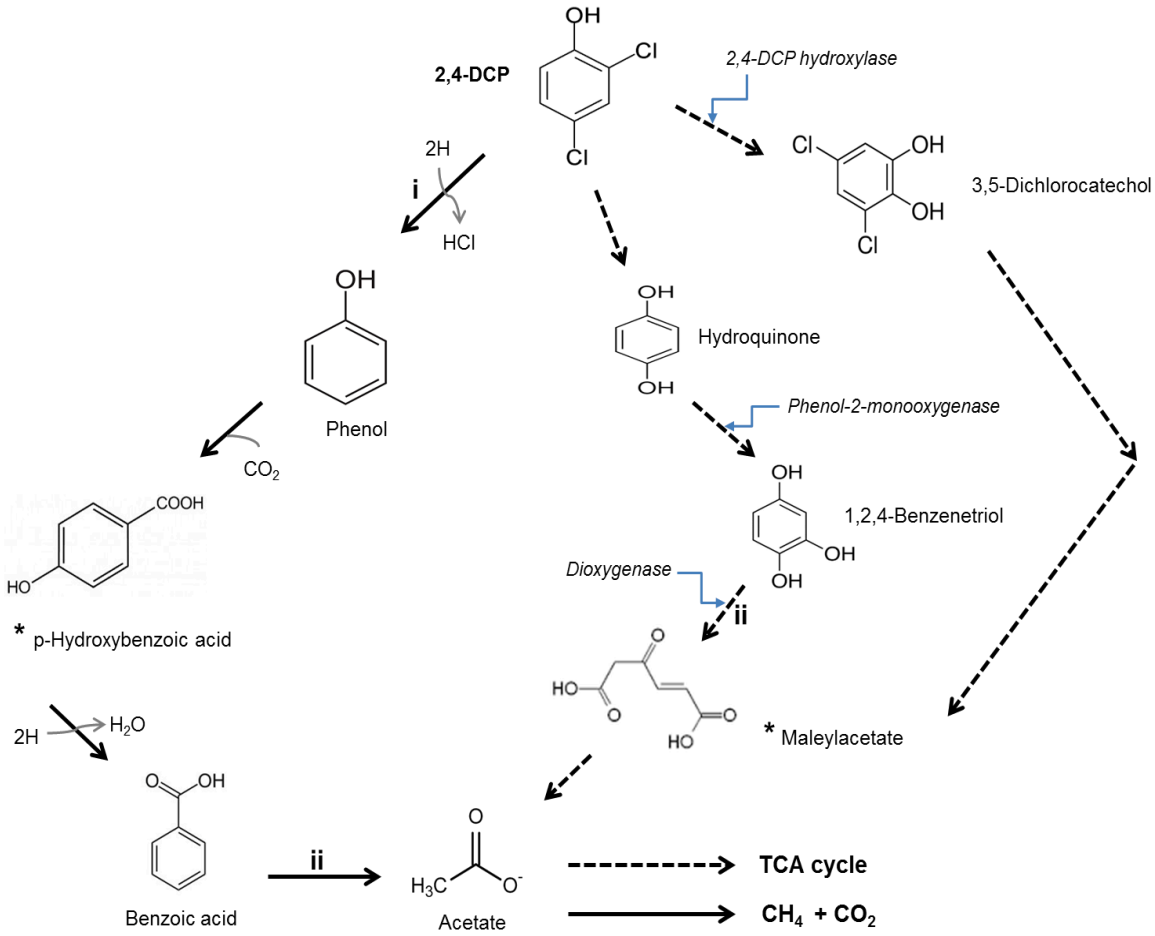

Figure 5.4 Proposed 2,4-DCP biodegradation pathway by dMFC. The solid arrows show the anaerobic degradation route and the dotted arrows show the aerobic degradation route. Compounds with star sign were not identified in the culture extract. Roman numbers represent the proposed reactions **(i)** reductive dehalogenation **(ii)** aromatic ring cleavage.

The 2-chloromaleylacetate was degraded into 3-oxoadipic acid by maleylacetate reductase enzyme [31] and finally transformed into CH_4 and CO_2. The LC-MS data for the final detected metabolite of 3-oxoadipic acid is provided in the **Supplementary Data**. On the other hands, the aerobic route by iMFC demonstrated that the 2,4-DCP was degraded into 3,5-dichlorocatechol and 4-chlorocatechol which both were further cleaved into 3-chloromuconic acid (not detected by the LC-MS). This metabolite was transformed into either 2-oxo-3-hexenedioate catalyzed by long reactions of isomerase enzyme [32] and/or 2-chlorodienelactone which subsequently cleaved into 2-chloromaleylacetate by dienelactone hydrolase enzyme [33].

With respect to the growth and biodegradation kinetics, the high 2,4-DCP degradation in dMFC could be associated with less complex biodegradation pathways as shown in Fig. 5.4. The simpler and less number of metabolites participating in the pathways resulted in low ability to generate electricity in MFC system (see Fig. 5.2). On the other hands, iMFC which achieved higher electric output yet low biodegradation rates, could be interpreted by the presence of freely ionic molecules of its metabolites through its complex pathways (Fig. 5.5).

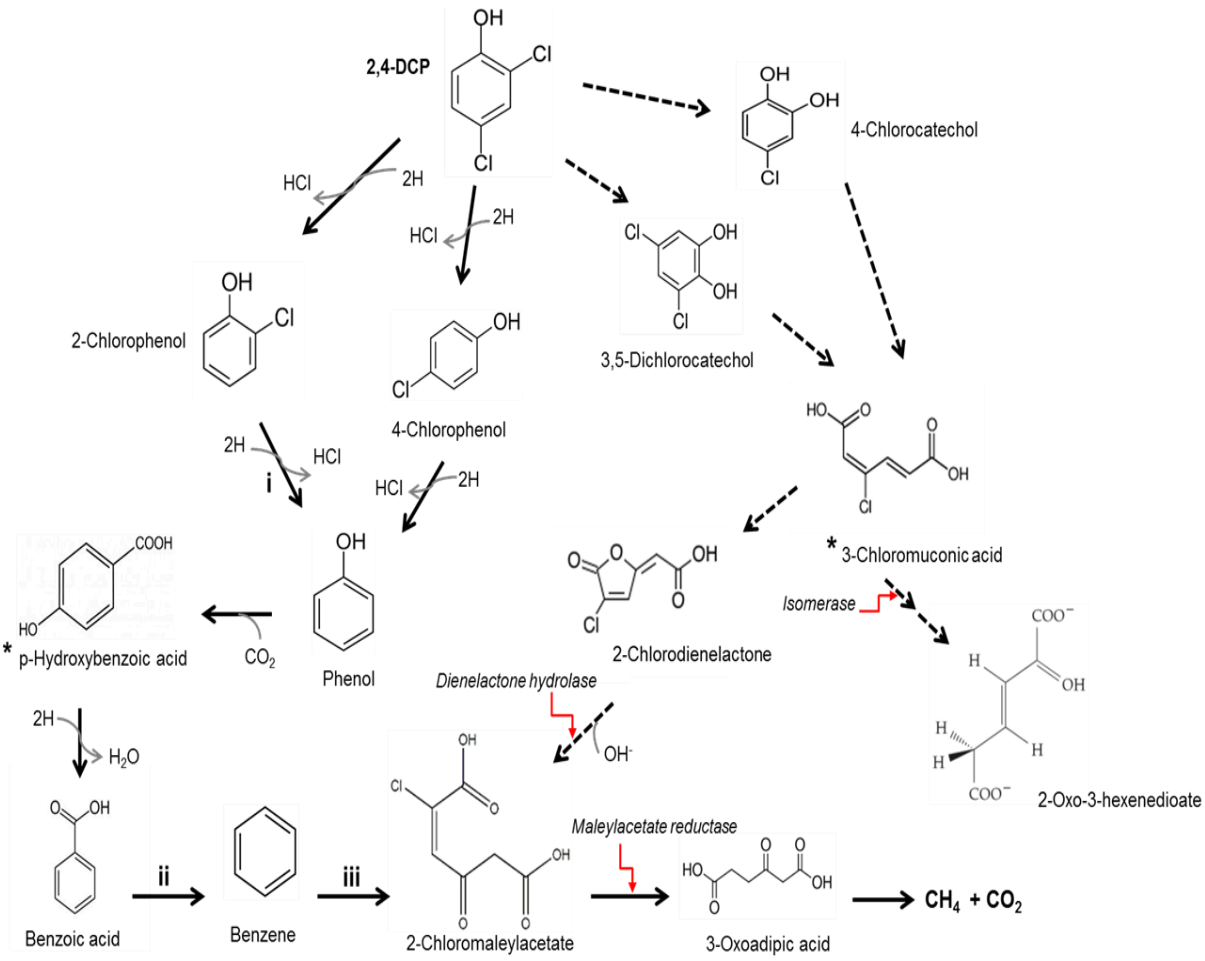

Figure 5.5 Proposed 2,4-DCP biodegradation pathway by iMFC. The solid arrows show the anaerobic degradation route and the dotted arrows show the aerobic degradation route. Compounds with star sign were not identified in the culture extract. Roman numbers represent the proposed reactions **(i)** reductive dehalogenation **(ii)** decarboxylation **(iii)** aromatic ring cleavage.

5.3.2.4 Metabolite Products

The metabolites produced from the 2,4-DCP degradation were analyzed using LC-MS. Figure 5.6 shows numerous metabolite compounds detected by the LC-MS using water-methanol and water-ACN solvents for both microbial consortia according to the MFC cultivation time. The number of excreted 2,4-DCP degradation metabolites in dMFC (Fig. 5.6 (A) and (B)) was lesser than that of iMFC (Fig. 5.6 (C) and (D)). It could also be suggested that most of the degradation metabolites were excreted during earlier biodegradation indicating that 2,4-DCP was well degraded by the microbial consortia rather promptly in the MFC system. The use of water-methanol and water-ACN solvents displayed slightly dissimilar detected metabolites except for 2/3/4-methylphenol (for both dMFC and iMFC), 1,2,4-benzenetriol (for dMFC) and benzoic acid and benzene (for iMFC). A number of metabolites degraded by the iMFC were produced at the later stage (final day) of the MFC operation as depicted in Fig. 5.6 (C) and (D) compared to that of dMFC. It could be stated that both microbial consortia degraded relatively diverse and complex metabolites irrespective to the degradation time of the MFC operations.

It is worthwhile to note that two degradation metabolites: 2/3/4-methylphenol and 3-hydroxyphencyclidine were found in iMFC and dMFC, but were not involved in the proposed biodegradation pathways. The methylphenol was expected to be bio-transformed by several aerobic bacteria in the degradation of higher chlorinated phenols [28]. *Mycobacterium* and *Rhodococcus* strains were found to be able to produce ortho-methylate from degradation of a variety of chlorophenols [34]. While for the 3-hydroxyphencyclidine, to the best of our knowledge, there is no previous evidence to link this compound with chlorophenol biodegradation. However, phenyl-piperidine which has been addressed in phenolic opioid kappa receptor selective antagonist activity [35] is one of the phencyclidine metabolites as assayed in gas-liquid chromatography technique [36].

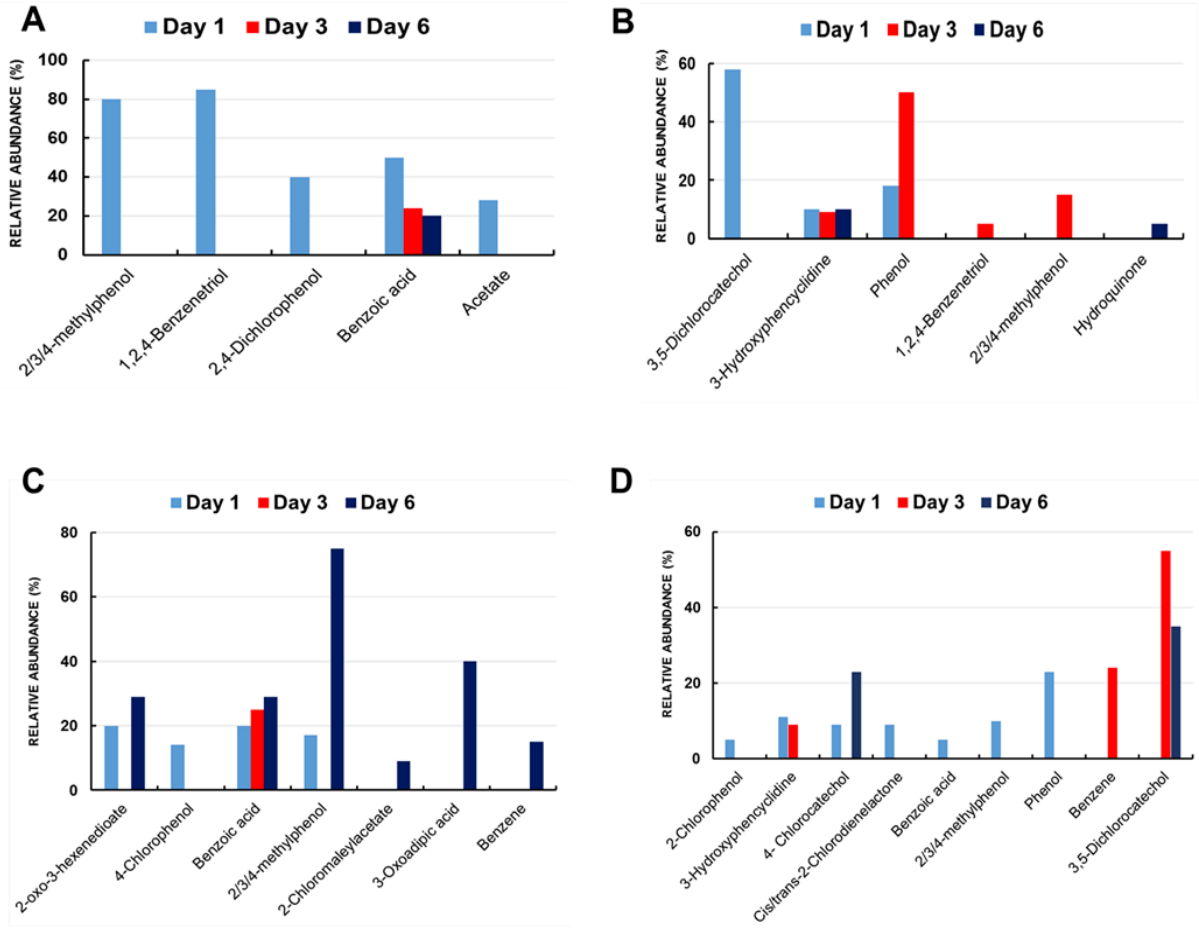

Figure 5.6 Percentage distribution of 2,4-DCP degradation in the biodegradation mixtures: **(A)** dMFC using water-methanol solvent **(B)** dMFC using water-ACN solvent **(C)** iMFC using water-methanol solvent **(D)** iMFC using water-ACN solvent.

5.3.2.5 Toxicology of End-Metabolites

The main objective for pollutant biodegradation process towards safe environment is to eliminate or minimize the overall toxicity caused by the parental toxic pollutant or toxic metabolites formed during the degradation [21]. The biodegradation of chlorophenol by mixed microorganisms can be very complex with respect to its synergistic and complete pathways. Considering the electron transfer activities with simultaneous chlorophenol degradation in the MFC, the proposed pathways reveal that the 2,4-DCP was efficiently degraded into less hazardous final metabolites by both consortia as shown in Table 5.3. The acetate and 3-oxoadipate are toxicologically categorized as very much less toxic compounds than 2,4-DCP. Acetate which was produced by dMFC turns to be less

hazardous with its simpler molecular structure as compared to 3-oxoadipate which was produced by iMFC. Acetate along with glucose, are simple biodegradable organic substrates usually used for fermentation, and are believed to reduce the toxicity and growth inhibition [37]. This simple substrate can also act as an inducing agent for biodegradative enzymes as well as an electron donor for bacterial growth [37]. Thus, the simpler pathways and better performance in biodegradation kinetic analysis demonstrated by dMFC could be explained with its capability to produce such an inducing agent of acetate as end metabolite product. In general, it can be deduced that our MFC cultivated with mixed bacteria is an efficient and potential system in transforming recalcitrant and toxic compounds into more acceptable products while generating significant current outputs.

Table 5.3 Toxicology of 2,4-DCP and final degraded metabolites by dMFC (acetate) and iMFC (3-oxoadipate) (adapted from Safety Data Sheet according to Work Health and Safety (WHS) and Australian Dangerous Goods Code (ADG) requirements).

Toxicological properties	**2,4-DCP**	**[a]Acetate (dMFC)**	**3-Oxoadipate (iMFC)**
Hazard alert code	[b]3	1 or 2	2
Toxicity	3	0	0
Chronic	0	0	0
Reactivity	1	1	1
Flammability	1	0 or 1	1
Body contact	3	0 or 2	2

[a] Acetate data is based on sodium acetate
[b] The numbers represent the hazard ratings i.e., 0 is the minimum level, 4 is the extreme.

5.4 Conclusions

2,4-DCP biodegradation kinetics and pathways have been analytically evaluated in the double chambered MFC systems using domestic and petrochemical industrial microbial consortia. Kinetic data revealed that both microbial consortia-inoculated MFCs fitted well to Haldane inhibition model, Michaelis-Menten and Hanes-Woolf models. Domestic microbial consortia-inoculated MFC (dMFC) was proven to achieve higher apparent kinetic values with maximum specific growth rate, μ_{max} of 0.037 h^{-1} and maximum biodegradation rate, V_{max} of 0.32 mg/L/h. However, industrial microbial consortia-inoculated MFC (iMFC) demonstrated higher bacterial growth profile at a lower initial 2,4-DCP concentration with relatively higher open-circuit voltage output than dMFC. Both dMFC and iMFC performed distinguished biodegradation kinetics and pathways, as well as final metabolites of acetate and 3-oxoadipate, respectively. The investigated dMFC and iMFC systems showed promising results of 2,4-DCP removal and its reduced toxicity through the simultaneous electric generation. The research approach and experimental data in this study can provide valuable insight for future study on biodegradation kinetic behaviors and relationship with bioelectrochemical activities employed in MFC system.

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Supplementary Data

Qualitative Compound Report

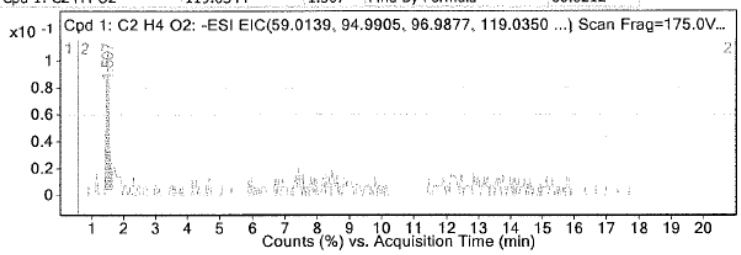
Data File	ChemEng_HH_24hDOM_21215_neg.d	**Sample Name**	24h DOM
Sample Type	Sample	**Position**	P1-F1
Instrument Name	Instrument 1	**User Name**	
Acq Method	PCInsitu-5-100ACN_neg_50_1700mz_20min.m	**Acquired Time**	12/2/2015 3:11:59 PM
IRM Calibration Status	Success	**DA Method**	Find-by-Formula.m
Comment			

Sample Group	**Info.**
Acquisition SW Version	6200 series TOF/6500 series Q-TOF B.05.01 (B5125.1)

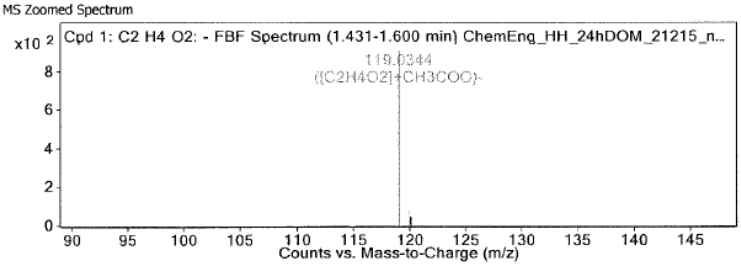
Compound Table

Compound Label	RT	Mass	Abund	Formula	Tgt Mass	Diff (ppm)	MFG Formula	DB Formula
Cpd 1: C2 H4 O2	1.507	60.0212	904	C2 H4 O2	60.0211	1.98	C2 H4 O2	C2 H4 O2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 1: C2 H4 O2	119.0344	1.507	Find By Formula	60.0212



MS Zoomed Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
119.0344	1	903.58	C2H4O2	(M+CH3COO)-
120.046	1	81.55	C2H4O2	(M+CH3COO)-

--- End Of Report ---

Figure S5.1 LC-MS spectra for final metabolite of acetate produced by dMFC.

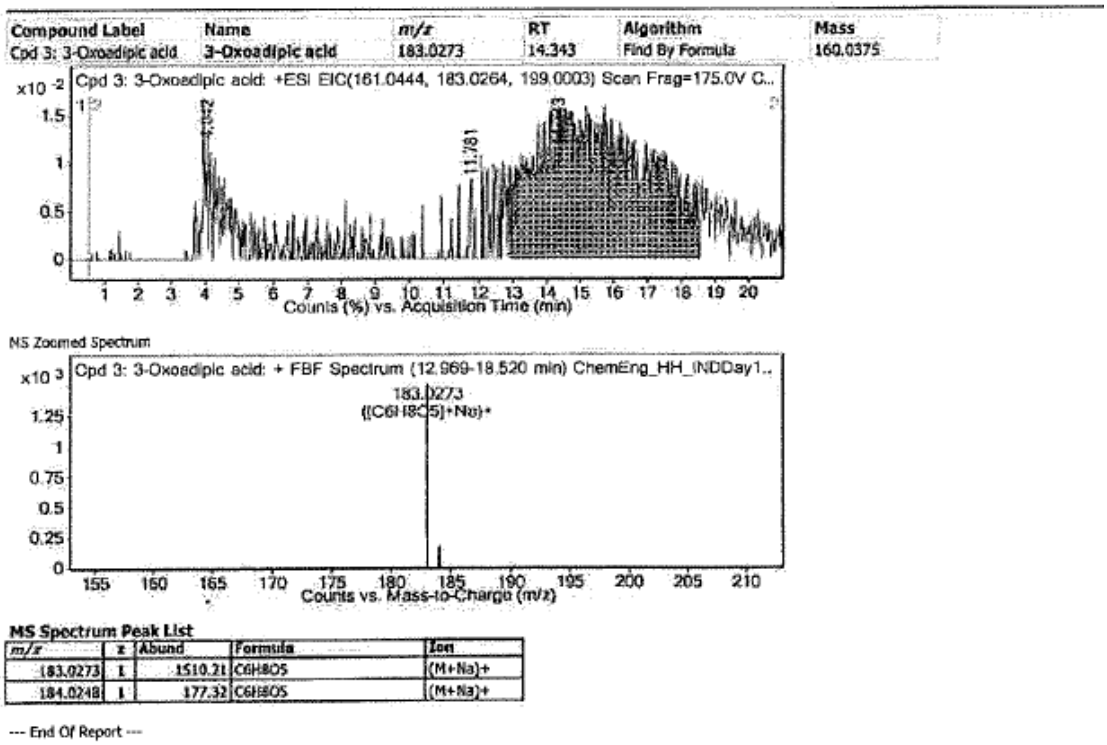


Figure S5.2 LC-MS spectra for final metabolite of 3-oxoadipate produced by iMFC.

CHAPTER 6

Statement of Authorship

Title of Paper	Application of polynomial models for optimizing microbial fuel cell system for electricity generation and dichlorophenol degradation
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Contribution to the Paper	Designing and performing the experiments, data analysis, and manuscript writing.
Overall percentage (%)	60 %
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 19/01/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Helping in conducting the experiments.		
Signature		Date	19/01/2017

6. Application of Polynomial Models for Optimizing Microbial Fuel Cell System for Electricity Generation and Dichlorophenol Degradation

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Chapter 6: Application of Polynomial Models for Optimizing Microbial Fuel Cell System for Electricity Generation and Dichlorophenol Degradation

Abstract

The interactions within microbial, chemical and electronical elements in microbial fuel cell (MFC) system can be crucial for its bioelectrochemical activities and overall performance. This study explored polynomial models by response surface methodology (RSM) to better understand interactions among the chemical and microbial species, aiming to optimize the MFC system for generation of electricity and degradation of 2,4-dichlorophenol. A statistical central composite design by RSM was used to develop the quadratic model designs. The optimized parameters were determined and evaluated by statistical results and the best MFC systematic outcomes in terms of current generation and chlorophenol degradation. Statistical results revealed that the optimum current density of 106 mA/m^2 could be achieved at anode pH 7.5, cathode pH 6.3-6.6 and 21 – 28 % for inoculum size. Anode-cathode pHs interaction was found to positively influence the current generation through intracellular electron transfer mechanism. The phenolic degradation was convinced to have lower response using these three parameters interactions. Only inoculum size-cathode pH interaction appeared to be significant where the optimum predicted phenolic degradation could be attained at cathode pH 7.6 and 29.6 % inoculum size.

Keywords: Polynomial optimization; Microbial fuel cell; *Bacillus subtilis*; 2,4-Dichlorophenol.

6.1 Introduction

Statistical optimization approach can be a promising tool to optimize complex bioelectrochemical systems such as microbial fuel cell (MFC), which can simultaneously produce electricity and remove chemical contaminants. Various optimization tools with varieties of statistical software are or could be used in the statistical optimization studies including central composite design (CCD), Box-Behnken and one-factor by response surface methodology (RSM), Plackett-Burman and Taguchi by factorial designs (FD) and Simplex methods of mixture designs. FD and CCD for example, are powerful statistical methods that have been widely used to determine the effect of several parameters on a response, simultaneously [1]. The commonly used statistical tool is analysis of variance (ANOVA) which results in systematic prediction models and algorithms that are useful for further modelling, simulation and scale-up purposes. The other aims of using systematic and uniform design are to reduce the experimental trials and thus operational time, attain cost-effective method by reducing the use of materials or chemicals, and apply efficient parameter interactions for optimizing the model terms, hence the responses.

These statistical approaches have proven that the current generation and waste reduction in MFC could be significantly enhanced through the positive influence of multi-variables parameters [2]. Several studies attempted to optimize MFC system with many operational parameters using experimental as well as modeling approach. These include buffer concentrations [1], temperatures [3], cultivation times [4], nutrients and substrate/organics concentrations [2, 5,], and pH [6, 7].

One of the most important parameters in MFC performance is pH in both anode and cathode chambers which has significant influence on MFC performance for current generation and COD removal. The pH level could entirely control the electron transfers rate and proton flows in the MFC circuit, thus improving the current generation profile in the system. With respect to the anolyte and catholyte, sufficient H^+ concentration could balance the amount of protons required for reduction reaction in the cathode, thus becoming the rate limiting factor in the MFC. The near neutral and higher pH were found to improve power output and COD removal [1, 7, 8]. In addition, it was reported that high level of buffer concentration with varying pH between 5.8 and 7.4 did not markedly influence the maximum power density and coulombic efficiency (CE). However, a low pH

of buffer solution could lead to higher power density [1]. Other operational and environmental factors such as temperature could also contribute to the enhanced MFC performance. A lower temperature resulted in a higher current and CE [9], but led to a reduced maximum power density and COD removal. Other than that, substrate nitrogen level was also observed to affect the current generation and COD removal [9]. Therefore, by considering the MFC's still-low power output especially for commercial feasibility, this optimization approach is extremely significant in the current and future MFC studies.

The main responses in the optimization studies normally include current or power densities, CE and COD removal. To date, there is no reported study on the optimization of the MFC system looking at phenolic degradation and electricity generation. Although several attempts to study the phenolic degradation in MFC have been reported [8, 10-13], the optimization of the operational parameters in overseeing phenolic degradation trend in MFC is significantly worthwhile to be further studied. Basak et al. [14] examined the optimization of 4-chlorophenol degradation by *Candida tropicalis* in a conventional biodegradation process using Taguchi approach and found that microbial growth and 4-chlorophenol could be significantly enhanced by 200%. This study pointed out that the selection of bacterial inoculum especially pure culture is greatly important in the optimization studies due to homogenous and synergistic interactions in terms of growth and utilizing the target pollutants. This result could support our hypothesis that phenolic degradation could be greatly enhanced using statistical optimization approach while maintaining the electricity generation in the MFC system using excellent phenol-degrading bacterial strain.

Therefore, our study is aimed to develop an integrated statistical approach for optimizing three key operational parameters; anode and cathode pHs, and bacterial inoculum size using a pure culture *Bacillus subtilis*. The optimized parameters were determined and evaluated by statistical results and the best MFC systematic outcomes in terms of current generation and chlorophenol degradation. RSM was utilized to design the polynomial model and the MFC performance outcomes were optimized through the interaction of the aforementioned parameters.

6.2 Materials and Methods

6.2.1 Microorganisms

Three bacterial cultures were used to determine the highest current generation and 2,4-dichlorophenol (2,4-DCP) degradation, namely, pure culture *Bacillus subtilis* (AWQC 111), domestic mixed culture and industrial mixed culture. *Bacillus subtilis* was provided by the Australian Water Quality Centre. The domestic mixed culture was derived from a domestic wastewater treatment plant at Glenelg, South Australia while the industrial mixed culture was derived from a petrochemical wastewater collected from Australia Mobil Oil Plant, South Australia as previously described (Chapter 4).

Bacillus subtilis was sub-cultured several times on a nutrient agar in a petri dish. The domestic and industrial mixed cultures inoculums (as previously described in Subsection 4.2.1) were pre-grown in the microbial fuel cell reactor for three days in order to develop biofilms on the anode surface. The biofilms on the anode surface was streaked by a sterilized inoculating loop and sub-cultured on the nutrient agar for several times. The sub-culture experiments for these three bacterial cultures were carried out in a biological safety cabinet (Class II, BH2000 Series) to maintain sterility. The inoculated bacteria were then incubated in an incubator at 37 ºC for 48 hours. The grown bacteria on the nutrient agar was then stored under 4 ºC in a refrigerator for further use.

Two to three streaked bacterial loops from the sub-cultured nutrient agar were inoculated and grown in a 250 mL of nutrient broth in a 500-mL shake flask for 48 hours on an incubator shaker (175 rpm) at 37 ºC. 10-mL sample was taken every two hours; 5 mL from this sample volume was for the optical density (OD) reading using UV-Vis Spectrophotometer (Shimadzu, UV-1601) at 660 nm wavelength. Another 5 mL sample was used to determine cell dry weight (CDW, g/L). The bacteria sample was filtered using pre-weighed filter paper (Whatman 110 mm) with the application of vacuum pump. The bacteria left on the filter paper was dried at 80 ºC for 24 hours. The dried filter paper was incubated in a desiccator for 24 hours prior to weighing. The CDW was determined using Eq. 6.1:

$$CDW = \frac{W_f - W_i}{V} \tag{6.1}$$

where W_f and W_i were the final and initial weight of filter paper (g) and V is the volume of sample (5 mL). The OD and CDW experiments were carried out in triplicate for all three bacterial cultures. The highest growth rate was determined from the OD and CDW data and become the basic start-up period for further optimization experiments using MFC.

6.2.2 Culture Medium and Catholytes

M9 medium was used as the growth medium for all three bacterial cultures, which was prepared according to Miller [15] with slight modification by adding yeast extract to supply vitamin and amino acid. The modified M9 medium was composed of 0.2% glucose and 1 g/L yeast extract in mineral salts (1 g/L NH_4Cl, 3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl, 1 mmol/L $MgSO_4$, and 0.1 mmol/L $CaCl_2$). The medium was autoclaved at 121 ºC for 15 min. The glucose solution was sterilized using 0.22 µm filter unit (Millipore membrane PVDF) and then added to the autoclaved medium. The culture and medium preparation was carried out in a biological safety cabinet to avoid culture contamination.

The catholytes used were phosphate buffer and acetate buffer according to the pH variables in the optimization experimental design. The pHs of both anolyte and catholyte were adjusted with NaOH or HCl and measured using a pH meter (Eutech Instruments).

6.2.3 MFC Reactor Setup

A dual-chambered membrane MFC reactor was constructed from two glass bottles (250 mL) joined with a glass bridge, which consists of a cationic exchange membrane (CMI-7000, gel polystyrene cross linked with divinylbenzene) as previously described [13]. Anode material was made up of 30 × 30-mm carbon cloth, while the cathode material was made of 40 × 30 × 2-mm platinised titanium (Pt/Ti) catalysts plate. Both electrodes were wired with titanium projecting outside connecting to an external electrical circuit.

The MFC glasses were autoclaved and the setup was carried out in the biological safety cabinet to avoid the glasses and culture contamination. The cathodic chamber was open for aeration. The MFC system was topped on a magnetic plate to ensure well mixing during operation and maintained in a room temperature.

6.2.4 Voltage-Current Data Acquisition and Phenolic Degradation

Voltage and current outputs were continuously recorded by a LabJack U6 recorder with an external load of 10 kΩ connected to a computer for 5 days of MFC operation. The current and anodic and cathodic potentials were measured with respect to operational time.

The model chlorophenol compound used in this study was 2,4-dichlorophenol (2,4-DCP) (Alfa Aesar), which is a chlorinated derivative of phenol with the molecular formula $C_6H_4Cl_2O$. 10 mg/L 2,4-DCP was fed into the anodic chamber under anoxic condition and the concentration was kept constant throughout the experiments. The 2,4-DCP concentration was examined using the UV-Visible spectrophotometer at a wavelength of 750 nm as described by Wilfred and Ralph [16]. Phenolic degradation was calculated based on the difference in 2,4-DCP initial and final concentrations.

6.2.5 Optimization Experimental Design

6.2.5.1 Preliminary Optimization Test

A preliminary optimization experiment was carried out using three bacterial cultures in order to compare their performance in current generation and 2,4-DCP degradation. The three cultures were cultivated in the MFC reactors for five days using constant conditions (anode and cathode pH of 7.0, room temperature and 30% v/v inoculum size). The bacterial culture with the highest current generation and phenolic degradation was selected and utilized in the succeeding optimization experiment using RSM. The optimum cultivation time was also determined from this preliminary result and used as a constant time factor in the subsequent optimization experiments.

6.2.5.2 Response Surface Methodology using Design Expert

Design Expert Software (Version 10) was used to examine the optimization model for current generation and phenolic removal in this study. The study type used was Response Surface Methodology (RSM) and the design type was Central Composite Design (CCD). 25 runs of experiments were carried out as developed by the software based on the three variable factors with three levels as shown in Table 6.1. The pHs of anolyte and catholyte are the most important parameter in enhancing MFC performance through the interactions and mechanisms of electron transfer process [1, 7]. In addition, the selection of suitable

bacterial culture with designated inoculum concentration could lead to well development of biofilm, thus impoving the mass and electron transfer in the bioelectrochemical activities in the MFC system. Therefore, pHs of anolte and catholyte, as well as bacterial inoculum size were systematically selected for the interaction variables in our optimization study. Two responses namely, current generation (mA/m^2) (R1) and phenolic degradation (%) (R2) were employed in this study. The polynomial design model was selected which comprised of quadratic design models for both R1 and R2.

The significance of a fitted model along with the terms in the model was analysed using analysis of variance (ANOVA) in combination with Fisher's statistical test (P-value < 0.05). The optimum value of a predicted response and the corresponding levels of the independent variables were calculated by solving the set of equations derived by the differentiation of the fitted model.

Table 6.1 Variable factors for optimization study using RSM.

Factor	Name	Units	Minimum	Maximum	Coded values	Std. Dev.
A	Anode pH	pH	5.5	8.5	-1.0 = 5.5 0.0 = 7.0 +1.0 = 8.5	1.0607
B	Cathode pH	pH	4	10	-1.0 = 4.0 0.0 = 7.0 +1.0 = 10.0	1.9365
C	Inoculum size	(v/v, %)	10	40	-1.0 = 10 0.0 = 25 +1.0 = 40	9.6825

6.3 Results and Discussion

6.3.1 Preliminary Optimization

Three microbial cultures namely *Bacillus subtilis*, domestic mixed culture and industrial mixed cultures were utilized in the preliminary experiment for searching the optimal operation time for a batch MFC mode. By using constant and moderate conditions in terms of pH in anode and cathode chambers as well as inoculum size, the best operation times to achieve the optimal performance of current generation and phenolic degradation were determined between 20 – 50 h (Fig. 6.1). The consistent evolution profiles suggest that the MFC systems using all three cultures were in agreement for their cultivation times in the MFC system. The extents of current generation and phenolic degradation were dramatically dropped after 60 hours, due to key nutrients limitations [17] and accumulation of inhibiting metabolites.

Bacillus subtilis-MFC reached the highest outcomes in terms of both current generation (80 mA/m^2) and phenolic degradation (48%), followed by industrial and domestic mixed cultures. The remarkable MFC performance shown by *B. subtilis* might be due to the synergistic effect of its pure bacterial culture in homogenously consuming the phenolic substrate while transferring electrons to the anode surface. Although similar level of cell densities was used for each of three bacterial cultures for the startup MFC period, the varying trend in both responses could be due to competition among participating diverse exoelectrogens with non-exoelectrogens in substrate consumptions as well as electron transfers. That is why the *B. subtilis*-MFC could reach higher current density and phenolic degradation due to this absence of arbitrary culture environment. Therefore, *B. subtilis* was selected to proceed the subsequent optimization experiment using polynomial design models by RSM.

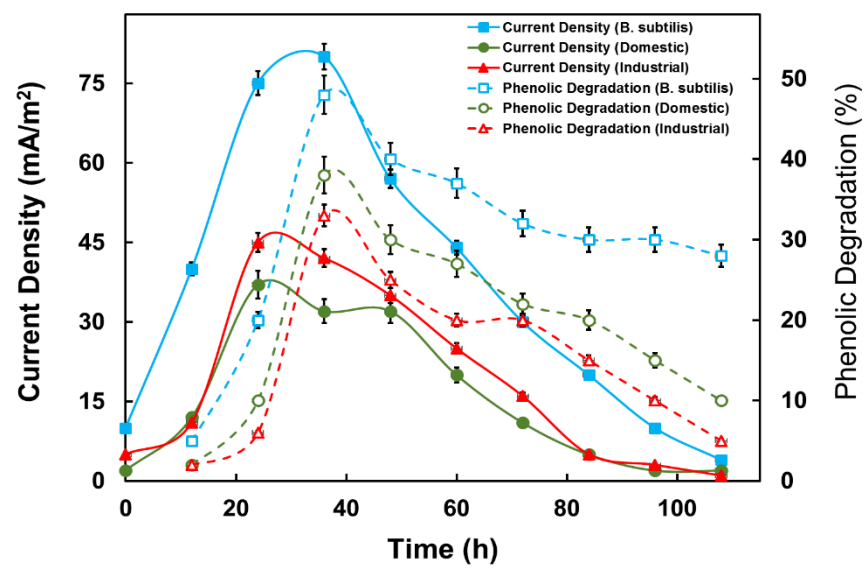

Figure 6.1 Preliminary results for optimizing the MFC cultivation time in terms of maximum current density and phenolic degradation by the selected bacterial cultures.

6.3.2 Design Summary

Analysis of variance (ANOVA) was used to analyze the optimization models for the two responses i.e., current generation and phenolic degradation. The data of the current generation and phenolic degradation measured from the 25 experimental runs are provided in the **Supplementary Data**. Table 6.2 summarizes the key experimental values obtained for the two responses using polynomial designs. The quadratic models for both current density (R1) and phenolic degradation (R2) responses required transformation as the ratio of maximum to minimum (15.3 and 14, respectively) was greater than 10 (normally ratios less than 3 the power transforms have little effect).

Table 6.2 Polynomial design data for the two responses.

Response	Minimum	Maximum	Mean	[a] Std. Dev.	Ratio	[b] Trans	Model
R1, Current Density (mA/m^2)	7.83	119.78	80.44	35.63	15.3	Square Root	Quadratic
R2, Phenolic Degradation (%)	5	70	31.44	18.06	14	Square Root	Quadratic

[a] Standard deviation; [b] Transformation

From Table 6.2, the optimum values for current density and phenolic degradation, 119.8 mA/m^2 and 70%, respectively, were increased significantly compared to the preliminary results. These results show that these optimization designs were potential statistical approach leading to efficient improvement of both responses. Then, the ANOVA was used (analyzed in the succeeding sections) to systematically select the optimum factor values and their interactions in order to achieve the optimized values of current density and phenolic degradation.

6.3.3 Current Density Response (R1)

6.3.3.1 Analysis of Variance for R1

Table 6.3 shows the ANOVA parameters for current density (R1) using quadratic model. The subplots show significant interactions within cathode pH (B), cathode pH-inoculum size (BC) and cathode pH-cathode pH (B^2). The R-squared (R^2) value of 0.88 was a good accordance to adjusted R^2.

Table 6.3 Analysis of variance for current density (R1).

Source	Term df	Error df	F	*p-value Prob > F	
Whole-plot	2	15.00	0.25	0.7855	Not significant
A-pH of anode	*1*	*15.00*	*0.27*	*0.6112*	
A^2	*1*	*15.00*	*0.22*	*0.6449*	
Subplot	7	15.00	12.58	< 0.0001	Significant
B-pH of cathode	*1*	*15.00*	*5.23*	*0.0372*	
C-inocculum size	*1*	*15.00*	*0.62*	*0.4434*	
AB	*1*	*15.00*	*0.13*	*0.7237*	
AC	*1*	*15.00*	*0.19*	*0.6658*	
BC	*1*	*15.00*	*44.58*	*< 0.0001*	
B^2	*1*	*15.00*	*20.17*	*0.0004*	
C^2	*1*	*15.00*	*1.10*	*0.3104*	

* *Values of Prob > F less than 0.050 indicate the model terms are significant*

The statistical data revealed that the cathode pH plays significant role in yielding positive effect on the quadratic optimization model for current density. Catholyte pH was found to have excellent contribution to the MFC performance in generating electricity [13, 18]. The pH in cathode chamber rectifies the electron transfer mechanism and enhance the rate of electron acceptance by the oxygen to become water and ultimately completing the electrical circuit of the MFC.

The empirical relationship between the current density response and variables in coded factors was quantified using quadratic expression (Eq. 6.2). The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor, and are useful for identifying the relative impact of the factors by comparing the factor coefficients. The model was further analyzed using the Anderson-Darling statistic to examine if the residuals are normally distributed [19]. According to this statistic, the residuals of current density model were normally distributed (data not shown).

$$\begin{aligned}\text{Current Density} = {} & + 10.14 + 0.17 * A - 0.80 * B + 0.28 * C \\ & + 0.14 * AB - 0.17 * AC + 2.62 * BC - \\ & 0.25 * A^2 - 2.84 * B^2 - 0.66 * C^2 \end{aligned} \quad (6.2)$$

6.3.3.2 Effect of A-B Model Terms Interactions for R1

Figure 6.2 illustrates the response surface plots for optimizing current density through the interaction between anode pH (A) and cathode pH (B). The contour plot displays a good interaction between these two factors as the predicted optimal current density (105.7 mA/m^2) was determined in the orange zone of the contour. The orange zone in the contour plot represents high response yielded by the specified factors. The optimized values of anode pH and cathode pH for this optimized current density were pH 7.5 and 6.6, respectively. It is noted that the optimized current density by *B. subtilis* favored neutral pH (~ pH 7.0 ± 0.5) for both anodic and cathodic MFC chambers. Previous findings reported that the higher buffer concentration at a pH 7.0 positively enhanced power generation in MFCs [20, 21]. Furthermore, Madani et al. [1] found that pH 7.4 resulted in the MFC power enhancement of nearly 1.3 times and Huang et al. [8] revealed that pH 7 yielded maximum current density followed by pH 6 and then pH 8 as the minimum current output.

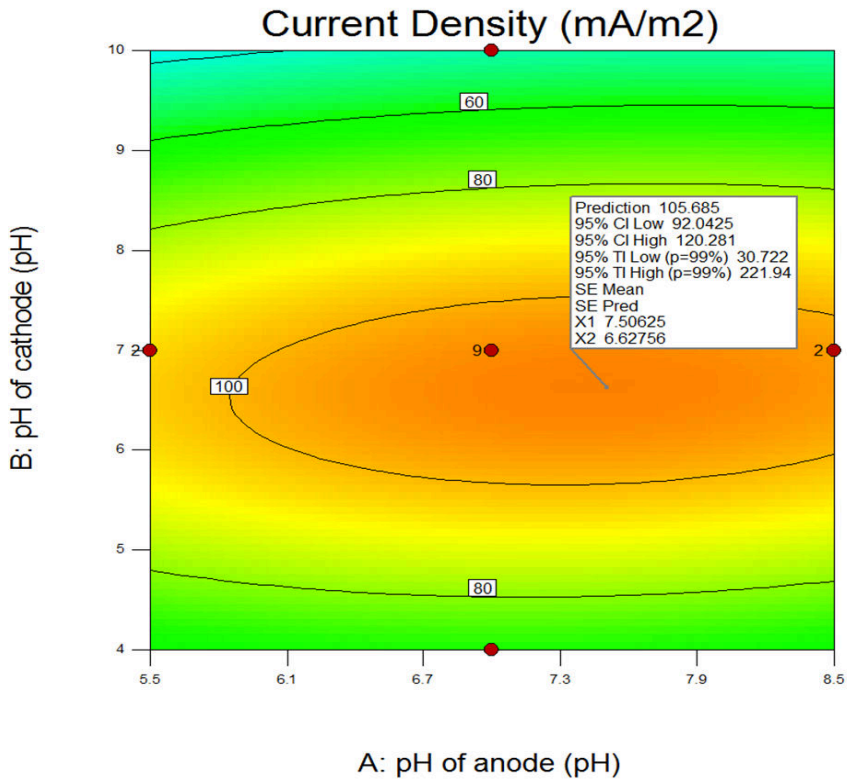

Figure 6.2 Response surface plot for optimizing the current density by anode pH-cathode pH (A-B) interaction. Notes: Orange indicates a high response; Green indicates a medium response; Blue indicates a low response.

The current density declined beyond a pH range between pH 5.5 – 7.8 in cathode chamber. This is due to the fact that the moderate level of pH would provide sufficient amount of protons, thus contributing to higher current generation in the MFC. Statistic results show that the best electricity generation could be obtained if the MFC was operated with a small favored pH range in cathode chamber and a broader pH range in anode chamber. This could indicate that anode pH was a slightly better model term compared to cathode pH as a very small pH change could hinder the MFC performance [13, 18]. This effect also may be attributed by the buffering capacity of the catholytes (phosphate and acetate buffer) used in the cathode chamber. It was found that the buffering property in cathode chamber could minimize the pH change in the MFC system thus improve the electron transfer leading to a higher current generation [13].

Considering the protons flow from anode to cathode chamber, a great pH change in catholyte (beyond neutral pH level) could have a negative influence on the electrochemically active bacteria in anodic chamber. *Bacillus subtilis*-MFC produces electrons and protons from degrading organic matter in the anodic chamber. A low pH in catholyte results in reducing the driving force for diffusion of protons through the membrane [1], thus making the protons accumulated in anolyte. This phenomenon may provide an unfavorable condition for electrochemical activities by *B. subtilis*, ultimately reduces the efficiency of MFC in generating electricity.

Generally, the pH effects on bioelectrochemical activities in MFC are associated with electron transfer pathways in anode chamber including intracellular electron transfer (IET), extracellular electron transfer (EET) and the diffusion of reduced 2,4-DCP to the anode electrode [22]. The step-wise potential losses can drive the electron flow from the electron donor to the anode, resulting in the generation of the electrical current [22]. In the IET process, half cell reaction (Reaction 6.1) should be considered for determining the electron production rates and the simultaneous anode potential.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad \text{(Rxn. 6.1)}$$

Nernst equation (Eq. 6.3) could be applied to derive the specific theoretical redox potential of the electron donor.

$$E = E^0 - \frac{RT}{nF}\ln\frac{[CO_2]}{[C_6H_{12}O_6]} + a\ \text{pH} \quad (6.3)$$

Since H^+ is involved in the cell reaction, the electrode potential, *E* depends on the pH. It is suggested that the redox potential of glucose increased when the anode pH was increased, indicating that the reducing ability of electron donor decreased as the pH increased [22]. That is the reason the contour slope of high response for current generation (Fig. 6.2) is pointing out to the increasing anode pH.

The half reduction reaction at the cathode chamber (Reaction 6.2) could also be affected by H^+. Therefore, the response of current generation by the electron transfer mechanism could be varied with pH in the cathode chamber. However, based on Fig. 6.2, the trend of cathode pH in responding to the optimum current generation is narrowed signifying that the cathode pH was less effective on the response than that of anode pH. This phenomena

could be interpreted by the influence of buffering capacity of the phosphate buffer catholyte used in this MFC experiment [13], which compensate the role of H^+ in the electron transfer pathway.

$$24H^+ + 24e^- + 6O_2 \rightarrow 12H_2O \quad \text{(Rxn. 6.2)}$$

In addition, it was reported that *B. subtilis* showed an optimum growth rate when the growth medium was adjusted at a narrow pH range of 6.5 - 7.0 [23 – 25]. Hence, the optimum electricity generation in the MFC system may be correlated with optimum growth of *B. subtilis* at this pH range. It is known that the pH of the medium could have significant influences on most of the biological processes that are required for microbial growth [14]. In addition, a broader anode pH range may be explained by the indefinite growth and biochemical activities by the *B. subtilis* which might affect the current generation. In other words, a high pH range in anolyte gave little influence on producing optimum current density.

6.3.3.3 Effect of A-C Model Terms Interactions for R1

The interaction between anode pH and inoculum size (C) was examined as illustrated in Fig. 6.3. The contour plot indicates that the optimum current density of 105.02 mA/m^2 was statistically determined at an optimum anode pH 7.5 and inoculum size of 28.1% (v/v). The optimum current density for this interaction model was also laid in the orange zone, specifying the response was highly favorable. However, the curvature peak seems to be enlarged which indicates that the optimum value positioned in a broader range of the model (approximately pH 6.5 - 8.0 and 22 – 34% for anode pH and inoculum size model terms, respectively). The anode pH-inoculum size interaction seemed to be linearly proportional to the current density. The current density increased when the anode pH and inoculum size were increased up to the aforementioned optimum values and vice versa. Figure 6.3 also demonstrates that optimum current density slightly favored inoculum size model term as compared with anode pH due to a slender shift of contour peak towards inoculum size model term.

There is lack of studies found in literature reporting the impact of inoculum size on MFC performance. Hence our results provide useful data especially on the optimization of the MFC system. These data imparted some values how the bacterial populations could

influence on the MFC performance in terms of current generation. A right bacterial inoculum size is needed to degrade organic matters and produce electrons and protons in order to stably complete the electrical circuit of MFC system. Excessive bacterial volumes may hinder the mass transport in biofilm thus inhibit the electron transfer from the bacteria to the anode surface. Furthermore, a congested bacterial community may create an unfavorable biofilm thickness and structure on anode surface which in turn decrease the efficiency of materials diffusivity and electron transfers, hence the current generation [26, 27]. The positive linear relationship between these two model terms collectively proves that the bacteria can carry out the electrochemical activities harmoniously in the moderate levels of pH and bacterium population size.

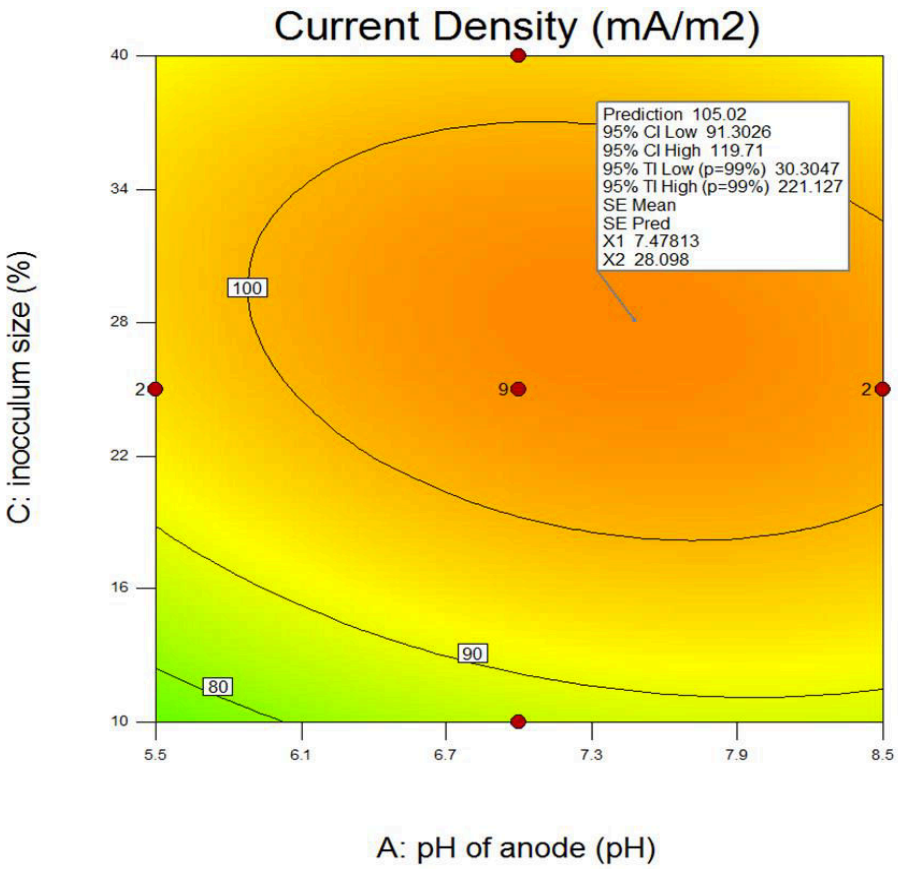

Figure 6.3 Response surface plots for optimizing the current density by anode pH-inoculum size (A-C) interaction. Notes: Orange indicates a high response; Green indicates a medium response; Blue indicates a low response.

6.3.3.4 Effect of B-C Model Terms Interactions for R1

A different trend was observed when inoculum size was interacted with cathode pH as demonstrated in Fig. 6.4. The stretched strips throughout inoculum size plot indicate that peak value of current density was found in a broader range of inoculum size but in a much smaller range for the cathode pH. It was found that cathode pH 6.2 and inoculum size of 20.9% contributed to the optimum current density of approximately 105.7 mA/m^2. Like previous interaction models, this interaction also indicates that the optimum current density was achieved in the orange region which represents high response for this model. However, it is obvious that an increased in the cathode pH and inoculum size would decrease the current density as demonstrated by the clear changing color of the contour plots from orange to green and finally to blue which represents the lowest response for this model.

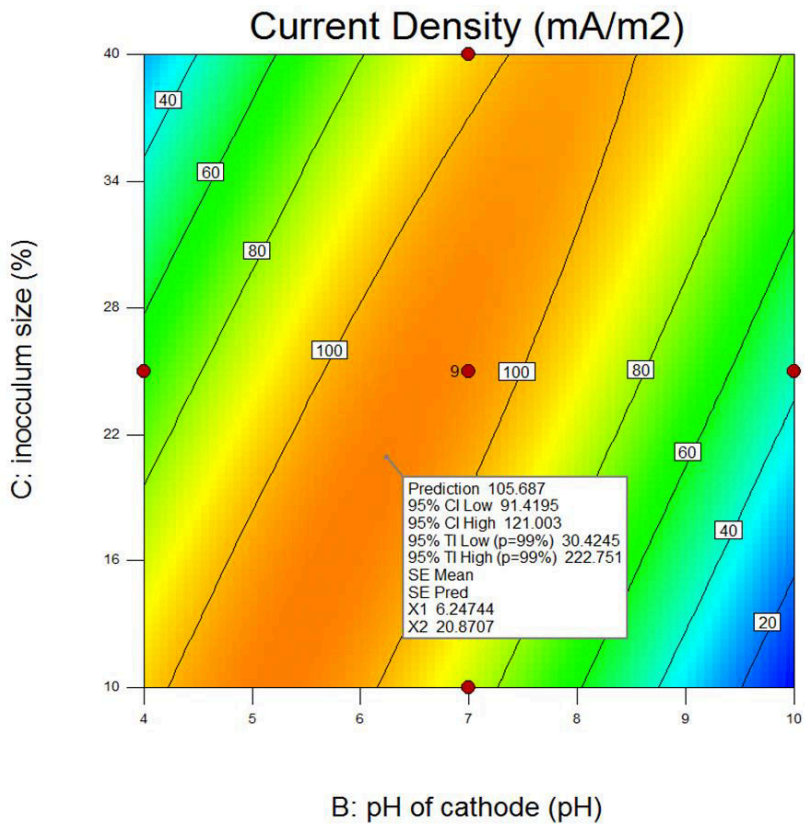

Figure 6.4 Response surface plots for optimizing the current density by cathode pH-inoculum size (B-C) interaction. Notes: Orange indicates a high response; Green indicates a medium response; Blue indicates a low response.

The interaction between inoculum size and cathode pH seemed to be more delicate compared with the other two interactions as discussed above. The range of inoculum size which contributed to the optimum current density (in the orange zone) was extended from the lowest level (-1 = 10%) to the highest level (+1 = 40%) or even slightly exceeded this range. This recommends that the selection of the bacterial inoculum size for this model could be less supportive and must be highly considered for future work. However, this inoculum size (~21%) was reliable since it is comparable with previous value (28%) and its interacted cathode pH (pH 6.2) and optimum current density (105.7 mA/m^2) were very much consistent with previous ones (pH 6.6, 105.7 and 105.02 mA/m^2, respectively).

6.3.4 Phenolic Degradation Response (R2)

6.3.4.1 Analysis of Variance for R2

Table 6.4 shows the ANOVA data for phenolic degradation (R2) using quadratic model. The whole-plot and subplot were both significant with anode pH (A) and cathode pH-cathode pH (B^2) interactions were significant model terms. The R^2 value was 0.73 lower than that of R1.

Table 6.4 Analysis of variance for phenolic degradation (R2).

Source	Term Df	Error df	F	p-value Prob > F	
Whole-plot	2	15.00	8.29	0.0038	Significant
A-pH of anode	*1*	*15.00*	*12.84*	*0.0027*	
A^2	*1*	*15.00*	*3.73*	*0.0725*	
Subplot	7	15.00	3.84	0.0136	Significant
B-pH of cathode	*1*	*15.00*	*2.06*	*0.1717*	
C-inocculum size	*1*	*15.00*	*3.18*	*0.0949*	
AB	*1*	*15.00*	*0.46*	*0.5067*	
AC	*1*	*15.00*	*1.82*	*0.1978*	
BC	*1*	*15.00*	*0.22*	*0.6455*	
B^2	*1*	*15.00*	*5.68*	*0.0309*	
C^2	*1*	*15.00*	*3.24*	*0.0920*	

The empirical relationship between the phenolic degradation response and variables in coded factors was quantified using quadratic expression (Eq. 6.4). The model was further analyzed using the Anderson-Darling statistic showing that the residuals of phenolic degradation model were normally distributed (data not shown).

$$\text{Phenolic Degradation} = + 5.92 + 1.18 * A + 0.52 * B + 0.64 * C + 0.28 * AB + 0.54 * AC - 0.19 * BC + 1.08\ A^2 - 1.55 * B^2 - 1.17 * C^2 \quad (6.4)$$

6.3.4.2 Effect of A-B Model Terms Interactions for R2

Figure 6.5 (a) demonstrates the interaction between anode pH and cathode pH (A-B) in defining the optimum 2,4-DCP degradation by the *B. subtilis*-MFC. The contour plot shows a slight arbitrarily ordered response at which the optimum phenolic degradation is rather challenging to be interpreted. Nonetheless, by looking at the colored region, the high response (orange curvatures) seems to be favorable towards high anode pH with cathode pH 7.8. Different trend was reported that a pH range of 6.0–7.0 was observed to be optimal for maximum chlorophenol degradation rates in conventional biological processes [28 – 30]. However, our data could be explained that at a higher anode pH, more positive cathodic potential would lead to more negative anodic potential resulting in a more suitable condition for *B. subtilis* to degrade 2,4-DCP [6]. In addition, a slightly lower cathode pH inhibits the over-potential and ohmic loss. Protons are a reactant in the cathodic reaction, and a low pH assures that protons are available in high concentration. Thus, the mass transfer loss, resulting from the cathodic over-potential, could be largely reduced under a low pH [6, 31] leading to an improved MFC performance.

A lower response can be observed in the contour plot of Fig. 6.5 (a) which might also show that the optimum phenolic degradation inclines towards lower pH. This could be explained by the consumption of protons in the degradation of 2,4-DCP into phenol through reductive dehalogenation process (Reaction 6.3). The more acidic conditions could therefore promote the 2,4-DCP degradation forward in a positive direction, although a low pH might be inhibitory to exoelectrogenic bacteria [8].

$$C_6H_4Cl_2O + 2H^+ \rightarrow C_6H_6O + 2Cl^- \quad \text{(Rxn. 6.3)}$$

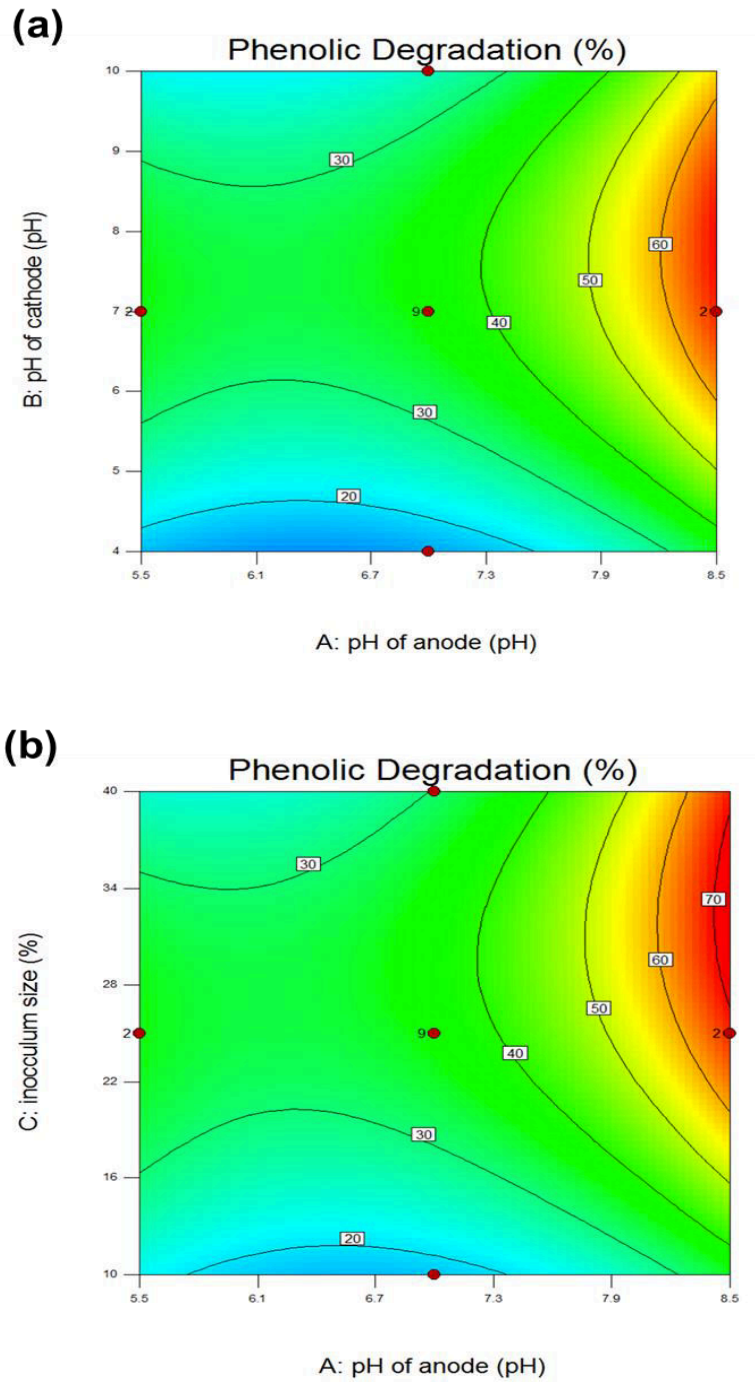

Figure 6.5 Response surface plots for optimizing the phenolic degradation by **(a)** anode pH-cathode pH (A-B) and **(b)** anode pH-inoculum size (A-C) interactions. Notes: Orange indicates a high response; Green indicates a medium response; Blue indicates a low response.

6.3.4.3 Effect of A-C Model Terms Interactions for R2

The same trend is observed when the anode pH was interacted with inoculum size (A-C) as illustrated in Fig. 6.5 (b). The optimum phenolic degradation was difficult to be determined as the peak contour or single curvature was absent in the plot. Like A-B interaction, this contour plot suggests that the phenolic degradation response could be split into low response and high response. High response (orange region) was again pointed towards high anode pH with 34% inoculum size. The low response (blue region) occurred at anode pH 6.5 and 10% inoculum size. Although a direct mechanism relating the pH and the phenolic degradation is apparently unspecified, the controlling H^+ concentration could alter the biological processes required for microbial growth as well as substrate degradation [14].

6.3.4.4 Effect of B-C Model Terms Interactions for R2

Interaction of cathode pH-inoculum size (B-C) model terms as shown in Fig. 6.6, provided more significant values as compared to the other two interactions. The contour plot shows that the optimum 2,4-DCP degradation laid in moderate response occurring at cathode pH 7.6 with 29.6% inoculum size. The phenolic degradation decreased when a lower cathode pH and a specified inoculum size were used in the MFC systems. Although a low cathode pH provides preferable MFC conditions by reducing over-potential and ohmic loss, the increasing cathode pH could be compensated by the higher inoculum size, thus yielded positive effect on the phenolic degradation response. It is known that ohmic loss is significantly counterbalanced because the sufficient protons are provided without the requirement of transfer through membrane to the cathode. The less ohmic loss results in a lower ohmic resistance, consequently enhancing electron transfer capability and MFC performance [6]. Figure 6.6 also displays an interesting fact that a small elevation in inoculum size will reduce the rate and extent of phenolic degradation. This might be attributed to the limitation in medium components and key nutrients like nitrogen and phosphorus [17].

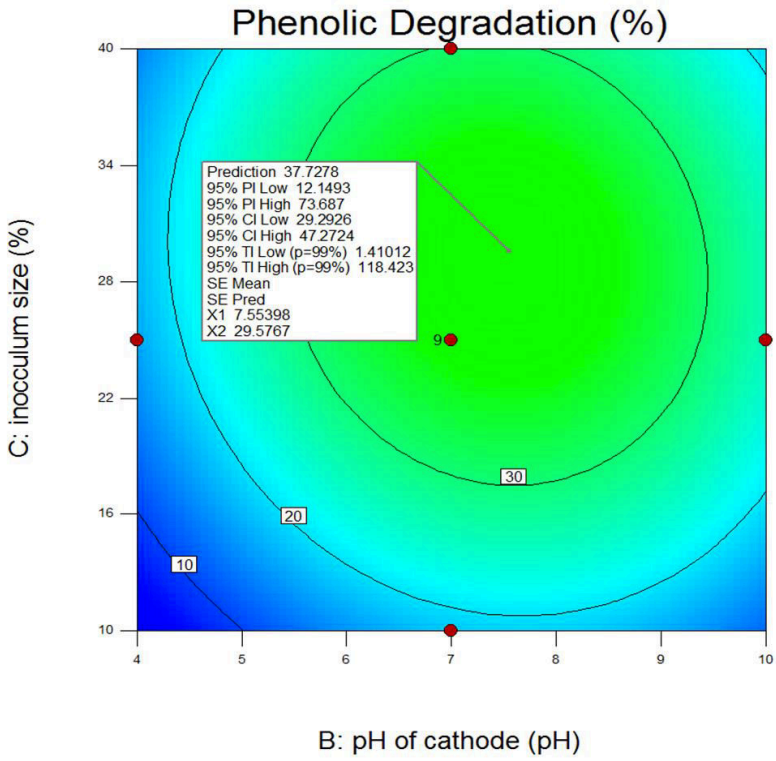

Figure 6.6 Response surface plot for optimizing the phenolic degradation by cathode pH-inoculum size (B-C) interaction. Notes: Orange indicates a high response; Green indicates a medium response; Blue indicates a low response.

6.3.5 Validation of Optimization for Combined Predicted Responses R1-R2

The two responses of the optimized models could be combined resulting in the predicted optimized parameters to be validated. The validation of the combined optimum values could provide the broad interaction mechanisms for further understanding of the interacted parameters. Table 6.5 shows the predicted values of the three model terms and the experimental results obtained from the triplicate trials of experiment. The predicted optimum current density of 110 mA/m^2 and optimum phenolic degradation, 73% with anode pH 7.6, cathode pH 6.8 and inoculum size of 27% lead to generation of 80% desirability for the optimization model.

Based on the validation table, the predicted optimized model terms values were replicable with relative differences of less than 15% and 10% for optimum current density and phenolic degradation, respectively. These small percentage discrepancies and high desirability suggest that the optimized models as quadratically expressed in Eqs. 6.2 and

6.4 were highly fitted for generating the optimum current density with the aforementioned phenolic degradation by *B. subtilis*-MFC system. The preferred values of anode and cathode pHs and bacterial inoculum size by the quadratic model could be further utilized in higher level of modelling approach leading to more comprehensive response interactions in optimization study.

Table 6.5 Validation of optimization of current density and phenolic degradation using predicted optimized parameters; anode pH= 7.6±0.1; cathode pH=6.8±0.1; inoculum size=27%. The desirability of these predicted optimized conditions is 80%.

Trial	Optimum Current Density, R1 (mA/m^2)		% Difference for R1	Optimum Phenolic Degradation, R2 (%)		% Difference for R2
	Experimental	Predicted		Experimental	Predicted	
1	119	110	8.2	68	73	6.8
2	124	110	12.7	70	73	4.1
3	115	110	4.5	66	73	9.6

6.4 Conclusions

Polynomial models by response surface methodology were used to better understand the interactions among anode pH, cathode pH and inoculum size for optimizing MFC system for current generation and 2,4-DCP degradation. Current generation response fitted well to quadratic model with R^2 0.88, and relatively lower R^2 (0.73) was achieved by phenolic degradation response using the quadratic model as well. The significant model terms of cathode pH (B), cathode pH-inoculum size (B-C) and cathode pH-cathode pH (B^2) interactions resulted in consistent optimum current generation (106 mA/m^2) by *B. subtilis*-MFC. However, the phenolic degradation demonstrated slightly arbitrary interactions among those three model terms, except for cathode pH-inoculum size. The intermediate inoculum size with neutral pH for both anode and cathode were found to have desirable interactions for current generation, while higher anode pH with the same level of inoculum sizes favors phenolic degradation optimization.

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Supplementary Data

Table S6.1 Data for responses current density and phenolic degradation with averaged values from duplicate trials.

Std.	Group	Run	Factor 1 A:Anode pH	Factor 2 B:Cathode pH	Factor 3 C:Inocculum size	Response 1 Current Density	Response 2 Phenolic Degradation
			pH	pH	%	mA/m^2	%
8	1	1	8.5	10	40	88.4	34
6	1	2	8.5	10	10	15.83	35
5	1	3	8.5	4	10	96.94	12
7	1	4	8.5	4	40	15.61	41
11	2	5	7	7	25	90	30
9	2	6	7	7	25	91.11	35
10	2	7	7	7	25	103	40
13	3	8	5.5	7	25	111	30
12	3	9	5.5	7	25	110	47
2	4	10	5.5	10	10	7.83	8
3	4	11	5.5	4	40	16.22	5
1	4	12	5.5	4	10	70.44	14
4	4	13	5.5	10	40	65.21	8
22	5	14	7	7	25	103	25
20	5	15	7	7	25	103	30
21	5	16	7	7	25	103	35
15	6	17	8.5	7	25	90	67
14	6	18	8.5	7	25	93.11	70
23	7	19	7	7	25	103	30
25	7	20	7	7	25	103	35
24	7	21	7	7	25	103	35
18	8	22	7	7	10	74.33	7
19	8	23	7	7	40	119.78	60
16	8	24	7	4	25	113.17	9
17	8	25	7	10	25	20.94	44

CHAPTER 7

~ CONCLUSIONS ~

Chapter 7: Conclusions

7.1 Conclusions

Electricity generation and 2,4-dichlorophenol (2,4-DCP) degradation by various microbial cultures using double chambered microbial fuel cell MFC have been the main subject of investigations in this project. The diversity of exoelectrogenic and bioelectrochemical activities of three selected microbial cultures including *Bacillus subtilis*, domestic and industrial petrochemical mixed consortia was utilized to promote the MFC performance in degrading the recalcitrant 2,4-DCP. The findings of the fundamental and systematic investigations based on topics (chapters) were concluded as the followings:

1- The MFC performance by *B. subtilis* was analyzed by employing various catholytes characteristics including oxidizing, buffering and salinity, for their impact on bioelectrochemical activities, electron transfer mechanisms as well as 2,4-DCP degradation. Potassium persulfate (PP) with its oxidizing property, appeared to be the best catholytes among others (M9, phosphate buffer (PB), NaCl and water) to increase current density significantly (64.0 mA/m^2) and 2,4-DCP degradation. Although NaCl was found to be promising catholytes for current generation in previous study, our study found that the NaCl along with water catholyte, attained lower electron transfer capabilities, thus current generation. The experimental data indicated that PP had high capability of solving diffusional and electrochemical restriction by *B. subtilis*. The electron transfer mechanism analysis demonstrated that PP, M9 and PB were vital catholytes in improving current generation and phenolic degradation through preventing large pH change that could negatively affect the MFC performance. In a nutshell, pure culture *B. subtilis* could be further employed in MFC to yield higher electricity and 2,4-DCP degradation with low concentrated, low-cost and low-toxicity catholytes especially persulfate-based variant catholytes.

2- The bioelectrochemical activities and microbial community changes towards 2,4-DCP degradation were investigated using domestic (DMC) and petrochemical industrial microbial consortia (IMC) in the Pt/Ti cathodic double chamber MFC systems. 2,4-DCP was greatly degraded by DMC (60%) with dominant *Arcobacter* and *Cloacibacterium* demonstrated positive response to 2,4-DCP degradation. IMC

which was dominated by *Bacillus*, was found to synergistically improve the MFC current outputs with relatively lower performance in 2,4-DCP biodegradation. CV and LSV analyses proved that the IMC was more capable in utilizing the organic compounds at the starting period and efficiently transferring the electrons in the MFC system, leading to higher current generation than that of DMC. Our MFC systems inoculated with DMC and IMC were also found to degrade 2,4-DCP with higher rate compared to anaerobic biodegradation system using the same microbial consortia. Therefore, it can be deduced that DMC- and IMC-MFCs with their diverse bacterial community improved 2,4-DCP biodegradation with simultaneous electricity generation.

3- The 2,4-DCP biodegradation and kinetic studies were evaluated using biofilms of domestic and petrochemical based industrial microbial consortia in the double chambered MFC systems. Both microbial consortia fitted well using Haldane inhibition model, Michaelis-Menten and Hanes-Woolf models. Domestic microbial consortia (dMFC) have been found to achieve higher apparent kinetic values with maximum specific growth rate, μ_{max} of 0.037 h^{-1} and maximum biodegradation rate, V_{max} of 0.32 mg/L/h. Similar with the above findings, the biofilm of *Bacillus*-dominated consortia (iMFC) demonstrated higher open-circuit voltage in all 2,4-DCP concentrations compared to biofilm of *Arcobacter*-dominated dMFC. The biodegradation pathways of 2,4-DCP revealed that both iMFC and dMFC could degrade 2,4-DCP into simpler and less toxic metabolites of 3-oxoadipate and acetate, respectively. This study offered valuable kinetic data to improve the biodegradation capability of 2,4-DCP by selected microbial strains associated with the bacterial growth and bioelectrochemical activities engaged in the MFC systems.

4- Polynomial optimization models of electricity generation and 2,4-DCP degradation by *B. subtilis* in MFC systems were constructed using central composite design of response surface methodology. Three model terms [anode pH (A), cathode pH (B) and inoculum size (C)] were best correlated in quadratic models with R^2 values of 0.88 and 0.73 for optimizing current generation and 2,4-DCP degradation, respectively. The significant model terms of cathode pH (B), cathode pH-inoculum size (B-C) and cathode pH-cathode pH (B^2) interactions resulted in consistent

optimum current generation (106 mA/m^2) by *B. subtilis*. Nevertheless, the quadratic 2,4-DCP degradation model demonstrated slightly randomized interactions among those three model terms, except for cathode pH-inoculum size. The neutral pH for both anode and cathode with intermediate level of inoculum size were the desirable interactions for current generation, while higher anode pH with the same level of inoculum sizes favors 2,4-DCP degradation optimization.

The exploration of electricity generation and 2,4-DCP degradation by these three bacterial consortia shows that they contributed significantly on improving MFC performance both in current outputs as well as chlorophenol degradation. The bioelectrochemical, biodegradation and kinetic data revealed that our MFC systems cultivated with pure *B. subtilis*, *Bacillus* and *Arcobacter* spp. could be further employed in toxic pollutant degradation with simultaneous energy production using sustainable technique of MFC as compared to conventional anaerobic biodegradation system.

7.2 Recommendations

Although this project comprised broad bioelectrochemical analysis for MFC performance in terms of electricity generation and 2,4-DCP degradation by various microbial cultures, there are some rooms for improvement and recommendations needed to be addressed in the future study undertakings.

Firstly, the performance of double chamber MFC systems could be enhanced by various factors including electrode materials, bioanode and/or biocathode exoelectrogenic microorganisms, catholytes materials, catalysts and membrane, MFC designs and physicochemical parameters. Our study data revealed that chemical characteristics of catholytes could alter current generation through pH change profiles and electron transfer mechanisms. Persulfate- and phosphate-variants of catholytes with high oxidizing and buffering characteristics should be taken into consideration when using double chambered MFC with specific pure microbial cultures. The relationship between the *B. subtilis* growth with respect to toxic substrate and catholyte roles should be developed to further understand the mechanism of electron transfer from anodic chamber to cathodic chamber in MFC, thus enhancing the MFC overall performance.

Secondly, our bacterial community analysis for both domestic and petrochemical industrial wastewaters recommend that *Cloacibacterium* sp. as a newly found exoelectrogenic bacteria, is worth to be further isolated and explored in MFC cultivation especially for chlorophenol degradation. Our study on the industrial microbial consortia has also supported the previous reported studies in which *Bacillus* strains, along with *Bacillus subtilis* play significant roles not only as a good phenols degrader, but also as promising exoelectrogenic bacteria in MFC systems. Therefore, *Cloacibacterium* and *Bacillus* should be utilized as leading bacterial culture in MFC cultivation for phenolic compound degradation.

Last but not least, more kinetic models are required to examine the rate of chlorophenol degradation in MFC such as Yano model, Aiba model, Edward and Webb models using statistical software for instance MATLAB. This modeling could be more accurately interpreted and comprehensively examined in a shorter time. Various models using statistical software could generate broad comparison of biodegradation and kinetic profiles, thus providing better solution in the kinetic study. The future MFC kinetic study should also be compared to conventional biodegradation system, so as to obtain wider understanding on the bioelectrochemical effect on the biodegradation systems. The modeling approach is also required to optimize wider range of physicochemical factors including cultivation temperature and time, pH of anode and cathode, concentration and type of substrates and inoculum, to ultimately increase the MFC performance.

APPENDIX

~ Published papers & conference proceedings in the journal styles ~

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Chemical impact of catholytes on *Bacillus subtilis*-catalysed microbial fuel cell performance for degrading 2,4-dichlorophenol

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HIGHLIGHTS

- *Bacillus subtilis* is capable of catalysing MFC bio-electrochemical reactions.
- *B. subtilis*-MFC shows high capability to generate electricity and degrade phenols.
- We studied electrochemical impact of non-aerated catholytes on MFC performance.
- Phenol feeding shows small influence on current generation using CV and LSV.

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ABSTRACT

This study was to explore a *Bacillus subtilis*-catalysed microbial fuel cell (MFC) system for electricity generation and dichlorophenol degradation. Our research focused on understanding the chemical interactions of various catholytes having distinctive properties (oxidizing, buffering, and salinity) and their impacts on the electrochemical activities, bacterial growth and phenolic degradation in the MFC system. Our experimental results revealed that *B. subtilis* is a potential exoelectrogenic bacterium for producing current density of 64.0 mA/m^2 while degrading 2,4-dichlorophenol in the MFC. Chemical properties of the catholytes and the pH change profiles could have significant impact on the bio-electrochemical activities, therefore the performance of the MFC system. Potassium persulfate was found to be the most suitable catholyte for generating the maximal power density of 9.5 mW/m^2 with a peak current of 1.11 mA over a potential of 0.45 V, while degrading over 60% 2,4-dichlorophenol. The *B. subtilis*-catalysed MFC could be a feasible technology for removal of hazardous phenol pollutants from industrial wastewater, while generating electricity.

1. Introduction

Microbial fuel cell (MFC) system is a unique sustainable biotechnology that is able to generate electricity through biodegradation of various organic compounds. MFC has received increasing research interests in broad fields; biochemistry, biochemical engineering and wastewater treatment. MFC has been recognised as a potential technology for the integration of electricity generation with wastewater treatment. MFC system relies on exoelectrogenic microorganisms to catalyse the electrochemical reactions occurring on electrode surfaces. To date, the applications of a number of pure or mixed cultures including *Clostridium* [1], *Pseudomonas* [2], *Shewanella* [3] and *Geobacter* [4] species have been reported.

Phenol and its derivatives are considered as refractory hazardous pollutants in wastewater. *Bacillus* species have been employed in many studies on the degradation of phenolic compounds. Pankaj [5] studied the decolourization of nitrophenol compound by *Bacillus subtilis* and reviewed the degradation of chlorophenols and their derivatives by various bacteria including *Bacillus* species [6]. *Bacillus cereus* isolated from a phenolic contaminated pond was found to be able to remove 2,4-dichlorophenol [7] and a strain of *B. subtilis* was able to oxidize mono- and dimethoxyphenols using laccase bound enzymes [8]. These phenolic biodegradation studies were carried out in the conventional biodegradation experiments and not in the MFC systems. To the best of our knowledge, there was no reported work so far in which the pure culture *B. subtilis* is used to degrade recalcitrant toxic compounds (particularly 2,4-dichlorophenol) through MFC systems. With the ability of *B. subtilis* to generate electricity in the

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MFC [9,10], we anticipate that this species could be a potential microorganism to catalyse MFC for electricity production while degrading organic hazardous waste including chlorophenols.

Many biochemical and systematic factors need to be considered in the development of MFC technology, including MFC design, microbial communities, electrodes and substrate chemistries [11]. Chemicals involved in the MFC system as catholytes, substrate, nutrients and reagent for pH control could have significant impact on the bio-electrochemical reactions and overall performance of the MFC system. Seeking suitable catholytes able to enhance the bio-electrochemical engineering performance of the MFC system is mainly empirical in previous studies. It should be noted that the diffusivity and transport mechanism of electrons in catholyte solutions can amplify current production in MFC systems. Previous studies used relatively high concentration of ferricyanide and permanganate as catholytes for investigating their effects on MFC performance [12,13]. However, these catholytes might be transformed into hazardous pollutants which impose negative impact on aquatic environments when discharged. Yongtae and Bruce (2013) found the use of saline catholyte to replace phosphate buffer in MFC resulted in high current production [14]. However, the concentration of saline catholyte needed to be relatively higher in order to offset the number of ions present in the catholyte solution that could produce greater electricity. High concentration of catholyte solution could also inhibit the performance of proton exchange membrane due to the concentration gradient imposed between the anode and cathode chambers. Therefore biochemical accessibility, electron transfer characteristics and costs of electrolytes need to be taken into consideration while studying MFCs.

Considering the significance of suitable microorganisms and catholytes used for MFC, this study aims at exploring *B. subtilis*-catalysed MFC system for simultaneous production of electricity and degradation of chlorophenol contaminant. Chlorophenol compounds are mostly disposed as organic wastes from palm oil, chemical processing and petrochemical industries. Our research focuses on studying how the chemicals of catholytes and chlorophenol as target hazardous pollutant could alter electrochemical mechanisms and affect the overall performance of the *B. subtilis*-catalysed MFC in terms of the electricity production and degradation of 2,4-dichlorophenol (2,4-DCP). Five catholytes, namely potassium persulphate (PP), M9 medium (M9), phosphate buffer solution (PB), saline NaCl (NaCl) and tap water (water), were examined in this study. The MFC experiments were carried out using low-concentrated catholytes with absence of air-purging so as to explore a low-cost and environmental-friendly operation mode. These experiments were expected to yield higher current generation and phenolic degradation.

2. Materials and methods

2.1. Microorganism, culture medium and catholytes

B. subtilis (AWQC 111) provided by the Australian Water Quality Centre, was used as a model bacterium in this study. The bacteria were grown in 100 mL of nutrient broth in a 250-mL shake flask for 20 h on an incubator shaker (175 rpm) at 37 °C. The bacterial cells were used as the seed culture for the MFC system.

M9 medium was used as the growth medium for the *B. subtilis*, which was prepared according to Miller [15] with slight modification by adding yeast extract to supply vitamin and amino acid. The modified M9 medium was composed of 0.2% glucose and 1 g/L yeast extract in mineral salts (1 g/L NH_4Cl, 3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl, 1 mmol/L $MgSO_4$, and 0.1 mmol/L $CaCl_2$). The pH of M9 medium was adjusted to pH 7.0 ± 0.2 with NaOH or HCl. The medium was autoclaved at 121 °C for 15 min. The glucose solution was sterilized using 0.22 μm filter unit (Millipore membrane PVDF) and then added to the autoclaved medium. The culture and medium preparation was carried out in a biological safety cabinet (Class II, BH2000 Series) to avoid culture contamination.

The MFC experiments were carried out using five catholytes with different initial pH (based on the original pH following the catholyte solutions preparation) and their distinctive characteristics were listed in Table 1. The pH changes of both anolytes and catholytes in the MFC systems were monitored throughout MFC operations using a pH meter (Eutech Instruments).

2.2. MFC reactor

A dual-chambered membrane MFC reactor was constructed from two glass bottles (250 mL) joined with a glass bridge, which consists a cationic exchange membrane (CMI-7000, gel polystyrene cross linked with divinylbenzene) with a 3-cm inner diameter. A 3.0 cm × 3.0 cm carbon cloth was used as the anode and cathode materials. Both electrodes were pierced with titanium wire projecting outside connecting to an external electrical circuit. The MFC glasses were autoclaved and the setup was carried out in the biological safety cabinet to avoid the glasses and culture contamination.

After transferring inoculum to the anodic chamber, air purging was immediately supplied in the anodic chamber to facilitate bacterial growth. The anodic chamber turned turbid after 12 h, indicating the bacterial growth. Then, air supply was disconnected at this stage and the joined parts of the anodic chamber were sealed with plasticine to block air penetration, thus creating an anoxic environment. The biofilm formation on the electrode surface was observed afterwards. The cathodic chamber was open for aeration. The MFC system was topped on a magnetic plate to ensure well mixing during operation and maintained in a room temperature. Fig. 1 shows a schematic diagram of the MFC setup used in this study with anolyte medium and 2,4-dichlorophenol, and various catholytes at the cathodic chamber under investigation. Various electrochemical reactions taking place in the MFC systems will be evaluated in the succeeding sections.

2.3. Biochemical analysis

2.3.1. Optical density vs. phenolic degradation

The optical density (OD) of bacterial growth was periodically determined using a UV–Visible spectrophotometer (Shimadzu,

Table 1
Characteristics of catholytes used in the *Bacillus subtilis*-catalysed MFC.

Catholytes (concentration)	pH*	Conductivity** (mS-cm)	Buffering property	Oxidizing property
Potassium persulfate, PP (50 mM)	3.0	11.79	–	√
M9	6.92	20.70	√	–
Phosphate buffer, PB (50 mM)	7.10	6.30	√	–
NaCl (50 mM)	5.83	11.15	–	–
Water	7.04	0.472	–	–

* Initial pH of catholyte measured before running the MFC experiment.
** Conductivity of catholyte measured at 25 °C.

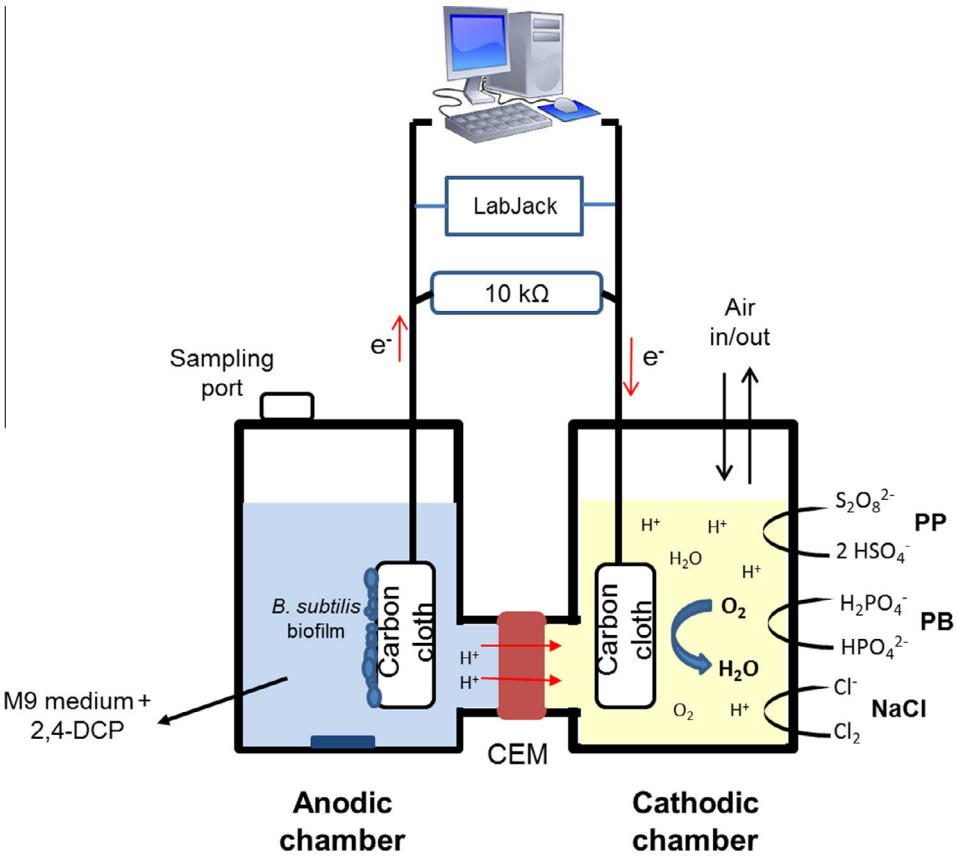

Fig. 1. Schematic diagram of double chamber MFC setup with various catholytes.

UV-1601) at a wavelength of 660 nm. The model chlorophenol compound used in this study was 2,4-dichlorophenol (2,4-DCP) (Alfa Aesar), which is a chlorinated derivative of phenol with the molecular formula $C_6H_4Cl_2O$. 10 mg/L 2,4-DCP was fed into the anodic chamber under anoxic condition. The 2,4-DCP concentration was examined using the UV-Visible spectrophotometer at a wavelength of 750 nm as described by Wilfred and Ralph [16]. Phenolic degradation was calculated based on the difference in 2,4-DCP initial and final concentrations.

2.3.2. Coulombic efficiency

To achieve a high power output MFC, it is expected to convert as much electrons stored in the substrate as possible to current [17]. The recovery of electrons is determined in terms of coulombic efficiency (CE) as described in Eq. (1),

$$CE = \frac{C_R}{C_{th}} \times 100\% \quad (1)$$

where C_R is the total coulombs through the external circuit in a complete circle by integrating current over time, and C_{th} is the theoretical amount of coulombs that can be calculated based on phenolic substrate removal.

2.3.3. Catholyte ionic change

The chemical changes of three selected catholytes namely potassium persulfate (PP), phosphate buffer (PB) and NaCl were analysed in terms of anionic reduction. The catholyte samples were taken before and after MFC operation. The concentrations of phosphate (PO_4^{3-}) and chloride (Cl^-) were measured using ion chromatography technique equipped with a HPIC pump (Waters, Model 515) and a conductivity detector (Waters, Model 430) with sample volume of 100 μL. The column used was anion column (Waters, IC-Pak A) with dimension of 4.6 × 50 mm, particle size of 10 μm and capacity of 30 ± 3 μeq/mL at a flow rate of 1.0 mL/min.

The concentration of persulfate ($S_2O_8^{2-}$) was measured using spectrophotometry technique equipped with UV–Vis spectrophotometer (Metertech, SP 8001). This spectrophotometry technique was based on procedures described by Liang et al. [18] with modified wavelength of 450 nm. The relevant data of ion chromatography and spectrophotometry techniques was provided in the Supplementary Data.

2.4. Electrochemical measurement

2.4.1. Voltage–current data acquisition

Voltage and current outputs were continuously recorded against time by a LabJack U6 recorder with an external load of 10 kΩ connected to a computer during 5 days operation of the MFC system. The current and anodic and cathodic potentials were measured with respect to operational time. To develop the polarization curve, different external loads were applied for a complete batch cycle, with a variable resistance ranging from 10 Ω to 100 kΩ.

2.4.2. Cyclic voltammetry and linear sweep voltammetry

Data for cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were recorded using a CHI 650 D bipotentiostat (CH Instruments, Inc., USA). The CV was conducted at a scan rate of 0.1 V/s ranging from 0 to 0.5 V (vs Ag/AgCl), while the LSV was conducted at a scan rate of 0.1 V/s ranging from 0.1 to 3.0 V (vs Ag/AgCl). To analyse the chemical impact of 2,4-DCP on the electrochemical activity with respect to various catholytes used, the CVs and LSVs

were performed before and after 2,4-DCP feeding into the anode solution.

The experiments were carried out in the MFC using three electrodes; working electrode, reference electrode (an Ag/AgCl electrode) and counter electrode. The working and counter electrodes were made of carbon cloths with the same size of 9.0 cm^2, pierced with titanium wires. All three electrodes were inserted into the MFC, avoiding any contact among these electrodes. The CVs and LSVs of the MFC with different catholytes were analysed during before and after 2,4-DCP feeding into the culture medium in order to examine the phenolic impact on the electrochemical activities of the *B. subtilis*-catalysed MFC system.

2.4.3. Electrochemical impedance spectroscopy

Data for electrochemical impedance spectroscopy (EIS) was obtained using the CHI 650 D bipotentiostat. The electrode materials were the same with CV and LSV as described above. To measure the anode impedance, the anode was connected to the working terminal, while the cathode and Ag/AgCl electrode were connected to the counter terminal and reference terminal, respectively. The working terminal was shifted to cathode and the counter terminal to anode when the cathode impedance was measured [19]. The EIS tests were carried out at a frequency range of 1.0–100 kHz with 0.005 V.

3. Results and discussion

3.1. Electricity generation

Electricity generation in term of current density (mA/m^2) was monitored in the *B. subtilis*-MFC reactors using five catholytes; PP, M9, PB, NaCl and water. Fig. 2 illustrates the electricity generation profiles in the MFC using five catholytes during 5 days operation. In general all catholytes-fed MFCs exhibited similar current generation profiles, implying similar biochemical activities occurred. During the first 20 h, *B. subtilis* seemed to adapt themselves to the new MFC environment showing a slow growth, resulting in low current densities. The bacterial growth was further maintained by feeding the glucose after 10 h as indicated by the solid arrow in Fig. 2. Thereafter, the MFC anodic chamber was fed with 10 mg/L of 2,4-DCP as shown by the dotted arrow in Fig. 2. This operation was to study the 2,4-DCP degradation associated with the current generation as well as the bacterial growth in the MFC.

The highest current density of 64.0 mA/m^2 was generated by PP-MFC, followed by M9-, PB-, NaCl- and water-MFCs with 32.0, 22.0, 4.6 and 4.0 mA/m^2, respectively. The high current density generation in PP-MFC suggests that the oxidizing and conductivity properties (Table 1) of potassium persulphate was able to

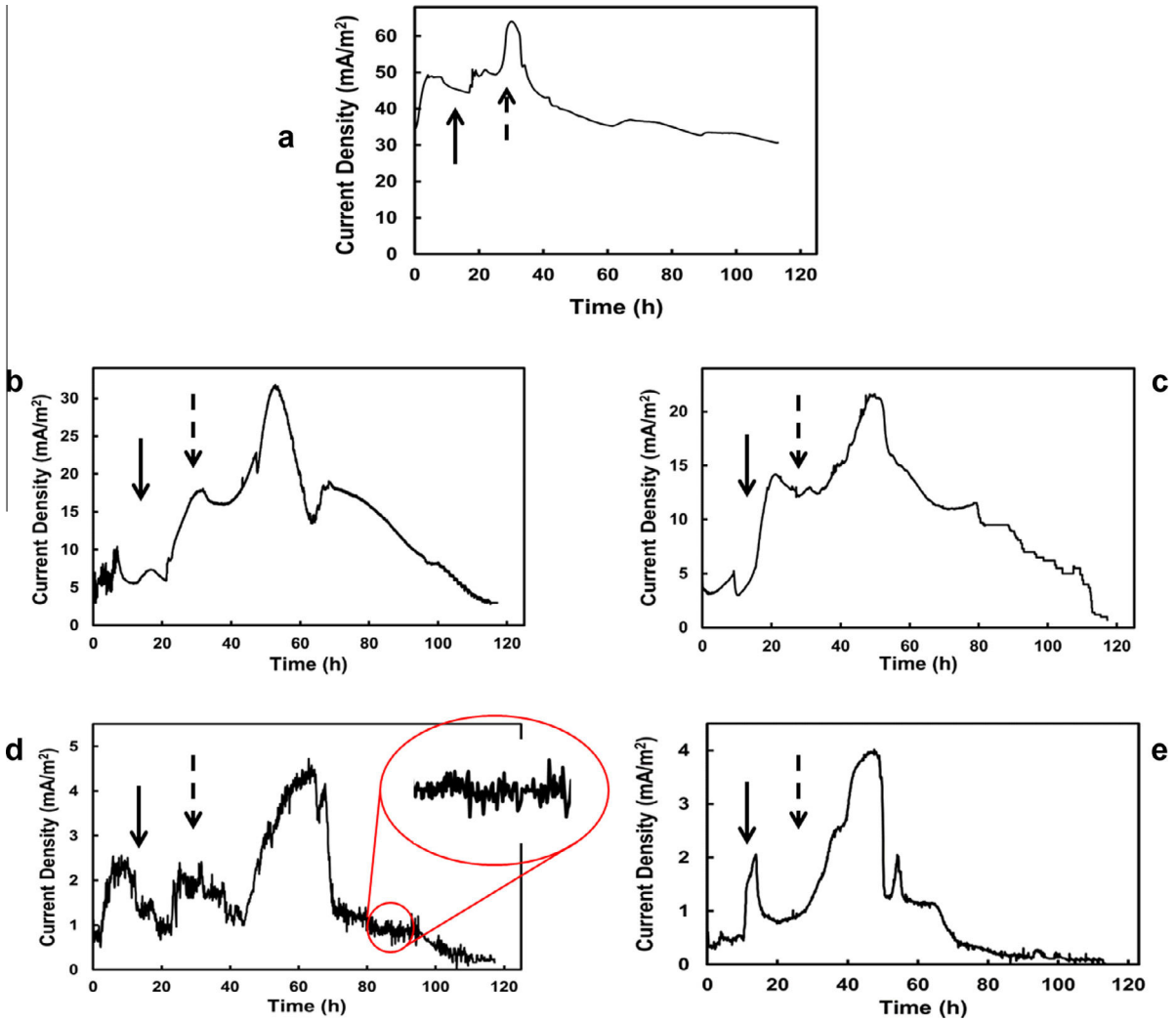

Fig. 2. Current density evolution profiles for *B. subtilis* MFCs using the catholytes of (a) PP, (b) M9, (c) PB, (d) NaCl and (e) water. The solid arrow shows the glucose feed while the dotted arrow shows 2,4-DCP feed.

accelerate the reduction reaction and electron transfer, thus intensifying the current production in the MFC system. After approximately 40–60 h of cultivation, PP-MFC (Fig. 2a) showed trivial decline of bacterial growth as evidenced by steadier drop of current density compared to other catholytes-fed MFCs that exhibited significant current decrease. This result demonstrated that there was a substrate limitation after a long cultivation, which affected the microbial metabolism and hence the current output [9]. It is also expected that the decline of current density for all MFC systems was due to the descending growth rate of *B. subtilis* in recalcitrant medium condition of 2,4-DCP.

It is interesting to note that the fluctuation profiles in current generation were observed in the NaCl- and water-MFC systems as compared to the other three MFC systems (see amplification of fluctuation trend in Fig. 2(d)). NaCl-MFC showed larger fluctuation in current generation as compared to water-MFC (Fig. 2(d) and (e)). This is because the Na^+ and Cl^- ions in the NaCl solution could interfere the flow of H^+ to the cathode chamber, thus misbalancing the proton concentration in the MFC systems leading to current fluctuation. Furthermore, NaCl- and water-MFCs showed a tremendous drop of current densities to almost below 1.0 mA/m^2 after 70 h. It can be deduced that NaCl, despite of its relatively high conductivity, showed inferior stability in the MFC, leading to inconsistency in current generation. In addition, it can be noted that the minimal conductivity level of water-MFC hindered the electron transfer between the MFC electrodes, thus resulting in its lowest current generation among all other catholytes-MFC systems.

It can be suggested that the PP, M9 and PB assisted the MFC to produce more stable electricity indicated by no current fluctuation in these systems. Moreover, PP-, M9- and PB-MFCs displayed insignificant current falls after 60 h. This can be inferred by the buffering capability for minimising pH changes as that could affect the electron transfer between MFC electrodes, thus inhibiting the current generation (in case of M9 and PB) [20]. Use of PP catholyte was found to be able to increase cathodic potential and reduce over potential during charge transfer process [21]. Moreover, the oxidizing property possessed by the PP could play an integral role in maintaining the redox reaction and electron transfer, thus stabilizing the current generation in the MFC.

3.2. Chemical impact of catholyte

3.2.1. pH Change profile

The pH changes (ΔpH) in the anodic and cathodic chambers of the catholyte-MFCs were monitored during the operation and the results are shown in Fig. 3(a). Since there was a cross-over of ions from anodic chamber to cathodic chamber through ion-exchange membrane, it is expected that the pH of anolytes and catholytes in the MFC were speckled throughout the cultivation period. In the anodic chamber, the microbes oxidized substrates such as glucose and some refractory organics (i.e., 2,4-DCP). For example, the oxidation of glucose generates electrons, protons and carbon dioxide (as shown in Reaction (2)). In the cathodic chamber, on the other hand, a reduction process occurs with the presence of oxygen molecules to form water as indicated by Reaction (3).

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad (2)$$

$$24H^+ + 24e^- + 6O_2 \rightarrow 12H_2O \quad (3)$$

Microbial reaction and phenolic degradation in the anodic chamber resulted negligible pH fluctuation (ΔpH < 0.09) in all MFC systems. Raghavulu et al. (2009) reported that the anodic environment generally controls the kinetics of electron transfer and thus current generation from microorganism to the electrode [12]. Wang et al. (2013) found that stable pH favours the fibre

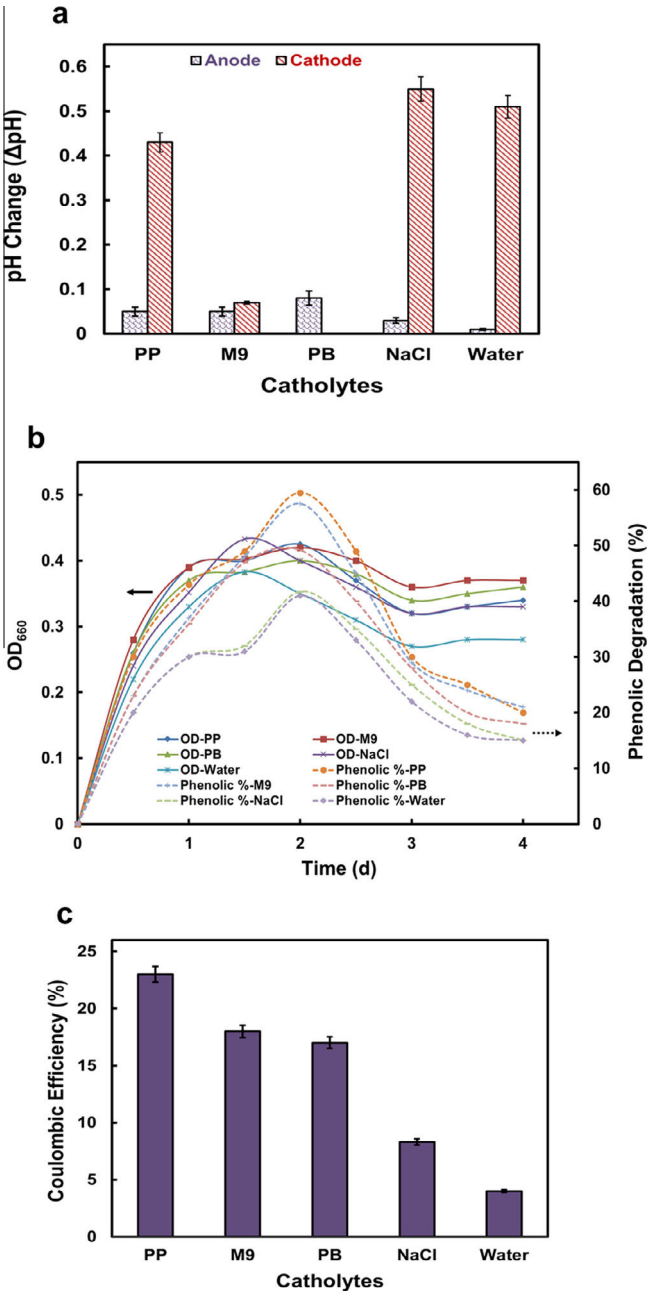

Fig. 3. (a) pH change (ΔpH) of the anodic and cathodic solutions of the MFCs with different catholytes. (b) Phenolic degradation with respect to growth profile of *B. subtilis* in the MFC with various catholytes. (c) Coulombic efficiency by *B. subtilis* using various catholytes.

degradation and electricity generation in mixed-rumen MFC [13]. These results further support that the unvaried pH in anodic chambers of our MFC systems can sustain the current generation throughout operations.

Unlike the anodic chambers, the catholyte solutions in the cathodic chambers showed diverse ΔpH among the five MFCs. A very low ΔpH measured in both anode and cathode of M9-MFC contributed to relatively higher current density among the other systems. The negligible ΔpH in both chambers of M9-MFC may be elucidated by the ions that are balanced between those chambers. This is due to the transfer of ions rather than H^+ and OH^- [14]. Furthermore, using PB, there was no ΔpH found in the cathodic chamber besides an extremely low ΔpH (~0.08) in its anodic chamber. It is clear to state that the negligible ΔpH in

M9- and PB-MFCs was due to the buffering characteristic of these catholytes, which obstructed ΔpH thus improving the MFC performance in electricity generation.

Nevertheless, the pH changes in PP-, NaCl- and water-MFC systems demonstrated contrary profiles as compared to the other two MFCs. The ΔpHs of the cathodic solution in the NaCl- and water-MFC showed the highest ΔpH (0.55 and 0.51, respectively), followed by PP-MFC with 0.43 ΔpH. This effect proposed that the fluctuations and low current production in those MFCs were due to the incapability (in case of NaCl and water) to maintain its initial pH throughout the operation. A similar performance was recorded by Yongtae and Bruce (2013), who found that the pH of a 240-mM saline catholyte-MFC dropped dramatically to almost pH 5.5 during cultivation, and identified that pH < 6 might inhibit current generation [14]. The relatively high ΔpH in PP-MFC was obviously due to the absence of buffering effect to maintain the pH of catholytes during reduction process.

The variance in pH change among these catholytes-fed MFCs was also due to the behaviours of the electron transfer and ionic diffusion through the ion-exchange membrane. That can be described by Nernst–Planck equation (Eq. (4)), where J_i is the ionic flux, D_i the diffusion coefficient for the ion, z_i the charge, ∇c_i the concentration gradient of the ion in solution, k Boltzmann's constant, e_o the elementary charge, E the electric field strength, and T the absolute temperature. It calculates the ion flux under the influence of both ionic concentration gradient and the electric field.

$$J_i = -D_i \nabla c_i + \frac{D_i z_i e_o E c_i}{kT} \quad (4)$$

The diffusion coefficients of PP, M9 and PB catholytes were relatively higher than that of NaCl and water (which contains trace level of ions): SO_4^{2-} ($\sim 16.0 \times 10^{-6}$ cm^2/s), PO_4^{3-} ($\sim 1.0 \times 10^{-5}$ cm^2/s), OH^- ($\sim 5.0 \times 10^{-9}$ cm^2/s) and Cl^- ($\sim 2.0 \times 10^{-9}$ cm^2/s). The small diffusion coefficients in NaCl and water could be favourable for the ion transport to the anode chamber through the membrane and increased the solution pH. It was reported that the pH decrease in the anodic chamber and pH increase in the cathodic chamber were due to less charge transferred by protons compared to other ions [14,20]. This further proved that the unbalanced protons present in the anode and cathode chambers for the NaCl- and water-MFCs resists the electron transfer in the circuit, hence lowering the current generation in these systems. Despite of the high diffusion coefficients (based on D_i of phosphate ions) of M9 and PB, the negligible ΔpH in M9- and PB-MFCs was due to the compensation of buffering effect in these solutions. This can result in stable and balanced protons and ions transfer between anodic and cathodic chambers, thus generating higher currents in MFC systems.

3.2.2. Anionic reduction in catholytes

PP, PB and NaCl were selected to study the reduction of their anions throughout the MFC operations in order to understand the relationship between the pH and anionic concentration changes. Ion chromatography data showed that these three catholytes endured minor changes in terms of their anions concentrations (data was provided in the Supplementary Data). 5.6% reduction of Cl^- concentration was found in the NaCl catholyte, followed by PP with its persulfate ($S_2O_8^{2-}$) of 1.05% and lastly PB (PO_4^-) with no notable change in concentration at all. These anionic reduction were in accordance to the pH change of the catholytes. The ΔpH of PP and NaCl (0.43 and 0.55, respectively) resulted in 1.05% and 5.6% of anionic reduction. The ΔpH in PB catholyte was minor in accordance to the absence of anionic reduction in the system. Although these anionic concentration changes were very small, the reduction reaction in catholytes might play vital role in associating the effect of pH change to the MFC performance.

The reduction in $S_2O_8^{2-}$ concentration in the catholyte gave precedence to its strong oxidizing and conductivity properties with an oxidation potential of 2.12 V (Reaction (5)). Its small concentration changes of 1.05% could be attributed to slow reaction kinetics at ordinary temperatures [22]. Under acidic conditions, persulfate anion can hydrolyse to form hydrogen peroxide (Reaction (6)).

$$S_2O_8^{2-} + 2H^+ + 2e^- \rightarrow 2HSO_4^- \quad (5)$$

$$S_2O_8^{2-} + 2H_2O \rightarrow H_2O_2 + 2HSO_4^- \quad (6)$$

The retaining capability of persulfate anion and its oxidizing property in the MFC catholytes might also be contributed by the presence of various oxidants and radicals with higher oxidation potentials including hydrogen peroxide (1.77 V) (Reactions (7)–(9)) [22,23]. The strong oxidants and free radicals build up in the catholyte might offset the presence of persulfate anions, thus minimising the anionic reduction reaction.

$$S_2O_8^{2-} + H_2O \rightarrow HSO_5^- + HSO_4^- \quad (7)$$

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{-\cdot} \quad (8)$$

$$SO_4^{-\cdot} + e^- \rightarrow SO_4^{2-} \quad (9)$$

For saline NaCl catholyte, the solubilisation of the saline in water produces sodium and chloride ions (Reactions (10) and (11)). The chloride anions were further reduced to chlorine Cl_2 which might affect the change of pH of the catholytes.

$$NaCl + H_2O \rightarrow Na^+ + Cl^- + H_2O \quad (10)$$

$$2Cl^- \rightarrow Cl_2 + 2e^- \quad (11)$$

Phosphate buffer system consists of dihydrogen phosphate ions ($H_2PO_4^-$) as hydrogen-ion donor and hydrogen phosphate ions (HPO_4^{2-}) as hydrogen-ion acceptor. These two ions are in equilibrium with each other as indicated by Reaction (12).

$$H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+ \quad (12)$$

The coming H^+ ions from anode did not affect much on the equilibrium state of phosphate anions since the value of molar concentration of H^+ ions is equal to the value of the equilibrium constant. The pH is equal to pK_a namely 7.21 (or in the range of 6.9–7.4). That is the main reason of the ΔpH absence in the PB catholyte, resulting in no notable change in phosphate anion concentration.

3.3. Bacterial growth vs. phenolic degradation and coulombic efficiency

The bacterial growth and 2,4-DCP degradation were monitored over four days of the MFC operation. Bacterial growth 2,4-DCP degradation profiles with respect to catholytes used are presented in Fig. 3(b). The highest 2,4-DCP degradation of 60% was obtained by the PP-MFC, followed by M9-, PB-, NaCl- and water-MFCs with 57.5%, 49.2%, 42.5% and 41.7%, respectively. The trend of phenolic degradation seems to follow the fashion of bacterial growth in the MFC systems. Day 1.5–2 denoted the highest growth with optical density (OD) of the range 0.4–0.45. This optimum cultivation time corresponded to the highest phenolic degradation as depicted in the figure. These results revealed that the best degradation of 2,4-DCP occurred when the *B. subtilis* reached the highest growth level. These results agree with the results from Silambarasan and Vangnai [24], where a 10 mg/L toxic 4 nitroaniline was completely removed by *Acinetobacter* at day 1–2 of cultivation when the cell density reached constant value of 0.45 OD. The growth and phenolic degradation profiles also indicate a short lag phase which corresponds to the rapid bacterial acclimatization of 2,4-DCP by

B. subtilis. The phenolic degradation started to decline almost sharply after day 2.5, however, the bacterial growth was steadily proceeded with small decreasing trend. These profiles suggest the efficient ability of *B. subtilis* in not only surviving in high toxicity of 2,4-DCP, but also reflect its prompt induction and response to 2,4-DCP when detoxification is required [24].

The OD vs. phenolic degradation profiles also suggest that there were relatively small difference of growth level and phenolic percentage degradation among the catholytes in which the PP resulted the highest and water catholyte achieved the lowest level for the two parameters. These findings raise intriguing questions regarding the nature and extent of catholytes affecting the MFC performance in terms of bacterial growth and phenolic degradation. A possible explanation for this might be the interaction between anolyte and catholyte which in turn assisted to yield varied MFC performance based on different catholyte. Nevertheless, future work is required to establish a mechanism of interaction especially between the phenolic or waste degradation with catholyte system in the MFC.

The coulombic efficiency (CE) of MFCs using five catholytes is depicted in Fig. 3(c). CEs of 23.0%, 18.0% and 17.0% in PP-, M9- and PB-MFCs, respectively, were much higher than that of NaCl- and water-MFCs with 8.3% and 4.0%, respectively. The degradation of substrate was expected to be highly favourable for the biochemical reactions in NaCl- and water-MFCs compared to maintaining the electron transfers between anode and cathode chambers. Possible oxygen transference from the cathode to anode could result in decreasing CE output in MFC [14]. In contrast, the relatively higher CEs in the M9- and PB-MFCs might be attributed to the anoxic condition of methanogenesis suppression for the controlled-pH catholytes (buffering properties of PB and M9) [25]. Furthermore, the use of PP and M9 could increase the system conductivity, resulting in a decrease of internal resistance. This improved the CE and current density production [14].

3.4. Polarization curve and power generation

Polarization curve is an essential parameter to evaluate the performance of the MFC system. In this study, polarization curves as depicted in Fig. 4 were developed in the period of stable current generation using external loads ranging from 10 Ω to 100 kΩ. The highest open circuit voltage (OCV) of 661 mV was obtained by PP-MFC (Fig. 4(a)), corresponding to current density of 7.34 mA/m^2, followed by M9-, PB-, NaCl- and water-MFCs with 264 mV, 238 mV, 72.0 mV and 60.2 mV, respectively. The linear trends of the polarization curves showed that the ohmic losses occurred using all catholytes. The internal resistances calculated from the polarization curves were found to be 700 Ω, 1.1 kΩ, 1.15 kΩ, 5.6 kΩ and 5.5 kΩ for PP-, M9-, PB-, NaCl- and water-MFCs, respectively. It is obvious that the internal resistances in NaCl- and water-MFCs were much higher than that in PP-, M9- and PB-MFCs which lead to lower electricity generation and phenolic degradation. Besides, the higher potential generated in PP-MFC may indicate that this MFC had lower mass transport limitation at its electrodes [9].

Fig. 4 also shows similar power density profiles of the MFCs using PP, M9 and PB as the catholytes. The maximum power density of 9.4 mW/m^2 was obtained by the PP-MFC at 32.4 mA/m^2 current density, followed by M9-, PB-, NaCl- and water-MFCs with 3.8, 3.4, 0.06 and 0.05 mW/m^2, respectively. The distinct advantage of PP-MFC over the other catholyte-fed MFCs is due to its oxidizing property and ability to produce more efficient electron transfer from the anode biofilm to the cathode, which yielded higher power density in the MFC system. The higher power densities obtained by the PP, M9 and PB catholytes may be attributed to the improved cathode dynamics with increasing electron acceptor concentration as compared to water catholyte [26].

NaCl and water (Fig. 4(d) and (e)) appeared to have insignificant effect on MFC performance in terms of power recovery. The dramatic decline of power density using these two catholytes could be elucidated by several factors. Firstly, the use of NaCl or water as catholytes could lead to severe over-potential for oxygen reduction without catalysis of noble metals (e.g. platinum) [20,27]. Secondly, biofilm formation during acclimating phase would also restrict the oxygen mass transfer, thus aggravating the over-potential in the MFC system [27]. Thirdly, the metabolic by-products excreted could impose adverse impacts on the power generation.

The overall results were consistent with the findings of Fan et al. [28], who proposed that the increase in the power density of the MFC could be attributed to the decrease in its internal resistance. The potential and power density obtained by Hongyan et al. [29] were also comparable with our results in which their 50 mM sodium bromate catholyte attained 640 mV and 0.7 W/m^3, respectively. Therefore, these data suggested that PP-, M9- and PB-MFC systems were favourable for solving diffusional transfer and electrochemical restrictions in MFC by *B. subtilis* compared to the other two catholyte systems.

3.5. Electrochemical activity and electron transfer mechanism

3.5.1. Cyclic voltammetry

Cyclic voltammetry (CV) characterizes the electrochemical activity of the redox compounds and reveals the performance of a catalytic process. Fig. 5 presents typical CVs of the five catholytes-fed MFCs. Generally, the CVs profiles show slight differences before and after 2,4-DCP feeding, except for the PP- and M9-MFCs (Fig. 5(a) and (b)). The CVs of before 2,4-DCP feeding for PP- and M9-MFCs show oxidation peaks of (1.11 mA/0.45 V), (1.12 mA/0.47 V) and reduction peaks of (−0.43 mA / 0.36 V), (−0.37 mA / 0.04 V), respectively, which were higher than these after 2,4-DCP feeding [oxidation peaks: (0.53 mA/0.36 V), (0.42 mA/0.42 V); reduction peaks: (0.07 mA/0.41 V), (−0.27 mA/0.04 V)], respectively. These CVs profiles in Fig. 5(a) and (b) suggest that the 2,4-DCP substrate marginally affected the performance of current generation in the MFC. It was also noted that the 2,4-DCP feeding in the anodic chamber slightly altered the pH of the medium for *B. subtilis* growth. When the pH was reduced from 7.0, the *B. subtilis* growth might be inhibited, leading to current decline. This also could be interpreted that a very small pH change could have significant effect on the MFC performance and thus *B. subtilis* growth. In addition, the increase in oxygen reduction peak current of after 2,4-DCP feeding proved that the existence of microbes with the function of transferring electrons donated by the electrode to oxygen, hence accelerating the reduction of oxygen [30,31].

In contrast, after 2,4-DCP feeding for PB- and NaCl-MFCs showed a slight increase in the current generation, as shown in Fig. 5(c) and (d). The oxidation cycle of PB-MFC occurred at 0.88 mA/0.17 V after 2,4-DCP feeding, while a lower points of 0.66 mA/0.17 V was found before 2,4-DCP feeding. This could be described by its buffering characteristic which compensates the electrons transfer mechanism, thus maintaining a high level of the current generation even after phenolic addition in its cultivation medium. Whereas, salt ions facilitated the electron transfer between anode and cathode chambers in NaCl-MFC, resulting in the small difference in current generation before and after phenolic feeding.

PP-, M9- and PB-MFCs showed vibrant oxidation and reduction peaks compared to NaCl- and water-MFCs which demonstrated inferior peaks. These profiles proved the presence of mediators in PP-, M9- and PB-MFCs, which could be reversibly oxidized and

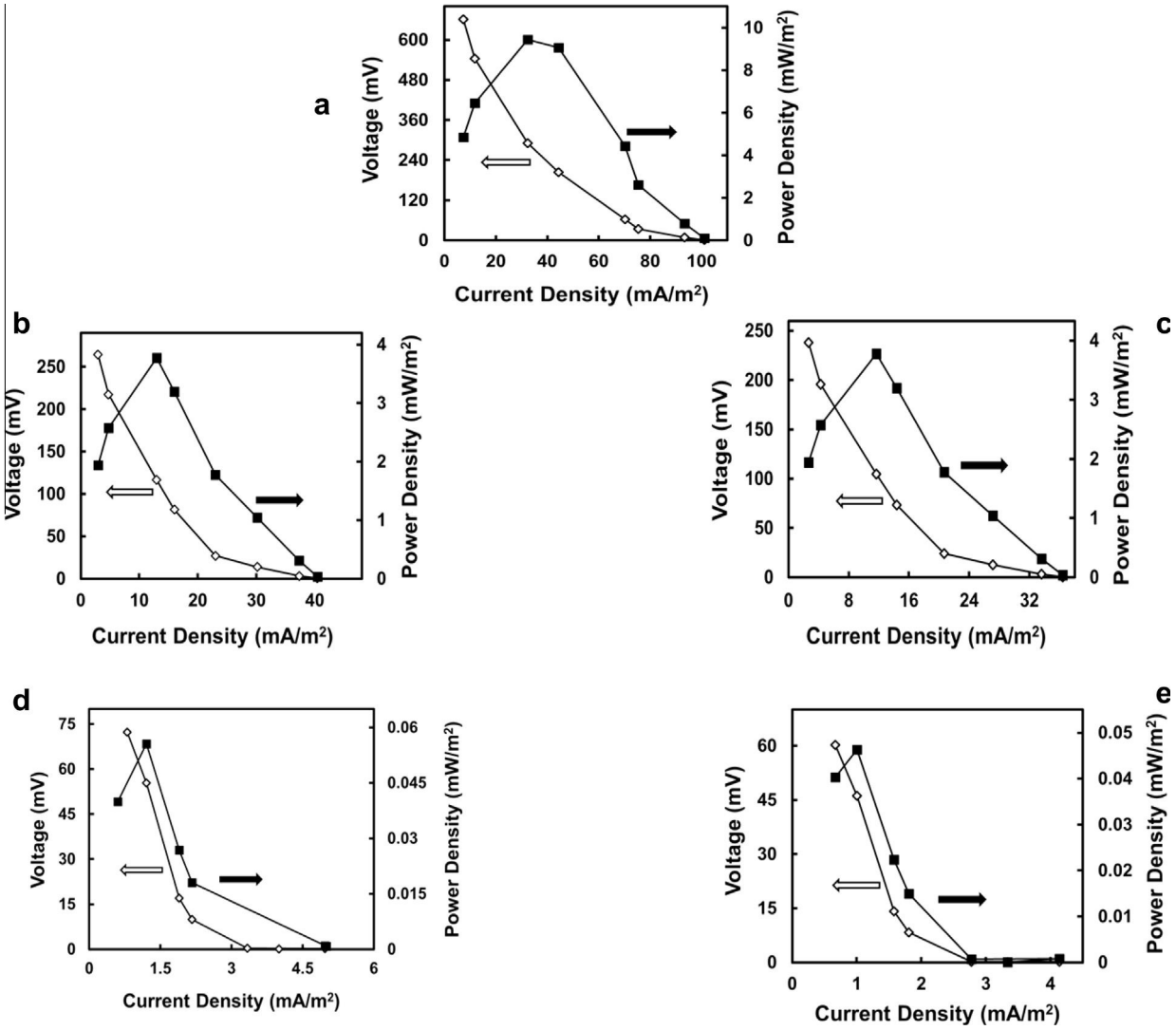

Fig. 4. Polarization curves and power generation using the five catholytes (a) PP, (b) M9, (c) PB, (d) NaCl and (e) water.

reduced during CV tests that demonstrated the presence of active redox compounds responsible for the electrochemical activity in the broth solutions [32]. These CV data were also comparable to the results reported by Vanita et al. [33], where the MFC operated at pH 7 showed the highest current generation of about 1.0 mA over the potential of 0.5 V. It is remarkably noted that the 2,4-DCP produced insignificant impact on the *B. subtilis* growth, therefore on the biochemical activities for the current generation in the MFC system.

3.5.2. Linear sweep voltammetry

To gain insight into the electrochemical characteristics of the cathode in the MFC, linear sweep voltammetry (LSV) at a scan rate of 0.1 V/s was performed to select the optimal operation parameters, such as operational current and potential. The analogous profiles of LSVs in Fig. 6 indicate that all catholytes exhibited similar biochemical activities in the systems [34]. The difference in the MFC performance using different catholytes was caused by the varied chemical reactions of the cathodes, rather than the anode as suggested by Zejie et al. [31]. The oxidation reaction started approximately at a voltage range of 0–0.8 (V vs. Ag/AgCl) for all catholyte-fed MFC systems. Then, linear increment of currents developed over subsequent potential flow which showed improvement in the microbial activities at this particular time and become constant (or slightly decreasing) afterwards.

PP-, M9- and PB-MFCs in Fig. 6(a)–(c) reveal significantly different LSV curves between before and after 2,4-DCP feeding operations. PP-MFC demonstrated the highest current generation of 28.9 mA at 2.11 V before the feeding. The same results were found in M9-MFC (Fig. 6(b)). However, PB-MFC exhibited contrary results with the same peak current of 1.0 mA, which were achieved at 1.34 V and 0.7 V, before and after the feeding, respectively. Increasing the applied voltage caused a little change in peak current for these experiments. The data of LSV for PB-MFC was slightly differed from its CV profile (Fig. 5(c)) might be due to some electrochemical interferences during scanning of linear region of potential sweep. However, both CV and LSV profiles achieved the same current peaks for before and after phenolic feeding. The similar profile can be observed from Fig. 6(d), showing a peak current of 3.53 mA achieved at 1.03 V and 1.14 V in the NaCl-MFCs before and after the feeding, respectively. Water-MFC (Fig. 6(e)), on the other hands, showed insignificant different performance before and after the feeding experiment where the current peak approximately occurred at 2.30 mA/0.7 V and achieved a constant current over further voltage spectrums.

The current increment over potential sweep from its initial electrochemical kinetic equilibrium region is a function of the avail-

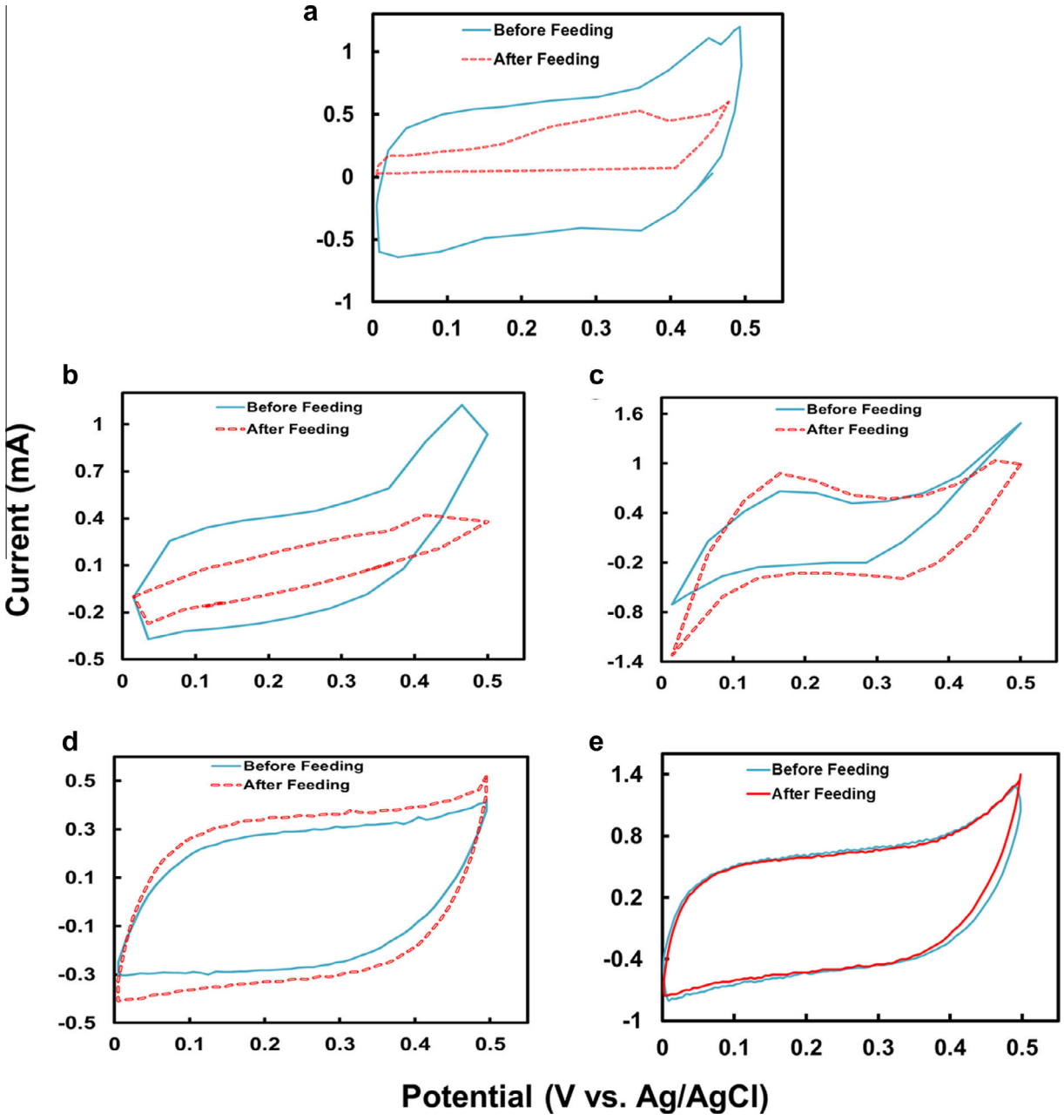

Fig. 5. Cyclic voltammograms (CV) of the different catholytes (a) PP, (b) M9, (c) PB, (d) NaCl and (e) water performed before and after phenolic compound feeding.

ability of organic substrates and the activity of electron transfer [35]. Our results revealed that any further increment in potential flow after the peaks could result in constant current for all catholytes fed MFCs. This phenomenon was due to the decrease in microbial activities associated with the microbial biofilm growth and the increasing diffusion for the substrate mass transport to the electrode surface [35]. The higher LSV current generation shown in Fig. 6(a) could be attributed to the combined effect of the higher redox potential and higher electron transfer number of the substrate reduction reaction compared to those in Fig. 6(c) and (e). A similar LSV profile was reported by Jun et al. [36], who used Fe- and K-based electrolytes and found no notable difference in electrolyte reactions as the electrolyte pH increased from 2.0 to 10.0.

The variance in current peaks with corresponding potential for all catholyte-MFC systems gives us a better understanding that the properties of catholytes are of significant factors in evaluating the performance of the MFC in electricity generation and phenolic degradation. Although the LSV curves for PP-, M9- and PB-MFCs could be rehabilitated by the 2,4-DCP feeding, their resilient oxidizing and buffering properties could otherwise tolerate the changes in maintaining the current generation and phenolic degradation in the MFC system.

3.5.3. Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) was used to analyse the internal resistances and electrochemical behaviour of anode and cathode individually or the whole MFC system. Fig. 7 shows the Nyquist plots of the MFC for anode and cathode before and after phenolic feeding. There are three resistances can be interpreted from the EIS curves: ohmic resistance (R_{ohm}), charge transfer resistance (R_{ct}) and diffusion resistance (R_d) [29]. In the high frequency region, the intercept of the curve and the real axis could be regarded as R_{ohm} [37]. In the low-frequency, the semicircle

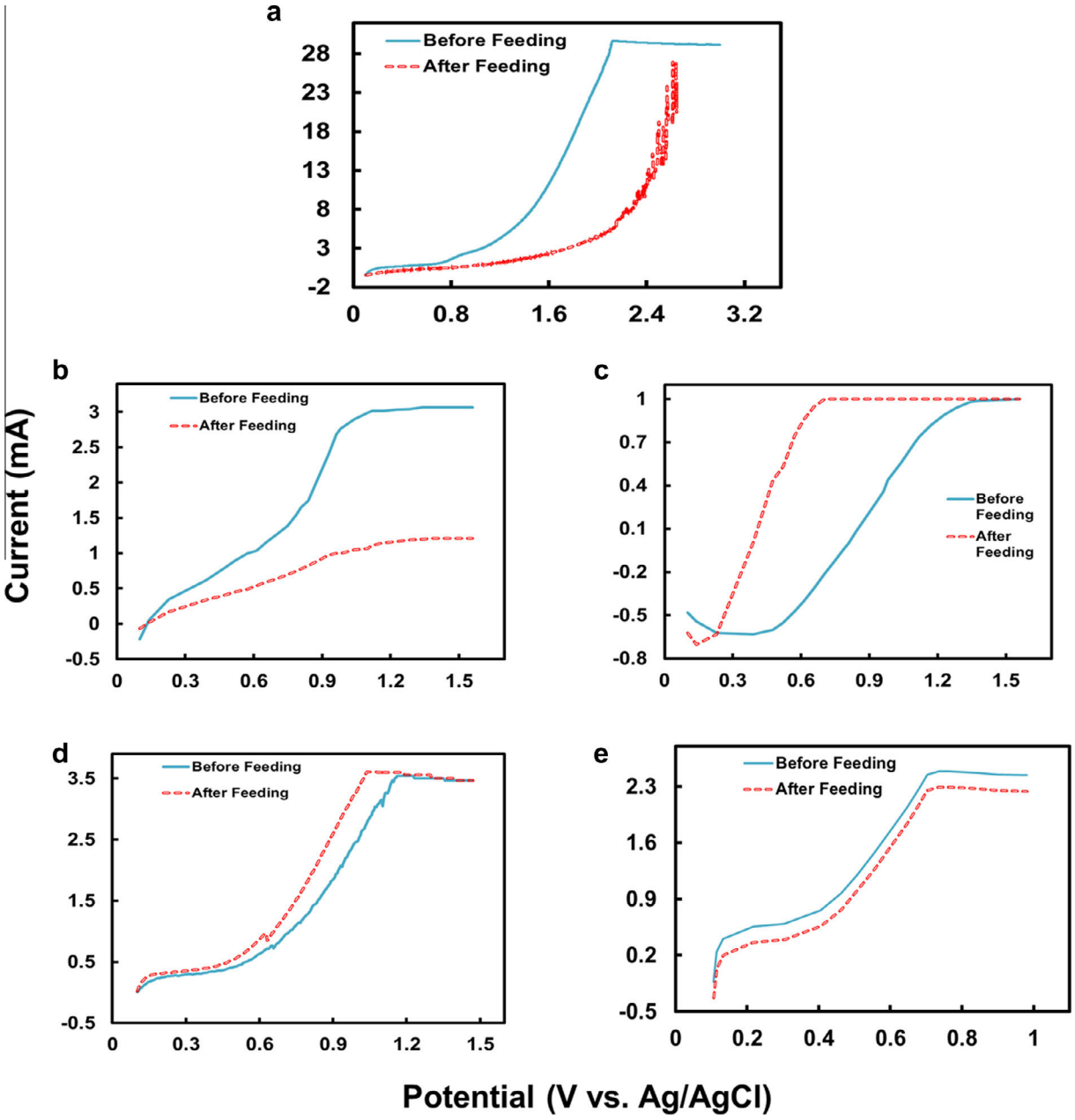

Fig. 6. Linear sweep voltammograms (LSV) of the different catholytes (a) PP, (b) M9, (c) PB, (d) NaCl and (e) water performed before and after phenolic compound feeding.

curves could be regarded as R_{ct}. Fig. 7 shows diverse profiles of Nyquist plots between anode (Fig. 7(a)) and cathode (Fig. 7(b)). The R_{ohm} values in anode were slightly varied and became significantly differed in the low-frequency region (R_{ct}). Water and NaCl show the highest R_{ct} with 800 and 700 Ω, respectively, followed by PP (520 Ω), M9 (500 Ω) and PB (410 Ω). The better result for PB which had the lowest R_{ct} value could be due to its buffering capability in maintaining pH stabilization in the MFC system. The significant variance in R_{ct} of anode could also be attributed by the biofilm development. The thicker anode biofilm during the process of cultivation could decrease charge transfer efficiency which resulted in high R_{ct} values.

On the contrary, cathode impedance curves in Fig. 7(b) showed similar trends which could be explained by analogous electrochemical activities occurred in the MFC catholyte systems. As a catholyte used in the MFC, water showed the highest R_{ct} of 512 Ω, while the lowest R_{ct} was given by PP and M9 with 400 and 383 Ω, respectively. These low values of R_{ct} were mainly due to excellent electrical conductivity of PP and M9 catholytes (Table 1), which increased the rate of electrochemical reactions in the MFC systems [21]. The lower overall impedance resistance values in cathodes as compared to anode gave us understanding that the electrochemical activities in cathode have higher influence on improving the MFC performance than that of anode. The results of internal resistance during EIS analysis were in a good accordance with the ones obtained from polarization methods with water and NaCl catholytes yielded the highest internal resistances, whereas the PP, M9 and PB resulted in lower resistances.

4. Conclusion and future works

In this study, we experimentally investigated double chamber MFC systems using pure culture *B. subtilis* with various catholytes for electric current production and 2,4-DCP degradation. *B. subtilis*

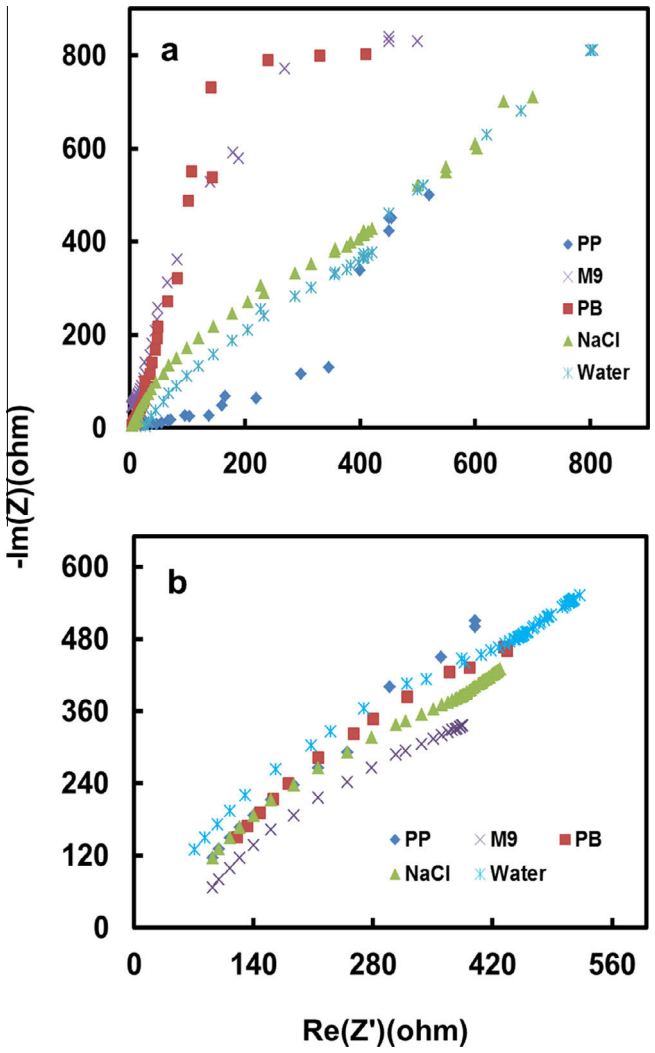

Fig. 7. Fitted Nyquist impedance spectra of (a) anode of before feeding (b) anode of after feeding (c) cathode of before feeding and (d) cathode of after feeding.

was found to have capability to degrade 2,4-DCP while generating electric current in the MFC system. Chemical characteristics of the catholytes have significant impact on the bio-electrochemical activities, and therefore the performance of the MFC system. The current density profiles show that PP-MFC exhibited significantly the highest current generation (64.0 mA/m^2) and 2,4-DCP degradation, while NaCl and water appeared to be less promising catholytes for MFCs. The phenolic degradation and CE data supported the findings that PP, M9 and PB were the potential catholytes. From the polarization curve data, PP had high capability of solving diffusional and electrochemical restriction by *B. subtilis*. The analyses of electrochemical and electron transfer activities using cyclic voltammetry and linear sweep voltammetry validated our initial hypothesis that PP, M9 and PB were vital catholytes in enhancing MFC performance to generate current as compared to NaCl and water catholytes. Therefore the results of this study proved that the low-concentrated, low-cost and low-toxicity catholytes could amplify the electricity generation with simultaneous 2,4-DCP degradation. The proposed system design is potentially very useful towards the development of low-cost and sustainable MFC applications.

In future works, the MFC performance could be studied using variants of the persulphate-based catholytes. Studies involving the utilization of mixed culture from wastewater could be carried out to explore the performance of the MFC in terms of phenolic degradation and current generation. Besides, future study is necessary to investigate the mechanism of interaction between catholytes and growth or waste degradation in the MFC system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2016.04.077.

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Formation of industrial mixed culture biofilm in chlorophenol cultivated medium of microbial fuel cell

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Formation of Industrial Mixed Culture Biofilm in Chlorophenol Cultivated Medium of Microbial Fuel Cell

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Abstract. The formation of microbial biofilm while maintaining the electricity output is a challenging topic in microbial fuel cell (MFC) studies. This MFC critical factor becomes more significant when handling with industrial wastewater which normally contains refractory and toxic compounds. This study explores the formation of industrial mixed culture biofilm in chlorophenol cultivated medium through observing and characterizing microscopically its establishment on MFC anode surface. The mixed culture was found to develop its biofilm on the anode surface in the chlorophenol environment and established its maturity and dispersal stages with concurrent electricity generation and phenolic degradation. The mixed culture biofilm engaged the electron transfer roles in MFC by generating current density of 1.4 mA/m^2 and removing 53 % of 2,4-dichlorophenol. The results support further research especially on hazardous wastewater treatment using a benign and sustainable method.

INTRODUCTION

Microbial fuel cell (MFC) is one of the popular engineering tools in harvesting potential sustainable biofuels. Numerous MFC designs, physicochemical characteristics, and electrochemical properties have been widely researched in recent years for generating optimal current or power output with simultaneous waste treatment capabilities. Although there are some researchers use pure culture in their MFC studies [4, 5], current MFC research trends have been shifting to the utilization of mixed microbial consortia derived from domestic and industrial wastewater [1, 2, 3]. One of the critical factor in enhancing MFC performance is the biofilm development.

Microbial biofilm plays a major role in MFC performance through electron transfer mechanisms. Principally, there are three mechanisms of electron transfer between bacteria and anode which lead to current generation. First and foremost is using outer-membrane redox proteins, for example cytochromes to mediate the electron transfer by direct contact between the bacterial biofilm and the anode surface. Secondly through self-excreting redox molecules (e.g. phenazine, quinones) as electron shuttles and lastly by producing nanowires as conductive appendages in exocellular transport of electrons [4]. Equations 1 and 2 depict the anodic and cathodic reactions in the MFC:

$$\text{Anode: } CH_3COO^- + 2H_2O \rightarrow 2CO_2 + 7H^+ + 8e^- \quad (1)$$

$$\text{Cathode: } O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \quad (2)$$

The electrons released by the oxidation of organic materials (for example, acetate) in the anodic chamber are transferred by the exoelectrogenic bacteria in the biofilms to the anode surface as shown in Eq. 1. These electrons flow through an external circuit to reach the cathode in the cathodic chamber where they are used for reduction of

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oxygen [6]. To maintain electroneutrality, protons migrate from the anodic chamber to the cathodic chamber to participate in Eq. 2 [6].

Maintaining the biofilm formation, stability and reliability while degrading refractory organic compounds becomes one of the debatable subject in MFC operation and performance [7, 8]. You et al. [9] recently studied the relationship between the anodic biofilm formations with different feedstock in the MFC. In that work, the authors found that the metabolic functional diversity of anodic biofilms increased through exposure to different feedstock conditions (acetate and casein). Read et al. [10] maintained the viability of biofilm nearest to the anode surface during closed MFC circuit operation using several pure cultures including *Geobacter* and *Pseudomonas*. In addition, the extent of biofilm growth and its coverage on the anodic surface showed direct influence on both power production and substrate degradation [11]. The aforementioned studies only investigated the biofilm development in the normal growth media consisting for instance acetate, nitrate and fumarate, without concerning the toxic wastes in the MFC media environment. To the best of our knowledge, there is still lack of data on literatures involving the investigation of biofilm formation and behaviors in toxic or recalcitrant cultivated environment of MFC (with respect to hazardous wastewater treatment).

Since the biofilm formation on the electrode is foremost to the electricity generation and substrate generation [7], the investigation of anodic biofilm roles in toxic and recalcitrant media become undeniably significant in generating optimal current output with simultaneous toxic substrate degradation in the MFC. This study explored the formation of anodic biofilms of industrial mixed cultures in recalcitrant chlorophenol-containing medium in a double chamber MFC. Characterization of biofilm formation on the anodic surface was performed. The evaluation of the current generation and phenolic degradation by the mixed cultures biofilms was carried out.

MATERIALS AND METHODS

Mix Culture and Growth Medium

Industrial wastewater was collected from a petrochemical plant, Australia Mobil Oil Pty Ltd in South Australia, and kept in a refrigerator at 4 °C before use. The oil layer of wastewater was separated using separating funnel. The wastewater (without oil) was used as inoculum in this study. The growth medium was made of artificial wastewater prepared with composition as follows: 0.5 % glucose in mineral salts [0.386 g/L $(NH_4)_2SO_4$, 0.149 g/L K_2SO_4, 3.31 g/L NaH_2PO_4, 10.31 g/L Na_2HPO_4, 1 g/L NaCl, 0.2 g/L $MgSO_4$ and 12.5 mg/L vitamin]. The pH of the solution was adjusted to 7.0 ±0.1 with NaOH or HCl. 2,4-dichlorophenol (2,4-DCP) with 10 mg/L concentration was fed into the growth medium after 10 h MFC cultivation (approximately during the exponential phase of the bacterial cultures) in order to study the phenolic degradation in the MFC system.

MFC Setup

A double chamber MFC reactor was constructed from two 250 mL-glass bottles joined together with a glass bridge consisting a cationic exchange membrane (CEM) with 3-cm diameter. The anode and cathode materials was made up of 3.0-cm × 3.0-cm carbon cloth. Both electrodes were pierced with titanium wire projecting outside connecting to an external electric circuit, through which the electrons were transported. The catholyte used was 50 mM phosphate buffer. The mixed cultures was cultivated in anoxic condition and the cathodic chamber was open for aeration.

Data Acquisition and Phenolic Removal

The electrode output voltage was continuously recorded against time by a LabJack U6 recorder with an external load of 10 kΩ connected to a computer. The MFC system was operated for 3 days, under the application of a single resistance. The current density was measured by the voltage divided by the resistant and surface area of the electrode.

The 2,4-DCP concentration was examined using a Shimadzu UV-Visible Spectrophotometer (UV-1601) at 750 nm as described by Wilfred and Ralph [12]. The percentage removal was calculated based on the difference in initial and final 2,4-DCP concentrations.

Characterization of Mixed Culture Biofilm

The characterization of mixed culture biofilms was carried out after the feeding of 2,4-DCP into the anode medium of MFC. The morphology of biofilms formed onto the anode surface was analyzed by a scanning electron microcopy (SEM) (Philips XL30). The carbon cloth-anode attached with mixed culture biofilm was aseptically removed from the MFC anodic chamber and washed with phosphate buffer saline (PBS) solution. Then the sample was heating dried at 80 °C for 24 h. The sample was coated using platinum for high resolution SEM imaging.

The microscopic images of biofilm formation were observed under a microscope (Motic, BA 400) equipped with Moticam 2000, 2.0 M Pixel. A drop of biofilm sample at the anode surface was taken aseptically at several time intervals by a micropipette and put onto a clean glass slide and covered with a glass slip. The microscope slide was placed on the microscope stage and fastened with the stage clips. Then, the biofilm microscopic images was observed according to the standard protocol of the microscope use.

RESULTS AND DISCUSSION

Biofilm Microscopic Characterization

The industrial mixed culture was cultivated using artificial wastewater in the MFC. After several hours of cultivation, the medium solution turned turbid implying the growth of the bacteria and biofilm was observed to form on the anode surface as depicted in Fig. 1.

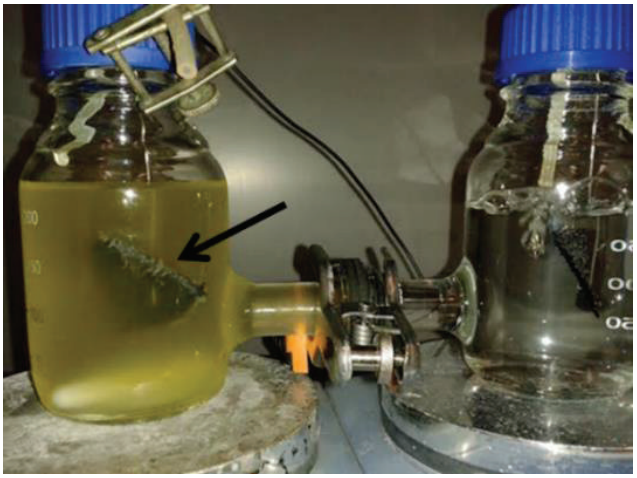

FIGURE 1. The formation of mixed culture biofilm onto the surface of anode in the MFC as indicated by the arrow

Figure 2 shows SEM images of mixed culture biofilm on the anode surface. Although the morphology of the biofilm may have been rehabilitated by the washing and drying processes, the SEM microphotographs offered good information demonstrating biofilm establishment and attachment. This gives us insight on degradation capabilities and electron mechanism behaviour through types of bacterial colony and their attachment on the anode surface.

It can be seen lots of bacteria colonies were spotted and spread out on the anode surface as displayed by Fig. 2 (A). This implied a good growth of bacteria biofilm in the chlorophenol containing medium. The SEM images at higher magnification (Fig. 2 B, C) provide further insight into the morphology of the biofilms formed on the anode surface. The coccal- and rod-like shapes of bacteria were seen in Fig. 2 (B) suggesting the *Pseudomonas* or *Bacillus* species might dominate in the system. This result suggested that these types of bacteria are resistant to phenolic environments. It is also interesting to note that *Pseudomonas* and *Bacillus* species have been found to degrade in highly toxic phenolic compounds in wastewater environment [13 - 16].

Further observation in Fig. 2 (C) clearly substantiates the presence of flagella in the biofilm formation. Flagella is a structure made of repetitive assemblies of covalently or non-covalently linked protein subunits, organized into filamentous polymers [17]. Flagella enable the bacteria to have strong motility and swarming across solid surfaces.

It was revealed that in many species a functional flagellar system is required for adhesion and biofilm formation [17]. The heavily presence of flagella in the environment also gave us understanding that the bacterial biofilm are resilient to the toxic environment as they function as sensory organelles being sensitive to chemical and temperatures outside the cells [18].

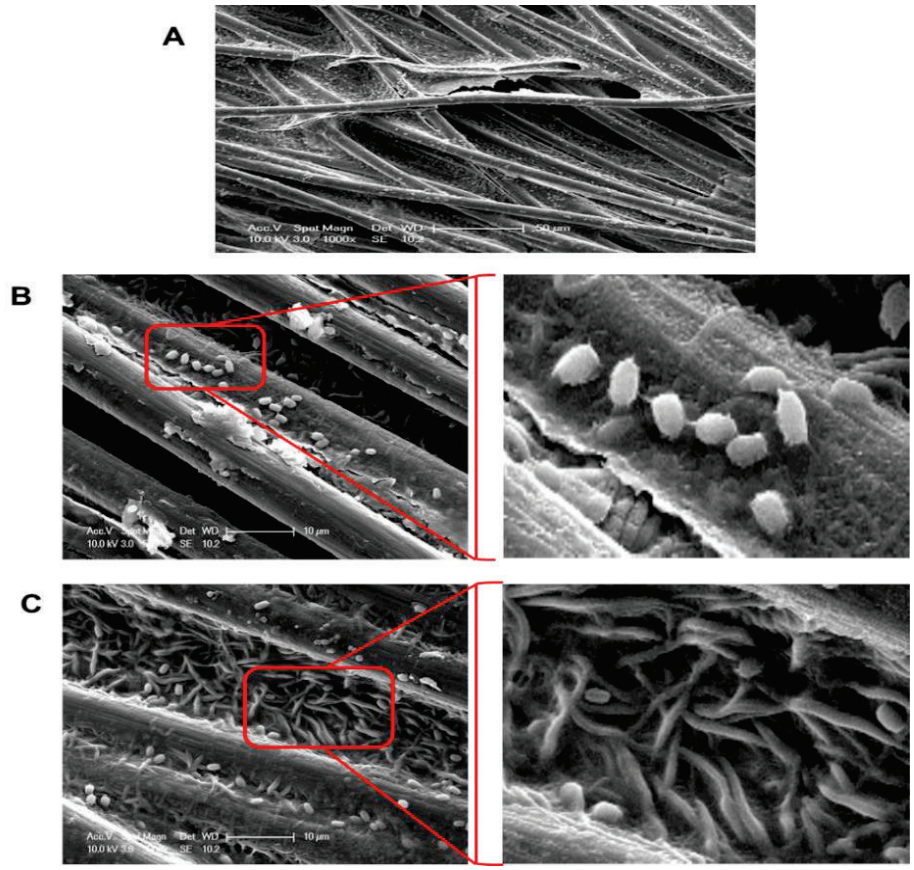

FIGURE 2. SEM images of mixed culture biofilms attached onto the anode surfaces at several magnifications; (A) 1000 X magnified image of carbon cloth spotted with lots of bacteria onto it (B) Coccal- and rod-like shapes bacteria on the anode surface (C) Flagella formation on the anode surface

The stages of biofilm formation in chlorophenol medium were further observed using the light microscope. Figure 3 shows images of establishment and dispersal of mixed culture biofilms. The establishment of mature biofilm in clumps can be observed in Fig. 3 (A), at which normally formed during exponential and stationary phases of bacterial growth. It is clearly seen that the bacteria colonies were grouped together inside a thick protein based polymer-like wall. These micro-colonies of bacterial cells encased in polysaccharide matrix are separated from each other by water channels [19]. Liquid flow occurs in water channels, allowing diffusion of nutrients, oxygen, and even antimicrobial agents.

In depth observation of the biofilm through higher magnification as depicted in Fig 3 (B) shows a clearer image of linkages made up of extracellular polymeric matrix (or exopolysaccharide). This matrix consists of abundant of the same or different bacterial species linked together. This matrix facilitates communication among the cells through biochemical signals and gene exchange. In this instance, the exopolysaccharide matrix maintains the biofilm strength for electronic transfer through trapping extracellular enzymes and keeps the cells in close vicinity. Furthermore, the matrix represents an external digestion system and allows for stable synergistic microconsortia of different species [20].

On the second day of cultivation, the biofilms were observed to disperse and shrink as displayed in Fig. 3 (C). Like all sessile organisms, the biofilms must release and disperse cells into the environment to colonize new sites [21]. Finally, the biofilm was seen to completely disperse and break down as micro-graphed in Fig. 3 (D). Until

recently, the mechanisms by which active bacterial dispersal from biofilms occurs remained almost completely unexplored, and little is known about the functions or regulatory pathways involved in the release of bacteria from biofilms [22]. The process and mechanisms of biofilm dispersal could be attributed by the enzyme-mediated breakdown of the biofilm matrix [23]. In addition, the production of surfactants which loosen cells from the biofilm could also be linked to the dispersal of biofilm [24].

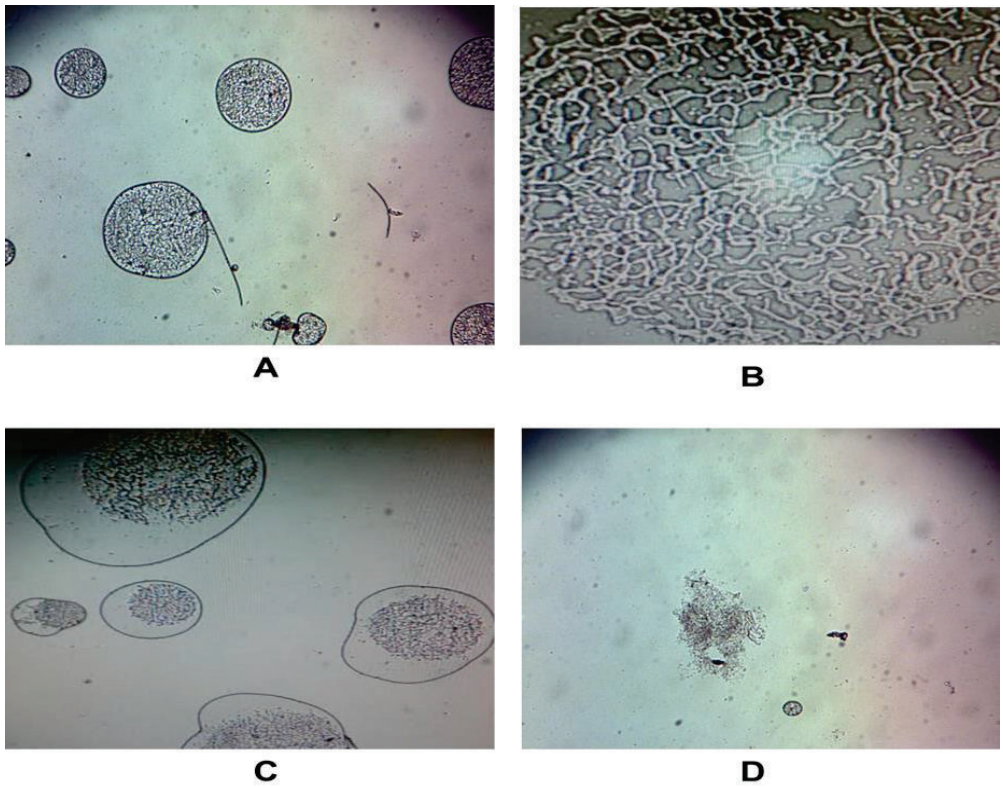

FIGURE 3. Microscopic images of mixed culture biofilm formation stages in chlorophenol containing medium; (A) Mature clumps of biofilms (B) Biofilm consists of plentiful linkages made of extracellular polymeric matrix (at 40 X magnifications) (C) Biofilms shrinkage and dispersal (D) Biofilm break down during the death phase

Current Density Generation and Phenolic Removal

Current density generated by the mixed culture in the MFC was monitored over 3 days of cultivation. As pictured in Fig. 4 (A), the 2,4-DCP was fed after the exponential phase of the bacterial growth (approximately after 10 h of cultivation). The highest current density recorded was 1.40 mA/m^2, and the current started to decrease gradually afterwards. This result is comparable with Read et al. [10] where *Pseudomonas* and *Enterococcus* biofilms produced 1.8 mA in the nitrate and fumarate containing anolyte. The decline of current output could be due to the nutrient deficiency over time as well as the effect of 2,4-DCP toxicity which lead to the microbial growth inhibition. It can be stated that the bacterial cultures continue to grow with slower rate in the chlorophenol environment and degraded the compound up to 53 % as indicated by Fig. 4 (B). The 2,4-DCP might be degraded into simpler compound by the bacteria through reductive dehalogenation process as suggested by Pankaj and Hanhong [25].

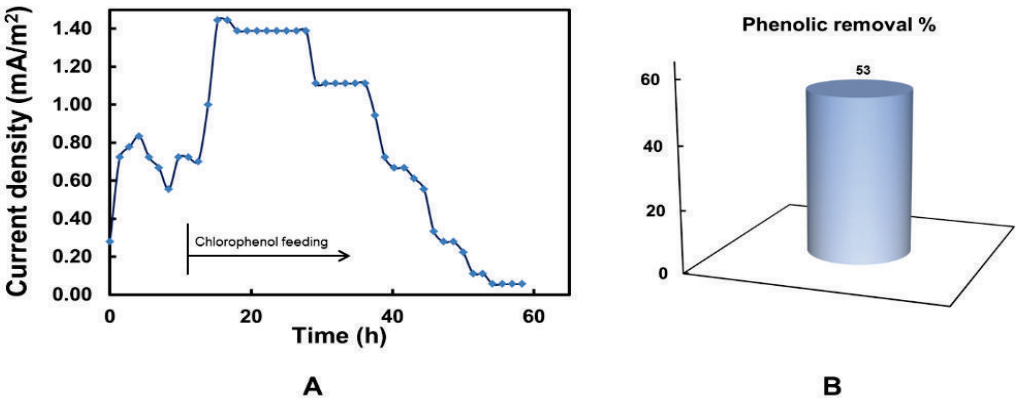

FIGURE 4. (A) Current density generated by the mixed culture in chlorophenol containing medium fed after 10 h cultivation (B) Phenolic removal by the mixed culture in MFC

CONCLUSION

This study explored the formation of industrial mixed culture biofilm on the anode surface in the MFC. The characterization of the biofilm using SEM and light microscope showed the establishment of the biofilm in chlorophenol containing medium. This suggests that the biofilm is flexible and conciliate in recalcitrant environments. The presence of *Pseudomonas*- and *Bacillus*-like species as well as flagella formation in the biofilm environment gave us understanding that the mixed culture biofilm developed distinctive roles. Examples of these roles are: facilitating cell motility, genetic communication, keeping the cell adhesion and proximity and presumably the electron transfer mechanism. This results were further supported by the culture capability to generate current of up to 1.4 mA/m^2 while removing 53 % 2,4-DCP. In conclusion, the outcomes of this investigation could be a backbone for further research in hazardous wastewater treatment through sustainable and green technological MFC technique.

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Degradation of 2,4-Dichlorophenol by *Bacillus Subtilis* with Concurrent Electricity Generation in Microbial Fuel Cell

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Abstract

The growing critical and toxic wastewater contamination problems seek various sustainable and environmental friendly treatment methods to be further investigated. 2,4-dichlorophenol (2,4-DCP) is one of the recalcitrant and hazardous contaminants normally found in industrial wastewater and in some cases in domestic wastewater as well. This study explored the capability of bacteria *Bacillus subtilis* to degrade the 2,4-DCP in a double chamber microbial fuel cell (MFC) system. The MFC enables the removal of contaminants by bacteria with concurrent electricity generation through electron transfer mechanisms. *B. subtilis* is found to be a good exoelectrogenic bacterium for generating an optimum potential of 95 mV with 12 mA/m^2 current density in MFC. *B. subtilis* was able to degrade ~60% of 2,4-DCP into acceptable simpler metabolites, thus could be further utilized in treating hazardous phenolic contaminants while generating electricity through benign and sustainable wastewater treatment method.

Peer-review under responsibility of the organizing committee of ICPEAM 2016

Keywords:

1. Introduction

Phenols and phenolic are a class of organic compounds, characterized by a hydroxyl (-OH) group attached to a carbon atom that is part of an aromatic ring. They can be synthesized industrially or produced in natural environment by plants and microorganisms and occur in high variety of derivatives [1]. Because of its great benefits for industrial applications, mainly in the petrochemical, oil refining, plastic and pharmaceutical industries,

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phenols reach a high global production. Consequently, the phenolic-based disposals to water bodies become more critical primarily due to its toxicity and harmful effects to the aquatic lives and human being even at very low concentrations (5 - 25 mg/l) [2, 3]. The World Health Organization (WHO) indicated substituted phenols as noxious substances for human health and valued some phenolic compounds with a maximum admissible concentration in drinking water. For example the maximum admissible concentration for 2,4-dichlorophenol (2,4-DCP) is 40 μg/L[4].

Nomenclature

MFC	Microbial fuel cell
2,4-DCP	2,4-dicholorophenol
CEM	Cationic exchange membrane
CV	Cyclic voltammetry
LSV	Linear sweep voltammetry
HPLC	High performance liquid chromatography

Due to their toxicity and recalcitrance characteristics, various treatment methods have been introduced for instance, chemical oxidation, adsorption onto activated carbon and other adsorbent materials and chromatography techniques. However, these techniques require high energy consumption and sophisticated equipment which could lead to high operational costs. One of the most preferable methods is biological degradation owing to its environmental friendly, relatively inexpensive and simple method. In addition, one of the most topical and important sustainable engineering tools in the wastewater treatment that utilizes waste degradation process is microbial fuel cell (MFC).

The MFC which consists of anode and cathode chambers separated by an ionic exchange membrane will produce electricity through the electron transfer mechanism between the two chambers. At the anode chamber, the microbes oxidize substrates such as glucose and some refractory organics. The oxidation of glucose, for example, generates electrons, protons and carbon dioxide as shown by Reaction 1. At the cathode chamber, on the other hand, a reduction process occurs with the presence of oxygen molecules to form water as indicated by Reaction 2.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad (1)$$

$$24H^+ + 24e^- + 6O_2 \rightarrow 12H_2O \quad (2)$$

This study investigated the degradation process of 2,4-DCP using a safe bacteria, *Bacillus subtilis* in the MFC. *B. subtilis* is a highly-resistant bacterium which is easily cultivated and has been found to be a promising species in degrading the phenolic contaminants. The MFC experiments were carried out in neutralized conditions (pH 7) so as to provide a good environment for the *B. subtilis* cultivation, thus expecting an excellent electricity output and high removal rate of 2,4-DCP.

2. Materials and Methods

2.1. Microorganism and culture medium

Bacillus subtilis (AWQC 111) was cultured and provided by Australian Water Quality Centre, and subsequently sub-cultured at the Bionanotechnology Laboratory, School of Chemical Engineering, the University of Adelaide. This bacteria was used to remove the 2,4-DCP in this study. The bacteria were grown in 100 mL of nutrient broth in a 250-mL shake flask for 20 h on an incubator shaker (175 rpm) at 35 °C. The grown bacterial cells were used as the seed culture in the MFC degradation study.

The M9 medium was used as bacterial growth medium and prepared according to Miller [5] with slight modification of composition as follows: 0.5 % glucose and 1 g/L yeast extract in mineral salts (1 g/L NH_4Cl, 3 g/L

KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl, 0.05 g/L $MgSO_4$, and 0.005 g/L $CaCl_2$). The pH of the medium was adjusted to pH 7.0 ±0.2 with NaOH or HCL. The medium was sterilized at 121 °C for 15 min. The glucose solution was filter-sterilized (Millipore membrane PVDF, 0.22 µm filter unit) and then added to the above autoclaved medium.

2.2. Experimental MFC Setup

A double chambered MFC reactor was constructed from two 250 mL-glass bottles joined together with a glass bridge consisting a cationic exchange membrane (CEM) with 3-cm inner diameter. The anode material was made up of 3.0-cm × 3.0-cm carbon cloth, and 12.56 cm^2 graphite fiber was used as cathode material. Both electrodes were pierced with titanium wire projecting outside connecting to an external electrical circuit, through which the electrons were transported. The catholyte used was M9 solution (without glucose and yeast extract). The MFC reactor setup is illustrated in Fig. 1.

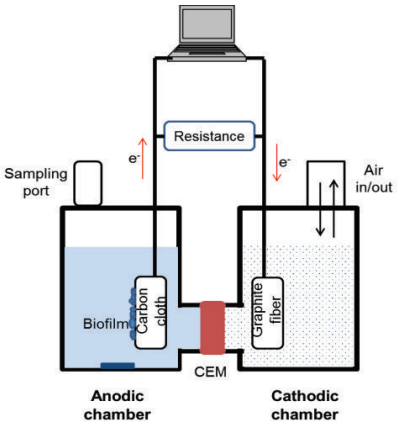

Fig. 1. MFC setup.

The *B. subtilis* was cultivated in an anoxic condition, in which the air penetration was blocked inside the anode chamber. The cathodic chamber was open for aeration. The MFC system was topped on a magnetic plate to ensure well mixing and operated at room temperature. After 10 h, the anodic chamber turned turbid, indicating the bacterial growth and the biofilm was observed to develop onto the surface of electrode. To maintain the growth of bacteria, glucose was added to the anodic chamber. Then, the chamber was subsequently fed with 10 mg/L 2,4-DCP for studying the contaminant degradation by the bacteria.

2.3. Data acquisition and electrochemical measurement

2.3.1. Voltage / current and polarization curve measurement

The electrode output voltage was continuously recorded against time by a computer. The anode and cathode were connected to a LabJack U6 recorder with an external load of 10 kΩ circuit connection. The MFC system was operated for 4 days, under the application of a single constant resistance. The current and anodic and cathodic potentials were measured with respect to time. To develop the polarization curves, different external loads were applied for a complete batch cycle, with a variable resistance ranging from 10 Ω to 100 kΩ.

2.3.2. Cyclic voltammetry (CV) and linear sweep voltammetry (LSV)

Cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were recorded using a CHI 650 D bipotentiostat (CH Instruments, Inc., USA). The CV was conducted at a scan rate of 0.1 V/s ranging from 0 to 1.0 V (vs. Ag/AgCl), while the LSV was conducted at a scan rate of 0.1 V/s ranging from 0.1 to 2.0 V (vs. Ag/AgCl). The experiments were carried out in a conventional three - electrode arrangement consisting of a working electrode, reference electrode (an Ag/AgCl electrode) and a counter electrode. The working electrode was made of carbon cloth and counter electrode was made of graphite fiber, pierced with titanium wires as discussed above. All three electrodes were inserted into the MFC, avoiding any contact among these electrodes.

2.4. Phenolic removal

The model phenolic compound used in this study was 2,4-dichlorophenol (2,4-DCP) (Alfa Aesar), which is a chlorinated derivative of phenol with the molecular formula $C_6H_4Cl_2O$. 10 mg/L 2,4-DCP was fed into the anodic chamber under anoxic condition. Samples of 5 mL were periodically withdrawn from the MFCs and centrifuged at 4000 rpm at 4°C for 10 min (Megafuge 1.0 R; Heraeus). The supernatant was used to analyze the phenolic degradation by *B. subtilis*. The 2,4-DCP degradation was analyzed qualitatively using two methods; difference in optical density values using UV-Visible spectrophotometry and chromatography technique using high performance liquid chromatography (HPLC).

2.4.1. UV-Visible spectrophotometry

The phenolic removal was examined using a Shimadzu UV-Visible Spectrophotometer (UV-1601) with optical wavelength of 750 nm as described by Wilfred and Ralph [6]. Phenolic degradation was calculated based on the difference in initial and final 2,4-DCP concentrations.

2.4.2. High performance liquid chromatography (HPLC)

The analysis of 2,4-DCP and metabolites were performed using the HPLC (VARIAN, ProStar) equipped with a C_{18} capillary column (Microsorp-MV 100-5, 150 x 4.6 mm). The column has been stored in 10% MeOH prior to use. The ultraviolet detector (VARIAN, ProStar) was set at 227 nm. The mobile phase was prepared by dissolving methanol in milli-Q water with 50:50 v/v ratio with 2% acetic acid preset at pH 3. The HPLC grade standards were pre-run at the same conditions in order to determine the expected metabolites from the 2,4-DCP degradation which were examined in conformity with Pankaj and Hanhong [7].

3. Results and discussion

3.1. Voltage generation and polarization curves

Voltage generated by *B. subtilis* with respect to time was recorded in MFC reactor over 4 days as depicted in Fig. 2 (A). The highest voltage obtained by the bacteria was 95 mV and found to be stationary over 24 hours. Then, the bacterial growth was dropped dramatically as demonstrated by the tremendous decrease of voltage after about 28 hours of MFC cultivation. This result suggested that there was a substrate inadequacy during the cultivation periods which led to the incline of voltage and microbial activities [8].

It was noted that 2,4-DCP was fed into the microbial medium during the log phase of bacterial cultivation and after the development of biofilm onto the surface of the electrode, which was occurred on day 1 of MFC operation. The subsequent considerably low voltage output afterwards (after 40 h) was due to the toxicity of 2,4-DCP which hindered the bacterial growth and electrochemical activities. The low voltage was maintained at the range of 5 – 10 mV and lasted for a total period of approximately 4 days. We supposed that the 2,4-DCP was degraded at slower rate during this low voltage output periods. In addition, the decrease of voltage output could be due to the increasing diffusion limitation with time, which might be attributed by the development of thick biofilm [9, 10].

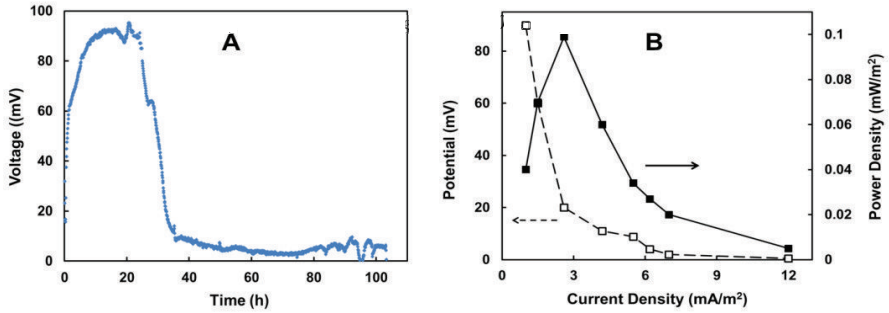

Fig. 2. (A) Voltage generated by *B. subtilis* with respect to time; (B) Polarization curves- potential and power density curves with respect to current density.

Fig. 2 (B) illustrates the potential and power density with respect to current density generated by *B. subtilis* in the MFC with varying external resistance ranging from 10 Ω to 100 kΩ. The inclining profile of closed circuit potential could be due to the utilization of the carbon source for microbial growth and metabolic activities. This is also might be owing to the effect of partial inhibition of microbial activities by the 2,4-DCP during early stage of phenolic feeding, although it would be degraded at slower rate at the later stage. At an infinite external resistance (open circuit conditions), the open circuit voltage generated by the bacteria was 89 mV at the corresponding current density of 1.0 mA/m^2. The linear region of the polarization curve represents ohmic loss. The internal resistance calculated from the polarization curve to be 44 Ω. The maximum power density obtained by the *Bacillus*-MFC system was 0.1 mW/m^2 at the corresponding current density of 2.6 mA/m^2 as shown in Fig. 2 (B). The power generation increased to the maximum value as the substrate availability continues and the electro-active biomass accumulates, as suggested by Picioreanu et al. [9] and Vanita et al. [10]. The lower inclining rate for the potential and power density curves after the corresponding current densities of 3.0 and 6.0 mA/m^2, respectively, lead to lower mass transport limitation at the electrode [8].

3.2. Electrochemical activity and electron transfer mechanism

Cyclic voltammetry (CV) characterizes the electrochemical activity of the redox compounds and reveals the performance of a catalytic process. To analyze the electrochemical behaviors of the bacteria in the medium containing 2,4-DCP and the impact on the redox reaction, we performed CV at a scan rate of 0.1 V/s. Fig. 3 (A) shows typical CV of the MFC system. The voltammogram shows a two-step change in the cathodic peak current, which is indicating the process of a two-electron transfer at two different peak potentials [10]. It is clear to note that the CV curve shows an oxidation peak (diamond symbol) of 0.34 mA / 0.12 V and a reduction peak (triangle symbol) of -0.35 mA / 0.63 V. This result is comparable to Vanita et al. [10] where the CV profile using glycerol containing medium at pH 7 showed the oxidation and reduction peaks were approximately at 0.5 mA / -0.1 V, and -0.3 mA / 0.30 V, respectively. The CV profile illustrates the presence of mediators in the MFC system, which could be reversibly oxidized and reduced during CV test, hence, demonstrating the presence of active redox compounds responsible for the electrochemical activity in the broth solutions [11]. Other than that, Freguia et al. [12] discussed the electron transfer mechanism in the CV electrochemical test which was due to diffusivity in the redox compounds, instead of being adsorbed on the electrode surface, diffuse towards and away from it.

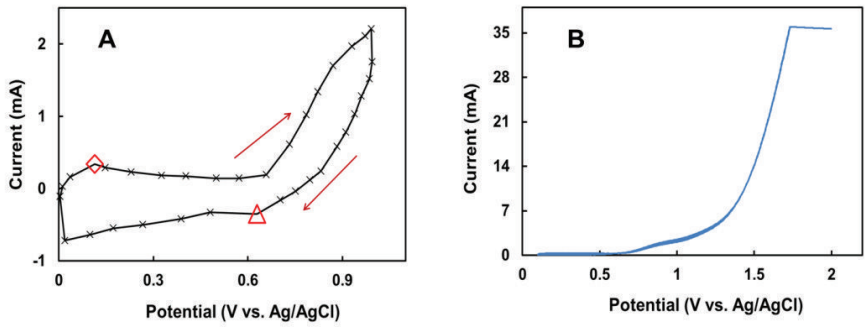

Fig. 3. (A) Cyclic voltammetry and (B) linear sweep voltammetry curves for *B. subtilis* electrochemical activities in the MFC.

To gain further insight into the electrochemical properties of the cathode in the MFC, linear sweep voltammetry (LSV) was performed at a scan rate of 0.1 V/s to select the optimal operating parameters. The LSV profile indicates the electrochemical activities in the cathodic chamber [13] which reflects the anodic biochemical action by the bacteria. The oxidation reaction started at a voltage range of 0.5 – 1.0 V (vs. Ag/AgCl) as depicted in Fig. 3 (B). Linear increment of currents developed over subsequent potential flow showing improvement in the microbial activities up to the current peak at 36.0 mA with corresponding potential of 1.73 V. The current increment over voltage sweep from its initial electrochemical kinetic equilibrium region was a function of the availability of organic substrates and the activity of electron transfer [14]. The further current flow over potential spectrum indicates a much smaller declining profile until reaching maximum potential at 2.0 V. This occurrence might be due to the declining phase of microbial metabolism corresponding to the microbial biofilm growth and the increasing diffusion for the substrate mass transport to the electrode surface [14].

3.3. 2,4-Dichlorophenol degradation analysis

The 2,4-DCP degradation by the *B. subtilis* in the MFC was measured over 4 days of cultivation. Fig. 4 (A) shows the HPLC peaks for the degradation of 2,4-DCP and its metabolites. It is interesting to observe that the concentration of 2,4-DCP was decreased from day 1 to day 4 proving the degradation process by *B. subtilis* was occurred. The highest 2,4-DCP removal of 60 % was obtained at day 4 as shown in Fig. 4 (B). Principally, the 2,4-DCP was degraded in the anoxic condition of MFC operation. It was reported that 2,4-DCP proceeds in a microbial biodegradation experiment via reductive dehalogenation in which chlorine atoms are replaced by hydrogen atoms [15, 16]. Several poly-chlorophenols are recalcitrant towards aerobic bacterial attack and can be reductively dehalogenated into lesser chlorinated phenols that could be further mineralized easily [7]. Previous studies also found that several bacterial species could degrade the 2,4-DCP (in the shake flask biodegradation experiments) into simpler metabolic products for instance *Pseudomonas sp.* DP-4 [17], *B. insolitus* [18] and *B. cereus* [19].

Based on this HPLC data, *B. subtilis* is found to dehalogenate 2,4-DCP into simpler chlorinated phenol molecules; 4-chlorophenol (99.0 % confirmation with the authentic HPLC-grade standard) and possibly to be cleaved via the formation of 3,5-dichlorocatechol (93.0 – 97.0 % confirmation with the authentic HPLC-grade standard) by a 2,4-DCP-hydroxylase [20]. Based on Fig. 4 (A), an unknown metabolite molecule was also detected at a retention time of 10.89 min. It is expected that this unknown metabolite which is probably having a molecular weight of smaller than 2,4-DCP and greater than 4-chlorophenol, could be another simpler phenolic product degraded metabolically by the *B. subtilis*.

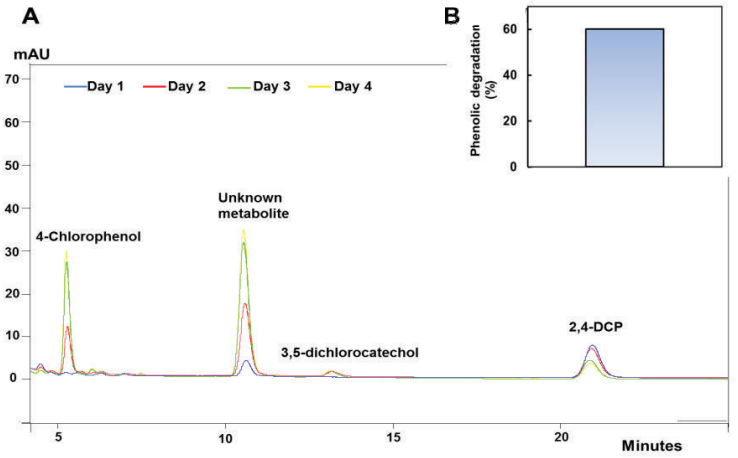

Fig. 4. (A) HPLC peaks for 2,4-DCP degradation metabolites; (B) percentage degradation of 2,4 DCP.

4. Conclusion

This study utilized safe bacteria, *B. subtilis* to treat hazardous and recalcitrant contaminant, 2,4-dichlorophenol in the MFC system. The voltage profile illustrated that the bacteria exhibited the highest voltage generation of 95 mV with current density of 12.0 mA/m^2. The CV test recorded the oxidation and reduction peaks at 0.34 mA / 0.12 V and - 0.35 mA / 0.63 V, respectively, and the LSV denoted the current peak which was at 36.0 mA with corresponding potential of 1.73 V (vs. Ag/AgCl). Although the voltage and polarization curves illustrate the tremendous incline phase of electrical outputs occurred during the early stage of phenolic feeding, the relatively lower inclining rate for the potential and power density curves succeeding the higher current densities lead to lower mass transport limitation in the system. The 2,4-DCP was degraded by the bacteria in the MFC reactor by 60 % and excreted simpler metabolite for instance 4-chlorophenol through reductive dehalogenation process; and 3,5-dichlorocatechol via the action of hydroxylase enzyme.

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