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SELECTION OF BACTERIAL COMMUNITY ON ELECTRODE BY ELECTRIC CURRENT FOR USE AS MICROBIAL FUEL CELL ANODE

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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Field of Study	Biotechnology
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กมล รอดอยู่ : การคัดเลือกชุมชีพแบคทีเรียบนขั้วไฟฟ้าด้วยกระแสไฟฟ้าเพื่อใช้เป็นแอโนดของเซลล์เชื้อเพลิงจุลินทรีย์ (SELECTION OF BACTERIAL COMMUNITY ON ELECTRODE BY ELECTRIC CURRENT FOR USE AS MICROBIAL FUEL CELL ANODE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.ศิริรัตน์ เร่งพิพัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.มานะ ศรียุทธศักดิ์, 92 หน้า.

วิทยานิพนธ์นี้ได้ทำการศึกษาผลการกระตุ้นด้วยกระแสไฟฟ้าบนแอโนด ต่อค่าแรงดันไฟฟ้า ความหนาแน่นกระแสไฟฟ้าและความหนาแน่นกำลังไฟฟ้าของเซลล์เชื้อเพลิงจุลินทรีย์ และได้เปรียบเทียบตัวแปรต่างๆที่ใช้ในการกระตุ้นซึ่งได้แก่ ระยะเวลาที่ใช้กระตุ้น อาหารเลี้ยงเชื้อที่ใช้ระหว่างกระตุ้น แหล่งคาร์บอนในเซลล์เชื้อเพลิงจุลินทรีย์ ชนิดและขนาดของกระไฟฟ้าที่ใช้ แหล่งของตัวอย่างดินหรือดินตะกอน และได้ศึกษาผลของอาหารเลี้ยงเชื้ออันได้แก่ nutrient broth (NB) และ phosphate buffer basal medium (PBBM) ที่ใช้ในระหว่างการกระตุ้นด้วยไฟฟ้ากระแสสลับต่อการเกิดกลุ่มแบคทีเรียบนแอโนด โดยเมื่อนำแอโนดที่ได้รับการกระตุ้นมาศึกษาภายใต้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (FESEM) พบกลุ่มของแบคทีเรียบนแอโนดในลักษณะไบโอฟิล์มที่มีลักษณะเป็นชั้นหนาเมื่อแอโนดถูกกระตุ้นด้วยกระแส 10-15 มิลลิแอมแปร์ และพบว่าไบโอฟิล์มบนแอโนดของอาหารเลี้ยงเชื้อทั้งสองชนิดมีลักษณะที่แตกต่างกัน หลังจากนั้นได้คัดแยกแบคทีเรียจากแอโนดไบโอฟิล์ม โดยสามารถคัดแยก *Shewanella putrefaciens* ซึ่งเป็นแบคทีเรียที่มีความสามารถในการถ่ายโอนอิเล็กตรอนให้กับแอโนดอย่างมีประสิทธิภาพได้ และพบว่าการกระตุ้นด้วยไฟฟ้ากระแสสลับที่ 15 มิลลิแอมแปร์ นั้น มีผลยับยั้งการเจริญของ *S. putrefaciens* สายพันธุ์ CU29 ทำให้มีจำนวนลดลงอย่างมาก (4 logCFU) ในการศึกษานี้ แรงดันไฟฟ้าวงจรเปิดสูงสุดที่ได้คือ 989 มิลลิโวลต์ โดยได้จากเซลล์เชื้อเพลิงที่ใช้โพรพิโอเนตเป็นแหล่งคาร์บอน และใช้แอโนดที่ไม่ได้กระตุ้นด้วยกระแสไฟฟ้า ใน PBBM เป็นเวลา 60 วัน อย่างไรก็ตามความหนาแน่นกระแสไฟฟ้าและความหนาแน่นกำลังไฟฟ้าสูงสุดคือ 72.9 มิลลิแอมแปร์ต่อตารางเมตร และ 13.4 มิลลิวัตต์ต่อตารางเมตร ได้จากเซลล์เชื้อเพลิงที่ใช้อะซิเตตเป็นแหล่งคาร์บอน และใช้แอโนดที่กระตุ้นใน PBBM เป็นเวลา 60 วัน ด้วยไฟฟ้ากระแสสลับที่ 5-10 มิลลิแอมแปร์ และพบว่าการกระตุ้นด้วยไฟฟ้ากระแสสลับสามารถนำไปใช้กระตุ้นกลุ่มของจุลินทรีย์ในตัวอย่างดินตะกอนจากแหล่งที่แตกต่างกันได้ เมื่อนำแอโนดไบโอฟิล์มที่ได้จากการกระตุ้นด้วยไฟฟ้ากระแสสลับมาศึกษาประสิทธิภาพในการลดค่า COD ของกากน้ำตาลและผลิตไฟฟ้าในเซลล์เชื้อเพลิงจุลินทรีย์ พบว่า แอโนดที่กระตุ้นด้วยไฟฟ้ากระแสสลับ 10 มิลลิแอมแปร์ สามารถลดค่า COD ได้ดีที่สุดถึง 60 เปอร์เซนต์และผลิตกระแสไฟฟ้าได้ 48 มิลลิแอมแปร์ต่อตารางเมตร จากผลการศึกษาวิจัยนี้พบว่าวิธีการกระตุ้นด้วยกระแสไฟฟ้า มีศักยภาพภาพที่จะนำไปใช้ในการคัดเลือกกลุ่มแบคทีเรียบนขั้วไฟฟ้า เพื่อใช้ในเซลล์เชื้อเพลิงจุลินทรีย์ต่อไป

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: MICROBIAL FUEL CELL / FERRIC REDUCING BACTERIA / ALTERNATIVE CURRENT STIMULATING ANODE

KAMOL RODYOU: SELECTION OF BACTERIAL COMMUNITY ON ELECTRODE BY ELECTRIC CURRENT FOR USE AS MICROBIAL FUEL CELL ANODE. ADVISOR: PROF. SIRIRAT RENGPIPAT, Ph.D., CO-ADVISOR: ASSOC. PROF. MANA SRIYUDTHSAK, D.Eng., 92 pp.

This dissertation studied the effect of anode electric current stimulation on open circuit voltage (Voc), current density and power density of MFC. Parameters which were used for electric current stimulation were stimulation periods, enrichment medium, carbon source in MFC, type and magnitude of electric current, and source of sediment. In addition, effect of nutrient broth (NB) and phosphate buffer basal medium (PBBM) on biofilm formation of stimulated anode were examined. Biofilm formation on anode was investigated under field emission scanning electron microscope (FESEM). It was found that thick biofilm was observed on AC stimulated anode at 10-15 mA. In addition, the different medium used in the enrichment of biofilm during AC stimulation led to the different characteristics of biofilm. Bacterial community on stimulated anode was isolated from biofilm anode. Electrochemical active bacteria (EAB) such as *Shewanella putrefaciens*, which effectively transferred electron to anode was isolated. The effect of AC stimulation on pure isolate showed that *S. putrefaciens* viable cell count was dramatically decreased (4 logCFU) when stimulated with 15 mA AC stimulation. In this dissertation, the highest Voc of 989 mV, produced from propionate-fed MFC with 60 days unstimulated anode in PBBM medium. However, the highest current density of 72.9 mA and power density of 13.4 mW m^{-2}, produced from acetate-fed MFC with 60 days, 5-10 mA AC stimulated anode in PBBM medium. Moreover, AC stimulation on anode can be used to stimulate different sources of sediments. Furthermore, application of stimulated anode for COD removal and electricity production was also investigated. It was found that the highest COD removal of 60% and 48 mA m^{-2} current density was obtained from molasses-fed MFC by using 10 mA AC stimulated anode. Thus, it could be concluded that electric current stimulation is a high potential tool for selecting effective bacterial community on electrode and using as anode for the MFC.

Field of Study: Biotechnology

Academic Year: 2015

Student's Signature .....

Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

MFC	=	microbial fuel cell
FRB	=	ferric reducing bacteria
EAB	=	electrochemical active bacteria
R	=	resistance
Voc	=	open circuit voltage
COD	=	chemical oxygen demand
%	=	percent
°C	=	degree Celsius
CFU	=	colony forming unit
gm	=	gram
min	=	minute
hr	=	hour
M	=	molar
mM	=	millimolar
mg	=	milligram
mL	=	milliliter
mV	=	milli-volt
mW	=	milli-watt
mA	=	milli-amperes
kΩ	=	kilo ohm
m	=	meter
m^2	=	square meter
cm	=	centimeter
cm^2	=	square centimeter
µm	=	micrometer

CHAPTER 1
INTRODUCTION

1.1 Rational

Fuel cell, an electrochemical energy conversion device is believed to be an alternative and renewable energy source for sustainable future energy needs. However, hydrogen production produced by electro-catalysis of water or fermentation process from biomass paid a lot of cost and energy consumption [1]. In order to convert biomass and organic compounds directly into electricity, the implementation of microorganism in fuel cell system was developed since 1911 [2]. Microorganism plays an important role in microbial fuel cell (MFC) by using organic compound as an electron donor in their catabolic pathway and transfers electrons to anode electrode. Due to lack of ability to facilitate electron transfer to anode electrode, the electricity output from Potter's MFC using Escherichia coli or Saccharomyces cerevisiae occurred in very low efficiency. The electron transfer of microbes in MFC can be enhanced by electron mediators, redox compounds, which have an electrochemical activity—oxidation and reduction of electron—and act as an electron shuttle between the microorganism and the anode electrode [3]. On the other hand, continuous additions of the electron mediators such as redox dyes may increase the operating cost and cause toxicity to living organism after release to the environment.

Microorganism directly transfer their electrons to anode of MFC was firstly proposed since 1999 [4]. *Shewanella putrefaciens* IR-1, a ferric reducing bacterium, was proved to have electrochemical activity against electrode and could use electrode as final electron acceptor to support their lactate catabolism in MFC [5]. In order to use MFC to treat wastewater, electrochemical active consortiums or biofilms on anode electrode are required [6]. The direct attachment of bacteria and biofilm formation on

anode in MFC can enhance the electron transfer greater than only bacterial cell suspension resulting in the increase of power density [7]. In addition, biofilm formation at anode can retain bacterial cells in anodic chamber which is required for continuous operation of the MFC for long periods [8]. Poised potential and current stimulation on anode could enhance electricity output, biofilm formation and COD removal in anode of MFC [9]. Poising electrical potential at anode gained more electricity output (current) than unpoised anode was found. Moreover, poised potential can reduce lag time of the pure culture [10] and mix consortium [11] in producing current; however, there is no difference in the maximum current output. Direct current stimulated at anode promoted biofilm formation and increased COD removal [12].

From my previous study [13], the assumption of bacteria that can resist and survive in electrical field should have an electrochemical activity for maintain themselves on electric stress conditions. Electric current could also be used to select electrochemically active isolates that can produce electricity in mediator-less MFC. In addition, many isolates that produced high electricity output were selected by electric current lower than 6 milli-amperes. Therefore, this research aims to search for a suitable electric current for selection of electrochemically active bacterial biofilm for use in anode compartment of MFC.

1.2 Objectives

1. To select bacterial community on anode using electric current

2. To isolate electrochemical active bacteria based on ferric reduction activity

3. To evaluate the electricity outputs of pure isolate and bacterial community in microbial fuel cell

4. To evaluate the electric current that appropriate to select bacterial community for use as anode for microbial fuel cell

CHAPTER 2
THEORY AND LITERATURE SURVEY

2.1 Fuel cell

Fuel cell, an electrochemical device that converts chemical energy to electrical energy was firstly developed by Sir William R. Grove in 1839 [14]. The fuel cell is comprised of anode and cathode side separated by proton exchange membrane (PEM). Hydrogen is reduced and catalyzed into proton and electron by platinum at anode electrode. Proton passes through PEM into cathode. Electron moves to cathode via external load. Oxygen in cathode oxidize proton and electron into water. Basic physical structure and the principle of PEM fuel cell is indicated in Figure 2.1. The reaction taking place in the anode and cathode, and the overall are reacted as shown in Equation 2.1, 2.2 and 2.3, respectively.

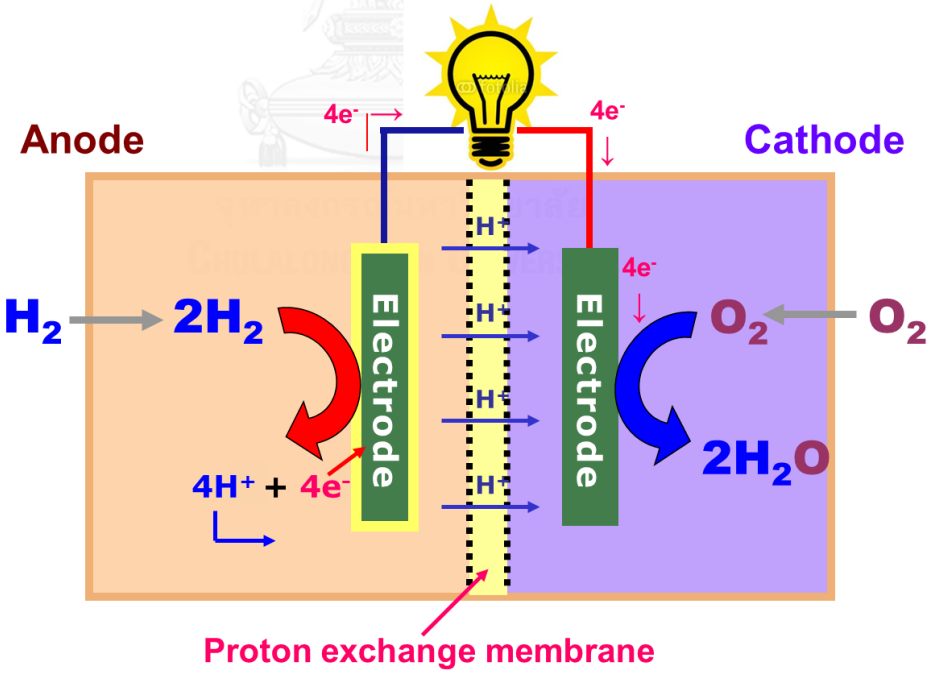


Figure 2.1 Schematic diagram of a PEM fuel cell

Anode reaction: $2H_2 \longrightarrow 4H^+ + 4e^-$ (2.1)

Cathode reaction: $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$ (2.2)

Overall reaction: $2H_2 + O_2 \longrightarrow 2H_2O$ (2.3)

2.2 Microbial fuel cell

Microbial fuel cell (MFC) was developed base on platform of fuel cell. It was firstly proposed by Potter in 1911 [2]. MFC is a bio-electrochemical device that can directly convert organic compounds into electricity through the catalytic activity of microorganisms. Microorganisms including yeast, *Saccharomyces cerevisiae* and bacteria, *Escherichia coli* were used in previous MFCs [2]. They performed oxidizing ability on glucose, starch and cane sugar that is later changed into electricity.

Generally, MFC is consisted of two separated compartments, anode and cathode, which are separated by the PEM. Microorganisms are used in the anode compartment to utilize organic compounds and reduce electron to electrode. Electrons move from the anode to the cathode through the external, while the hydrogen ions transport from the anode across through the PEM into the cathode compartment to combine electron with oxygen to form water. The schematic diagrams of MFC are shown in Figure 2.2.

However, the electron transfer to the anode electrode of microbes depends on electrochemical activity of cell. The electron transfer to the anode electrode of microbes that lack of electrochemical activity will occur in very low efficiency that reflects in low electric current production. The electron transfer of microbes to anode electrode in MFC can be enhanced by electron mediators [3]. Electrochemically active bacterium that can self-mediate electron transfer to the anode electrode was firstly proposed by Kim *et al*. (1999) [4, 5]. *S. putrefaciens*, ferric reducing bacterium (FRB), can directly transfer electron to the anode electrode [15]. Many FRB, which have the ability to use soluble and insoluble ferric ion (Fe^{3+}) as an electron acceptor in anoxic environment, express electrochemical activity by using graphite electrode of MFC as an electron acceptor [4, 8, 16-18]. Electrochemical activity of bacteria can be determined by cyclic voltammetry

[4] which is used to characterize oxidation and reduction of redox compounds including redox protein such as cytochromes on bacterial cell membrane.

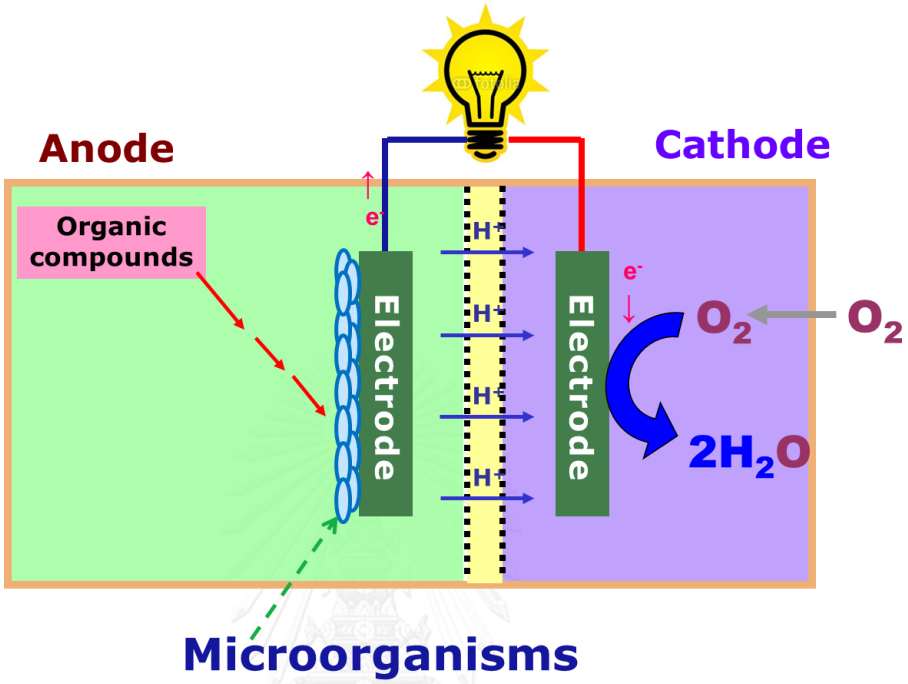


Figure 2.2 Schematic diagram of a MFC

There are several reports mentioned that membrane-bound cytochromes of *S. putrefaciens* cultured in anoxic condition are present on its outer membrane [19, 20]. It was believed that membrane-bound cytochromes involved in electron transfer of *S. putrefaciens* to electrode [15]. Phenomena of electron transferring via periplasmic c-type cytochromes of *S. oneidensis* MR-1 biofilm closely attached anode simulated the reduction reaction of insoluble Fe(III) which served as an electron acceptor [21, 22].

In some cases, microorganisms such as *Pseudomonas aeruginosa* could produce their own mediators, phenazine pyocyanin, that can be used to facilitate the electron transfer to electrode [23]. Pyocyanin compounds such as phenazine cause pathogenic effect on human and inhibitory effect on other bacteria [24]. Not only, *Ps. aeruginosa* can use phenazine as electron mediator, but other bacteria such as *Lactobacillus amylovorus*, *Entrococcus faecium* [25] and *Brevibacillus* sp. [26] can also use phenazine for their electron transfer to electrode.

Moreover, *Geobacter sulfurreducens* used electrical pilli or nanowires to transfer electrons to electrode or insoluble ferric hydroxide as an electron acceptor for their respiration [27, 28]. In addition, *S. oneidensis* MR-1 also have bacterial nanowires for transferring electrons to electrode [29], but the size of its nanowires is different from *G. sulfurreducens*.

In summary, there are three proposed mechanisms of self-mediate electrode transfer of bacteria that used in mediator-less MFC as shown in Fig. 2.3 First, electrons are transferred via membrane bound cytochromes to electrode that closely attached (green). Second, bacteria utilize self-produced electron mediator for use as electron shuttle between cell and electrode (blue). Third, electrons are transferred through electrical pilli or "nanowires" that directly contact with electrode (purple). Other bacteria (brown) in biofilm can transfer electron through electrochemical active bacteria to anode [30].

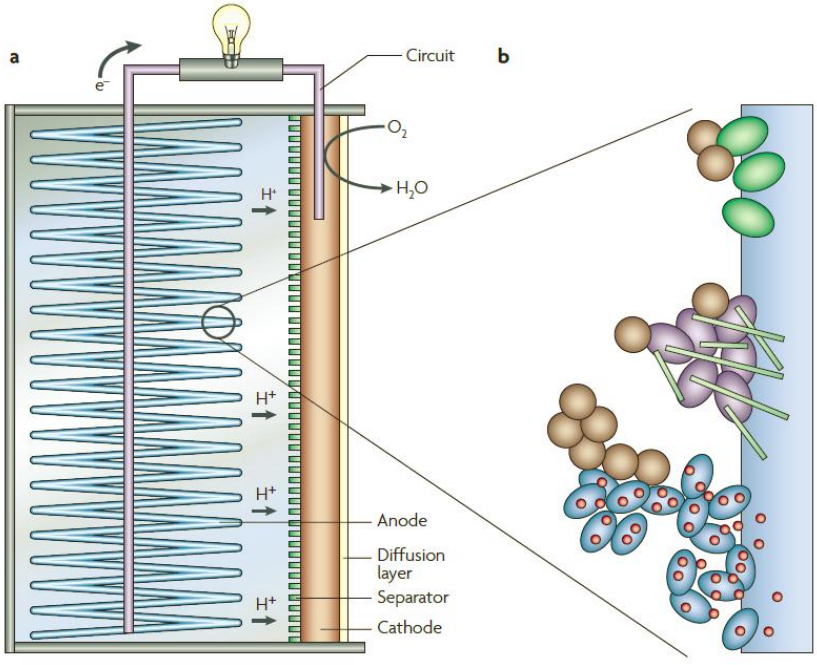


Figure 2.3 Mechanisms of electron transfer of bacteria in MFC

2.3 Ferric reducing bacteria as biocatalysts in mediator-less MFC

Ferric reducing bacteria (FRB) play a vital role in the iron nutrient cycling on the Earth's crust [31]. They can reduce ferric ion (Fe^{3+}) into ferrous ion (Fe^{2+}) by using ferric ion (Fe^{3+}) as terminal electron acceptor in anaerobic respiration. The ferric ion reduced by microorganisms may be in soluble form such as ferric citrate or insoluble form for example; limonite (FeOOH), goethite ($Fe_2O_3 .H_2O$) and hematite (Fe_2O_3). Furthermore, many reports indicated that *Shewanella* sp. and other FRB can be operated effectively in mediator-less MFC. FRB holds great promise as microbes that be used in mediator-less MFC because they have the potential for use graphite electrode as a final electron acceptor in their respiratory system. Culture of *S. putrefaciens* that grown in an anaerobic condition possessed electrochemical activity greater than aerobically grown culture but this electrochemical activity did not belong to both anaerobically and aerobically-grown *E. coli* [15]. Many researchers used pure culture of FRB such as *S. putrefaciens* [15], *S. oneidensis* [29], *Clostridium butyricum* [17], *Geobacter sulferreducens* [8], *Aeromonas hydrophila* [18] and *Rhodoferrax ferrireducens* [16], as biocatalysts in mediator-less MFC. But not all ferric reducing bacteria could produce electricity current. FRB, *Pelobacter carbinolicus* could reduce ferric oxide but couldn't use electrode as an electron acceptor therefore none of electricity was produced [32].

2.4 Biofilm on anode

Biofilm is a structure of microbial community on surface that microorganisms in community secrete extracellular polymeric substances (EPS) to prevent them from the environment. EPS was typically composed of protein, polysaccharides, DNA and enzymes. EPS had many functions such as adhesion, aggregation of bacterial cells and protective barrier depend on microorganisms and environment [33].

Biofilm on anode functions as electron donor, electron acceptor and conductive material on electrode surface. Biofilm matrix on anode comprises of bacterial community, EPS and filamentous structure. EPS mainly compose of protein, polysaccharides and humic substances [33]. Benefit of anode biofilm was the ability to share component (self-producing mediator) or structure (nanowires) of electrochemical

active bacteria to transfer their electrons to electrode [30]. The direct attachment and biofilm formation on anode in MFC can enhance the electron transfer greater than the ones from only bacterial cell suspension resulting in the increase of power density [7]. Biofilm of co-culture of *Ps. aeruginosa* and *E. faecium* could generate current higher than single culture biofilm [34]. Modeling study of anode biofilm showed that the thinness and thickness of biofilm could affect electron transfer of biofilm [35].

2.5 Effect of poised potential stimulation on anode of MFC

In MFC system, Cho and Ellington (2007) [10] investigated the impact of positive poised potential on current production of aerobically grown *S. oneidensis* inoculums. The effect of various positive potential from 0-500 mV that poised into anode chamber on lag period prior current production and current output were observed. When poised potential increased lag period was decreased from 90 to 5 hour before current production but not significantly different in maximum current productivity. They suggested that aerobically grown cells could be adapted for current production in anaerobic condition by poised potential that supplied into anodic compartment of mediator-less MFC. Higher poised potential above 750 mV inhibited the growth and current production of *S. oneidensis* in MFC were previously claimed [10].

Wang et al, (2009) [11] investigated the effect of positive poised potential at +200 mV on anode of MFC using domestic waste water mixed with anaerobic sludge as inoculum. Positive poised potential could reduce startup time of MFC from 59 days to 35 days compare to control (unpoised potential). But power output of poised potential MFC did not significantly different from control.

Srikanth et al, (2010) [9] studied the influence of positive poised potential from 200-1,000 mV on MFC using anaerobic mixed consortia as inoculum. They found that poised potential at 600 mV could effectively remove COD in synthetic waste water and generate the highest power output of 79.33 mW m^{-2}.

Torres et al, (2009) [36] used poised potential from -150, -90, +20 and +370 mV to select electrochemical active bacteria. They found that negative potential could select

Geobacter-dominated biofilms but positive potential could select other bacteria including *Geobacter* and other non-electrochemical active bacteria.

Commault et al, (2015) [37] used negative poised potential to select *Geobacter*-dominated biofilms. They used poised potential at -360 mV to select biofilm from three different inoculum sources. It was found that *Geobacter* sp. were dominated species in anode biofilm of all inoculums.

2.6 Effect of current stimulation on anode of MFC

Lin et al, (2013) [12] investigated the effect of direct current (DC) stimulation on anode of MFC using river sediment, activated sludge and anaerobic sludge as inoculums. They found that +2V DC stimulation of river sediment could generate highest power output of 98 mW m^{-2} and it could stimulate biofilm formation at anode better than unstimulated anode.

CHAPTER 3
MATERIALS AND METHODS

3.1 Instruments

1. 4-Digit precision weighting balance model AG 204 (Mettler Toledo, Switzerland)
2. Anaerobic jar model GENbox (Mitsubishi chemical, Japan)
3. Autoclave model ES-315 (Tomy Seiko Co. Ltd, Japan)
4. Digital camera model X-pro1 and X10 (Fujifilm, Japan)
5. DNA thermo cycler model TP 600 (TaKaRa Bio Inc., Japan)
6. Electrophoresis chamber set model Mupid-exU (TaKaRa Bio Inc., Japan)
7. Field emission scanning electron microscope model JSM-7610F (JOEL, Japan)
8. Gel Documentation system (Bio-Rad, USA)
9. Hot air oven model UC 30 (Memmert, Germany)
10. Hot plate stirrer model MSH 20D (Wisestir, Korea)
11. Incubator (Memmert, Germany)
12. Kubota Refrigerated Microcentrifuge model 6500 (Kubota, Japan)
13. Laminar flow 'clean' model V6 (Lab service, Thailand)
14. Micropipette model P2, P20, P100, P200, and P1000 (Gilson, France)
15. Microscope model CH 30RF200 (Olympus, Japan)
16. Multi-parameter bench photometer (Hanna, Japan)
17. Personal computer model X2000 (HP, USA)
18. Pipette tip model 0.01 ml, 0.2 ml, and 1 ml (Axygen Scientific, USA)
19. pH meter (Mettler-Toledo, USA)
20. Syringe (Nipro, Thailand)
21. Syringe filter membrane pore size 0.45 µm (CTI group, Thailand)
22. Volt meter model pico ADC11 parallel (pico Technology, UK)
23. Vortex mixer model G-560E (Scientific Industries, USA)
24. Water bath model WB14 (BecThai, Thailand)

3.2 Material, test kit, and chemicals

1. Agarose (Vivantis, Malaysia)
2. API 20E test kit (bioMérieux, France)
3. Bacto agar (Difco Laboratories, USA)
4. Dipotassium hydrogen phosphate (A.R. grade) (Merck KGaA, Germany)
5. Ferric citrate (analytical grade) (SRL, India)
6. Glucose (analytical grade) (Merck KGaA, Germany)
7. Medium Range COD reagent (Hanna, Japan)
8. Neosepta® PEM (CMS C-1502) (ASTOM, Japan)
9. Nutrient Broth (Oxoid, UK)
10. Paraffin oil (A.R. grade) (Carlo erba, France)
11. Potassium dihydrogen phohosphate (A.R. grade) (Merck KGaA, Germany)
12. Potassium hexa cyanoferrate (III) (A.R. grade) (May and Baker, UK)
13. Sodium acetate (A.R. grade) (Merck KGaA, Germany)
14. Sodium chloride (A.R. grade) (Merck KGaA, Germany)
15. Sodium propionate (A.R. grade) (Sigma, USA)

3.3 Experimental procedures

3.3.1 Sample collection

Sub-sediments from fresh water and sea water were collected. Five samples were collected from individual source as shown in Table 3.1. All samples were stored at 4 °C before use.

3.3.2 Alternative current (AC) stimulation of bacterial biofilm on anode

A 5 gm sample was inoculated into 250 ml glass bottle containing 100 ml of nutrient broth (NB) or phosphate buffer basal medium (PBBM), having 3 cm X 3 cm carbon cloth electrode immersed in the solution. The solution was covered with paraffin oil in order to generate an anaerobic condition. Subsequently, the various electrical current 0, 5, 10, and 15 milli-amperes (mA) was biased respectively, in each system by connecting with Modified Howland's circuit AC current source (Bio-Electronic Research Laboratory) to the copper wire of carbon cloth electrode as shown in Figure 3.1. Each

experiment was performed in triplicate. After incubation for 15 days, electrodes were transferred to anode chamber of MFC.

Table 3.1 Source of sub-sediment and sub-surface soil collected from various sources

Sample	Collecting site	Depth (m)	Location (Province)	Denoted as
1	Sub-sediments inside the cave	0.3	Trang	Trang
2	Sub-surface soil from shrimp farming pond	0.5	Chachoengsao	Cha
3	Sub-sediment from Ko Sichang	0.25	Chonburi	Si
4	Sub-surface soil	2	Khonkaen	Khon
5	Sediment under flood way of Chulalongkorn University	2	Bangkok	CU

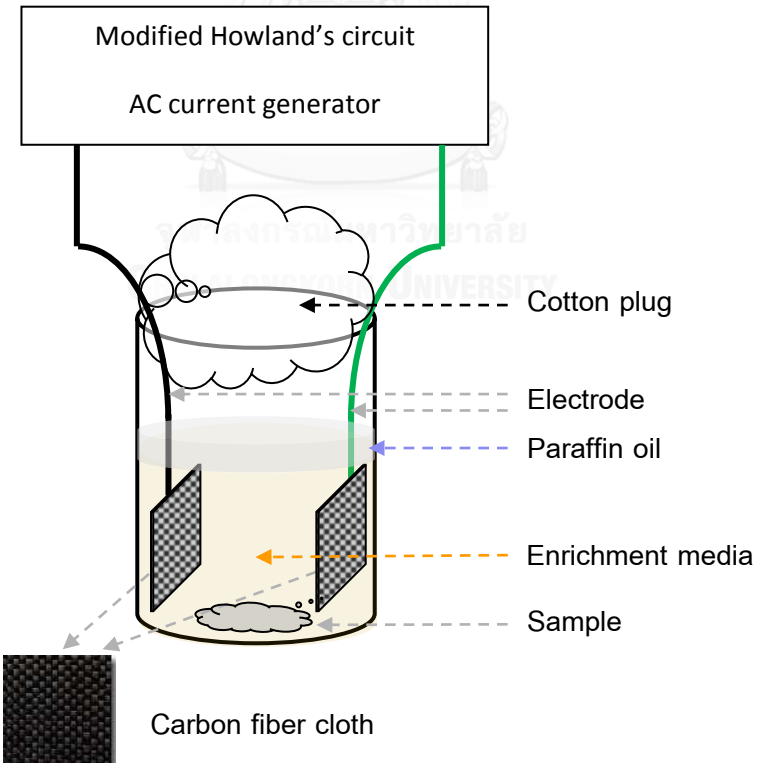


Figure 3.1 Configuration of AC stimulation on anode experiment

3.3.3 Effect of electric current stimulation parameters on electricity output of MFC

A 5 gm sample was inoculated into 250 ml glass bottle containing 100 ml of nutrient broth (NB) or phosphate buffer basal medium (PBBM) or fresh water medium (FWA), having 3 cm X 3 cm carbon cloth electrode immersed in the solution. Paraffin oil was put to cover the solution surface in order to generate an anaerobic condition. Subsequently, the various AC or DC current 0, 5, 10, and 15 mA was biased respectively, as mentioned in section 3.3.2. After incubation for 15 or 60 days, electrodes were transferred to anode chamber of MFC.

3.3.4 Evaluation of electricity output of MFC using electric current stimulated anode

3.3.4.1 Configuration of MFC

MFC chamber was designed and constructed from 100 ml glass bottle (Duran, Germany) that connected with glass socket for sampling port and the opposite side of sampling port was connected with cylindrical glass, 1.8 cm in diameter, for the connection anode to cathode compartment as shown in Figure 3.2 . All glassware sets were sterilized by autoclaving at 121 ºC for 15 minutes before use. Neosepta® PEM was installed between the anode and cathode compartments and sterilized by autoclaving at 110 ºC for 10 minutes. A 3 cm X 3 cm carbon fiber cloth (ACELAN, Korea)—surface area (18 cm^2)—was used as electrodes in both compartments.

3.3.4.2 Electrolyte solution in MFC

Anode chamber contained phosphate buffer basal medium (PBBM) with 10 mM of sodium acetate, sodium propionate or glucose. Cathode chamber contained 1 mM potassium ferric cyanide ($K_3Fe(CN)_6$), as electron acceptor.

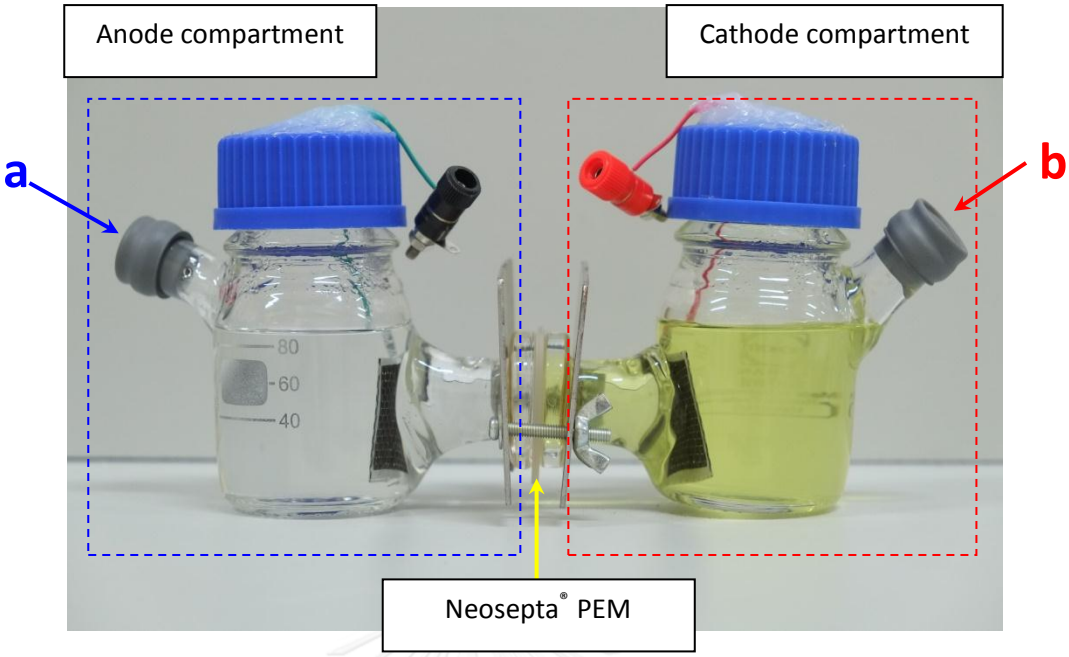


Figure 3.2 Configuration of MFC, (a) and (b) show injecting and sampling port

3.3.4.3 MFC operated with stimulated anode

Stimulated anode of each AC electric current (0, 5, 10, 15 mA) was placed in anode chamber of MFC. After that silicone sealant was filled on the top of cap where wire of anode out of the chamber.

3.3.4.4 MFC operated with pure isolate

Facultative anaerobic isolates were cultured in nutrient agar (NA) (Difco®) incubated at room temperature. Bacterial cultures were prepared by scraping colony on nutrient agar and resuspended in 5 ml of 0.85% sodium chloride solution until concentration equal to 0.5 McFarland (approximate cell count 1.5 x 10^8 CFU ml^{-1}). Cell suspension of 5 ml was inoculated via injecting port. For mono-culture and co-culture of isolate in MFC, glucose concentration was determined by Somogyi-Nelson method [38].

3.3.4.5 Electrical parameter measurement and calculation

MFC performances were evaluated in term of voltage, current density and power density that supplied to external load or resistor. MFC system was connected

to pico ADC-11 data acquisition unit (pico technology, UK), the voltage output from MFC was recorded every 60 seconds and transferred to the personal computer via parallel port. The open circuit voltage (*V*oc) was obtained when the MFC was disconnected from load or resistor. Various external resistance load(*R*), 0.1, 0.5, 0.75, 1, 2, 5.1, 10, 51 and 100 kΩ, were connected to the MFC to create different load condition. Current density and power density were obtained from the output voltage measurement when the load was connected. The current density (i) (mA m^{-2}) was calculated as shown in Equation 1 where V is the voltage (volts) and *a* is electrode surface area (m^2). The power density (P) (mW m^{-2}) was calculated as indicated in Equation 2 [39].

Current density: $$i = (V / R) / a \quad (1)$$

Power density: $$P = (I \times V) / a \quad (2)$$

Anode stimulating current (0-15 mA) vs. voltage (open circuit), current density (i), and power density (*P*), were plotted to investigate the maximum power condition.

3.3.5 Preparation of anode for observing biofilm under scanning electron microscope

Anode was fixed in 2.5% glutaraldehyde in 0.1 M Phosphate buffer pH 7.2 for 2 hr. The anode was rinsed twice with phosphate buffer and once with distilled water for 10 min. Then it was subsequently dehydrated with a graded series of ethanol (30%, 50%, 70%, 95% for 10 min per each and 100% for 3 times, 5 min per times). Anode was dried in critical point dryer (Quorum model K850, UK) then mounted and coated with gold using sputter coater (Balzers model SCD 040, Liechtenstein). Anode was observed under a field emission scanning electron microscope (Joel, model JSM-7610F, Japan)

3.3.6 Isolation of electrochemical active bacteria from anode biofilm

Electrochemical active bacteria on anode were isolated based on their ferric reducing activities. Biofilm was resuspended in PBBM and serially diluted in 0.85% NaCl solution and spread on PBBM agar with 20 mM of ferric citrate, 10 mM of sodium

acetate, and incubated in anaerobic jar (GENBox anaer), at room temperature for 5-7 days. The change of PBBM with ferric citrate from the reddish-brown color to the light-green color was used to evaluate the ferric reducing activity. Single colony on PBBM was restreaked until pure colony was obtained. Pure isolate of FRB was restreaked on nutrient agar, incubated at 37°c in aerobic condition for observation of their growth condition.

3.3.7. Identification of isolated bacteria

3.3.7.1 Morphological examination and biochemical tests

Bacterial identification was classified based on morphology, Gram's straining, and nutrient utilization by using rapid identification kit API® 20E. Results from API kit were interpreted by using program API®WEB (bioMérieux, France).

3.3.7.2 Molecular technique based on 16s rDNA sequencing

Genomic DNA of overnight cultures were extracted using GF-1 nucleic acid extraction kit (Vivantis, Malaysia). DNA extraction procedure was prepared by following the manufacturer's instructions manual. The PCR amplification of 16s rRNA gene was amplified by using two universal primer 16F27 (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16R1492 (5'-TAC GGC TAC CTT GTT ACG ACT T-3') as described in Bayane *et al*. (2006) [40]. About 1,500 base-pairs of PCR amplicons were performed under the following conditions: denaturing 94 °C for 1 minute, annealing 55 °C for 1 minute and elongating 72 °C for 2 minutes by PCR thermal cycler TP600 (TaKaRa Bio Inc., Otsu, Shiga, Japan) for 35 cycles. PCR products were submitted for sequencing at 1st Base (Singapore). Bacterial similarity was obtained after DNA sequence compared with the GenBank database of the *National Center for Biotechnology Information* (NCBI) using BLASTn (for nucleotide sequence) algorithm.

3.3.8 Effect of stimulating current on pure isolates

Bacterial isolates were inoculated into 250 ml flask containing 100 ml of nutrient broth (NB). 3 cm X 3 cm carbon cloth electrode was immersed in the solution. The solution was then covered with paraffin oil to generate anaerobic condition.

Subsequently, the various electrical current 0, 5, 10, and 15 mA was introduced respectively, as mention in section 3.2.2. To study the effect of AC stimulating current on bacteria, bacterial concentration was determined by drop plate technique for 48 hr.

3.3.9 Electricity production and COD removal of MFC of stimulated anode biofilm

AC stimulated anode of sediments sample from CU in PBBM and NB medium was only performed their utilization of molasses instead of glucose. Molasses were adjusted COD to nearly equal to COD of 10 mM glucose in PBBM medium. Stimulated anode was transferred to anode chamber of MFC contained PBBM and molasses. Initial COD and final COD was measured by medium range COD reagent (HANNA). Culture broth from anode was centrifuged at 8,000 rpm for 10 min, after that supernatant was filtered by using 0.45 μm syringe filter. Supernatant was pipetted 2 ml into the COD reagent and mixed thoroughly. Put reagent tube on heater at 150°c and heat for 2 hr. After heating for 2 hr, reagent tube was mixed thoroughly and cooled down. After cooling down, COD was measured by HANNA bench meter.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Effect of alternative current (AC) stimulation and enrichment medium on anode biofilm formation

In this experiment, the effect of enrichment medium on anode biofilm formation was studied using PBBM and NB as enrichment medium during AC stimulation.

4.1.1 AC stimulation on anode in PBBM medium

After AC stimulation on anode in PBBM for 15 days, color of the medium stimulated at 10 and 15 mA were changed to be more blackish-color than the ones at 0 and 5 mA. Figure 4.1 shows a typical color of the medium with of the CU sediment after 15 days stimulation. All sediment samples in this research showed the same color changing patterns.

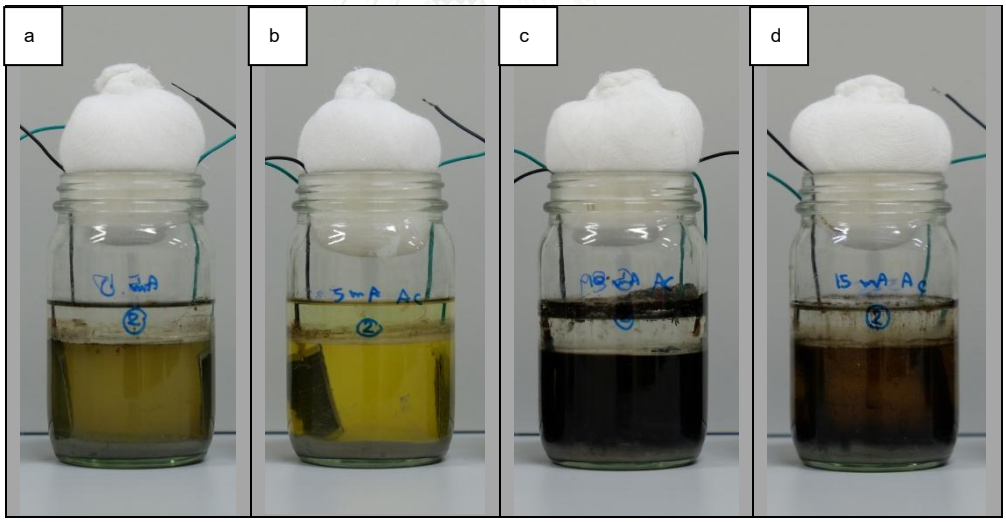


Figure 4.1 AC stimulation of CU sediment after incubation for 15 days:

(a) Enrichment 1: PBBM without AC stimulation (0 mA)

(b) Enrichment 2: PBBM with AC stimulation at 5 mA

(c) Enrichment 3: PBBM with AC stimulation at 10 mA

(d) Enrichment 4: PBBM with AC stimulation at 15 mA

Before transferring the stimulated anode to MFC, the anode was rinsed in PBBM to remove unattached cell, only biofilm on anode was then transferred to anodic chamber of MFC. Biofilm on anode was shown in Figure. 4.2.

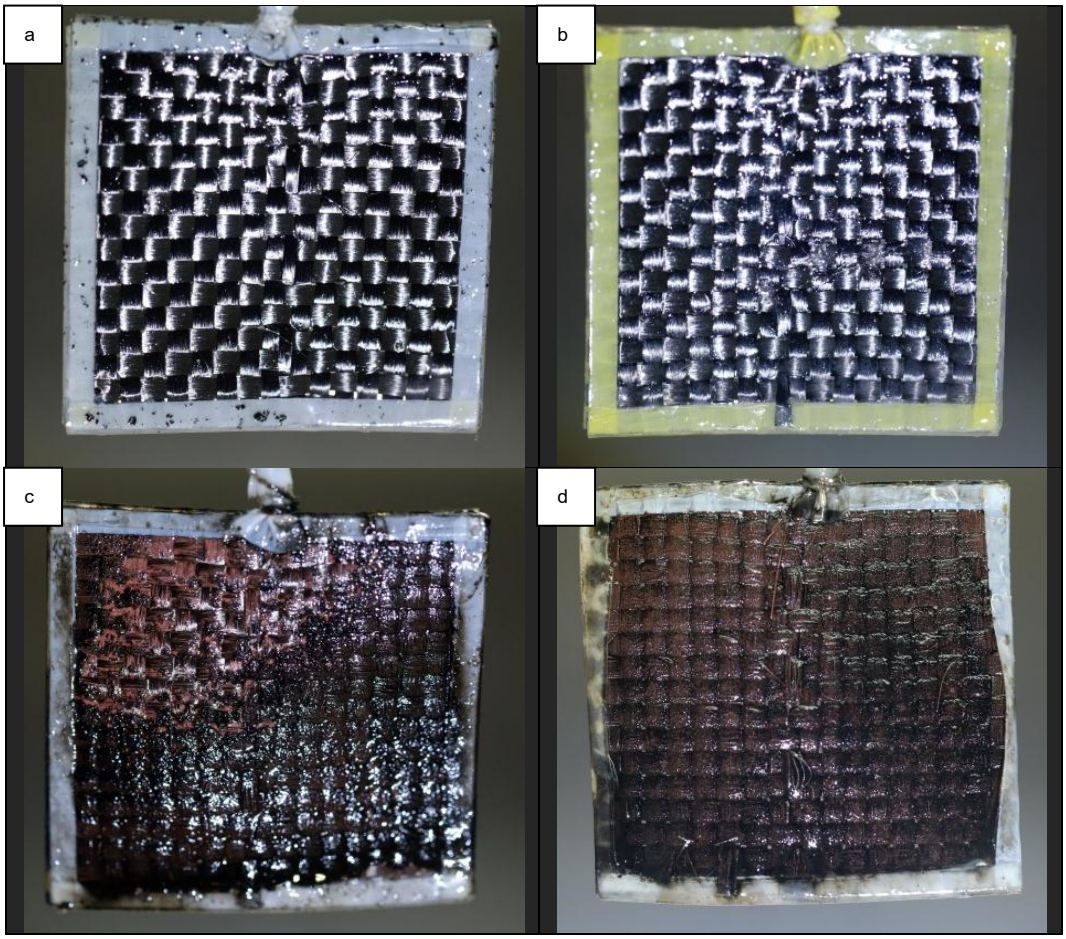


Figure 4.2 Biofilm formations on AC stimulated anode of CU sediment after incubation for 15 days in PBBM

(a) Stimulated anode without AC stimulation (0 mA)

(b) Stimulated anode with AC stimulation at 5 mA

(c) Stimulated anode with AC stimulation at 10 mA

(d) Stimulated anode with AC stimulation at 15 mA

4.1.2 AC stimulation on anode in NB medium

AC stimulation on anode in NB medium was only performed using sediment sample from CU in order to compare the effect of enrichment medium on biofilm during

AC stimulation. After AC stimulation in NB for 15 days, red color was observed in the medium which were stimulated at 10 and 15 mA, as shown in the bottom of Figure 4.3(c&d).

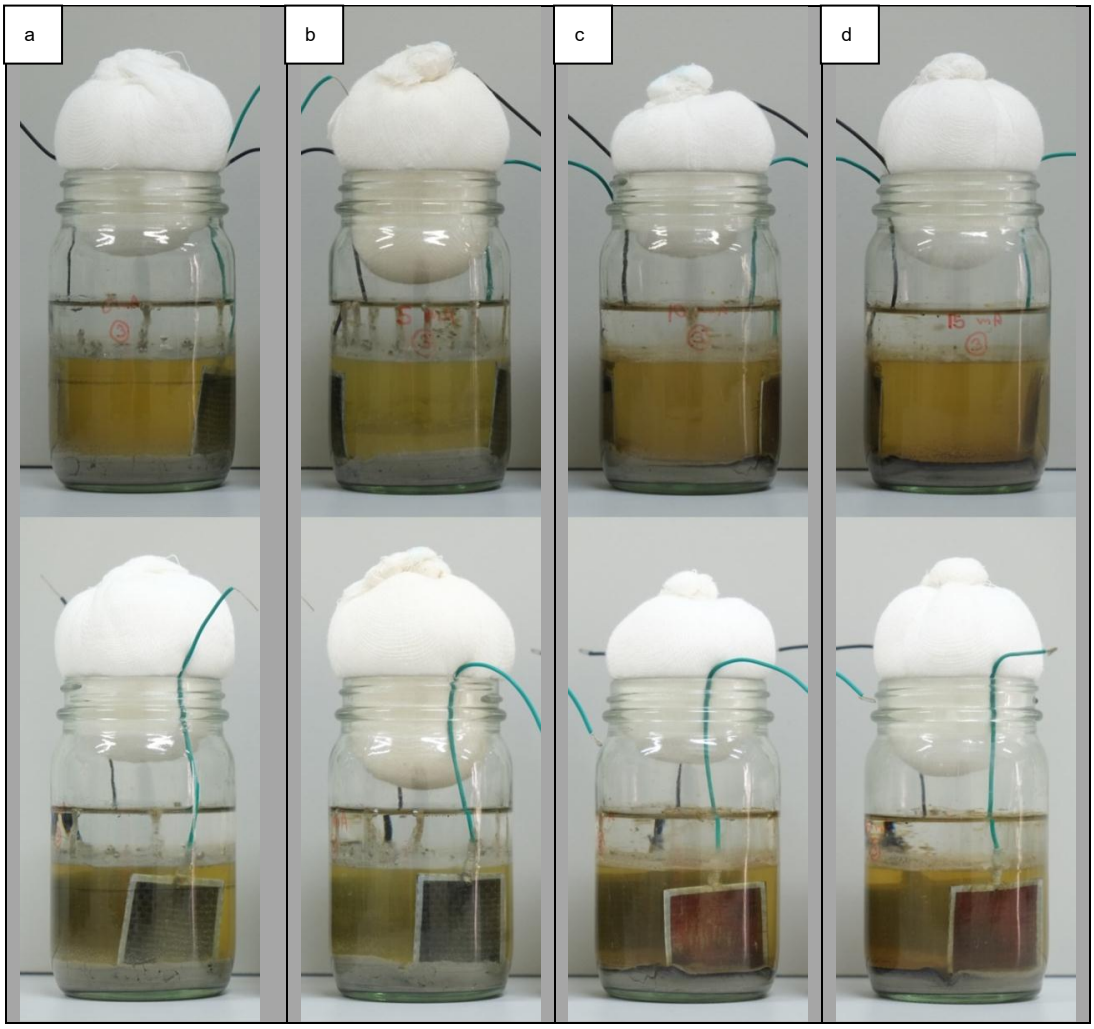


Figure 4.3 AC stimulation of CU sediment in nutrient broth (NB) Top: front view Bottom: side view after incubation for 15 days:

(a) Enrichment 1: NB without AC stimulation (0 mA)

(b) Enrichment 2: NB with AC stimulation at 5 mA

(c) Enrichment 3: NB with AC stimulation at 10 mA

(d) Enrichment 4: NB with AC stimulation at 15 mA

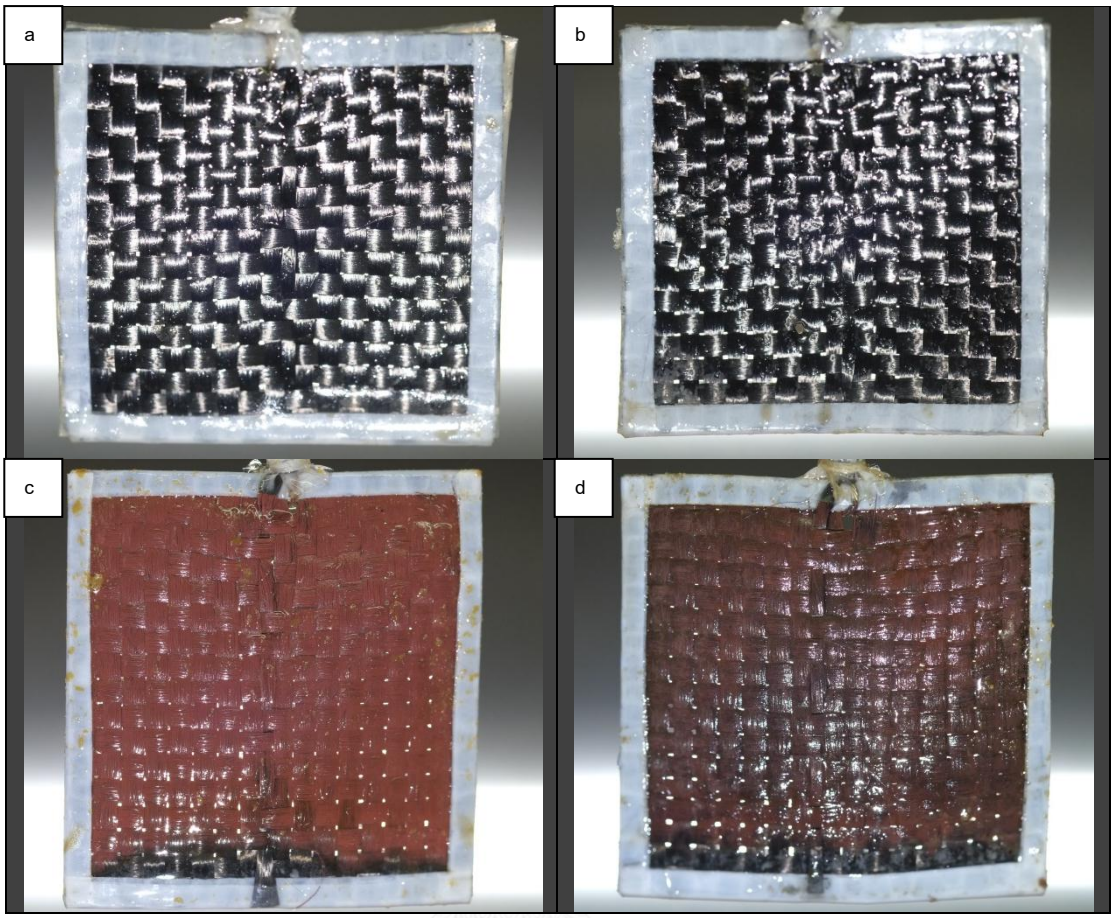


Figure 4.4 Biofilm formations on AC stimulated anode of CU sediment after incubation for 15 days in NB

(a) Stimulated anode without AC stimulation (0 mA)

(b) Stimulated anode with AC stimulation at 5 mA

(c) Stimulated anode with AC stimulation at 10 mA

(d) Stimulated anode with AC stimulation at 15 mA

According to the difference of medium used during AC stimulation, biofilm was formed on anode with 10 and 15 mA in Figure. 4.2(c&d) and 4.4(c&d) which can be seen with naked-eyes but biofilm on anode with 5 mA and without (0 mA) AC stimulation could not be clearly observed. From these results, the different medium used in the enrichment of biofilm during AC stimulation led to the different characteristics of biofilm. Stimulated anode was cut and examined for the characteristics of biofilm under scanning electron microscope.

4.1.3 Biofilm on stimulated anode under scanning electron microscope (SEM)

A piece of stimulated anode from CU sediment in either PBBM or NB was examined under field emission scanning electron microscope (FESEM) at Scientific and Technological Research Equipment Center, Chulalongkorn University (STREC). Biofilm on anode enriched by either PBBM or NB was shown in Figure 4.5-4.11 and appendix B.

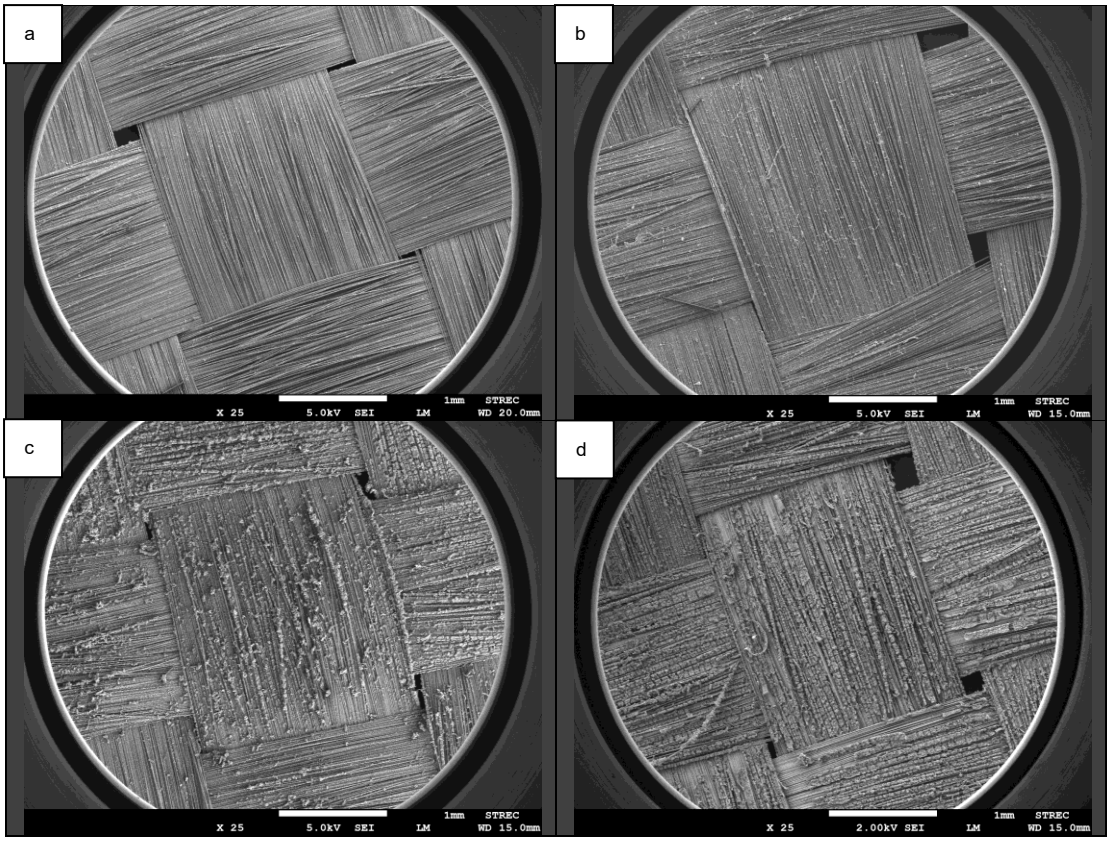


Figure 4.5 SEM micrographs at 25x magnification of stimulated anode from CU sediment enriched in PBBM

(a) Stimulated anode without AC stimulation (0 mA)

(b) Stimulated anode with AC stimulation at 5 mA

(c) Stimulated anode with AC stimulation at 10 mA

(d) Stimulated anode with AC stimulation at 15 mA

Figure 4.5 depicted SEM micrographs at 25x magnification. The carbon fibers were thickening when anode stimulating AC current was increased. The thickest anode biofilm was observed at 15 mA stimulated anode.

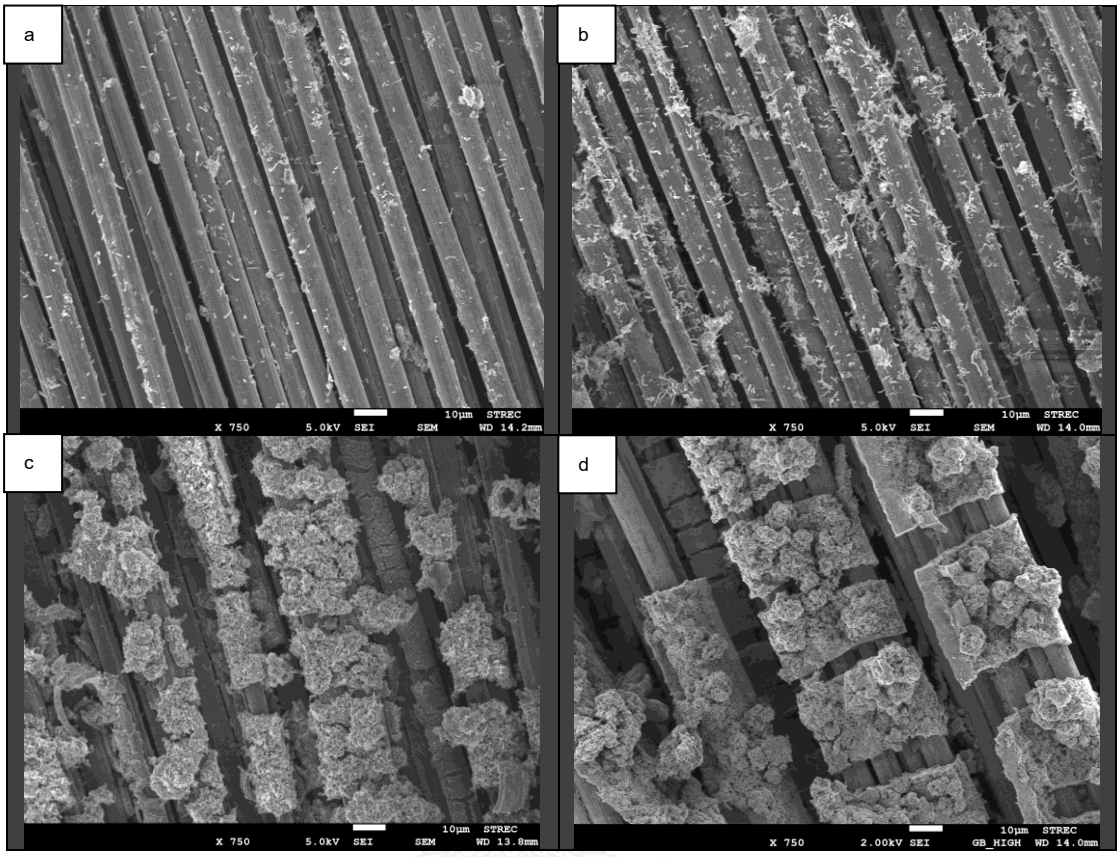


Figure 4.6 SEM micrographs at 750x magnification of stimulated anode CU sediment enriched in PBBM

(a) Stimulated anode without AC stimulation (0 mA)

(b) Stimulated anode with AC stimulation at 5 mA

(c) Stimulated anode with AC stimulation at 10 mA

(d) Stimulated anode with AC stimulation at 15 mA

Figure 4.6 showed SEM micrographs at 750x magnification. In Figure 4.6(c&d), biofilm matrix formed sheath structure on the carbon fiber. From Figure 4.6(d), the 15 mA stimulated anode illustrated that biofilm matrix formed sheath structure on the carbon fiber was larger and thicker than anode with AC stimulation at 10 mA as found in Figure 4.6(c).

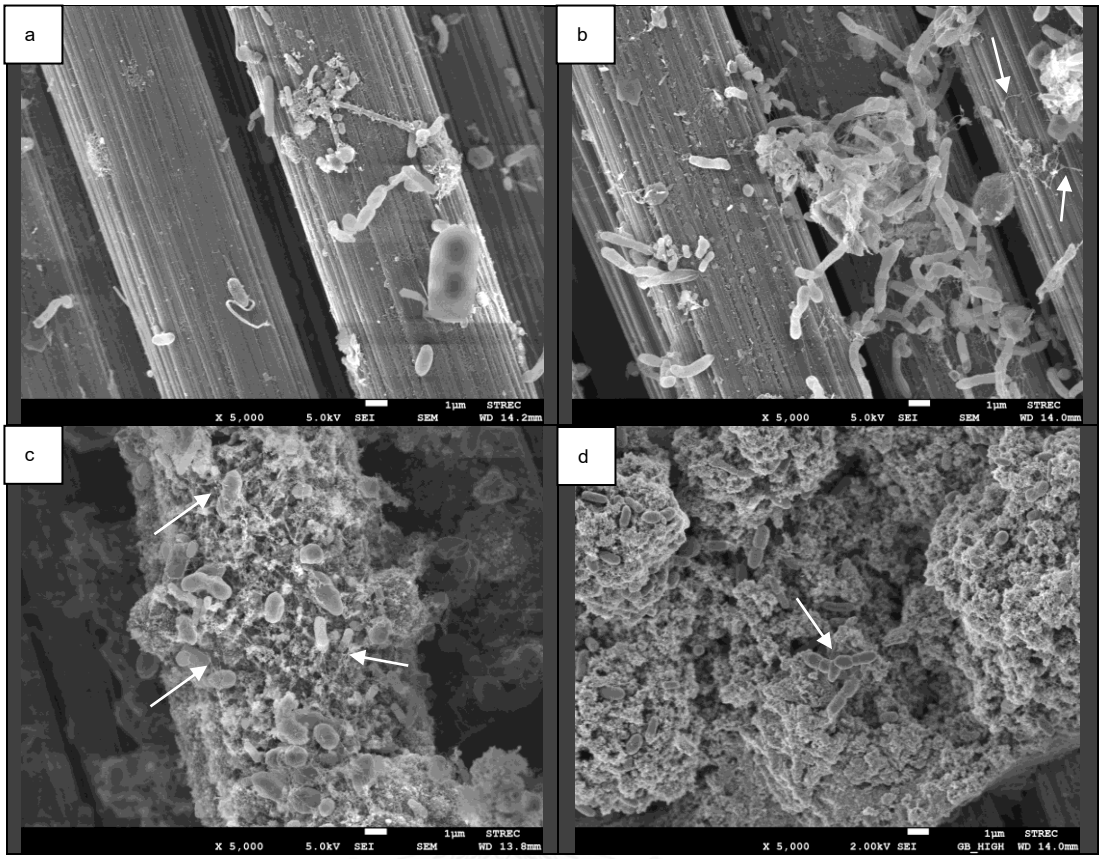


Figure 4.7 SEM micrographs at 5,000x magnification of stimulated anode CU sediment enriched in PBBM

(a) Stimulated anode without AC stimulation (0 mA)

(b) Stimulated anode with AC stimulation at 5 mA

(c) Stimulated anode with AC stimulation at 10 mA

(d) Stimulated anode with AC stimulation at 15 mA

SEM micrographs at 5,000x magnification illustrated bacterial cells on carbon fiber anode. Bacterial cells density increased by AC stimulation on anode as compared with the non-stimulation (0 mA). Biofilm matrix on anode stimulated at 10 and 15 mA which comprised of bacterial cell surrounded with extracellular polymeric substances (EPS) and filamentous structure of bacterial cells (indicated by arrow) were illustrated in Figure 4.7(c&d).

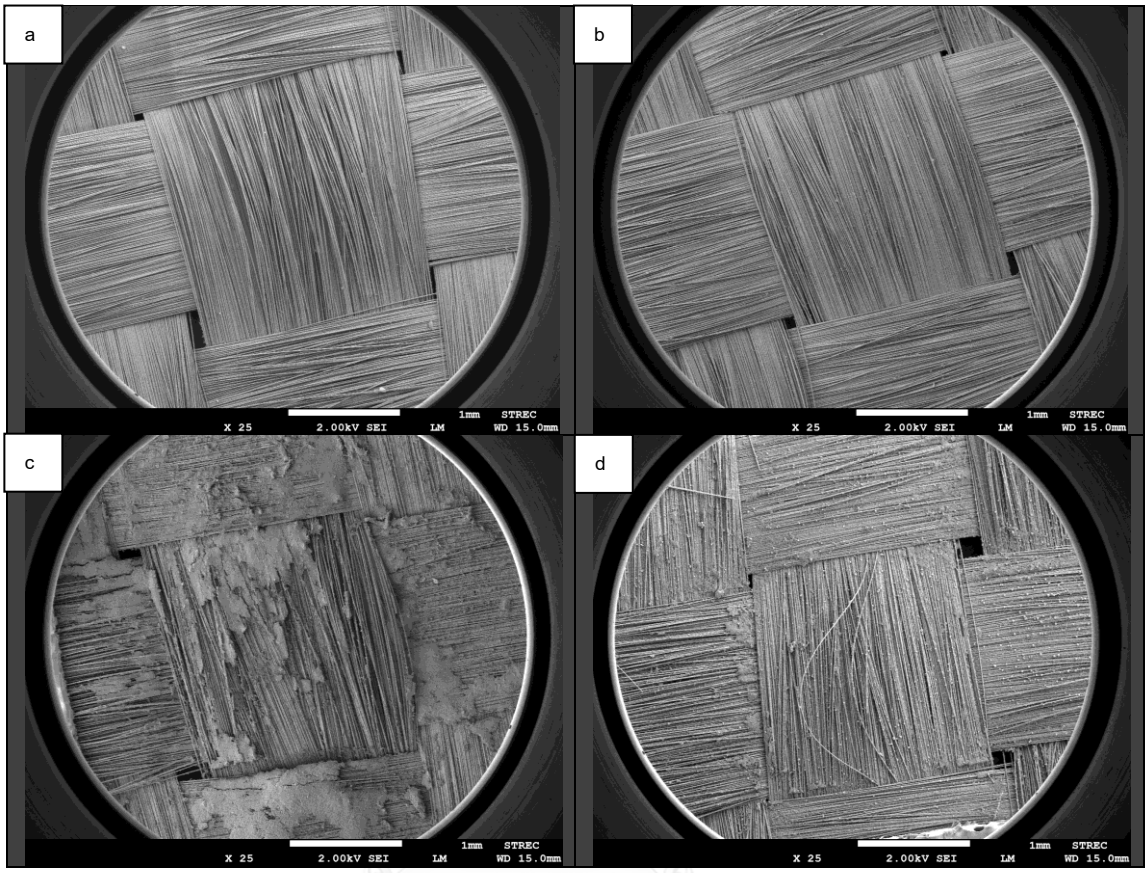


Figure 4.8 SEM micrographs at 25x magnification of stimulated anode CU sediment enriched in NB

(a) Stimulated anode without AC stimulation (0 mA)

(b) Stimulated anode with AC stimulation at 5 mA

(c) Stimulated anode with AC stimulation at 10 mA

(d) Stimulated anode with AC stimulation at 15 mA

Figure 4.8 showed SEM micrographs at 25x magnification. The carbon fibers were thickened with biofilm when stimulating AC current was increased. The anode biofilm was clearly seen on the 10-15 mA stimulated anode.

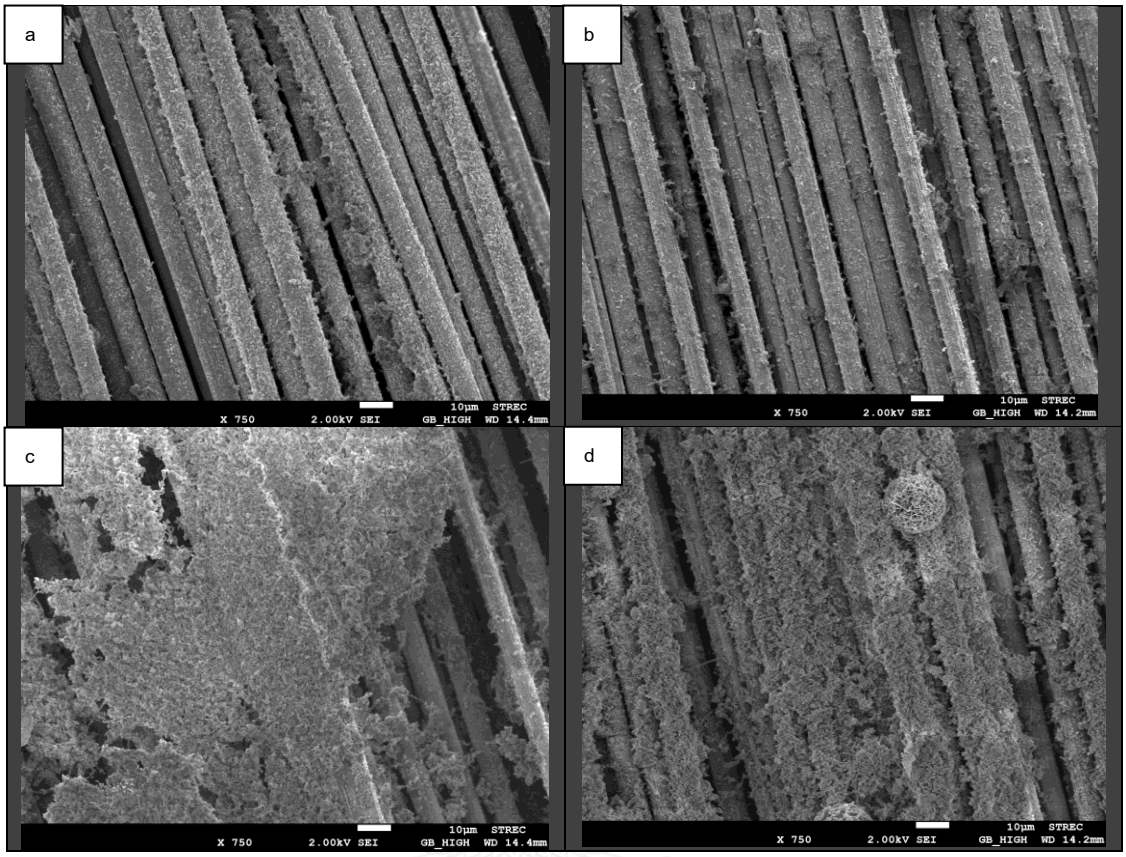


Figure 4.9 SEM micrographs at 750x magnification of stimulated anode CU sediment enriched in NB

(a) Stimulated anode without AC stimulation (0 mA)

(b) Stimulated anode with AC stimulation at 5 mA

(c) Stimulated anode with AC stimulation at 10 mA

(d) Stimulated anode with AC stimulation at 15 mA

Figure 4.9 showed SEM micrographs at 750x magnification. The anode biofilm was clearly seen at 10-15 mA stimulated anode. The thickest biofilm was observed on anode stimulated at 15 mA as found in Figure 4.9(d).

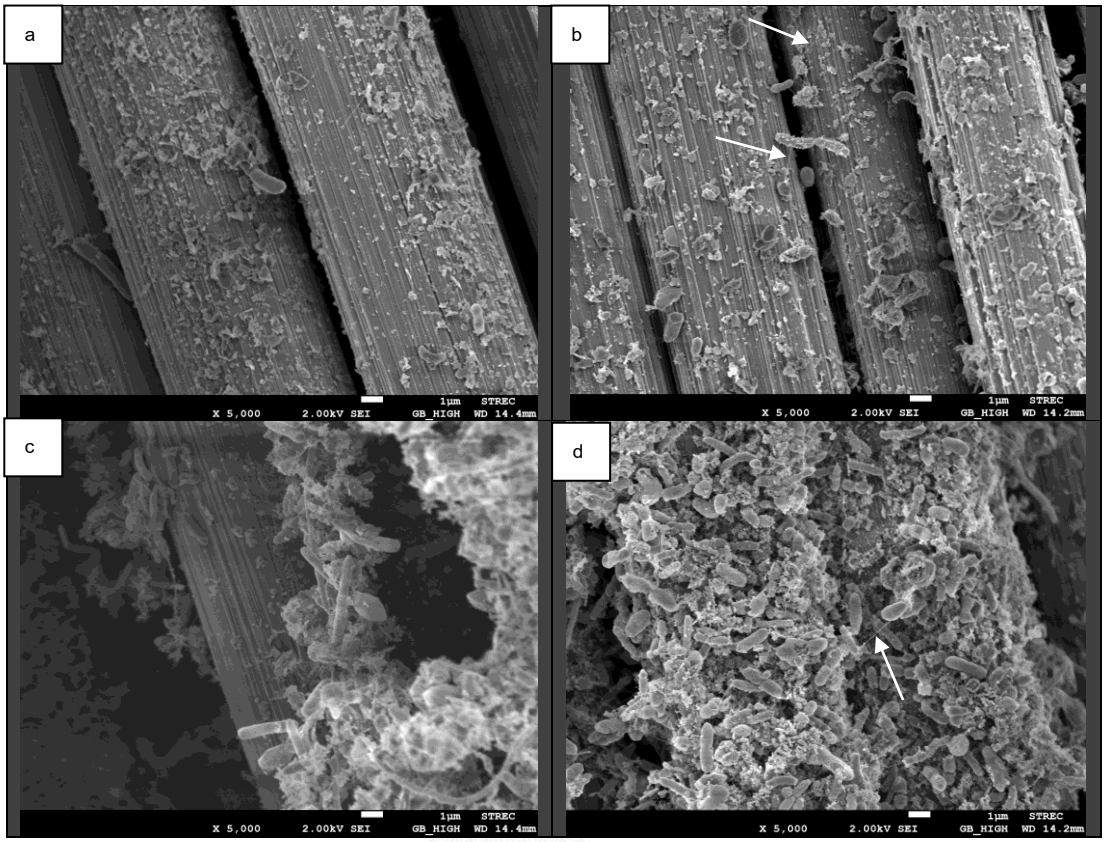


Figure 4.10 SEM micrographs at 5,000x magnification of stimulated anode CU sediment enriched in NB

(a) Stimulated anode without AC stimulation (0 mA)

(b) Stimulated anode with AC stimulation at 5 mA

(c) Stimulated anode with AC stimulation at 10 mA

(d) Stimulated anode with AC stimulation at 15 mA

Figure 4.10 showed SEM micrographs at 5,000x magnification. In Figure 4.10(d), biofilm matrix of the 15 mA stimulated anode was thicker than anode which was stimulated at 5 and 10 mA as shown in Figure 4.10(b&c). Biofilm matrix comprised of bacterial cell surrounded with extracellular polymeric substances (EPS) and filamentous structure of bacterial cells (indicated by arrow) is shown in Figure 4.10(d).

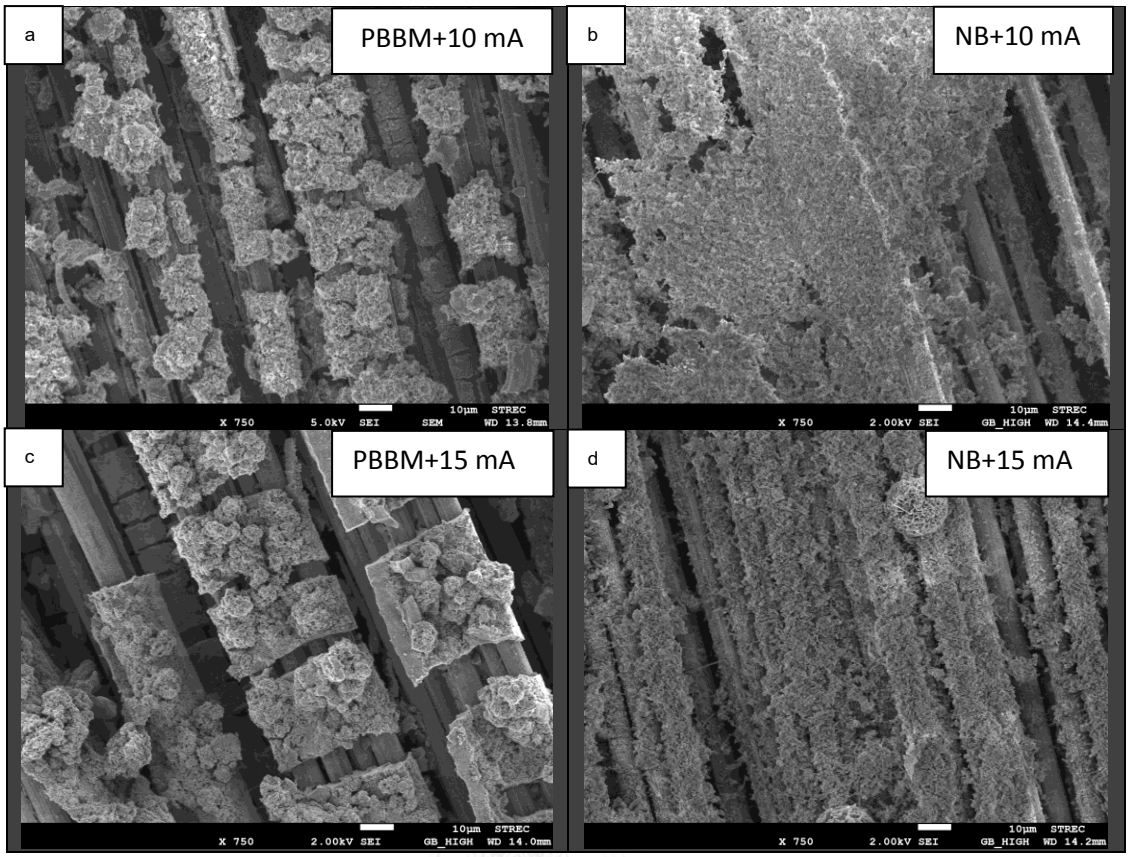


Figure 4.11 SEM micrographs at 750x magnification of stimulated anode CU sediment enriched in PBBM (a-b) and NB (c-d)

(a) Stimulated anode with AC stimulation at 10 mA in PBBM

(b) Stimulated anode with AC stimulation at 15 mA in PBBM

(c) Stimulated anode with AC stimulation at 10 mA in NB

(d) Stimulated anode with AC stimulation at 15 mA in NB

Figure 4.11 showed SEM micrographs at 750x magnification. It showed the effect of enrichment medium (PBBM and NB) and AC stimulation (10 and 15 mA) on anode biofilm. Biofilm enriched in the PBBM medium formed sheath structure on carbon fiber as shown in Figure 4.11(a, c). However, the biofilm enriched in the NB medium covered along the length of carbon fiber and had more coverage than biofilm in the PBBM medium as shown in Figure 4.11(d). The medium composition of PBBM is different from NB. PBBM is minimal medium, while NB is complex medium. PBBM has a limit of carbon and nitrogen source for the bacteria growth, so that biofilm in the PBBM

medium is less coverage than biofilm in the NB medium. These results agreed with the report of Daniel et al, 2016 [41], that complex medium was achieved thicker biofilm of *Pseudomonas veronii* 2E than the one from cultivating in minimal medium. Moreover, biofilm structure of *Ps. veronii* 2E cultivated in minimal medium was formed as a monolayer on coverslip.

According to the results, it can conclude that the different kinds of enrichment medium for AC stimulation will induce the difference of biofilm formation on anode. In addition, the thickness of biofilm will increase when the intensity of AC stimulation is increased.

4.2 Effect of AC stimulation periods on electricity outputs of MFC

To evaluate the performance of stimulated anode in MFC, voltage of MFC was measured and used to calculate current density and power density output of MFC. Three parameters are necessary for electrical device. First, voltage or electric potential (in unit of volt) is necessary for device such as light-emitting diode (LED) that requires voltage (~1.2-1.5 V) rather than current in emitting the light. Second, electric current (in unit of ampere) is necessary for device such as candescent light which requires current rather than voltage in lighting. Third, electric power (in unit of watt) is necessary for heating devices which require electric power (both current and voltage) to supply devices.

Open circuit voltage (Voc) was the maximum voltage of MFC before MFC was connected to the external resistors. The current density and power density output were measured and calculated after connecting the various external resistance loads across the anode and cathode, 100 kΩ to 100 Ω. After MFC was connected to the external resistor, the actual voltage that system supplied to individual resistance load was measured by digital volt meter that connected to computer. The actual voltage from each resistance load was collected and used to calculate the current density and power density by using the averaged actual voltage for one hour before connecting to other load. For example, the maximum voltage (Voc) and the maximum current density were determined from the plot of voltage vs current density of MFC as shown in Figure 4.12.

Moreover, power density was determined from the plot of power density vs current density of MFC as shown in Figure 4.13.

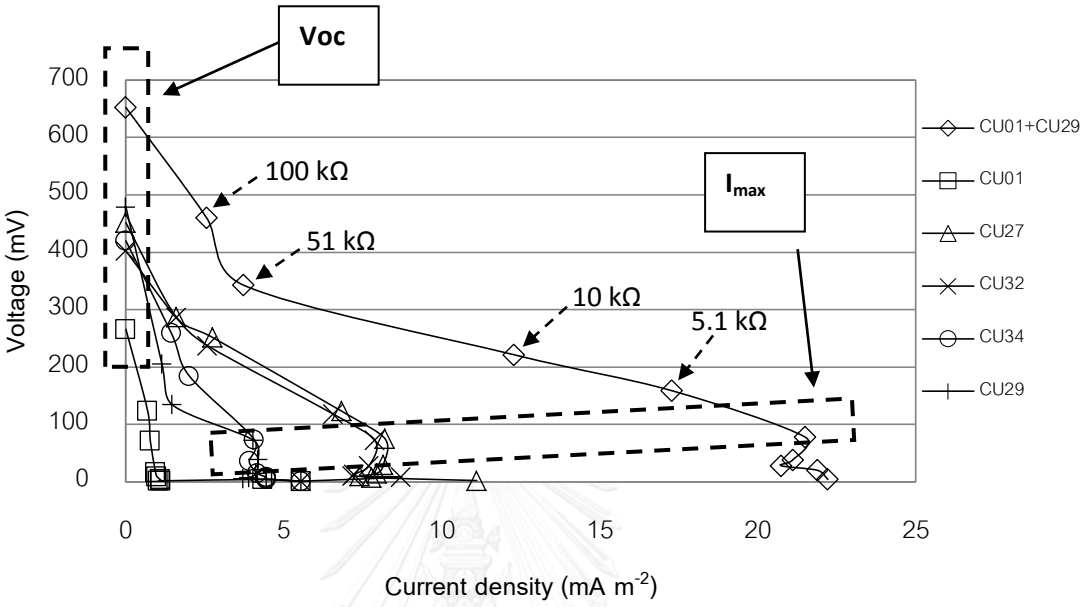


Figure 4.12 Voltage vs current density of MFC using isolates from CU sediment

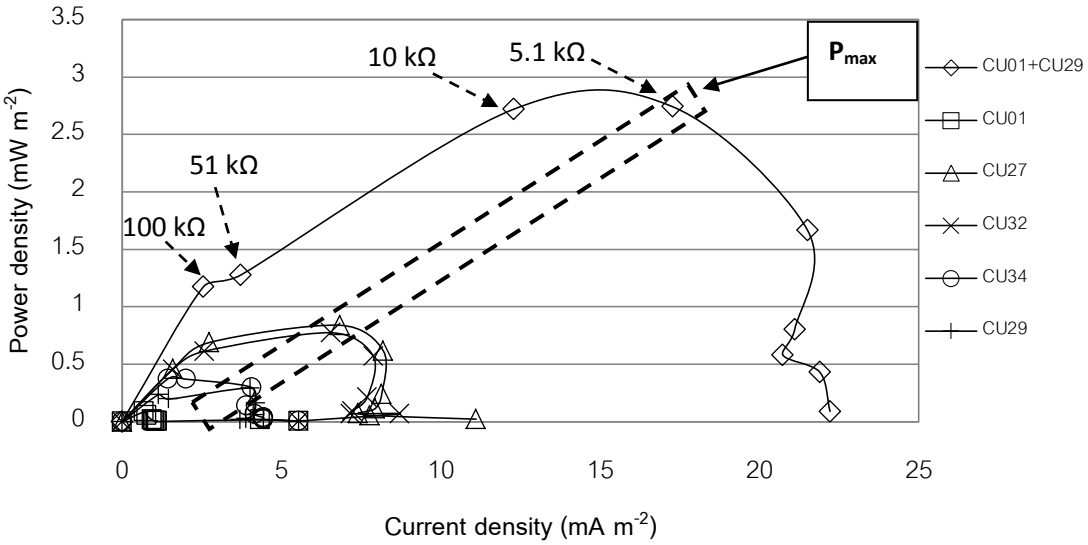


Figure 4.13 Power density vs current density of MFC using isolates from CU sediment

In this experiment, the effect of AC stimulation periods on electricity outputs of MFC was studied using CU sediment which is enriched with PBBM and NB medium for 15 and 60 days. In order to remove enrichment medium and unattached microbial cells, the stimulated CU sediment anode in the PBBM and NB medium were resuspended in PBBM medium. Then, the stimulated anode (containing biofilm) was transferred into anodic chamber of MFC containing PBBM and 10 mM of glucose. Maximum voltage (open circuit voltage), maximum current density and maximum power density of individual MFC were calculated and plotted as shown in Figure 4.14-4.16.

In Figure 4.14(a), it was found that Voc of MFC with 15 days stimulated anode in PBBM decreased when stimulating current was increased, however Voc of MFC with 60 days stimulated anode in PBBM was rather stabilized when stimulating current increased. Current and power density of MFC with 60 days stimulated anode in PBBM tentatively increased when stimulation period was increased as shown in Figure 4.15(a) and 4.16(a). However, current density and power density of MFC with stimulated anode in NB enrichment medium did not increase when stimulation period was increased as shown in Figure 4.15(b) and 4.16(b). Moreover, Voc of MFC with stimulated anode in NB tentatively decreased when stimulation period was increased as shown in Figure 4.14(b).

Highest current density and power density of MFC of 47.8 mA m^{-2} and 11.6 mW m^{-2} were obtained from 60 days 10 mA stimulated anode in PBBM medium. 60 days stimulated anode in PBBM offered stable Voc and could increase current and power density for ~3 times and ~6 times, respectively. From these results, stimulation period for 60 day was used to study the effect of three enrichment mediums; NB, PBBM and FWA together with the different types of stimulating current source; alternative current (AC) and direct current (DC) on electricity output of MFC.

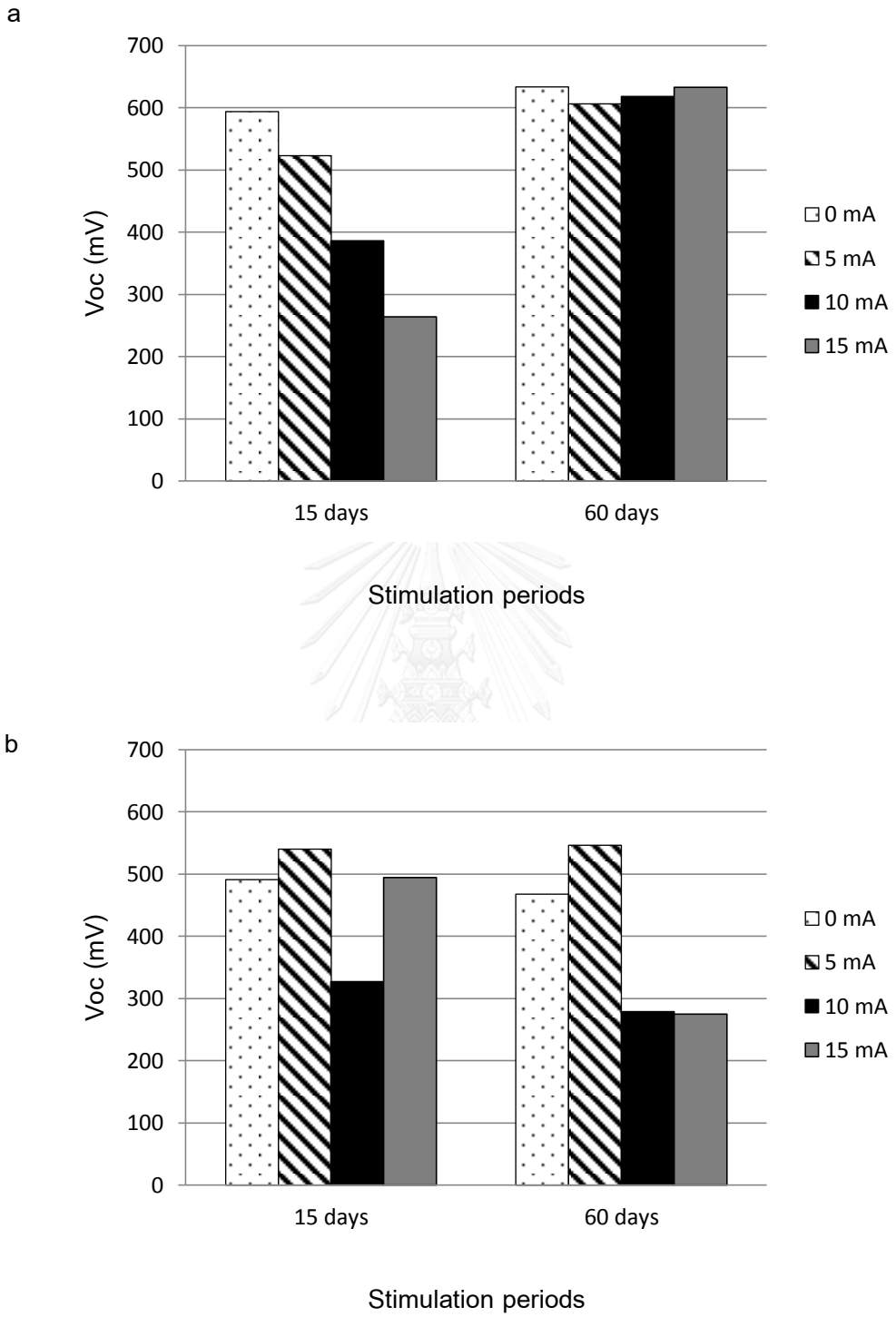


Figure 4.14 Open circuit voltage of MFC with AC stimulated anode (0-15 mA) of CU sediment which was enriched for 15 days and 60 days in (a) PBBM and (b) NB medium.

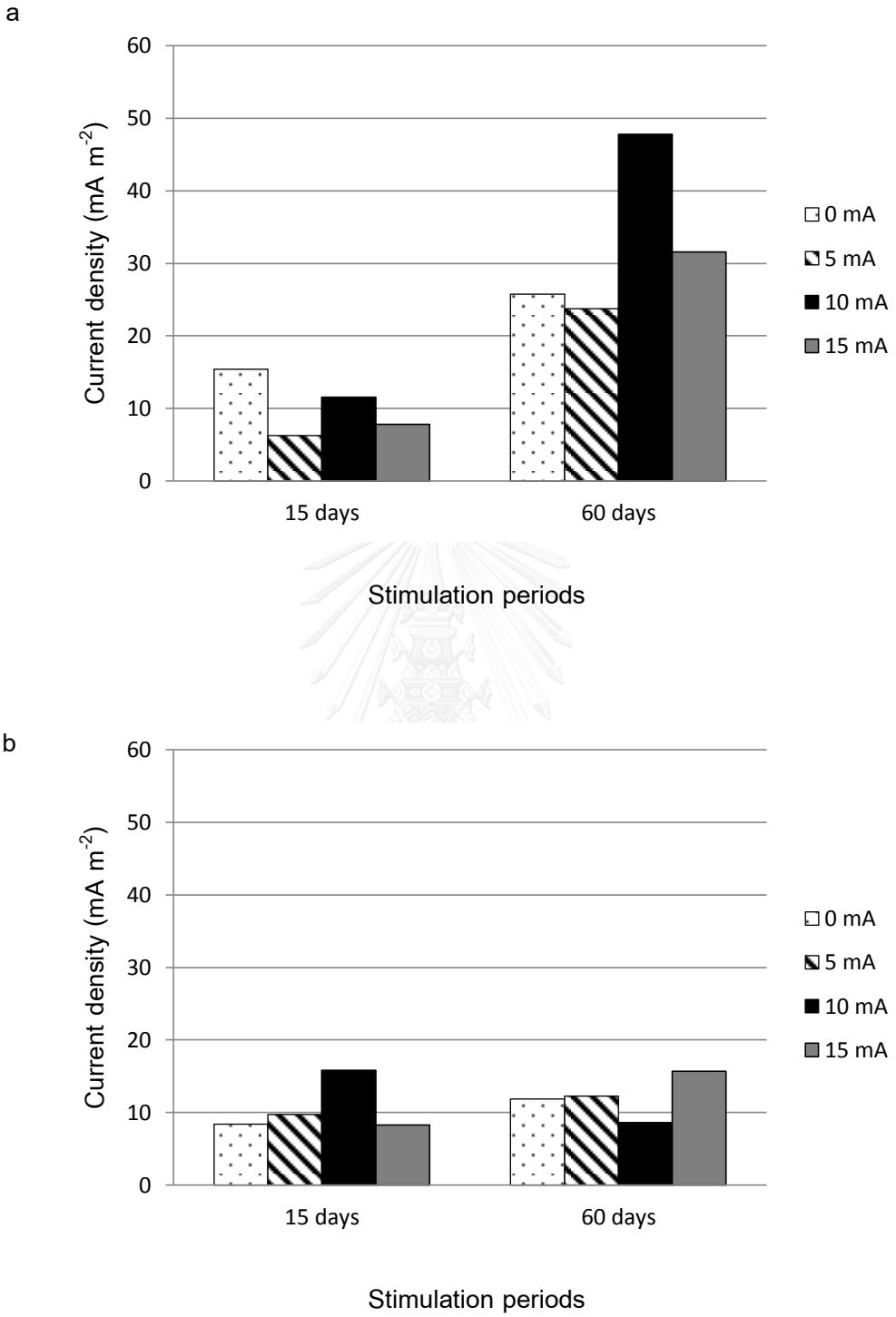


Figure 4.15 Current density of MFC with AC stimulated anode (0-15 mA) of CU sediment which was enriched for 15 days and 60 days in (a) PBBM and (b) NB medium.

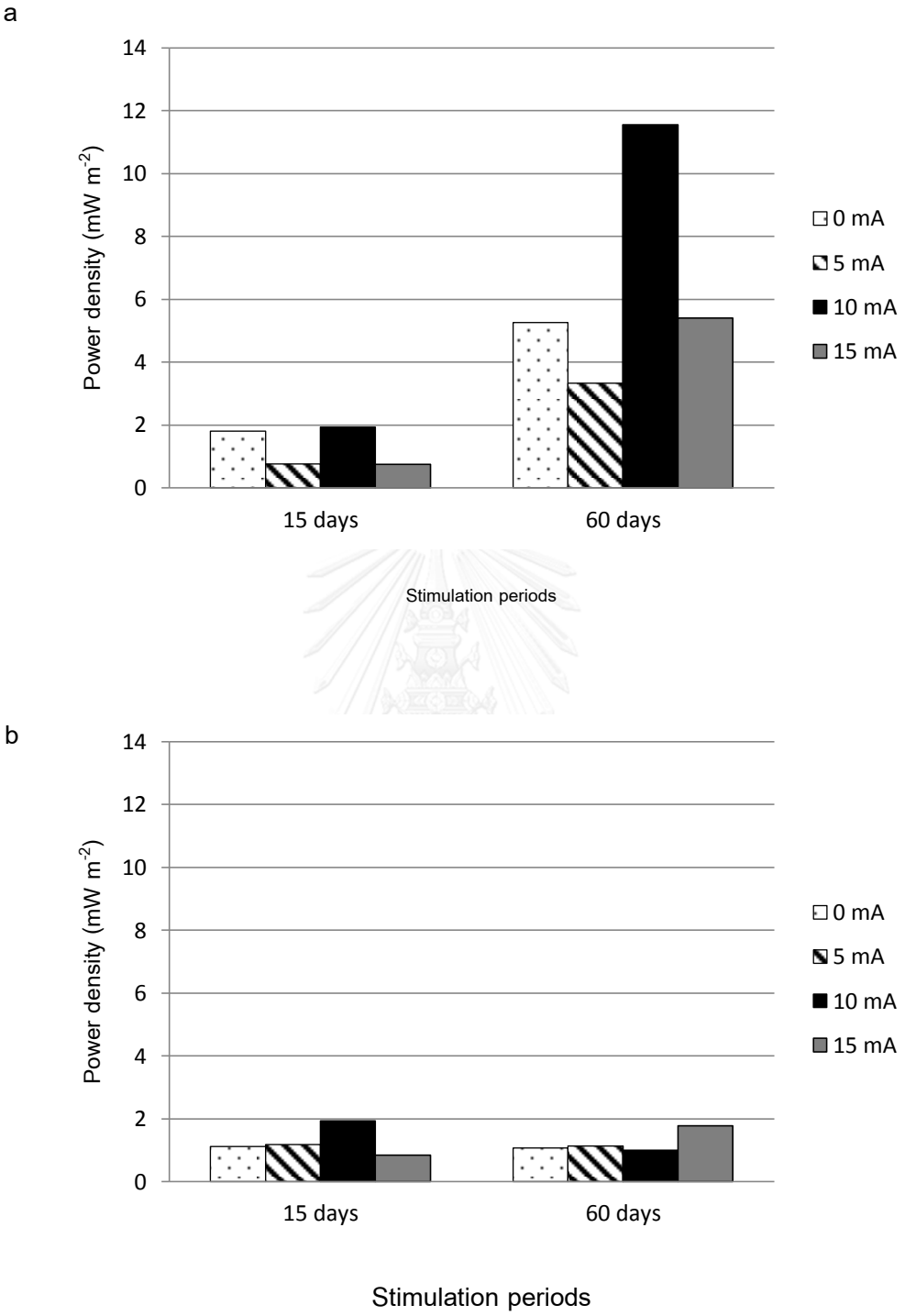


Figure 4.16 Power density of MFC with AC stimulated anode (0-15 mA) of CU sediment which was enriched for 15 days and 60 days in (a) PBBM and (b) NB medium.

4.3 Effect of enrichment medium vs AC and DC stimulation on electricity outputs of MFC containing stimulated anode

After anode was stimulated by AC and DC in PBBM, NB and FWA for 60 days, stimulated anode (containing biofilm) was transferred to anodic chamber of MFC contained PBBM and 10 mM of sodium acetate. Electricity outputs of MFC in this experiment were plotted as illustrated in Figure 4.17-4.19. AC stimulated anode which was enriched in PBBM showed higher electricity outputs of MFC than enrichment with NB and FWA. In PBBM enrichment, MFC with 5 and 10 mA AC stimulated anode had current density greater than anode without AC stimulation. Moreover, MFC with 5 and 10 mA AC stimulated anode also delivered power density greater than anode without AC stimulation. Furthermore, Voc, current density and power density of MFC dramatically decreased when anode was stimulated with AC current at 15 mA in PBBM. DC stimulated anode and enrichment with PBBM showed higher electricity outputs of MFC than enrichment with NB and FWA. Although anode was enriched in PBBM, anode without DC stimulation still had electricity outputs greater than anode with DC stimulation at 5-15 mA.

Comparison between AC and DC stimulation on MFC output, current density and power density of the AC stimulation was higher than the DC stimulation. The highest Voc and current density were obtained from 5 mA AC current stimulated anode which was enriched in PBBM, with the value of 719 mV and 72.9 mA m^{-2} respectively. The highest power density of 13.4 mW m^{-2} was obtained from 10 mA AC current stimulated anode which was enriched in PBBM. In DC stimulation, the highest Voc, current density and power density of 716 mV, 65.2 mA m^{-2} and 11.2 mW m^{-2} respectively, were obtained from anode enriched in PBBM without DC stimulation.

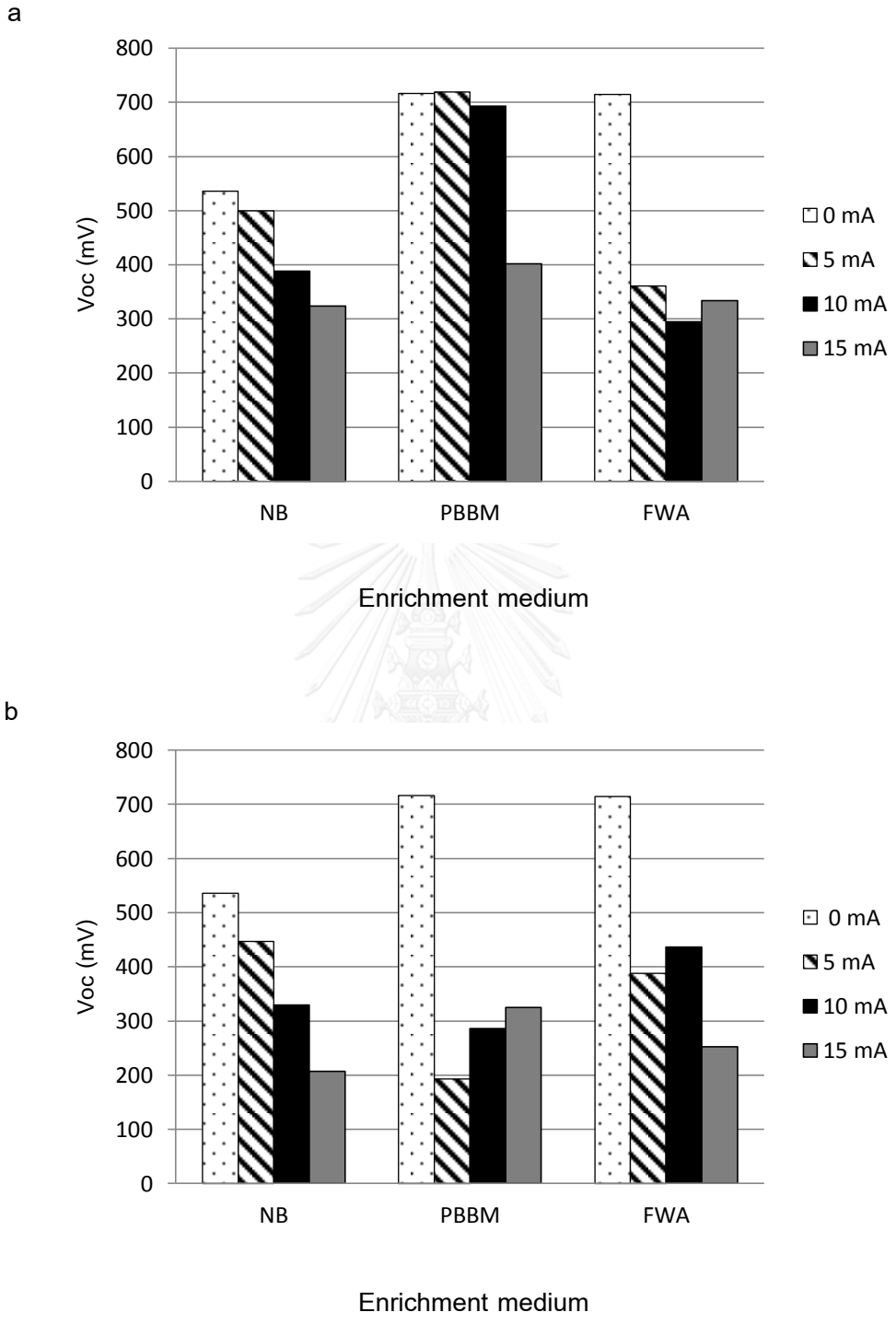


Figure 4.17 Open circuit voltage of acetate-fed MFC containing (a) AC stimulated anode at 0-15 mA and (b) DC stimulated anode at 0-15 mA of CU sediment enriched in NB, PBBM and FWA.

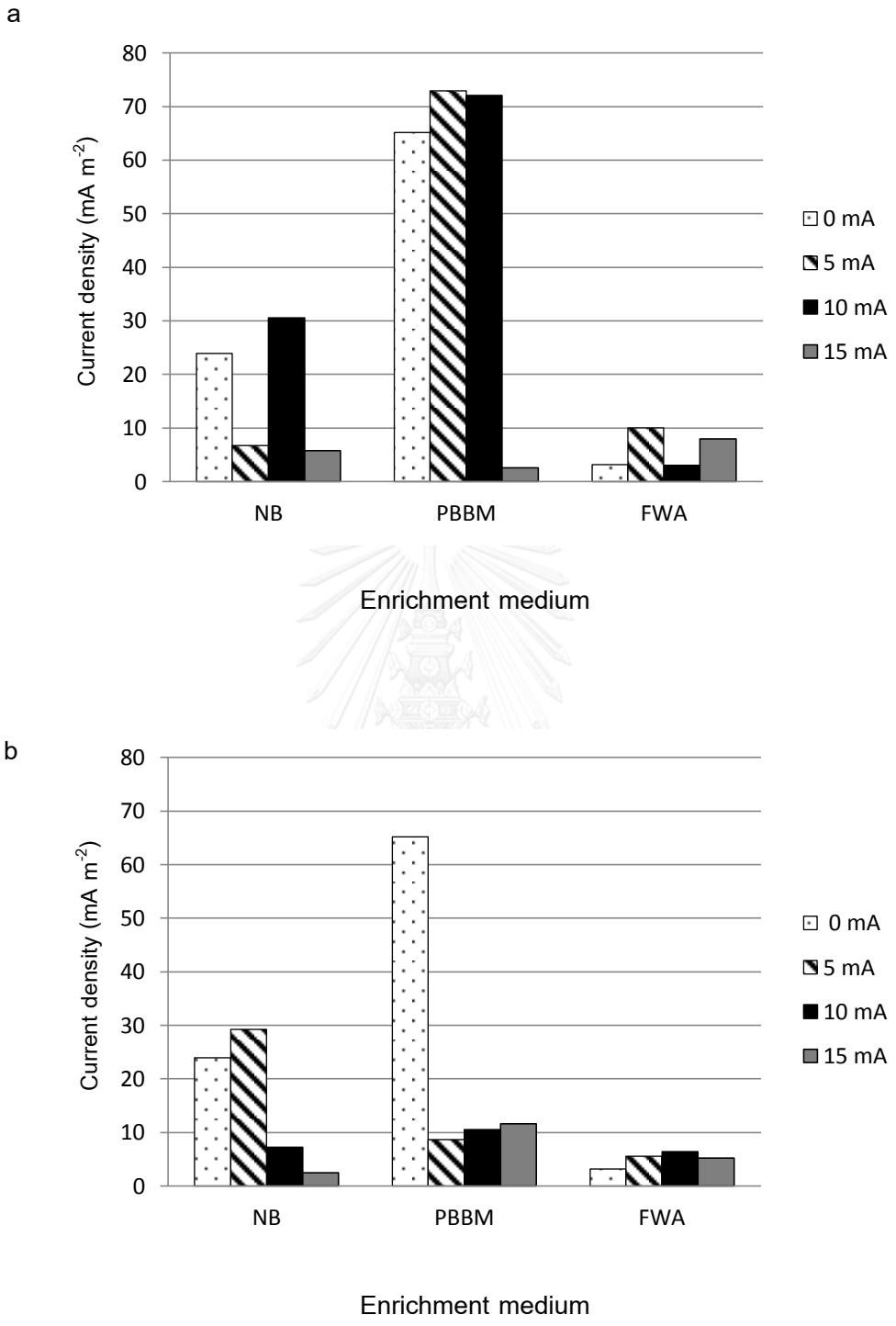


Figure 4.18 Current density of acetate-fed MFC containing (a) AC stimulated anode at 0-15 mA and (b) DC stimulated anode at 0-15 mA of CU sediment enriched in NB, PBBM and FWA.

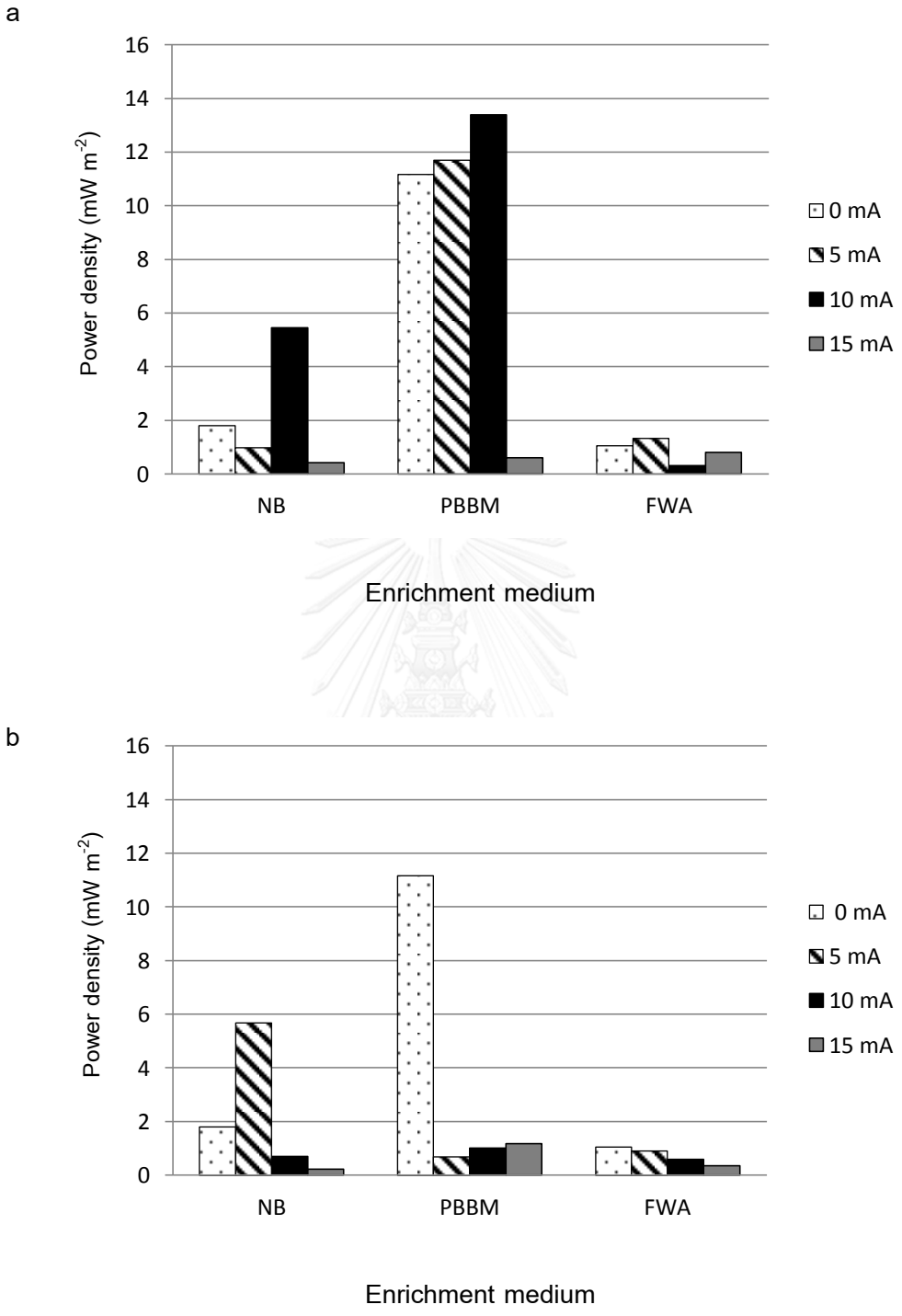


Figure 4.19 Power density of acetate-fed MFC containing (a) AC stimulated anode at 0-15 mA and (b) DC stimulated anode at 0-15 mA of CU sediment enriched in NB, PBBM and FWA.

The results from using different enrichment medium agreed with Wang et al., (2010) [42] that PBBM could be used to enrich ferric reducing bacteria in anode of MFC and the increase in current output of MFC would be obtained. Generally, FWA medium is used to study bacteria that involved in iron cycling. Nevertheless, FWA could also enrich bacterial consortium in group of aerobic and facultative anaerobic bacteria, fermentative bacteria anaerobic bacteria better than ferric reducing bacteria [43]. For NB, this medium was a general medium used to culture non-fastidious microorganisms, so it could cultivate facultative anaerobic ferric reducing bacteria such as *Proteus* sp. and *Bacillus* sp. as mentioned earlier [13].

From these results, it could be concluded that PBBM was a suitable enrichment medium for AC stimulation. In addition AC stimulation was better than DC stimulation.

4.4 Effect of carbon source vs AC and DC stimulation on electricity outputs of MFC containing stimulated anode

To study the effect of carbon source in MFC on electricity output of MFC, CU sediment was enriched in PBBM, and then was stimulated with AC and DC current for 60 days. After stimulation ended, stimulated anode (containing biofilm) was transferred into MFC containing PBBM and 10 mM of sodium acetate. After electricity output of current stimulated anode in acetate-fed MFC was collected, PBBM with acetate was removed from anode chamber and replaced with PBBM and 10 mM sodium propionate. Cathode solution was also removed and replaced with PBBM with 1 mM of $K_3Fe(CN)_6$. After electricity output of current stimulated anode in propionate-fed MFC was collected, PBBM with 10 mM glucose would be replaced as the same procedure performed in propionate-fed MFC. Electricity outputs of MFC in this experiment were plotted as illustrated in Figure 4.20-4.22.

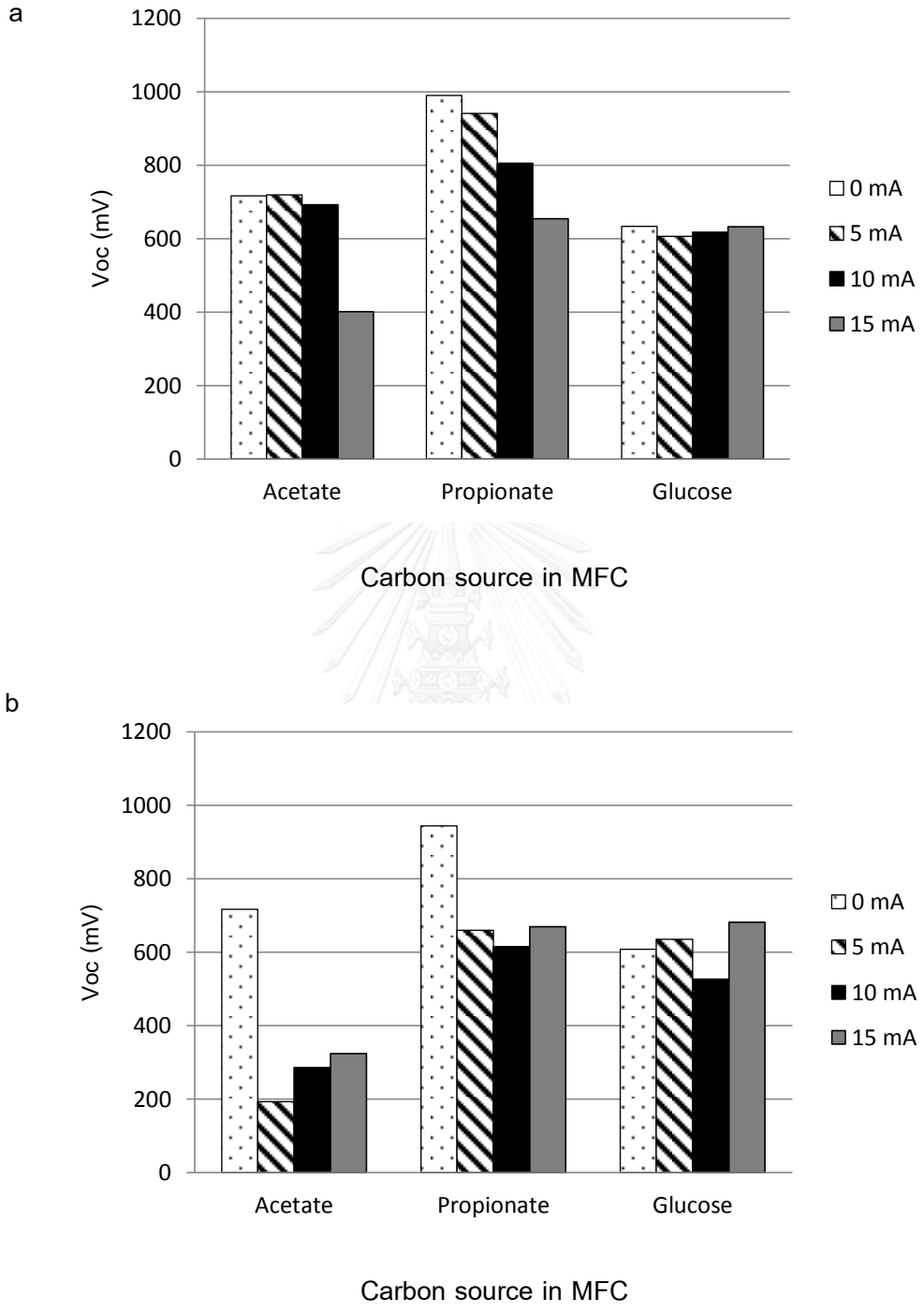
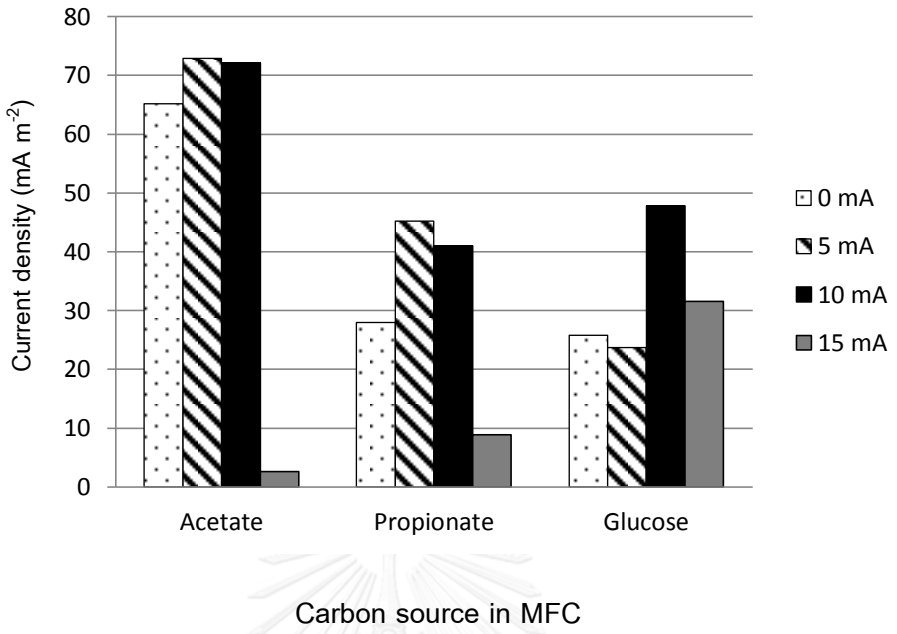


Figure 4.20 Open circuit voltage of MFC containing acetate, propionate and glucose as carbon source, and (a) AC stimulated anode at 0-15 mA and (b) DC stimulated anode at 0-15 mA of CU sediment

a



b

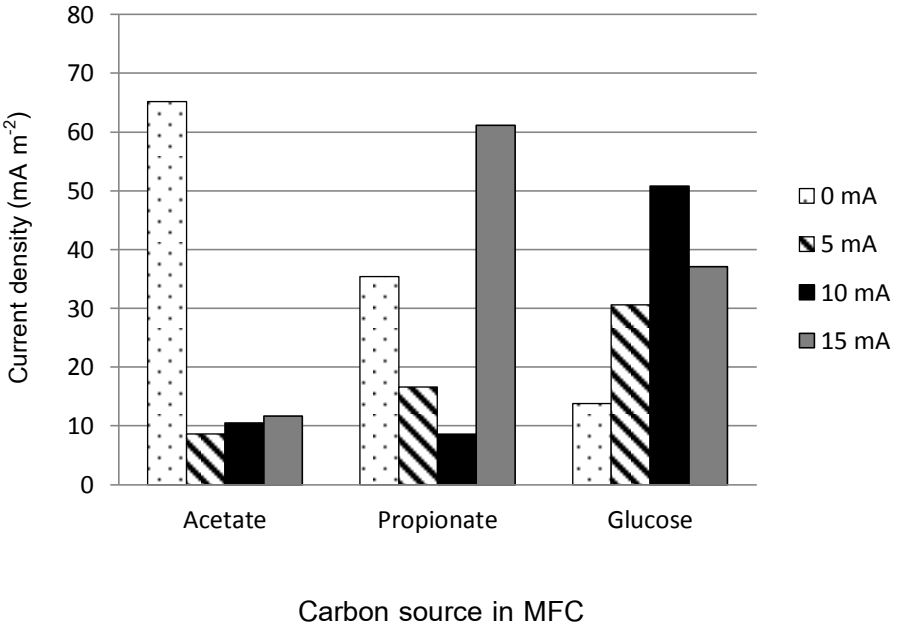


Figure 4.21 Current density of MFC containing acetate, propionate and glucose as carbon source, and (a) AC stimulated anode at 0-15 mA and (b) DC stimulated anode at 0-15 mA of CU sediment

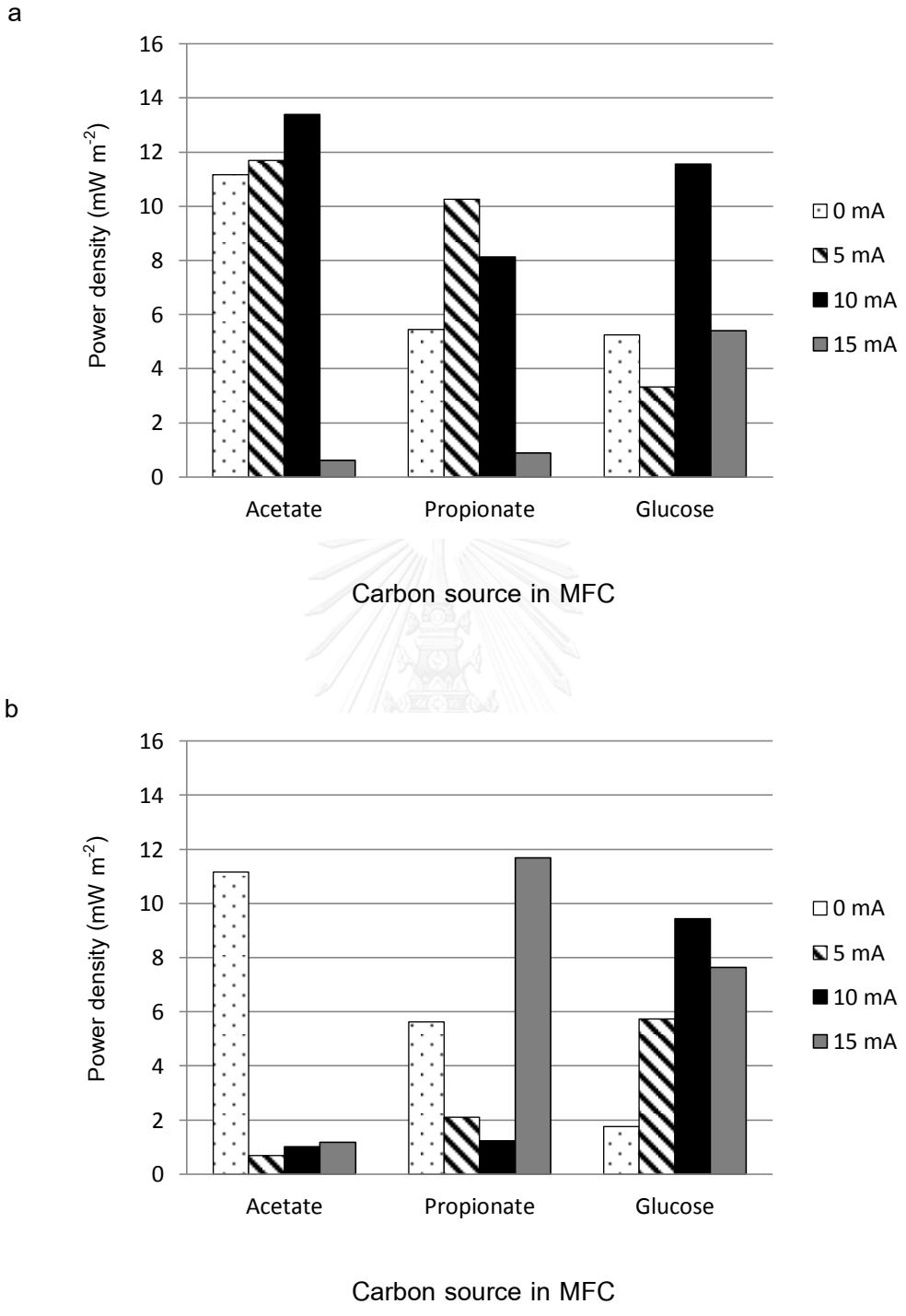


Figure 4.22 Power density of MFC containing acetate, propionate and glucose as carbon source, and (a) AC stimulated anode at 0-15 mA and (b) DC stimulated anode at 0-15 mA of CU sediment

In AC current stimulation, the Voc of propionate-fed MFC was higher than the ones of acetate-fed and glucose-fed MFC. The highest Voc of 989 mV was obtained from propionate-fed MFC without AC stimulation. Voc of propionate-fed MFC decreased when AC stimulating current was increased as shown in figure 4.20(a). In DC stimulation, propionate-fed MFC also had higher Voc than acetate-fed and glucose-fed MFC. Voc of propionate-fed MFC with anode without DC stimulation was higher than propionate-fed MFC anode with DC stimulation at 5-15 mA, as shown in figure 4.20(b).

For AC stimulation, acetate-fed MFC had higher current density and power density than propionate-fed and glucose-fed MFC, and also higher than DC stimulation. In Figure 4.21(a), acetate-fed MFC with 5 and 10 mA AC stimulated anode delivered current density of 11.8 % and 10.6 % higher than anode without AC stimulation. Moreover, acetate-fed MFC with 5 and 10 mA AC stimulated anode also gave power density 4.46 % and 19.6 % greater than anode without AC stimulation as shown in Figure 4.22(a). However, current density and power density of acetate-fed MFC dramatically decreased when anode was stimulated with AC current at 15 mA.

From these results, AC and DC stimulated anode among three carbon sources performed utilizing activity and produced electricity output in MFC. It could be concluded that anode without stimulation in propionate-fed MFC was suitable for producing Voc, while 5-10 mA AC stimulated anode in acetate-fed MFC was suitable for producing current density and power density.

4.5 Electricity output in MFC of AC stimulated anode of sediments from various places

To determine the effect of AC stimulation in various sediments, 5 sediments from different source were used and enriched in PBBM medium. They were sediments from Chulalongkorn University (denoted as CU), Trang province (denoted as Trang), Chachoengsao province (denoted as Cha), Khonkaen province (denoted as Khon) and Ko Sichang (denoted as Si). All the sediments were stimulated for 15 days. The stimulated anode of each of sediment was transferred into anodic chamber of MFC containing PBBM and 10 mM of glucose. Open circuit voltage, the maximum current density and the maximum power density of individual MFC were calculated and the

average of the three MFCs with the same AC stimulation was plotted as depicted in Figure 4.23(a-c).

Figure 4.23, the highest Voc of 704 mV and the maximum current density of 26.8 mA m^{-2} were obtained from Cha sediment with AC stimulation of 5 mA. Power density of 3.83mW m^{-2} was obtained from Khon sediment with 15 mA stimulation. In Figure 4.23(a), the 10 and 15 mA AC stimulated anode had a low Voc for all sediments. For the 5 and 10 mA AC stimulated anode, the power density seemed to be higher than the anode without AC stimulation. However, the power density of 15 mA AC stimulated anode was lowest as shown in Figure 4.23(c). These results indicated that an appropriated AC stimulation on anode could be used to stimulate biofilm on anode in various sources of sediment. It should be noted that electricity output of Si sediment did not depend on the effect of AC stimulation. This might be due to the fact that Si sediment was sampled from the seashore. The PBBM medium may not be suitable for enrichment the sea-sediment and buffering capacity of PBBM might be interfered by a high salinity of sea-sediment.

4.6 Isolation and identification of anode biofilm

To study bacterial community on stimulated anode, AC stimulated anode of CU sediment which was enriched in PBBM medium was used in this experiment. Bacterial consortium from biofilm on anode was isolated based on ferric reducing activity. It was reported that the ferric reducing bacteria (FRB) could directly and effectively transfer electron to the anode, in consequence, electricity output was higher than the ones from non-FRB [36]. Due to that fact that FRB can reduce ferric ion (Fe^{3+}) into ferrous ion (Fe^{2+}). Therefore, reducing activity of ferric (Fe^{3+}) can be observed on PBBM agar with 20 mM of ferric citrate by monitoring the changing of the reddish-brown color into the light green-colored (Figure 4.24).

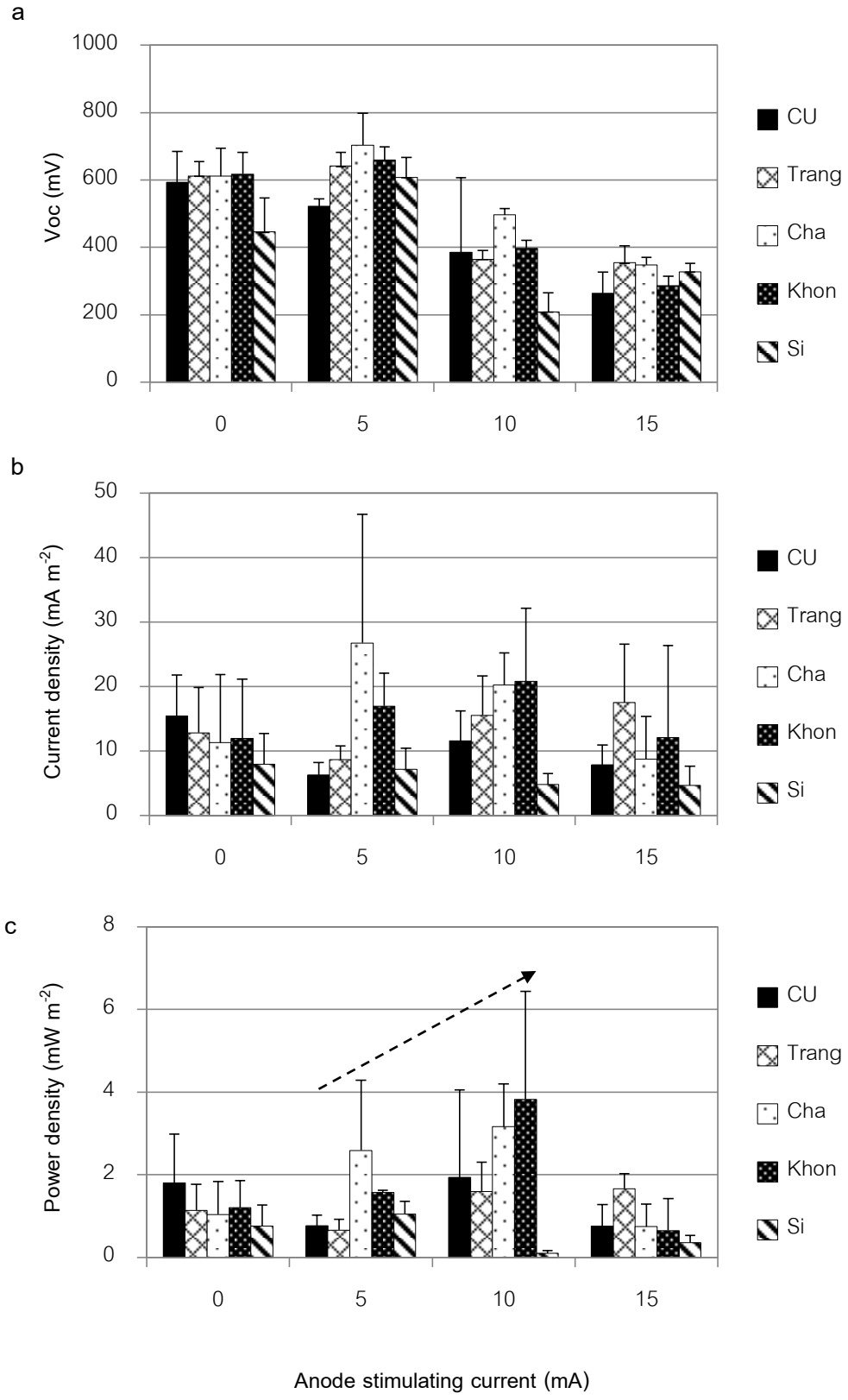


Figure 4.23 Open circuit voltage (a), current density (b) and power density (c) of MFC containing stimulated anode of 5 sediments in PBBM

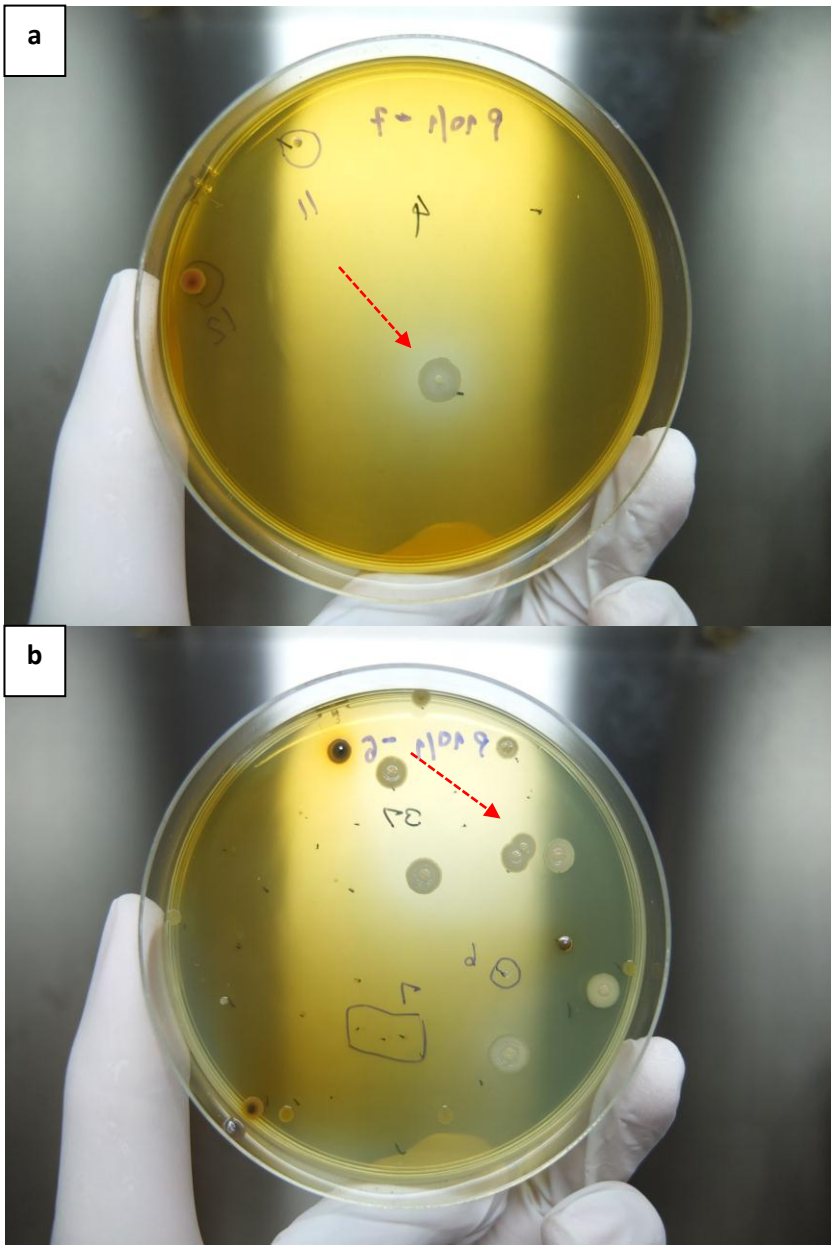


Figure 4.24 Ferric reducing activities of (a) single colony and (b) multiple colonies of bacteria (indicated by arrow) on PBBM agar containing ferric citrate

Pure culture of 35 isolates of FRB from biofilm on the anode was 26 obligate anaerobic bacteria and 9 facultative anaerobic bacteria. Twenty six of obligate anaerobic bacteria were identified by 16s rDNA sequencing comparing with GenBank database as shown in Table 4.1. Facultative anaerobic bacteria of 9 isolates were identified by API 20E test kit based on biochemical test of non-fastidious bacteria as shown in Table 4.2.

Table 4.1 Identification of isolates from biofilm by using 16s rDNA sequencing

Isolate	Identification	% Similarity	Accession Number
CU01	*Clostridium saccharoperbutylacetonicum*	99	CP004121.1
CU02	*Bacteroides graminisolvens*	99	NR041642.1
CU03	*Clostridium aciditolerans*	99	NR043557.1
CU04	*Desulfotomaculum* sp.	99	KF601944.1
CU05	*Pleomorphomanas oryzae*	99	NR114056.1
CU06	*Aeromonas punctata*	99	FJ407187.1
CU07	*Bacteroides graminisolvens*	99	NR113069.1
CU08	*Dysgonomonas termitidis*	99	AB971823.1
CU09	*Clostridium* sp.	99	LC020511.1
CU10	*Propionicimonas paludicola*	99	NR104769.1
CU11	*Macellibacteroides fermentans*	99	NR117913.1
CU12	Uncultured bacterium	99	KM251010.1
CU13	Uncultured bacterium	99	FN436190.1
CU14	Uncultured bacterium	99	JN245875.1
CU15	*Psychrobacter pulmonis*	99	HQ202844.1
CU16	*Clostridium* sp.	98	HQ222294.1
CU17	*Cellulomonas fimi*	97	NR074509.1
CU18	*Sanguibacter soil*	99	NR044276.1
CU19	*Enterobacter hormaechei*	99	JQ660194.1
CU20	*Pleomorphomonas koreensis*	99	AB127971.1
CU21	*Cellulomonas hominis*	99	JQ353816.1
CU22	*Aeromonas sanarelli*	99	NR116584.1
CU23	*Cellulomonas chitinilytica*	97	NR041511.1
CU24	*Cellulomonas bogoriensis*	98	NR114941.1
CU25	*Cellulomonas carbonis*	98	NR118030.1
CU26	*Cellulomonas flavigena*	97	KF040991.1

Table 4.2 Identification of isolates from biofilm by using API20E test kits

Isolate	Identification	% Similarity	T value
CU27	*Aeromonas hydrophila*	99.6	0.84
CU28	*Citrobacter koseri*	97.2	0.18
CU29	*Shewanella putrefaciens*	99.9	0.3
CU30	*Pantoea* spp.	84.1	0.54
CU31	*Chryseobacterium indologenes*	92.3	0.21
CU32	*Alcaligenes* spp.	82.3	0.51
CU33	*Pseudomonas fluorescens*	94.5	0.43
CU34	*Pseudomonas aeruginosa*	99.9	0.36
CU35	*Enterobacter cloacae*	91.3	0.86

One of bacteria found from biofilm on the anode was identified as *S. putrefaciens*. This bacterium was previously reported to possess electrochemical activity in transferring electron to anode [15]. *A. hydrophila* was also claimed as electrochemical active bacteria (EAB) that could attach and directly transfer electron to anode [18]. Furthermore, *Ps. aeruginosa* was claimed by Rabaey et al. (2004) [44] that it could produce pyocyanin acted as electron mediator for transferring electron to anode. Nevertheless, not only EAB and FRB were isolated from the biofilm, but non-EAB such as *Alcaligenes* sp. was also isolated from the biofilm as reported by Rabaey et al. (2004) [44]. *Alcaligenes* sp. was reported to be a major population in anode biofilm of glucose-fed MFC but it did not have an electrochemical activity to transfer electron to electrode. In addition, *E. cloacae* could utilize cellulose and effectively transferred electron to anode [45]. Lastly, *Desulfotomaculum* sp. a sulfate-reducing-bacteria could reduce sulfate and transfer electron to electrode [46]. Thus it could be concluded that AC stimulation on anode can enrich consortium of FRB and EAB in biofilm for use as MFC anode.

4.7 Effect of AC stimulation on bacterial isolates

According to the effect of electrical current stimulation on anode, the AC stimulation at 5-10 mA had a potential for stimulation of biofilm on anode. To study the effect of AC stimulation on bacteria, facultative anaerobic bacteria isolated from biofilm on anode were selected and stimulated at 0-15 mA in NB medium. Facultative anaerobic isolates, *A. hydrophila* (CU27), *S. putrefaciens* (CU29) and *Ps. aeruginosa* (CU34) previously reported as EAB [15, 18, 44] and non-EAB, *Alcaligenes* sp. (CU32) [44] were selected to study in this experiment.

It was found that stimulating current at 10-15 mA could inhibit bacterial growth but not for the 5 mA stimulation. After culturing for 48 hr, viable cells count of *A. hydrophila* CU27, *S. putrefaciens* CU29, *Alcaligenes* sp. CU32 and *Ps. aeruginosa* CU34 decreased 3, 1.5, 1.5 and 2 logCFU, respectively, by stimulating current at 10 mA and decreased 4, 3, 1 and 2 logCFU, respectively, by stimulating current at 15 mA. Whereas viable cells count of *Alcaligenes* sp. CU32 was slightly decreased by stimulating current as shown in Figure 4.25(c).

From these results, AC stimulating current more than 5 mA might be harmful to bacterial cell. Suitable intensity of electric current should be applied to stimulate bacterial cell not only the formation of biofilm on anode but also used to stimulate bacterial cell metabolism [47]. However, high intensity of electric current may cause lethal effect on bacterial cell [48]. In addition, AC current at 15 mA could inhibit growth of EAB, such as *A. hydrophila* (CU27) and *S. putrefaciens* (CU29), more than non-EAB such as *Alcaligenes* sp. (CU32). It implied that AC current intensity at 15 mA for more than 48 hr might decrease the population of EAB which led to the lower current and power density of MFC with 15 mA AC stimulated anode.

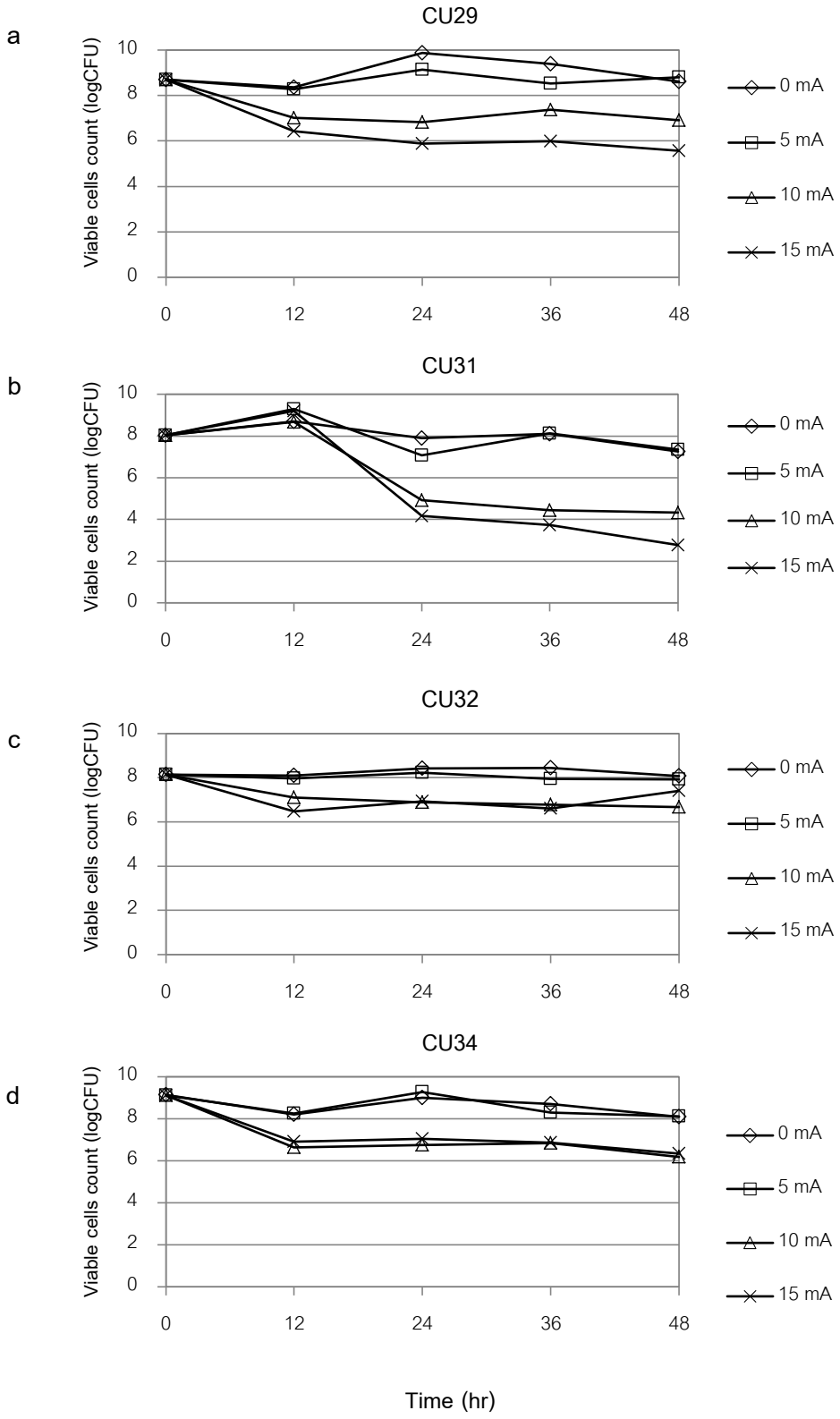


Figure 4.25 Viable cells count (logCFU) vs. time (hr) of isolates during AC stimulation at 0-15 mA for 48 hr

4.8 Electricity output of isolates

Five isolates were selected to determine electricity output of MFC with 10 mM glucose as shown in Table 4.3. In this experiment, *Cl. saccharoperbutylacetonicum* CU01 was selected to evaluate electricity output in MFC, since it was a major population of FRB in anode biofilm as shown in Figure 4.24. Mono-culture of isolates produced low voltage, current density and power density. Electricity output of CU01 was the lowest among 5 isolates. According to the physiology of *S. putrefaciens* (CU29) and *Alcaligenes* sp. (CU32), they could not utilize glucose. Therefore, their concurrent results of no-consumption of glucose were detected in MFC. While CU01, *A. hydrophiila* (CU27) and *Ps. aeruginosa* (CU34) could consume glucose but could not effectively transfer electron to electrode. However co-culture of CU01 and CU29 produced highest output. The maximum voltage of 652 mV, current density and power density of 22 mA m^{-2} and 2.70 mW m^{-2}, respectively, were obtained. From these results, it implied that co-culture could consume glucose greater than mono-culture in MFC. *S. putrefaciens* CU29 could utilize byproducts from *Cl. saccharoperbutylacetonicum* CU01 and effectively transferred electron to the electrode. In addition, these implied that mix-culture on biofilm anode could perform cascade utilization of glucose which led to decrease byproducts of glucose and increase the electricity outputs of MFC.

Table 4.3 The maximum Voltage, maximum current density and power density of isolates of MFC with glucose as carbon source

Isolate	Identification	V_{max} (mV)	I_{max} (mA m^{-2})	P_{max} (mW m^{-2})	Glucose consumption (%)
CU01	*Cl. saccharoperbutylacetonicum*	266	4	0.09	28.21
CU27	*A. hydrophila*	451	8	0.84	65.38
CU29	*S. putrefaciens*	479	4	0.29	1.3
CU32	*Alcaligenes* sp.	402	9	0.77	2.88
CU34	*Ps. aeruginosa*	420	4	0.37	84.94
CU01+ CU29	*Cl. saccharoperbutylacetonicum* and *S. putrefaciens*	652	22	2.70	69.87

4.9 Application of MFC with stimulated biofilm anode for electricity production and COD removal

AC stimulated anode which used sediments sample from CU in PBBM and NB medium were investigated in the utilizing molasses instead of glucose in MFC. Molasses were adjusted to have COD to nearly equal to COD of PBBM with 10 mM glucose of MFC. Electricity outputs and COD removal of MFC by CU sediment were shown in Figure 4.26 and 4.27.

Electricity outputs of MFC with stimulated anode in PBBM medium were greater than the one in NB medium as shown in Figure 4.27. Maximum voltage of 811 mV was obtained when PBBM was used without stimulation (0mA). Maximum current density of 48 mA m^{-2} was obtained from when PBBM was used under 10 mA stimulating current. Maximum power density of 6.8 mW m^{-2} was obtained from when PBBM was used without stimulation (0 mA). COD removal of MFC in PBBM medium was greater than the one in NB medium as shown in Figure 4.27. The highest COD removal was found from the MFC with 10 mA stimulated anode in PBBM. It could remove COD in molasses for 60%.

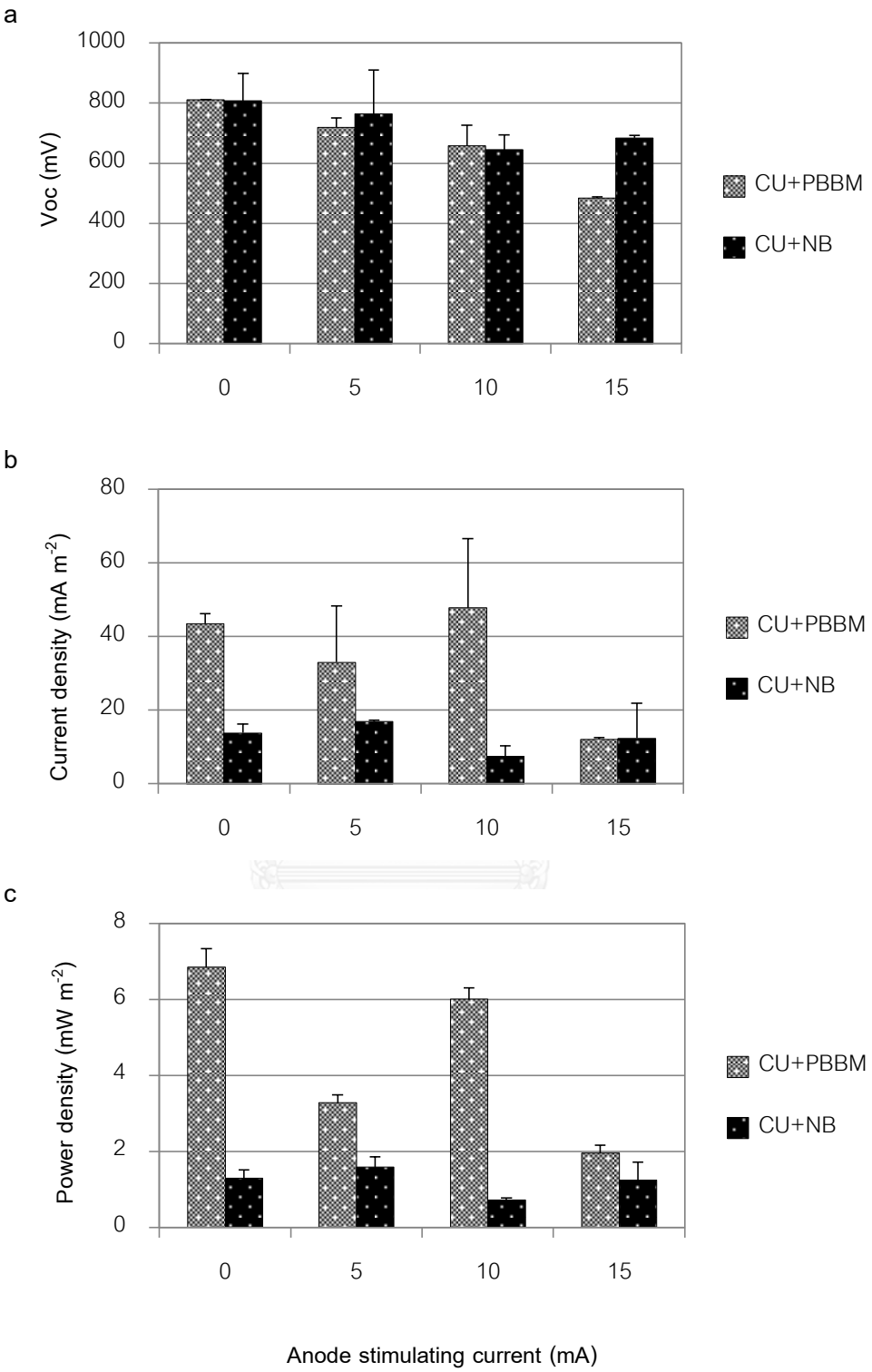


Figure 4.26 Open circuit voltage (a), current density (b) and power density (c) of molasses-fed-MFC from the different enrichment medium and anode AC stimulation of sediment from CU

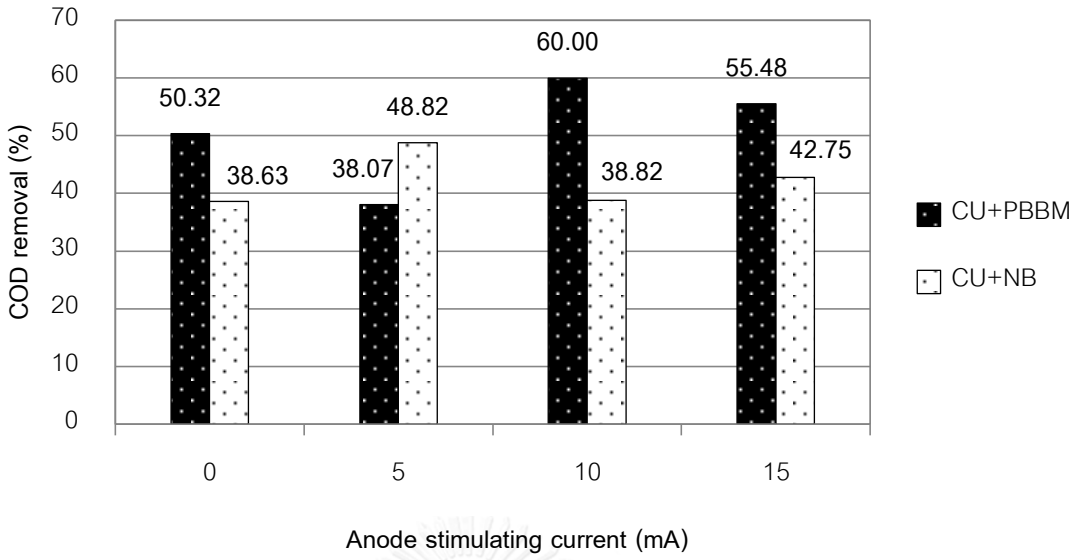


Figure 4.27 COD removal from molasses-fed-MFC from the different enrichment medium and anode AC stimulation of sediment from CU

From these results, it could be concluded that MFC with AC current stimulated anode could be used to produce electricity and removed COD from molasses. Anode which was stimulated in PBBM with 10 mA could be used to select bacterial consortium for further usage as MFC anode.

Comparison with the research of Lin et al, (2013) [12], DC current stimulated on anode was employed. The maximum power density of their MFC was 98 mW m^{-2} and their MFC configurations were indicated in Table 4.4. The major difference between this research and Lin et al, (2013) [12] was air cathode and platinum coated cathode. The platinized cathode was used to catalyze oxidizing ability that lead to produce higher power density. However, cost of the platinized cathode is high.

In order to increase electricity outputs many physical parameters should be optimized including both of anode and cathode surface area, distance between electrode and PEM, PEM surface area, cathode electrolyte, cathode electron acceptor, etc. These parameters affected electron transfer of MFC system which was analyzed by Ouitrakul, (2007 and 2008) [39, 49].

Table 4.4 The difference between MFC of this research and MFC of Lin et al, (2013)

MFC	(Lin et al, 2013)	This research
Stimulating current	DC current	AC current
Stimulating period	30 days	60 days
Anode volume	150 ml	100 ml
Anode electrode surface area	20 cm^2	18 cm^2
Architecture	glass tube-type	glass bottle
Cathode	air cathode	$K_3(FeCN)_6$
Catalyst on cathode	platinum coated on cathode	-
Power density	98 mW m^{-2}	13.4 mW m^{-2}

CHAPTER 5
CONCLUSION

Bacterial community on electrode by electric current stimulation for microbial fuel cell (MFC) was isolated and studied in this dissertation. Effect of electric current stimulation on biofilm formation on anode was demonstrated. Many parameters used for electric current stimulation such as stimulation periods, enrichment medium, carbon source in MFC, type of electric current, source of sediment and intensity of electric current on biofilm formation were experimented. In addition, the effect of electric current stimulated anode on open circuit voltage (Voc), current density and power density of MFC was examined. Moreover, ferric reducing bacteria isolated from biofilm anode were selected and identified. As well as the effect of AC stimulating current on these bacteria was studied. Electricity output produced from mono-culture and co-culture of these isolates were evaluated. Lastly, application of stimulated biofilm anode for COD removal of molasses including electricity production was investigated. All results of this work were summarized as following.

5.1 Effect of electric current stimulation on biofilm formation

Alternative current (AC) stimulation on anode and enrichment in PBBM and NB medium could promote biofilm formation on anode. Field emission scanning electron microscope (FESEM) was used to observe the characteristics of biofilm anode. It was found that the thickness of biofilm will increase when the intensity of AC stimulation is increased. In addition, the different medium used in the enrichment of biofilm during AC stimulation led to the different characteristics of biofilm.

5.2 Effected parameters of electric current stimulation on electricity output of MFC

5.2.1 Stimulation periods

AC stimulation periods were investigated in NB and PBBM medium for 15 days and 60 days. It was found that the increasing of stimulation periods in PBBM affected electricity outputs of MFC. 15 days stimulated anode in PBBM and NB gave rather different electricity output of MFC. However, 60 days stimulated anode in PBBM offered

a stable Voc and could increase current and power density for ~3 times and ~6 times as compared with a stimulation for 15 days.

5.2.2 Enrichment medium

Three enrichment media, NB, PBBM, and FWA were used to enrich bacterial community in sediment during AC and DC stimulation for 60 days. It was found that PBBM was a suitable enrichment medium for use during electric current stimulation. Both AC and DC stimulation with PBBM enrichment gave higher current and power density than NB and FWA enrichment. In addition, when PBBM medium was used, AC stimulation gave the higher current and power density than DC stimulation.

5.2.3 Carbon source in MFC

Acetate, propionate, and glucose were used as carbon source of stimulated biofilm anode in MFC. It was found that the use of propionate as carbon source of anode biofilm gave Voc higher than others carbon source. In addition, the use of acetate as carbon source gave current and power density higher than the others. Unstimulated anode in propionate-fed MFC gave the highest Voc in this dissertation. But the increasing of AC current intensity, Voc of propionate fed MFC would decrease. However, 60 days AC stimulated anode at 5 and 10 mA in acetate-fed MFC gave the highest current density and power density, respectively.

5.2.4 Type of electric current

AC current and DC current were used to stimulate anode in this study. It was found that Voc, current density and power density of MFC with AC stimulated anode was greater than DC stimulated anode.

5.2.5 Source of sediment

Five sources of sediment were used as inoculum of AC stimulation with PBBM enrichment. It was found that AC stimulation on anode can be used to stimulate different sources of sediments especially freshwater sediment and sub-surface soil. AC stimulation on anode at 5-10 mA tentatively increased current density and power density higher than unstimulated anode.

5.2.6 Intensity of electric current

Various intensity of electric current at 0, 5, 10 and 15 mA were used to stimulate biofilm anode. It was found that the reduction trend of Voc of MFC would occur during the increase of AC stimulation. However, AC stimulated anode at 5 and 10 mA gave higher current and power density than unstimulated anode and AC stimulated anode at 15 mA. It could be noted that the appropriate intensity of AC stimulation should be used to stimulate anode biofilm in order to increase current and power density.

5.3 Bacterial community in biofilm anode

Bacterial consortium was isolated from biofilm anode based on ferric reducing activity. It was found that electrochemical active bacteria (EAB) were enriched on biofilm anode. Ferric reducing bacteria (FRB) reported as EAB were isolated from stimulated anode biofilm such as *Shewanella putrefaciens*, *Aeromonas hydrophila,* and *Pseudomonas aeruginosa*. It could be concluded that electric current stimulation could be used to enrich EAB on biofilm anode. Direct and effective electron transferring of EAB to anode led to increase current and power density of MFC.

5.4 Effect of AC stimulating current on viable cell count of bacteria

FRB were selected and stimulated with AC current at 0, 5, 10 and 15 mA in NB medium. It was found that AC stimulation had an effect on viability of bacterial cells. AC stimulating current more than 5 mA could inhibit bacterial growth. The highest inhibitory effect on bacterial growth was AC stimulation at 15 mA. In addition, AC current at 15 mA could inhibit growth of EAB such as *A. hydrophila* (CU27) and *S. putrefaciens* (CU29) more than non-EAB such as *Alcaligenes* sp. (CU32). It could be concluded that AC stimulation at 15 mA might decrease EAB population more than non-EAB led to the lower of current and power density of MFC with 15 mA AC stimulated anode. Suitable intensity of electric current should be applied to dominate EAB population in biofilm anode rather than non-EAB.

5.5 Electricity outputs of FRB isolates

Five FRB were selected to determine electricity outputs in glucose-fed MFC. Effectiveness of electron transferring of isolate to anode led to increase current density and power density of MFC. *Clostridium saccharoperbutylacetonicum* (CU01) could consume glucose but could not effectively transfer electron to anode, so it produced the lowest electricity outputs among 5 isolates. On the other hand, *S. putrefaciens* (CU29) could effectively transfer electron to anode but could not consume glucose. It was found that co-culture of CU01 and CU29 could produce the highest output and consume glucose greater than mono-culture of each isolate. It implied that *S. putrefaciens* CU29 could utilize byproducts from *Cl. saccharoperbutylacetonicum* CU01 and effectively transferred electron to the electrode.

5.6 Application of stimulated anode on COD removal and electricity production

AC stimulated anode was used to investigate the COD removal and electricity production in molasses-fed MFC. The highest COD removal was 60% of the initial COD and the highest current density obtained from 10 mA AC stimulated anode. It was found that AC stimulated anode could reduce COD of molasses in MFC and produce electricity better than unstimulated anode.

5.7 Summary

This dissertation aims to investigate the use of electric current for selection of electrochemical active bacterial community on electrode for use as anode of MFC. Parameters using during electric current stimulation were also investigated on the electricity outputs of MFC. It could be concluded that electric current stimulation had the opportunity to apply for selecting effective bacterial community on electrode using as anode of MFC. The highest Voc of 989 mV, produced from propionate-fed MFC with 60 days unstimulated anode in PBBM medium. The highest current density of 72.9 mA m^{-2} and power density of 13.4 mW m^{-2} produced from acetate-fed MFC with 60 days alternative current (AC) stimulated anode at 5-10 mA in PBBM medium. Moreover, application of stimulated anode for COD removal and electricity production was also

investigated. It was found that the highest COD removal of 60% in molasses-fed MFC was performed by AC stimulated anode at 10 mA.

5.8 Future Suggestion

1. Strictly anaerobic system may be required to maintain anaerobic bacteria.

2. Optimizations of the physical and chemical parameters are required for improving electricity production of MFC with AC stimulated anode.

3. Application of electric current stimulation for selection of electrochemical active bacterial community in waste water, anaerobic sludge from waste water treatment plant should be used to stimulate with AC current and investigate electricity output of MFC during waste treatment.

4. For continuous operating of MFC, viability and stability of biofilm on the stimulated anode should be investigated.

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APPENDIX

Appendix A

Media for microorganisms

A1. Modified PBBM (Nelson & Zeikus, 1974) (per L)

$(NH_4)_2SO_4$	0.45	g
NaCl	0.90	g
$MgSO_4.7H_2O$	0.18	g
$CaCl_2.2H_2O$	0.10	g
NH_4Cl	0.50	g
KH_2PO_4	1.50	g
K_2HPO_4	2.19	g
Trace mineral solution	9	ml
Vitamin solution	5	ml

A2. Freshwater medium for enrichment (per L)

$NaHCO_3$	2.5	g
$CaCl_2.2H_2O$	0.1	g
KCl	0.1	g
NH_4Cl	1.5	g
$NaH_2PO_4.H_2O$	0.6	g
Trace mineral solution	9	ml
Vitamin solution	5	ml
Sodium acetate	10	mM
Sodium propionate	10	mM
Glucose	10	mM

A3. Trace Mineral Solution (per L)

Nitrilotriacetic acid	15	g
$FeSO_4.7H_2O$	0.1	g
$MnCl_2.4H_2O$	0.1	g
$CaCl_2.6H_2O$	0.17	g
$CaCl_2.2H_2O$	0.1	g
$ZnCl_2$	0.1	g
$NiSO_4.6H_2O$	0.026	g
$CuCl_2.2H_2O$	0.02	g
H_3BO_3	0.01	g
$NaMoO_4.2H_2O$	0.01	g
NaCl	1.0	g
Na_2SeO_3	0.016	g

A4. Vitamin Solution (per L)

Biotin	1	mg
Folic Acid	1	mg
B_6 Pyridoxine HCl	5	mg
B_1 Thiamine HCl	2.5	mg
B_2 Rhiboflavin	2.5	mg
Nicotinic acid (Niacin)	2.5	mg
Panthothenic acid	2.5	mg
B_{12} Cyanocobalamin	0.05	mg
Para aminobenzoic	2.5	mg
Lipoic acid	2.5	mg
DDW	500	ml

A5. Nutrient Broth or Nutrient Agar (Oxoid) (per L)

Lab-Lemco powder	1.0	g
Yeast extract	2.0	g

Peptone	5.0	g
Sodium Chloride	5.0	g
Agar (for Nutrient Agar)	15.0	g

Appendix B

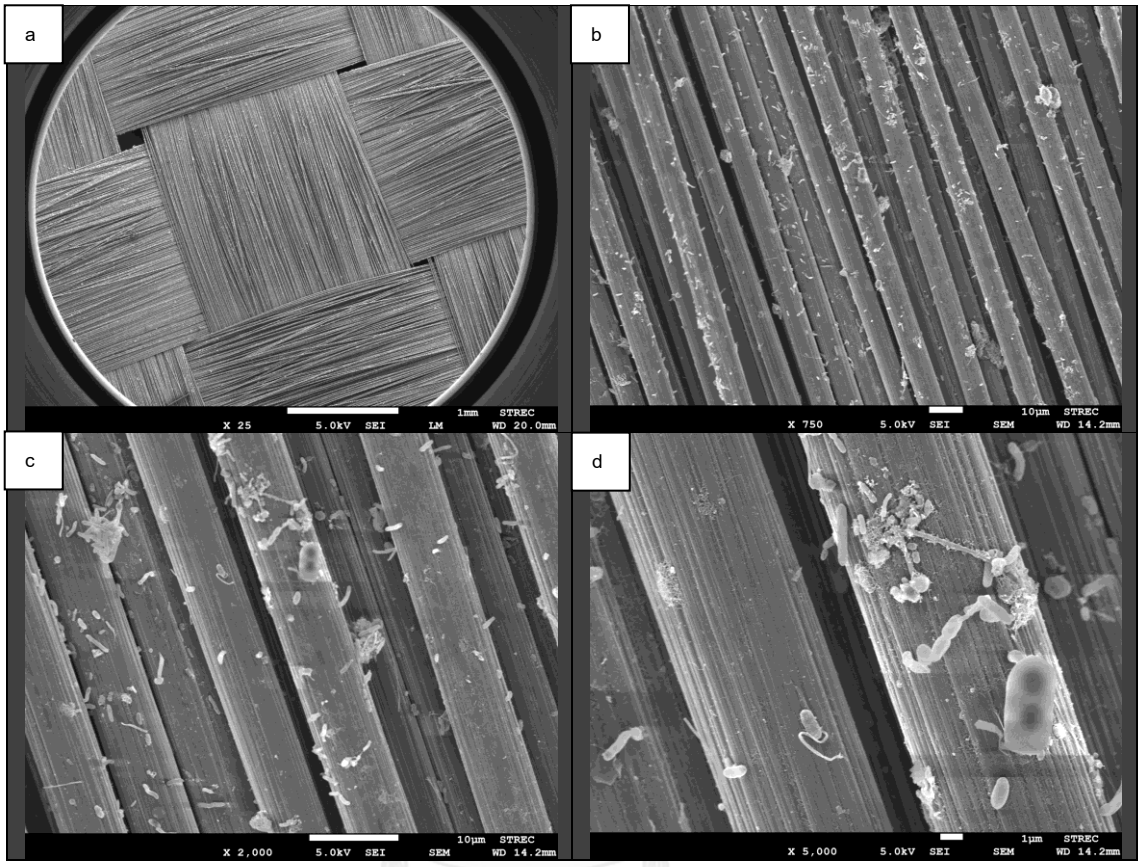

Figure B1 SEM micrographs of stimulated anode of sediment from CU enriched in PBBM without AC stimulation at 0 mA

(a) Stimulated anode at 25x magnification ratio

(b) Stimulated anode at 750x magnification ratio

(c) Stimulated anode at 2,000x magnification ratio

(d) Stimulated anode at 5,000x magnification ratio

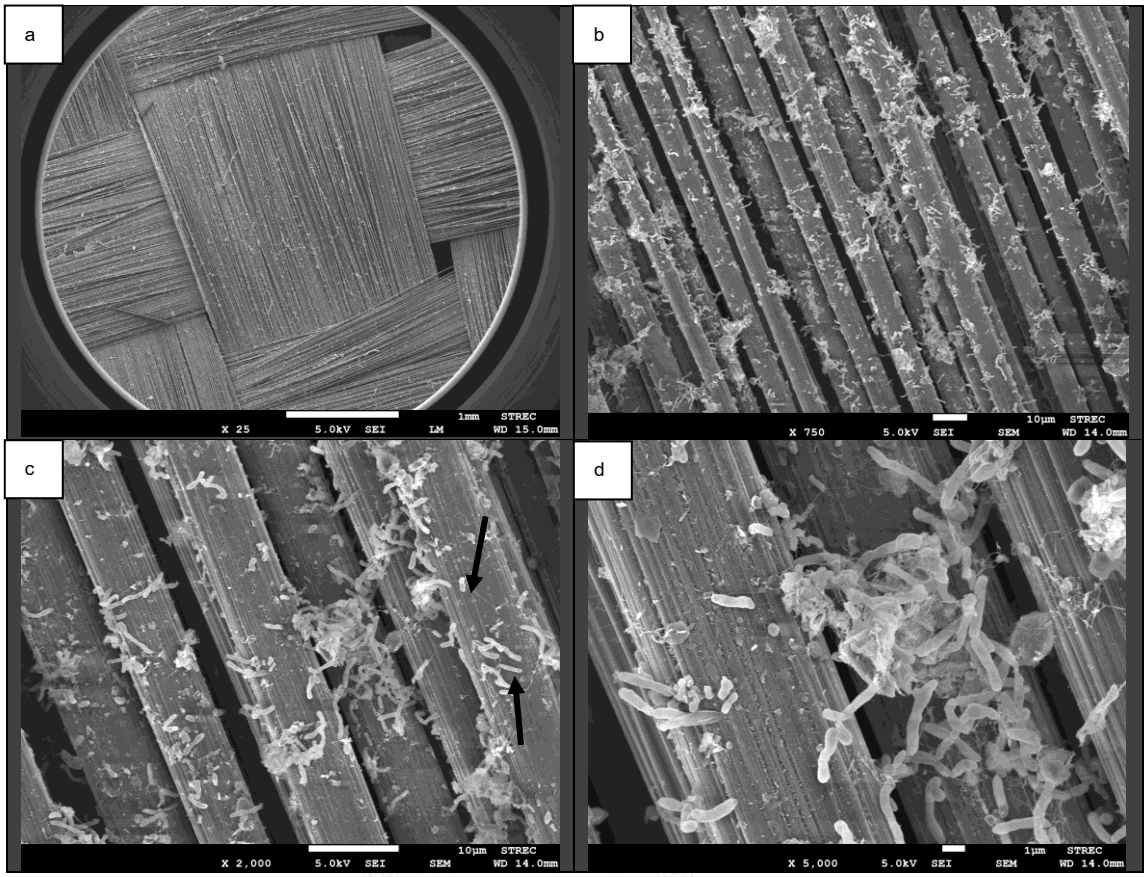

Figure B2 SEM micrographs of stimulated anode of sediment from CU enriched in PBBM with AC stimulation at 5 mA

(a) Stimulated anode at 25x magnification ratio

(b) Stimulated anode at 750x magnification ratio

(c) Stimulated anode at 2,000x magnification ratio

(d) Stimulated anode at 5,000x magnification ratio

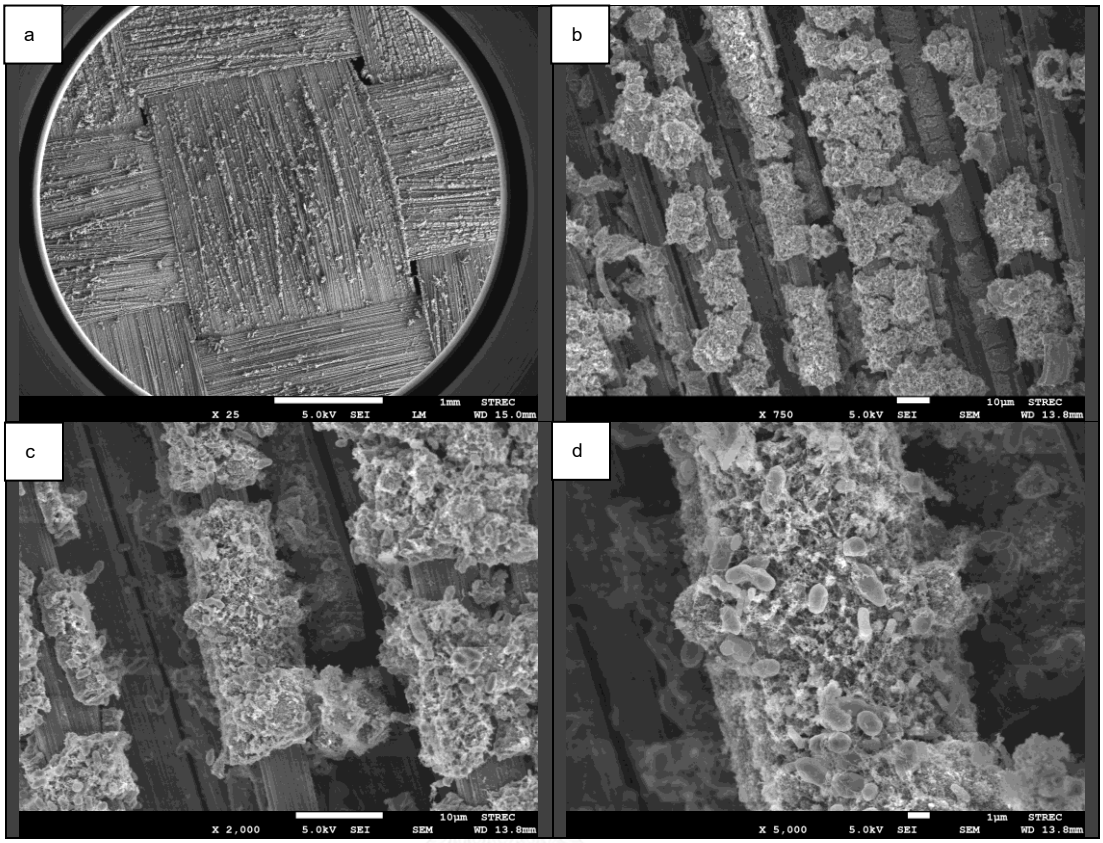

Figure B3 SEM micrographs of stimulated anode of sediment from CU enriched in PBBM with AC stimulation at 10 mA

(a) Stimulated anode at 25x magnification ratio

(b) Stimulated anode at 750x magnification ratio

(c) Stimulated anode at 2,000x magnification ratio

(d) Stimulated anode at 5,000x magnification ratio

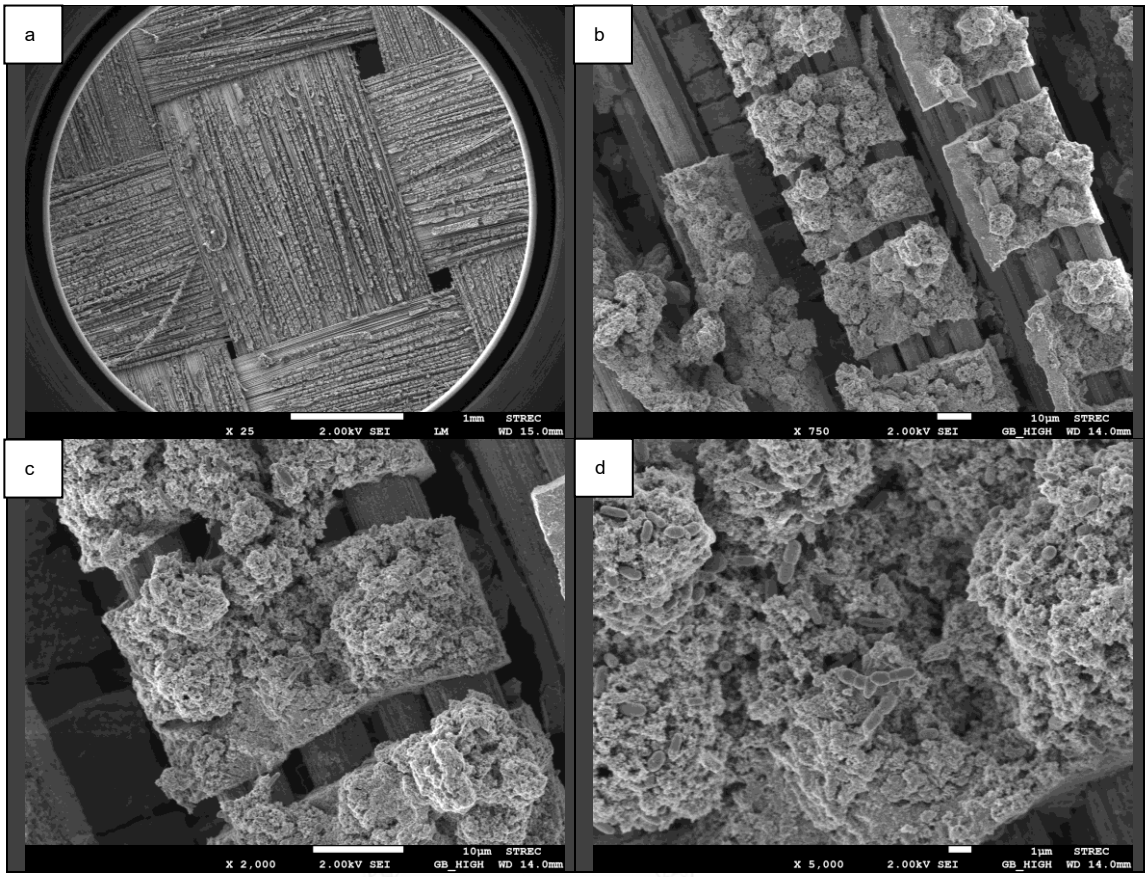

Figure B4 SEM micrographs of stimulated anode of sediment from CU enriched in PBBM with AC stimulation at 15 mA

(a) Stimulated anode at 25x magnification ratio

(b) Stimulated anode at 750x magnification ratio

(c) Stimulated anode at 2,000x magnification ratio

(d) Stimulated anode at 5,000x magnification ratio

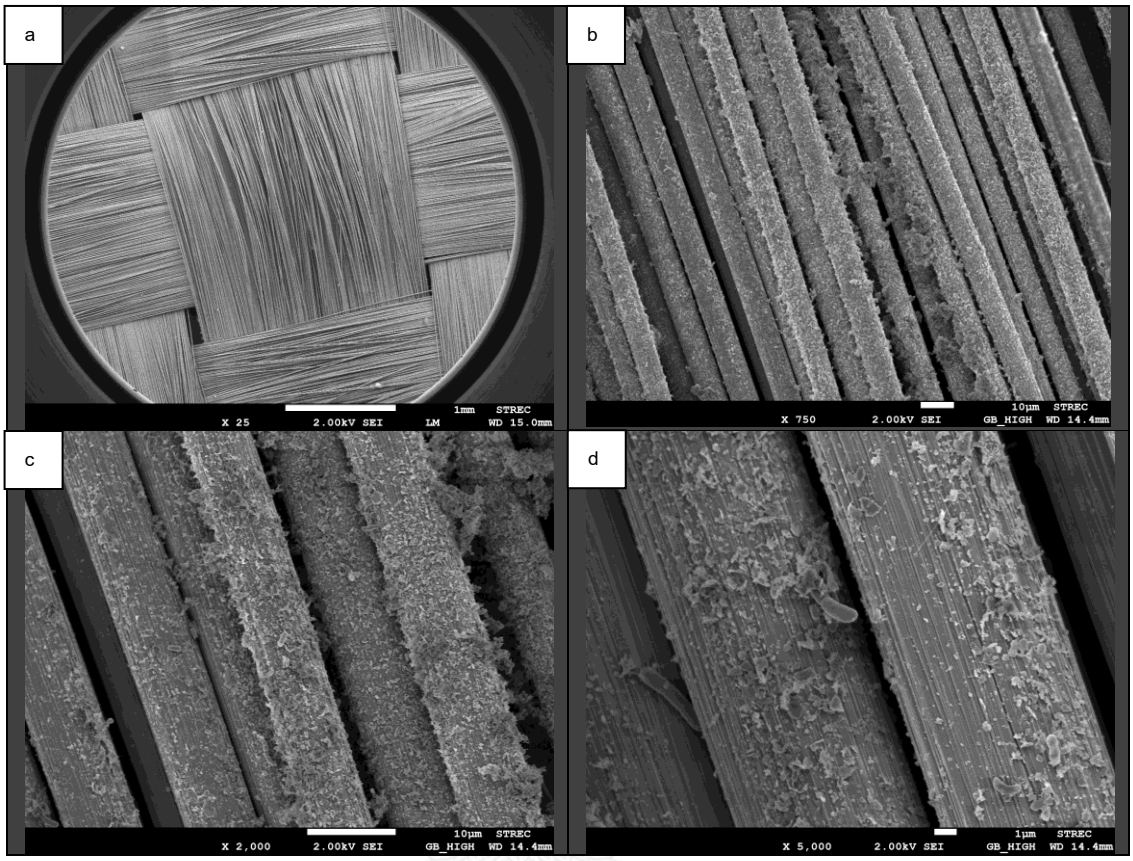

Figure B5 SEM micrographs of stimulated anode of sediment from CU enriched in NB without AC stimulation (0 mA)

(a) Stimulated anode at 25x magnification ratio

(b) Stimulated anode at 750x magnification ratio

(c) Stimulated anode at 2,000x magnification ratio

(d) Stimulated anode at 5,000x magnification ratio

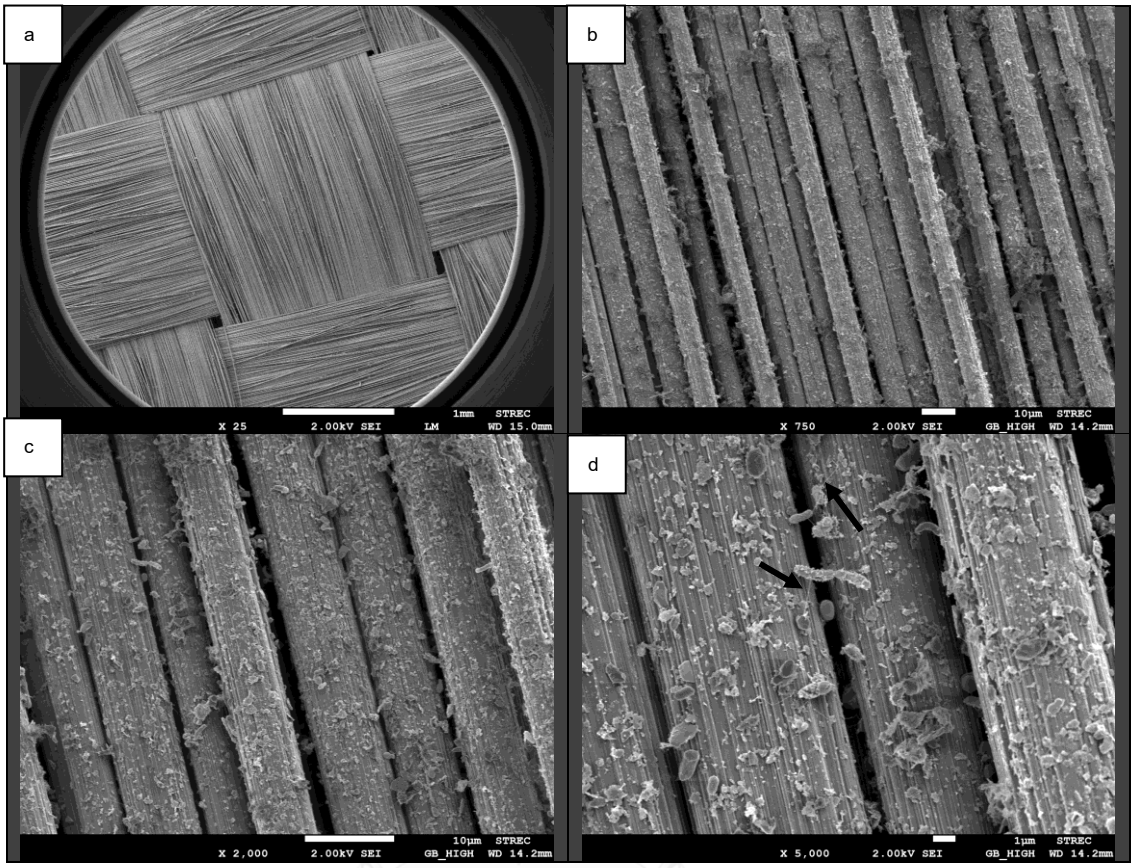

Figure B6 SEM micrographs of stimulated anode of sediment from CU enriched in NB with AC stimulation at 5 mA

(a) Stimulated anode at 25x magnification ratio

(b) Stimulated anode at 750x magnification ratio

(c) Stimulated anode at 2,000x magnification ratio

(d) Stimulated anode at 5,000x magnification ratio

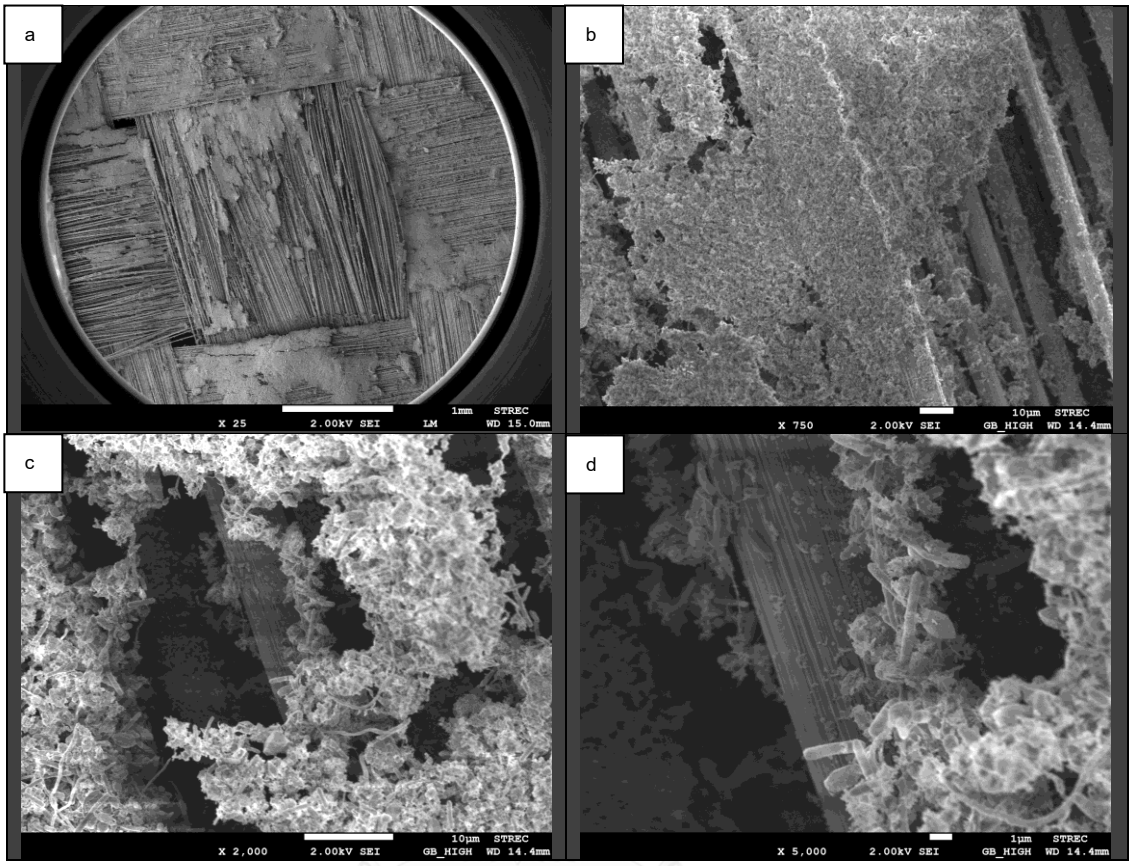

Figure B7 SEM micrographs of stimulated anode of sediment from CU enriched in NB with AC stimulation at 10 mA

(a) Stimulated anode at 25x magnification ratio

(b) Stimulated anode at 750x magnification ratio

(c) Stimulated anode at 2,000x magnification ratio

(d) Stimulated anode at 5,000x magnification ratio

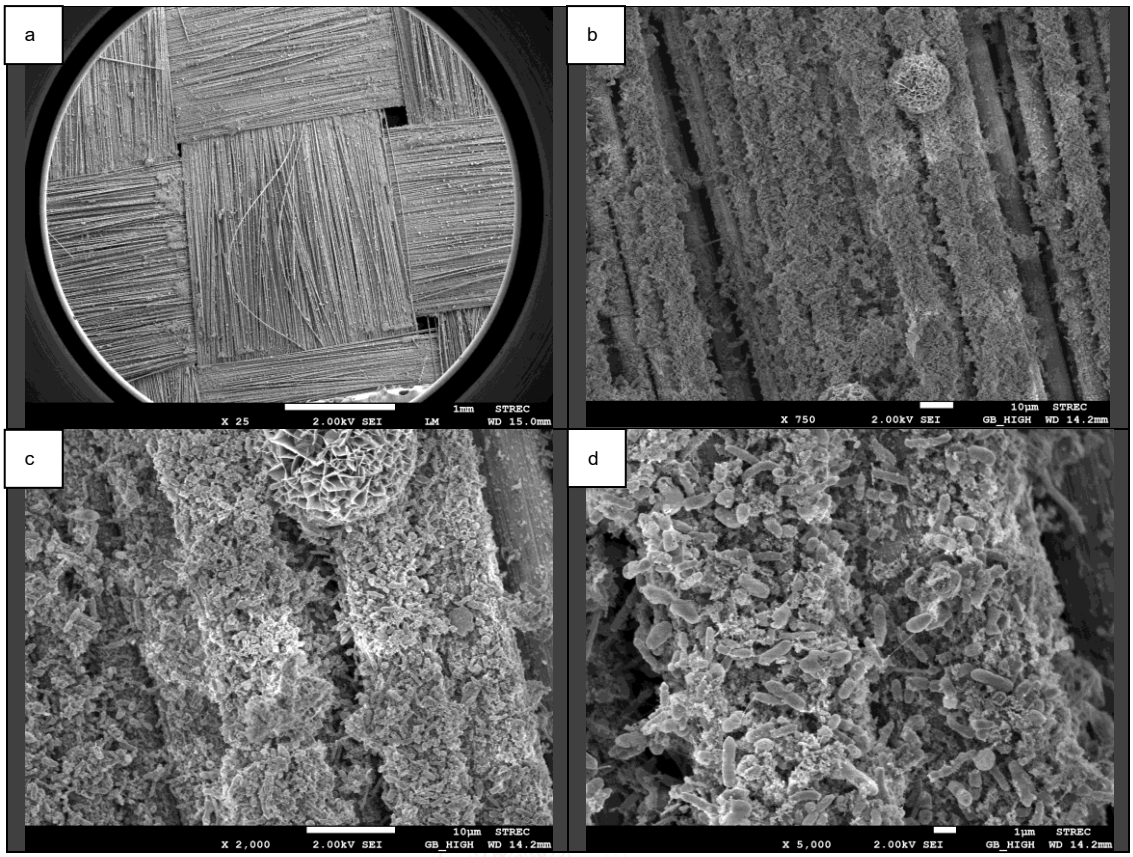

Figure B8 SEM micrographs of stimulated anode of sediment from CU enriched in NB with AC stimulation at 15 mA

(a) Stimulated anode at 25x magnification ratio

(b) Stimulated anode at 750x magnification ratio

(c) Stimulated anode at 2,000x magnification ratio

(d) Stimulated anode at 5,000x magnification ratio

Appendix C

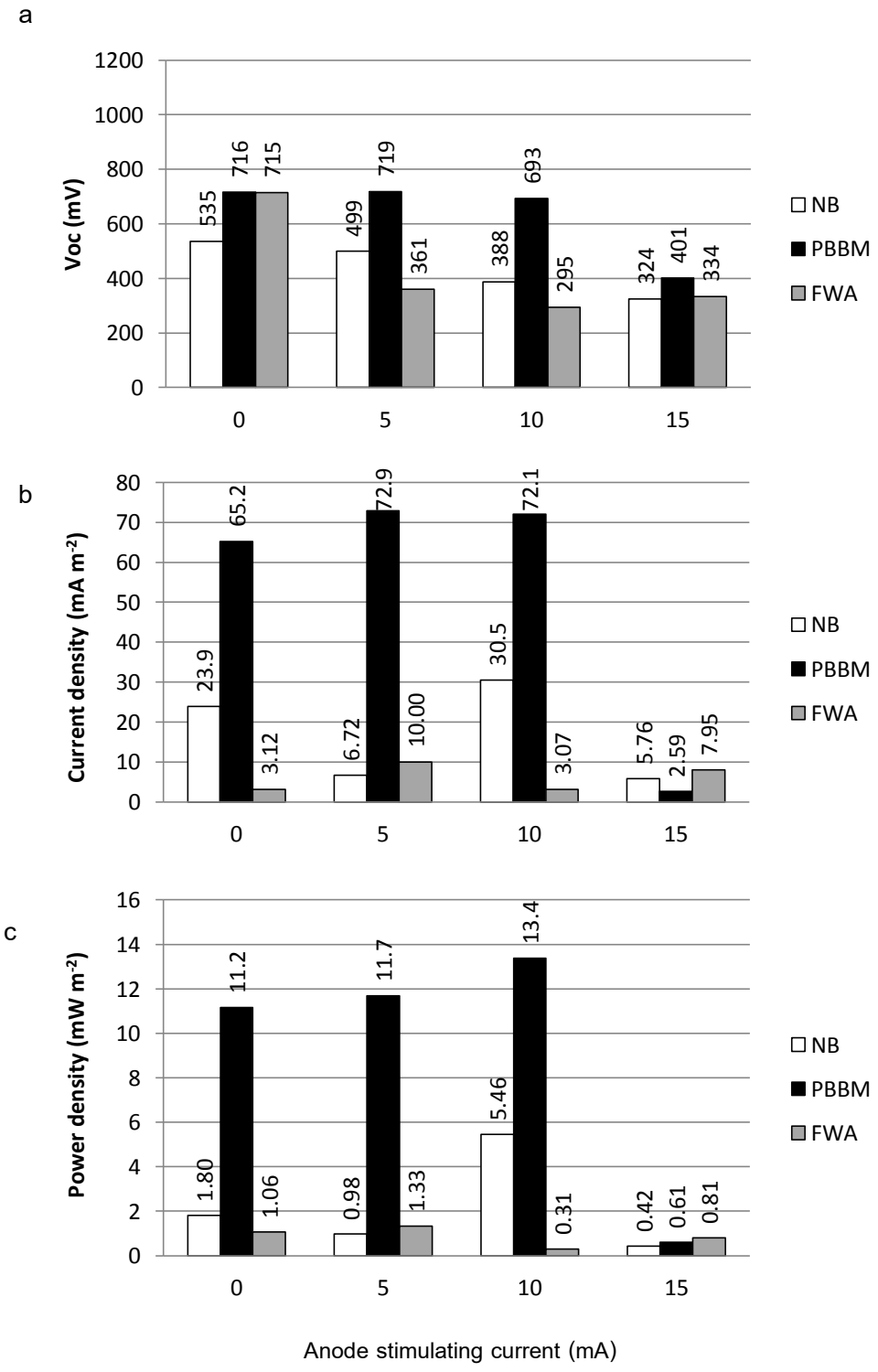

Figure C1 Open circuit voltage (a), current density (b) and power density (c) of acetate-fed MFC from the different enrichment medium and anode AC stimulation

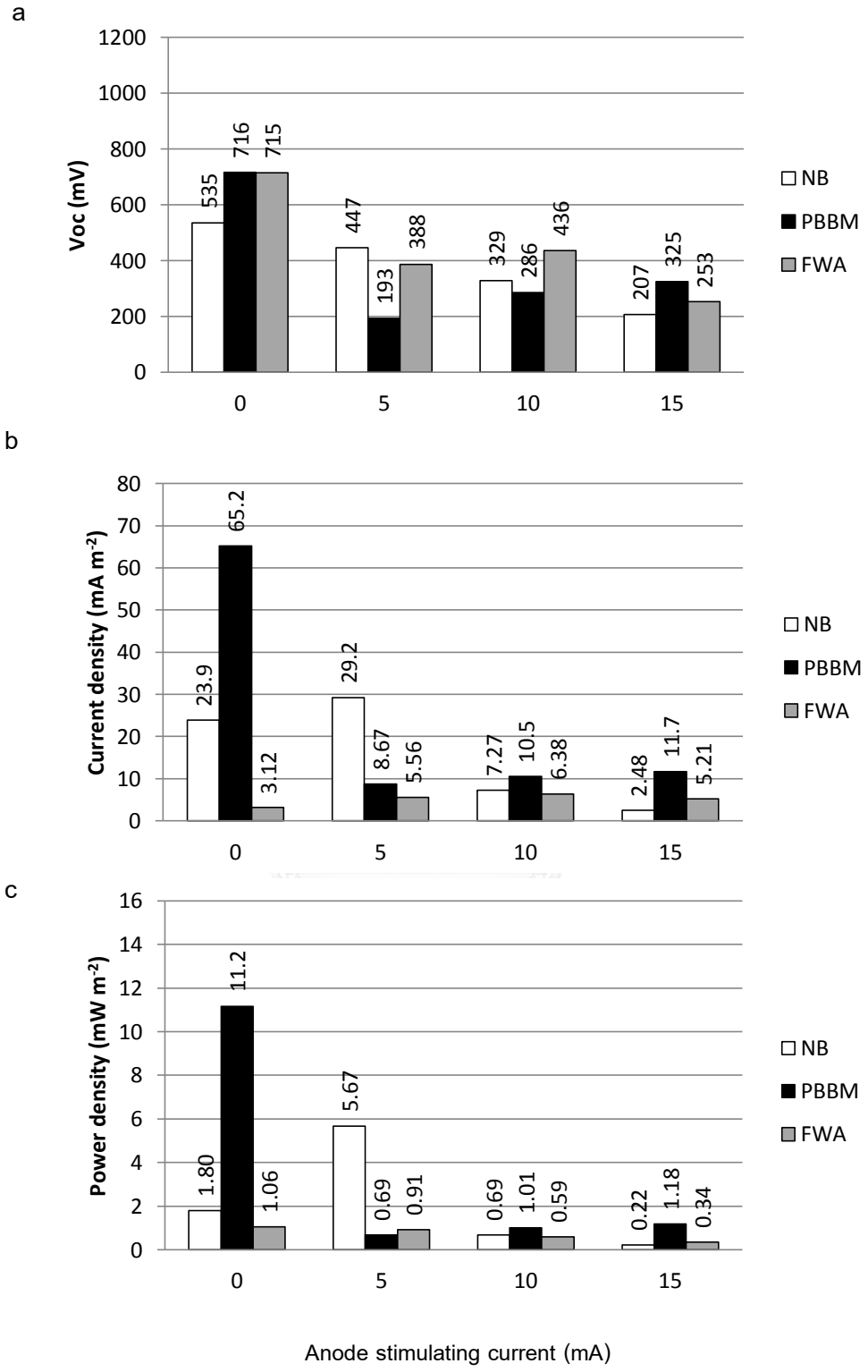

Figure C2 Open circuit voltage (a), current density (b) and power density (c) of acetate-fed MFC from the different enrichment medium and anode DC stimulation

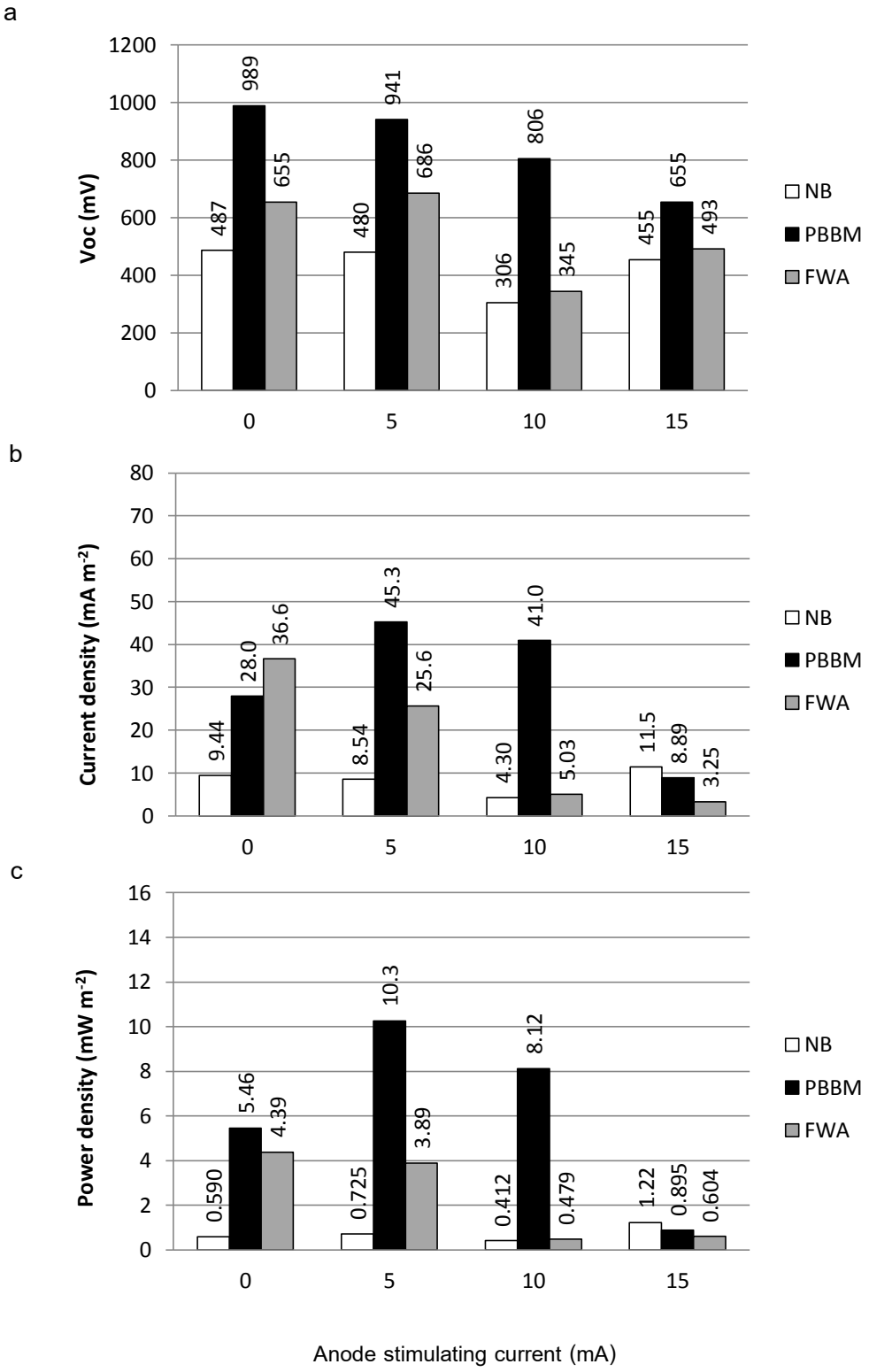

Figure C3 Open circuit voltage (a), current density (b) and power density (c) of propionate-fed MFC from the different enrichment medium and anode AC stimulation

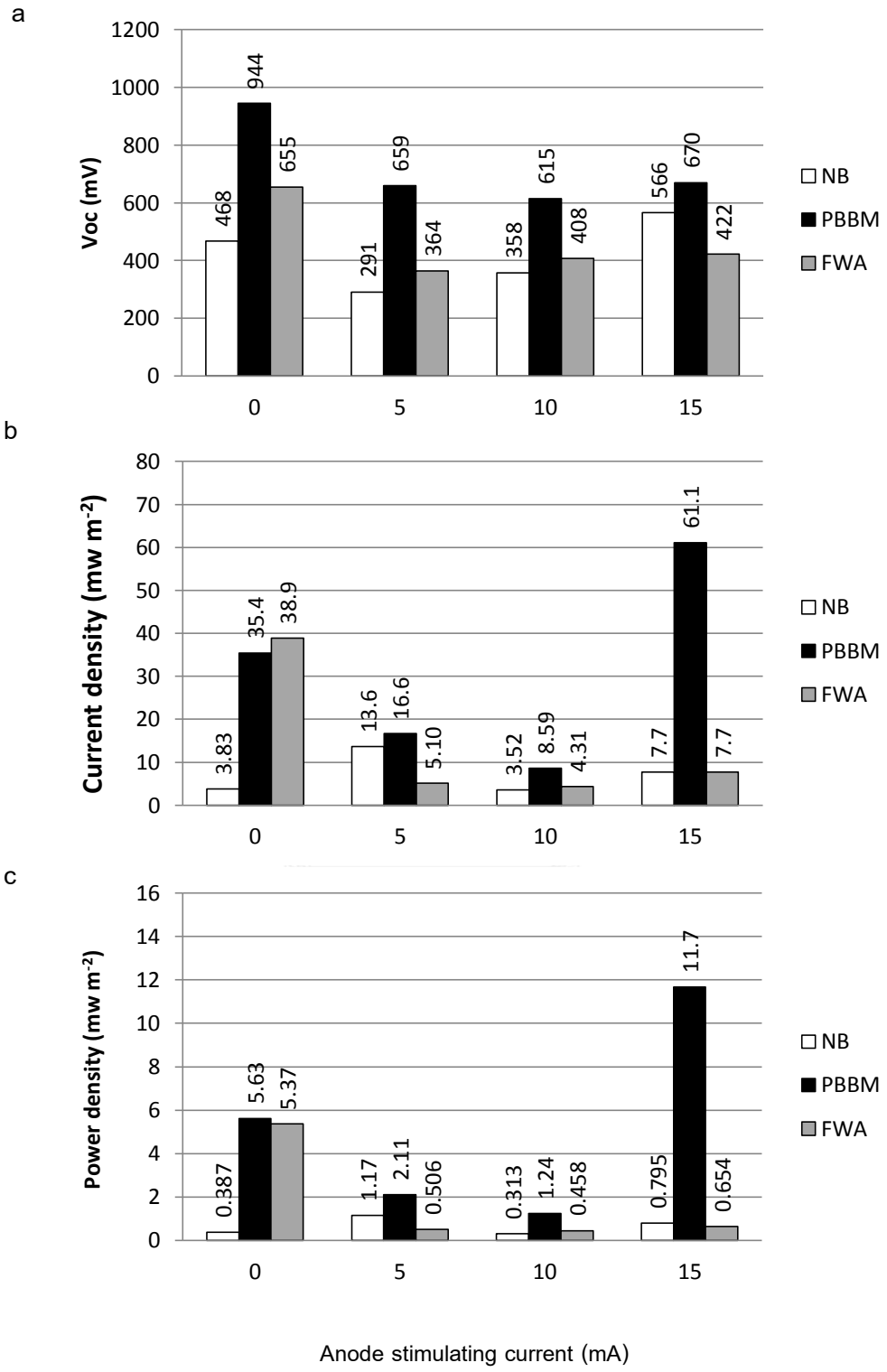

Figure C4 Open circuit voltage (a), current density (b) and power density (c) of propionate-fed MFC from the different enrichment medium and anode DC stimulation

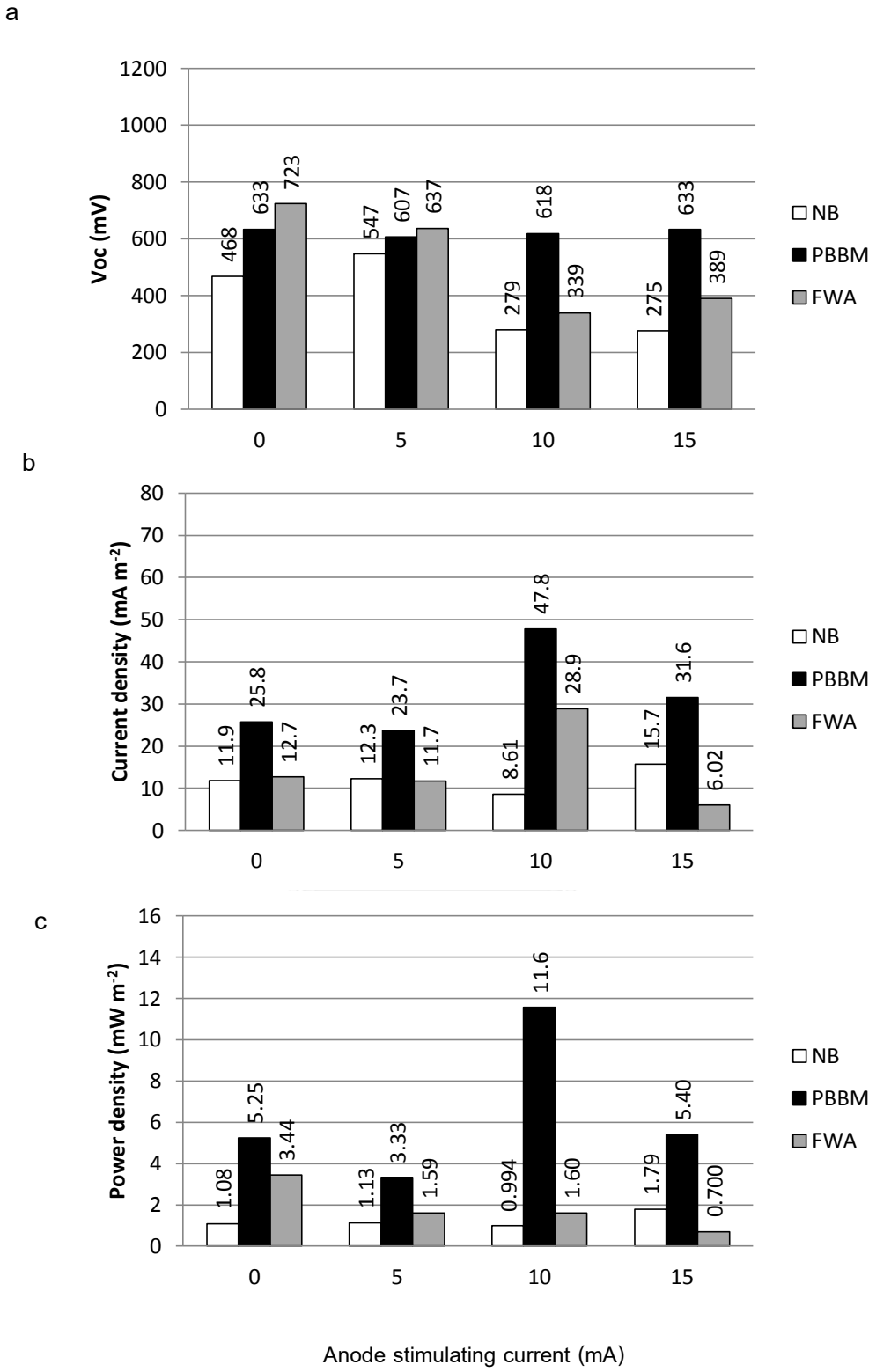

Figure C5 Open circuit voltage (a), current density (b) and power density (c) of glucose-fed MFC from the different enrichment medium and anode AC stimulation

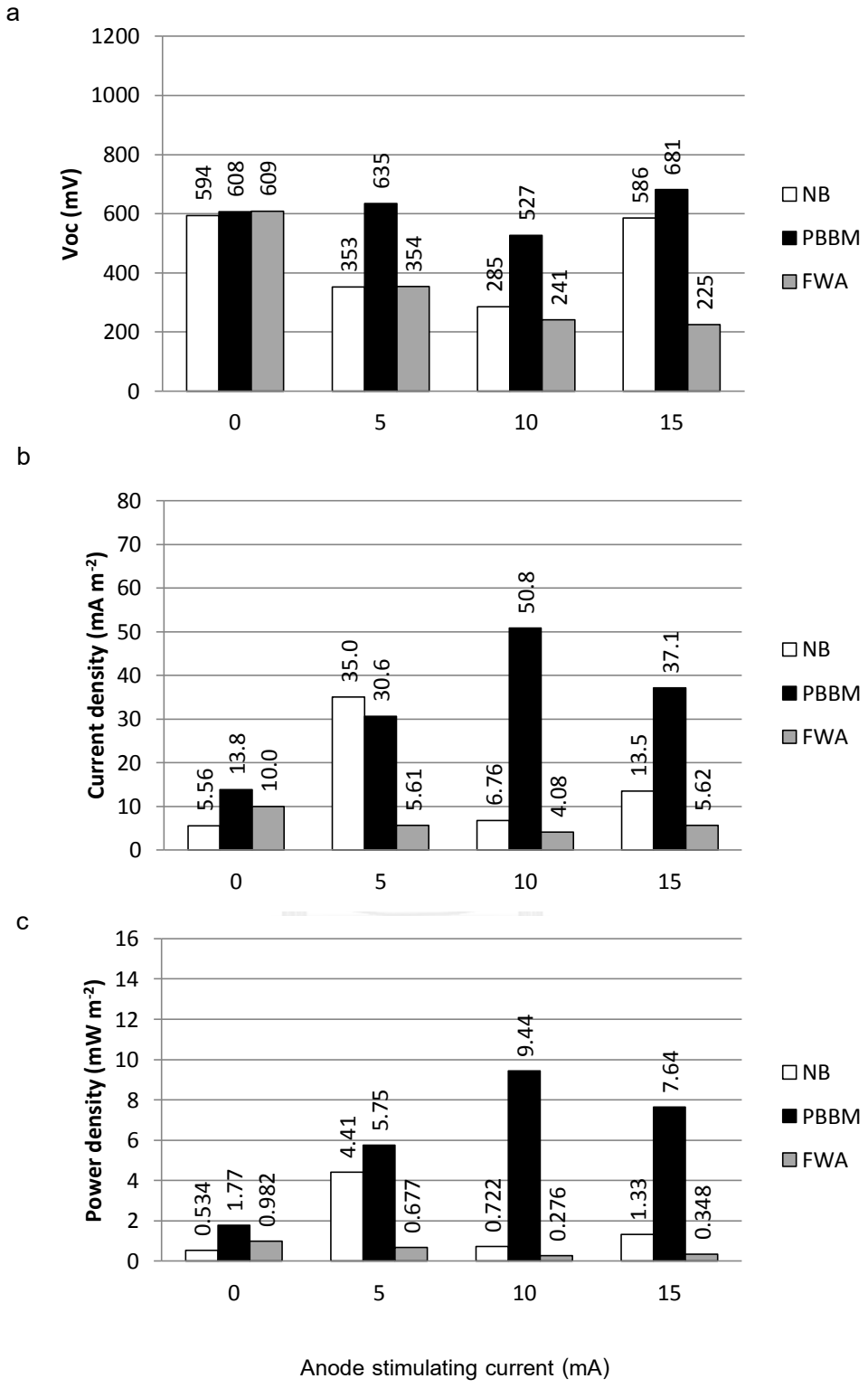

Figure C6 Open circuit voltage (a), current density (b) and power density (c) of glucose-fed MFC from the different enrichment medium and anode DC stimulation

Appendix D

Estimation of Glucose by Somogyi-Nelson method

Table D1 O.D. at 520 nm of various concentrations of glucose by Somogyi-Nelson method

Tube	1 mg/ml glucose (ml)	DW (ml)	Final conc. of glucose (µg/ml)	O.D. at 540 nm			Average of O.D. at 540 nm
1	-	1.0	0	0	0	0	0.000
2	0.02	0.98	20	0.117	0.106	0.107	0.110
3	0.04	0.96	40	0.231	0.233	0.232	0.232
4	0.06	0.94	60	0.349	0.35	0.35	0.350
5	0.08	0.92	80	0.469	0.464	0.469	0.467
6	0.10	0.90	100	0.585	0.581	0.589	0.585
7	0.12	0.88	120	0.722	0.724	0.72	0.722
8	0.15	0.85	150	0.953	0.951	0.949	0.951
9	0.18	0.82	180	1.202	1.188	1.185	1.192
10	0.20	0.80	200	1.324	1.336	1.288	1.316

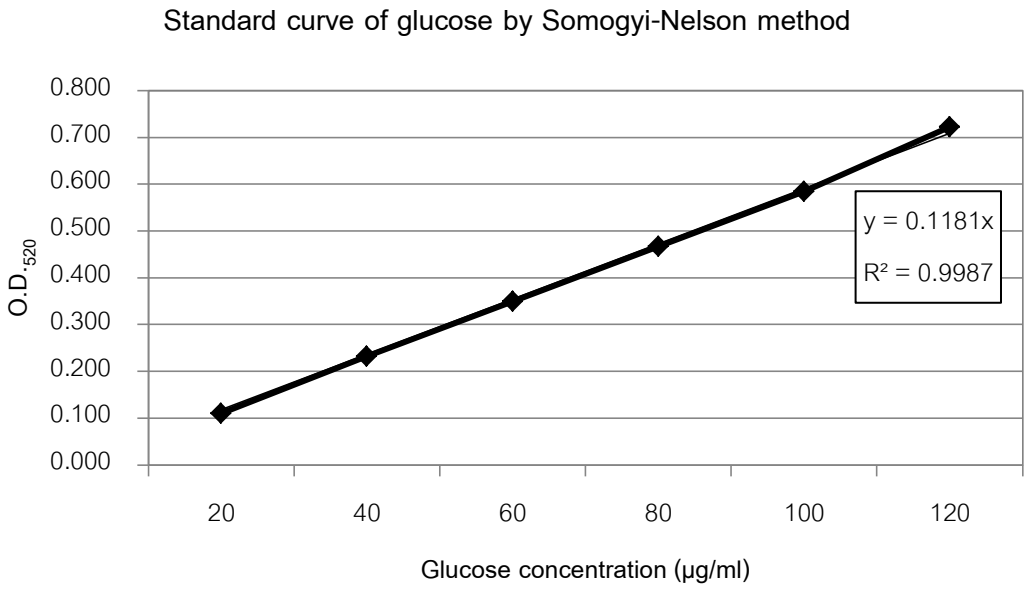

Figure D1 Standard curve of glucose by Somogyi-Nelson method

Determination of glucose concentration by Somogyi-Nelson method can be calculated as follow:

$$[\text{Glucose}]\ (\mu g/ml) = \frac{\text{O.D. x dilution factor}}{\text{Slope}}$$

Appendix E

Bacterial identification by API 20E kit

Table E1 Ingredient utilization pattern of strains CU27 and CU28 using API 20E

Active ingredients	Isolate number	
	CU27	CU28
β-galactosidase	+	+
Arginine dihydrolase	+	+
Lysine decarboxylase	+	-
Ornithine decarboxylase	-	+
Citrate utilisation	-	+
H_2S production	-	-
Urea hydrolysis	-	-
Tryptophan deamination	-	-
Indole production	+	+
Acetoin production	+	-
Gelatin hydrolysis	+	-
Glucose fermentation	+	+
Mannitol	+	+
Inositol	+	-
Sorbitol	+	+
Rhamnose	-	+
Sucrose	+	+
Melibiose	-	+
Amygdalin	+	+
Arabinose	-	+
Cytochrome-Oxidase	+	-
NO_2 production	+	+
Reduction to N_2 gas	-	-
Motility	+	+
MacConkey medium	+	+
Glucose (API OF Medium): OF-O	+	+
Glucose (API OF Medium): OF-F	+	+
Accession number	704772557	334457357

+ = positive; - = negative

Table E2 Ingredient utilization pattern of strains CU29 and CU30 using API 20E

Active ingredients	Isolate number	
	CU29	CU30
β-galactosidase	-	+
Arginine dihydrolase	-	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Citrate utilisation	+	-
H_2S production	+	-
Urea hydrolysis	-	-
Tryptophan deamination	-	-
Indole production	-	+
Acetoin production	-	-
Gelatin hydrolysis	+	-
Glucose fermentation	-	+
Mannitol	-	+
Inositol	-	+
Sorbitol	-	+
Rhamnose	-	+
Sucrose	+	+
Melibiose	-	+
Amygdalin	-	+
Arabinose	+	+
Cytochrome-Oxidase	+	-
NO_2 production	+	-
Reduction to N_2 gas	-	-
Motility	+	-
MacConkey medium	+	+
Glucose (API OF Medium): OF-O	-	+
Glucose (API OF Medium): OF-F	-	+
Accession number	060202651	104477307

+ = positive; - = negative

Table E3 Ingredient utilization pattern of strains CU31 and CU32 using API 20E

Active ingredients	Isolate number	
	CU31	CU32
β-galactosidase	+	-
Arginine dihydrolase	-	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Citrate utilisation	-	-
H_2S production	-	-
Urea hydrolysis	+	-
Tryptophan deamination	-	-
Indole production	+	-
Acetoin production	+	+
Gelatin hydrolysis	-	+
Glucose fermentation	-	-
Mannitol	-	-
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	-	-
Melibiose	-	-
Amygdalin	-	-
Arabinose	-	-
Cytochrome-Oxidase	+	+
NO_2 production	-	+
Reduction to N_2 gas	-	-
Motility	-	-
MacConkey medium	+	+
Glucose (API OF Medium): OF-O	+	-
Glucose (API OF Medium): OF-F	+	-
Accession number	105100407	000300411

+ = positive; - = negative

Table E4 Ingredient utilization pattern of strains CU33 and CU34 using API 20E

Active ingredients	Isolate number	
	CU33	CU34
β-galactosidase	-	-
Arginine dihydrolase	+	+
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Citrate utilisation	+	+
H_2S production	-	-
Urea hydrolysis	-	+
Tryptophan deamination	-	-
Indole production	-	-
Acetoin production	+	+
Gelatin hydrolysis	-	+
Glucose fermentation	-	+
Mannitol	-	-
Inositol	-	-
Sorbitol	-	-
Rhamnose	-	-
Sucrose	-	-
Melibiose	-	-
Amygdalin	-	-
Arabinose	-	+
Cytochrome-Oxidase	+	+
NO_2 production	+	-
Reduction to N_2 gas	-	+
Motility	-	+
MacConkey medium	+	+
Glucose (API OF Medium): OF-O	+	+
Glucose (API OF Medium): OF-F	-	-
Accession number	220100413	221700663

+ = positive; - = negative

Table E5 Ingredient utilization pattern of strains CU35 using API 20E

Active ingredients	Isolate number
	CU35
β-galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	+
Citrate utilisation	+
H_2S production	-
Urea hydrolysis	-
Tryptophan deamination	-
Indole production	-
Acetoin production	+
Gelatin hydrolysis	-
Glucose fermentation	+
Mannitol	+
Inositol	+
Sorbitol	+
Rhamnose	+
Sucrose	+
Melibiose	+
Amygdalin	+
Arabinose	+
Cytochrome-Oxidase	-
NO_2 production	+
Reduction to N_2 gas	-
Motility	+
MacConkey medium	+
Glucose (API OF Medium): OF-O	+
Glucose (API OF Medium): OF-F	+
Accession number	330577357

+ = positive; - = negative

Appendix F

Electricity output of isolates

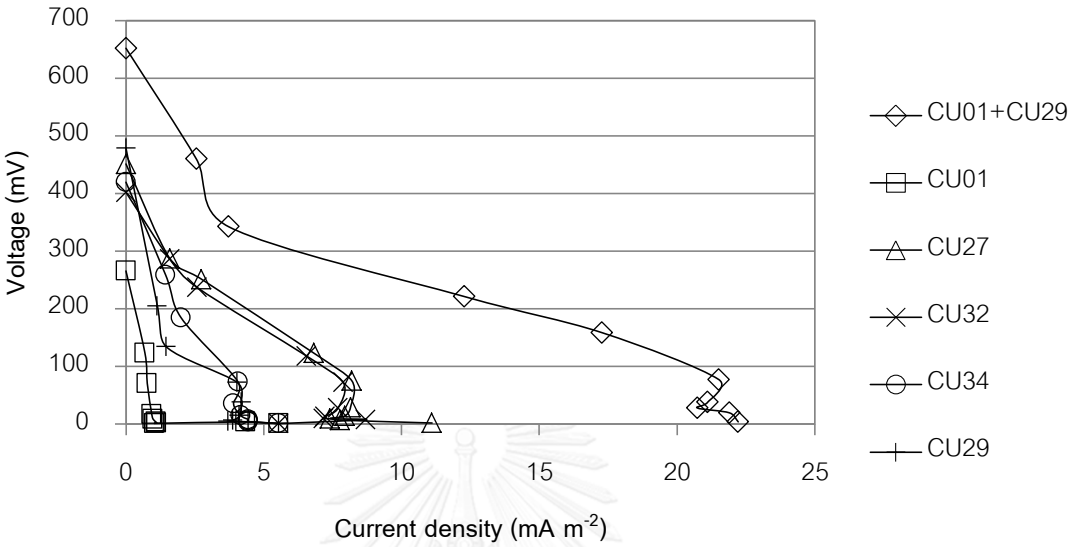

Figure F1 Voltage vs.current density of MFC using different isolates

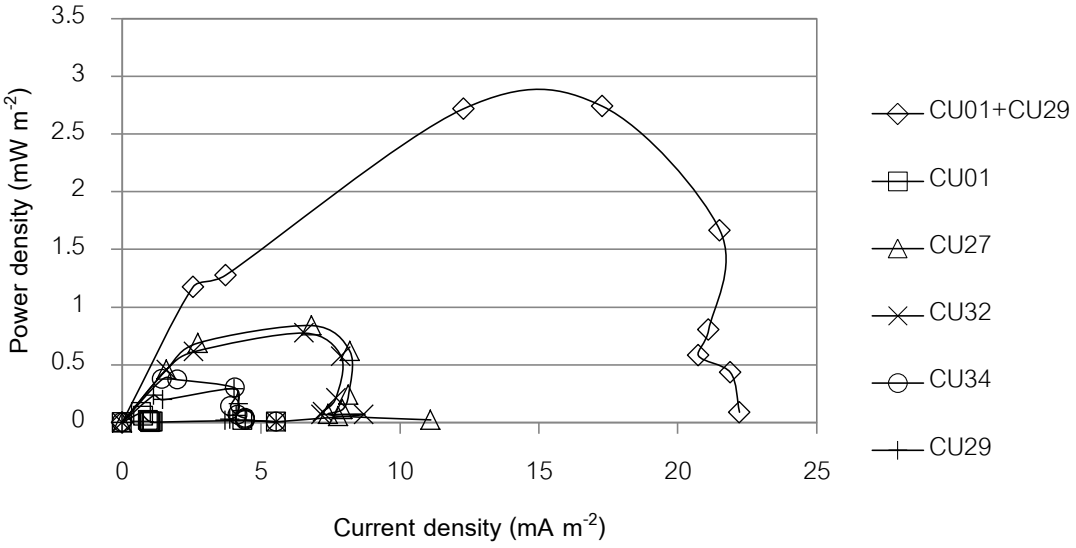

Figure F2 Power density vs.current density of MFC using different isolates

VITA

Mr. Kamol Rodyou was born in February 21, 1983, Bangkok, Thailand. He graduated from Department of Microbiology, Faculty of Science, Chulalongkorn University in 2005 with the Bachelor degree of Science (Microbiology) and Master degree of Science (Industrial Microbiology) in 2008. Recently, he has pursued for Doctoral degree of Science (Program in Biotechnology) from the same institute and expected to finish by the academic year of 2015.

Scientific Presentation

Kamol Rodyou, Mana Sriyudthsak and Sirirat Rengpipat. Electricity production in microbial fuel cell containing bacteria isolated from sub-sediment from Ko Lan, Thailand. Poster presenting in 18th Biological Sciences Graduate Congress (BSGC), 6-8th January 2014, University of Malaya, Kuala Lumpur, Malaysia.