

Coupling Indonesian indigenous *Citrobacter freundii* and *Chlorella pyrenoidosa* strain on the anode of microbial fuel cell with various substrates

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Manuscript received: 6 February 2022. Revision accepted: 23 April 2022.

Abstract. Fauzan IA, Meryandini A, Ridwan R, Fidriyanto R, Agustini NWS, Santosa DA. 2022. Coupling Indonesian indigenous *Citrobacter freundii* and *Chlorella pyrenoidosa* strain on the anode of microbial fuel cell with various substrates. *Biodiversitas* 23: 2471-2481. Microorganism plays a crucial role in the development of MFC (Microbial Fuel Cell) systems. Indigenous to Indonesia, *Citrobacter freundii* GBH253 is a potential exoelectrogenic bacterium that could be developed into an MFC system. Coupling *C. freundii* GBH253 with potentially electricity-producing microalgae indigenous to Indonesia, such as *Chlorella pyrenoidosa* INK, in the anode of an MFC, could result in a more stable and higher electricity output. This study used *C. freundii* GBH253 and *C. pyrenoidosa* INK to produce electricity in various substrates. This research was conducted using a Factorial Randomized Block Design and Tukey's test to determine significant differences between treatments. The result shows that electricity was generated in all treatments. The Bacterium-microalgae combination in acetate substrate can generate power density up to 211,97 mW m⁻² and is the most stable compared to others. Bacterium dominates the electricity production in this combination, but the microalgae also play a role in producing electricity and increasing Chemical Oxygen Demand. The pH value of all treatments was higher than 7. Volatile Fatty Acids, like acetate and phenol, were produced in all treatments, whereas butyric acid and propionic acid were produced in several treatments. The Pearson correlation showed that some VFAs are highly correlated with power density.

Keywords: *Chlorella pyrenoidosa* INK, *Citrobacter freundii* GBH253, Indonesian indigenous microorganism, microbial fuel cell, substrate

Abbreviations: MFC: Microbial Fuel Cell; PEM: Proton Exchange Membrane; NBAF: Nutrient Broth Acetate-Fumarate; HSM: High Salt Medium; COD: Chemical Oxygen Demand; CE: Coulombic Efficiency; VFA: Volatile Fatty Acid.

INTRODUCTION

The Microbial Fuel Cell (MFC) is a bio-electrochemical system using microorganisms to generate electricity from different organic substances. In recent years, MFC innovations worldwide have significantly increased since MFC could be an alternative source of electricity in the future. Researchers worldwide are working hard to develop MFCs from several features and components to generate higher and more stable electricity and be more environmentally friendly. For example, in the non-biotic aspect, MFCs have developed in the proton exchange membrane (PEM) and chamber. Poly (4-styrene sulfone acid)/bacterial PEM membrane was designed to replace PEM with an affordable and environmentally friendly membrane (Vilela et al. 2020). Besides, Sulfonated Polyether Ether Ketone (SPEEK) has also been developed to get a more affordable membrane in MFC (Permana et al.

2018). Furthermore, supercapacitor paper for the chamber has been designed to replace plastic or acrylic to obtain a more environmentally-friendly MFCs system (Santoro et al. 2019). Several studies have also created MFCs by integrating them with diverse techniques, such as the drop-hydroponic system (Yadav et al. 2020). Furthermore, they have tested MFCs in harsh environments, such as the eutrophicated coastal sea (Kubota et al. 2019) and Indonesian local agriculture soil (Mulyono et al. 2020). MFC has also been developed by optimizing various waste and toxic compounds as carbon sources, such as human urine (Ozdemir et al. 2019), rice mill wastewater (Raychaudhuri and Behera 2020), food waste (Hou et al. 2020), oil refinery wastewater (Meshram and Jadhav 2019), and wastewater treatment plant sludge (Passos et al. 2016).

In addition to the strategies mentioned above, the development of MFCs by considering the microorganisms

is also critical. Microorganisms in the dual-chamber MFC systems are usually present in the anode. Microorganisms play an essential role in the MFC system, which transfers electrons from organic materials to the cathode (Xu and Liu 2011). The transfer of electrons from the anode to the cathode generates an electric current. Many potential microorganisms are used in the anode of MFC, with the most used being bacteria, especially exoelectrogen bacteria. Exoelectrogen bacteria can transfer electrons extracellularly after gaining them from the substrate (Mishra 2017). One of the most potent and viable exoelectrogen bacteria for the MFC system is *Citrobacter* sp. They could create power in a wide range of substrates (Huang et al. 2015), including citrate (Xu et al. 2015), acetate (Venkidusamy et al. 2018), and glucose (Matsena et al. 2020), glycerol (Sultana et al. 2019). *Citrobacter freundii* GBH253, which belongs to the genus *Citrobacter*, is a potential exoelectrogenic bacterium for the MFC system. *Citrobacter freundii* GBH253 is an indigenous bacterium isolated from hydrocarbon-contaminated sediment in Bunyu Island, North Kalimantan, Indonesia. *Citrobacter freundii* GBH253 has a thin cell wall that facilitates the transfer of electrons through the cell membrane with c-type cytochrome protein and capable of generating a power density up to 15.93 mW m⁻² but not stable during incubation (Hasanah 2020). The MFC system with *C. freundii* GBH253 should be developed to obtain higher and more stable electricity. The most effective way to develop MFC with *C. freundii* GBH253 is by combining it with another electricity-generating microorganism, such as potentially electricity-generating microalgae species.

In the MFC system, Microalgae are usually used at the cathode as a biocathode due to their ability to produce oxygen, one of the best electron acceptors (Sevda et al. 2019). Several studies showed that microalgae could be placed on the anode of MFC and help exoelectrogen bacteria to generate electricity. For example, a previous study by Nishio et al. (2013) reported that *Chlamydomonas* sp. could be coupled with *Geobacter* sp. in the anode of the MFC system and help *Geobacter* sp. in the MFC system by providing organic compounds via photosynthesis. However, the microalgae could not generate electricity; therefore, microalgae needed bacterium to enhance their electrical power output. Coupling electricity-generating microalgae species could also help the exoelectrogen bacterium in the MFC system by assisting them with organic compounds and generating electricity along with the bacterium. Unfortunately, only a few microalgae species can produce electricity, and one of them is *Chlorella pyrenoidosa*.

Chlorella pyrenoidosa, as a pure culture, could generate electricity in the anode of MFC. Although it is not fully known, electron leakage from mitochondria is highly suspected as the primary factor for the electrical production of *C. pyrenoidosa* in MFC (Xu et al. 2015). *Chlorella pyrenoidosa* strain INK, isolated from a freshwater pond in Cianjur, West Java -Indonesia (Kusmiati et al. 2020), can be combined with *C. freundii* GBH253 to generate electricity. In this study, *C. freundii* GBH253 and *C. pyrenoidosa* INK were coupled together in the anode of

dual-chamber MFC to determine their capability to co-producing electricity and aid each other. Several substrates were used to evaluate the best substrate for producing electricity from *C. freundii* GBH253, *C. pyrenoidosa* INK, and the combination of the two. This study also aimed to find out further about *C. freundii* GBH253 and *C. pyrenoidosa* INK metabolism in each substrate after being incubated in the MFC chamber, as indicated by their volatile fatty acid production (VFA).

MATERIALS AND METHODS

Preparation of chemical and microorganism cultivation

Chemicals

Media used in this study were high salt medium (for microalgae cultivation), NBAF (Nutrient Broth Acetate-Fumarate) medium (for bacterium cultivation), and NBAF modified medium (as anolyte in MFC chamber). The composition of the high salt medium per liter of deionized water is 5 mL salt solution (100 g L⁻¹ NH₄Cl; 4 g L⁻¹ MgSO₄·7H₂O, 4 g L⁻¹ CaCl₂·2H₂O), 5 mL phosphate solution (288.0 g L⁻¹ K₂HPO₄; 144.0 g L⁻¹ KH₂PO₄), and 1 mL Hutner's trace elements. These chemicals were mixed and added with deionized water to 1 L. The composition NBAF medium composition are 2,5 g L⁻¹ NaHCO₃, 1,5 g L⁻¹ NH₄Cl, 0,6 g L⁻¹ NaH₂PO₄, 0,1 g L⁻¹ KCl, 10 mL Wolfe's mineral solution (3 g L⁻¹ MgSO₄·7H₂O; 1,5 g L⁻¹ C₆H₉NO₆; 1 g L⁻¹ NaCl; 0,5 g L⁻¹ MnSO₄·H₂O; 0,1 g L⁻¹ CaCl₂·2H₂O, FeSO₄·7H₂O, ZnSO₄·7H₂O; and Alk(SO₄)₂·12H₂O, CuSO₄·5H₂O, H₃BO₃, Na₂MoO₄·2H₂O, Na₂SeO₃, NaWO₄·2H₂O, NiCl₂·6H₂O, each 0,01 g/l), 10 mL Wolfe's vitamin solution (0,01 g L⁻¹ pyridoxine.HCl; 0,005 g L⁻¹ p-aminobenzoic acid, lipoic acid, nicotinic acid, riboflavin, thiamin. HCl, calcium DL-pantothenate; 0,002 g L⁻¹ biotin, folic acid; 0,0001 g L⁻¹ cobalamin), 0,82 g L⁻¹ sodium acetate (10 mM, as carbon source) and 8 g L⁻¹ sodium fumarate (10 mM, as electron acceptor). The composition of the NBAF modified medium was similar to the NBAF medium. The difference between the modified NBAF and NBAF was the carbon source compound and its concentration (20 mM for anolyte in MFC) and electron acceptor concentration (20 mM).

Cultivation of microorganisms

Citrobacter freundii GBH253 and *Chlorella pyrenoidosa* INK culture were obtained from the Biotechnology Culture Collection, Research Center for Biotechnology-BRIN (Cibinong, Indonesia). Bacteria colonies were cultivated anaerobically in the NBAF medium that has been flushed out by Oxygen Free-Nitrogen (N₂) and carbon dioxide (CO₂) gas. *Chlorella pyrenoidosa* INK was cultivated in a sterilized bottle with an aerator and 600 Lumens lamp as the light source. Both cultures were cultivated at controlled temperature (30 ± 3 °C) until it reached OD₆₀₀ = 0,5 (for *C. freundii* GBH253) and OD₆₇₀ = 2 (for *C. pyrenoidosa*) prior to use in a microbial fuel cells. The optical density was measured by Shimadzu UV-1800 Spectrophotometer (Shimadzu, Japan).

The growth of microorganisms on different carbon sources

This step was conducted to determine if *C. freundii* GBH253 and *C. pyrenoidosa* INK could grow in NBAF with different carbon sources. The carbon sources used in this step were glucose, citrate, acetate, and glycerol. *Citrobacter freundii* GBH253 and *C. pyrenoidosa* INK were cultivated separately in the test tube (10 mL) sealed with a rubber cap. The medium used in the test was NBAF medium containing different carbon sources mentioned before. The test tube was flushed out with Oxygen Free-Nitrogen (N₂) and carbon dioxide (CO₂) gasses before inoculating the microorganism. Microorganisms were incubated for 120 hours near the T5 lamp, and every 24 hours, OD₆₀₀ (bacterium) and OD₆₇₀ (microalgae) were measured using a Spectronic 21D spectrophotometer.

MFC configuration and preparation

MFC configuration

The MFC chambers used in this study consisted of two identical cube chambers (220 cm³ volume each chamber) separated by a 9 cm² activated Nafion® 117 Proton exchange membrane (PEM) (Lynntech, United States) (Fig 1). PEM was activated by boiling it (100°C) for 60 minutes in 500 mL of deionized water, and after that, PEM was boiled (100°C) for 60 minutes in 500 mL of 3% H₂O₂, and PEM was boiled again (100°C) in 500 mL 1 M H₂SO₄ (Mazzapioda et al. 2019). The electrodes were square and made from carbon fiber (5 cm x 5 cm). The electrodes were placed in the anode and cathode chamber and connected to an external resistor (1000 Ω) through copper wires. A sampling port was designed on the left corner of the anode chamber lid and sealed with a rubber cap to maintain anaerobic conditions.

MFC Preparation

Both anode and cathode chambers were sterilized with alcohol (70%) and placed under a UV sterilization lamp for 30 minutes before being used. The anode chamber was then filled with NBAF modified medium (pH=7) with four different carbon sources (glucose, sodium acetate, sodium citrate, glycerol; each 20 mM) and inoculated with microorganisms (10% (v/v)), whereas the cathode chamber was filled with KMNO₄ (pH=7). Both anode and cathode solution were dissolved in Phosphate-Buffered Saline. The initial volume of the anode and cathode was 180 ml. Nitrogen (N₂) and carbon dioxide (CO₂) gas were used to bubble the anode chamber before the inoculation of the microorganism to get an anaerobic condition. MFCs run at controlled temperature conditions (30 ± 3°C) and near 600 Lumens with T5 lamps as the light source. The cathode was covered with black duct tape to avoid lamplight.

Parameters analysis

Electrochemical analysis

The electrochemical parameters in this study were close-circuit voltages, electric current, power density, and Coulombic efficiency. Close-circuit voltages (V) were

measured by connecting copper wires from the anode and cathode across an external resistor (1000 Ω) and digital multimeter. Data were collected from the 12th hour at 12-hours intervals to ascertain that the voltages resulted from microorganism metabolism and minimized data ambiguity. Voltages were observed until 72 hours, and from the close circuit voltage data, electric current (I) can be calculated by Ohm's Law (eq.1).

$$\text{Electric Current (A)} = V / R_{\text{ext}} \quad (1)$$

Where:

V : close-circuit voltages (volt)

R_{ext} : external resistance (1000 Ω)

The close circuit voltages and electric current were used to determine the power density (P) by using eq. 2

$$\text{Power Density (mW/m}^3\text{)} = V^2 / R_{\text{ext}} \times A \quad (2)$$

Where:

A: surface area of the electrode in the anode (m²)

The Coulombic efficiency (CE) was measured by integrating the electric current relative to the theoretical current based on the COD value. CE could be determined by using eq. 3

$$\text{CE} = (M_s \times I / F \times b \times q \times \Delta\text{COD}) \times 100\% \quad (3)$$

Where:

M : relative molecular mass

I : current density (mA/m²) per hour

F : Faraday's Constanta (96.485 C/mol e⁻)

b : electron per substrate

q : anolyte volumes

ΔCOD : COD changes in some periodic (Logan 2009).

Where M represents relative molecular mass, I represent current density (mA/m²) per hour, F represents Faraday's Constanta (96.485 C/mol e⁻), b represents an electron per substrate, q represents anolyte volumes, and ΔCOD represents COD changes in some periodic (Logan 2009).

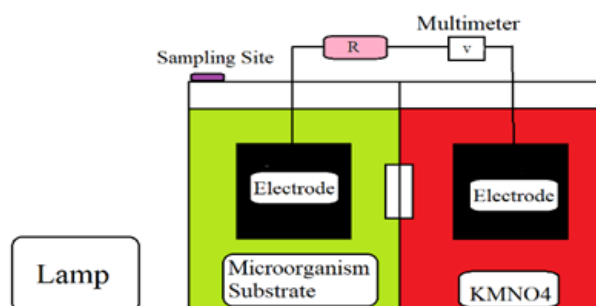


Figure 1. Schematic design of MFC chamber

Chemical analysis

The chemical parameters observed were pH, chemical oxygen demand (COD), and volatile fatty acid. Determination of pH was performed using a digital pH meter (BenchTop Professional pH meter BP3001), and COD was measured using a spectrophotometry method (Shimadzu UV-1800 Spectrophotometer) using 0.25 N $K_2Cr_2O_7$ and 96% H_2SO_4 as reagents at $\lambda = 600$ nm. Volatile fatty acids (VFAs) obtained from MFC sludge at the end of running time were analyzed using a GCMS Shimadzu QP2010 SE comprising AOC 20i+s autosampler and MEGA WAX MS column (length, 30 m; i.d., 0.25mm; and film thickness, 0.25 μ m). Ultra-high purity helium (99.99%) was used as the carrier gas with 3 ml/min flow GCMS condition: split ratio of 50:1 solvent cut time 3 min. The injection, transfer line, and ion source temperatures were 250°C.

Experimental design and data analysis

Experimental design

Randomized Block Design Factorial was used in this study. The first factor is carbon source (glucose, sodium acetate, sodium citrate, glycerol), and the second factor is microorganism combination (bacterium only, microalgae only, bacterium + microalgae); hence there were 12 treatments. Blocks in this study were the repetition of the treatment as described before. There were five blocks in this study, and each block ran for 72 hours.

Data analysis

ANOVA (analysis of variance at 95% confidence level) was conducted to determine any statistically significant differences between treatments. In addition, the Tukey test was conducted for variables that have significant differences in the ANOVA analysis ($p < 0,05$). Minitab 19 (Minitab® Statistical Software) was used for the ANOVA and Tukey's test.

Data label

All data below are described as an average of five replicates. The label for each treatment is B for the bacterium (*C. freundii* GBH253), M for microalgae (*C. pyrenoidosa* INK), and B+M for bacterium and microalgae combination.

RESULTS AND DISCUSSION

Microorganisms growth in various carbon sources

The growth test of *C. freundii* GBH253 and *C. pyrenoidosa* INK on various substrates was carried out to determine whether these bacteria and microalgae could grow on all tested substrates and determine the length of the incubation period that would be used in the MFC system later. The results (Figure 2) showed that these two microorganisms could grow well on all tested carbon sources. This result confirmed the ability of *C. freundii*

GBH253 to grow in a wide range of substrates. In addition, these results also showed that *C. pyrenoidosa* INK was not inhibited by the tested substrate and could potentially use the substrate as an additional carbon source besides photosynthesis.

The growth curve of *C. freundii* GBH253 (fig 2) shows that at 72 hours, this bacterium has already in the stationary phase, while *C. pyrenoidosa* INK has not been in the stationary phase until 120 hours. *Citrobacter freundii* GBH253 reached the stationary phase the fastest in the glucose and glycerol treatment, at 24 hours. Based on this result, the MFC treatment will be carried out for 72 hours because electricity in the MFC system will mostly be produced in the logarithmic phase and decrease in the stationary phase.

Electricity production

Table 1 shows that all treatments significantly differ for all variables except for CE ($p > 0,05$). All treatments could generate voltage throughout the incubation time (Figure 3). The highest voltage was observed in the B+M treatment with glucose as a carbon source (Figure 3c) in the first 12 hours (763,8 mV), then drastically decreased in the next 12 hours and only had a voltage of 113 mV at the end of incubation time. The most stable voltage during incubation time was generated by B+M with acetate as a carbon source (Figure 3b), with the lowest voltage being 528,2 mV at 12 hours of incubation and the highest voltage was 727,4 mV at 26 hours of incubation. This result is in line with the voltage generated from *C. freundii* GBH253 without *C. pyrenoidosa* INK (B-treatment), where the treatment with acetate produced the most stable voltage but decreased within 24 hours of incubation (Figure 3b). By utilizing acetate, *C. freundii* GBH253 could generate 399,8 mV in 12 hours as the highest voltage of B treatment. At the end of incubation time (72 hours), *C. freundii* GBH253 (B) with acetate as a carbon source could generate higher electricity than other media (383 mV). The highest voltage on *C. pyrenoidosa* INK (M treatment) was 400,8 mV on citrate medium (Figure 3a), as the best carbon source for *C. pyrenoidosa* INK to generate electricity.

All treatments showed a decrease in voltage as increasing incubation until 72 hours, except for treatment of B+M with acetate. (Figure 3) The reduction of voltage can be caused by the depletion of the carbon source during the incubation time, as indicated by the decrease in COD (Figure 4). In M and B+M treatments, the COD was decreased throughout the incubation time, although it increased at some point. Microalgae could increase COD due to COD increase in effluent through photosynthesis (Ramaraj et al. 2016). An increase in COD was also observed in the B+M treatment. The rise in COD of the bacterium and microalgae combination could happen because microalgae fix the CO_2 from the air and convert it into soluble carbon, causing an increase in COD in the effluent (Ramaraj et al. 2015).

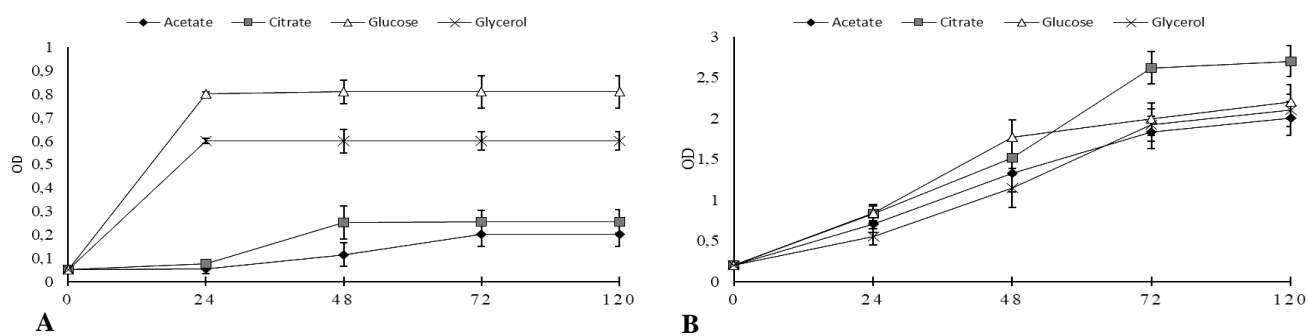


Figure 2. Growth curve of *Citrobacter freundii* GBH253 (A) and *Chlorella pyrenoidosa* INK (B) on various carbon sources | ◆: acetate; ■: citrate; △: glucose; x: glycerol

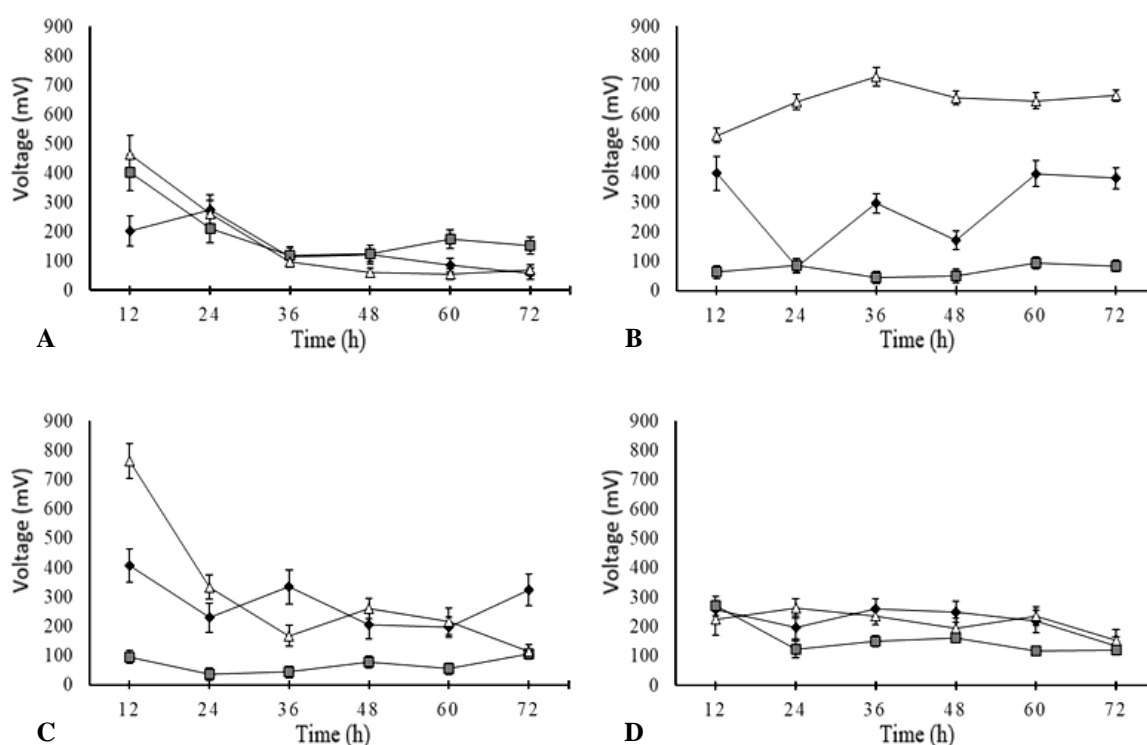


Figure 3. The voltage on different substrates: A. Citrate, B. Acetate, C. Glucose, D. Glycerol | ◆: bacterium; ■: microalgae; △: bacterium+microalgae

Table 1. The effect of treatment combination between carbon source and microbes on voltage, power density, pH, COD, and CE

Treatments	Variables				
	Voltage (mV)	Power Density (mW m ⁻²)	pH	COD (mg L ⁻¹)	Coulombic Efficiency (%)
B-Citrate	142,5 ^{de}	13,57 ^{cd}	7,89 ^a	1422,7 ^{bc}	4,83
B-Glucose	283,4 ^{bc}	38,02 ^{bcd}	7,24 ^c	1554,6 ^{abc}	17,8
B-Acetate	288,2 ^{bc}	48,2 ^{bc}	7,7 ^a	1366,9 ^c	7,17
B-Glycerol	220,1 ^{bcd}	30,52 ^{cd}	7,62 ^{ab}	1463,1 ^{abc}	7,83
M-Citrate	196,9 ^{bcd}	21,23 ^{cd}	7,71 ^a	1642,6 ^{abc}	7,17
M-Glucose	69,5 ^e	4,25 ^d	7,34 ^{bc}	1826 ^a	11,5
M-Acetate	70,23 ^e	2,9 ^d	7,8 ^a	1484,6 ^{abc}	1,8
M-Glycerol	157 ^{de}	13,16 ^{cd}	7,61 ^{ab}	1761,3 ^{ab}	8,83
B+M-Citrate	167,9 ^{cde}	23,24 ^{cd}	7,75 ^a	1636,7 ^{abc}	7,33
B+M-Glucose	309,5 ^b	70,4 ^b	7,26 ^c	1714,3 ^{abc}	13,17
B+M-Acetate	644,3 ^a	168,01 ^a	7,79 ^a	1578,6 ^{abc}	16,5
B+M-Glycerol	217,9 ^{bcd}	21,44 ^{cd}	7,66 ^{ab}	1708,4 ^{abc}	7,67
P-Value	<0,05	<0,05	<0,05	<0,05	0,865

Note: a, b, c, d Superscripts show significant differences (p<0,05).

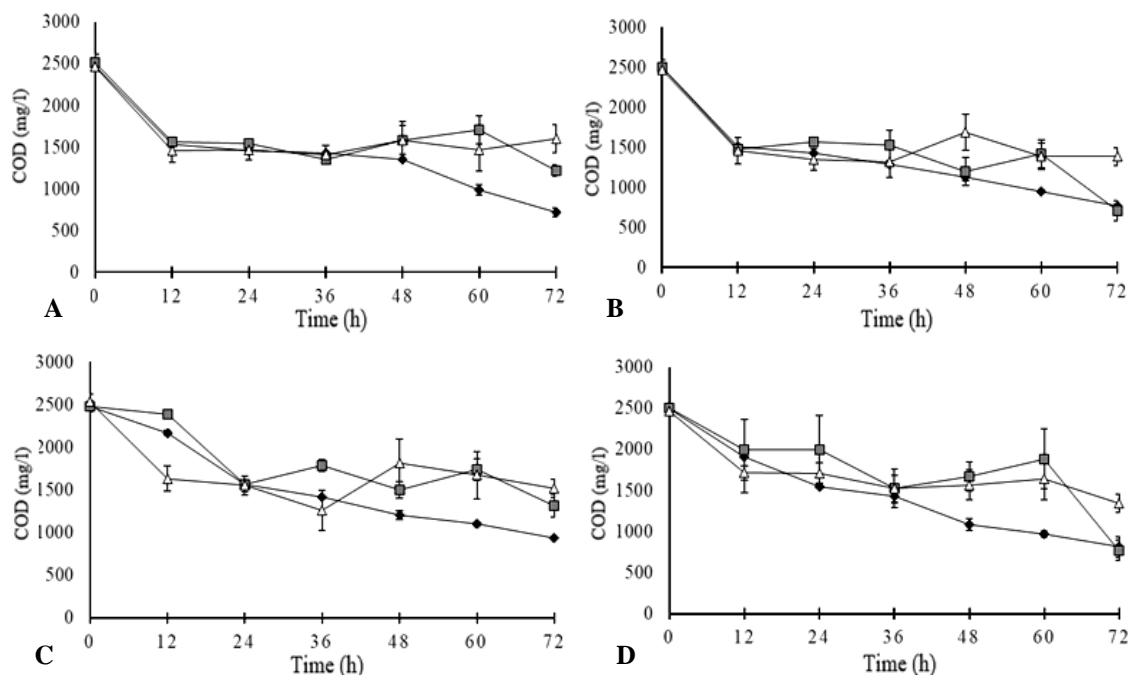


Figure 4. COD on different substrates: A. Citrate, B. Acetate, C. Glucose, D. Glycerol | ◆: bacterium; ■: microalgae; △: bacterium+microalgae

The highest power density was generated from B+M-glucose (243,73 mW m⁻²) (Figure 5a). On the other hand, the highest average power density, which means the most stable along incubation time, was generated on B+M with acetate (168,01 mW m⁻²) (Figure 5b). The highest average power density from the B treatment was also developed in the acetate medium (48,18 mW m⁻²). Meanwhile, the citrate medium produced the highest average power density in the M treatment (21,23 mW m⁻²). However, the lowest average power density in B treatment was generated in the citrate medium (21,23 mW m⁻²), and the lowest average power density in M treatment was in the acetate medium (13,57 mW m⁻²) (Figure 5b). Table 1. showed that the highest voltage and power density was obtained from the combination treatment of *C. freundii* GBH253, *C. pyrenoidosa* INK, and acetate (B+M-acetate), and it was significantly different ($p < 0,05$) from others. In addition, *C. freundii* GBH253 with acetate as carbon source (B-acetate) produced significantly higher voltage and power density ($p < 0,05$) compared to *C. pyrenoidosa* INK with acetate (M-acetate).

These results showed contradictory results for *C. freundii* GBH253 and *C. pyrenoidosa* INK in different carbon sources treatment, especially acetate and citrate. *C. freundii* GBH253 could generate higher power density with acetate because acetate was a low molecular compound and the end product of several metabolic pathways. Therefore, acetate is hardly diverted to alternative microbial conversions at room temperature (Das 2018). As a result, *C. freundii* GBH253 could metabolize acetate more into electricity than other metabolic processes and enhance electricity production. Table 1. showed that B-acetate treatment had the lowest COD of other treatments, indicating that acetate could easily be decomposed for electricity production by *C. freundii* GBH253 rather than

other compounds. *Citrobacter freundii* GBH253 could also generate high electricity because acetate was the only sugar that could not be fermented in this study. As the sole carbon source for the bacterium, acetate would be assimilated into acetyl-CoA, essential for the TCA cycle (Kim et al. 2012). Therefore, the metabolism of *C. freundii* GBH253 is more likely to be oxidatively phosphorylative than fermentative. In this type of metabolism, bacteria can transfer and store electrons in their electron acceptors, i.e., KMNO₄, in the cathode through electrodes in the anode. The flow of electrons from the anode to the cathode generates current and voltage, which are then converted into electricity in the MFC system (Kumara Behera and Varma 2016). In the fermentative metabolism, the electricity generated in the MFC system tends to be lower because the electrons reside in the fermentation product (Kumar et al. 2015).

High power density generated by *C. pyrenoidosa* INK in citrate treatment could be caused by microalgae's ability to convert citrate to speed up their metabolism, driving faster cell growth rather than converting it into lipid. Several studies have proved these phenomena. A previous study by Marudhupandi et al. (2014) showed that increasing citrate concentration caused decreasing total lipid content in microalgae, but the carbohydrate content was increased up to 29,43%. Meanwhile, the low electricity from the acetate substrate by *C. pyrenoidosa* INK in this study happened because microalgae were more likely to produce lipid and slow down metabolism when there was acetate as a carbon source. *Chlorella* sp. utilize acetate to accumulate fatty acids in their cell wall (Leyva et al. 2014). Due to *C. pyrenoidosa* INK producing more lipids, the number of microalgae cells in the acetate treatment was lower than in the other treatments. This condition could result in lower electricity generated by the acetate-microalgae treatment.

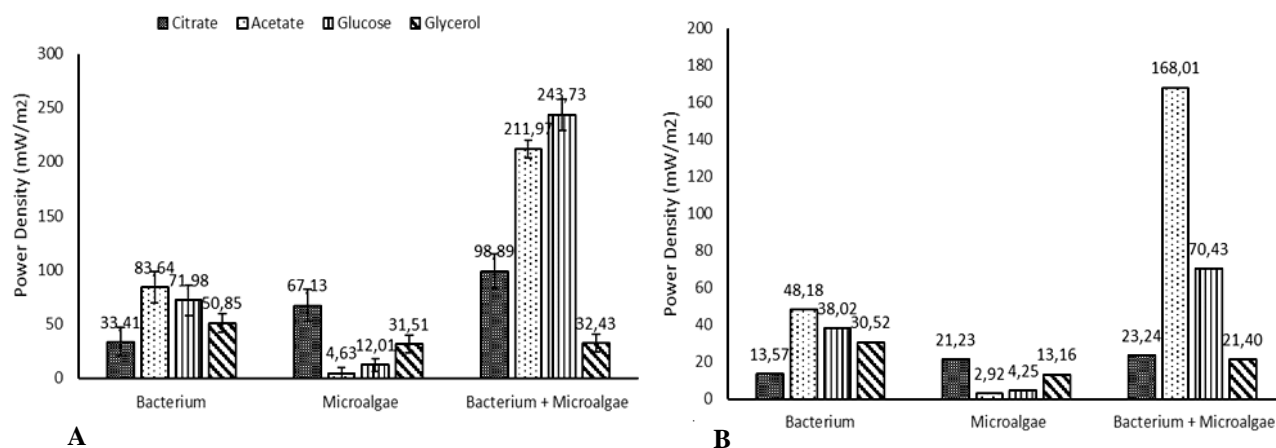


Figure 5. Maximum power density (A) and average power density (B) at 72 hours of incubation

The average power density from the B+M treatment had a similar trend with the B treatment, but the opposite with the M treatment. This result indicates that the bacterium dominated electricity production from *C. freundii* GBH253 and *C. pyrenoidosa* INK. It might be caused by bacterium's faster and more efficient ability to utilize substrate than microalgae. On the other hand, the energy source of *C. pyrenoidosa* INK probably depends on photosynthesis than substrate consumption. Therefore, based on this character, *C. pyrenoidosa* INK can be categorized as an autotrophic microorganism. However, *C. pyrenoidosa* INK still benefits the bacterium in this symbiosis because B+M treatment has higher and more stable voltage and power density than bacterium alone.

As previously mentioned, microalgae could provide an alternative carbon source from photosynthesis for the bacterium (Kazamia et al. 2012). *Chlorella pyrenoidosa* INK can provide alternative carbon sources for *C. freundii* GBH253. The bacterium used it as an additional carbon source to grow and produce more electricity when the initial carbon source decreased. *Chlorella pyrenoidosa* INK also donated electrons from their cell due to photosynthesis and substrate breakdown. The COD result of this study might prove this theory (Figure 3). The COD value of B+M treatment increased up to 48 hours in citrate, acetate, and glucose treatment, and the COD value increased at 60 hours in glycerol treatment. The COD value increased at some point, indicating the addition of substrate to the medium. Interestingly, the CE value (Figure 6) was stable when COD was increased on acetate, glycerol, and glucose treatment. It indicated that the soluble carbon source from *C. pyrenoidosa* INK could also be used for electricity production.

Coloumbic efficiency (CE)

Table 1 showed that CE was not significantly different in all treatments ($p > 0,05$). However, CE decreased in all treatments with increasing incubation time, and CE was low at the end of incubation time (Figure 5). This result is relatively in line with electricity generation (except for B+M-acetate) and COD results.

The highest CE was in the B-glucose treatment (84%), which means only 16% or fewer electrons of substrate consumption were used for biomass or other metabolic compounds production. CE on B+M is mostly higher and more stable than B and M treatment in all substrates. A small percentage of CE indicates that substrates were likely converted into metabolic compounds for bacterium and microalgae rather than electricity. Substrates could also be transformed into mediator compounds, used in electricity, or lost through the extracellular membrane (Logan 2009).

pH value in MFC effluent

Another indicator for electricity generation was the increase in pH (Figure 7). The rise in pH value was observed in all treatments, although there was a slight decline in the glucose at 12 and 24 hours incubation time probably caused by fermentation. The increase of pH in MFCs could affect the MFC system to generate higher electricity (Puig et al. 2010). Proton accumulation in an anode due to electrons transferred to the cathode can cause an increase in pH in MFC (Ortiz-Martínez et al. 2015).

Volatile fatty acids production

Volatile fatty acids (VFAs) analyses showed that acetate and phenol were produced in all treatments. In contrast, propionic acid was not produced in B-citrate, B-glycerol, M-acetate, and Butyric acid, but only produced by B-glucose and B+M-glucose (Figure 8). Acetic acid was the most abundant VFA produced from all treatments because MFC effluent was alkaline at the end of incubation. Alkaline conditions ($pH > 7$) could enhance phosphate acetyltransferase (PTA) and acetate kinase (AK) enzymes, which were more active in alkaline conditions (Yang et al. 2015). These enzymes were responsible for acetate production so that the production of acetate becomes the dominant VFA. PTA and AK were also responsible for the breakdown of acetate into other compounds for metabolism (Ingram-Smith et al. 2006). High activity of PTA and AK could help *C. freundii* GBH253 break down acetate rapidly, which probably led to increased and stable electricity in this study.

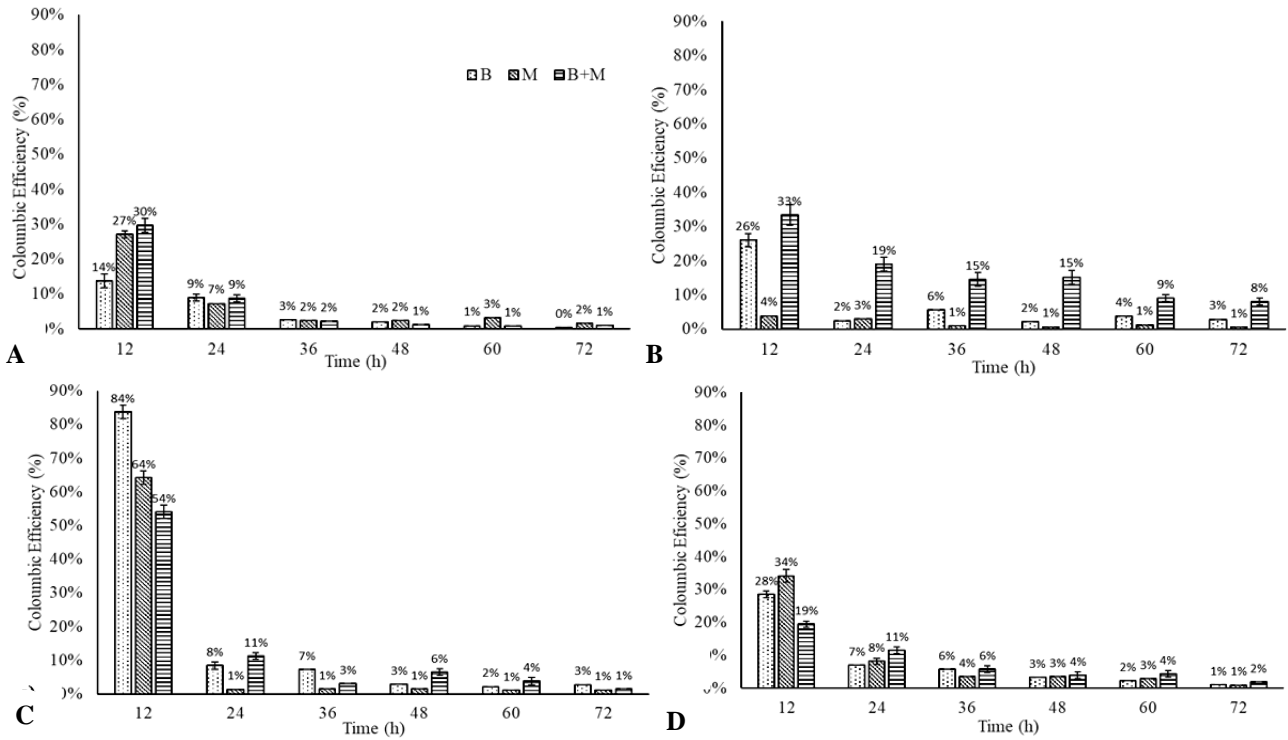


Figure 6. Coulombic Efficiency (CE) on various substrates: A. Citrate, B. Acetate, C. Glucose, D. Glycerol. Note: B: Bacterium, M: Microalgae, B+M: Bacterium + Microalgae

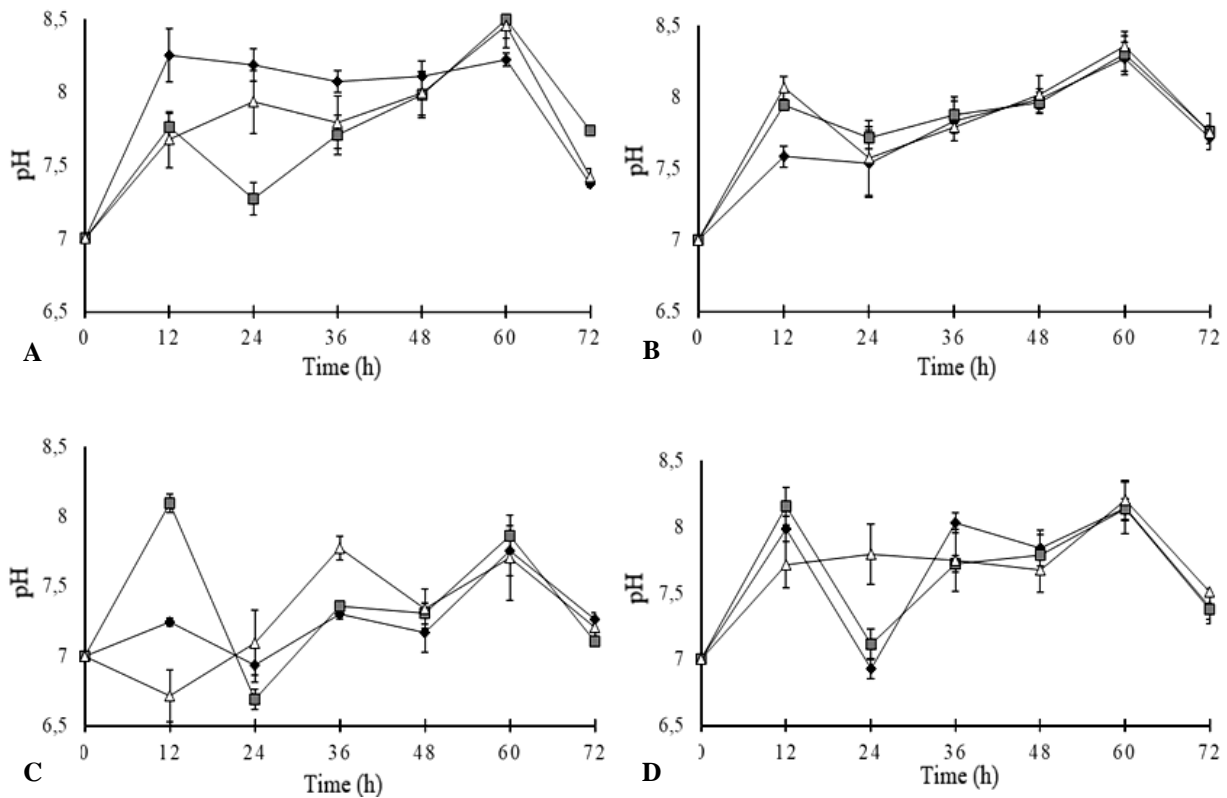


Figure 7. The pH value on various substrates: A. Citrate, B. Acetate, C. Glucose, D. Glycerol. ◆: bacterium; ■: microalgae; △: bacterium+microalgae

Propionic acid was produced almost in every treatment, except B-citrate, B-glycerol, and M-acetate. Propionic acid was a secondary metabolite produced from acetyl-CoA, an essential compound for the TCA cycle. *Citrobacter freundii* GBH253 could use citrate directly into the TCA cycle, which later converts into acetate due to PTA and AK activity explained before. The use of citrate by *C. freundii* GBH253 caused an absence of propionic acid in B-citrate. In B-glycerol treatment, *C. freundii* GBH253 would convert glycerol into pyruvate, which is crucial for the TCA cycle (da Silva et al. 2009). Pyruvate might also be converted into acetyl-Coa and further into pyruvate rather than propionic acid. M-acetate treatment also did not produce propionic acid. In acetate medium, microalgae were more likely to form lipid in their cell wall and speed down their metabolism.

Butyric acid was only produced in B-glucose and B+M-glucose in small amounts, i.e., 1,95 and 2,81 mM, respectively. Bacteria can use simple sugar, such as glucose, to produce various VFA because sugar can be easily used in many metabolic pathways. Acidification in the MFC medium at 24-36 hours incubation time could enhance butyric acid production by *C. freundii* GBH253. Butyric acid was produced due to the presence of the enzymes phosphate butyryltransferase (PTB) and butyrate kinase (BK), which were active in an acidic medium (Feng et al. 2009). The presence of glucose in acidic conditions (24-36 hours) caused the formation of butyric acid and was used by *C. freundii* GBH253 for another metabolic process.

Based on the results of the Pearson correlation (Table 2), showed propionic acid has the highest correlation value with the power density (0,811), which means that the higher the propionic acid produced by *C. freundii* GBH253 in MFC, the higher the power density produced. Therefore, *C. freundii* GBH253 could produce high power density by

using compounds that can be converted into propionic acid. Lactate was the potential substrate because lactate could be converted into propionic acid by *C. freundii* GBH253.

and 0,947, respectively) (Table 2). Like *C. freundii* GBH253, *C. pyrenoidosa* INK could produce high power density by using a substrate converted into acetate and phenol. Citrate can increase the production of acetate and phenol in *C. pyrenoidosa* metabolism. Acetate and phenol were produced from compounds in the TCA cycle, so by increasing the speed of the TCA cycle, the electricity produced by *C. pyrenoidosa* would be higher. The highest correlation of power density in B+M treatment was phenol (correlation value -0,765) (Table 2). A negative value in a Pearson correlation means that two parameters were reversely correlated. The higher power density generated in *C. freundii* GBH253 and *C. pyrenoidosa* community, the lower phenol concentration in the MFC sludge. It means that phenol could be used by *C. freundii* GBH253 and *C. pyrenoidosa* INK as a potential carbon source in the MFC system to get a high power density. Acetic acid and phenol were highly correlated with power density in the microalgae treatment (correlation values 0,821

Table 2. Correlation Matrix for power density and VFA fermentation

VFA	Correlation with Power Density		
	Bacterium (B)	Microalgae (M)	Bacterium+Microalgae (B+M)
Acetic acid	0.057	0.821	-0.295
Propionic acid	0.811	0.079	-0.461
Butyric acid	0.228	*	-0.340
Phenol	0.247	0.947	-0.765

Note: *: not detected.

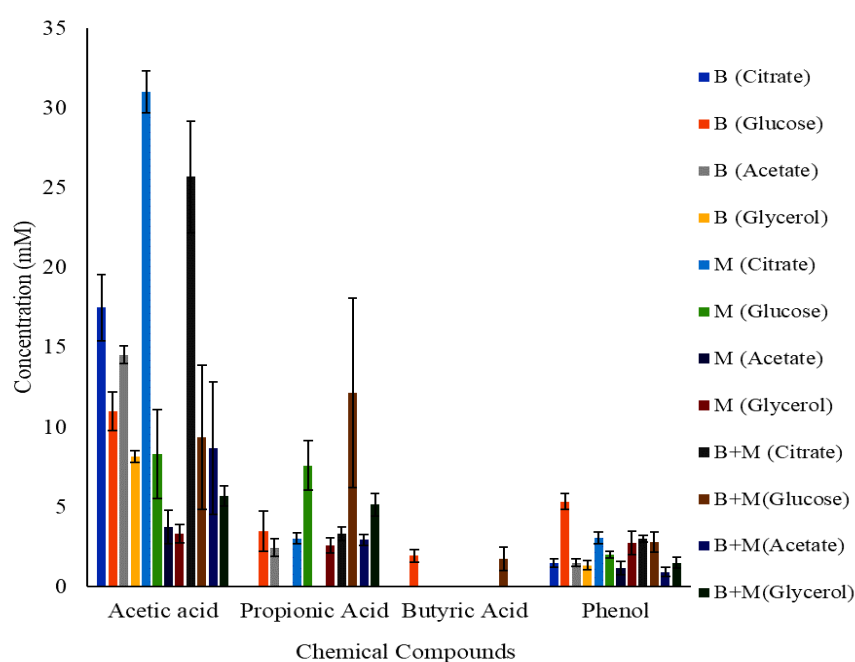


Figure 8. Volatile Fatty Acids (VFAs) concentration in different treatments

Discussion

The results showed that *C. freundii* GBH253 was compatible with *C. pyrenoidosa* INK to generate electricity in the MFC system. The interaction between the two microorganisms had a beneficial impact on the bacterium because microalgae provide dissolved carbon and possibly other essential molecules, making it possible for the bacterium to proliferate more efficiently. It was indicated by the higher COD value in the bacterium and microalgae combination than in the bacterium without microalgae. The increase in COD value proves that the carbon source in the medium increases due to the photosynthesis by microalgae.

The acetate as a carbon source resulted in significantly higher power density and voltage than other treatments in the MFC system. Because acetate cannot be fermented, the breakdown process of acetate was more focused on bacterial growth than on producing other metabolites due to the inability of acetate to be fermented. It results in a higher amount of power generated than other carbon sources. The inability of acetate to be fermented results in an alkaline pH, which is an indicator of the ability of microorganisms to generate electricity. It could be seen in the acetate treatment with the bacterium and bacterium + microalgae, which have higher pH. The CE parameter showed that the bacterium and bacterium + microalgae with acetate as a carbon source had the most stable CE during the incubation time than other carbon sources, although they were not significantly different. A decrease in CE value in all treatments was due to the depletion of carbon sources, as indicated by decreasing COD values.

The results of the PVAs analysis showed that acetate was present until the end of the incubation time in the treatment of the bacterium and the bacterium + microalgae in the acetate medium. The acetate residue in the bacterium and bacterium + microalgae treatments were 14.54 mM and 8.68 mM, respectively. These results indicate that *C. freundii* GBH253 and *C. pyrenoidosa* have not fully utilized acetate during 72 hours of incubation. The presence of residual acetate during bacterial incubation before inoculation on the MFC anode can also induce this. Before being inoculated on the MFC anode, the bacterium was incubated in the acetate medium at 20 mM acetate concentration. After reaching the stationary phase (OD = 0.5), the bacterium was cultured at 10% (v/v) in MFC medium. That method indicates that the acetate in the MFC medium could have an additional concentration up to 1.8 mM, but more likely lower than that because acetate was used in the incubation process. The remaining acetate in the bacterium + microalgae treatment was less than in the bacterium treatment; it indicates that *C. pyrenoidosa* INK also utilized acetate. In addition, these results also suggest that *C. freundii* GBH253 grew better in combination treatment because of the increased consumption rate of acetate, which correlated with the number of bacterial cells.

The results of this study can be used as a basis for further research on the utilization of *C. freundii* GBH253 and *C. pyrenoidosa* INK, which are indigenous electricity-producing microorganisms from Indonesia. In addition, acetate, which is the best substrate in this study, can be used as a basis for future research and development of the

MFC system. The development of this research can be done by applying a combination of these two microorganisms to waste containing acetate, such as industrial pineapple waste. In addition, this system can also be applied to other wastes containing citrate, glucose, and glycerol because it has been proven that these two microorganisms can generate electricity with these carbon sources. A fed-batch system also needs to be done to get more stable electricity.

In conclusion, *C. freundii* GBH253 and *C. pyrenoidosa* INK could grow together in the NBAF medium, creating favorable conditions for the bacterium. As a result, electricity could be generated in all MFC treatments, although the incubation time decreases as the substrate decomposes. The best treatment is *C. freundii* GBH253 combined with *C. pyrenoidosa* INK with acetate as a carbon source, which could generate maximum power density up to 211,97 mW m⁻² and almost generated steady electricity during the incubation time and is significantly different than other treatments. The electricity production from *C. freundii* GBH253 and *C. pyrenoidosa* INK combination is probably dominated by the bacterium; however, microalgae also produce electricity and help increase COD to promote bacterial growth. Due to the metabolism of the bacterium and microalgae at high pH, acetate and phenol were the only VFA produced in all treatments.

ACKNOWLEDGEMENTS

The authors are grateful to Research Center for Biotechnology, the Research Organization for Life Sciences-National Research and Innovation Agency (BRIN), and the Indonesian Center of Biodiversity and Biotechnology (ICBB) for conducting and finishing this research. In addition, the authors acknowledge the scientific and technical support and the permission to use the facilities in Characterization Biotechnology Laboratories, National Research, and Innovation Agency through E- Layanan Sains, Badan Riset dan Inovasi Nasional, Indonesia.

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