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Isolation and Electrochemical Evaluation of Electrogenic Bacteria from the Sediment of Two Waterlogged-Ecosystems

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Abstract

Electrogenic microbes have been exploited in the microbial fuel cell (MFC) system for harvesting bioelectricity. Electrogens are widely distributed in various environments, but the exploration of this microbial group from ecosystems in Indonesia is still limited. This present study aimed to isolate the electrogenic bacteria from sediments of two waterlogged ecosystems (dam and paddy field) using serial dilution into extinction method prior to streak plate method on the solid thioglycollate media supplemented with Fe^{3+}. Electrochemical evaluation was conducted in glucose-fed dual-chamber MFC by using Arduino UNO-based data logger for the accurate monitoring of electricity production in the term of open circuit voltage (OCV). A total of 54 electrogens were successfully isolated from these two ecosystems, ranging from weakness to strongest electrogens (OCV >800 mV) and ranging from microaerophilic, aerotolerant and facultative anaerobes, to obligate anaerobes. This result also suggested that sediment of waterlogged ecosystems rich in electron donor and solid acceptor electron compounds could potentially host electrogenic microbes. The exploration of electrogens from many other waterlogged ecosystems in Indonesia, both natural and anthropic ecosystems, could be conducted to collect genetic resources of novel electrogenic bacteria for the development of MFC technology in Indonesia.

Keywords: Bioelectricity, electron acceptor, electron donor, microbial fuel cell, thioglycollate media

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Introduction

Living organisms depend on electron flow for various cellular processes that generate energy. A continuous flow of electrons is essential to establish an electrochemical gradient within the cell that allows for the synthesis of ATP. Microbes obtain energy through respiration by catalyzing redox reactions or degrading organic and inorganic compounds. Electrons generated from substrate degradation will be captured by NADH (nicotinamide adenine dinucleotide), $FADH_2$ (flavin adenine dinucleotide), and other mobile electron carrier compounds (Mohan et al., 2019). Some microbial groups can transfer electrons outside their cells and they have been utilized in various microbial electrochemical technology (MET) systems, one of which is for microbial fuel cells.

Microbial fuel cell (MFC) is a microbial-based fuel cell that utilizes microorganisms as biocatalysts to convert chemical energy into electrical energy through catalytic processes (Logan *et al*., 2006). MFC generally consists of an anodic chamber and a

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cathodic chamber separated by a membrane (i.e. proton exchange membrane). In the anodic chamber, anaerobic degradation of organic substrates takes place so that carbon dioxide, protons, and electrons are produced and then transferred to the outside of the microbial cell (Lovley, 2006). The protons generated will diffuse into the cathodic chamber through the proton exchange membrane. At the same time, electrons will flow through the external electrical circuit (connecting the anode electrode and cathode electrode) into the cathodic chamber (Rabaey & Verstraete, 2005). This flow of electrons can be harvested as electricity from the MFC system. Studies on MFC technology are increasing along with the awareness toward environmental issues, such as the need for eco-friendly wastewater treatment (Gude, 2016), renewable energy and electricity generation (Rahimnejad et al., 2015), bioremediation (Kubota et al., 2019), polluted-soil detoxification (Rodrigo et al., 2014), and biosensors (Adekunle et al., 2021).

The microbial groups commonly exploited in MFC technology are electrogen or electrogenic microbes, also known as electroactive (Sydow *et al.,* 2014) or electrochemically active microorganisms (Chang *et al*., 2005). Electrogenic microbes are known to be widely distributed in various ecosystems. Knowledge of the ecology that hosts electrogen is important for the development of MFC technology (Sreelekshmy, 2020). Various ecosystems have been explored so far to obtain electrogen, both natural and anthropic ecosystems, including extreme ecosystems (Chabert *et al*., 2015). However, until now there has not been much exploration of electrogenic microbes from ecosystems in Indonesia. Some electrogens that have been isolated from ecosystems in Indonesia include *Aeromonas salmonicida*, *Pseudomonas* sp., *Vibrio gazogenes*, *Photobacterium lipolyticum*, and *Salinivibrio siamensis* isolated from mangrove sediments (Wiryawan *et al*., 2014), *Aeromonas hydrophila*, *Acinetobacter* sp. and *Bacillus marinus* isolated from marine sediments (Riyanto *et al.*, 2011), *Micrococcus* sp. ICBB9556 and *Acinetobacter baumannii* ICBB9557 isolated from detritus sediments (Indriyani, 2017), *Staphylococcus saprophyticus* ICBB9554 isolated from paddy field sediments (Indriyani, 2017), *Citrobacter freundii* ICBB9763 isolated from petroleum-contaminated soils (Hasanah, 2020), and *Bacillus altitudinis* AC11.2 isolated from aquaculture pond sediments (Indriyani *et al.*, 2024c).

Some MFC studies in Indonesia focused on the use of laboratory culture collection or bacterial culture that have previously been identified as electrogenic microbes, as well as the use of bacterial consortia from various sediments or sewage sludge (Indriyani, 2023). However, the utilization of bacterial consortia is often without further isolation and electrochemical analysis on single isolates within the consortium to distinguish between electrogen and non-electrogen isolates. The use of pure bacterial culture as a biocatalyst is crucial for the study and development of MFC technology, since the use of bacterial consortium has several weaknesses, such as the aspect of reproducibility and the presence of non-electrogenic microbes which have the potential to act as competitors in the use of substrates, thus affecting the performances of MFC.

Indonesia is one of the countries in the tropics with high biodiversity (mega-biodiversity), possessing various waterlogged ecosystems where sediments are rich in nutrients and electron acceptors, the potential ecosystems as habitats for electrogenic microbial groups. Hence, this present study aimed to isolate electrogenic bacteria from the sediments of two waterlogged ecosystems in Indonesia that potentially host electrogene, namely the dam ecosystem (Waduk Saguling) and reductive paddy field ecosystem in Lebak, Banten.

Material and Methods

Sediment Samples

Sediment samples used for the isolation of electrogenic bacteria were the sediment of Saguling dam ecosystem located in Galanggang village, Batujajar subdistrict, Bandung, West Java, Indonesia (6.91ºS and 107.49ºE), and the reductive sediment of paddy field ecosystem located in Pasirtangkil village, Warunggunung subdistrict, Lebak, Banten, Indonesia (6.33ºS and 106.14ºE) (Figure 1). Sediment samples were taken in

August and October 2022 for dam sediment and paddy field sediment, respectively. These sampling locations were determined using purposive sampling method and the number of subsample taken per sampling area was determined based on ISO Norm 10381-4 for microbiological analysis purposes. The composited-sediment sample was kept in anaerobic condition and stored under -4ºC prior to further analysis.

Isolation of Electrogenic Bacteria

A total of 10 g composited-sediment from each ecosystem was then serially diluted into 90 mL of NaCl 0.85% solution and the dilution factor of suspension was made up to 10^{-8}. For initial evaluation of whether the sediment of the dam and paddy field ecosystem potentially hosts electrogenic bacteria, a total of 1 mL sample solution (from serial dilution of 10^{-5}-10^{-7}) was solely injected into 9 mL of liquid thioglycollate media enriched with Fe^{3+} ($FeCl_3$) 0.1% w/v, in triplicate repetition. Isolation of electrogens was conducted (from serial dilution of 10^{-3}-10^{-8}) using the pour plate method on solid thioglycollate medium supplemented with Fe^{3+} 0.1% w/v, in duplicate repetition (Indriyani *et al*., 2024c). The grown colonies after incubation period of 24-48 hours were then quadrant streak plated on solid thioglycollate medium (without supplementation of Fe^{3+}) to get pure bacterial isolate. The single colony was then morphologically characterized in terms of colony size, color, shape, margin, elevation, appearance, texture, and optical property, as well as Gram staining and oxygen demand.

Evaluation of Electrochemical Capability

The evaluation was conducted in glucose-fed dual-chambered MFC and the bacterial isolates' electrochemical capability was evaluated in the terms of open circuit voltage (OCV). Arduino Uno-based data logger was used for monitoring the OCV production during 75 hours and the OCV data was collected per 30 minutes following Indriyani *et al.* (2024b). The dimension of MFC reactor, electrode, and membrane separator, as well as the composition of anodic and cathodic solutions, was following Indriyani *et al.* (2023, 2024a, 2024b). The MFC chamber and the monitoring system used in this study are depicted in Figure 2.

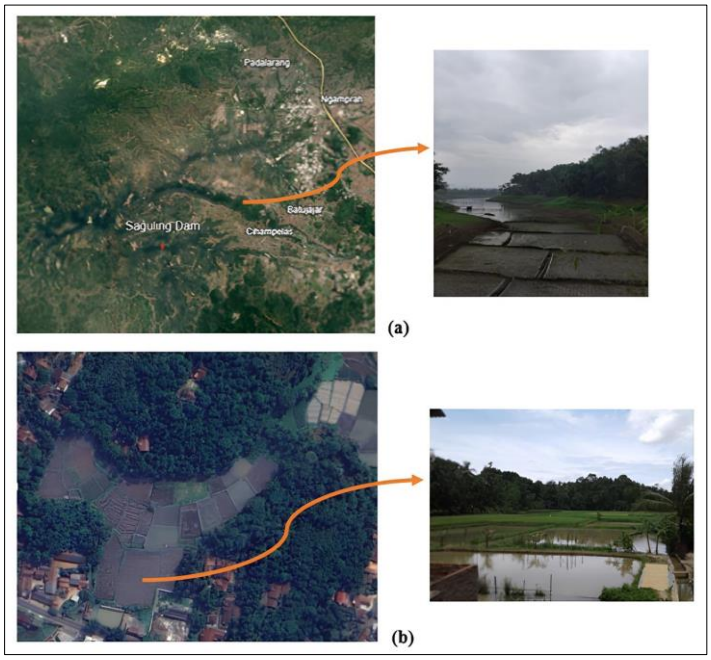

Figure 1. Sampling sites of (a) Saguling dam captured through Google Earth (left) and actual field conditions during sampling (right), (b) paddy field ecosystem in Warunggunung, Lebak, captured through Google Earth (left) and actual field conditions during sediment collection (right).

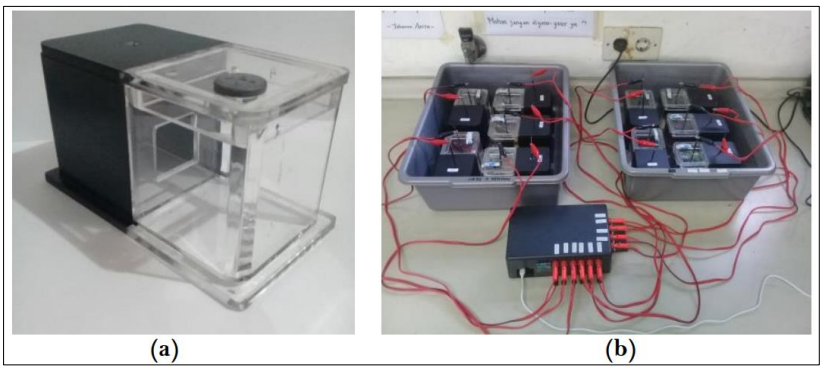

Figure 2. (a) MFC reactor in dual chamber model, (b) The monitoring of MFC electricity production using Arduino UNO-based data logger.

Results and Discussion

Pure Bacterial Isolates Isolated from the Sediment of Dam and Paddy Field Ecosystem

Initial evaluation utilized liquid thioglycollate medium enriched with Fe^{3+} 0.1% w/v showed that sediments from dam and paddy fields ecosystems have the potential to host electrogenic microbes. It can be seen from the color change in the Fe^{3+} enriched-thioglycollate medium after inoculation of bacterial suspensions after ±48 hours (Figure 3), indicated that the sediment contains microbes possessing anaerobic respiration and having the capability to use external electron acceptor (Fe^{3+}). Thioglycollate medium, designed by Brewer for both aerobic and anaerobic microbial cultivation (Brewer, 1940), is a reductive medium containing several reducing compounds (such as sodium thioglycollate) and oxidation-reduction indicators (i.e. resazurin). The medium will turn yellow when reductive conditions increase (NCCLS, 2004). The use of thioglycollate media for the isolation of electrogenic bacteria has not been widely reported. This present study demonstrated the potential of using enriched-thioglycollate media to isolate electrogenic microbes in a simple yet relatively efficient way, as did our previous study that successfully isolated electrochemically active bacteria from aquaculture pond sediments utilized this media (Indriyani *et al.*, 2024c).

Ecosystems as potential habitats for electrogens are environments where electron donor and solid electron acceptor compounds are naturally abundant (Yee *et al.*, 2019). Previous study (Wang *et al.*, 2015) reported that soil properties such as C-organic content, ammonium, and Fe, significantly affect the presence and diversity of electrogenic microbes. Sediments of Saguling dam and Banten paddy fields are rich in electron donor and acceptor compounds, containing relatively high C-organic content and total Fe: (1) C-organic content of 3.51% and 3.93% for Banten sediment and Saguling sediment, respectively, and (2) total Fe of 47835.00 ppm and 47578.67 for Banten sediment and Saguling sediment, respectively. C-organic is a carbon source and electron donor for microbes. Iron is an abundant element in nature, and in the form of Fe^{2+} is found in primary minerals and phyllosilicates (Colombo *et al.*, 2013). In oxygen-poor environments (i.e. waterlogged ecosystems), iron in the form of Fe^{3+} can be used as a terminal electron acceptor in the respiration of certain groups of microbes (Lovley *et al.*, 2004).

A total of 23 bacterial isolates from Saguling sediment (Table 1) and 31 isolates from Banten sediment (Table 2) were successfully isolated using thioglycolate solid media enriched with Fe^{3+} 0.1% w/v. Figure 4 shows the colony color and morphological characteristics of several isolates grown on thioglycollate solid media.

Figure 3. Colour changes in liquid thioglycollate media: (a) before the inoculation of bacterial suspension and (b) after the bacterial inoculation and incubation for ±48 hours. Pink and yellow color indicated the oxidative and reductive condition of the media, respectively.

Table 1. Morphological of the bacterial colony of isolates isolated from sediment of dam Saguling

Isolate	Morphological characteristics of bacterial colony							
	Size (mm)	Color	Shape	Elevation	Margin	Appearance	Optical property	Texture
LGf1	0.5-1	yellow	circular	convex	entire	glistening	translucent	smooth
LGf2	1-2	cream	circular	raised	entire	glistening	translucent	smooth
LGf3	0.5	white	circular	convex	entire	glistening	translucent	smooth
LGf4	0.5-1	white	circular	flat	undulate	dull	opaque	dry
LGf5	2-3	transparent	irregular	flat	undulate	glistening	translucent	smooth
LGf6	1-2.5	transparent	irregular	flat	undulate	glistening	translucent	smooth
LGf7	4-9	white	circular	flat	undulate	glistening	translucent	mucoid
LGf8	2-5	cream	circular	raised	entire	dull	opaque	smooth
LGf9	2-3	yellow	circular	convex	undulate	dull	opaque	smooth
LGf10	1-2	transparent	circular	convex	entire	glistening	transparent	smooth
LGf11	0.5-1	orange	circular	raised	undulate	dull	opaque	smooth
LGf12	1-2	yellow	circular	flat	undulate	dull	opaque	smooth
LGf13	1-2	orange	circular	convex	entire	glistening	translucent	smooth
LGf14	3-5	yellow	irregular	umbonate	curled	dull	opaque	dry
LGf15	1-2	white	circular	flat	undulate	dull	opaque	dry
LGf16	2-5	cream	circular	raised	entire	glistening	translucent	mucoid
LGf17	1-3	transparent	circular	convex	entire	glistening	translucent	mucoid
LGf18	1-2	yellow	circular	convex	entire	dull	opaque	smooth
LGf19	0.2	yellow	circular	convex	entire	dull	opaque	smooth
LGf20	0.5-1	white	circular	convex	entire	dull	opaque	smooth
LGf21	0.5-1	yellow	circular	raised	entire	glistening	translucent	smooth
LGf22	2-3	white	irregular	flat	undulate	dull	opaque	rough
LGf23	1-1.5	white	circular	flat	entire	dull	opaque	smooth

Table 2. Morphological of the bacterial colony of isolates isolated from sediment of Banten paddy field

Isolate	Morphological characteristics of bacterial colony							
	Size (mm)	**Color**	**Shape**	**Elevation**	**Margin**	**Appearance**	**Optical property**	**Texture**
SBf1	0.5-1	yellow	circular	umbonate	entire	dull	opaque	dry
SBf2	1.5-2.5	orange	circular	flat	entire	dull	opaque	dry
SBf3	0.5-1	yellow	circular	flat	entire	dull	opaque	smooth
SBf4	2-3	cream	circular	raised	curled	glistening	translucent	mucoid
SBf5	1-1.5	yellow	circular	flat	entire	dull	translucent	dry
SBf6	1-2	white	circular	raised	entire	glistening	opaque	smooth
SBf7	0.5-1	cream	circular	raised	entire	glistening	opaque	smooth
SBf8	1.5-2	yellow	circular	raised	entire	glistening	translucent	smooth
SBf9	1-1.5	white	circular	raised	entire	glistening	opaque	mucoid
SBf10	1-1.5	orange	circular	convex	entire	glistening	translucent	smooth
SBf11	0.5-1	yellow	circular	raised	entire	dull	opaque	smooth
SBf12	0.5-1	orange	circular	flat	entire	dull	opaque	smooth
SBf13	0.5-1	yellow	circular	convex	entire	dull	opaque	smooth
SBf14	2-2.5	orange	circular	flat	entire	dull	opaque	smooth
SBf15	2-3.5	white	irregular	flat	undulate	dull	opaque	dry
SBf16	2-2.5	white	circular	raised	curled	glistening	translucent	mucoid
SBf17	1.5-2	cream	circular	raised	entire	glistening	opaque	mucoid
SBf18	0.5-1	white	circular	raised	entire	glistening	opaque	smooth
SBf19	1-1.5	orange	circular	convex	entire	glistening	translucent	smooth
SBf20	1-2	cream	circular	raised	entire	glistening	opaque	smooth
SBf21	1-2	yellow	circular	raised	entire	dull	opaque	rough
SBf22	3-3.5	white	irregular	flat	curled	dull	translucent	smooth
SBf23	2-3	white	circular	raised	entire	glistening	opaque	mucoid
SBf24	0.5-1	orange	circular	convex	entire	glistening	opaque	smooth
SBf25	1-2	orange	circular	convex	entire	glistening	opaque	smooth
SBf26	2-3	white	irregular	raised	curled	glistening	opaque	smooth
SBf27	1-1.5	yellow	circular	convex	entire	glistening	translucent	smooth
SBf28	2-3	white	circular	raised	entire	dull	opaque	mucoid
SBf29	1-1.5	yellow	circular	convex	entire	glistening	translucent	smooth
SBf30	2-2.5	orange	circular	raised	entire	glistening	opaque	smooth
SBf31	1-1.5	white	irregular	flat	undulate	dull	opaque	rough

Microbial characterization based on Gram staining showed that most of the bacteria successfully isolated from both ecosystems were Gram negative bacteria. Only five isolates from Saguling sediment and seven isolates from Banten sediment belong to Gram positives. This result was in line with the phylogenetic analysis of many studies that state most electrogens are Gram-negative groups (Modestra & Mohan, 2014). In contrast to Gram-negatives that electron transfer mechanism is known (such as Geobacter and Shewanella), the electron transfer mechanism in the Gram-positives is not well known (Carlson *et al*., 2012). Gram-positive bacteria do not have an outer membrane, so it is assumed that this bacterial group does not have an extracellular electron transfer system like the cytochrome-porin system in Gram-negative bacterias (Lovley & Holmes, 2022). In addition, the thicker the cell wall, the more limited the ability of bacteria to transfer electrons extracellularly (White *et al.,* 2016).

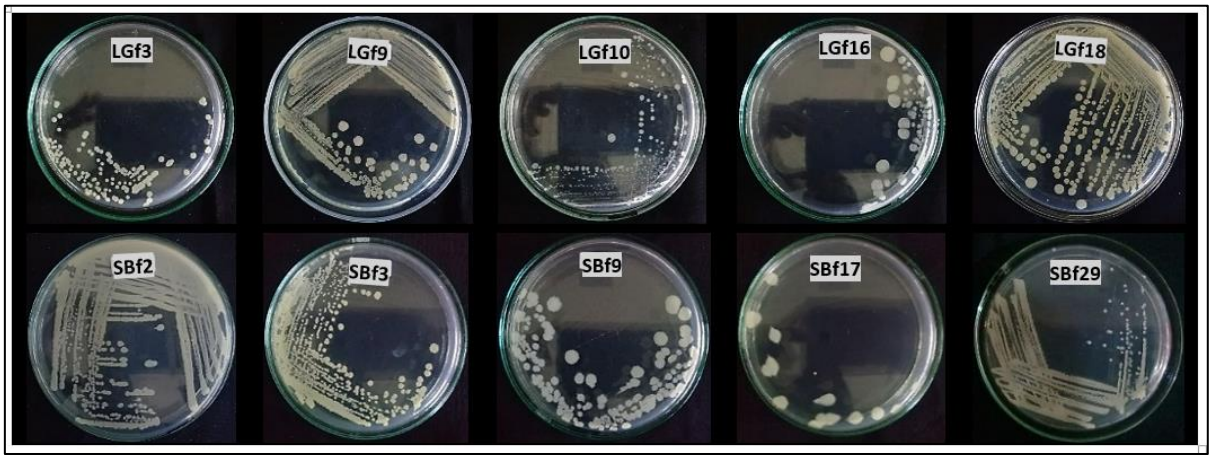

Figure 4. Colony color and morphological characteristics of several bacteria isolated from the dam and paddy field sediment, grown on solid thioglycollate media after incubation period of ±48 hour at temperature of 27±2ºC.

Electrochemical Performance of MFCs Powered by Electrogenic Isolates

In this study, the electrochemical capability of all bacterial isolates was evaluated in terms of open circuit voltage (OCV), which was measured without attaching an external resistor to the MFC electrical circuit. OCV is the potential difference between the electrodes in the anodic and cathodic chambers, and describes the highest voltage value that can be obtained by an MFC system (Erensoy *et al*., 2022). Theoretically, the maximum OCV that can be attained by an MFC system is 1.1-1.2 V if oxygen is used as an electron acceptor (An & Lee, 2014). In practical terms, the maximum OCV that an MFC cell can achieve is only ±0.8 V (Liu *et al*., 2005), due to losses that occur within the microbial cell (Chandrasekhar & Mohan, 2012).

Evaluation of the isolates' performance as biocatalysts in glucose-fed MFC confirmed that all 54 isolates from paddy field and dam sediments were electrogenic microbes with diverse electrochemical abilities. Five out of 23 bacteria isolated from dam sediment were able to produce the highest and relatively stable OCV values in the range of 600-800 mV, namely isolate LGf1, LGf11, LGf15, LGf20, and LGf22 (Figure 5). Figure 6 also shows that the 31 isolates from paddy field sediments have a diverse ability in electrical output (30-816 mV). Eight isolates were able to produce the highest OCV >600 mV, namely isolate SBf3, SBf10, SBf11, SBf13, SBf20, SBf23, SBf28, and SBf30. The fluctuating electrical output of MFC is a natural phenomenon, considering that MFC is a biological system catalyzed by living microbes with dynamic metabolic processes. An increase in the electrical value measured by the data logger is likely to occur when microbes perform anaerobic substrate breakdown so that electrons are produced, which are then transferred outside the cell and then captured by the electrode in the anodic chamber of the MFC. The decrease in electricity that occurs may be due to the absence of substrate breakdown activity by microbes.

Sediment of dam Saguling was dominated by facultative anaerobe electrogens (11 isolates) (Figure 5c) and sediment of Banten paddy field was dominated by aerotolerant anaerobe electrogens (10 isolates) and microaerophilic electrogens (10 isolates) (Figure 6d). In this study, the classification of microbes into microaerophile, facultative anaerobe, aerotolerant anaerobe, and obligate anaerobe was based on the bacterial growth characteristic in liquid thioglycollate media according to the microbial need for oxygen. The categorization of microbes into these groups is also related to the presence of two specific enzymes, namely catalase (EC 1.11.1.6) and superoxide dismutase (EC 1.15.1.1) (Brioukhanov *et al*., 2002).

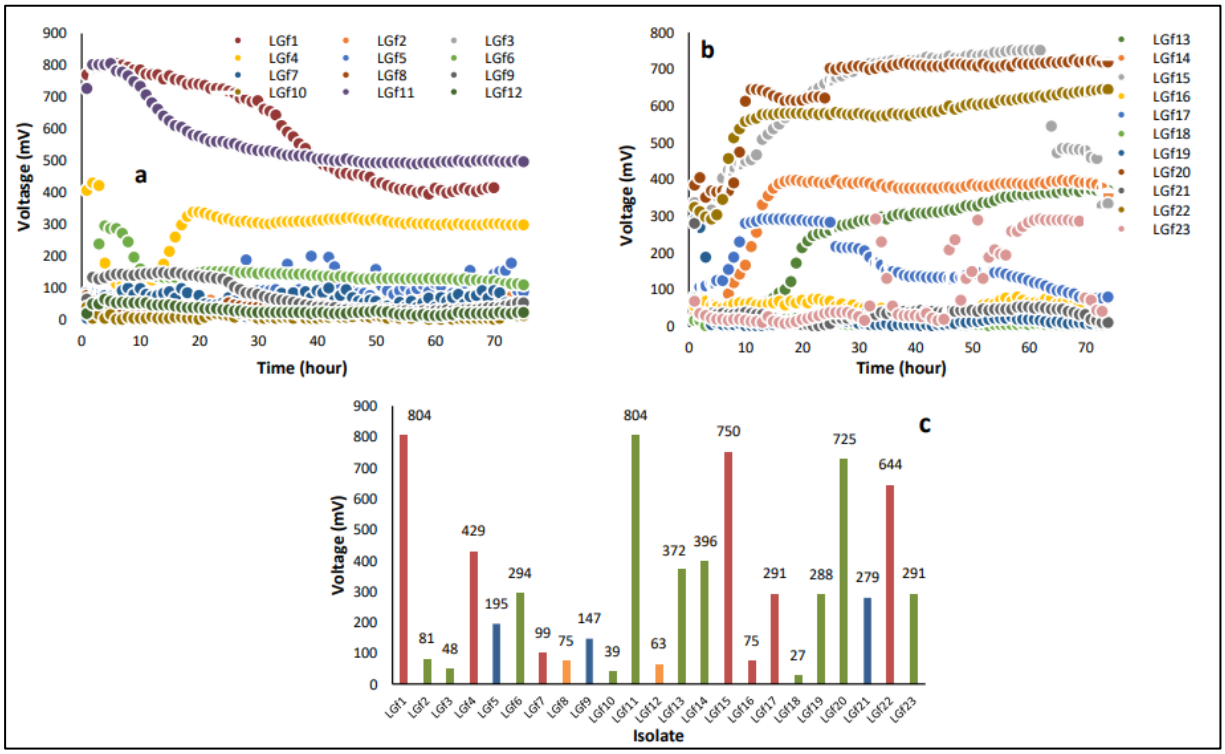

Figure 5. OCV output during 75 hours generated by MFCs powered by (a) isolate LGf1-LGf12, and (b) isolate LGf13-LGf23. (c) Highest OCV output attained by all electrogens isolated from Saguling sediment. These electrogens varied in the need of oxygen and depicted in different color: ■ aerotolerant anaerobe, ■ facultative anaerobe, ■ microaerophilic, ■ obligate anaerobe.

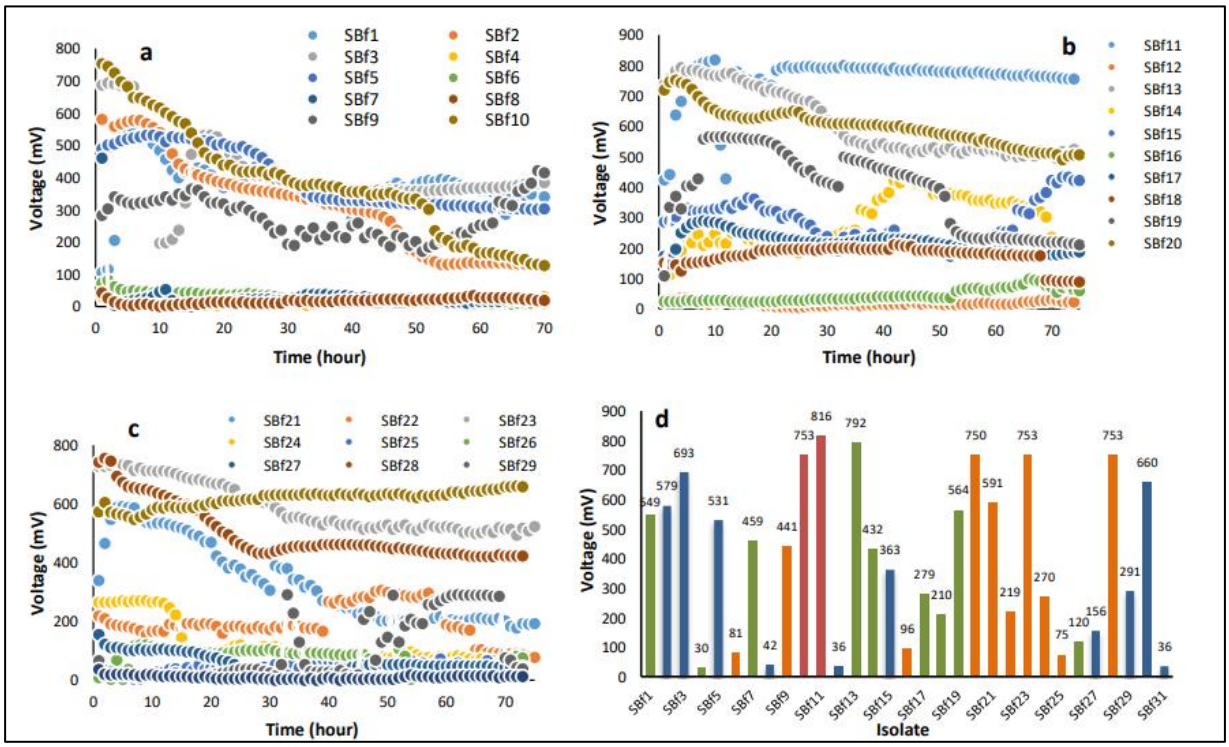

Figure 6. OCV output during 75 hours generated by MFCs powered by (a) isolate SBf1-SBf10, (b) isolate SBf11-SBf20, and (c) isolate SBf21-SBf31. (d) Highest OCV output attained by all electrogens isolated from Lebak sediment. These electrogens varied in the need of oxygen and depicted in different color: ■ facultative anaerobe, ■ microaerophilic, ■ obligate anaerobe, ■ aerotolerant anaerobe.

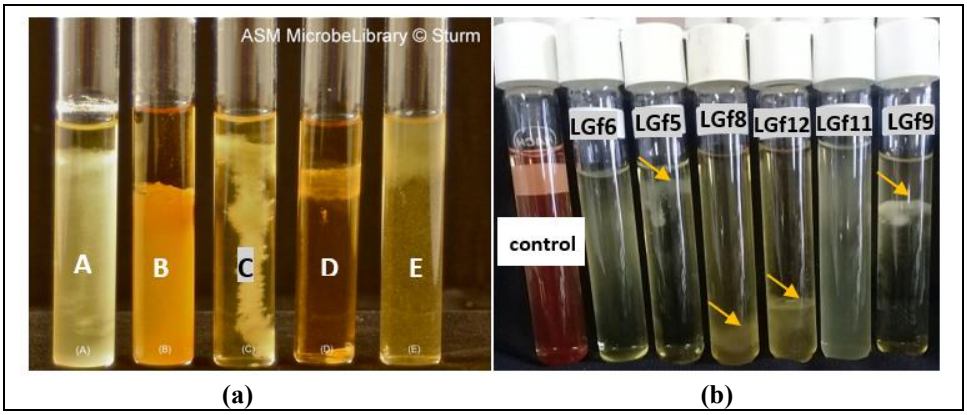

Figure 7. (a) Growth characteristics of microbes in liquid thioglycollate media: A. *Escherichia coli* (facultative anaerobe), B. *Clostridium butrycium* (obligate anaerobe), C. *Staphylococcus aureus* (facultative anaerobe), D. Neisseria *sicca* (microaerophilic), E. *Pseudomonas aeruginosa* (obligate aerobe) (Adapted from ASM MicrobeLibrary); **(b)** Growth characteristics of some bacteria isolated from the sediment of Saguling dam in thioglycolate liquid medium: LGf6 and LGf11 facultative anaerobes, LGf5 and LGf9 microaerophiles, LGf8 and LGf12 obligate anaerobes.

In thioglycollate liquid media, obligate aerobes will only grow on the surface of the media where high concentration of oxygen diffuses. Facultative anaerobes can grow in all parts of the media, whether there is oxygen or not. However, cell accumulation will be found on the surface of the media because this microbial group prefers the presence of oxygen. Aerotolerant anaerobes can grow in all parts of the media uniformly, but this microbe prefers anaerobic conditions. Microaerophiles will grow near the surface of the media where low concentrations of oxygen diffuse into the media. Meanwhile, obligate anaerobes will only grow if there is no oxygen diffusing into the media and will only grow in the 1/4 to ½ bottom of the media (Merck, 2021). Figure 7 shows the growth characteristics of bacterial isolates according to their need for oxygen.

Conclusion

This study revealed that dam and paddy field sediments were habitats of electrogenic microbes having various electrochemical capabilities that can be exploited for microbial fuel cells. This finding also suggested that nutrient-rich (in terms of donor and acceptor electrons)-sediments from waterlogged ecosystems in Indonesia could be one of the research foci to explore and isolate other indigenous and novel electrogenic microbes, as genetic resources for the development of MFC technology in Indonesia.

Some bacterial isolates obtained in this study showed the ability to produce relatively high and stable electricity (in terms of OCV or open circuit voltage), making them potential to be exploited for further analysis, both for molecular identification and evaluation of bioelectroactivity and efficiency.

Acknowledgments

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