Proceedings of Science and Mathematics
Faculty of Science, Universiti Teknologi Malaysia

Vol. 13, 2022, page 196- 205

Isolation of Electrogenic Bacteria from POME Anaerobic Sludge

Zawani Nadzirah Zamani, Mohd Firdaus Abdul-Wahab*
Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia,
81310 Johor Bahru, Johor, Malaysia
*Corresponding author: firdausw@utm.my

Abstract
Microbial fuel cell (MFC) is a bioelectrochemical system that uses electrogenic bacteria either as mixed or pure culture to transform the chemical energy into electrical energy in an organic compound. MFC can be used as a treatment for wastewater such as Palm Oil Mill Effluent (POME) with the help of the microbes. In this study, isolation of the electrogenic bacteria from anaerobic sludge was carried out using double-chambered MFC. Acetate was chosen as the carbon source to develop the electrogenic biofilm. The biochemical activity of the microbes in the MFC was observed via cyclic voltammetry after a month-long operation shows the biochemical activity of the electrogenic microbes, despite the inconsistency. A pure colony of the isolated electrogenic bacteria was characterized via three biochemical tests and morphological analysis and the results showed that it was positive in catalase test, oxidase and indole test. Besides that, microscopic analysis revealed the isolated colonies as Gram- negative bacteria and rod-shaped. The maximum power density and current density generated in the MFC were 53117.10 mW/m2 and 18.17 mA/m2, respectively. The COD removal obtained was 47.62%.
Keywords: Electrogenic bacteria; POME anaerobic sludge; Microbial fuel cell

Introduction

Malaysia has been known to be one of the world's largest exporters and producers of palm oil. The number of Malaysian palm oil mills has increased considerably almost every year due to the competition with other oils such as sunflower and olive oil (Abdullah et al., 2013). Apart from that, there is a demand on the production of palm oil due to the high usage of this product in both non-food and food industries. As a result, the local industry generated a large number of wastes, including shell (5%), mesocarp fibre (12%), empty fruit bunch (EFB) (23%), and palm oil mill effluent (POME) (60%) (Baharuddin et al., 2010). It is calculated there is a rise for the discharge of these wastes almost every year. Kamyab et al., (2018) mentioned that Malaysia had produced approximately 44 million tons of POME and 99.85 million tons of fresh fruit bunch per year.

Among all of these wastes, POME is the most toxic organic residue as it contains high chemical oxygen demand (COD), biological oxygen demand (BOD), and suspended solids (SS) value (Zainal et al., 2017). The release of untreated POME can pose a hazard to the environment as it has an unpleasant odour combined with other dissolved organic compounds that can pollute the river (Hamzah et al.,

2020). Nevertheless, this issue can be prevented by treating this POME with biological treatment such as pond systems (facultative or anaerobic or aerobic). Malaysia had been using these techniques for the last couple of decades (Abdurahman et al., 2013). Nowadays, there are another treatment of POME that received an increasing attention among the researchers. That treatment used the application of bacteria in the microbial fuel cell as one of the alternative ways (Alkhair et al., 2018).

Microbial fuel cell is considered as one of the renewable energies as it uses the concept of green energy. It can be obtained from organic sewage treatment that connects with generated energy (waste to energy concept) (Albarracin-Arias et al., 2021). Within such a concept, sewage treatment methods that are based on bacterial activity like anaerobic digestion (AD) have been investigated for biomass and wastewater treatment, whereas the chemical energy is harvested concurrently. In this way, wastewater with a high amount of organic matter is suitable for both generating electricity and feeding the MFCs. Furthermore, it utilizes the electrogenic bacteria to transform the chemical energy in an organic compound directly into electrical energy.

This research aims to isolate and screen potential electrogenic bacteria from POME anaerobic digested sludge. This microbe will generate energy via the oxidation which take places in the anode chamber. Therefore, it will produce a biofilm on the surface of the anode, which will be collected and examined for identification later on. In this case, POME anaerobic digested sludge will be used as an inoculum after assembling the double-chambered MFC. POME is chosen because of its high content of complex organic compounds and allow the stimulation of bacterial development, hence lowering the strength of pollution (Baharuddin et al., 2010). Apart from that, Albarracin-Arias et al. (2021) has stated that POME can be utilized as an electron donor for the generation of electricity and obtained the removal of COD with over 96.5% via double chamber MFC. Moreover, the electrogenic bacteria is mostly anaerobic, and the source of growth is mainly from wastewater. Thus, it is possible to isolate the electrogenic bacteria from POME anaerobic sludge as POME is considered wastewater.

Materials and methods

Sample collection

POME anaerobic digested sludge sample was collected from Felda Maokil Palm oil mill factory and stored in the refrigerator at 4℃ for further investigation. This was to prevent the microbes in the sample from breaking down throughout transit and conserved the quality of the samples.

Pre-treatment of Nafion membrane

The Nafion membrane will be immersed in 0.1 M H_2SO_4, and subsequently 0.1 M H_2O_2. After that, the membrane is rinsed with deionized water. Within 60 minutes, these procedures will be performed at 60°C. Before operating the MFC, Nafion membrane will be kept in a container of deionized water overnight. Whenever the MFC is not being used, the anode and cathode chambers are filled with deionized water to keep the membrane's conductivity intact.

Construction and operation of MFC analysis

The double chambers of MFC (cathode and anode) with the working volume of 100 mL are constructed using Perspex sheets with a capacity of around 125 mL (5 cm x 5 cm) and carried out at room temperature. These compartments will be separated with proton exchange membrane (PEM) which in this case, Nafion 115 membrane. A carbon felt electrode will be used in both chambers that will be connected via copper wires. Then, 90 mL of synthetic media (acetate as carbon source) will be placed in the anode chamber. Next, 10 mL of the inoculum that consists of the overnight culture of POME anaerobic digested sludge is also added into the anode chamber. At the cathode chamber, 0.1 M potassium ferricyanide in 0.1 M potassium phosphate buffer is placed inside it. After setting up the MFC, sparging nitrogen gas/ degassing about 10 minutes was done before fully seal the instrument's hole. This instrument

is inoculated under a fed-batch mode of operation. There will be a weekly replacement and it will be operated for about 30 days. Last but not least, both electrodes will be connected with an electrical multimeter to measure the voltage generated from day one to the final day at a regular interval.

Electricity Generation Analysis

Labjack U3-HV was used to analyse the generation of electricity throughout the MFC operation. Moreover, DAQFactory Express software was used to record the voltage (mV) of the MFC every 15 minutes. The current generated by the MFC was calculated using Ohm's Law, I = V/R where I = current in mA, V = voltage in mV and R = external resistance. The power generated by the MFC was calculated from the current and voltage, P = VI where P = power in mW, V = voltage and I = current. Afterward, the power density was calculated with the formula, $P = VI/A_{an}$ where P = power density in mW/m^2, V = voltage, I = current and A_{an} = surface of the electrode in m^2. Lastly, the current density was calculated with the formula $J = I/A_{an}$ where J = current density in mW, I = current and A_{an} = surface of the electrode in m^2.

Isolation of electrogenic bacteria from the anode plate

In this experiment, the electrogenic bacteria were chosen due to their potential for producing a biofilm on the anode electrode. Enrichment cultures are prepared by incubating the overnight culture of POME sludge in nutrient broth (10% v/v) at 30°C with shaking at 150 rpm. The anode plate is collected aseptically from the MFC after 30 days. It will be removed and soaked in 0.1 M phosphate buffer before being vigorously shaken to obtain the bacteria that produced the biofilm on the surface of the anode. Then, the anode side will be scraped and resuspended in the buffer as well. The samples are then cultured in Nutrient agar using a serial dilution technique and a spread plate technique to isolate pure bacteria colonies. Moreover, the gram staining technique and biochemical tests were performed to identify the isolated bacteria.

Screening of the potential electrogenic bacteria

Cyclic voltammetry analysis will be carried out to identify the electrochemical characterization of the potential microbes in the anode chamber. To compare with the inoculated media, sterile media is used. In this study, a classic three-electrode setup is utilized, which is the anode consisting of biofilm (working electrode), reference electrode, and counter electrode. These electrodes are inserted into the electrolyte and allowed to run until a cyclic voltammogram is formed. The reference electrode in liquid form would be Ag/AgCl and the scan rate (mV/s) used in this analysis was 100 mV/s. The start and stop potential were -0.1 V and +0.1 V respectively while the number of stop crossing was 2.

Result and discussion

The electrochemical behaviour of the double-chambered MFC was described via the cyclic voltammetry analysis. To be specific, this analysis was performed to determine whether the bacteria released the compound of the electroactive presence or not in the media. As shown in Figure 1 (A), the cyclic voltammogram (CV) curve depicted the information about the reduction and oxidation reaction. From the voltammogram, the anodic peak potential (E_{pa}) was 610 mV, while the cathodic peak potential (E_{pc}) was -800 mV. The redox couple's mid-point potential ($E_{1/2}$) in this analysis was calculated as −0.095 V, and the peak separation was approximately 1410 mV. It can be predicted that there is a bioelectrochemical activity of the bacteria in the media. It can be said that the cyclic voltammogram obtained from the potentiostat is not a good result as it does not produce a good curve like the 'duck-shaped' cyclic voltammogram. Apart from that, there might be some presence of oxygen in the MFC, which might disturb the result of this analysis. When preparing the MFC, a process known as degassing/sparging with an inert gas such as nitrogen was done to remove its oxygen. This is because the oxygen molecule is electrochemically active, which will generate an unwanted redox process and be interrupted with the result of CV if it is not remove

A

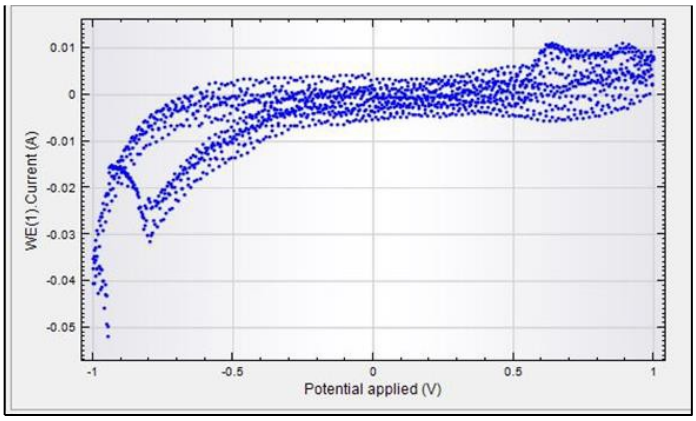
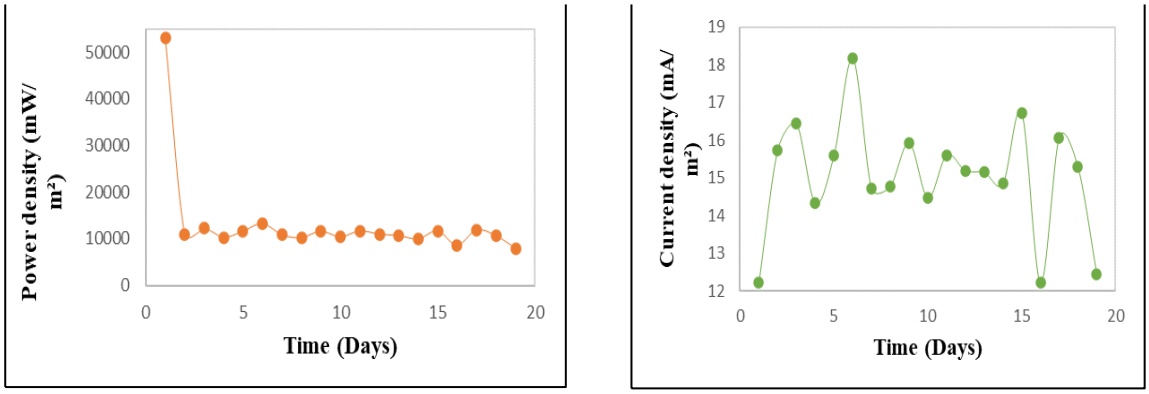


Figure 1 Result of cyclic voltammetry

The power density generated by the anaerobic sludge in the MFC is shown in Figure 2 (a), while the current density is generated as shown in Figure 2 (b). The maximum power density achieved by the MFC was 53117.10 mW/m^2, whereas the maximum current density achieved was 18.17 mA/m^2. It can be seen that power density had dropped drastically after day 1. This might be due to other groups of microbes that used up the available compounds from anaerobic sludge resulting in continuous generation of electricity. Instead, the electricity generation had gradually decreased from day 2 and onwards. The MFCs were operated in a fed-batch mode where the accessible organic compounds would decrease within time, blocking further power generation. To compare, the maximum power density achieved from this experiment was said to be higher than the previous study by Nor et al. (2015) which the power density achieved only about 85.12 mW/m^2 and Khater et al. (2017) with the maximum power density of 80.50 mW/m^2. On the last day (day 19), the voltage generated depleted. The performance of this instrument may be affected by the external resistance used. The external resistance used in this experiment was 1000 Ω. Khater et al. (2017) stated that 500 Ω gave a better performance than 1000 Ω. They concluded that the higher the external resistance used, the lower the current generated.



(a) (b)

Figure 2 (a) Power density generated, (b) Current density generated

Next, the pH of the anolyte was analysed every batch. The pH in the anode chamber showed changes in every batch of the MFC operation, as shown in Table 1. The pH considered one of the important factors and needs to be controlled so the microbial reactions can run effectively. To begin with, the pH of the synthetic media (acetate as carbon source) and anaerobic sludge was 7.14.

The pH of the anolyte increased on the 2nd batch before it gradually decreased to 6.80, which was on the 6th batch. When the pH of the anolyte becomes slightly acidic, the generation of power is depleted at the same time. To justify this statement, Halim et al. (2021) conducted an experiment which compared different pHs in the MFC where the pH involved was pH 6 to pH 10. They deduced that the lower the pH, the lower the voltage produced. Another finding from the same journal mentioned that the maximum power obtained would be between pH 6.5 and pH 8. The MFC with pH 6 produced lower electricity generation, whereas the pH 8 produced the highest electricity generation. The reason behind this is because higher pH may affect the bacteria growth and result in depleting the current generation. Moreover, bacteria need an environment which is close towards neutral for the optimal growth.

Table 1 Result of pH analysis in 6 batches of MFC operation

	1st batch	2nd batch	3rd batch	4th batch	5th batch	6th batch
MFC 2	**7.14**	**7.34**	**7.15**	**5.47**	**6.55**	**6.80**

Chemical oxygen demand (COD) measures the amount of oxygen required to chemically oxidize the organic compounds in the wastewater. The treatment of POME anaerobic sludge was assessed by comparing the COD levels before and after every batch operated. In this experiment, the samples were diluted at 2X dilution with deionized water, and the result was observed. Based on Figure 3, it was depicted that the COD value had decreased after every batch. The highest COD value obtained was 3000 mg/L, indicating a high oxygen demand. So, dissolved oxygen (DO) levels are likely depleted. A reduction of DO will cause harm to the aquatic life form if the wastewater is released into the river or sea. The COD removal efficiency was calculated with the formula given. As a result, the maximum COD removal efficiency obtained was 47.62%. Some studies have been made, concluding that the higher power density generated in MFC had a higher COD value. It was because there were more accessible electrons from the POME compound. This analysis gave successful information regarding the metabolism of the bacteria in the anode chamber. Nevertheless, the changes in COD value do not provide information on the type of bacteria that consume the oxygen. Owing to the fact that there was a presence of non-electrogenic microbes apart from the electrogenic microbes in the anaerobic sludge (Ullah and Zeshan, 2020).

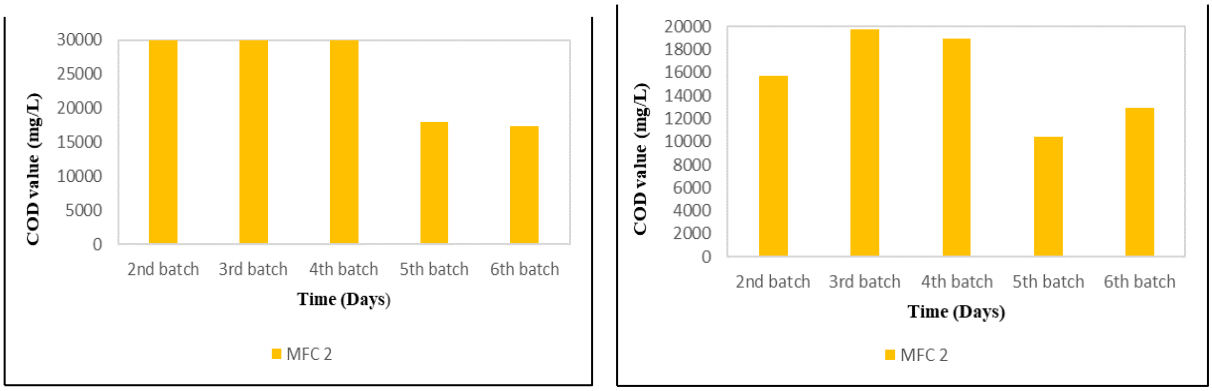
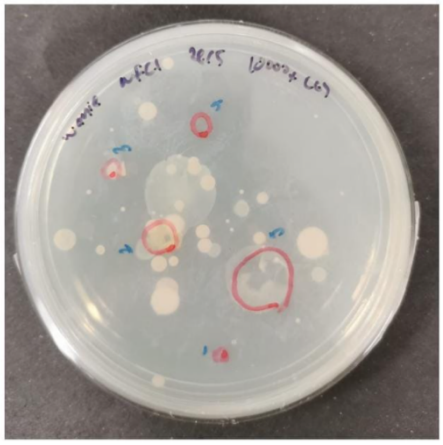
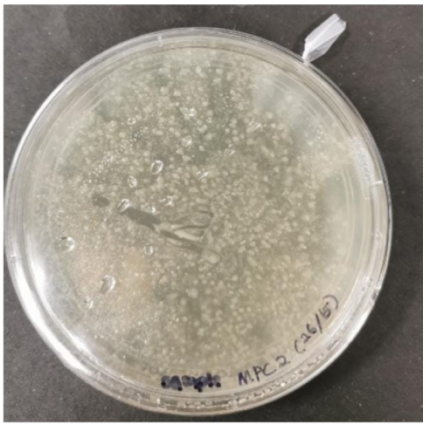


Figure 3 The COD value before and after every batch during MFC operation

At the end of the MFC operation, biofilm was successfully formed, which can be seen after the anode electrode was removed from the instrument to isolate the potential electrogenic microbes. After transferring the culture on the nutrient agar plate via serial dilution and spreading technique, pure bacteria colonies were obtained. A total of two isolates from the anaerobic sludge, isolate A and isolate B, were shown in Table 2.

Table 2 Two isolates namely isolate A and B on the nutrient agar plates

Isolate A	**Isolate B**
	

Based on the Table 3, the colony morphological of the isolated bacteria was characterised based on colony size, colour, form, opacity, elevation and edge. The colony on the nutrient agar plate appeared to have round form and entire edged.

Table 3 Colony morphological of isolate A and B

	Isolate A (1)	**Isolate A (2)**	**Isolate A (3)**	**Isolate A (4)**	**Isolate A (5)**	**Isolate B**
Size	Tiny	Medium	Small	Small	Large	Tiny
Colour	White	Yellow	Creamy	White	White	Creamy
Form	Round	Round	Round	Round	Round	Punctiform
Opacity	Opaque	Opaque	Opaque	Transparent	Transparent	Opaque
Elevation	Flat	Raised	Raised	Raised	Flat	Flat
Edge	Entire	Entire	Entire	Entire	Entire	Entire

Next, the isolated microbes proceeded with gram staining analysis to know whether they were gram-negative or gram-positive microbes. Hans Christian Gram had developed a technique to differentiate between Gram-negative and Gram-positive bacteria via crystal violet and safranin (counter stain). Under a microscope, the colour of the Gram-positive microbes will give off purple, which was the stain from crystal violet. In contrast, Gram-negative will give off pinkish colour from the counter-stain safranin (Breijyeh et al., 2020). After the gram-staining procedure had been done, all the 6 isolates (Figure 4) appeared pinkish and possessed rod- shaped. So, it made the isolated microbes as Gram-negative.

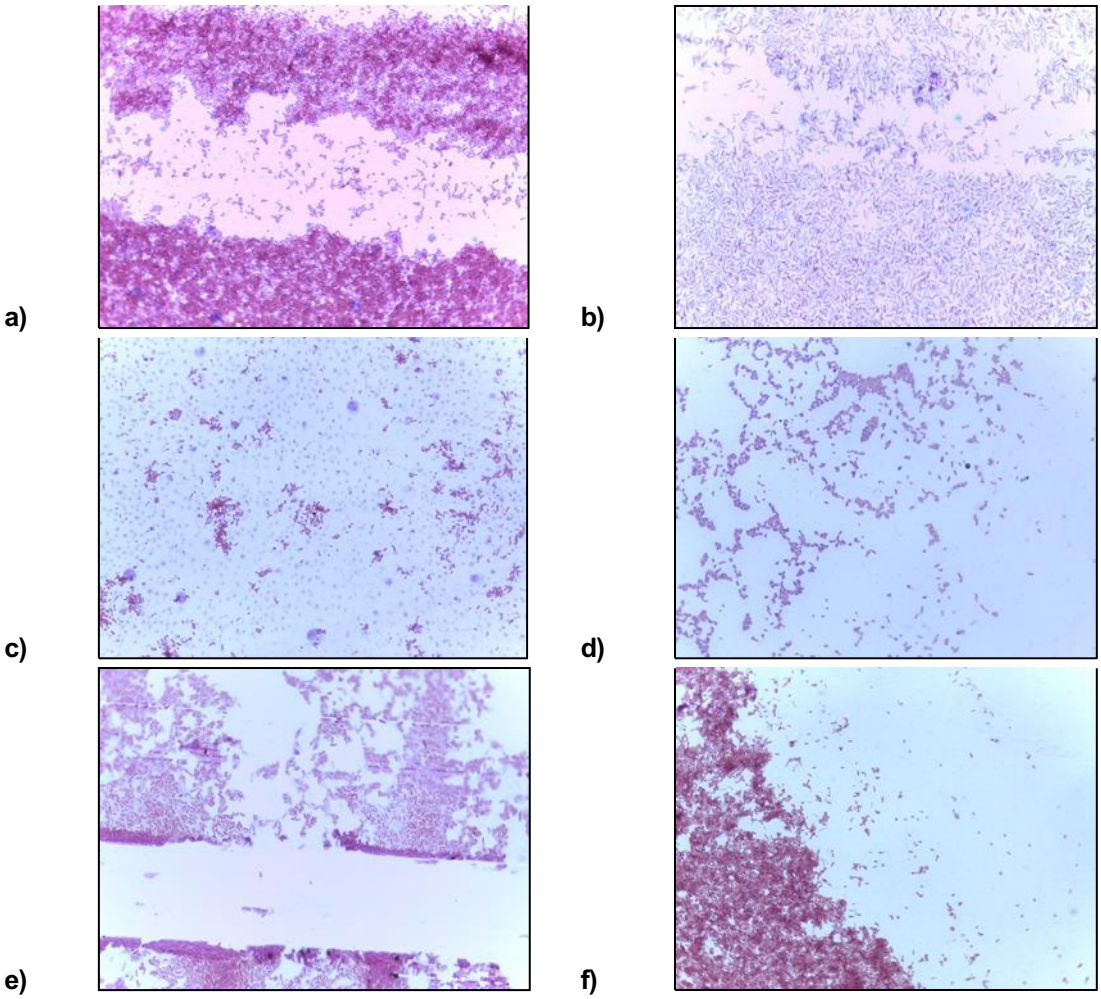
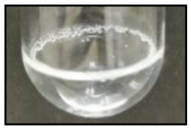
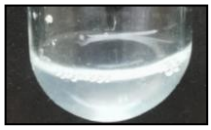
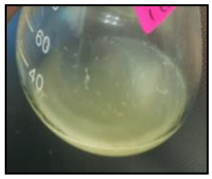
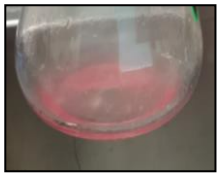
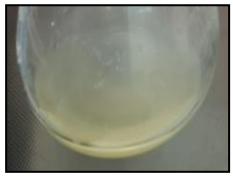


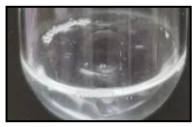
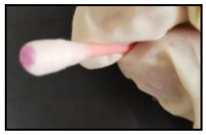
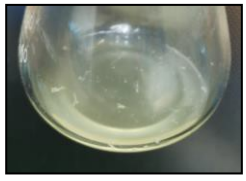
Figure 4 a) Isolate A1, b) A2, c) A3, d) A4, e) A5 and f) B under 100x microscope

Biochemical tests depicted that the isolated microbes possessed many metabolic activities. Therefore, for this experiment, the biochemical tests performed on the isolate microbes were the catalase, oxidase, and indole. The results obtained were tabulated in Table
4.4 shown above. Out of 6 isolates, 5 were positive in the catalase test, and 3 were positive in the oxidase and indole test.

First and foremost, the catalase test aided in detecting the enzyme catalase in the bacteria. The gas bubble production confirmed the presence of catalase that will break down the hydrogen peroxide (H_2O_2) into oxygen and water. So, the positive test indicated that the bacteria could produce catalase, while the negative test indicated that the bacteria could not produce catalase. Next, the oxidase test detected the presence of a cytochrome c oxidase system involved in the electron transport chain. When the test is positive, it will change the colour to purple, whereas the negative one remains colourless within a limited time. Last but not least, the indole test demonstrated the ability of the bacteria to produce tryptophanase to catalyse the deamination reaction and form the product indole. A positive indole test showed a pink or red colour formation, indicating that bacteria could produce the indole and the negative one remained colourless after the addition of the Kovac's reagent.

Table 4 Biochemical tests for Isolate A and B

	Isolate A (1)	**Isolate A (2)**	**Isolate A (3)**
Catalase test	Positive 	Positive 	Positive 
Oxidase test	Negative 	Positive 	Positive 
Indole test	Negative 	Positive 	Negative 

	Isolate A (4)	**Isolate A (5)**	**Isolate B**
Catalase test	Positive 	Negative 	Positive 
Oxidase test	Negative 	Negative 	Positive 
Indole test	Positive 	Positive 	Negative 

Conclusion

The biofilm was successfully developed in the double-chambered MFC with the acetate as the carbon source. It had been isolated from the anode electrode and then proceeded to identify the characteristics of the isolates. For the colony morphological analysis, it can be seen that most of the colonies appeared round-shaped, had the entire edge and were opaque. Moreover, the culture bacteria obtained can be characterised as Gram-negative bacteria, and it possessed rod-shaped that can be seen under the microscope. Out of the 6 isolates, 5 were positive for the catalase test, and 3

were positive for the oxidase and indole test. Moving on to the cyclic voltammetry, there is the biochemical activity of the bacteria in the sludge, yet the results obtained showed that it is not consistent. Last, the chemical oxygen demand (COD) in the anaerobic sludge decreased gradually after the MFC operation. The maximum COD removal obtained was 47.62%, which indicated the sludge had been successfully treated

Acknowledgement

This project was supported by Faculty of Science, Universiti Teknologi Malaysia (UTM).

References

Abdullah, N., Yuzir, A., Curtis, T. P., Yahya, A., & Ujang, Z. (2013). Characterization of aerobic granular sludge treating high strength agro-based wastewater at different volumetric loadings. *Bioresource Technology*, 127, 181–187. https://doi.org/10.1016/j.biortech.2012.09.047

Abdurahman, N. H., Rosli, Y. M., & Azhari, N. H. (2013). The Performance Evaluation of Anaerobic Methods for Palm Oil Mill Effluent (POME) Treatment: A Review. In *International Perspectives on Water Quality Management and Pollutant Control*. InTech. https://doi.org/10.5772/54331

Albarracin-Arias, J. A., Yu, C. P., Maeda, T., Valdivieso Quintero, W., & Sanchez-Torres, V. (2021). Microbial community dynamics and electricity generation in MFCs inoculated with POME sludges and pure electrogenic culture. *International Journal of Hydrogen Energy*, 46(74), 36903–36916. https://doi.org/10.1016/j.ijhydene.2021.08.218

Alkhair, K. B., Hassan, O. H., Mohamed, S. A. S., Andrew, Y. K. C., Rahman, Z. A., & Yahya, M. Z. A. (2018). Palm oil mill effluent's microbial fuel cell's optimisation procedure by using two-level factorial design method and chemical oxygen demand treatment. Journal of Oil Palm Research, 30(3), 503–514. https://doi.org/10.21894/jopr.2018.0040

Baharuddin, A. S., Hock, L. S., Zulkhairi, M., Yusof, M., Aini, N.', Rahman, A., Kalsom Md Shah, U., Hassan, M. A., Wakisaka, M., Sakai, K., & Shirai, Y. (2010). Effects of palm oil mill effluent (POME) anaerobic sludge from 500 m 3 of closed anaerobic methane digested tank on pressed-shredded empty fruit bunch (EFB) composting process. *African Journal of Biotechnology*, 9(16), 2427–2436. http://www.academicjournals.org/AJB

Breijyeh, Z., Jubeh, B., & Karaman, R. (2020). Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. Molecules, 25(6). https://doi.org/10.3390/MOLECULES25061340

Friman, H., Schechter, A., Ioffe, Y., Nitzan, Y., & Cahan, R. (2013). Current production in a microbial fuel cell using a pure culture of Cupriavidus basilensis growing in acetate or phenol as a carbon source. Microbial Biotechnology, 6(4), 425–434. https://doi.org/10.1111/1751-7915.12026

Halim, M. A., Rahman, O., M., Ibrahim, M., Kundu, R., & Kumar, B. (2021). Study of the effect of pH on the performance of microbial fuel cell for generation of bioelectricity. 10.21203/rs.3.rs-151072/v1.

Hamzah, M. A. F., Abdul, P. M., Mahmod, S. S., Azahar, A. M., & Jahim, J. M. (2020). Performance of anaerobic digestion of acidified palm oil mill effluent under various organic loading rates and temperatures. *Water (Switzerland)*, 12(9). https://doi.org/10.3390/w12092432

Kamyab, H., Chelliapan, S., Din, M. F. M., Rezania, S., Khademi, T., & Kumar, A. (2018). Palm Oil Mill Effluent as an Environmental Pollutant. In *Palm Oil*. InTech. https://doi.org/10.5772/intechopen.75811

Khater, D. Z., El-Khatib, K. M., & Hassan, H. M. (2017). Microbial diversity structure in acetate single chamber microbial fuel cell for electricity generation. Journal of Genetic

Engineering and Biotechnology, 15(1), 127–137. https://doi.org/10.1016/j.jgeb.2017.01.008

Nor, M. H. M., Mubarak, M. F. M., Elmi, H. S. A., Ibrahim, N., Wahab, M. F. A., & Ibrahim, Z. (2015). Bioelectricity generation in microbial fuel cell using natural microflora and isolated pure culture bacteria from anaerobic palm oil mill effluent sludge. *Bioresource Technology*, 190, 458–465. https://doi.org/10.1016/j.biortech.2015.02.103

Ullah, Z., & Zeshan, S. (2020). Effect of substrate type and concentration on the performance of a double chamber microbial fuel cell. Water Science and Technology, 81(7), 1336–1344. https://doi.org/10.2166/WST.2019.387

Zainal, N. H., Jalani, N. F., Mamat, R., & Astimar, A. A. (2017). A review on the development of palm oil mill effluent (POME) final discharge polishing treatments. *Journal of Oil Palm Research*, 29(4), 528–540. https://doi.org/10.21894/JOPR.2017.00012