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Research paper



Identification of Bacterial Community in Wastewater and Their Kinetic Growth in Microbial Fuel Cells

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Abstract

Microbial fuel cell (MFC) is an alternative energy system comprise of bio-electrochemical reactor with wastewater as raw material and electrochemically active microorganisms as the main biocatalyst that utilize and convert chemical energy within organic matters to electrical energy. Sequence analysis by BLAST indicated that five species existed which were *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Enterobacter aerogenes*, *Leucobacter luti* and *Bacillus cereus*. *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Enterobacter aerogenes* are biofilm and exoelectrogens that responsible to generate electricity in the MFC for both aerated or with the addition of ferricyanide. However, *Leucobacter luti* and *Bacillus cereus* are also among the suspended growth bacteria that can be found in the MFC reactor. As for the rate of removal of COD, it showed that with aerated cathode, the COD removal rate was 51.30 % while with the addition of ferricyanide in the cathode showed a COD removal rate of 93 %. The highest voltage generated by the additional of ferricyanide was 0.841 V while for the aerated cathode, the highest voltage of 0.589 V has been generated. As for the kinetic growth of the bacteria, specific maximum growth rate, μ_{net} of the biomass was 0.003 h^{-1} and 0.004 h^{-1} for biofilm in the aerated tank while with the addition of ferricyanide, μ_{net} for biomass was 0.002 h^{-1} and 0.001 h^{-1} for biofilm. Yield growth, Y for biomass in the aerated tank had the highest growth which was 2.035 g.cell/g.substrate while biofilm only had limited growth of 0.1213 g.cell/g.substrate. As for the addition of ferricyanide, Y for biomass was 1.206 g.cell/g.substrate and 0.3268 g.cell/g.substrate for biofilm.

Keywords: *bacterial community; kinetic; microbial fuel cell; wastewater.*

1. Introduction

Microbial fuel cell (MFC) is an alternative method of renewable energy by applying the concept of direct conversion of organic waste to electricity by microorganism, called exoelectrogens. Exoelectrogens can transfer electrons outside of their cell to an electron acceptor to generate electricity in either mixed culture or pure cultures. Previous studies had shown that mixed culture of activated sludge was the best community of bacteria for pollutants removal and simultaneously producing electricity using microbial fuel cell [1-4]. Due to these findings, an in-depth study about the process of activated sludge also need to be done. Zuhairi et al. [5] found that microwave treated sludge can improve the productivity of electricity generation from microbial fuel cell. Microbial fuel cell (MFC) represents the latest study for alternative and renewable energy for generating electricity from wastewater using bacteria. Microorganisms oxidize organic matters in MFC, producing electrons that travel through a series of respiratory enzymes in the cell and make energy for the cell in the form of ATP (Adenosine triphosphate). The electrons are then released to a terminal electron acceptor (TEA) which accepts the electrons and becomes reduced. However, we have now discovered that some bacteria can transfer electrons exogenously or out of the cell to a TEA such as a metal oxide. These bacteria that can exogenously transfer the electrons are called exoelectrogens which can produce power in a MFC. The rate of the bio-energy generation is highly affected by the biological activities of the microorganisms in the anodic chamber. Exoelectrogens bacteria are a group of bacteria that were able to directly transfer electrons to function in an MFC for electricity generation [6]. *Escherichia coli* and *Saccharomyces* are among the very first exoelectrogens discovered by Potter [7]. MFC has been occupied by bacteria with different genetic groups which range from *ß-Proteobacteria* such as *Rhodoferax* [8], *γ-Protobacteria* such as *Shewanella* and *Pseudomonas* [9-11], *δ-Protobacteria* such as *Aeromonas*, *Geobacter*, *Geopsychrobacter* and *Desulfobulbus* [12,13], *Firmicutes* such as *Clostridium* [14], and also *Acidobacteria* such as *Geothrix* [15]. It has been identified that Fe(III)-reducing bacteria such as *Shewanella* and *Geobacter* are the major contributors to power generation in MFC [9,12,16,17] including *Shewanella oneidensis* DSP10 [18], *Shewanella putrefaciens* [19], *Geobactor sulfurreducens* [20], *Geobactor metallireducens* [21] and *Rhodoferax ferrireducens* [22]. Ishii et al. (2013) [23] in their study at three different MFC operational conditions found that inoculated wastewater which dominated by members of *Epsilonproteobacteria*, *Gammaproteobacteria*, and *Bacteroidete*s species; the electricity-generating biofilms in MFC and reactors were dominated by *Deltaproteobacteria* and *Bacteroidetes*. Ismail et al (2013) [24] studied the actual domestic wastewater as substrate and alternatively inoculated with activated sludge and *Bacillus subtilis*. It was found that *Bacillus subtilis* as a single pure culture was able to produce higher power and better COD removal efficiency compared to the mixed cultures in activated sludge.

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2. Materials and methods

2.1. MFC construction and operation

Two-chamber MFC with two equal volume of anode and cathode chambers (1L capacity) were separated by membrane (Nafion™, Alfa Aesar, USA) which act as proton exchange membrane (PEM). Nafion™ was pretreated by boiling in H_2O_2 (30%) and deionized water, followed by 0.5M H_2SO_4 and deionixed Reactor was connected to pump (Masterflex® Digital Pump Drive) and self-fabricated reactor (Figure 1). Anode chamber was sealed with bottle cap to keep it free from oxygen and was recirculated continously at 30 ml/min to provide effective contact between the mixed consortia and substrate. Electrodes were both made of carbon paper (2.5 x 5 cm) and connected by copper wire while exposed metal were sealed with epoxy. Prior to use, electrodes were soaked in deionized water for 24h. Anode chamber was filled with synthetic wastewater that contained 1g/L $C_6H_{12}O_6$, 50.00 mg/L NH_4NO_3, 52.7 mg/L $MgCl_2.6H_2O$, 0.29 mg/L $MnCl_2.4H_2O$, 0.29 mg/L $FeCl_3.6H_2O$ and 4.29 mg/L $CaCl_2.2H_2O$. Vitamin and mineral composition had been obtained from previous study (Balch et al., 1979). Two MFC were operated seperately to compare the efficiencies between aerated (MFC_{AC}) and ferricyanide (MFC_{FC}) in the kinetic growth relative to the power generation. MFC_{AC} cathode chamber was continuosly sparged with air by using air pump. MFC_{FC} cathode chamber was filled with ferricyanide [50 mM $K_3Fe(CN)_6$ in phosphate buffer (50 mM HPO_3)]. The reaction occured in anode and cathode chambers of MFC with glucose as substrate under complete oxidation were as indicated in Eqs. (1) – (3).

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

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

Ferricyanide catholyte: $4Fe(CN)_6^{3-} + 4e^- \rightarrow 4Fe(CN)_6^{4-}$ (3)

The pH for both catholytes were maintained at 7. MFC were operated for 10 days while 200 mL sample was taken from anode chamber every 2 days for COD and biomass test. 200 mL of fresh synthetic feed was reloaded after each obtained of sample. Cell voltage outputs were measured by voltmeter connected to the computer which recorded the data at an intervals of 10 s. Voltage were then intepreted into interval of 1 h for 240 h during MFC operation.

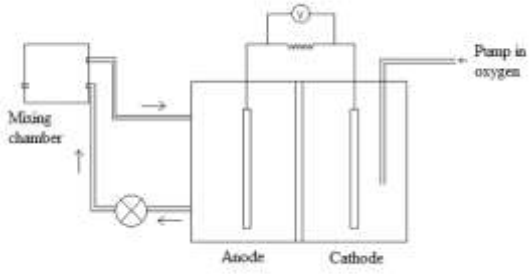


Fig.1: Self fabricated reactor.

2.2. Cultivation and isolation of bacteria

Samples were obtained from the scratched of the anode electrode for biofilm and taken directly after mixed throughout for suspended growth bacteria before and after MFC operation. Samples were then diluted with the dilution factor of 10^{-6}. 100 µL of bacteria from each group of isolation were pipetted into nutrient agar and spread plate methods were conducted. After 24 hours of incubation at 37 ºC, plates were observed under light microscope. Gram staining was then being conducted to identify the morphological and gram classification of the respective bacteria. Isolates were cultivated in nutrient broth (NB, Oxoid) at 37 ºC for 24 h. DNA was extracted (Vivantis GF-1 bacterial DNA extraction kit) according to manufacturer's instruction.

2.3. PCR amplification of 16S rRNA gene sequences

Amplification of bacterial 16S rRNA was performed using universal primer set proposed previously (Kim et al., 2006). Sequences of the primers were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTAGGACTT-3'). Polymerase chain reaction (PCR) consisted of initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C at 1 min/kbs, with final extension at 72°C for 5 min prior to cooling at 4°C. Master mix of PCR reaction consisted of Green Go Taq® Master Mix (Promega, Madison, WI, USA), sterile ultrapure water, primers, $MgCl_2$ solution, nucleotide mix of 10mM and extracted DNA. All PCRs were performed using Mastercycler (epgradient S, Eppendorf, Version 3.608).

2.4. Gel electrophoresis

Gel electrophoresis of PCR products was carried out with 1% agarose gels (16cm x 16cm gel, 1mm thickness). Electrophoresis was conducted using a 1 x Tris-acetate-EDTA (TAE) buffer at 1.5 kbs and 65 V for 8 h. Gel was then stained with ethidium bromide in 1 x TAE buffer for 15 min and destainded in 1 x TAE for 10 min. Products were visualized under UV transilluminator.

2.5. Sequence analysis

PCR products were sent to 1st BASE Laboratories Sdn. Bhd for sequencing. The sequences were then subjected to Basic Local Alignment Search Tool (BLAST) at the homepage of The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)

3. Result and discussion

3.1. MFC community 16S rRNA identification and analyse

20 isolated bacteria were observed under microscope. 5 final groups of bacteria were obtained based on morphological and gram stain result of the respective colonies. Gram stain result indicated that 3 gram-negative bacteria and 2 gram-positive bacteria presented with the shapes of either coccus or bacillus. Figure 2 illustrates the agarose gel electrophoresis profiles of 16S rRNA gene fraction for each labeled strain. Each band represents a specific species in microbial community and the staining intensity represents the abundance of the corresponding microbial species

Table 1: 16S rRNA gene identifications

						Isolation source during MFC operation			
						Before		After[b]	
Strain	*Accession no.*	GenBank closest match	Identity (%)[a]	Gram	Morphology	Bio-film	Suspended bio-mass	Bio-film	Suspended bio-mass
S1	HQ 425293.1	*Pseudomonas aeruginosa*	99	-ve	Bacillus	√	**X**	√	√
S2	GQ 259887.2	*Klebsiella pneumoniae*	98	-ve	Coccus	√	**X**	√	√
S3	AY 335554.1	*Enterobacter aerogenes*	98	-ve	Bacillus	**X**	√	√	√
S4	AM 072819.1	*Leucobacter luti*	98	+ve	Coccus	**X**	√	**X**	√
S5	JF 414767.1	*Bacillus cereus*	99	+ve	Streptobacilli	**X**	√	**X**	√

[a] The value represent the similarities between identified strain with the closest match of GenBank.
[b] Result for both MFC_{AC} and MFC_{FC}.

[25]. Table 1 shows the result of gene identification based on 16S rRNA. BLAST analysis of gene sequences showed that S1 is 99% identical to *Pseudomonas aeruginosa* while S2 is 98% identical to *Klebsiella pneumonia*. *Pseudomonas aeroginosa* and *Klebsiella pneumonia* can be detected at the biofilm before and after MFC operation with some of them were detected in suspended biomass as well. It is believed that these species can be detected in suspended biomass due to the continuous flow of synthetic wastewater in the reactor that had reduce the attraction ability of the bacteria to the electrode. Both species imply the electron exchange mechanism where no direct touches to the electrode are needed to generate electricity in MFC. *Pseudomonas aeruginosa* is proved to generate electricity with excreted redox mediators called pyocyanin in both mixed culture and pure culture without the addition of exogenous mediator [11]. *Klebsiella pneumonia* is an electrochemically active Fe(III)-reducing bacterium and for the first time, Deng et al. (2010) [26] had proved that the species excreted an electrochemically active mediator called 2,6-DTBBQ. However, S3 is 98% identical with *Enterobacter aerogenes* which has been identified before and after on electrode as biofilm. The result of the study was proved by Zhuang et al. (2010) [27] which used pure colony of *Enterobacter aerogenes* in MFC operation to generate electricity, had showed that a layer of biofilm present after being observed under microscope. Besides, *Leucobacter luti* and *Bacillus cereus* are among the bacteria that can be found in the suspended biomass before and after MFC operation. There is no any past research that proved the role of these bacteria in electricity generation. They are said to act in balancing the ecosystem of the synthetic wastewater in the reactor.

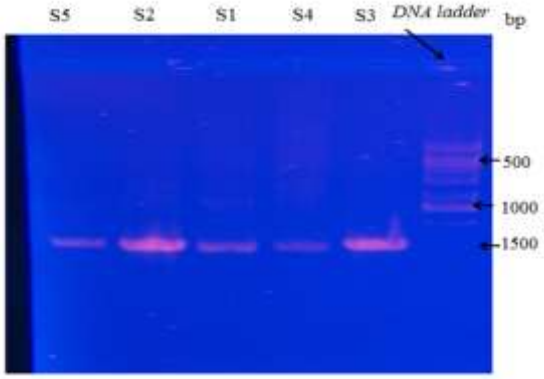


Fig.2: Agarose gel electrophoresis of PCR products.

3.2. Voltage generation

The results of voltage measurement for time 0 to 240 h are shown in Figure 3. At the beginning of the MFC operation, voltage generation for the MFC_{AC} changed in a much consistent way throughout 10 days of MFC operation. As for MFC_{FC}, a drastic dropped of voltage generation was observed until 75 h. This phenomenon has proved that biofilm is more adapted to the aerated condition as compared to the addition of ferricyanide in the reactor that might had poisoned the bacteria in the anode chamber. After around 84h of MFC operation, MFC_{FC} showed a steadier voltage generation which indicates that bacteria are trying to adapt to the condition in the MFC and thus, variation of voltage generation was minimize. When it reached the 172 h of the study, it can be observed that voltage in MFC_{FC} was started to increase slowly until end of the study and these indicate that more time are needed to capture the real potential of MFC in voltage generation. It was found that replacing of oxygen with ferricyanide had successfully increased the voltage generation for 1.4 times from the highest voltage for MFC_{AC} which was 0.589V to 0.841V, the highest voltage generated in MFC_{FC}. Oh et al. (2006) had proved that the result was rational with the increament of 1.8 times of voltage generation when ferricyanide is replacing the aerated condition as catholytes.

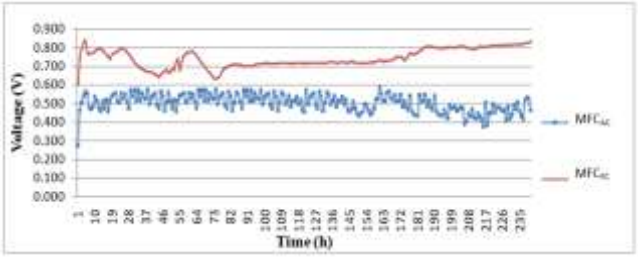


Fig.3: Voltage generation during MFC operation

3.3. COD removal efficiency

COD showed the substrate concentration in the reactor as higher content of COD indicates that the carbon component in synthetic wastewater are in great concentration. From the analysis, we observed that MFC_{FC} had the COD removal efficiency which wass steadier than MFC_{AC}. As illustrated in Figure 4 and Table 2, MFC_{AC} was found to achieve the highest COD removal efficiency of 51.30 % while MFC_{FC} noted a higher removal rate of 93 %.

Table 2: COD removal efficiency

Catholytes	Time (h)	COD (mg O_2/L)	COD removal efficiency (%)
MFC_{AC}	0	2028	
	48	2028	0.00
	96	1768	12.82
	144	1768	12.82
	192	1872	7.70
	240	988	51.30
MFC_{FC}	0	2268	
	48	1968	13.23
	96	324	85.71
	144	324	85.71
	192	270	88.10
	240	162	92.86

Table 3: Comparison of COD removal efficiency in this study with result from researchers

Anode	Cathode	PEM	COD removal efficiency (%)		References
			Aerated	Ferricyanide	
Carbon paper	Carbon paper with platinum	Nafion 117	86		Min et al., 2005
Graphite	Graphite	Nafion 117	74.15	74.2	Mohan et al., 2008a
Carbon cloth	Carbon cloth with PTFE	Nafion 117	71 - 91		Tunc et al., 2008
Graphite	Graphite	Nafion 117	61.11		Mohan et al., 2008b
Graphite	Graphite	Nafion 117	66.13	68.28	Mohan et al., 2008c
Carbon paper	Carbon paper	Nafion™	51.30	92.86	This study

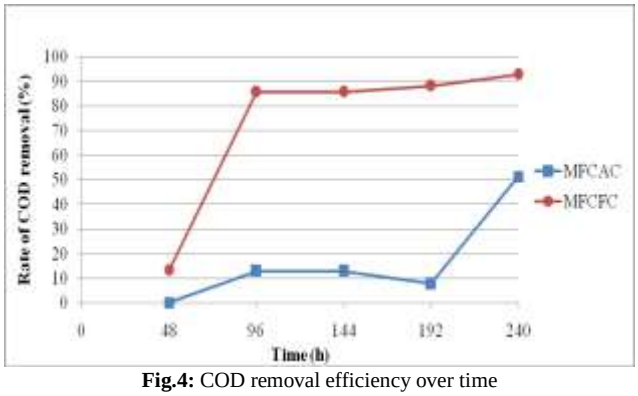


Fig.4: COD removal efficiency over time

From the result, Mohan et al. (2008a) [28] in his study did showed that a reactor with the addition of ferricyanide marked a much higher COD removal efficiency compared to the one with aerated condition. This is due to the time required for aerated reactor to become exhausted is higher compare to the condition with the addition of ferricyanide. Table 3 which indivates the comparison between the result in this study with previous reserchers, showed that the COD removal efficiency in this study marked a higher rate that never have been achieved before.

3.4. Kinetic growth of bacteria

Kinetic growth of bacteria as indicated in Table 4 showed the maximum specific growth rate, μ_{net} for suspended growth biomass was 0.003 h^{-1} while 0.004 h^{-1} for biofilm in MFC_{AC}. As for MFC_{FC}, μ_{net} for biomass was 0.002 h^{-1} while 0.001 h^{-1} for biofilm. Yield, Y for biomass at MFC_{AC} was 2.035 g.cell/g.substrate while Y for biofilm was only 0.1213 g.cell/g.substrate. As for MFC_{FC}, Y for biomass was 1.206 g.cell/g.substrate and 0.3268 g.cell/g.substrate for biofilm. Results show that bacteria are more adapted to aerated condition compared to the ferricyanide catholyte. Kinetic growth of biomass showed that μ_{net} for biofilm at MFC_{AC} was 1.7 times higher than MFC_{FC}. Yet a totally varied condition occurred for voltage generation where MFC_{FC} had 1.4 times greater voltage generation than MFC_{AC}. However, the kinetic growth of biofilm MFC_{FC} were found to be 2.7 times greater than MFC_{AC}. Hence, the biofilm kinetic growth was directly proportionate to the voltage generation and it is said to play a significant role in electricity generation of MFC. Besides, it also proved that the growth rate of biomass which do not directly proportionate to the electricity generation had no influence to the electrivity generation in MFC.

Table 4: Kinetic growth of bacteria in MFC reactor

Reactor	Maximum specific growth rate, (μ_{net}), h^{-1}		Yield, (Y) , g.cell/g.substrate		Substrate utilization rate, L^{-1} jam^{-1}	
	Biomass	Biofilm	Biomass	Biofilm	Biomass	Biofilm
MFC_{AC}	0.003	0.004	2.035	0.121	0.003	0.003
MFC_{FC}	0.002	0.001	1.206	0.326	0.004	0.004

4. Conclusion

BLAST analysis showed that 5 species that can be found in the wastewater are *Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobacter aerogenes, Leucobacter luti* and *Bacillus cereus. Pseudomonas aeruginosa, Klebsiella pneumonia* and *Enterobacter aerogenes* are biofilm and exoelectrogens that played role in voltage generation of MFC. However, *Leucobacter luti* and *Bacillus cereus* are biomass that acted to balance wastewater ecosystem.

MFC performance showed that when oxygen has been replaced with ferricyanide, it increases the voltage generation for 1.4 times with 0.841 V as the highest voltage for MFC_{FC} while 0.589 V for MFC_{AC}. COD removal efficiency for reactor with ferricyanide was almost 100% which is 93%. While under aerated condition, the COD removal rate was merely 51.30 %. In a nutshell, the performance of MFC with the additional of ferricyanide are very much better than aerated condition.

Kinetic growth of biofilm MFC_{FC} were found to be 2.7 times greater than MFC_{AC} which also directly proportional to the increament of voltage generation. However, the kinetic growth of biomass was found deviate with the voltage generation in MFC. So, it can be conclude that biofilm played a significant role in voltage generation in MFC.

Acknowledgement

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