ACID RED 27 BIODECOLOURISATION AND BIOGENIC ELECTRICITY GENERATION IN STACKED MICROBIAL FUEL CELL BY *Citrobacter freundii* A1 AND *Enterococcus casseliflavus* C1

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I would like to dedicate my deepest appreciation to the following peoples, people who are very meaningful in my life. People who always inspires, supports and prays for my success in life.

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ABSTRACT

Microbial fuel cell (MFC) is an electrochemical system which utilises microorganisms to generate electricity via its catalytic activities. Recently, the capability of MFC in generating electricity has been assimilated with wastewater treatment as an alternative approach for a sustainable and eco-friendly technology. Although the MFC has the potential to be synchronised with both the wastewater treatment and electricity generation application, the amount of electricity generated from this technology is still insufficient. This study employed *Citrobacter freundii* A1 and *Enterococcus casseliflavus* C1 bacterial consortia that have been previously isolated and identified in the assessment of azo dye biodecolourisation and biogenic electricity generation in dual-chamber salt bridge MFC. Initially, the feasibility of sequential facultative anaerobic-aerobic treatment for complete dye degradation was evaluated using Acid Red 27 (AR-27) dyes where 98% decolourisation was achieved using 0.5 g/L glucose and 1.0 g/L nutrient broth as co-substrates under static condition for the non-MFC study. Ultra Violet-Visible spectroscopy and Fourier Transform Infrared (FTIR) spectroscopic analyses confirmed that the azo linkage was cleaved after the decolourisation occurred. The cyclic voltammetry analyses also showed that the decolourisation of AR-27 by *C. freundii* A1 and *E. casseliflavus* C1 was an electrochemically irreversible reaction while the detection of oxidation reaction during aerobic treatment proved that the process of mineralisation took place. The degradation of AR-27 was also confirmed by the decrease in catechol concentration detected through High-Performance Liquid Chromatography (HPLC) analysis. Simultaneous electricity generation and wastewater treatment were conducted by connecting two individual MFC in parallel with optimised 5000 Ω external resistance and 3.0 M sodium chloride salt bridge concentration. The maximum voltage recorded by the open circuit voltage and close circuit voltage was 595 mV and 84 ± 15 mV, respectively. While the power and current density generated by the optimised MFC system was 10.15 ± 2 mA/m^2 and 0.86 ± 0.3 mW/m^2. The use of higher concentration of sodium chloride salt bridge and parallel configuration in MFC was able to improve the MFC performance by generating higher current and output power. Scanning Electron Microscope image and bacterial cell number analysis revealed the surface morphology and biofilm development during the MFC operation with the adhesion of microorganisms on the electrode surface. Besides, FTIR analysis on the MFC electrode after operation also showed the presence of biofilm with the detection of extracellular polymeric substances (EPSs) functional groups on the electrode surface. In conclusion, *C. freundii* A1 and *E. casseliflavus* C1 consortium has the potential to be used in simultaneous azo dye wastewater treatment and biogenic electricity generation using the MFC technologies.

ABSTRAK

Sel bahan api mikrob (MFC) merupakan satu sistem elektrokimia yang menggunakan mikroorganisma untuk menjana tenaga elektrik melalui aktiviti pemangkinnya. Baru-baru ini, keupayaan MFC dalam menjana tenaga elektrik telah diasimilasikan dengan rawatan air sisa sebagai kaedah alternatif untuk teknologi yang mampan dan mesra alam. Walaupun MFC ini mempunyai potensi untuk diselaraskan dengan kedua-dua aplikasi rawatan air sisa dan penjanaan tenaga elektrik, jumlah tenaga elektrik yang dihasilkan oleh teknologi ini masih lagi tidak memadai. Kajian ini menggunakan konsortia bakteria *Citrobacter freundii* A1 dan *Enterococcus casseliflavus* C1 yang sebelum ini telah dipencilkan dan dikenal pasti keboleh upayaannya dalam penyahwarnaan bio dan penjanaan tenaga elektrik biogenik di dalam MFC dwi ruang yang menggunakan jambatan garam. Pada mulanya, kebolehlaksanaan rawatan fakultatif anaerobik–aerobik secara berurutan telah diuji untuk degradasi sempurna pewarna Asid Merah 27 (AR-27) yang mana 98% penyahwarnaan telah dicapai dengan menggunakan 0.5 g/L glukosa dan 1.0 g/L kaldu nutrien sebagai substrat bersama dalam keadaan statik untuk kajian yang tidak menggunakan MFC. Analisis spektroskopi Ultra Lembayung-Nampak dan Fourier Infra-Merah (FTIR) telah mengesahkan bahawa ikatan azo telah diputuskan semasa proses penyahwarnaan berlaku. Analisis kitaran voltammetrik menunjukkan bahawa penyahwarnaan AR-27 oleh *C. freundii* A1 dan *E. casseliflavus* C1 merupakan tindak balas elektrokimia yang tidak berbalik sementara pengesanan tindak balas pengoksidaan semasa rawatan aerobik membuktikan proses mineralisasi telah berlaku. Degradasi pewarna AR-27 juga dipastikan melalui penurunan kepekatan katekol yang dikesan melalui analisis Kromatografi Cecair Prestasi Tinggi (HPLC). Penjanaan tenaga eletrik dan rawatan air sisa telah dilakukan secara serentak dengan menghubungkan dua MFC secara selari dengan menggunakan jumlah rintangan luaran 5000 Ω dan kepekatan jambatan garam sodium klorida sebanyak 3.0 M yang telah dioptimakan. Jumlah maksimum voltan yang telah direkodkan dalam litar terbuka dan litar tertutup adalah masing-masing sebanyak 595 mV dan 84 ± 15 mV. Manakala, jumlah ketumpatan kuasa dan arus yang dihasilkan oleh MFC yang telah dioptimumkan adalah 10.15 ± 2 mA/m^2 dan 0.86 ± 0.3 mW/m^2. Penggunaan sodium klorida dengan kepekatan tinggi dan sambungan selari dalam MFC telah membolehkan peningkatan prestasi MFC dalam menjana jumlah arus dan kuasa yang tinggi. Imej mikroskopi elektron pengimbasan dan analisa jumlah sel bakteria telah menunjukkan morfologi permukaan elektrod dan pembentukan biofilem semasa operasi MFC melalui pelekatan bakteria pada permukaan elektrod. Di samping itu, analisis FTIR ke atas elektrod selepas operasi MFC turut menunjukkan kehadiran biofilem dengan pengenalpastian kumpulan berfungi bagi bahan polimer ekstrasel (EPSs) pada permukaan elektrod. Kesimpulannya, konsortia *C. freundii* A1 dan *E. casseliflavus* C1 mempunyai potensi untuk diaplikasikan bagi rawatan air sisa pewarna azo dan penghasilan tenaga elektrik biogenik secara serentak dengan menggunakan teknologi MFC.

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LIST OF ABBREVIATION / SYMBOLS

%	-	Percent
μA	-	Microampere
μL	-	Microlitre
μm	-	Micrometre
μV	-	Microvolt
A	-	Surface area
Abs	-	Absorbances
Abs_{600nm}		Absorbance at the wavelength of 600 nm
A_f	-	Final absorbance
Ag_2SO_4	-	Silver sulphate
A_i	-	Initial absorbance
AR-27	-	Acid Red 27
BOD	-	Biological oxygen demand
C/N	-	Carbon per nitrogen
CCV		Close circuit voltage
CFU/mL	-	Colony forming unit per milli litre
Cm	-	Centimeter
COD	-	Chemical oxygen demand
$CuSO_4$	-	Copper sulphate
CV	-	Cyclic voltammetry
DET	-	Direct electron transfer
DNS	-	Dinitrosalicyclic
e^-	-	Electrons
EDX	-	Energy dispersive X-ray
EPSs	-	Exopolysaccharides
FADH	-	Flavin adenine dinucleotide
$FeCl_2$	-	Ferrous dichloride

FTIR	-	Fourier Transform Infrared spectroscopy
G	-	Gram
g/L	-	Gram per liters
g-unit	-	G – Force
h	-	Hours
H^+	-	Hydrogen ion
H_2SO_4	-	Sulphuric acid
$HgSO_4$	-	Mercury sulphate
HPLC	-	High performances liquid chromatography
I	-	Current
J	-	Current density
$K_2Cr_2O_7$	-	Potassium dichromate
K_2HPO_4	-	Dipotassium hydrogen phosphate
KCl	-	Potassium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
kPa	-	Kilopascal
$kW \cdot h \cdot m^{-3}$	-	Kilowatt hour per metre cube
L	-	Litre
LC-MS	-	Liquid chromatography – mass spectrometry
M	-	Molar
mA	-	Milliampere
MFC	-	Microbial fuel cell
mg/mL	-	milligram per millilitre
mL	-	Milliliters
mL/min	-	Millilitre per minute
mM	-	Millimolar
mm	-	Millimetre
$MnCl_2.2H_2O$	-	Manganese (II) chloride dehydrate
MP5 medium	-	Modified P5 medium
mS/cm	-	Milli siemens per centimetre
mV	-	Millivolt
mV/s	-	Millivolt per second
mW/m^2	-	Milliwatts per metre square

MΩ	-	Mega ohm
Na_2CO_3	-	Sodium carbonate
NaCl	-	Sodium chloride
NADH	-	Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide adenine dinucleotide phosphate
$NaMoO_4.2H_2O$	-	Molybdic acid sodium salt dehydrate
nm	-	Nanometre
Ø	-	Diameter
^oC	-	Degree Celsius
OCV	-	Open circuit voltage
OD	-	Optical density
OM	-	Outer membrane
P	-	Power density
PEM	-	Proton exchange membrane
R_{ext}	-	External resistance
RPM	-	Revolution per minutes
SS	-	Suspended solid
TOC	-	Total organic carbon
TPP	-	Total polyphenol
UV-Vis	-	Ultra violet visible
V	-	Volt
v/v	-	Volume to volume
w/v	-	Weight to volume
λ	-	Wavelength
λ_{max}	-	Maximum wavelength
Ω	-	Ohm

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CHAPTER 1

INTRODUCTION

1.1 Background Study

The production of sustainable energy and wastewater treatment has introduced an area of interest among the global community due to the facts that the world is facing severe environmental problems and global energy crisis. In term of energy crisis, issues related to fossil fuel depletion and non-renewable energy resource shortages have been debated as it is crucial for the industrialisation and urbanisation activities (Karthikeyan and Kanchana, 2014). Intensive effort has been proposed and developed in order to obtain a more sustainable treatment in handling the pollution issues which corresponds to the rapid industrialisation and urbanisation. This modernisations activities results in severe global issues such as the discharge of large amounts of waste into the environment either through the water body, ground or air which in turn creates more pollution (Karthikeyan and Kanchana, 2014). This situation leads to environmental concerned as their biorecalcitrance might carry potential toxicity effects on plants, animals and humans being (Martin *et al.,* 2002).

Currently, water pollution which is due to the discharged of wastewater into water bodies is one of the major concerns among the global community. It was reported that many industrial activities have been discharging their manufacturing waste into the water body without properly treating their waste before releasing it into the environment. One of the components that cause water pollution is coloured effluents consisting of dyes which are released by the textile dyestuff and dyeing industries (Idris *et al.,* 2007). Such pollution is particularly associated with the reactive dyes, which accounts for a significant proportion of the total dye market (Karthikeyan and Kanchana, 2014). Moreover, in recent reactive dyeing processes, it is estimated that up to 50% of the dye used was lost to the wastewater (Mu *et al.*, 2009; Saranraj, 2013; Saranraj and Sivasakthivelan, 2014) due to the relatively low dye fiber fixation.

Azo dye is an aromatic compound containing one or more azo bond (–N=N–) and it is widely used as major components by the industry (Chen *et al.*, 2003). However, these dyes are highly stable and resistant to microbial degradation which makes it not easily degradable under natural condition or by conventional wastewater treatment process (Stolz, 2001). Current treatment such as Physio-chemical method has been applied to remove dye from textile wastewater, but its application are expensive and ineffective (Pandey *et al.*, 2007). Furthermore, this method will cause secondary pollution problems and producing concentrated sludge as by-products in which difficult to be disposed (Pearce *et al.*, 2003).

Thus, a more convenient and environmentally friendly wastewater treatment had been introduced using biological approach which involves microorganisms (Zhang *et al.*, 2004; Saratale *et al.*, 2011; Abdul-Wahab *et al.*, 2013). It was found that a variety of bacterial species that are not only capable of decolourising, but also able to completely mineralise many reactive dyes under certain conditions (Kumar *et al.*, 2012). Bacterial degradation of dyes is often initiated under static/anaerobic conditions by an enzymatic transformation reaction for dye decolourisation which results in the formation of aromatic amines (Kumar *et al.*, 2012). Then, this aromatic amine ares further oxidised and mineralised to form a simpler non-toxic by-product under aerobic conditions (Chan *et al*. 2012c; Kumar *et al.*, 2012).

Recently, fuel cells technology has received attention as an alternative approach for renewable energy generation due to the high energy density, up scaling applicability and simple modular use (Evan *et al.*, 2012). This technology will enables the conversion of electrochemical energy for electricity generation and storage (Evan *et al.*, 2012). The electricity produced in fuel cell is obtained through the reaction between the fuel (anode) and oxidant (cathode) in the presence of an electrolyte (Evan *et al.,* 2012). Compared to batteries, fuel cell requires the reactant (anode) to be replenished due to the electrochemical reaction consumption. One of the example of fuel cell that is currently been study for electricity generation is Microbial Fuel Cell (MFC). MFC uses the principle of converting organic matter into electrical energy through the microorganism's catalytic activities (Chaudhuri and Lovley, 2003). In MFC, the electrochemically active microorganisms in anode oxidise the organic co-substrates that eventually generate electron (Logan *et al.,* 2006; Lai *et al.,* 2017). This electron is then transferred to the MFC cathode through an external circuit with the assistance of the microorganisms to generate current. Ion exchange membrane is fixed in the MFC to allow proton migration while separating the anode and cathode chamber (Logan *et al.,* 2006; Solanki *et al.,* 2013; Lai *et al.*, 2017). Hence, it is plausible for azo dyes to be introduced into the MFC as an anolyte (anode analyte) for the simultaneous azo dye treatment and electricity generation based on the electron generated by the metabolic activity of azo degrading bacteria (Sun *et al.,* 2011). The application of this technology has also the potential to provide an alternative clean and renewable form of energy in the near future.

1.2 Problem Statement

The textile industry is one of the fastest growing industries and contributes significantly to the economic growth in Malaysia. According to previous report, Malaysia is the ninth largest producer and exporter of textile fiber in the Asian region in 2008 which rise to seventh in 2011 (Pang and Abdullah 2013; Esho, 2015). Although the textile industry contributes positively toward the Malaysian economic growth, it was found that the industry pose a significant threat to the environmental quality, especially in terms of liquid effluent pollution and high energy consumption operational system. Moreover, this untreated textile wastewater may cause harm to the environment due to its xenobiotic and carcinogenic properties (Kumar *et al.*, 2012). In response to the increasing cost of energy, the Malaysian government has focused on strengthening its conservation policies. The government is also continually reviewing its energy policy to ensure sustainability of the energy resources (Mohamed and Lee, 2006) as it was estimated that the primary energy consumption would triple by 2030 (Gan and Li, 2008). Furthermore, the global warming issues and exhaustion of fossil fuels together with unstable petroleum prices in the global market have encouraged the Malaysian government to start focusing on renewable energy as a promising sources in the global energy mix in line with the National Energy Policy (1979). As an alternative (with the agreement of the National Renewable Energy Policy and Action Plan (2009) that encourages the innovation and the invention of Malaysian renewable energy sources), a novel process, i.e., MFC technology was chosen to be adapted for electrical energy production in wastewater treatment. As an energy source, wastewaters show a plausible outcome to be utilised as the MFC anolyte due to diverse types of organic substrate (Rahimnejad, 2015).

Although currently the idea of MFC being a power generator is not sufficient for large or industrial scale due to its low power output, especially by using single unit MFC (Logan, 2008; Gurung and Oh, 2012), the production of energy from the wastewater treatment in MFC should be given applause. This pilot study focused on the increase in electricity generation and enhance in wastewater quality analysis by using synthetic textile wastewater model. Several studies have recommended optimisation which includes the electrogenic azo degrading bacteria and the MFC system itself. The optimisation is crucial, especially for the application of MFC in textile wastewater treatment due to the fact that the system has to treat the wastewater efficiently while simultaneously produce electrical energy. For example, the concentration of co-substrates use by azo degrading bacteria must be sufficient to treat the textile wastewater and an ideal operating system must be developed to enhance the MFC performance. However, materials cost for MFC, the proton accumulation within the biofilm and over potential at the MFC are just a few problems that need solutions. Besides, the maximum power production is limited by internal resistances, ohmic losses in the solution, electrochemical losses at the electrodes, and bacterial metabolic losses (Ter Heijne *et al.,* 2011).

Earlier, Chan *et al.,* (2011) has isolated, identified and developed azo degrading bacterial consortia called NAR-I which composed of *Enterococcus casseliflavus* C1 and *Enterobacter cloacae* L17. This NAR-I bacteria consortium has the ability to achieve 95% decolourisation using Acid Orange 7 (AO7) dyes within

60 minutes incubation. Later, a novel azo degrading bacteria consortia was formed with the name NAR-II consisting of *Citrobacter freundii* A1, *Enterococcus casseliflavus* C1 and *Enterobacter cloacae* L17 which possessed the ability to achieve nearly 100% decolourisation within 30 minute incubation using Acid Red 27 (AR-27) dyes. These two distinctive azo dye decolourisation studies were performed under facultative anaerobic condition with the addition of co-substrates (glucose and nutrient broth) and synthetic dyes.

To date, the NAR-II bacteria consortium performance has been assessed for electricity generation by using a dual chamber (H-type) MFC for azo dye decolourisation using glucose (5.0 g/L) and nutrient broth (10.0 g/L) as co-substrates (Kardi *et al.,* 2016). The results showed the potential of simultaneous electricity generation in MFC and azo dye removal by achieving maximum voltage of 0.950 V for open circuit voltages (OCV), maximum power density 951 mW/m^2 (300 Ω) and 93% decolourisation using 0.3 g/L AR-27 within 24 hours at fixed the temperature of 30ºC (Kardi *et al.*, 2016).

However, the performance of decolourisation of azo dye and bioelectricity generation via stacked microbial fuel cell (MFC) by using a bacteria combination of *C. freundii* A1 and *E. casseliflavus* C1consortia has yet been studied. *Citrobacter* sp. strain A1 was isolated from a sewage oxidation pond, which is characterised as a Gram-negative enteric coccobacillus, facultative aerobe and mesophilic dye-degrading bacterium (Chan *et al*., 2012a). This organism degrades azo dyes efficiently *via* azo reduction and desulfonation, followed by the successive biotransformation of dye intermediates under aerobic environment (Chan *et al*., 2012a). In contruct, *Enterococcus* sp. strain C1 is a Gram-positive facultative anaerobe which was co-isolated with *Citrobacter* sp. strain A1 from a sewage oxidation pond (Chan *et al*., 2012b) and could degrade azo dyes very efficiently via azo reduction and desulfonation in a microaerophilic environment (Chan *et al*., 2012b).

Hence, this study focused on the azo dye treatment using an novel azo degrading bacterial consortium consisting *C. freundii* A1 and *E.*

casseliflavus C1 while simultaneously performing a series of optimisation for the salt bridge MFC system in order to increase the bioelectricity generations in the form of stacked microbial fuel cell (MFC). In this study, MFC that use salt bridge for the proton exchange was initially optimised before being tested for stacked MFC. The setup of a stacked MFC involves the connection of multiple units of individuals MFC through serial or parallel connection configuration. Therefore, it is crucial to fully grasp the basic operation for the application of stacked microbial fuel cell for electricity generation.

1.3 Objective of Research

Based on current understanding and recent study on azo dye decolourisation and MFC application, this study was performed to assess the potential of azo dye degrading bacteria in an optimised stacked MFC. This includes the determination of azo dye removal by the bacteria consortium and biogenic electricity performance in the MFC. Hence, these objectives were established in the research to achieve the research aim:

a). To investigate the performance of azo dye decolourisation by using *Citrobacter freundii* A1 and *Enterococcus casseliflavus* C1 bacterial consortium with Acid Red 27 (AR-27) as the dye model.

b). To design, construct and optimise the operating condition of MFC for bioelectricity generation and wastewater treatment.

c). To characterize the biofilm formation on MFC anode electrode during the MFC operation based on AR-27 decolourisation.

1.4 Scope of Research

The main scope of this research was to assess the performance of *the C. freundii* A1 and *E. casseliflavus* C1 bacteria consortium in stacked salt bridge MFC. Hence, these scopes were established to accomplish the azo dye decolourisation and biogenic electricity study.

This study investigated the performance of *C. freundii* A1 and *E. casseliflavus* C1 bacteria consortium in azo dye decolourisation and degradation by using modified P5 (MP5) medium with AR-27 as the dye model under sequential facultative anaerobic-aerobic conditions. Here, the effect of co-substrates concentration (glucose and nutrient broth) in modified P5 medium was optimised by lowering the concentration of the substrates based on previous studies (Chan *et al.*, 2011; Chan *et al.*, 2012c; Kardi *et al.*, 2016).

For the MFC study, the constructions of dual chamber salt bridge MFC for biogenic electricity generation was initially conducted in which several parameters were evaluated such as external loads, salt bridge concentration and connection configuration (series/parallel).

Next, the study continues on the performance of optimised stacked salt bridge MFC for azo dye decolourisation in terms of wastewater treatment and biogenic electricity generation under sequential facultative anaerobic - aerobic conditions. Hence, the study demonstrated the first generation of stacked salt bridge MFC operated under sequential facultative anaerobic-aerobic conditions.

Lastly, the morphological study of biofilm formation on the anode surface area throughout the MFC operation was performed in order to monitor the biofilm development during the MFC operation.

1.5 Significance of Research

Based on earlier studies, the biogenic electricity generation by MFC using azo dye was usually centred on the azo dye treatment using an MFC system which implemented single or stacked proton exchange membrane (MFC) for power generation. However, this study focused on determining the biogenic electricity generation performance of salt bridge stacked using *azo degrading* bacteria consortium for azo dye decolourisation and dye removal using AR-27 dye. The main idea for the study was to use the *C. freundii* A1 and *E. casseliflavus* C1 bacteria in the form of consortium as these bacteria combination has yet been studied for the decolourisation and electricity generation in MFC. Furthermore, this study attempted to assess the stacked MFC potential for a higher electricity generation. Hence, the performance of the bacteria consortium were initially evaluated at an optimised co-substrates concentration using synthetic wastewater under sequential facultative anaerobic-aerobic condition before being applied into the MFC systems. Then, the optimised synthetic textile wastewater was introduced into the optimised salt bridge stacked MFC for the assessment of biogenic electricity generation. Therefore, the problem of low voltage production by salt bridge MFC can be theoretically solved by using the selected optimum conditions for higher voltage generation. This study could provide a solution for the current treatment of textile effluent and as an alternative green energy in the future.

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