BIODEGRADATION OF AR-27 DYE AND ELECTRICITY GENERATION BY *Enterobacter cloacae* NF2015

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DEDICATION

Dedicated to all people that I treasure most in life…

To my Beloved Father and Mother

Yahya bin Abdullah and Norhayati binti Othman

To my Beloved Father and Mother in Law

Mohamad bin Jusoh and Zainab binti Ali

my Beloved Husband and daughter

Ahmad Zainal bin Mohamad and Nuur Hasya Ammara Hawani bt Ahmad Zainal

To my respectful supervisor

Dr. Norahim bin Ibrahim

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ABSTRACT

One of the major concerns of developing countries worldwide is wastewater treatment and renewable energy generation. Wastewater by-product from production activity is the cause of pollution by industries. Microbial degradation is considered a good alternative to replace wastewater treatment approaches using physical and chemical method that are less desirable due to high costs and sludge production. This study was carried out to screen and identify the potential of unidentified bacterial strains POS, F2B, PCO OIL, and B1, previously isolated from Iraqi crude oil reservoir to decolorize azo dye and implementing the system in Microbial Fuel Cell (MFC) for bioelectricity generation. The selected bacterium was identified using 16S rDNA method. The effect of co-substrates was studied to determine the minimal conditions required for maximum decolorization of AR-27 dye. Analysis of degradation were performed using UV-Vis, FTIR, CV, COD, DNS, TPP content and HPLC under facultative anaerobic and sequential facultative anaerobic-aerobic condition. Bioelectricity study was conducted using two-chambered MFC using agar salt bridge and graphite felt electrodes. The effect of different diameter of salt bridge and electrode size to electricity generation was studied. Bacteria B1 identified as *Enterobacter cloacae* NF2015 was found to be the most dominant decolorizing bacteria that could decolorize 99 % of 100 mg/L of AR-27 within 2 h under facultative anaerobic condition, pH 7.0, and temperature of 29 ^{o}C ± 2. Yeast extract and glucose at 0.5 g/L each was found to be the optimum co-substrates for AR-27 decolorization. The disappearance of peak in the UV–Vis spectra at 521 nm indicated the biodegradation of azo dye. The presence of oxidation and reduction peak by cyclic voltammetry analysis showed an irreversible redox reaction during the degradation. FTIR-ATR analysis confirmed that the azo linkage was cleaved after decolorization had occurred. The reduction in concentration of metabolite catechol detected by HPLC analysis confirmed a successful degradation of AR-27 dye. There was 87.6 % and 100 % COD removal after 72 hours treatment in facultative anaerobic and facultative anaerobic-aerobic condition, respectively. Optimization of the MFC voltage using 4.5 cm diameter salt bridge and 25 cm^2 electrode surface area showed that the maximum OCV and CCV obtained were 809.7 ± 12 mV and 108.3 ± 19 mV, with current density and power density at 1.93 ± 0.3 mA/m^2 and 0.21 ± 0.04 mW/m^2. SEM and FTIR analysis revealed biofilm and extracellular polymeric substances (EPSs) functional groups development on the electrode surface during MFC operation. In conclusion, bacteria *E. cloacae* NF2015 from crude oil reservoir has the potential to be used in simultaneous azo dye wastewater treatment and bioelectricity generation by MFC technologies.

ABSTRAK

Salah satu kebimbangan utama negara-negara membangun di seluruh dunia ialah rawatan air sisa dan penjanaan tenaga boleh diperbaharui. Air sisa yang terhasil daripada aktiviti pengeluaran merupakan penyebab pencemaran daripada perindustrian. Degradasi mikrob dianggap alternatif yang baik untuk menggantikan pendekatan semasa rawatan air sisa menggunakan kaedah fizikal dan kimia yang kurang diidami akibat kos tinggi dan pengeluaran enapcemar. Kajian ini dijalankan untuk menilai dan mengenal pasti potensi bakteria-bakteria yang belum dikenalpasti iaitu POS, F2B, PCO OIL, dan B1 yang dipencil daripada minyak mentah Iraq untuk menghuraikan pewarna azo dan seterusnya melaksanakan sistem tersebut dalam Sel Bahan Api Mikrob (MFC) untuk penjanaan bioelektrik. Bakteria yang dipilih telah dikenalpasti menggunakan kaedah rDNA 16S. Kesan substrat bersama telah dikaji untuk menentukan keadaan minimum yang diperlukan untuk penghuraian pewarna AR-27 oleh bakteria. Analisis degradasi telah dilakukan menggunakan UV-Vis, FTIR, CV, COD, DNS, kandungan TPP dan HPLC di bawah keadaan anaerobik fakultatif dan anaerobik fakultatif-aerobik berturutan. Kajian bioelektrik dijalankan menggunakan MFC dua ruang dengan jambatan garam dan elektrod karbon. Kesan diameter jambatan garam dan saiz elektrod yang berbeza kepada penjanaan elektrik telah dikaji. Bakteria B1 dikenalpasti sebagai *Enterobacter cloacae* NF2015 yang didapati sebagai penghurai paling dominan dapat menjernihkan 100 mg/L AR-27 sebanyak 99 % dalam masa 2 jam di bawah keadaan anaerobik fakultatif, pH 7.0, dan suhu 29 ^{o}C ± 2. Ekstrak yis dan glukosa masing-masing pada 0.5 g/L didapati substrat yang optimum untuk penghuraian AR-27. Kehilangan puncak dalam spektrum UV-Vis 521 nm menunjukkan biodegradasi pewarna azo. Kehadiran puncak pengoksidaan dan penurunan yang dikesan dalam analisis kitaran voltametri menunjukkan reaksi redoks tidak terbalikan berlaku semasa degradasi. Analisis FTIR-ATR mengesahkan bahawa rangkaian azo telah dipecahkan selepas penjernihan berlaku. Pengurangan kepekatan metabolit katekol yang dikesan oleh analisis HPLC mengesahkan degradasi pewarna AR-27. Terdapat 87.6 % dan 100 % penyingkiran COD selepas 72 jam rawatan dalam keadaan anaerobik fakultatif dan anaerobik-aerobik fakultatif. Pengoptimuman voltan MFC menggunakan jambatan garam berdiameter 4.5 cm dan luas permukaan elektrod 25 cm^2 menunjukkan maksimum OCV dan CCV iaitu 809.7 ± 12 mV dan 108.3 ± 19 mV, kepadatan arus dan ketumpatan kuasa sebanyak 1.93 ± 0.3 mA/m^2, dan 0.21 ± 0.04 mW/m^2, masing-masing. Analisis SEM dan FTIR mendedahkan pembentukan kumpulan berfungsi biofilm dan bahan polimer ekstrasel (EPS) pada permukaan elektrod semasa operasi MFC. Sebagai kesimpulan, bakteria *E. cloacae* NF2015 dari takungan minyak mentah mempunyai potensi untuk digunakan dalam rawatan air sisa pewarna azo serentak dengan penjanaan bioelektrik oleh teknologi MFC.

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LIST OF ABBREVIATIONS

Abs	-	Absorbances
Abs_{600nm}	-	Absorbance at the wavelength of 600 nm
A_f	-	Final absorbance
A_i	-	Initial absorbance
AR-27	-	Acid Red 27
Asa	-	Anode total surface area
BOD	-	Biological oxygen demand
CCV	-	Close circuit voltage
CDM	-	Chemically defined media
COD	-	Chemical oxygen demand
CV	-	Cyclic voltammetry
C_i	-	Initial COD
C_f	-	Final COD
DNA	-	Deoxyribonucleic acid
DNS	-	Dinitrosalicyclic
EDX	-	Energy dispersive X-ray
EDTA	-	Ethylenediaminetetraacetic acid
FA	-	Facultative anaerobic
FA-A	-	Sequential facultative anaerobic-aerobic
FADH	-	Flavin adenine dinucleotide
$FMNH_2$	-	1,5-dihydro flavin mononucleotide
FTIR	-	Fourier transform infrared spectroscopy
gDNA	-	Genomic DNA
H_2SO_4	-	Sulphuric acid
$HgSO_4$	-	Mercury sulphate
HPLC	-	High performance liquid chromatography
I	-	Current
ID	-	Current density
$K_2Cr_2O_7$	-	Potassium dichromate
K_2HPO_4	-	Dipotassium hydrogen phosphate

KCl	-	Potassium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
MFC	-	Microbial fuel cell
Na_2CO_3	-	Sodium carbonate
NaCl	-	Sodium chloride
NADH	-	Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NB	-	Nutrient broth
$(NH_4)_2SO_4$	-	Ammonium sulphate
OCV	-	Open circuit voltage
OD	-	Optical density
P	-	Power
PD	-	Power density
PEM	-	Proton exchange membrane
R	-	Resistance
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
SEM	-	Scanning electron microscope
sp	-	species
TPPC	-	Total polyphenolic content
TOC	-	Total organic carbon
UV-Vis	-	Ultra violet visible

LIST OF SYMBOLS

%	-	Percent
µA	-	Microampere
µL	-	Microlitre
µm	-	Micrometre
µM	-	Micromolar
A	-	Ampere
cm	-	Centimetre
cm^2	-	Centimetre square
e^-	-	Electrons
g	-	Gram
g-unit	-	G-Force
g/L	-	Gram per litre
h	-	Hours
H^+	-	Hydrogen ion
kΩ	-	Kilo ohm
kg	-	kilogram
kPa	-	Kilopascal
L	-	Litre
M	-	Molar
mA	-	Milliampere
mA/m^2	-	Milliampere per metre square
mg/mL	-	Milligram per millilitre
mL	-	Millilitre
mL/min	-	Millilitre per minute
mM	-	Millimolar
mV	-	Millivolt
mW	-	Miliwatt
mW/m^2	-	Miliwatts per metre square
min	-	minute
nm	-	Nanometre

^oC	-	Degree Celsius
pmol	-	Picomoles
rpm	-	Revolution per minute
sec	-	second
V	-	Volt
v/v	-	Volume per volume
w/v	-	Weight per volume
λ	-	Wavelength
λmax	-	Maximum wavelength
Ω	-	Ohm

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The management of industrial waste continues to be a major challenge in urban areas throughout the world, particularly in the rapidly growing cities and towns of the developing world. The lack of an effective and efficient industrial waste management system has had negative impact on the environment. According to Department of Environmental (DOE)'s Environmental Quality Report 2013, industrial effluent coming from manufacturing industries is one of the major sources of water pollution in Malaysia (DOE, 2013). The rise in the number of industries in Malaysia, including textile dyeing operations, has seriously increased the pollution that the country is experiencing. Textile industry is the major source of wastewater and accounts for 22 % of the total volume of industrial wastewater produced in Malaysia (Idris *et al.*, 2007).

Water pollution issue coming from industrial discharging effluents is life threatening. Among the various stages of textile production, the operations in the dyeing plant, which include pre-treatments, the dyeing and finishing, produce the most pollution. Previous report showed that the textile industry which contains various not eco-friendly dyes and organic compounds that are not eco-friendly is a major source of wastewater (Idris *et al.*, 2007). It also contains higher chemicals, high concentration of heavy metals, Biological Oxygen Demand (BOD), and also dissolved solid (Sharma *et al.*, 2007). The textile dyeing wastes are often of strong color and may also be of high temperature. When disposed into water bodies or onto land, these effluents result in the deterioration of ecology and aquatic life.

The biggest dye consumer is textile industry (Asad *et al*. 2007). The dye used in the printing and dyeing of textile product and discharging about approximately thirty thousand tons of the dye material annually (Anjaneya *et al*., 2011). 80% of annual commercial dyes production comes from azo dye in most countries due to their easiness of manufacturing methodology. There are over ten thousand dyes available commercially with a production of exceeding 7×10^5 tons per year (Fu & Viraraghavan, 2001). Industries such as paper, textile, and food had major usage of azo dye (Bazin *et al*., 2012; Anjaneya *et al*., 2011). Their presence in wastewater affects transparency and photosynthesis by limiting the oxygen and light passing through (Sun *et al*., 2009). Besides, the dye itself and the degradation products could be fatal and harmful to aquatic organisms which eventually affect human life via the food chain. Most of the azo dyes are either nontoxic or inert, but become toxic, mutagenic and carcinogenic upon their biotransformation (Kodam *et al*., 2006).

Azo dyes are aromatic compounds that contain one or more azo bonds –N=N- or chromophore which make up the color (Anjaneya *et al*. 2011). Azo dyes are electron-deficient xenobiotic compounds as they contain the electron-withdrawing groups azo (N=N) and sulfonic ($-SO_3^-$), causing the molecule to be an electron deficiency and subsequently less likely to undergo oxidative catabolism by bacteria (Sahasrabudhe & Pathade, 2012). Hence, azo dyes tend to persist under aerobic environmental conditions. Biological approach was chosen for wastewater treatment instead of other approaches such as physicochemical methods which involve the process of coagulation–flocculation, chemical precipitation, membrane filtration, and ion exchange (Kurniawan *et al.*, 2006) due to the costly operation and complicated process needed. In addition, biological approach is more environment-protective and economically feasible in wastewater treatment process.

In accordance with the Act and Environmental Rules (1997) it is mandatory for textile dyeing factories to install effluent treatment plants (ETPs) to treat wastewater before it leaves the factory premises. International pressure for effluent treatment is also increasing and many international buyers are now showing more concern over whether or not textiles are produced in an environmentally friendly way. Considering both factors of environmental pollution and serious health-risk caused by azo dyes, searching alternative ways to treat synthetic dyes are essential particularly for small scale textile industries. This is due to the fact that most of the textile dyeing and processing industries are located in developing countries whereby rivers are the main source of daily activities and drinking water. Most of the textile industries in Malaysia use conventional treatment methods to treat the textile effluents. the methods are effective in reduce the COD reading and removing the fiber of the wastewater however, they are not effective to decolorize the wastewater due to the presence of recalcitrant sulpho and azo groups in the zo dyes (Kulla *et al*., 1983). This resulted in the remaining color in the treated textile wastewater.

Recently, fuel cells technology has received an exceptional attention as a new process for alternative wastewater treatment and renewable energy generation due to the high energy density, up scaling applicability and simple modular use (Evan *et al*., 2013). In view of the overwhelming energy crisis, dependency upon fossil fuels has become a major concern. Among other sustainable and renewable resources, this bioenergy approach is considered as the most efficient way. A device that converts chemical energy from organic and inorganic matter into electrical energy through catalytic activities of microorganisms is called a microbial fuel cell (MFC). Electrons produced by the bacteria from substrates degradation are transferred from the anode to the cathode that is connected by a conductive material containing resistor. The protons in the anode migrate to the cathode and then combine with the electron and reduced by a catholyte such as oxygen to form water at the cathode surface.

The merging of the generation of power from wastewaters with the oxidation of organic or inorganic compounds is one of the vital areas of MFC research. Since the MFCs research in the pilot scale has reached a novelty success, it has boosted a research improvement globally in recent years. Even though MFC can directly generate electricity from the breakdown of organic matter, but optimization of MFC are required to increase and achieve sustainable power output.

1.2 Problem Statement

One of the fastest growing industry that is significantly contributed to Malaysia's economic growth is the textile industry Previous report stated that Malaysia is the seventh largest producer and exporter of textile fibre in the Asian region in 2011 (Esho, 2015). Although the textile industry contributes positively toward the Malaysian economic growth, it was found that the industry poses a significant threat to the environmental quality, especially in terms of liquid effluent pollution and high energy consumption operational system. For the past ten years, it shows that total amount of scheduled waste generated by textile industry in Malaysia increased sharply from 744 tons in 2007 to 1559 tons in 2009 with the total water consumption in a textile industry reach as high as 3000 m^3/day (Pang & Abdullah, 2012).

The Malaysia government has focused on improving its conservation policies in response to the increasing cost of energy. The government is also continually revising its energy policy to ensure sustainability of the energy resources (Mohamed & Lee, 2006) as it was estimated that the main energy consumption would increase by 2030 (Gan & Li, 2008). Besides, the depletion of fossil fuels and global warming issues together with unstable petroleum prices in the global market have encouraged the Malaysian government to start searching on renewable energy as an alternative source in line with the National Energy Policy (1979). The idea of treating the textile wastewater with simultaneous electricity generations in Microbial fuel cell ensure sustainability of the energy resources due to increasing cost of energy (Mohamed and Lee, 2006) and at the same time solving the water pollution problem in respond National Renewable Energy Policy and Action Plan (2009). Wastewaters show a convincing outcome as an energy source to be utilised as the MFC anolyte due to diverse types of organic substrate (Rahimnejad, 2015).

Hence, this pilot study focused on to enhance in wastewater quality analysis and to increase in electricity generation by using synthetic textile wastewater model. Several studies have recommended optimization which includes the electrogenic azo degrading bacteria and the MFC system itself. The optimization of textile wastewater treatment is crucial prior to MFC due to the fact that the system has to treat the wastewater efficiently while simultaneously produce electrical energy. The slow rate of substrate degradation is the main obstacle in biodegradation, so it is important for the newly isolated microbes to be acclimatized and the condition need to be optimized for the decolorization in flask. It was found that there is far greater diversity of electrogens which are electrochemically active biofilms in MFCs. Novel electrogenic bacteria remain undiscovered and unidentified. Meanwhile, many strains which have been identified by 16S rRNA studies remain to be cultured in the laboratory and their contribution to electricity generation have not yet to be confirmed (Zhang *et al*., 2011). Besides, the maximum power production is limited by internal resistances, bacterial metabolic losses, electrochemical losses at the electrodes, and ohmic losses in the solution (Sleutels *et al.*, 2012).

Previously, and unpublished research by Bushra (Phd) has isolated and developed unidentified bacteria strains from sour crude oil. These bacteria strains have the ability to desulfurize the hydrocarbon. This finding had become a stepping stone for further implementation of the bacterial strains in wastewater treatment using synthetic textile wastewater and azo dye AR-27 (contain sulphur) used as a model.

The performance of decolorization of azo dye and bioelectricity generation via microbial fuel cell (MFC) by using *E. cloacae* bacteria from crude oil has yet been studied. Hence, this study focused on the azo dye treatment using a novel azo decolorizing bacterial strain *E. cloacae* NF2015 while simultaneously performing a series of optimization 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 and the graphite felt electrode were optimized.

1.3 Research Objectives

The main objective of this study is to screen the newly isolated bacteria from crude oil for AR-27 dye decolorization and optimize its condition to improve biodecolorization, biodegradation, and bioelectricity performance. The objectives of this study were as follows.

- To select and identify bacteria isolated from Iraqi crude oil for their ability to decolorize Azo dye (AR-27) wastewater.

- To revise AR-27 dye decolorization by the selected bacteria strain through optimization of co-substrates and treatment condition.
- To improve bioelectricity generation by the selected bacteria using different salt bridge diameter and electrode size in double-chambered microbial fuel cell.

1.4 Research Scope

The purpose of the current investigation is to identify that crude oil hydrocarbon degrading bacteria, and to elucidate their potential to degrade another form of hydrocarbon which is the azo dye AR-27. Four bacteria species POS, POS M2, B1, and F2B which were previously isolated from Iraqi crude oil reservoir were screened for the ability to decolorize Chemically Defined Media (CDM) containing azo dye Acid Red (AR) 27. The screening was performed using mixed bacteria culture under facultative anaerobic condition. The analysis of the azo dye degradation present in the synthetic wastewater sample was based on the absorbance reading at wavelength 521nm.

Out of these bacteria, the most potential azo dye decolorizing bacteria were characterized and identified using biochemical and molecular techniques. The effects of co-substrates (carbon and nitrogen source) were optimized by selecting the suitable carbon and nitrogen sources and adjusting the concentration thus improving decolorization efficiency simultaneously. The efficiency of selected bacteria to biodegrade azo dye medium was determined by analyzing the media before and after treatment using Ultraviolet-visible spectroscopy (UV-Vis), Fourier-transform infrared spectroscopy (FTIR), and High-performance liquid chromatography (HPLC) as well as the analysis of Chemically oxygen demand (COD) reduction, Total polyphenolic (TPP) content, total nitrogen, and reducing sugar. The electrochemical analysis was done using cyclic voltammetry (CV) analysis.

Finally, the biodegradation of azo dye by bacteria was applied in MFC to produce electricity. In order to test for the robustness of the MFC when treating with azo dye wastewater, the important limiting factors of MFC operation were suggested to improve MFC performance. The factors highlighted that increase power output in MFCs are the diameter of salt bridge (proton exchange system) and size of electrode at anode and cathode. The performance of MFCs was analyzed by calculating the power densities, current densities, voltage output with external resistance, and polarization curve. Besides, the surface morphology of electrodes had been examined using Scanning Electron Microscope (SEM).

1.5 Significance of Research

This study focused on establish a bacterial culture capable in azo dye AR-27 removal and further applied in double chamber salt bridge-microbial fuel cell to generate electricity. The main idea for this study was to characterize and identify unknown strain on their ability to remove azo dye AR-27. The selected bacterial strain was further optimized on the effect of co-substrates to the efficiency of dye removal. The understanding on these potential isolates to degrade azo dye will increase the possibilities of developing strategies and models for azo dye pollutants removal from the environment focusing on the textile wastewater. Hence, this study could provide new idea for the current treatment of textile effluent and as an alternative green energy in the future

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Yahya, N.F.A.B., Khiavi, N.D., & Ibrahim, N. (2016). Green Electricity Production by *Epipremnum aureum* and Bacteria in Plant Microbial Fuel Cell. Journal of Advanced Research in Applied Sciences and Engineering Technology, 5(1), 22-31