CHROMOBACTERIUM VIOLACEUM R1-FERRICYANIDE MEDIATED BIOSENSOR FOR DETERMINATION OF BIOCHEMICAL OXYGEN DEMAND

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ABSTRACT

Biochemical oxygen demand (BOD) is an important organic pollution parameter in water system because it indicates the amount of organic matters in water sample. Since the standard method of BOD measurement (BOD_5) takes 5 days to complete, it is not practical for rapid environmental pollution monitoring. In the present study, an effective ferricyanide-mediated microbial biochemical oxygen demand (BOD) biosensor was constructed and used for rapid BOD determination in a water system. Prior to biosensor fabrication, microorganisms were first isolated from various water systems and then screened for their ability to use a redox mediator for organic degradation. The selected microorganism was immobilized onto the surface of the electrode tip for BOD measurement. The performance of the developed biosensor was then optimized, characterized and validated using synthetic organic solutions and real water samples. Several different types of microorganisms were isolated from different organic-rich environmental sources and their ability to use ferricyanide during organic (standard glucose-glutamic acid solution) degradation were effectively assessed using ferricyanide-mediated BOD assay. *Chromobacterium violaceum* R1 (isolated from river water) was found to be a potential microorganism to be used as a biological sensing element in the biosensor. The technology of ultramicroelectrode (UME) was incorporated into the biosensor during transducer fabrication. The electrode tip (transducer) was fabricated in two-electrode configuration (10-µm Pt working electrode and 1-mm Pt counter electrode). Living *C. violaceum* R1 cells were immobilized onto the surface of the UME working electrode by using calcium alginate gel and further enclosed by a layer of polyamide membrane. Glucose-glutamic acid (GGA) solution and OECD synthetic wastewater were used as the standard solutions. The amperometric measurement was optimized at +450 mV operating potential and 30 mM ferricyanide in a 0.1 M phosphate buffer (pH 7.0) at 26°C. The developed biosensor exhibited a linear response ranging from 20–225 mg O_2 L^{-1} BOD_5 for standard GGA solution and 25–230 mg O_2 L^{-1} BOD_5 for OECD synthetic wastewater, with a response time of 30 min. Repeatability and reproducibility of the biosensor were within the limits set by the APHA—less than 15.4%. The biosensor is applicable for rapid BOD measurement of samples with a high content of fast and easily assimilated compounds. When used to estimate the BOD of various wastewaters, the developed biosensor gave values comparable to those obtained using the conventional BOD_5 method. Hence, a biosensor has been successfully developed for rapid determination of BOD in water samples.

ABSTRAK

Permintaan oksigen biokimia (BOD) merupakan parameter pencemaran organik yang penting dalam sistem pengairan kerana BOD mampu menunjukkan jumlah bahan organik yang terkandung di dalam sampel air. Oleh sebab kaedah standard untuk penentuan BOD (BOD_5) memerlukan masa selama lima hari untuk mengeluarkan bacaan BOD, maka kaedah tersebut dianggap sebagai tidak praktikal untuk memantau pencemaran alam sekitar dengan cepat. Dalam kajian ini, biopenderiaan BOD yang berkesan dalam penentuan BOD dalam sistem air yang pesat telah dibina. Sebelum biopenderiaan BOD ini dibina, mikroorganisma telah diasingkan daripada pelbagai sumber alam sekitar dan dinilai dengan menggunakan analisis pengantara feriksianida-BOD yang digunakan sebagai elemen biologi dalam biopenderiaan BOD. Mikroorganisma yang telah dipilih, kemudiannya dilekatkan pada permukaan tip elektrod kerja untuk penentuan BOD. Akhir sekali, prestasi biopenderiaan BOD ini dioptimumkan dan dicirikan serta disahkan dengan menggunakan larutan-larutan organik sintetik dan sampel-sampel air. *Chromobacterium violaceum* R1 (dipencilkan daripada air sungai) merupakan mikroorganisma yang berpotensi untuk digunakan sebagai elemen biologi bagi biopenderiaan BOD. Seterusnya, transduser biopenderiaan telah dibina dengan menggunakan teknologi ultramikro elektrod, iaitu tip biopenderiaan ini dihasilkan daripada sistem dua elektrod yang mengandungi 10 μm elektrod kerja platinum dan 1 mm elektrod kaunter platinum. Sel-sel *C. violaceum* R1 yang hidup telah berjaya dilekatkan pada permukaan elektrod kerja dengan menggunakan gel kalsium alginat dan ditutup oleh satu lapisan membran poliamida. Larutan glukosa-glutamik asid (GGA) dan air sisa sintetik Pertubuhan Kerjasama Ekonomi dan Pembangunan (OECD) telah digunakan sebagai larutan piawai. Pengukuran biopenderiaan dilakukan secara amperometri yang telah dioptimumkan dan beroperasi pada potensi 450 mV, manakala fosfat penampan (0.1 M, pH 7.0) yang mengandungi 30 mM ferisianida turut digunakan. Biopenderiaan ini (*CV_{R1}-FM-BOD biosensor*) berjaya mengeluarkan rangsangan yang linear daripada larutan-larutan yang berkepekatan antara 20 hingga 225 mg O_2/L BOD_5 bagi larutan GGA, manakala 25 hingga 230 mg O_2/L BOD_5 bagi air sisa sintetik OECD dalam masa 30 minit. Kebolehulangan biopenderiaan ini berada pada had yang telah ditetapkan oleh APHA, iaitu kurang daripada 15.4%. Biopenderiaan ini juga sesuai digunakan untuk mengukur BOD dengan cepat bagi sampel yang mengandungi sebatian yang mudah diasimilasikan. Apabila digunakan untuk menentukan BOD bagi pelbagai sisa air buangan, biopenderiaan ini didapati mampu memberi nilai-nilai BOD yang setanding dengan nilai-nilai yang diperoleh apabila menggunakan kaedah BOD_5 yang konvensional. Oleh iu, sejenis biopenderiaan untuk penentuan BOD secara cepat dalam sistem air telah berjaya dibina.

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LIST OF ABBREVIATIONS & SYMBOLS

Abs	-	Absorbance
Ag/AgCl	-	Silver/Silver Chloride
APHA	-	American Public Health Association
ATP	-	Adenosine Triphosphate
AWW	-	Artificial Wastewater
BART	-	Biological Activity Reaction Tests
BLAST	-	Basic Local Alignment Search Tool
BOD	-	Biological Oxygen Demand
BOD_{eq}	-	Biological Oxygen Demand equivalent
bp	-	base pair
$CaCl_2$	-	Calcium chloride
CFU	-	Colony Forming Unit
COD	-	Chemical Oxygen Demand
CV	-	Cyclic Voltammetry
CV_{R1}-FM-BOD-		*Chromobacterium violaceum* R1-ferricyanide Mediated Biochemical Oxygen Demand
DM	-	Double Mediator
DNA	-	Deoxyribonucleic Acid
DO	-	Dissolved Oxygen
DOC	-	Dissolved Organic Carbon
e^-	-	electron
E. coli	-	*Escherichia coli*
EtBr	-	Ethidium Bromide
ETC	-	Electron Transport Chain System
Eqn.	-	Equation
$FeCl_3$	-	Ferric Chloride
$[Fe(CN)_6]^{3-}$	-	Ferricyanide

Fe^{3+}	-	Iron(III) ion
FM-BOD	-	Ferricyanide Mediated-BOD assay
FSE	-	Food Service Establishments
g	-	Gravity
GGA	-	Glucose-Glutamic Acid
H^+	-	Proton
HAB	-	Heterotrophic Aerobic Bacteria
HCl	-	Hydrochloric Acid
HCF(II)	-	Ferricyanide
HCF(III)	-	Ferrocyanide
HQ	-	Hydroquinone
H_2O	-	Water Molecule
i_{lim}	-	limiting current
JIS	-	Japanese Industrial Standard
kb	-	kilobase
KCl	-	Potassium Chloride
KH_2PO_4	-	Potassium dihydrogen orthophosphate
K_2HPO_4	-	Dipotassium hydrogen orthophosphate
$K_3[FeCN]_6]^{3-}$	-	Pottasium Ferricyanide
$MgSO_4.7H_2O$	-	Magnesium Sulphate Heptahydrate
M	-	Molar
mg/L	-	Milligram per litre
min	-	Minute
mL	-	Mililitre
mM	-	Milimolar
mm	-	Milimeter
mV/s	-	Milivolt per second
NA	-	Nutrient Agar
nA	-	Nanoampere
NaOH	-	Sodium Hydroxide
nm	-	Nanometer
NaCl	-	Sodium Chloride
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NCBI	-	National Center for Biotechnology Information

NH_4Cl	-	Ammonium Chloride
nm	-	Nanometer
ng	-	Nanogram
OD_{600}	-	Optical Density at 600nm
OECD	-	Organisation for Economic Co-operation and Development
OH^-	-	Hydroxide ion
ON	-	Organic Nitrogen
O_2	-	Oxygen
P	-	Petroleum Wastewater
PA	-	Pineapple Waste
PBS	-	Phosphate Buffer Saline
PCR	-	Polymerase Chain Reaction
PDB	-	Potato Dextrose Broth
POME	-	Palm Oil Mill Effluent
ppm	-	Parts per million
Pt	-	Platinum
PVA	-	Polyvinyl acetate
Q	-	p-Quinone
R	-	River
RM	-	Ringgit Malaysia
RNase	-	Ribonuclease
rpm	-	Revolution per minute
rRNA	-	Ribosomal Ribonucleic Acid
RSD	-	Relative Standard Deviation
*r*2	-	Correlation Coefficients
SbQ	-	Quaternized Stilbazol
SPV	-	Surface Photo Voltage
SS	-	Suspended Solid
SWW	-	Synthetic Wastewater
TAE	-	Mixture of Tris Base, Acetic Acid and EDTA
TAN	-	Total Ammoniacal Nitrogen
TCA	-	Tricarboxylic Acid Cycle
TOC	-	Total Organic Carbon
TS	-	Textile Sludge

TSB	-	Tryptic Soy Broth
UME	-	Ultramicroelectrode
UV	-	Ultra Violet
µL	-	Microlitre
µm	-	Micrometer
Vis	-	Visible
v/v	-	Volume per volume
w/v	-	Weight per volume
Y	-	Yeast
°C	-	Degree Celsius

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CHAPTER 1

INTRODUCTION

1.1 Background of study

River waters are the main water supply to the daily activities of citizen in Malaysia. However, the percentage of polluted rivers increased significantly between 1987 and 2009 and at present river pollution still remains one of the most serious issues in Malaysia (Tortajada, 2013). The rivers that have experienced heavy pollution include Sungai Kelang, Sungai Selangor, Sungai Linggi, Sungai Langat, Sungai Melaka, Sungai Muda, Sungai Juru, Sungai Muar, Sungai Segamat, Sungai Johor, Sungai Terengganu, and Sungai Kelantan (Hussain & Ismail, 2001). In 1989, eateries industry contributed around 42% of total pollution to the rivers water in Malaysia, and the rest were contributed by several different types of industries such as rubber industry, chemical industry, palm oil industry, raw rubber industry, textile industry and paper industry (Hussain & Ismail, 2001). More recently, reports from local newspapers indicate that pollution of water system in Malaysia still persists and this poses serious risk to human health and the environment (New straits times, 2014). Rapid urbanization and rapid industrial development appear to be the dominant factors contributing to the pollution of water system in Malaysia.

Biochemical oxygen demand (BOD) is one of the important indicators for organic pollution in industrial wastewater effluent or natural water. This BOD value indicates the amount of the oxygen used to biodegrade organic materials and oxidizes inorganic material such as sulphides and ferrous iron (Liu & Mattiasson, 2002). The standard method for BOD analysis is the conventional 5-day biochemical

oxygen demand (BOD_5) method described by American Public Health Association (APHA) Standard Methods Committee (Chen *et al.*, 2008) and Japanese Industrial Standard (JIS) Committee (Yoshida *et al.*, 2001).

BOD_5 measures the quantity of dissolved oxygen consumed for microbial oxidation of organic matter under specific conditions over a 5-day incubation period (APHA, 1992). Although BOD_5 is a universal method of measuring most wastewater samples and the required equipment is inexpensive, it is time-consuming and requires complicated procedure and skilled analysts to obtain reproducible results (Yoshida *et al.*, 2001). In addition, BOD_5 is not suitable for *in situ* determination or on-line process monitoring. Thus, it is necessary to develop an alternative method that could circumvent the weakness of BOD_5.

Fast determination of BOD could be achieved by biosensor-based methods. A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological sensing element which is immobilized onto a transducer (Turner, 1991). Due to the broad range of biodegradable compounds, microorganisms have been used as a biological sensing element in the most developed BOD biosensors (Liu & Mattiasson, 2002). Most developed microbial BOD biosensors have focused on measuring the remaining dissolved oxygen (DO) levels or concentration after the immobilized microbes utilized DO to degrade the organic compounds in the sample over a certain period. Due to the poor solubility of oxygen (O_2) in water (8.7mg/L at 25 °C), O_2 rapidly becomes the limiting reactant in the biodegradation process. Subsequently the amount of organic compounds biodegraded in the short time is small, which represents only a small fraction of the total organic content (Pasco *et al.*, 2004). This results in narrow response ranges and poor reliability of microbial BOD biosensor.

So far, BOD biosensor development has focused on the use of a ferricyanide-mediated BOD approach to overcome the oxygen limitation problems. In this approach, O_2 is replaced by the ferricyanide ion which serves as an alternative electron acceptor. During the metabolic oxidation of organic substances by microbes, ferricyanide is reduced to ferrocyanide. The ferrocyanide is then reoxidized at a

working electrode which is held at a sufficiently high electric potential. As a result, a current is generated and detected using the electrode system, in which the limiting current is related to the amount of ferricyanide reduced and thus amount of organics (Yoshida *et al.*, 2000). Due to the highly soluble properties of ferricyanide, it allows the use of much higher microbial populations without rapid depletion of the electron acceptor, the incubation time is subsequently reduced and the need for excessive dilution of samples is greatly reduced (Catterall *et al.*, 2001).

However, there still remain restrictions of the present BOD biosensors, and consequently these weaknesses limit their applications such as insufficient reliability for wastewater samples with varied compositions or high content of polymers, insufficient resistance to various toxic compounds in the wastewater, lacking robustness for field service, complicated requirements of maintenance, and restrictions due to the lack of standardization and legislation in most countries (Liu & Mattiasson, 2002). Furthermore, in terms of reliability, large size of equipment, complicated preparation and handling of the biosensor limit their application.

To our knowledge there has yet to be work reported on the use of ultramicroelectrode (UME) in Ferricyanide-mediated (FM)-BOD biosensor. Ultramicroelectrode is an electrode with at least one dimension made small (≤ 25 μm) until it exhibits the special properties of UME. Most of the previously developed redox-mediated BOD biosensors involved immobilizing the biological sensing element on the tips of conventionally sized working electrodes, thus eliminating the entry of the biological sensing element into the sample solution [Nakamura *et al.*, 2007; Trosok *et al*., 2001; 2002, Yoshida *et al*., 2000; 2001]. Interestingly, Morris *et al*. (2001) used UME technology in their redox-mediated BOD assay; however, it needs to be noted that in Morris's BOD detection system, the biological sensing element (mixed microbial culture) was not immobilized on the UME.

The UME of the biosensor is capable of providing highly sensitive and accurate measurements. Due to the small size and relatively large diffusion layers of UME, highly accurate measurements can be made even in non-polar solvent or

resistive solutions without deliberately adding supporting electrolyte (Aoki, 1993). Furthermore, the small size of UME permits *in situ* characterization and avoid disturbance of the sample's equilibria. Thus this UME-incorporated BOD sensor becomes more reliable than conventional electrode-incorporated biosensor.

In view of this, the present project aimed to develop a two-electrode BOD biosensor tip which incorporated the UME technology and mediator system for BOD measurement. The proposed fabrication method for this biosensor tip was based on the self fabricated ultramicroelectrode (UME) tip with a single microorganism (biological sensing element) immobilized on the surface of the UME working electrode. Subsequently, amperometric circuitry was used to monitor the current response due to an electrical potential applied between an electrode surface and an electrolyte solution. The current response of the sensor eventually was correlated to BOD_5.

1.2 Problem Statement

According to Environmental Quality (Industrial Effluent) Regulations 2009, BOD is an important parameter to monitor the quality of water system in Malaysia. However, since the standard method for BOD measurement ($BOD_{5)}$ is time consuming and involves complicated procedures, it is not practical for rapid environmental pollution monitoring and industries to store the wastewater for several days before. Thus, it is necessary to develop a reliable BOD biosensor locally to provide a rapid BOD measurement with simple operating procedure and maintenance. In addition, the cost of the sensor can be made affordable and accessible to the local industries.

1.3 Aims and Objectives

The aim of this project was to fabricate a redox-mediated amperometric BOD sensor for rapid detection of organic pollution in water systems with several objectives:

1. To isolate and screen for microorganisms able to use redox mediator during biochemical degradation of organics
2. To design and fabricate the redox-mediated-BOD biosensor using the isolated microorgasnism as biosensing element
3. To characterize and optimize the performance of the fabricated redox-mediated-BOD biosensor
4. To validate the performance of the redox-mediated-BOD biosensor on simulated and real water systems.

1.4 Scope of Study

In this study, microorganisms were isolated from different environmental sources and their abilities to be used as an effective biological sensing element for FM-BOD biosensor were screened. Subsequently, it was immobilized onto previously fabricated tip. During the electrode tip fabrication, the technology of UME was incorporated into the biosensor by making the working electrode into 10 µm radius. The electrode tip was made in two-electrode configuration and the performance of the electrode tip was verified using standard commercial electrodes prior to be used as the transducer of the biosensor. After the biological sensing element was successfully immobilized onto the fabricated electrode tip, several parameters (thickness of gel, immobilized biomass and concentration of ferricyanide) were optimized conventionally. This was followed by the characterization of the biosensor where the performances of biosensor (linearity, influence of dissolved oxygen, lifetime, repeatability and reproducibility) were determined. In the end of the study, the biosensor was used to determine the BOD of several pure synthetic organic solutions and real wastewaters and the results obtained were verified and compared to the conventional BOD_5 assay.

1.5 Significance of Study

The present work is an attempt to locally produce BOD biosensor tips for organic pollution detection. The tips of biosensor were fabricated in UME size and locally isolated potential microorganism was immobilized onto the tips as biological sensing element of the biosensor. The anticipated developed biosensor tips enable fast determination of BOD. This is due to conventional BOD_5 requires a 5-day incubation period and substantial experienced and skilled operator to obtain reproducible results. The measurement of the developed FM-BOD biosensor tips is also more reliable than oxygen electrode-incorporated BOD biosensors because the response of the sensor is not affected by the concentration of dissolved oxygen (DO) in the samples.

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