DEVELOPMENT OF ELECTROCHEMICAL BIOSENSOR FOR DETERMINATION OF FORMALIN IN FISH SAMPLES; MALABAR RED SNAPPER (*Lutjanus Malabaricus*) AND LONGTAIL TUNA (*Thunnus Tonggol*)

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BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2016

DECLARATION

I hereby declare that the material in this thesis is of my own effort, except for quotations, excerpts, equations, references and summaries, which have been duly acknowledged and cited clearly its sources.

09 October 2016

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CERTIFICATION

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DEGREE	:	**MASTER OF SCIENCE (Biotechnology)**
VIVA DATE	:	**20 SEPTEMBER 2016**

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Assoc. Prof. Dr. Md. Shafiquzzaman Sidiqquee Signature

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In the name of Allah, the Beneficent, the Merciful. Praise to Allah, the Lord of the world and sequel is for those who keep their duty onto Him. Blessing and salutation be upon the most honorable Prophet and Messenger, his family all his disciplines, and those who follow them in goodness till the Day of Judgment. All praise and thanks due to Allah the master of the universe for giving me the strength to endure all challenges and complete this study.

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ABSTRACT

Formaldehyde is used as preservative in fish by fisherman, in order to maintain its fresh look and prevent microbial spoilage, is a big risk to consumer's health. Formaldehyde is carcinogenic. Thus a simple, sensitive and rapid device for its determination is necessary based on electrochemical biosensor coupled with an enzyme and formaldehyde dehydrogenase (FDH). A novel electrochemical biosensor was developed by depositing an ionic liquid (IL) (e.g., 1-ethyl-3-methylimidazolium trifluoromethanesulfonate; [EMIM][Otf]), ZnO nanoparticles (ZnONPs), CaO nanoparticles (CaONPs), Au nanoparticles (AuNPs) and chitosan (CHIT) on a glassy carbon electrode (GCE) for determination of formaldehyde in fish. The enzyme requires ß-nicotinamide adenine dinucleotide (NAD^+) as a cofactor which is then reduced to NADH during enzymatic reaction. Formaldehyde Dehydrogenase (FDH) was immobilized through the entrapment technique and measured based on the reduction of NAD^+ under a potential of 0.4 V. The morphological characteristics of microstructures of CHIT, NPs/[EMIM][Otf]/CHIT and FDH/NPs/[EMIM][Otf]/CHIT were observed under scanning electron microscopy (SEM). Methylene blue was used as a redox indicator to increase in the electron transfer capacity and stable in both the redox reaction. The enzyme electrode showed optimum response when operated at 25^0C in 50 mM Tris-HCl buffer, pH 6.5 within 5 s. The storage stability was done for 5 weeks. A relative standard deviation for the slopes of 0.32% (n = 5) was obtained for reproducibility. Under the optimal conditions, the FDH/AuNPs/[EMIM][Otf]/CHIT/GCE was showed wider linear range of formaldehyde concentrations from 0.01 to 10 ppm within 5 s, with the detection limit of 0.1 ppm. The FDH/AuNPs/[EMIM][Otf]/CHIT was found more sensitive than FDH/ZnONPs/[EMIM][Otf]/CHIT and FDH/CaONPs/[EMIM][Otf]/CHIT. FDH/AuNPs/[EMIM][Otf]/CHIT was used for the detection of formalin on-site in fish sample; *Lutjanus malabaricus* and *Thunnus tonggol* which were found below the safety level (5 ppm). The developed biosensor is a simple, rapid, economically, highly efficient, and high accuracy when compared to the existing technique.

ABSTRAK

Formaldehid digunakan sebagai pengawet dalam ikan oleh nelayan, untuk mengekalkan ikan kelihatan segar dan mencegah kerosakan mikrob, adalah satu risiko yang besar kepada kesihatan pengguna. Formaldehid adalah karsinogenik. Oleh itu peranti mudah, sensitif dan cepat bagi penentuannya itu adalah perlu berdasarkan biosensor elektrokimia ditambah pula dengan enzim dan formaldehid dehidrogenase (FDH). Biosensor elektrokimia telah dibangunkan dengan menempatkan cecair ionik (IL) (contohnya, 1-etil-3-methylimidazolium trifluoromethanesulfonate; [EMIM][OTF]), partikel nano ZnO (ZnONPs), partikel nano CaO (CaONPs), partikel nano Au (AuNPs) dan chitosan (Chit) pada elektrod karbon berkaca (GCE) untuk penentuan formaldehid dalam ikan. Enzim memerlukan ß-nicotinamide adenin dinucleotide (NAD^+) sebagai kofaktor yang kemudiannya dikurangkan kepada NADH semasa tindak balas enzim. Formaldehid dehidrogenase (FDH) telah bergerak melalui teknik pemerangkapan dan diukur berdasarkan pengurangan NAD^+ di bawah potensi 0.4 V. Ciri-ciri morfologi mikrostruktur Chit, NPS/[EMIM][OTF]/Chit dan FDH/NPS/[EMIM][OTF]/Chit diperhatikan di bawah mikroskop elektron pengimbas (SEM). Metilena biru digunakan sebagai penunjuk redoks meningkatkan kapasiti pemindahan elektron dan penstabilan dalam kedua-dua tindak balas redoks. Elektrod enzim menunjukkan tindak balas optimum apabila beroperasi pada 25^0C di 50 mM Tris-HCl penampan, pH 7.0 dalam tempoh 5 s. Kestabilan penyimpanan telah dilakukan selama 5 minggu. Sisihan relatif standard untuk lereng 5% (n = 5) telah diperolehi bagi kebolehulangan. Dalam kes kebolehulangan, biosensor yang mengekalkan 11% daripada kepekaan awal selepas giliran kelima. Dibawah keadaan optimum FDH/AuNPs/[EMIM][OTF]/Chit/GCE telah menunjukkan julat linear yang lebih luas daripada kepekatan formaldehid 0.01-10 ppm dalam masa 5 saat, dengan had pengesanan 0.1 ppm. FDH/AuNPs/[EMIM][OTF]/Chit didapati lebih sensitif daripada FDH/ZnONPs/[EMIM][OTF]/Chit dan FDH/CaONPs/[EMIM][OTF]/Chit. FDH/AuNPs/[EMIM][Otf]/CHIT telah digunakan untuk mengesan formalin di lokasi dalam sampel ikan; *Malabaricus Lutjanus* dan *Thunnus tonggol* yang didapati di bawah tahap keselamatan (5 ppm). Biosensor yang dibangunkan adalah mudah, pantas, ekonomi, sangat berkesan, dan tinggi ketepatan bila dibandingkan dengan teknik yang sedia ada.

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LIST OF ABBREVIATIONS AND SYMBOLS

CV	-	Cyclic voltammetry
DPV	-	Differential pulse voltammetry
IARC	-	International Agency for Research on Cancer
EPA	-	United States Environmental Protection Agency
CE	-	Counter electrode
RE	-	Reference electrode
WE	-	Working electrode
GCE	-	Glassy carbon electrode
ANOVA	-	Analysis of variance
SPSS	-	Statistical Package for the Social Sciences
mL	-	Millilitre
µl	-	Microliter
%	-	Percentage
v/v	-	volume per volume
^{o}C	-	Degree Celsius
Mg	-	Milligram
G	-	Gram
M	-	Molar
Ppm	-	Parts per million
Rpm	-	Revolutions per minute
NAD^{+}	-	Oxidised β-nicotinamide adenine nucleotide
NADH	-	Reduced β-nicotinamide adenine nucleotide
FA	-	Formaldehyde
FDH	-	Formaldehyde dehydrogenase

CHIT	-	Chitosan
AuNPs	-	Gold nanoparticles
CaONPs	-	Calcium oxide nanoparticles
ZnONPs	-	Zinc oxide nanoparticles
AgCl	-	Silver chloride
Pt	-	Platinum

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1 $R = \frac{\text{Total amount detected} - \text{Original amount without spike}}{\text{Amount spiked}} \times 100\ \%$ 44

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CHAPTER 1

INTRODUCTION

1.1 Background of study

The biggest source of protein in Malaysia is mainly fish and seafood. The composition of fish which includes fat, free amino acids and water makes it vulnerable to deterioration by microorganism and biochemical reactions (Ismail, 2005). Moreover, the flesh of fish can spoil quickly and it should either be consumed on the day of capture, or preserved immediately to avoid spoiling the fish. Depending on the fish species, some can be kept in ice for 8 days and up to 14 days (Ashie et al., 1996).

Due to the high nutritional value of fish (omega 3 fatty acid or docosahexaenoic acid (DHA)), careful handling of fish is required to retain the nutrients. Although there are many alternative ways to handle fish that are available in the market such as ultra-high pressure and modified atmosphere packaging, fishermen usually prefer a low cost method. Hence, they tend to use formaldehyde as a preservation agent since it is a well-known food preservative. Recent news and research reported that the use of formalin in fish preservation is very popular especially in Asian countries (Respi Leba, 2012; Andrews, 2013). Islam (2015) reported that formaldehyde tend to contaminate most of the commercial imported fish. Local fish has a longer shelf life and excellent organoleptic characteristics.

Formaldehyde is known to protect fish from spoilage by microbial contamination. The high activity, high chemical purity and comparative cheapness make formaldehyde one of the most important commercial chemicals. Formaldehyde has the capability to create bond between soluble and structural protein. The resulting structure retains its cellular components in their *in-vivo* relationships to each other, giving it point of mechanical strength that enables it to

withstand consequent processing (Environmental & Occupational Health and Safety Service, 2004).

Formaldehyde is the simplest aldehyde which is in a very reactive gas state of chemical. It is colourless but strong smelling, irritating and is highly flammable. Meanwhile formalin is in a liquid state of chemical and is commonly used as bath treatment in aquaculture and the preservation of any biological samples. Recently, formaldehyde-caused hazard has been discovered. This chemical has been proven to be dangerous to human health in many ways (Duong et al., 2011; Noordiana et al., 2011; Latorre et al., 2011). If large doses of formaldehyde are used, serious problems like vomiting, coma and possible death may occur. The oral dosage limit in Malaysia is not exceeding 5 mg kg^{-1} (Food Safety and Quality Department, 2014) but the limit suggested by the United States Environmental Protection Agency is 0.2 mg kg^{-1} (Yeh et al., 2013). The effort to monitor the use of formaldehyde in food need to be further expanded.

Chitosan (CHIT) becomes a solid-liquid phase as the pH is slightly acidic. One of the advantages of using CHIT is that it allows simple electrodeposition (Wu et al., 2002). The deposition of nanoparticles and FDH are simply adaptable once CHIT is used as the platform for biosensor. Dehydrogenase-based sensor has drawn attention because of the ubiquity of this enzyme. Lobo et al. (1997) and Herschkovitz et al. (2000) have identified approximately 250 NADH-dependent NADH and over 150 NADH-dependent enzymes. The enzymatic reactions and transducer probably gave enormous importance and efficacy to biosensor. In this study, the development of enzyme biosensor could detect specific substrate (formaldehyde) for formaldehyde dehydrogenase.

Many analytical methods are available for formaldehyde determination such as high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), Nash test and other chemical-based biosensor (Bechmann, 1996; Ngamchana and Surareungchai, 2004; Noordiana et al., 2011; Ye et al., 2013). Biosensor has enabled researches to develop formaldehyde biosensor that is

simple, fast, high selectivity and sensitivity as compared to other analytical methods.

Several modifications have been constructed onto the glassy carbon electrode and the results yield are found to be more favourable. The use of chitosan (CHIT) for enzyme immobilization is low cost, has zero toxicity (Kumar, 2000; Krajewska, 2004) and is easily controlled by pH manipulation (Rinaudo, 2006). Nanoparticles (AuNPs, ZnONPs, or CaONPs) are immobilized onto the membrane along with FDH enzyme to increase the large surface area for electrochemical reaction. Research has applied ionic liquid in electrochemical fabrication to produce high sensitivity and specificity. FDH is found to specifically react to formaldehyde (Masuda et al., 2013) where the presence of a cofactor NAD^+ will provide higher stability of the reaction (Ngamchana and Surareungchai, 2004).

The aim of this research is to develop electrochemical enzyme biosensor using formaldehyde dehydrogenase (FDH) and electrochemically modified based on nanomaterial's-coated chitosan since there is inadequate number of devices for real time determination of formaldehyde in real fish samples. This study sought to determine the reproducibility, repeatability, sensitivity, selectivity and storage stability of the modified (FDH/NPs/[EMIM][Otf]/CHIT/GCE) glassy carbon electrode for formaldehyde detection in fresh fish samples; Malabar Red Snapper (*Lutjanus malabaricus)* and Longtail Tuna *(Thunnus tonggol*). Therefore, it is also important to investigate the formaldehyde content in fish in order to better understand the risks of consumption, to manage the risks of consumption and to provide additional information in food safety.

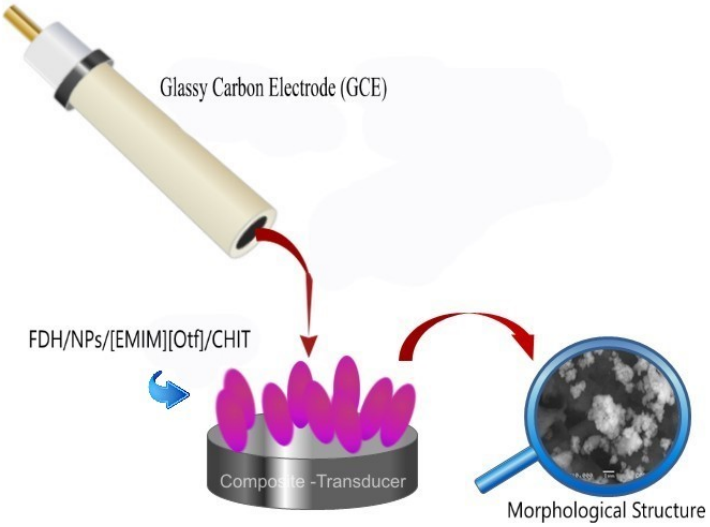

Figure 1.1: The summarization of the research.

1.2 Problem statement

Nowadays, the amount of fish caught exceed the consumer demand. The excessive amount of fish leads to fish vendors resorting to use formalin to preserve fish for a certain period of time. The usage of formalin could be above permissible level. The conventional methods available to detect formalin such as HPLC, GC and Gravimetric have some drawback as they require more time, high cost and skilled manpower. The electrochemical biosensor is developed to determine formalin level with user friendly, simpler and faster procedures, low cost and zero pollution. The developed biosensor is able to detect a wider range of concentration as low as 0.01 ppm. The methylene blue, NAD^+, nanoparticles and ionic liquid are also used.

Based on literature study, no research is found on the fast determination of formalin presence in fish using the developed formaldehyde biosensor (FDH/NPs/[EMIM][Otf]/CHIT/GCE) in Malaysia particularly in Sabah (Noordiana et al., 2011; Marzuki et al., 2012). Due to the lack in data on level of formaldehyde presence in fish in the country, the study on the detection of formaldehyde concentrations in real fish samples and development of the biosensor methods have yet to be further studied. Thus, this study can be a starting point for more research on the development of biosensor methods to detect the formaldehyde concentrations from fish consumption particularly in Sabah.

This study will also lead to the development of formaldehyde device that can be applied for the detection of formaldehyde in fish samples in any wet market in Malaysia. Hence, by using the formaldehyde chips that are being developed, this study can create public awareness on the negative effects of formaldehyde and the concentration of formaldehyde that might be consumed. Next, the data for the development of biosensor methods in this study can help researchers to find best method to assess the concentration of formaldehyde in fish samples.

In this study, we plan to; (i) develop modified biosensor that presents the highest performance and (ii) utilize nanomaterials on GCE surface of the designated electrode assembled with FDH/AuNPs/[EMIM][Otf], FDH/ZnONPs/[EMIM][Otf], and FDH/CaONPs/[EMIM][Otf] for determination of substances produced by enzymatic reaction and (iii) develop a biosensor with high sensitivity and is long lasting in determining formaldehyde in real samples.

1.3 Research objectives

a. To characterize the developed formaldehyde biosensor based on FDH/AuNPs/[EMIM][Otf]/CHIT, FDH/ZnONPs/[EMIM][Otf]/CHIT and FDH/CaONPs/[EMIM][Otf]/CHIT onto glassy carbon electrode.

b. To determine the sensitivity, repeatability, reproducibility, storage stability and detection limit of formalin based on each developed biosensor.

c. To detect and analyze the presence of formaldehyde levels in real fish samples; Malabar Red Snapper *(Lutjanus Malabaricus) and* Longtail Tuna *(Thunnus Tonggol).*