EFFECTIVENESS OF PLANT ESTERASE IMMOBILIZED ON MULTI-WALLED CARBON NANOTUBE SCREEN-PRINTED ELECTRODE FOR DETECTION OF PESTICIDES

BY

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A thesis submitted in fulfillment of the requirement for the degree of Master of Science in Biotechnology Engineering.

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APRIL 2024

ABSTRACT

Organophosphorus compounds (OP) account for the most toxic substances used to destroy pests by inhibiting acetylcholinesterase (AChE) of the central nerve. Since OP resulted in the accumulation of its residue and finally increased the exposure to humans, therefore, various acetylcholinesterase (AChE) based biosensors from animal sources had been developed for this purpose. However, Alpha naphthyl acetate esterase (ANAE) enzyme from wheat flour was more economical alternative due to its simpler procedure of extraction and purification. OP has an inhibitory effect on ANAE as also with AChE, thus ANAE based biosensors' sensitivity is comparable to the AChE biosensors. The crude enzyme extract was first filtered and purified using an aqueous two-phase separation system (ATPS). A purification fold of enzyme 4.36 was obtained with an enzyme yield of 56.89% of atta flour. The molecular weight of the target esterase was found to be around 63 (kDa). The incubation time within 15 minutes at 30°C with pH 6.5 of phosphate buffer are the optimum condition at which ANAE resulted in the highest activity. The Michaelis-Menten parameters of the purified enzyme were 9.765 mM and 0.084 $mMmin^{-1}$, respectively for K_m and V_{max}. To analyze and characterize the immobilization of ANAE on functionalized MWCNT, Fourier transform infrared (FTIR) spectroscopy was utilized to verify the presence of the functional groups. The confirmation of ANAE immobilization on functionalized MWCNT was based on the observation of specific peaks at 3646.81 cm^{-1} and 3850.91 cm^{-1}, indicating the formation of amide linkages between the carboxylic acid groups and the amine group on the MWCNT. The kinetic constants which are k_3 (phosphorylation rate constant), and k_i (dissociation constant of enzyme-inhibitor complex) of an irreversible inhibition model were 0.2223 $mMmin^{-1}$ and 0.4816 $mMmin^{-1}$ respectively for varying incubation times, and different concentrations of the inhibitor (OP). The detection limit for dichlorvos was found to be 0.005 $\mu g/L$. Lowering the concentration of pesticides resulted in increasing the CV responses.

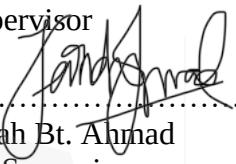
ملخص البحث

تمثل مركبات الفسفور العضوي (OP) أكثر المواد سمية حيث تستخدم لتدمير الآفات عن طريق تثبيط إنزيم الأسيتيل كولينستريز (AChE) للعصب المركزي. نظرًا لزيادة تعرض البشر لمركبات الفسفور العضوي بسبب زيادة تراكمه، تم تطوير العديد من أجهزة الاستشعار الحيوية المستندة إلى الأسيتيل كولينستريز (AChE) من المصادر الحيوانية لهذا الغرض. ومع ذلك، كان إنزيم ألفا نفثيل أسيتات إستراز (ANAE) المستخرج من دقيق القمح هو البديل الاقتصادي، نظرًا لسهولة استخلاصه وتنقيته. الفسفور له تأثير مثبط على ANAE كما هو الحال أيضًا مع AChE، وبالتالي فإن حساسية أجهزة الاستشعار الحيوية المعتمدة على ANAE قابلة للمقارنة مع مستشعرات AChE الحيوية. تم ترشيح مستخلص الإنزيم الخام وتنقيته أولاً باستخدام نظام فصل مائي ثنائي الطور (ATPS). ثم تم الحصول على نسبة تنقية 4.3 من الإنزيم بإنتاجية إنزيمية تبلغ 56.89% من دقيق atta. تم العثور على الوزن الجزيئي للإستريز المستهدف بحوالي 63 (كيلو دالتون). خلال وقت حضانة 15 دقيقة عند 30 درجة مئوية مع درجة حموضة 6.5 لمحلول الفوسفات هي الحالة المثلى التي يؤدي فيها ANAE إلى أعلى نشاط. كانت مؤشرات Michaelis-Menten للإنزيم النقي 9.765 ملمولار و 0.084 ملمولار / دقيقة، على التوالي لـ *Km* و *Vmax*. لتحليل وتوصيف تجميد ANAE على MWCNT الوظيفية، تم استخدام التحليل الطيفي للأشعة تحت الحمراء (FTIR) لتحويل فوريه للتحقق من وجود المجموعات الوظيفية. استند تأكيد تجميد ANAE على MWCNT الوظيفية إلى ملاحظة قمم محددة عند 3646.81 سم$^{-1}$ و3850.91 سم$^{-1}$، مما يشير إلى تكوين روابط أميد بين مجموعات حمض الكربوكسيل ومجموعة الأمين على MWCNT. كانت الثوابت الحركية التي هي k_3 (ثابت معدل الفسفرة)، و*ki* (ثابت تفكك مركب مثبط الإنزيم) لنموذج تثبيط غيرعكسي هي 0.2223 ملمولار دقيقة$^{-1}$ و0.4816 ملمولار دقيقة$^{-1}$ على التوالي لأوقات حضانة مختلفة، وتركيزات مختلفة للمثبط. وتبين أن حد الكشف عن الدايكلورفوس هو 0.005 ميكروغرام/لتر. وهو الحد الذي أدى لخفض تركيز المبيدات الحشرية وزيادة الاستجابة.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Biotechnology Engineering

…….
Faridah Bt.Yusof
Supervisor



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ACKNOWLEDGEMENTS

All glory is due to Allah, the Almighty, whose Grace and Mercies have been with me throughout the duration of my study. Although, it has been tasking, His Mercies and Blessings on me ease the herculean task of completing this thesis.

I am most indebted to by supervisor, Professor Faridah binti Yusof and Dr. Farah binti Ahmad, whose enduring disposition, kindness, promptitude, thoroughness and friendship have facilitated the successful completion of my work. I put on record and appreciate their detailed comments, useful suggestions and inspiring queries which have considerably improved this thesis. Their brilliant grasp of the aim and content of this work led to his insightful comments, suggestions and queries which helped me a great deal. Despite their commitments, they took time to listen and attend to me whenever requested. The moral support they extended to me is in no doubt a boost that helped in building and writing the draft of this research work.

Lastly, my gratitude goes to my husband and family for their prayers, understanding and endurance while away.

Once again, we glorify Allah for His endless mercy on us one of which is enabling us to successfully round off the efforts of writing this thesis. Alhamdulillah

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LIST OF ABBREVIATIONS

ANAE	A-Naphthyl Acetate Esterase
SPE	Screen Printed Electrode
MWCNT	Multiwall Carbon Nanotube
OP	Organophosphorus
AChE	Acetylcholinesterase
CV	Cyclic Voltammetry
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry
CNT	Mass Spectrometry
PTFE	Polytetralenefluoroethylene
FTIR	Fourier-transform infrared spectroscopy
SDS	Dodium dodecyl sulfate

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

In recent years, as a way to secure the quality and quantity of crops from pests, hundreds of various chemical pesticides are utilized in agriculture, which is a major economic industry. (Carvalho et al., 2006). However since pesticides were used extensively, their residue accumulated, polluting soils and water, crops, the food chain, and ultimately increasing human exposure (Sankararamakrishnan, Sharma, and Sanghi et al., 2005). This is a growing concern due to the harmful effects it may bring to human health such as liver damage and also leads to death in infants and young children.

Organophosphorus (OP) compounds such methyl parathion and dichlorvos are included in the multiple categories of pesticides (Vidal et al., 2006). Acetylcholinesterase (AChE), an important enzyme for nerve transmission and a potential source of cholinergic toxicity in humans, is inhibited by OP pesticides, which can lead to neurological issues (Schulze et al., n.d.). In order to implement strict monitoring procedures and eradication measures, pesticide residues detected in food products and vegetables is a warning symptom (Chauhan, Narang, & Jain, 2016).

Pesticides detection may be accomplished using a variety of techniques, including chromatographic ones like gas chromatography (GC) and high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS). However, these procedures caused significant negative aspects since they need expensive and specialized equipment as well as labor-intensive sample preparation procedures (Chawla et al., 2018). Hence, it is now more critical than ever to quickly determine and accurately quantify OP

chemicals (Costa et al., 2006). Enzyme-based electrochemical biosensors represent the promise for on-site pesticide detection. The detecting activity may be evaluated for the detection of variable substrates or enzymatic reaction products using a variety of transduction methods, such as amperometry, potentiometry, spectrometry, and thermometry.

The simplicity and reliability of amperometric biosensors that utilize enzyme inhibition have generated significant interest (Malhotra et al., 2017). The change in oxidation current in these devices enables to assess the inhibition of enzyme activity. The main theory behind the progress of amperometric biosensors is based on the relationship between the toxicity of pesticides and the reduction in enzyme activity brought on by OP inhibition. As a result, measuring the activity of enzyme quantitatively when exposure to a pesticide is essential for the development of these biosensing devices (Silva et al., 2004).

The acetylcholinesterase (AChE) is an enzyme that had been reported for the fabrication of pesticides biosensor. AChE is normally extracted from animal tissue such as the head of the fly *Musca domestica*, electric eel, and human erythrocytes (Tanimoto de Albuquerque and Ferreira et al., 2007). Due to complex and tedious extraction procedures and extra care needed while handling the enzyme, it is often hard to utilize a pure enzyme for the bio-sensing application (Wang, et al., 2012). These drawbacks can limit the wide applications of the AChE enzyme.

Thus, in this study, esterase extracted from plants could be used to detect OP pesticides because esterase had shown to have similar sensitivity as AChE in the detection of OP through enzymatic α-naphthyl acetate inhibition. Moreover, it could be extracted from low-cost feedstock requires simpler methods for extraction, purification, and preservation (Li J.K et al., 2009). The electro-active α-naphthol was created as a result of ANAE's interaction with the substrate of α-naphthyl acetate when it was immobilized on the working electrode surface. The current produced by α-naphthyl acetate could

potentially be utilized as an analytical indicator of enzyme activity and a biomarker for assessing the biological impact of OP pesticides, which are responsible for the inhibitory action. (Cai and Du et al., 2008).

Enzyme kinetics is the study of the rates of enzyme-catalyzed chemical reactions. In enzyme kinetics, the rate of the reaction is measured, and the consequences of changing the reaction's parameters are explored. The maximum rate of reaction is characteristic of a particular enzyme at a particular concentration and is known as the maximum velocity V_{max}. The substrate concentration that gives you a rate that is halfway to V_{max} is called the K_m and is a useful measure of how quickly the reaction rate increases with substrate concentration. K_m is also a measure of an enzyme's affinity (tendency to bind to) for its substrate. A lower K_m corresponds to a higher affinity for the substrate, while a higher K_m corresponds to a lower affinity for the substrate. Unlike V_{max} which depends on enzyme concentration, K_m is always the same for a particular enzyme characterizing a given reaction although the "apparent," or experimentally measured, K_m can be altered by inhibitors. Understanding the kinetics of enzyme inhibition by enzyme inhibition assay will assist in improving the sensitivity and effectiveness of the enzyme biosensor.

Multi-walled carbon nanotubes (MWCNT) have become highly valuable in diverse industries because of their distinctive qualities, including excellent electrical conductivity. These qualities make MWCNT an excellent choice as a support material for enzymes (Favero et al., 2015). The creation of Amperometry biosensors may benefit from the potential of MWCNT to enhance electron-transfer processes. MWCNT functionalized with carboxyl group by acid treatment sulfuric acid and nitric acid to form a peptide bond with amino group existed in ANAE modified on screen printed electrode (SPE) is an attempt to fabricate an ANAE biosensor (Vatanpour et al., 2014). Overall, this study aims to explore and quantify the effectiveness of an immobilized plant esterase (alpha-naphthyl acetate esterase, ANAE) on a multi-walled carbon nanotube (MWCNT) coated on Screen Printed Electrode (SPE) for efficient detection of OP pesticides.

1.2 PROBLEM STATEMENT

OP compound pesticides bio-accumulate in living things and have long-term effects on them, which can lead to environmental issues like water contamination. Pesticide residues that may exist the food chain through the air, water, or soil are to blame for these environmental issues since they might disrupt the ecosystem and result in a number of health issues for both humans and animals. OP causes respiratory, infertility, bone marrow, and nervous system issues. (Jokanović et al., 2018).

Due to their sensitivity and effectiveness, chromatographic techniques such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) have historically been utilized for pesticide residue analysis. However, chromatographic methods are time-consuming and arduous, and they frequently result in trash that contains organic solvents that needs to be treated after the analysis. Enzymatic biosensors offer an advantageous approach for detecting these pesticides due to their ability to combine the selectivity of enzymatic reactions with easy operational detection methods. This results in a quick, easy, and selective technique for pesticides analysis (Zhao et al., 2018).

Acetylcholinesterase (AChE) enzyme is a common enzyme for detection of pesticide based on inhibition of AChE activity. Unfortunately, AChE is extracted from animal tissue requires a perfect technique of low-temperature processing and costly (J. Wang, Xia, Zhang, Hu, & Lin, 2012). To counter this problem, plant-esterase which is α-naphthyl acetate esterase (ANAE) was chosen as sensing material rather than AChE because ANAE can be extracted from atta, wheat, soybean, corn, rice and other grains which is easier to get and less expensive. In addition, it is proven that an ANAE has similar sensitivity as AChE.

With their distinct mechanical, thermal, and electrical characteristics as well as for their biocompatibility, carbon-based nanomaterials, such as carbon nanotubes (CNT), have gained substantial interest among nanostructured materials as support materials for the immobilization of enzymes. According to recent research (Putzbach and Ronkainen et al., 2013), the enzyme activity was much greater when immobilized on functionalized CNT than it was when graphene oxide derivative was utilized as the immobilization support, which appears to be correlated to the enzyme loading. MWCNT has been claimed to have several benefits that make it suitable as a support material in this project, including its stable and robust structure and exceptional thermal conductivity

1.3 RESEARCH SCOPE

This study involves the extraction of plant esterase α-naphthyl acetate esterase (ANAE) from atta flour and purified by PEG1000 aqueous two-phase separation. The characterization conducted in terms of optimum temperature, pH, and concentration of substrate. The kinetic model for the inhibition of ANAE in the presence of dichlorvos pesticides by enzyme inhibition assay was analyzed to determine the kinetic parameters. Using sulphuric acid and nitric acid in a reflux system, a straightforward approach for adding carboxyl groups to the surfaces of multi-walled carbon nanotubes (MWCNT) is provided. Then, enzyme activity for ANAE immobilized on MWCNT is examined by carrying out an enzyme activity assay to find out the residual activity of an enzyme. Fourier transform infrared (FTIR) spectroscopy is employed to analyze and identify the functional groups present on the MWCNT (Zhao et al., 2013).

Next, an amperometric biosensor is developed by drop casting of functionalized MWCNT-COOH-ANAE on SPE (Mohamed et al., 2016). The MWCNT-COOH-ANAE-SPE biosensor was employed for the detection of dichlorvos by the cyclic voltammetry

(CV) method. The amperometric detection of pesticides monitors the inhibition reaction by measuring the oxidation current of the enzyme product (Hayat & Marty et al, 2014).

1.4 RESEARCH OBJECTIVES

This project aims to study the effectiveness of the immobilized ANAE on a multi-walled carbon nanotube (MWCNT) coated on a screen-printed electrode for the detection of pesticides. A few objectives that carried out to achieve the above aim are listed below:

i. To analyze the performance of ANAE towards α-naphthyl acetate in different reaction condition.

ii. To study enzyme kinetic inhibition reaction with different OP concentrations by applying a linear regression graph.

iii. To functionalize the MWCNT with carboxyl group and immobilize the enzyme with covalent bonding.

Iv To evaluate the electrochemical performance of immobilized ANAE in detecting different OP concentrations by amperometric measurements.

1.5 THESIS ORGANIZATION

This thesis consists of five chapters which have an introduction and a summary.

Chapter 1 portrays the main focus of this research which includes the background of a study problem statement, scope of the study, and research objectives. This chapter contains a recent study of the effect of pesticides and an overview of current technology to detect pesticides by using a more convenient enzyme which is α-naphthyl acetate esterase (ANAE) rather than the Acetylcholinesterase (AChE) enzyme.

Chapter 2 is the literature review which includes a theoretical explanation and past research on all the elements in this study such as a detailed explanation of the extraction and purification of the enzyme. In addition, this chapter summarizes and compare the methodology of immobilization and detection of pesticide used in this study.

Chapter 3 contains the materials and methods used in this study which includes a flow chart that summarizes the whole flow of this study. This chapter includes the preparation of crude enzymes, functionalization of MWCNTs, immobilization method, optimization of condition parameters, characterization, a kinetic study of enzyme inhibition, and detection of pesticides using plant esterase.

Chapter 4 comprises experimental results and discussions on findings from experiments conducted. All experiments were done in triplicates. The validity of the results obtained was supported by past research on similar findings and was discussed in detail to provide proper understanding of the nature of the results obtained.

Chapter 5 concludes the whole research based on the findings obtained. Recommendations and suggestions were also included to enhance this study through further research.

CHAPTER TWO

LITERATURE REVIEW

2.0 INTRODUCTION

In order to maintain the environment and ensure consumer safety, the increasing use of pesticides needs advancement of successful techniques for residue detection in agriculture. A quick and accurate biosensor test was created as a consequence. Because of its cheap cost, ease of extraction, convenience of preservation, sensitivity, and accuracy, an esterase isolated from plants may respond to OP while achieving important role in the quick identification of OP residues (Li J.K., 2009). This chapter discusses every aspect of this research, from the type of enzyme employed through the implementation of the herbicide detection method.

2.1 PESTICIDE

Pesticides are the biological agent likes a virus, bacterium, antimicrobial, or disinfectant that highly used in killing pests. They are highly benefit in agriculture in order to destroy insects, harmful animals or plants, and vectors of disease. There are many types of pesticides, which are insecticides, rodenticides, herbicides, and fungicides. Each of these types have different and specific function in agriculture. For example Herbicides kill weeds and other plants that grow where they are not wanted and Insecticides kill insects and other arthropods (Achparaki et al., 2012).

Pesticides include herbicides for destroying weeds and other unwanted vegetation, insecticides for controlling a wide variety of insects, fungicides used to prevent the growth of molds and mildew, and rodenticides are pesticides that kill rodents, including mice and rats which are often formulated as baits with attractive substances like peanut butter or molasses (Nicolopoulou-Stamati, Maipas, Kotampasi, Stamatis, & Hens, 2016). The extensive application of pesticides in the food industry will affect the people with a small amounts of pesticide residues through their diet. Pesticides have greatly benefited human life by improving crops and controlling infectious diseases, but their widespread use is now causing adverse effects on human health, regardless of occupational or environmental exposure. (Nicolopoulou-Stamati et al., 2016).

2.1.1 Insecticide

Insecticides are agents of chemical substances that have been used globally for pest control in agriculture, horticulture, and forestry. There are 2 categories of insecticides which are natural and synthetic (Zaim & Guillet, 2002).

Natural pesticides might be chemical, biological, or mineral-based in composition which function to kill, repel, or otherwise disturb pest behavior (Tu & SATOH, 2001). All natural pesticide products must adhere to federal and state laws involving registration, sale, transportation, usage, storage, and disposal in order to be considered pesticides for this purpose. In systems that are certified as organic, some natural pesticides may be used provided that extra federal organic requirements are satisfied (Isbn, Publication, This, & Union, 2011).

Synthetic pesticides are manufactured from substances that don't naturally exist. Depending on their intended usage, they are divided into several groups. Inorganic

insecticides are those that do not contain carbon. Usually they are white crystals in their natural state, resembling the salts (Chowański, Kudlewska, Marciniak, & Rosiński, 2014). They are stable chemicals, do not evaporate, and are usually water soluble. Meanwhile, Organic pesticides are produced from natural ingredients. However, that does not conclude these pesticides are completely free from chemicals. The only difference is these chemicals are derived from botanical and natural sources (Ara & Haque, 2021).

There are four classes of organic insecticides which are organophosphates, organocarbamates, organochlorides and pyrethroids (R. Kaur, Mavi, Raghav, & Khan, 2019). This study primarily focuses on organophosphate insecticides, specifically organic insecticides (Hassaan & El Nemr, 2020). Figure 2.1 shows the classification of insecticides into sub-classes.

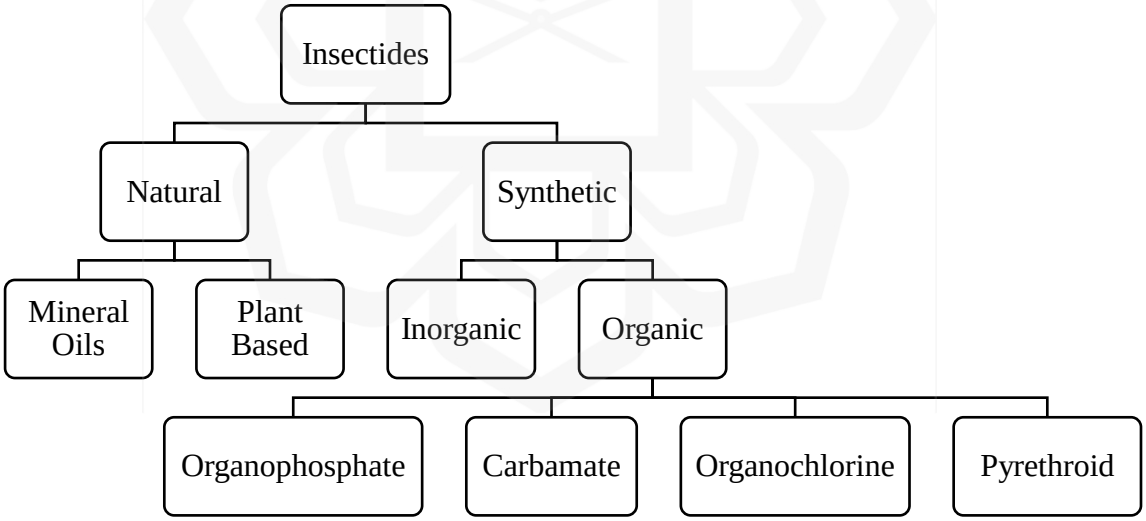


Figure 2.1 Classification of insecticides into sub-classes

2.1.2 Organophosphorus Compound

Organophosphates (OP) are a class of organophosphorus compound with the general structure as shown in Figure 2.2. They can be considered as esters of phosphoric acid. Organophosphates are found in a wide variety of substances, including key biomolecules. (Farahat et at., 2003). Since Organophosphates (OP) are a unstable chemical nature, thus they are very effective and lack of persistence in the environment (J. Kaur & Singh, 2020). As an alternative to chlorinated hydrocarbons they are very useful in pest control.

Organophosphates are poisonous to both insects and mammals which they can destroyed the enzyme acetylcholinesterase (AChE) and inactivate all functions of the central nervous system (CNS) (Singh and Walker et al., 2006). There are many reports published on harmful effects of pesticide suffered on agricultural workers every year in the third world countries (Alavanja et al., 2009). Hence, it is significant to create a biosensor that is sensitive, fast, and accurate for detecting organophosphate. Figure 2.2 shows the organophosphate functional group.

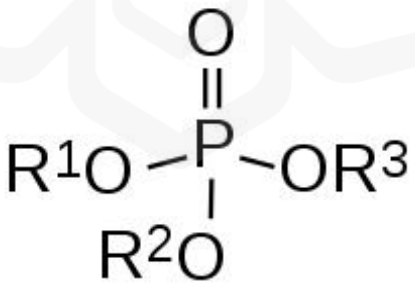


Figure 2.2 Organophosphate functional group (Singh and Walker et al, 2006)

2.2 ENZYME

The acetylcholinesterase (AChE) is an enzyme that had been reported for the fabrication of pesticides biosensor. AChE is normally extracted from animal tissue. In this study, esterase extracted from plants could be used to detect OP pesticides because esterase had shown to have similar sensitivity as AChE in the detection of OP.

2.2.1 Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) is a crucial enzyme found in the muscular tissue and sensory system of various animals, reptiles, and insects. It controls rapid hydrolytic degradation of the neurotransmitter acetylcholine (AChE) into the inactive product which are choline and acetic acid (Sassolas, Prieto-Simón, and Marty et al., 2012). AChE is one of various physiologically essential neurotransmitter agents. It involves in the transmission of nerve impulse to effector cells at cholinergic, synergic and neuromuscular junctions (Chauhan et al., 2018).

The presence of AChE has been shown in an abundant of animal tissues and enzymes from various distinctive sources, including erythrocyte insects, mammalian brain and other tissues**.** These enzymes have been purified and characterized. However, as it is taken from animal blood or tissue, AChE's high price and limited accessibility severely restrict its widespread use. Figure 2.3 shows the hydrolytic degradation chemical equation of the neurotransmitter Acetylcholine (AChE) into the inactive product choline and acetic acid.

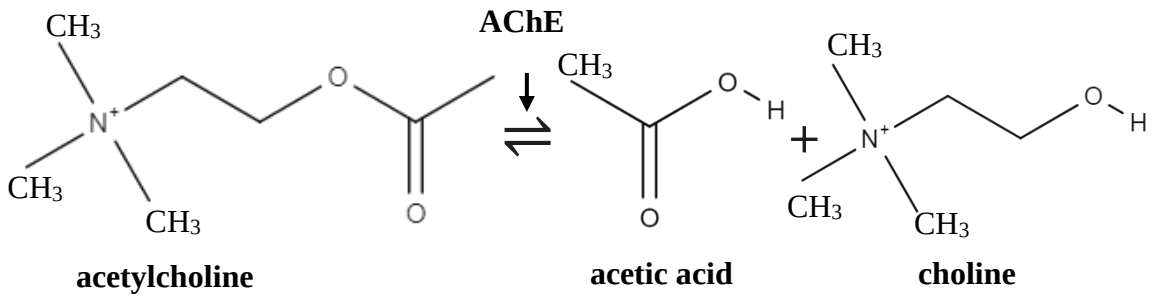
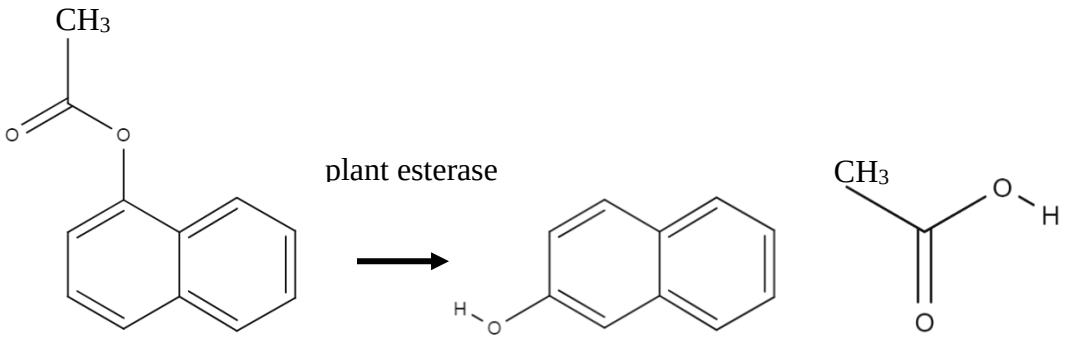


Figure 2.3 Equation of the Acetylcholine (ACh) into choline and acetic acid (Sassolas, Prieto-Simón, and Marty et al., 2012)

2.2.2 Alpha naphthyl acetate esterase (ANAE)

Alpha naphthyl acetate esterase (ANAE) is a plant esterase which catalyze the hydrolysis of α-naphthyl acetate into α-naphthol (Bayraktaroğlu et al., 2015). In large quantities, soybean, wheat, sorghum, rice, and other crops all contain the esterase enzyme known as plant-esterase. It is important for numerous biological processes, including signal molecule activation and the control of endogenous product bioactivity (Bao et al., 2015).

Other studies have much attention to plant esterase because their comparable behavior with animal esterase which is acetylcholinerase, AChE in inhibition mechanism with OP pesticides (Hou et al. 2012). The enzyme's activity was calculated using the linear connection between time and the absorbance of a purple diazonium dye. The principle behind ANAE detection is similar to the AChE, whereby ANAE hydrolysis of α-naphthyl acetate into α-naphthol and acetic acid will be inhibited by pesticides. Figure 2.4 represents the reaction scheme for the plant esterase catalyzed hydrolysis of α-naphthyl acetate (Bao et al., 2015)



1-Naphthyl acetate **1-Naphtol** **acetic acid**

Figure 2.4 Reaction scheme for the plant esterase catalyzed hydrolysis of α-naphthyl acetate (Bao et al., 2015)

+

2.3 SUPPORT MATERIAL

The development of effective biosensors with the necessary capabilities, including high sensitivity, high selectivity, quick reaction times, and high repeatability, appears to depend heavily on enzyme immobilization. Immobilized biomolecules must remain securely bonded to the surface, preserve their structure and function, keep their biological activity following immobilization, and not be desorbed while being used as a biosensor. Magnetic particles, mesoporous materials, ceramic materials, carbon nanotubes, and graphene are some recent examples of materials used in biotechnology that have various shapes and sizes, porous and non-porous structures, and binding properties.

2.3.1 Magnetic nanoparticles (MNP)

Magnetite (Fe_3O_4), maghemite (-Fe_2O_3), and cobalt ferrite ($CoFe_2O_4$) are the magnetic elements and their oxides that make up magnetic nanoparticles (MNP). Due to its wide surface area and the hydroxyl groups on its surface, which allow for facile

functionalization and robust binding of the enzyme molecule, it has undergone substantial research, particularly in biomedical and protein or enzyme immobilization (Nguyen, Lee, Lee, Fermin, & Kim, 2019).

Magnetic nanoparticles (MNPs) are among the potential choices for carrier-bound enzyme immobilization and show promise due to their magnetic characteristics, biocompatibility, and lack of toxicity. Yeast alcohol dehydrogenase and lipase have been immobilized using MNPs with different surface modifications as the support material.

2.3.2 Mesoporous

Numerous microporous and mesoporous materials have been successfully introduced during the past 20 years, contributing to the astonishing rise of porous materials. Because of their distinctive properties, including their sizable surface area, increased pore volume, and variable pore size, non-toxicity, and chemical and thermal durability, mesoporous materials are thought to provide potential substrates for the immobilization of enzymes. Encapsulation or covalent bonding are two ways that enzymes can be immobilized on mesoporous materials. Diffusional restrictions must also be taken into account when the enzyme is positioned in the carrier's pores since the transfer of substrates and products is constrained. Figure 2.5 shows IUPAC classification of porous materials

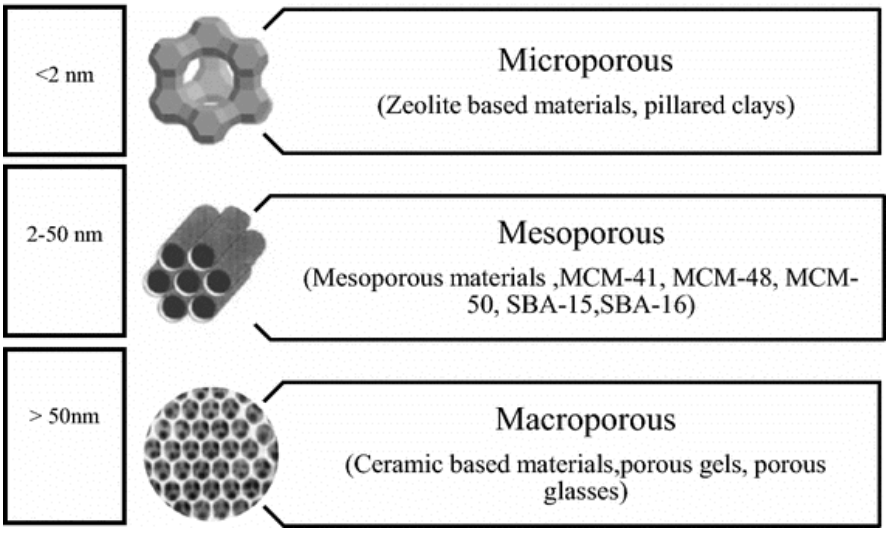


Figure 2.5 IUPAC classification of porous materials (Suib, 2017)

2.3.3 Ceramic Materials

In terms of extended service life, strong chemical, mechanical, and thermal resistance, large surface area, sufficient pore size, biocompatibility, inertness, and simple regeneration following enzyme inactivation, ceramic supports have several benefits. Alumina, zirconia, titania, and silica are a few examples of ceramic materials that are employed (Kelly & Benetti, 2011).

Due to their availability in a wide variety of porosities, low cost, and adaptability to various procedures, ceramic materials properties make it possible to utilize immobilized enzymes in various reactor types (Pilathadka, Vahalová, & Vosáhlo, 2007). Additionally, ceramic supports provide high mechanical stability. As a result, when the enzymes lose their ability to catalyze, they may be quickly restored and employed to immobilize a new biocatalyst. However, the lack of reactive groups on the surface of ceramic materials restricts the immobilization of enzymes (Kelly & Benetti, 2011).

2.3.4 Nanomaterial

The best support materials, at least in one dimension, are nanomaterials, which have sizes smaller than 100 nm. This indicates that they are much smaller than microscale. Nanomaterials can be created using a variety of techniques.

On the basis of their form, size, characteristics, and constituents, there are several types of nanomaterials. They include lipid-based nanomaterials, nanoparticles made of metals, semiconductor nanomaterials, polymeric nanomaterials, and nanomaterials based on carbon. Due to their electrocatalytic activity and large surface area, nanomaterials can be extremely useful when used with electrochemical transducers. A wide different of nanomaterials, including graphite, grapheme, and carbon nanotubes, as well as gold, silver, silicon, and copper nanoparticles, are employed to construct biosensor immobilization (Putzbach & Ronkainen, 2013). Since carbon nanotubes (CNTs) offer special characteristics including a high surface area ratio, superior biocompatibility, and low density, they have been employed in this work as a support for sensors.

2.3.4.1 Carbon Nanotube

CNTs are hexagonally arranged, nanoscale tubes consisting of hybridized carbon atoms. They are created by coiling one or more layers of graphene sheets at a certain rotation angle around the central axis.

The immobilization of enzymes or several enzymes on CNTs by physical adsorption or cross-linking makes CNTs an appealing platform. Because the lateral

connections between neighbouring adsorbed proteins are suppressed, CNTs can shield enzymes from inactivation in hostile conditions.

The CNT are mainly divided into two categories: single walls CNT (SWCNT) and multiple walls CNT (MWCNT) as in Figure 2.6. In this research, the multiwall carbon nanotubes (MWCNT) were used as the best raw materials which introduced highly stable and strong support material for enzyme immobilization (Saifuddin & Juniazah, 2013).

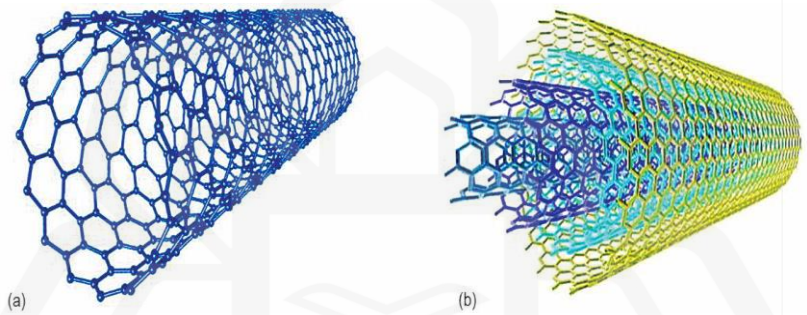


Figure 2.6 (a) SWCNT, (b) MWCNT (Favero, Fusco, Mazzei, Tasca, & Antiochia, 2015)

2.4 FUNCTIONALIZATION OF CARBON NANOTUBE

SWNTs have been regarded as one of the most well-liked nanomaterials because of their exceptional and distinctive mechanical, electrical, optical, and thermal capabilities. However, SWNTs frequently form bundles and have a relatively low solubility in common solvents. They are now more challenging to control and purify as a result. For synthetic chemists and materials scientists, the technique of "chemical functionalization" is a method for getting beyond these obstacles (Yu, Wu, Zhao, Wei, & Lu, 2015).

Chemical functionalization is a well-known method for adding functional groups like carboxyl and amine groups to the surface of nanomaterials to give them the required qualities. These techniques may be divided into two broad categories: covalent functionalization and non-covalent functionalization. A covalent bond is created between functional entities and the carbon skeleton of nanotubes during the process of covalent functionalization. These carboxylic groups may have already been present on the CNTs when they were being produced or they may have been added during oxidative purification (Abuilaiwi, Laoui, Al-Harthi, & Atieh, 2010).

Non-covalent functionalization is mostly accomplished by supramolecular complexation, which employs a number of adsorption forces, including the Van der Waals force, hydrogen bonds, electrostatic force, and p-stacking interactions. Non-covalent functionalization offers benefits over chemical functionalization in that it may be used under very moderate reaction conditions and can preserve the excellent graphitic structure of CNTs. Figure 2.7 shows the visual structure of functionalized carbon nanotube with carboxyl.

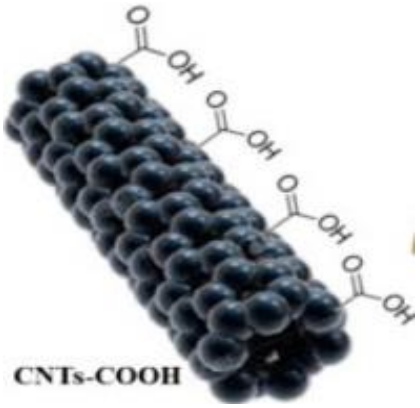


Figure 2.7 CNT Molecular structures for Functionalized with carboxylic groups(-COOH)(Peng et al., 2003)

2.5 BIOSENSOR

Recently, a biosensor is one of the useful devices used to detect biological and chemical processes. There are many different ways that biosensors may be used to enhance quality of life. This area includes their application for a different of purposes, including environmental monitoring, disease detection, defense, safety of meals and drug development. There five important elements in biosensor which are analyte, biological recognition element or bioreceptor, transducer, and signal detector as shown in Figure 2.8.

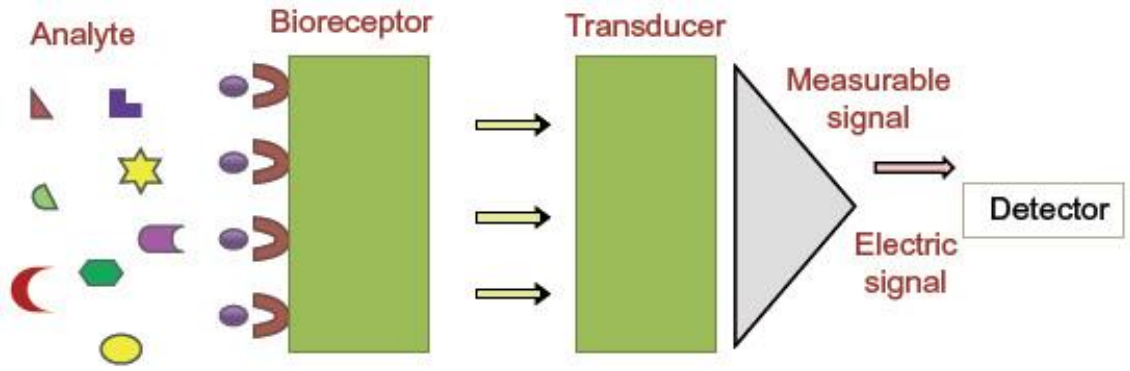


Figure 2.8 Enzyme based electrochemical biosensor (Malhotra, Verma, Tyagi, & Kumar, 2017)

An analytical substance is one that demands detection. One such "analyte" in a biosensor intended to detect glucose is glucose. Enzyme, antibodies and DNA are the examples of bioreceptor which used to identify any analyte attached particularity. When the bioreceptor detect any analyte the signal will produce, this process called Bio-recognition. The transducer's function in a biosensor is to transform a bio-recognition event into a detectable signal (Chauhan, Narang, & Jain, 2018). Most transducers produce optical or electrical signals that are generally proportional to the level of interactions occurring between the analyte and the bioreceptor. The transducer then detects the changes and converts them into quantifiable signals that can be used to calculate the concentration of analyte in the sample. Biosensor become the most effective device with several advantages including high specificity and sensitivity. In this study, the biosensor used to focus on detection of pesticide using plant esterase as bioreceptor (Bilgi & Ayranci, 2018).

2.6 ENZYMES INHIBITION

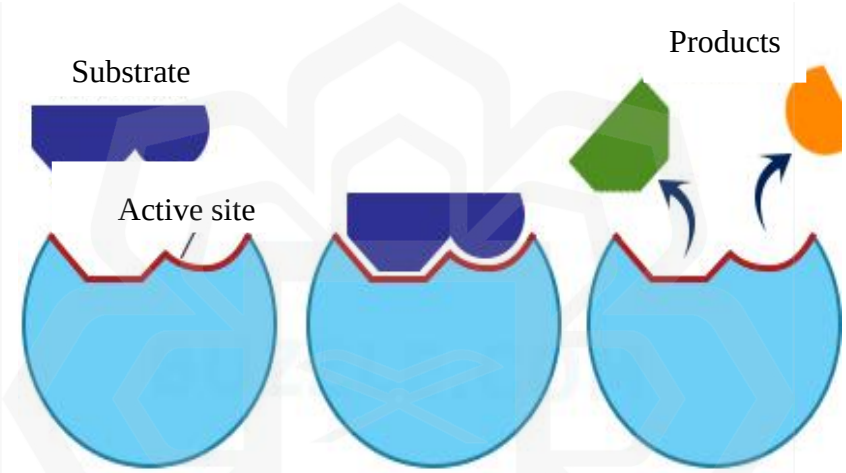
Pesticides as chemical substances work in a wide range of ways and some are more viable than others. Organophosphates (OP) commonly work as a destroyer of the enzyme known as acetylcholinesterase (AChE) that is a basic enzyme involved with muscle control in all animals (Amine, Arduini, Moscone, & Palleschi, 2016). AChE acts to inactive a chemical nerve messenger responsible for muscle contraction known as acetylcholine (ACh). Muscles in animals contract because of the acetylcholine signal released by nerves and the job of acetylcholinesterase is to break down acetylcholine and allow the muscle to relax (Mangas et al., 2017).

Basically, pesticides work by destroying the acetylcholinesterase and prevent muscle relaxation. In this manner, the muscles controlling breathing never work again and the insects rapidly asphyxiate and die due to paralysis and absence of oxygen. Pesticides serve as analytes whose concentrations must be determined. A non-covalent intermediate is formed between the enzyme and the OP compound in a reversible step, and this intermediate is then converted into an inactive complex in an irreversible step by the enzyme encasing the OP compound with a "key in lock" fit. This is how ANAE inhibition by OP develops as a "progressively irreversible" kinetics (Silva et al., 2004).

2.7 KINETIC STUDY OF ENZYME

Enzyme kinetics is the study of chemical reactions that are catalyzed by enzymes. The reaction rate of enzyme and different conditions affecting the enzymatic reactions is investigated.

Knowledge of the kinetics of an enzyme could assist in understanding the structure of an enzyme, suggesting models on how substrates and products bind during catalysis and changes that occur during the reaction can be used in designing a more sensitive, stable and efficient biosensor. Enzymes are typically protein entities that interact with their substrates or other compounds. The enzymatic process is a set of steps that transforms these target molecules into products when they attach to an enzyme's active site showed in Figure 2.9.



(a) Enzyme (b) Enzyme-substrate complex (c) Enzyme

Figure 2.9 Enzyme kinetic mechanism (Bean et al., 2002)

2.7.1 Michaelis-Menten kinetics

One of the most well-known models of enzyme kinetics is Michaelis-Menten kinetics. Initial velocity measurements at various substrate concentrations are used to calculate the enzyme kinetic constants (V_{max} and K_m). The highest rate that the system may attain at saturating substrate concentration is denoted by the term V_{max}. The substrate concentration

at which the reaction rate is half of V_{max} is numerically equivalent to the Michaelis constant K_m (Bean et al., 2002).

The Michaelis-Menten equation are specifically used to calculate the kinetics of enzyme reactions (Doran, 2012) as shown in Eq. (2.1):

$$V = \frac{V_{max}S}{K_m + S} \tag{2.1}$$

v is the volumetric rate of reaction, S is the substrate concentration, V_{max} is the maximum rate of reaction, and K_m is the enzyme kinetic constant. Figure 2.10 shows the Michealis-Menten plot which is represented by Eq. (2.1).

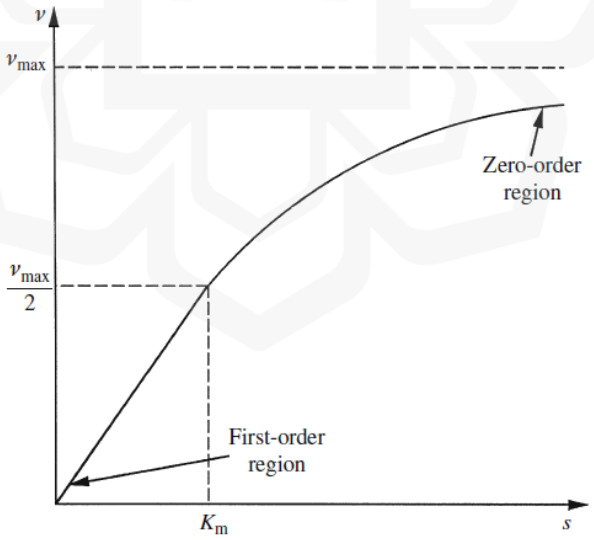


Figure 2.10 Michaelis-Menten Plot

All of the enzyme is attached to the substrate as an enzyme-substrate complex when $V = V_{max}$. In this study, only initial substrate concentrations data is collected for several batch experiments. The response rate is assessed at time zero using each batch of data. To estimate V_{max} and K_m, the beginning rates, V, and initial substrate concentrations, s, were utilized as pairs that could be displayed in a variety of ways.

The derivations of the Michaelis-Menten equation using Lineweaver-Burk method, Hanes-Woolf method and Eadie-Hofstee method were established to linearize the Michaelis-Menten equation which makes it easier to obtain accurate V_{max} and K_m values.

2.8 ENZYMES BASED BIOSENSORS

Biosensor is generally divided into two parts, a bio receptor and a transducer. A bio receptor is an immobilized biological element (DNA, enzyme, antibody) sensitive enough to recognize the analyte (enzyme substrate, DNA, antigen). Amongst biological elements, enzymes are the most commonly used biosensors. While a transducer is used to transform the signal produced when analyte interacts with a sensing device into a detectable electrical signal.

An analytical tool known as an enzyme biosensor creates a signal that is proportional to the concentration of the analyte or the rate of enzymatic turnover when an enzyme is in contact or close proximity with the transducer. The signal is often produced by a change in the protons' concentration, the release or absorption of gases, the emission of light, or the emission of heat (Bucur, Munteanu, Marty, & Vasilescu, 2018). The

transducers use electrochemical, thermal, and optical methods to turn these signals into quantifiable responses like current, potential, temperature fluctuations, or light absorption.

The signals generated are then amplified and sorted for the future usage. Enzymes are immobilized for the production of biosensors by adsorption, covalent attachment, and entrapment in gels, electrochemically produced polymers in bilipid membranes, or in solution behind a membrane of choice. The transducers frequently used with the enzymes are electrochemical and fiber optic transducers (Malhotra et al., 2017).

2.9 ELECTROCHEMICAL BIOSENSOR

According to the kind of transducer being utilized, electrochemical biosensors are categorized as amperometric (current), potentiometric (potential), and impedimetric (impedance) (Chauhan et al., 2018). The electrochemical approach for pesticide detection uses amplimetric transduction the most frequently. Pesticide-induced enzyme oxidation or decrease in inhibition results in current generation in an amperometric biosensor (Cai and Du et al., 2008). The amount of current generated at the working electrode's surface varies inversely with the amount of enzyme.

Amperometric biosensor work in three-electrode configurations which are counter, reference and working electrode. Current passes between the working and the counter electrode, and voltage is supplied between the reference and working electrodes. Additionally, the electrochemical biosensor needs to be selective. The biosensor's electrochemical response ought to be distinct to a certain target analyte (Bilgi and Ayranci et al, 2018).

2.10 CARBON NANOTUBE MODIFIED BIOSENSOR

A new acetylcholinesterase biosensor based on immobilised enzyme membrane-electrode separating sensor was suggested in which electrode was modified with multiwall carbon nanotubes in order to construct a biosensor for detecting the residues of organophosphorus pesticides (D. Chen et al., 2017). The amperometric measurement of AChE multiwall carbon nanotubes was employed for detection because the enzyme membrane was stuck to the surface of the tubes during the detection process.

In addition, because they may offer quick, sensitive, easy, and affordable on-field detection, carbon nanotubes (CNT) based biosensors are more effective than currently used conventional approaches. Enzymatic and non-enzymatic detection can be used as the foundation for the measurement techniques. In order to create ultrasensitive biosensors for the detection of OPs, AChE is combined with CNT using a variety of immobilization techniques (Zhu et al., 2017). As a method of quantifying OPs, this approach depends on determining the level of enzyme inhibition.

2.11 SCREEN PRINTED CARBON ELECTRODES

Screen Printed Carbon Electrodes (SPCE) has been extensively employed as matrix for biosensing applications due to the easiness to produce and mainly because they are portable, which facilitates in situ applications. Carbon is one of the most extensively employed materials used in these devices due to its versatility and low cost. With screen-printed technology, the geometry of the sensor has the benefits of flexible design, automated processes, high repeatability, and a variety of materials (Bilgi & Ayranci, 2018).

Working, counter, and reference electrodes are the three electrodes that are typically included in SPCE. These electrodes were all printed on various plastic or ceramic substrates, which may be readily customized using a wide range of store-bought or home-made inks. Silver ink and carbon ink are the pastes that are most frequently used while printing SPE. Because it is affordable, versatile, and chemically inert, carbon paste is a widely used substance.

The selectivity and sensitivity needed for each study are determined by the makeup of the different inks used to print on the electrodes (Tanimoto de Albuquerque & Ferreira, 2007). As analytical tools for food analysis in the control of food spoilage that enable quick screening at any point of the food production chain, preventing the occurrence of foodborne illnesses, and reducing food waste, SPCE, as shown in Figure 2.11, have attracted growing interest.

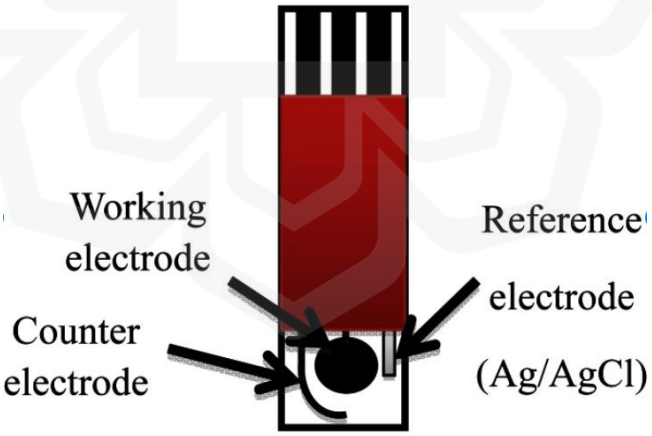


Figure 2.11 Screen printed electrode (Neelam et al., 2016)

2.12 ENZYME IMMOBILIZATION

In comparison to bulk solid materials, enzyme immobilization on nanostructured materials has various benefits, including a greater surface area that can result in increased enzyme loading, effective nanoscale dispersion, and practicable surface functionalization. Additionally, an immobilized enzyme can only travel a minimal amount of space, or even none at all (Putzbach & Ronkainen, 2013). Examples of immobilization methods are physical adsorption, physical entrapment, covalent coupling, and electropolymerisation.

2.12.1 Physical adsorption

Van der Waals force, ionic bonds, and hydrogen bonding between solid materials and enzymes are all examples of physical adsorption. The physical technique preserves the majority of the enzyme's activity while being convenient and seldom changing the structure of the enzyme. The enzyme leakage caused by this approach is a downside (Mubarak et al., 2014).

2.12.2 Physical Entrapment

The active ingredients are physically trapped inside a film, gel, fibre, coating, or microencapsulation as part of the physical entrapment process. An enzyme or active molecule can be combined with a polymer to create this approach, and the polymer is then cross-linked to create a lattice structure that traps the enzyme. This is a one-step process that is carried out at a low temperature, is quick and easy, and doesn't interfere with the enzyme's function (Vidal, Esteban, Gil, & Castillo, 2006).

2.12.3 Covalent Coupling

In covalent coupling, a strong covalent link is created between the support and the enzyme, preventing the enzyme from being leached. The enzyme is also immediately available for interacting with the analyte, resulting in a rapid reaction time. Covalent immobilization often guarantees the strongest link between support and enzyme, minimizing leakage problems, among any of these techniques. Additionally, covalent attachment permits the greatest improvement in operational stability, particularly with respect to heat, pH, organic solvents, and also regarding storage, since it often does not interfere with the mass transfer of reagents or products (Vidal et al., 2006).

In this study, amine groups from the enzyme and carboxylic groups on the functionalized MWCNT formed a covalent bond that allowed the enzyme to be immobilized onto MWCNT.

2.13 MWCNT-SPE BIOSENSOR

A biosensor, created based on the multi-walled carbon nanotubes (MWCNT) modified on a screen-printed terminal (SPCE) can be used for the detection of many types of pesticides (Bayraktaroğlu et al., 2015). Besides, a SPE displayed favorable material properties which can be connected to the in-situ recognition contrast with common electrode.

In order to improve the electron transfer at lower potential, the biosensor was created using N,N-dimethylformamide (DMF) (Costa et al., 2006). A 10 µL of that solution was cast on the carbon electrode surface of the sensor strip and the coating was dried at 50°C for one hour. The surface of the printed carbon electrode was then

completely covered by a thin-layer of CNTs so that the screen-printed carbon only serves as a conducting base for the carbon nanotube electrode (Sun, Fu, Lin, & Huang, 2002).

2.14 ANAE BASED BIOSENSOR

Amperometry detection of pesticides monitor the inhibition reaction by measuring the oxidation current of the enzyme product (Hayat and Marty et al., 2014). In the biosensor system, ANAE hydrolyzes the α-naphthyl acetate to produce α-naphthol and acetic acid, which produces the initial current response by its oxidation at the electrochemical transducer at constant potential. When pesticides react with OPs the normal ANAE activity is changed. This will reduce the response signal of the biosensors, which can be related to the concentration of pesticide (Bucur et al., 2018). Table 1 summarizes the different of biosensors and the detection limits**.**

Table 2.1: Summarization of different biosensors and the detection limits

Analyte	Immobilization support	Electrochemical biosensor	Limit of detection	Reference
Organophosphorus hydrolase (OPH)	Carbon nanotube	amperometric	6 nA/μM	(Prakash et al., 2005)
acetylcholinesterase (AChE)	Graphene oxide	amperometric	0.15 ng/mL	(Li et al., 2017)
acetylcholinesterase	multi-walled carbon nanotubes	cyclic voltammetry (CV)	0.05 μg/L.	(Chen et al., 2017)
acetylcholinesterase	multi-walled carbon nanotubes (MWCNTs)	amperometric	1.4 1mol L^{-1} 0.95 1mol L^{-1}	(Cesarino et al., 2012)
α-naphthyl acetate esterase (ANAE)	Chitosan/Gold Nanoparticles	cyclic voltammetry (CV)	0.19 nM 1.51 nM	(Bao, et al., 2015)
OPH and AChE with a set of cushion- ing bilayers consisting of polyethyleneimine (PEI) and DNA	multi-walled carbon nanotubes (MWCNTs)	cyclic voltammetry (CV)	0.5 for OP pesticide paraoxon and 1 μM for non-OP pesticide carbaryl	(Y. Zhang et al., 2015)
Anti- parathion antibody	Graphene-NH_2	Electrochemical Impedance Spectroscopic (EIS)	52 pgL^{-1}	(Mehta et al., 2016)

2.15 SUMMARY

This chapter reviews every expect relating to this research, supported by theoretical explanations and findings from past research. In this study, we focused on ANAE enzyme from wheat flour which is low cost, convenient preservation and requires simpler methods for extraction and purification. The most promising alternative to detect pesticides, enzyme-based electrochemical biosensors, have promised a quick identification and accurate quantification for OP substance. Employing the transduction technique of amperometry for the detection of various enzyme substrates or reaction products allows for the measurement of the detection activity. The main idea behind developing amperometric biosensors is based on the relationship between pesticide toxicity and a decline in enzyme activity.

CHAPTER THREE

RESEARCH METHODOLOGY

3.1 INTRODUCTION

Alpha naphthyl acetate esterase (ANAE) is a plant esterase extracted from a wheat flour and purified in an aqueous two-phase system (ATPS) for investigation. Two phase solution formed by using PEG1500 will be measured their absorbance using spectrometry for further calculation of enzyme activity assay. For immobilization of purified ANAE, multi-walled carbon nanotubes (MWCNT) doped screen printed electrode (SPE) are used as a support. The presence of functional groups which are carboxyl group and amine group on the MWCNTs characterized by fourier transform infrared (FTIR) spectroscopy. Next, the amperometric detection of pesticides monitor the inhibition reaction by measuring the oxidation current of the enzyme product (Hayat & Marty et al, 2014).

3.2 FLOWCHART

Figure 3.1 illustrate the stepwise methodology of experiment to be carried out to achieve all the objectives.

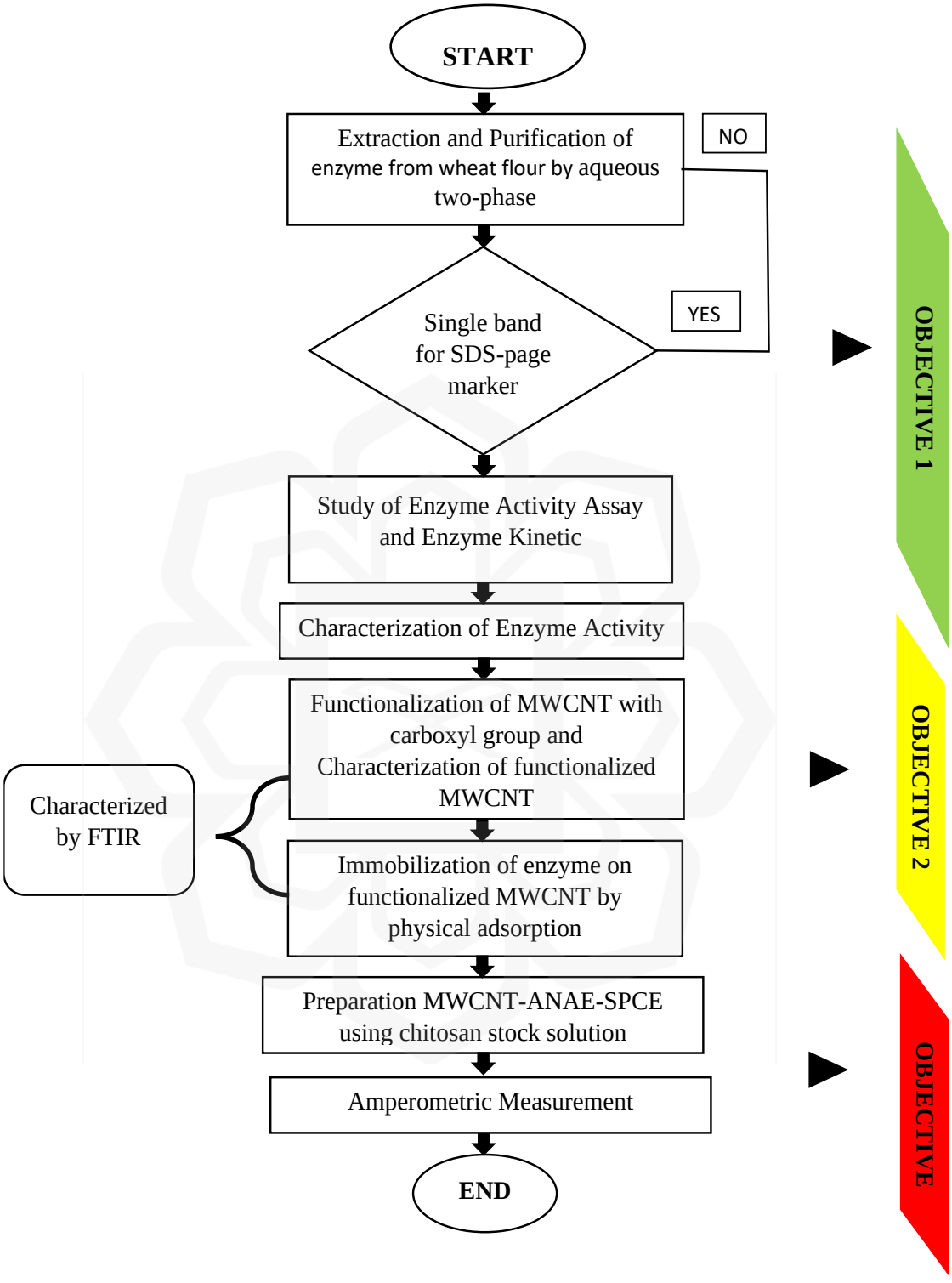


Figure 3.1 Flowchart

3.3 MATERIALS AND APPARATUS

Disposable screen-printed carbon electrodes which is suitable for working with microvolumes of samples were purchased from Parque Technológico de Asturias Lanera (Asturias) in Spain. Cyclic voltammetry (CV) measurement was performed by using a model PGSTAT 30 Autolab Electrochemical System equipped with GPES 4.9 software (Eco Chemie, Utrecht, The Netherlands). The presence of certain functional groups in a molecule characterized by fourier transform infrared (FTIR) spectroscopy at the International Institute for Halal Research and Training (INHART) International Islamic University Malaysia (IIUM). Thermo Scientific Multiskan Go Spectrophotometer from Environmental Lab International Islamic University Malaysia (IIUM) used for measuring absorbance.

Additional items that were used for the experiments are Falcon tubes (15 ml and 50 ml), pipette (10 µl – 10000 µl), pipette (0.5 ml – 5 ml), cuvettes, beakers and centrifuge tubes.

3.4 CHEMICALS

ANAE enzyme extracted from wheat flour purchased from local market, Giant. Polyethylene glycol PEG 1000, protein marker, coomassie blue R-250,1-Naphthyl acetate (1-NA) and Fast Blue B salt, Acrylamide and dichlorvos were purchased from Sigma Aldrich. sulfuric acid, nitric acid, disodium hydrogen phosphate, ethanol, ammonium dihydrogen phosphate, sodium hydroxide pellets, sodium dodecyl sulfate (SDS) and hydrochloric acid were obtained from biotechnology engineering laboratory of IIUM.

3.5 METHODOLOGY

3.5.1 Extraction and Purification ANAE

The ANAE enzyme extracted from wheat flour source by weighing 2 g of wheat flour and mix it with 10 mL distilled water. The mixture was left in the flask for 25 minutes with the magnetic stirrer inside the shaker. Then, stand overnight at $4℃$. Two layers of mixture formed and centrifuged at 4000 rpm for 10 minutes before the supernatant, the crude extract is collected.

Next, pipette out the supernatant and filtered using 0.22 μL PES non-protein binding cellulose membranes using vacuum pump. This was referred as ANAE crude extract. The crude extract then stored at 4℃ and used within 10 days. The crude extract was used to obtain ATPS1 by mixing with 2.7 g PEG1500, 13 g disodium hydrogen phosphate, and 5 ml of distilled water. The solution adjusted to pH 5 by adding the sodium hydroxide to increase pH or hydrochloric acid to lower the pH. An aqueous two-phase formed after centrifuge for in 20 minutes. Then, it repeated to obtain ATPS2 by adding top phase of ATPS1 and 0.6 g of ammonium dihydrogen phosphate without crude extract to replace the conventional salting-out step in purification. The bottom phase of ATPS2 was used for further dialysis to remove all buffer and get the purified ANAE (Yang et al., 2010).

3.5.2 Sodium Dodecyl Sulphate-Gel Electrophoresis (SDS-PAGE)

Characterization of ANAE for its molecular weight was conducted by using Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by following Deutscher's method

(Yang et al., 2010), using a 30% polyacrylamide slab gel. The purified ANAE was used to check the molecular weight of the enzyme and check the purity in the SDS-PAGE system. The revolving and stacking gel containing 30% acrylamide undergo electrolysis at 120 V, for 70 minutes. Then, that gel removed and stained with staining solution destained in methanol (Hou et al., 2012).

3.5.3 Enzyme activity assay of ANAE

ANAE activity determined by the colorimetric method (Hemingway, et al., 1962). The stop solution prepared by mixing the fast blue B salt (1%) and SDS (5%) with 2:5 ratios. Each sample which are crude extract, ATPS1, ATPS2 and after dialysis will be added with 1.95 mL of buffer, with pH 8.0 and 0.05 *mL* of α-naphthyl acetate (α-NA) used as substrate. The mixture consisting of phosphate buffer (1.95 mL), enzyme solution (0.5 *mL*) and α-NA (0.5 *mL*) was incubated at 30 ◦ C for 15 min in a water bath.

The hydrolysis of α-NA was terminated by adding 0.5 mL of stop solution, it produces a purple coloured diazonium enzyme. The absorbance at 595 nm measured by a UV/Vis microplate spectrophotometer after 5 min (J. Wang et al., 2012). The value of absorbance further used to calculation of naphtol concentration, enzyme activity (*EA*), protein concentration (*PC*), specific activity (*SA*), purification factor (*PF*) and yield (*Y*) (Yang et al., 2010). One unit of enzyme (*u*) is defined as the amount of enzyme that will produce 1 mol of 1-naphtol per minute under the assay conditions.

ANAE activity is calculated using Eq. (3.1):

$$\text{ANAE Activity } \left(\frac{Units}{ml}\right) = \frac{Naphtol\ concentration\ (mmol) \times 3ml\ of\ assay}{0.5ml\ of\ enzyme \times 15min} \quad (3.1)$$

Protein Concentration (*PC*) calculated based on equation BSA Standard Curve second polynomial, equation (3.2):

$$\text{PC } \left(\frac{mg}{ml}\right) = 107.66(\text{absorbance at } 562nm)^2 + 864.83(\text{absorbance at } 562nm) - 11.591 \quad (3.2)$$

Specific Activity (*SA*) is calculated according to Eq. (3.3):

$$SA\left(\frac{U}{mg}\right) = \frac{ANAE\ activity\ (U/ml)}{Protein\ Concentration\ (mg/ml)} \quad (3.3)$$

Purification Factor (*PF*) is calculated according to Eq. (3.4):

$$PF = \frac{Specific\ Activity}{Initial\ Specific\ Activity} \quad (3.4)$$

Yield (*y*)

The yield is calculated according to Eq. (3.5):

$$y\ (\%) = \frac{ANAE\ Activity}{\frac{Initial\ ANAE}{Activity}} \quad (3.5)$$

3.5.4 Characterization Enzyme Activity

3.5.4.1 Optimal pH

The optimum pH value of the enzyme was determined by measuring its activity for 15 min incubation time at 30 *℃*. The mixture consists of 0.5 *mL* of purified ANAE, 0.05 *mL* of 16 *mM* α-NA and 1.95 *mL* of 0.04 *mM* sodium phosphate buffer with different pH values, *3.5, 4.5, 5.5, 6.5, 7.5, 8.5* and *9.5*. The pH was adjusted using a solution of H_2SO_4 or NaOH (Ye et al., 2019).

3.5.4.2 Optimal temperature

To get the optimum temperature, 0.5 *mL* of purified ANAE was mixed with 0.05 *mL* of 16 *mM* of α-NA and 1.95 *mL* of 0.04 *M* sodium phosphate buffer pH 6.5. The mixture was incubated for 15 minutes for different temperatures 20 *℃,* 30 *℃,* 40 *℃,* 50 *℃*. The enzyme activity was assayed as mentioned above (Hou et al., 2012b).

3.5.5 Enzyme Kinetic Study

Initial rates of hydrolysis of α-NA (4, 16, 32, 48, 60 and 76 *mM*) by the purified ANAE in 0.5 *mL* solution were determined at room temperature. Incubations were performed for different reaction time (2, 5, 10, 15, 20, and 25 minutes) and for each substrate concentration. All absorbance were

read using spectrometer (Yang et al., 2010). The Michaelis-Menten kinetic constants V_{max} and K_m were estimated by using the Langmuir linear plot.

3.5.6 Kinetic Study of Inhibition Reaction

Inhibition reaction was carried out by mixing 1.95 mL of $I_1(5x10^{-4}$ *mg/ml*) Dichlorvos with 0.5 mL of ANAE by inverting the centrifuge tube twice. The reaction mixture was incubated at 26^oC for 1 minutes and repeated with different incubation times 2, 3, 4, 5 minutes. Then, the hydrolysis of α-NA was occurred by adding 0.05 *mL* α-naphthyl acetate. The reaction was stopped by adding fast blue B salt (1%) and SDS (5%). The absorbance in the supernatant was measured at 562 *nm* using Thermo Scientific Multiskan Go Spectrophotometer within 10 minutes. The values of absorbance further used for the calculation of naphthol concentration, residual enzyme activity before inhibition, residual enzyme activity after inhibition. All steps were repeated using different concentration of Dichlorvos $I_2(5x10^{-5}$ *mg/mL*), $I_3(5x10^{-6}$ *mg/mL),* $I_4(5x\ 10^{-7}$ *mg/mL*), $I_5(5x10^{-8}$ *mg/mL*).

3.5.6.1 Linear Standard curve

The standard curve graphs were plotted with residual enzyme activity after inhibition, E_r (U/*mL*) against time (mins) for each concentration of pesticide. Residual enzyme activity before inhibition, E_0 (U/*mL*) was obtained by extrapolated the graph to time zero. The residual enzyme activity before inhibition, E_0 (U/*mL*) was recorded for 1, 2, 3, 4, and 5 minutes and repeated for each concentration of pesticide. The rate of inhibition reaction of enzyme derived by Eq 3.6 in which E_r is residual enzyme activity after inhibition, E_0 is initial enzyme activity before inhibition and t is the reaction time. The solved equation (Kitz and Wilson, 1962) may be written as Eq. 3.6:

$$\ln \frac{E_r}{E_0} = - \frac{k_3 t}{1 + {k_i}/{I}} \quad (3.6)$$

Slope of the regression ln $[E_r/E_0]$ over incubation time yields an apparent phosphorylation rate constant, k_{app} derived by Eq. 3.7 and 3.8 (Silva et al., 2004).

$$kapp = \ln \frac{E_r}{E_0} / t \quad (3.7)$$

$$kapp = \frac{k_3}{1 + {k_i}/{I}} \quad (3.8)$$

Equation 3.9 can be linearized with the reciprocals by derived equation 3.8. Two linear regressions equation 3.7 and 3.8 made it possible to calculate the inhibition kinetic constants (Silva et al., 2004).

$$\frac{I}{kapp} = \frac{k_i}{k_3} \cdot \frac{1}{I} + \frac{1}{k_3} \quad (3.9)$$

The linear standard graph of the reciprocal of the apparent rate constant (1/ k_{app}) versus the reciprocal of different Dichlorvos concentrations, (1/I) was plotted for each different concentration of pesticide from equation 3.9 to calculate the phosphorylation rate constant, k_3 and affinity constant, k_i. All kinetic constants involved in this kinetic study of enzyme inhibition.

3.5.7 Functionalization of MWCNTs-COOH

In a round bottom flask, 1 g of MWCNT was combined with 60 mL of concentrated sulfuric acid and 20 *mL* of concentrated nitric acid in a 1:3 (v/v) ratio to achieve functionalization. In order for the combination to flow through the reflux system in 15 hours, the heating mantle was adjusted to 60°*C*. This mixture was diluted with 600 *mL* of distilled water to cease the reaction. It was filtered using a 0.45 *m* PTFE membrane with a vacuum filtering system repeatedly using 6*L* of deionized water after chilling at ambient temperature due to the concentrated acid's low pH, until it reached neutral pH 7. After that, the collected mixture was vacuum-dried overnight at 90°*C* (Z. Zhao et al., 2013). The untreated MWCNT in the mixed acid was designated as MWCNTs-COOH (Z. Zhao et al., 2013).

3.5.8 Immobilization of ANAE

By using the physical adsorption approach, the enzyme's immobilization portion was made to adhere to the functionalized MWCNT. 2 *mL* of 0.05 M phosphate buffer at pH 7 was added to 4 *mg* of weighted functionalized MWCNT to create the 2 *mg/mL* combination. Then, to dilute the ANAE, 2 *mL* of 0.05M phosphate buffer at pH 7 was used to create a 4 *mg/mL* ANAE solution. The produced solution is made up of functionalized MWCNT, ANAE, and phosphate buffer, which were combined in a centrifuge tube and incubated for 2.5 hours at 30 °*C* at 200 *rpm* (Mubarak et al., 2014).

The mixture was centrifuged at 3800 *rpm* for 12 minutes following incubation. The mixture was periodically dispersed into new phosphate buffer solution and centrifuged at least 4-5 times to wash and eliminate the unattached protein. The collected conjugated, designated as MWCNT-

COOH-ANAE, can be utilized for further analysis after being allowed to air dry for 24 hours (Mubarak et al., 2014).

3.5.9 Characterization of functional groups using FTIR

An infrared spectrum of an object's absorption or emission was obtained using the Fourier-transform infrared spectroscopy (FTIR) method. The functional groups on functionalized MWCNT, which were carboxyl groups, can be characterized using FTIR. At the INHART laboratory, IIUM, Gombak, the materials that underwent FTIR were designated as pristine MWCNT, MWCNT-COOH, ANAE, and MWCNT-COOH-ANAE. With wavelength 400 to 4000 cm^{-1}, new functional group appearances were considered.

3.5.10 Preparation of MWCNT/ANAE/SPCE

Chitosan flakes were dissolved in an aqueous solution of acetic acid (2.0 $molL^{-1}$) to create chitosan stock solution (0.50%, w/v), and the pH was then brought to 5.0 by adding concentrated NaOH solution. When not in use, the chitosan solutions were kept in a refrigerator (4 *°C*). To create cross-linked chitosan with free CHO groups, 5.0 mL of glutaraldehyde was fully combined with 1.0 *mL* of chitosan solution while stirring for two minutes.

After adding 1.2 *mg* of the immobilized MWCNT to 1.0 *mL* of the mixture, the immobilized chitosan-multiwall carbon nanotubes composite (ANAE-CMC) was extensively sonicated to create a homogenous suspension. Then, 2.0 *L* of the mixture was added to an SPCE

that had already been treated, and it was allowed to react at 20 °*C* for 4 hours. To get rid of extra glutaraldehyde, the modified SPCE was carefully washed in double-distilled water (Cai and Du et al., 2008). The ANAE-CMC/SPCE gathered data can be used for amperometric measurement.

3.5.11 Enzyme Inhibition Reaction

In order to achieve a steady curve, the resulting MWCNT-ANAE-SPCE was first activated in pH 7.0 PBS using cyclic voltammetric sweeps from 0.1 to 1.0 V. The pretreated MWCNT-ANAE-SPCE was transferred to the electrochemical cell of 1.0 *mL* pH 7.0 PBS containing 16 mM -naphthyl acetate to study the electrochemical response by cyclic voltammetry (CV) for the measurements inhibition of dichlorvos after being submerged in the PBS solution for 15 min (Cesarino et al., 2012). The inhibition of dichlorvos was calculated as follows:

$$\text{Inhibition (\%)} = (\text{IP control} - \text{IP exp}) / \text{IP control} \times 100\% \quad (6)$$

IP, control is a current before inhibitor. Then, (IP, exp) is a current after incubation with inhibitor (Vald et al., 2008).

3.6 SUMMARY

This chapter includes the chemicals, materials, equipment and facilities used throughout this research. This chapter also portrays the whole experimental work of this research starting from preparation of Alpha naphthyl acetate esterase (ANAE). The procedure for each experiment was based on past research work with minor modification wherever necessary.

The experimental work starts with preparation of Alpha naphthyl acetate esterase (ANAE) from extraction wheat flour for inhibition study of pesticide. The next part fulfills the first objective of this study which is to purify the enzyme and study about characterization of enzyme. Also, to determine the kinetic study of enzyme using Michaelis-Menten kinetics.

The second objective involves functionalization of MWCNTs-COOH and immobilization of MWCNTs-COOH-ANAE. FTIR was used to characterize the functional groups on functionalized MWCNT which were carboxyl group.

The third objective involves the preparation of MWCNT/ANAE/SPCE using chitosan solution. The collected conjugated denoted as ANAE-CMC/ SPCE can be used for amperometric measurement. The last part of the experimental work is the measurements inhibition of Dichlorvos, the pretreated MWCNT-ANAE-SPCE using amperometric measurement.

CHAPTER FOUR

RESULT AND DISCUSSION

4.1 INTRODUCTION

This chapter presents all the results and findings from the experimental works for this research. All the results obtained are discussed and justified while supported by past research and theoretical facts. The residual activity of enzyme was analyzed for assessing the kinetic parameters of ANAE. The extracted enzyme was characterized by SDS page, where it may further immobilized on SPE and analyzed by FTIR spectrometry and cyclic voltammetry.

4.2 EXTRACTION AND PURIFICATION OF ANAE

In this work, a plant esterase called alpha naphthyl acetate esterase (ANAE) was employed to identify pesticides. A wheat flour was used to extract this enzyme, which was then filtered using 0.22 L PES membranes that don't attach to proteins. In order to conduct research, ANAE is purified using an ATPS (aqueous two-phase system). In order to make plant-esterase suitable for its targeted application in OPs detection, it is necessary to remove the salts by dialysis method.

Following the purification procedure, the bottom phase of the ATPS was dialyzed for 48 hours at $4℃$ (with four buffer changes) using 0.05M sodium phosphate buffer, pH 6.5. By

incorporating dialysis into the ATPS purification process, salts may be removed from the plant-esterase without endangering it. Thus, the plant esterase's purity may be preserved.

The enzyme activity assay was conducted to determine the content of plant esterase in a wheat flour by the colorimetric method. Enzyme activity assay for each sample can be measured using Eq. 4.1. From Table 4.1, it showed the enzyme activity, protein concentration, specific activity, purification factor, and yield for each sample which are crude enzyme, after purification ATPS1 and ATPS2, then the sample after dialysis.

Table 4.1 Purification of ANAE extracted from atta flour at different steps

Samples	Enzyme Activity (U/ml)	Protein Conc (mg/ml)	Specific Activity (U/mg)	Purification Factor	Yield (%)
Crude ANAE	3.223	2.395	1.346	1.00	100.00
ATPS1	2.757	0.857	3.216	2.39	85.53
ATPS2	1.834	0.313	5.863	4.36	56.89
DIALYSIS	0.750	0.102	7.366	5.47	23.25

A summary of the ANAE activity for each step of purification is presented. The enzyme activity and protein concentration decreasing as the purification steps indicate that it removed unnecessary proteins. Since the molecules that surround the enzyme can form intermolecular bonds with the enzyme, the components in the buffer solutions used in the purification process inevitably affect the enzyme (Van Dyk & Pletschke, 2012). The rate of reaction catalyzed by an enzyme is stated in units of micromoles of substrate converted into product per minute, and these units are used to assess enzyme activity. The enzyme activity for the crude ANAE is 3.223 U/*ml*. Other study had obtained 3.99 U/*ml* for enzyme activity (J. Chen, Zhang, Wu, & Zhang, 2013). The value slightly different since different methods were used to purify the crude enzyme.

The specific activity calculated on how much of the total protein is the enzyme to isolate. Therefore, its value should increase as the purification progresses. Based on the Table 4.1, the specific activity showed an increasing value from crude to dialysis part which are 1.346 U/*mg* to 7.366 U/*mg*. Purification factor used to measure the relative purity, so increasing values indicate increasing purity. The purification factor for the starting material equals 1. The purification factor resulted increasing value starting with 1, 2.39, 4.36 and 5.47 assigned to crude ANAE, ATPS 1, ATPS2 and after dialysis part respectively. The yield in the homogenate is defined as 100 %. Since each purification step will result in some loss of ANAE activity, hence the yield is always less than 100 %. At the first step of ATPS PEG 1000/NaH_2PO_4, the ANAE was isolated a yield of 85.53 %. Then, the last step of ATPS (PEG 1000/NaH_2PO_4 $(NH_4)_2SO_4$) resulted yield of 56.89%. Followed by dialysis part, the yield shifted to 23.25%.

4.3 ESTIMATION OF MOLECULAR WEIGHT BY SDS-PAGE

SDS-PAGE is an analytical technique to separate proteins based on their molecular weight. When proteins are separated by electrophoresis through a gel matrix, smaller proteins migrate faster due to less resistance from the gel matrix. Other influences on the rate of migration through the gel matrix include the structure and charge of the proteins. SDS PAGE detects the presence of target proteins by their molecular weights. The molecular weight of the protein for ANAE was identified by SDS PAGE. Figure 4.2 shows the bands of ANAE protein by their molecular weight. The most visible band was detected at molecular weight of 65 *kDa*. Similar results were obtained by Yang (2010) and Bao et al. (2005) for plant esterase, where they detected a band at 50-75 *kDa*. Thus, it can be concluded that the presence of ANAE in wheat flour through an aqueous two phase purification process. The different bands in molecular weight for plant esterase might differ based on the proteins purity since different methods are used to purify the crude protein (Bao et al., 2015)(Hou et al., 2012a)

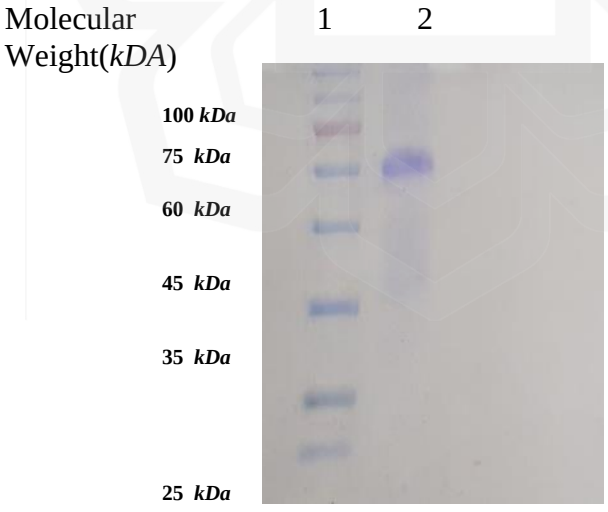


Figure 4.2 1-Protein marker 2-Purified ANAE

4.4 CHARACTERIZATION OF ANAE

ANAE was characterized to observe its performance in different reaction conditions. Characterization includes determining the optimum working temperature and pH for catalyzation of enzyme.

4.4.1 Optimum pH

Environmental factors such as too much acid or too much alkaline might cause an enzyme's molecular structure to change and cause it to lose activity. The external pH may affect the enzyme molecules, particularly the necessary group in the active center of the degree of dissociation and the quality of the catalytic group the sub donor or the proton acceptor required ionized state. They may also affect the degree of dissociation of the substrates and the coenzyme, affecting the binding of the enzyme and the substrate (Ye et al., 2019).

Because enzymes are proteins, their molecular structure is determined by interactions between the charges of the amino acids that compose the protein chains. These interactions take the form of hydrogen bonds, which are altered by pH. These positive charges impact the charges of the amino acids inside the protein, making the enzyme more or less active depending on the enzyme's optimum pH. Because enzymes are proteins, their molecular structure is determined by interactions between the charges of the amino acids that compose the protein chains. These interactions take the form of hydrogen bonds, which are altered by pH. These positive charges impact the charges of the amino acids inside the protein, making the enzyme more or less active depending on the enzyme's optimum pH. Different pH values of phosphate buffer which are 3.5,

4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 used to examine the activity of ANAE against pH values showed in Figure 4.3.

The effect of pH on the ANAE activity was studied using 16*mM* of α-NA as the substrate. It shown in Figure 4.3 the enzyme activity has increase gradually at pH range between 3.5 and 6.5 and decrease at pH 7.5 to 9.5. ANAE achieve the optimum pH value at pH 6.5 with 100% of relative enzyme activity. Plant-esterase shows considerable pH stability in slightly acidic environment due to carboxylic acids, as it is hydrolyzed. In other research, (J. Chen et al., 2013; Hou et al., 2012a) also have similar optimum pH value for ANAE activity which is 6.5. Besides, (J. L. Wang, Xia, Zhang, Hu, & Lin, 2012) obtained optimum pH value 6.38.

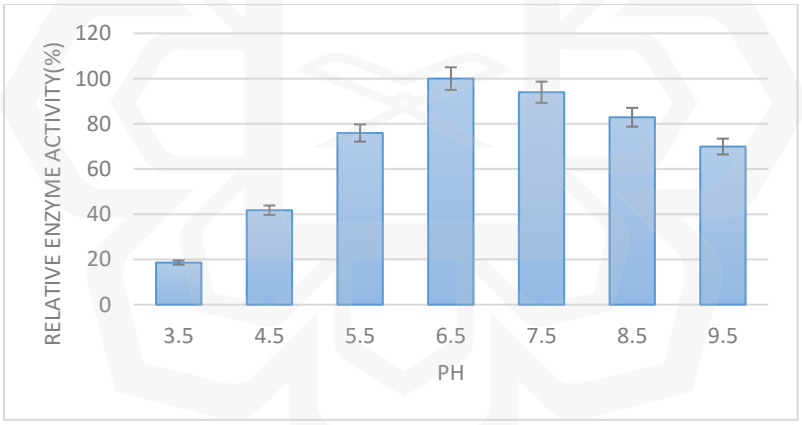


Figure 4.3: Effect of pH on ANAE activity

4.4.2 Optimum Temperature

Temperature is one of the parameters in order to know enzymatic reaction which may affect the sensitivity and accuracy of enzyme activity. The architecture of the enzyme and overall stability are both influenced by a key factor called temperature. It is commonly known that enzyme reaction rates rise with temperature, but at a certain temperature, denaturation causes an enzyme's activity to decline. The assay was incubated for a set of temperature ranging from 20 to 50°*C* within 15 min while keeping other parameter in constant value. The result of ANAE activity against temperature represented in Figure 4.4.

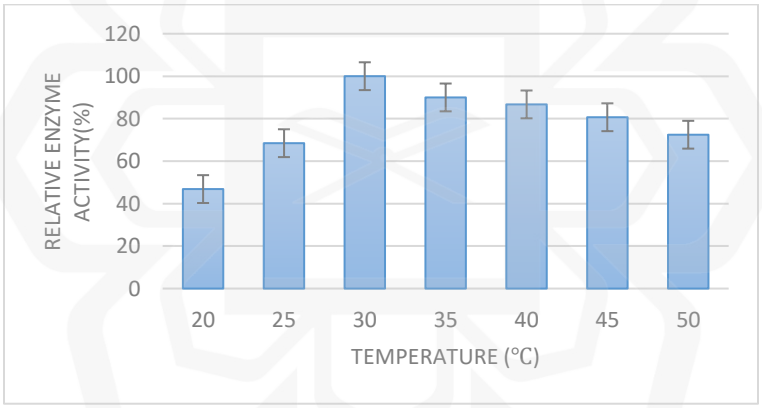


Figure 4.4: Effect of temperature on ANAE activity

It shown in Figure 4.4, the ANAE maximum enzyme activity is 100% at 30°*C*. As a result, 30°*C* was the ideal temperature for ANAE to hydrolyze -NA. Most of biochemical reactions are carried out at lower temperatures because enzymes are not stable at these higher temperatures and will denature after a few minutes (Dong, He, & Huo, 2020). The stability of the enzyme gradually

decreased as the temperature was raised from 35°*C* to 50°*C*. Similar result has obtained which is 30°*C* for optimum temperature (Hou et al., 2012a; Ye et al., 2019). Overall, results show that pH 6.5 and 30°*C* are the optimum condition at which ANAE showed the best performance.

4.5 ENZYME KINETIC STUDY

The Michaelis-Menten kinetics describes the rate of enzymatic reactions by correlating the concentration of a substrate, [S], with the rate of product creation reaction rate. The greatest rate that the system can attain at saturating substrate concentration is represented by the V_{max}. The Michaelis constant K_m is an inverse indicator of the substrate's affinity for the enzyme, and its value is numerically equal to the substrate concentration at which the reaction rate is half of V_{max}. The V_{max} and K_m of ANAE were determined for each Michaelis-Menten plot using microsoft excel and the best model plot was chosen based on the R^2 value which is closer to 1.

4.5.1 Determination of Kinetic Parameters

The best plot chosen was Langmuir linear graph by plotting [S]/υ versus [S] in Figure 4.5 with R^2 of 0.9929. The Michaelis constant K_m and V_{max} of the ANAE were determined using equation from Langmuir graph.

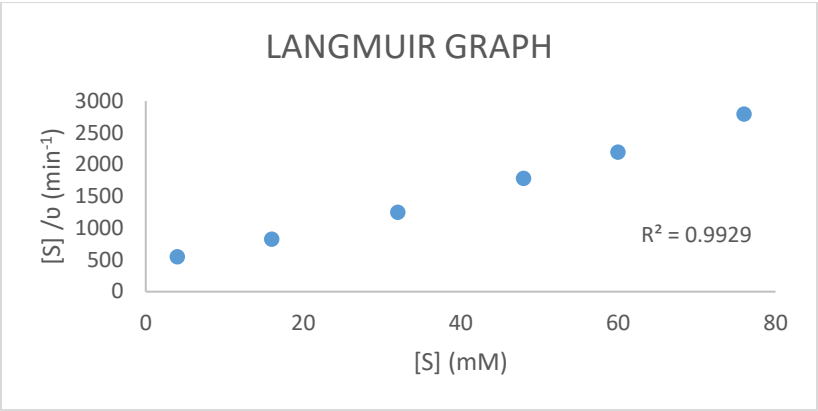


Figure 4.5: Langmuir graph of ANAE activity

Langmuir equation 4.1:

$$\frac{s}{v} = \frac{Km}{V\max} + \frac{s}{V\max} \tag{4.1}$$

Equation 4.2 from Langmuir graph Figure 4.5:

$$y = 11.905\,x + 111.25 \tag{4.2}$$

$$\frac{1}{V\max} = 11.905$$

$$V\max = 0.084$$

$$\frac{Km}{V\max} = 116.25$$

$$Km = 9.765$$

At saturating substrate concentrations, the ANAE reaches its maximal response rate, or V_{max}. The substrate concentration at which the reaction rate is half of V_{max} is numerically equivalent to the Michaelis constant K_m. The kinetic constants values were found to be 0.084mM/min and 9.765mM, respectively for V_{max} and K_m. Low value of K_m is favorable as it indicates that only a low amount of substrate is required for the process to achieve half of V_{max} (Khushairi, Abdul, Nurul, & Abdul, 2020).

Catalytic efficiency (V_{max}/K_m) is the measure of how efficiently an enzyme can convert a subtrate into a product. The ratio of V_{max}/K_m obtained in this study ($8.60 \times 10^{-3}\ min^{-1}$) was similar to that of pectin methyl esterase extracted from lime peel at $2.88 \times 10^{-3}\ min^{-1}$, where the Michaelis–Menten kinetics were linearized through Lineweaver-Burk plot (Khushairi et al., 2020). This result confirmed by plotting the Michaelis–Menten saturation curve in Figure 4.6 for an enzyme reaction showing the relation of the substrate concentration, [S], and rate of reaction, υ.

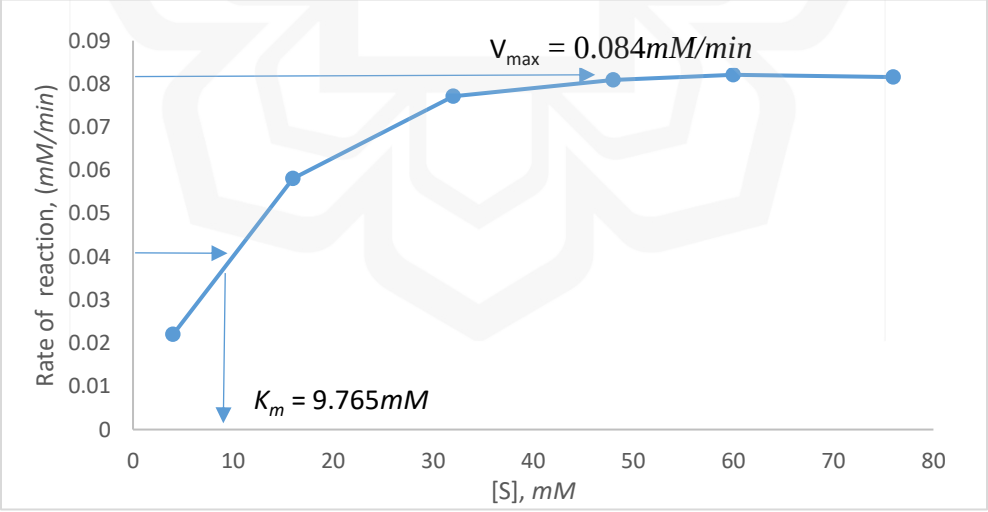


Figure 4.6: The Michaelis–Menten saturation curve of ANAE activity

4.6 KINETIC STUDY OF ENZYME INHIBITION

A molecule that attaches to an enzyme and inhibits its activity either by attaching to the enzyme's active site, preventing the substrate from attaching, or by attaching to another place on the enzyme, preventing the enzyme from catalyzing the process. Enzyme inhibitors may bind reversibly or irreversibly. Irreversible inhibitors form a chemical bond with the enzyme such that the enzyme is inhibited until the chemical bond is broken. By contrast, reversible inhibitors bind non-covalently and may spontaneously leave the enzyme, allowing the enzyme to resume its function.

Dichlorvos is one of the enzyme-inhibitor that have been used in this study to investigate the kinetic of irreversible inhibition reaction of enzyme. Following the substrate reaction while the inhibitor is present and evaluating the kinetic constants for the inhibitor at various inhibitor doses and incubation durations (OP). The chemical equation that describes the inhibition mechanism is:

$$[E] + [I] \underset{\longleftrightarrow}{^{\mathrm{ki}}} [EI] \underset{\longleftrightarrow}{^{\mathrm{k3}}} [E - I]$$

The kinetic constants, k_i is a dissociation constant, and k_3 is a phosphorylation rate constant dissociation constant of enzyme-inhibitor complex. The linearity of the regression between ln (E_r / E_0) and the incubation time plotted for each concentration of dichlorvos showed in Figure 4.7, which indicated a first order reaction between enzyme and inhibitor.

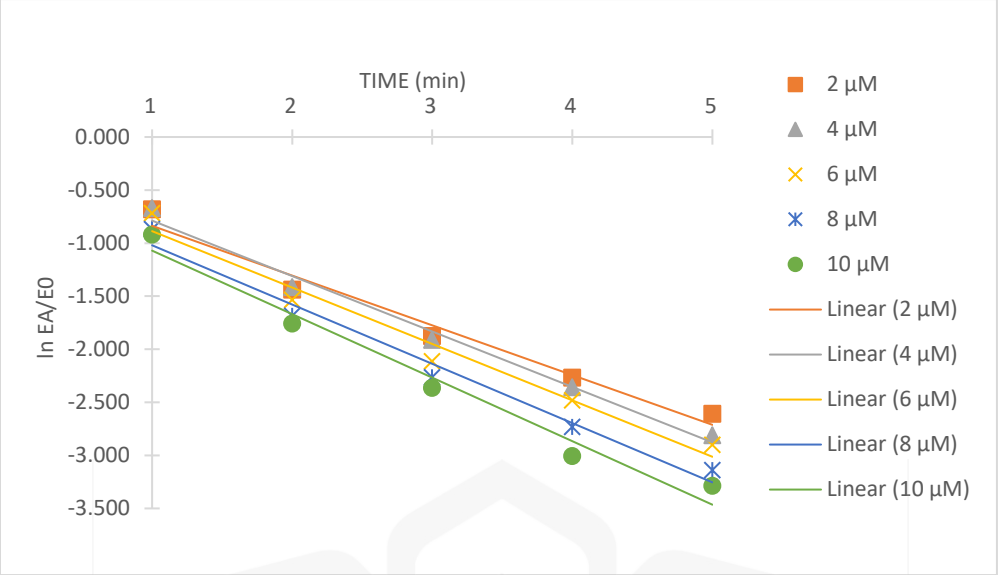


Figure 4.7: Inhibition of ANAE by dichlorvos

The rate of inhibition reaction of enzyme derived by equation 4.3 which E_r is residual enzyme activity after inhibition, E_0 is initial enzyme activity before inhibition and t is the detection time (JR & KB., 1990).

$$\ln \frac{E_r}{E_0} = - \frac{k_3 t}{1 + {k_i}/{I}} \qquad (4.3)$$

Slope of the regression ln $[E_r/E_0]$ over incubation time yields an apparent phosphorylation rate constant, k_{app} derived by equation 4.4 and 4.5 (Silva et al., 2004). k_{app} values are 0.5954, 0.583, 0.5682, 0.5536, 0.5366 respectively for concentration of dichlorvos $5X10^{-4}$*mg/mL*, $5X10^{-5}$*mg/mL*, $5X10^{-6}$*mg/mL*, $5X10^{-7}$*mg/mL*, $5X10^{-8}$ *mg/mL*.

$$kapp = \ln \frac{E_r}{E_0} / t \quad (4.4)$$

$$kapp = \frac{k_3}{1 + {}^{k_i}/_I} \quad (4.5)$$

Equation 4.5 may be linearized with the reciprocals by derived equation 4.6. Two linear regressions equation 4.5 and 4.6 made it possible to calculate the inhibition kinetic constants (Silva et al., 2004).

$$\frac{I}{kapp} = \frac{1}{k_3} + \frac{k_i}{k_3} \cdot \frac{1}{I} \quad (4.6)$$

The linear standard graph of the reciprocal of the apparent rate constant (1/ k_{app}) versus the reciprocal of different dichlorvos concentrations, (1/I) plotted for each different concentration of pesticide as shown in Figure 4.8. From equation 4.6 the phosphorylation rate constant, k_3 and dissociation constant, k_i can be calculated. Based on the graph the equation 4.7, the value of k_3 is 0.4816 *min-1* and k_i is 0.2223 μM. the reaction between AChE and pesticide would occur with no limiting step, which means that the equilibrium of the reaction forming the inhibitor-enzyme intermediary and the rate of the enzyme phosphorylation that stabilizes it correlate in a way that makes the concentration of the intermediate compound *EI* very low (Silva et al., 2004).

$$y = 0.4615x + 2.0763 \quad (4.7)$$

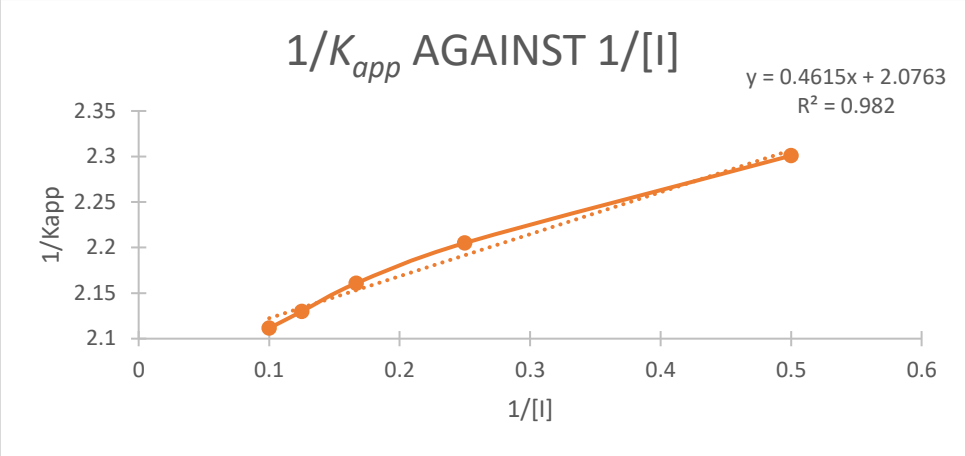


Figure 4.8 Linear standard graph

4.7 FTIR ANALYSIS

4.7.1 Functionalization of MWCNT

The MWCNT's functional groups were investigated using FTIR Figure 4.9 displayed the unfunctionalized MWCNTs and their natural counterparts in an FTIR spectrum with wavelengths between 400 and 4000 cm^{-1}.

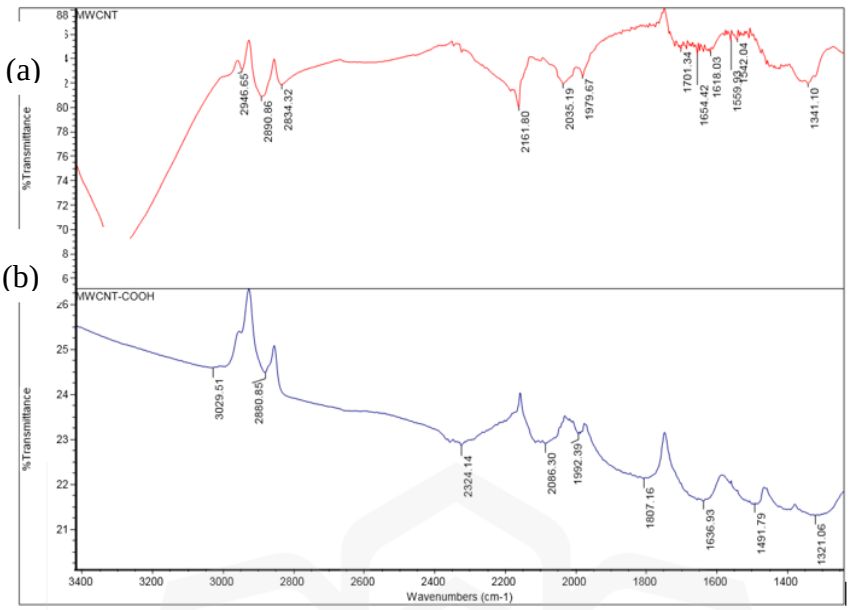


Figure 4.9 FTIR spectra: (a) pristine MWCNT and (b) functionalized MWCNT

According to Figure 4.9(a), the emergence of a peak at 1416.57 cm^{-1}, which denotes the stretching of the carbon double bonding (C=C) on the side wall defect in MWCNTs. The peak for C=C is between 1400 and 1600 cm^{-1}, according to (Chauhan et al., 2016). The peak at 1807.16 cm^{-1} and 1992.39 cm^{-1} in Figure 4.9(b) was moved, and this may be attributed to the C=O symmetric stretching. While the peaks at 2086.30 cm^{-1} and 2324.14 cm^{-1} can be associated to the O-H stretch from highly hydrogen-bonded COOH, the peak at 3029.51 cm^{-1} is related to the O-H stretch from carboxyl groups (O=COH and COH). The carboxyl group's HC stretch modes were produced via an absorption peak at 2880 cm^{-1}. The results of this investigation and those reported by (Z. Zhao et al., 2013) for the carboxyl group are pretty comparable at 2885 cm^{-1}. These findings thus imply that carboxylic acid groups have successfully been added by acid treatment onto the surfaces of MWCNT.

4.7.2 Immobilization of ANAE

Figure 4.10 demonstrates FTIR spectra for ANAE Figure 4.10(a) and functionalized MWCNT-ANAE in Figure 4.10(b) with the wavelength between 400 to 4000 cm^{-1}.

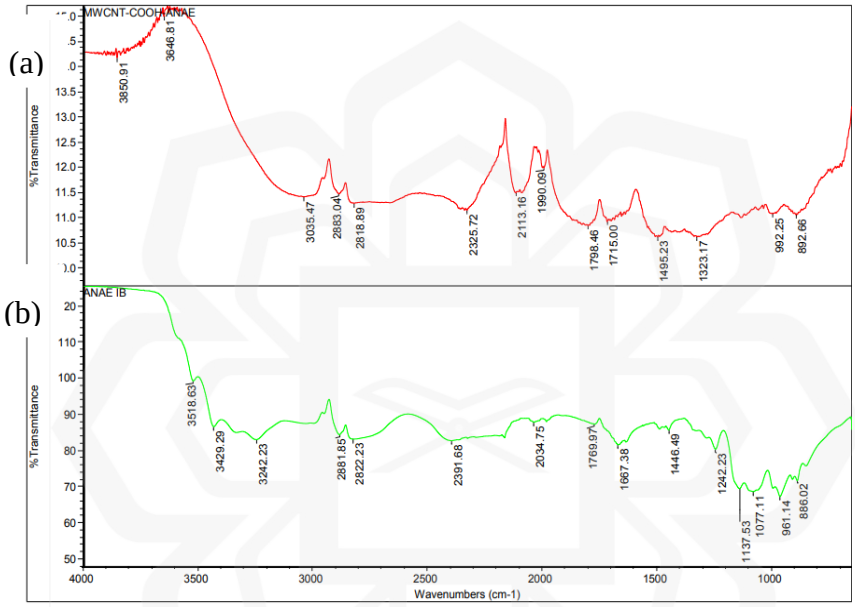


Figure 4.10 FTIR spectra: (a) Functionalized MWCNT–ANAE and (b) ANAE

The FTIR spectra were collected in an FTIR spectrometer to verify that the ANAE was immobilized on the functionalized MWCNT. Several notable peak regions at various wavelengths were seen in order to compare both the unaltered ANAE and the immobilized MWCNT-COOH. The peak 1242.23 cm^{-1} might be attributed to C-N stretching based on Figure 4.10(a). Then, the in-plane amide N-H deformation mode was characterized by the peaks at 1667.38 cm^{-1}. The large peak at 1769.97 cm^{-1} can then be attributed to the NH_2

scissoring mode after that. In addition, asymmetric and symmetric CH_2 stretching are responsible for the peaks at 2822.23 cm^{-1} and 2881.85 cm^{-1}, respectively. Next, the overlap of the OH and NH stretching bands is what causes the peaks to move to 3200-3900 cm^{-1} (Majumdar et al., 2008). The peaks 3646.81 cm^{-1} and 3850.91 cm^{-1} on the functionalized MWCNT, which can be seen in Figure 4.10(b), introduced the strong amide connections between carboxylic acid groups and amine groups.

4.8 CYCLIC VOLTAMMETRY OF ENZYME INHIBITION REACTION

The CV responses were examined after the exposure of MWCNT-ANAE-SPCE to different concentration of pesticide added which are I_3($5x10^{-4}$ g/L), I_2($5x10^{-5}$ g/L), I_1($5x10^{-6}$ g/L) in Figure 4.11. when inhibitors are present, there will be a significant reduction of product, or even no product will be formed. Under the influence of applied voltage, the product is oxidized and the anodic oxidation current, whose strength depends on the concentration of pesticides in the sample and the duration of the exposure, is electrochemically measured. The electrochemical response of the enzymatic biosensor was determined using cyclic voltammetry (CV).

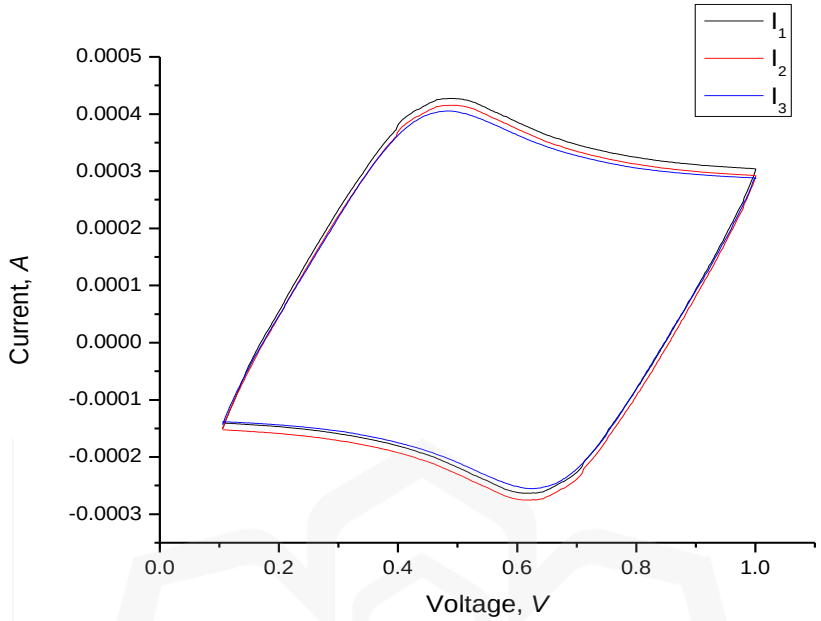


Figure 4.11 Cyclic voltammetry of enzyme inhibition reaction

Figure 4.11 showed that lowering the concentration of pesticides resulted increasing the redox peak current. The peak current of different concentration of pesticides likely occurred as a result of blocking serine hydroxyl groups of ANAE by covalently bound of pesticides, which invariably reduced the overall charge of the catalytic active site. The relationship between inhibition rate and dichlorvos concentrations plotted based on the peak current results. The results showed in Figure 4.12 the inhibition rate increase with increasing concentration of dichlorvos. The detection limit for dichlorvos were found to be 0.005 $\mu g/L$. Other study had obtained 0.08 $\mu g/L$ for detection limit (Chen et al. 2017). The value slightly different because the pesticide which used is chlorpyrifos. It showed that the present MWCNT-ANAE-SPCE exhibited a wider range and lower detection limit. This indicated that the MWCNT-ANAE-SPCE not only improved the electron transfer rate, but also decreased the detection limit in the quantitative analysis of pesticides.

Therefore, MWCNT-ANAE-SPCE biosensor would be an excellent platform for the detection of pesticides.

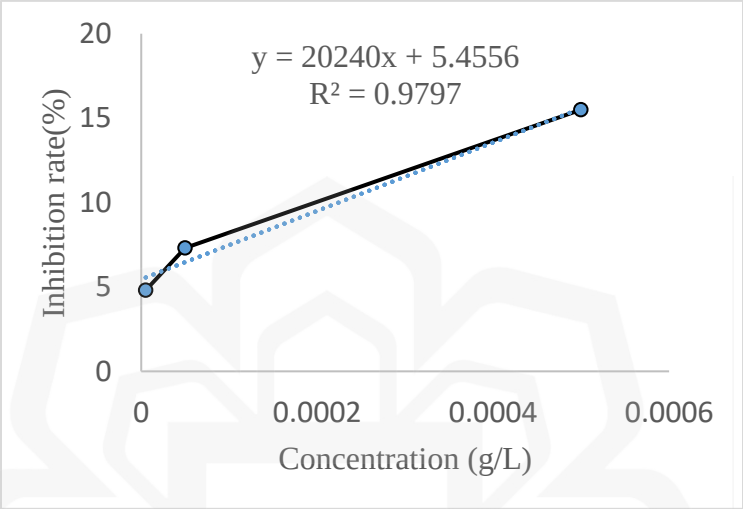


Figure 4.12 Relationship between inhibition rate and dichlorvos concentrations

4.9 SUMMARY

This investigation demonstrated that crude esterase extracts can fulfil the criteria for detecting organophosphorus pesticide residues using a pesticide sensitivity test. The temperature and pH level have an impact on the activity of the same kind of crude esterase extract. Results indicate that the esterase catalytic process occurs under ideal circumstances. Determining the best conditions for the enzyme utilized in the enzyme inhibition test of organophosphorus pesticide residues is therefore important.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

Food goods and vegetables containing pesticide residues should be thoroughly monitored, and appropriate eradication measures should be taken. The detection of pesticides may be accomplished using a variety of techniques, including chromatographic ones like gas chromatography (GC) and high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS). Despite being dependable, it is expensive and time-consuming. Furthermore, it requires highly skilled professional method from the operators. The detection concept of the enzyme inhibition test, a quick and inexpensive technique of detection, is as follows: Under specific circumstances, esterase catalyzes the hydrolysis of acid esters. Pesticides, however, may prevent esterase from performing this catalyzed hydrolysis, and the rate of inhibition is strongly connected with pesticide concentrations.

The acetylcholinesterase (AChE) is an enzyme used for the fabrication of pesticides biosensor normally extracted from animal tissue such as the head of the fly *Musca domestica*, electric eel and human erythrocytes. Due to the extra care needed while handling the enzyme, and complex and tedious extraction procedures, it is often hard to obtain a pure enzyme for the bio-sensing application. These factors can limit the wide applications of the AChE enzyme. Thus, in this study an esterase, extracted from plants could react with OP pesticides used because it is proven that an esterase has a similar sensitivity as AChE

We investigated the purified plant-esterase made from wheat flour's catalytic characteristics. Plant esterase is stable between pH 5.5 and 6.5 and active between 30°C and pH 6.5. The Michaelis-Menten parameters of the purified enzyme were 9.765 mM and 0.084 mMmin-

1, respectively for Km and Vmax. Fourier transform infrared (FTIR) spectroscopy was utilized to verify the presence of the functional groups. The confirmation of ANAE immobilization on functionalized MWCNT was based on the observation of specific peaks at 3646.81 $cm^{-1}$and 3850.91 cm^{-1}, indicating the formation of amide linkages between the carboxylic acid groups and the amine group on the MWCNT.

The kinetic constants which are k3 (phosphorylation rate constant), and ki (dissociation constant of enzyme-inhibitor complex) of an irreversible inhibition model were 0.2223 *mMmin-1* and 0.4816 *mMmin-1* respectively for varying incubation times, and different concentrations of the inhibitor (OP). The detection limit for dichlorvos was found to be 0.005 $\mu g/L$. Lowering the concentration of pesticides resulted in increasing the CV responses.

To improve sensitivity and accuracy for the detection of OPs in food, future research should concentrate on the purification of ANAE, the structural characterization of the target enzyme, and the discovery of the inhibitory mechanism.

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APPENDICES

APPENDIX I: LIST OF EQUIPMENT AND MATERIALS

Table A.1: Equipment and materials used for this research

No	Equipment/ Glassware	Usage
1	Centrifuge (Hettich)	Separate solid and liquid phases
2	Thermometer	To measure the temperature
3	Water bath	To heat solution
4	Spectrophotometer (Thermo Scientific Multiskan Go Spectrophotometer)	To measure the light intensity after it passes through sample solution
5	Incubator Shaker	To incubate samples
6	Weighing Balance (Mettler Toledo)	To measure weight of materials used
7	Sonicator	To agitate particles in sample
8	Drying oven (Memmert)	To dry immobilize nanomaterials
9	Vortex mixer (ERLA)	To mix samples
10	Refrigerator	To cool samples

11	Hot plate magnetic stirrer (ERLA)	To mix and heat samples
12	Vacuum filter	To separate precipitant from solution mixture
13	Nicolet iS50 FTIR spectrometer	FTIR analysis
14	Fume hood	Used when working with dangerous chemicals
15	SDS-PAGE	To characterize molecular weight
16	Vacuum pump	To provide suction to drive the filtration

APPENDIX II: EXPERIMENTAL WORK

1) Preparation of ANAE enzyme samples

Figure B.1 Crude enzyme

Figure B.2 ATPS1

Figure B.3: ATPS2

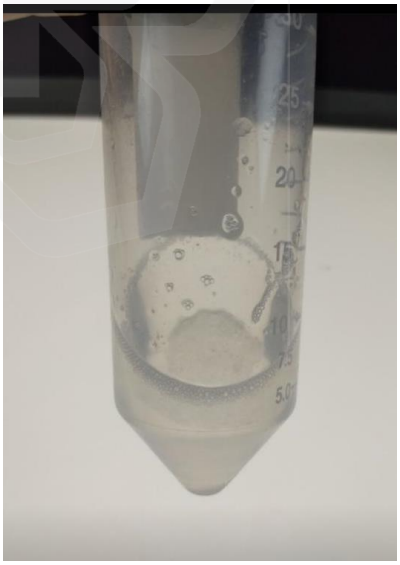

Figure B.4: ANAE after dialysis

2) Characterization of Enzyme

Figure B.5: Sodium Dodecyl Sulphate-Gel Electrophoresis

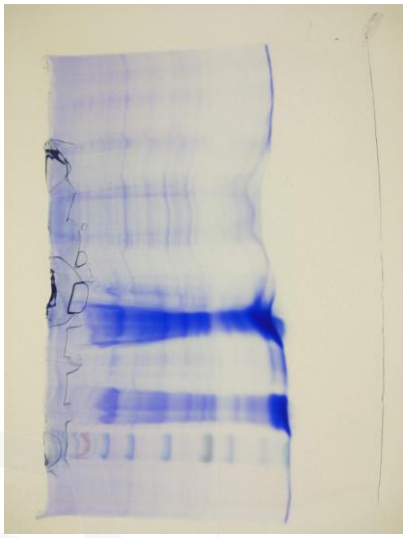

Figure B.6: Sodium Dodecyl Sulphate-Gel Electrophoresis

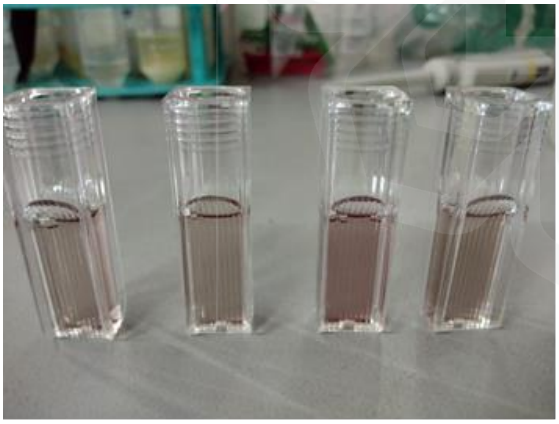

Figure B.7: Samples for enzyme activity assay

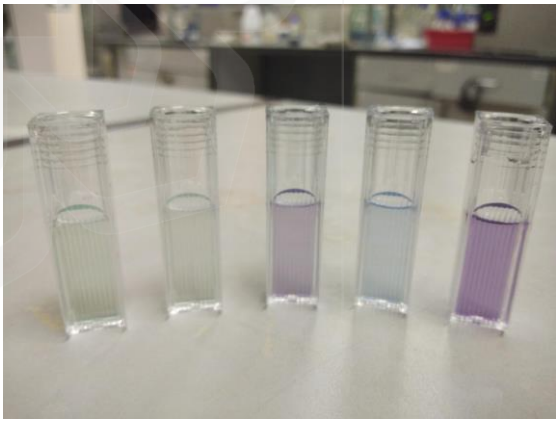

Figure B.8: Samples for enzyme characterization

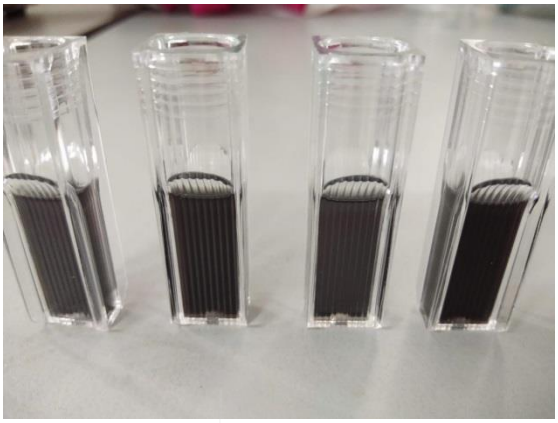

Figure B.9: Samples for enzyme inhibition

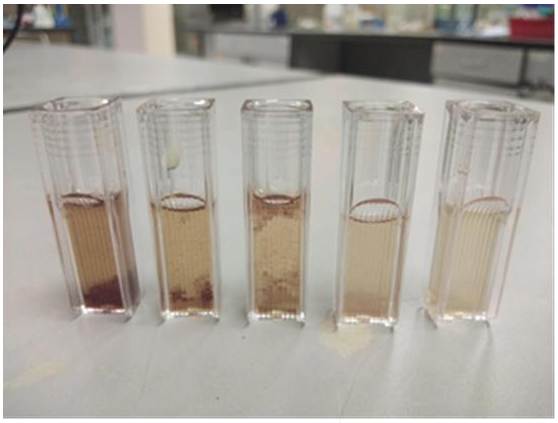

Figure B.10: Samples for enzyme immobilized enzyme

3) Functionalization of MWCNT

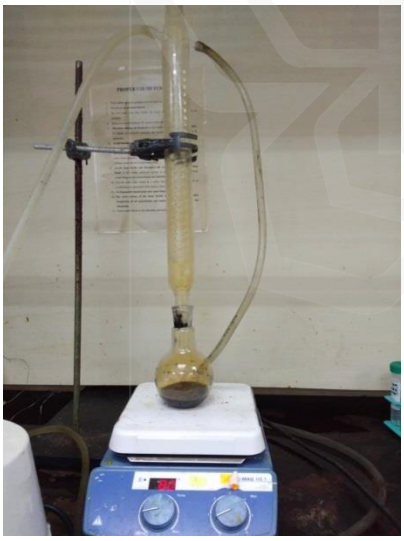

Figure B.11: Functionalization of MWCNT

Figure B.12: Filtration of functionalized MWCNT

Figure B.13: Samples for MWCNT

Figure B.14: Immobilization of MWCNT