INVESTIGATION OF HALOPEROXIDASE PROPERTIES EXTRACTED FROM *SARGASSUM BINDERI* SONDER EX J. AGARDH (SARGASSACEAE, FUCALES)

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Name of Candidate: Thillai Punitha Segaran

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Field of Study: Marine Biotechnology

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INVESTIGATION OF HALOPEROXIDASE PROPERTIES EXTRACTED FROM *SARGASSUM BINDERI* SONDER EX J. AGARDH (SARGASSACEAE, FUCALES)

ABSTRACT

Vanadium haloperoxidases (V-HPOs) are key enzymes that catalyze halides in the presence of hydrogen peroxide (H_2O_2) producing halogenated compounds. These HPOs have been characterized into vanadium chloroperoxidase (V-CPO), vanadium bromoperoxidase (V-BPO) and vanadium iodoperoxidase (V-IPO). Previous studies show that V-CPO and V-BPO have also been identified in eukaryotic species. In the present study haloperoxidase from a marine brown alga (seaweed) *Sargassum binderi* Sonder ex J. Agardh. was extracted and its properties were investigated. This brown seaweed collected from a fringing coral reef, at Cape Rachado, Port Dickson, west coast of Peninsular Malaysia produces HPO, that was extracted using two- phase aqueous system developed by Vilter (1994). The crude extract incubated with sodium metavanadate exhibits enhanced haloperoxidase activity, showing the presence of vanadium haloperoxidase. In addition the rate of activity was also increased by the addition of H_2O_2.The enzymatic activity was stabilized by introducing the optimized pH, buffer concentration and temperature. Substrate and enzyme concentration were used to determine the initial rates of reaction. Increased interest in these enzymes are a result of their increasing importance in the pharmaceutical industry.

Keywords: Vanadium-haloperoxidase, Vanadium-chloroperoxidase, Vanadium-bromoperoxidase, Vanadium-iodoperoxidase.

MENGKAJI SIFAT HALOPEROKSIDA YANG TELAH DIEKSTRAK DARIPADA *SARGASSUM BINDERI* SONDER EX J. AGARDH (FUCALES)

ABSTRAK

Vanadium haloperoksida (V-HPO) adalah enzim utama yang menjadi pemangkin halida dengan kehadiran hidrogen peroksida (H_2O_2) menghasilkan sebatian berhalogen. HPO ini telah dicirikan kepada tiga iaitu, vanadium kloroperoksida (V-CPO), vanadium bromoperoksida (V-BPO) dan vanadium iodoperoksida (V-IPO). Kajian terdahulu menunjukkan bahawa V-CPO dan V-BPO juga telah dikenal pasti dalam spesies eukariot. Dalam kajian ini, V-HPO telah diekstrak dari alga marin (rumpair laut) yang berwarna perang, *Sargassum binderi* Sonder ex J. Agardh dan dikaji sifatnya. Rumpair laut perang ini telah dikutip dari terumbu karang pinggiran, di Cape Rachado, Port Dickson, Pantai Barat Semenanjung Malaysia menghasilkan HPO yang telah diekstrakan dengan kaedah dua fasa sistem akueus yang dibangunkan oleh Vilter (1994). Enzim mentah ini telah diinkubasi dengan natrium metavanadate menunjukkan peningkatan aktiviti haloperoksida, mengesahkan kehadiran vanadium haloperoksida. Di samping itu, kadar tindak balas aktiviti juga meningkat dengan penambahan H_2O_2. Aktiviti enzim telah distabilkan dengan pH, kepekatan penimbal, dan suhu yang tepat. Manakala, kepekatan substrat dan enzim digunakan untuk menentukan kadar awal tindak balas. Kepentingan terhadap enzim ini semakin meningkat
dalam industri farmaseutikal.

Kata Kunci: Vanadium-haloperoksida, Vanadium- kloroperoksida, Vanadium-bromoperoksida, Vanadium-iodoperoksida.

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LIST OF SYMBOLS AND ABBREVIATIONS

H_2O_2	:	Hydrogen Peroxide
HPO	:	Haloperoxidase
BPO	:	Bromoperoxidase
IPO	:	Iodoperoxidase
CPO	:	Chloroperoxidase
ROS	:	Reactive Oxygen Species
KBr	:	Potassium bromide
KI	:	Potassium iodide
AnI	:	*Ascophyllum nodosum I*
AnII	:	*Ascophyllum nodosum II*
V-HPO	:	Vanadium Haloperoxidase
SOD	:	Superoxide dismutase
PCA	:	Principal Component Analysis
PO	:	Peroxidase
MnO_2	:	Manganese oxide
DCMU	:	(3-(3, 4-dichlorophenyl)-1, 1-dimethylurea)
R	:	Respiration

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CHAPTER 1: INTRODUCTION

1.1 Background

Algae are photosynthetic organisms divided into microalgae and macroalgae, and are widely distributed in aquatic ecosystems. Marine macroalgae also known as seaweeds, are generally classified into three types which are the brown (Phaeophyceae), green (Chlorophyta) and red (Rhodophyta) seaweeds, identified approximately with 1 800, 1 200 and 6 000 species, respectively (Almeida *et al*., 1998). Brown seaweeds date more than 200 million years ago (Mya) are multicellular photosynthetic organisms surrounded by cell walls (Silberfeld *et al*., 2010). They are morphologically diverse in comparison with green and red seaweeds, occurring from the largest sized kelp (*Macrocystis pyrifera*, order *Laminariales*) to the smallest, microscopic, filamentous *Ectocarpales* order such as *Laminariocolax* spp. or *Microsyphar* spp., that are found at subtidal regions which only receive 0.01% of the photosynthetic active radiation (PAR) (Matsuda *et al*., 2015). *Ectocarpus siliculosus* was the first brown seaweed to have its genome sequenced and successfully published in 2010 by overcoming the past failures with advancements in macroalgal genomics (Coelho *et al*., 2011). Brown seaweeds have similar genes and metabolic pathways with green plants and metazoans and horizontal transfer of bacterial genes had occurred in their evolutionary history (Cock *et al*., 2010; Michel *et al*., 2010).

Brown seaweeds are cultivated commercially for phycocolloid (alginates) production and in recent years as a potential source of bioactive compounds. These compounds have been reported to possess properties such as antioxidant (Yuan & Walsh, 2006); anti-viral (Artan, 2008); anti-allergic (Li Yi, 2008); anti-inflammatory (Kang, 2008; Kim, 2009); anti-cancer (Kong, 2009); and anti-coagulant (Pushpamali, 2008). Based on these diverse beneficial

properties, brown seaweeds have been used in many medicinal applications. Starting from the 4th century in China and the 19th century in Europe, the brown seaweed, *Laminaria* has been used in powder form to cure goiter disease, although this is no longer practiced due to other less costly medicine. Genera like *Laminaria, Ecklonia*, and *Sargassum* were also used in Chinese medicine to treat other diseases such as tumours, edema and throat infections (Dharmananda, 2002).

Recently, brown seaweeds have been found to produce haloperoxidases (HPOs) (punitha *et al*., 2018). Haloperoxidases (HPOs) are enzymes that catalyze the oxidation of a halide by hydrogen peroxide (Wever & Hemrika, 2001; Leblanc *et al.,* 2006). HPOs are haemoproteins, and have ferriprotoporphyrin IX as a prosthetic group (Suthiphongchai *et al.,* 2008). HPOs occur in nature (Neidleman & Geigert, 1986), *inter alia* in bacteria (Neidleman, 1975; Suthiphongchai *et al.* 2008) and algae (Fenical, 1975), and function as a catalyst in halogenation reactions (Suthiphongchai *et al*., 2008). Haloperoxidases have two main functions in the algae, namely as a defense mechanism and as a way of regulating reactive oxygen species (ROS), through the production of halogenated compounds. The halogenated compounds when released into the atmosphere, are responsible for the breakdown of the ozone layer (Hossaini *et al*., 2015). Therefore, although HPOs are listed under potential sources of bioactive compounds, they may also contribute significant impacts on climate change (Syrpas *et al*., 2014). Brown seaweeds are one of the major contributors for the release of halocarbons through haloperoxidase (HPO) activity (punitha *et al*., 2018). Though reports on HPOs by temperate seaweeds are available, reports from the tropics are relatively scarce. Keng *et al*. (2013) reported that the tropical *Sargassum binder*i was dominant in the fringing coral reefs at Port Dickson, west coast Peninsular Malaysia, and was found to emit a suite of halocarbons together with other brown seaweed species of *Turbinaria* and *Padin*a.

Therefore the brown seaweed *Sargassum binderi* was selected for investigation on the production and properties of haloperoxidase.

1.2 Problem Statement

There are many algal species that produce various types of haloperoxidases. For example, several nonheme vanadium BPOs (V-BPOs) have been isolated from brown algae *Fucus distichus* and *Macrocystis pyrifera* (Soedjak & Butter 1991) and the Laminariaceae (Almeida *et al*., 2001), and red algae *Corallina pilulifera* (Krenn *et al*., 1989), *C. officinalis* (Yu & Whittaker, 1989), *Cvancou veriensis* (Everett *et al*., 1990), *Gracilaria fisheri* (Kongkiattikajorn & Pongdam, 2006). Although there have reports of haloperoxidases from temperate brown seaweeds, the production of haloperoxidases from tropical brown seaweeds like *Sargassum* has not been studied. To date there is still no clear understanding of the production pathways and functions of the haloperoxidases. Much research has to be conducted to provide information for understanding their role in ozone depletion and climate change, as well as identifying their potential therapeutic applications.

The increase in halocarbon emissions by brown seaweed is one of the major contributors to ozone depletion. This study on the commonly found brown seaweed *Sargassum binderi* would produce an important profile of tropical seaweed haloperoxidases that will lead to a better understanding of the mechanism responsible for halocarbon emission from the tropics. Peroxidases have medicinal applications; for example, mammalian peroxidases (myeloperoxidase, eosinophil peroxidase and lactoperoxidase) have been implied in host defensive system where they catalyze the formation of bactericidal products in vivo (Pourtois *et al.,* 1992). The first peroxidase application was for oral care; the enzyme has the property of killing bacteria of dental diseases (Kessler and Rosenbaum 1984) and can activate

antimicrobial activity in oral dentrifice with lactoperoxidases (Montgomery, 1994). Meanwhile, Allen (1992) proposed a method using haloperoxidase which has high potential in therapeutic or prophylactic activities by selectively binding to the target, due to the hydrophobic properties and strong charge of the peroxidase. The haloperoxidase catalyzes the halide oxidation and initiates the disproportionation of peroxide to singlet molecular oxygen at the surface of the target microbes and kills them (Allen, 1996). Thus peroxidases has antimicrobial properties, and the extracted HPO from seaweeds can be further characterized to be used as an antibody, where the resulting hypohalides could kill specific target cells such as tumour cells (Wever, unpublished).

1.3 Research Questions

The following questions are set to guide the research on identifying the types and functioning of the haloperoxidases to be extracted from the tropical *Sargassum binderi*.

(i) What types of haloperoxidases are expressed in the crude extracts of *Sargassum binderi?*

(ii) What are the factors that influence the activity of the crude extracts?

(iii) What are the limitations (such as temperature and substrate concentrations) for the activity of the enzymes?

1.4 Objective

The main objective of this project is to characterize the haloperoxidase of *Sargassum binderi* Sonder ex J. Agardh. This can be done by:

1) To extract and determine the type of haloperoxidase produced from tropical brown seaweed *Sargassum binderi* Sonder ex J. Agardh
2) To determine the effect of temperature and substrate concentrations on haloperoxidase extracted from *Sargassum binderi* Sonder ex J. Agardh.
3) To determine the conditions under which the enzyme will show the highest possible degree of stability and activity.

1.5 Thesis outline

This thesis is divided into six chapters described as follows:

i) Chapter 1 introduces the background of this study and addresses the associated problems and research question with appropriate objectives.
ii) Chapter 2 defines relevant terms, describe research scopes and previous work by referring to the literature.
iii) Chapter 3 provides the experimental design and methodology from the beginning of seaweed collection to the expression of the enzyme.
iv) Chapter 4 presents the experimental results in proper format and validates the model of study.
v) Chapter 5 discusses the results in comparison with relevant literature accordingly.
vi) Chapter 6 gives an overview of the research presented and concludes the findings and their contributions.

CHAPTER 2: LITERATURE REVIEW

Algae are rich with bioactive compounds and have been utilized in the agrochemical and pharmaceutical industries (Syrpas *et al*., 2014). There are various bioactive compounds found but the most interesting are halogenated compounds due to their biological properties such as antifungal, anti-inflammatory, antifeedant, cytotoxic, insecticidal, and antibacterial activity (Syrpas *et al*., 2014). These halogenated compounds are released through biohalogenation mechanisms which are mediated by action of haloperoxidases (HPOs) from these marine organisms (Syrpas *et al*., 2014). The screening of HPOs primarily focused on marine macroalgae or better known as seaweeds, and recently on diatoms, an abundantly found type of marine microalgae (Syrpas *et al*., 2014). HPOs catalyze the oxidation of halides by hydrogen peroxide producing oxidized halogen intermediate. Halogens specifically iodine, bromine and chlorine are vastly found in the ocean (Matsuda *et al*., 2015). This intermediate will halogenate an organic substrate and synthesize organohalogens (Soedjak *et al*., 1995; Everett *et al*., 1990) along with the release of large quantity of halometabolites (Cabrita *et al*., 2010; Gribble, 2003).

HPOs are grouped into three types according to the cofactor, which are nonheme and nonmetal HPOs, heme HPOs, and vanadium HPOs (Matsuda *et al*., 2015). Most of the macroalgae have been shown to produce vanadium-haloperoxidases (V-HPOs), which were further classified into three groups following the selectivity of catalyzing halides oxidation (Fournier *et al*., 2014):

1) Vanadium chloroperoxidases (V-CPOs) mostly found in terrestrial fungi and bacteria, and can oxidize chloride, bromide and iodide.

2) Vanadium bromoperoxidases (V-BPOs) rich in all classes of macroalgae such as brown, red and green seaweeds, and can catalyze oxidization of bromide and iodide.
3) Vanadium iodoperoxidases (V-IPOs) have been detected in brown alga (*Laminaria digitata*) and can oxidize iodide.

During the enzymatic reaction either substrate X− (halide and pseudohalide) or enantioselective (prochiral) sulfide will be oxidized as shown in Eqs. (1a) and (1b), respectively (Rehder, 2014). The resulting unstable bromination will catalyze the oxidation of many other organic substrates. In the absence of substrates VHPO releases singlet oxygen, Eqs. (1c).

$$X + H_2O_2 + H^+ \rightarrow HO^-X + H_2O \quad (1a)$$

$$RR'S + H_2O_2 \rightarrow RR'S{=}O + H_2O \quad (1b)$$

$$HOX + H_2O_2 \rightarrow O_2 + H_2O + HX \quad (1c)$$ (Rehder, 2014)

2.1 Distribution and Structural Characteristics of HPOs

The HPOs from *Aschophyllum nodosum*, first seaweed to be studied, have been structurally characterized into two isoenzymes namely VBrPO (AnI) and VBrPO (AnII), which share 41% sequence homology (Rehder, 2014). There have been reports on the active site of the bromoperoxidases of *A. nodosum* and *Corallina pilulifera*, as well as on phosphate variants (i.e. the apo-enzyme reconstituted with phosphate) of *C. pilulifera* and *C. officinalis* have been reported earlier (Rehder, 2014). The table below shows some information of the selected characterized species.

Table 2.1: The detailed molecular information of haloperoxidase of selected seaweeds

Phylum	**Species**	**Types of Peroxidase**		**Quaternary structures**	**Number of amino acids per subunit**	**Molecular mass**	**Binding characteristics**	**References**
Brown algae	*Ascophyllum nodosum*	VBrPO (*An*I)	Homodimer	Covalently bound dimers	557 amino acids per subunit	136 kDa,		(Reihder, 2014)

Table 2.1 Continued

	Ascophyllum nodosum	VBrPO (*An*II)	Hexamer		641 amino acids & from putative mature protein 620 amino acids	420 kDa	Binding 0.5 equivalent of vanadate per 67.4 kDa-	(Reihder, 2014)
Red algae	*C. pilulifera*	VBrPO (*Cp*)	Homododecamer (hexamer of homodimers)	Self rearrange into dodecamers	595 amino acids per subunit		stabilized by one Ca^{2+} per subunit	(Reihder, 2014)

V-HPOs tertiary structures with high conservation and identical arrangement of amino acids residues at vanadium active site have similar evolutionary history with bacterial phosphatases (Fournier *et al*., 2014a). These two main structures suggest that they originated from a common ancestor.

2.1.1 Recombinant HPOs

BPO genes have been isolated from various macroalgae such as brown algae, *Fucus distichus* (AF053411) and *Ectocarpus siliculosus* (Cock *et al*., 2010), as well as from the red algae *Chondrus crispus* (Collén *et al*., 2013), *Corallina pilulifera* (Shimonishi *et al*., 1998), *Corallina officinalis* (Carter *et al*., 2002), *Gracilaria changii* (Baharum *et al*., 2013), and *Delisea pulchra* (Sandy *et al*., 2011) in order to understand in detail of its catalytic properties and to explore their molecular engineering using recombinant proteins. The mutated recombinant BPOs at a single amino acid, in *C. pilulifera* and *G. changii*, showed enhanced halide affinity and activity; indicating that BPOs can be utilized for producing novel organohalogens (Sandy *et al*., 2011).

2.2 HPOs and Reactive Oxygen Species (ROS)

2.2.1 The production of H_2O_2

Hydrogen peroxide (H_2O_2) is a small, relatively long-lived compound (half- life is 1 ms) and is able to migrate through cell membranes (Petrov & Van Breusegem, 2012). These properties allow H_2O_2 to traverse cellular membranes and migrate into and from various compartments, which supports its signaling functions (Bienert *et al*., 2006).

H_2O_2 is mostly produced from photosynthetic processes involving the thylakoid electron transport chain and photorespiration (Tarakhovskaya *et al*., 2015). Photorespiration is usually suppressed in algae by action of CO_2 concentrating mechanisms. Cell wall and plasmalemma- located oxidases and peroxidases can also generate ROS (Tarakhovskaya *et al*., 2015) which can diffuse out of the cell (Miller *et al*. 2010). This H_2O_2 is produced more by a class of cell membrane NADPH-dependent oxidases that function like respiratory burst oxidase homologues (Rboh) (Potin, 2008; Slesak *et al*., 2007).

In most cases, H_2O_2 is formed after reduction of superoxide radicals in a reaction catalyzed by superoxide dismutase (SOD). Different sources of H_2O_2 are found in different plant cell compartments. These are the result of increased metabolism such as photorespiration and fatty acid oxidation in peroxisomes and glyoxysomes respectively, as well as over-energization of the electron transport chains in chloroplasts and mitochondria (Apel & Hirt, 2004; Gechev *et al*., 2006).

The H_2O_2 produced as a result of metabolic processes are in small quantity compared to oxidative stress condition. The VHPOs are more resistant to high concentration of H_2O_2 (Rehder, 2014). Hydrogen bonding interaction stabilized coordination of geometry changes from trigonal-bipyramidal towards strongly distorted tetragonal- pyramidal, mediated by H_2O, between apical oxido (2-) ligand and the apical histidine (Rehder, 2014). The concentration of H_2O_2 in open ocean water can go up to 0.25 μM (Rehder, 2014).

2.2.2 Factors triggering ROS and release of halocarbons

2.2.2.1 Effect of Temperature

A study conducted by Abrahamsson *et al*., (2003), showed that algal growth can be enhanced under high temperature at the northern brackish Baltic Sea (Snoeijs and Prentice, 1989; Snoeijs, 1992). This abiotic change can cause high release of volatile halocarbons from coastal areas. In their study, six algal species (the diatom *Pleurosira laevis,* the brown alga *Fucus vesiculosus* and four filamentous green algae, *Cladophora glomerata, Enteromorpha ahlneriana, E. flexuosa and E. intestinalis*) were exposed to temperature variations to evoke oxidative stress (Abrahamsson *et al*., 2003).

Principal component analysis (PCA) on data collected from 42 samples of the six algal species, showed that the five macroalgal species (incubated at 23 °C, but sampled in the field at 12 - 23 °C) emitted 16 volatile halocarbons (Abrahamsson *et al*., 2003). Production of $CHBr_3$ increased with temperature in *E. ahlneriana* and *E. flexuosa*, but decreased in *E. intestinalis*. This indicates that temperature does not have a universal influence on halocarbon emission, which is species-specific, inconsistent and varied (Abrahamsson *et al*., 2003). Temperature has less influence on distribution pattern and elicited different responses from the algae. Thus, quantitative composition of VHC released are highly dependent upon the type of species involved. The presence of H_2O_2 induces release of brominated compounds $CHBr_3$, CH_2Br_2, $CHBrCl_2$ and CH_2BrCl, as well as C_3H_7I and C_4H_9I. *C.glomerata* produced lower $CHBr_3$, due to less H_2O_2 after being scavenged by the enzymes (Abrahamsson *et al*., 2003). So, low temperature and high radiation have evoked oxidative stress from the macroalgae *C. glomerata* and *E. ahlneriana* (Snoeijs, 1992, 1999; Abrahamsson *et al*., 2003).

In Forsmark, *C. glomerata* exposed at temperature changing from 23 °C to 12 °C or from 12 °C to 23 °C increased H_2O_2 production six- to eightfold and CH_2I_2 production two- to fivefold. During cross incubation, the high release of H_2O_2 in *C. glomerata* resulted in iodine uptake for halocarbons being doubled, from 13-23 pmol I to 43- 56 pmol I (g ADW)-1 h 1 (Abrahamsson *et al*., 2003) .

Vilter *et al*., (1983) reported that in brown algae, peroxidase activity (PO) was seasonally variable with decreasing amounts of PO in the order of months, April > March > July > September, probably responding to the change in temperature (cited in Hartung *et al*., 2008).

2.2.2.2 Effect of pH

Algae grown in media containing MnO_2, showed a big variation in VHC production due to decrease in pH. The conditions reduced extracellular H_2O_2 concentration, and increased VHC production in comparison with algae treated with NaN_3 and maintained under pH 8.8. NaN_3 has the characteristics of inhibiting heme- containing catalase and peroxidase and induced more H_2O_2 (Mtolera *et al*., 1996; 2010).

According to Wuosmaa & Hager (1990), the S- adenosyl methyl transferase catalyzes formation of methyl halides in the order iodide > bromide > chloride. This catalytic process is influenced by small changes in the pH as observed in the red alga *Endocladia muricata* (Postels & Ruprecht) J. Agardh (Endocladiaceae). Although incubation of the whole cells produces only methyl halides (Mtolera *et al*., 1996), an increase in pH from 8.0 to 8.8, resulted in a doubling of the major iodinated VHC, diiodomethane with very little release of methyl iodide.

2.2.2.3 Effect of Light

The influence of light intensity on *Ulva lactuca*, was reported by Manley & Barbero (2001). The production of bromoform was nearly tripled in the light (376 ± 92 pg cm^{-2} h^{-1}) compared to the dark (114 ± 70 pg cm^{-2} h^{-1}). The differences in the observation are probably due to physiological (photosynthetic, respiratory, or nutritional state) and environmental influences (Manley & Barbero, 2001). The availability of light allowed photosynthesis to occur and provide energy for the halogenation process.

To understand the influence of light intensity in *Ulva lactuca*, the inhibitor DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea) was used to control the production of $CHBr_3$ in the light. DCMU stops electron flow to PSII by binding to the secondary acceptor quinone. Eventually $CHBr_3$ production and photosynthesis are inhibited (Manley & Barbero, 2001). Respiration (R) was high for a few days after exposure, but after day 4, $CHBr_3$ production become less and correlated with R. Respiration by Ulva was observed to be inhibited by 36% and $CHBr_3$ production decreased 38% by rotenone (Manley & Barbero, 2001), which inhibits mitochondrial electron transport acting between the flavoprotein and non- heme proteins of complex I (Manley & Barbero, 2001). Electron flow increases and O_2 reduced due to uncoupling of oxidative phosphorylation from mitochondrial electron transport. In the presence of DNP (which acts as an uncoupler), Ulva respiration rate increased together with increases in electron flow, O^{2-}, H_2O_2 and $CHBr_3$. External H_2O_2 either activates or inhibits $CHBr_3$ production depending on the availability of light. At low H_2O_2 (1 mM) level, $CHBr_3$ production was inhibited in both light and dark conditions, associated with inhibition of photosynthesis and respiration respectively. However with addition of 100 µM H_2O_2 $CHBr_3$ production was inhibited in the light but was activated in the dark (Manley & Barbero, 2001).

The possible reason for the inhibition of PS and R could be due to membrane destruction by peroxidation. At high H_2O_2 level, $CHBr_3$ was reduced probably due to loss of internal BPO activity caused by membrane disruption. Bromoform was still produced in minor quantities, with the assumption that BPO was still active. (Manley & Barbero, 2001; Leedham *et al*., 2015). Light has a positive effect on production of halocarbons. The effect of this factor also interacts with temperature.

2.2.2.4 Effect of Salinity

Freshwater macrophytes *Pistia stratiotes* and *Salvinia molesta* released high H_2O_2 and lipid peroxidation due increase of salinity (Vpadhyay & Panda, 2005; Maharana *et al*., 2015). In another study by Kumar *et al*. (2010), the red algae *Gracilaria corticata* grow at a salinity range of 25-34 psu which gives a hypo or hyper- salinity stress, inducing oxidative stress and release antioxidants in response. Also in the first 2 to 3 hours of exposure to desiccation caused water loss with increase of antioxidant and ROS (Kumar *et al*., 2010; Maharana *et al*., 2015). Although the range of salinity was low (30 to 34.2 psu) it synergized with other abiotic stress factors and caused a rise in antioxidants. The elevated levels of antioxidants assist colonization of this species in the rocky intertidal zone under harsh conditions (Maharana *et al*., 2015).

2.2.2.5 Interaction between abiotic factors

The interaction among the abiotic factors such as photon fluence rate, temperature, salinity and also others have great influence on the release of iodine and bromine at atmospheric in a global scale. For instances Laturnus *et al*. (2000), in their study compared standard culture conditions with influences of abiotic factors on release of iodine and bromine to atmosphere for the Antarctic red macroalgal species *Gymnogongrus antarcticus*. The standard culture conditions released iodine and bromine at 46.9 x 10^6 g yr^{-1} and 41.6 x 10^6 g yr^{-1} respectively. The increase of photon fluence rate, decreased 7.1 x 10^6 g yr^{-1} iodine release and increased 67.9 x 10^6 g yr-1 of bromine compared to standard. An increase in temperature gave lower release of the halogens than the standard culture conditions that are 21.3 x 10^6 g yr^{-1} reduction in iodine transfer and 12.3 x 10^6 g yr^{-1} bromine transfer (Laturnus *et al*., 2000). However, decrease in salinity and nutrient levels caused an increase in transfer rates compared to standard culture conditions. The iodine transfer and bromine transfer changes due to salinity was 94.4 x 10^6 g yr^{-1} and 64.6 x 10^6 g yr^{-1}, followed by for nutrients at 56.7 x 10^6 g yr^{-1} and 86.2 x 10^6 g yr^{-1} respectively (Laturnus *et al*., 2000). In another study, *Eucheuma denticulatum* was incubated at different range of light intensities. In response this species actively released bromoform (310 ± 25 µg kg $DW^{-1}h^{-1}$) and diiodomethane (182 ± 9 µg kg $DW^{-1}h^{-1}$) which are the dominant volatile halocarbons (VHCs) compared with the other released halocarbons (Mtolera *et al*., 1996). In their study, Mtolera *et al*. (1996) found that higher production occurred at a photon flux density of 1500 than at 400 µmol photon $m^{-2}s^{-1}$. But the pH influence was minimal at 400 µmol photon m^{-2} s^{-1} (Mtolera *et al*., 1996).

2.2.2.6 Effect of biotic interaction

The continuous exposure to the green alga *Acrochaete operculata* which is a parasite of *Chondrus crispus*, causes it to exhibit oxidative bursts (Dring, 2005). *C. crispus* has the ability to sense microbe associated molecular patterns (MAMPs), known as general or exogeneous elicitors. Weinberger (2007) explained the mechanism, that the parasitic alga released L-asparagine, which acts as a substrate for L-amino oxidase in the apoplasm of the H_2O_2 released by the host. The ROS (H_2O_2) acts in preventing parasitic infection and the H_2O_2 will be scavenged subsequently by host-specific peroxidases (Bischof & Rautenberger, 2012b).

2.2.2.7 Effect of Metal

Metals such as Fe, Mn and Cu in combination with superoxide dismutase (SOD) enzymes play roles in the photosynthetic activity of eukaryotic algae (Asada & Takahashi 1987; Maharana *et al*., 2015) and in stimulating ROS production (Moenne *et al*., 2016). The content of metals in seaweeds depends on photosynthetic activity and bioavailability in the surrounding water (Jothinayagi & Anbazhagam, 2009). In their study, the content of metal was negatively correlated with biomass in different seasons; like during pre-monsoon (May) the biomass was high but metal content was low in the seaweeds (Maharana *et al*., 2015).

At the South Andaman Island, *Acetabularia calyculus* from Wandoor was observed to have accumulated more of essential and non- essential metals (Mn, Pb, Zn, Cd, Cu and Cr) in comparison with other algal species (Karthicka *et al*., 2012; Maharana *et al*., 2015). The high content of non- essential trace metals i.e. Cd, Pb, Hg and As, could evoke oxidative stress in their tissue (Kumar *et al*., 2010; Collen *et al*., 2010; Maharana *et al*., 2015). Metals (iron and copper specifically) accelerate lipid peroxidation during

oxidative stress (Fenton reaction), involving mechanism of producing hydroxyl radicals by converting hydrogen peroxide, and in corresponding reaction, they split hydroperoxides, giving alkoxyl, and more peroxyl radicals. An uninterrupted fatty acid side chain and carbon oxidation results in loss of membrane integrity (rupture of lysosomal or central vascular membranes) (Halliwell, 2006). For an example, copper is one of the metals that serve as micronutrients for all life forms. It acts as a cofactor for various enzymatic systems and functions in important physiological process such as photosynthesis and respiration (Maksymiec, 1998; Ritter *et al*., 2010). Copper in presence of other chelating metals becomes toxic in high concentration (Sueur *et al*., 1982), although algal cell walls contain charged sulfated polysaccharides which function as primary ion filters (Witvrouw & De Clercq, 1997).The oxylipin signaling pathways related to detoxification mechanisms are triggered by Cu-induced ROS production in *Laminaria digitata* (Ritter *et al*., 2008).

2.2.3 The effects of ROS production

Elevated ROS is a result of increased reduction of NADPH and ferredoxin (Asada, 1999). This occurs in shallow waters with polar and cold-temperate algae when exposed to excess of light or decreased CO_2 assimilation. High levels of photosynthetically active radiation (PAR) supplies more electrons into the photosynthetic electron transport chain; (ETC) (Colle´n & Davison 1999b, 2001; Becker *et al*., 2009, 2010 ; Bischof & Rautenberger, 2012a) in the absence of adequate dissipation of electrons to NADPH formation and CO_2 assimilation, electrons will flow to molecular oxygen via the Mehler reaction. This will eventually increase the O^{2-} concentration (Bischof & Rautenberger, 2012a; Becker *et al*., 2009, 2010). The excretion of ROS caused reduction in photosynthetic carbon assimilation (photoxidation) through the Carbon- Benson cycle (Becker *et al*., 2010).

2.2.4 The purpose and functions of ROS production

Seaweeds have developed their own mechanisms to defend against potentially lethal physiological damage occurring during environmental changes and to maintain their physiological integrity (Davison & Pearson, 1996). In the photosynthetic reaction, due to high irradiance triplet state formed cause of over excitation of chlorophyll a molecules (Ledford & Niyogi 2005). Thus, algae dissipate excess radiation energy through the xanthophyll cycle to prevent the formation of triplet chlorophyll ($3Chl^{+}$), which is a strong photosensitizer of reactions leading to singlet oxygen and thus formation of other ROS. In the xanthophyll cycle, energy dissipates as heat by interconversion of specialized xanthophylls located in the antennae (Jahns *et al*., 2009). In addition, triplet chlorophyll is also deactivated through carotenoids associated with photosynthetic reaction centers. In the case of extreme radiation, ROS are formed (Bischof & Rautenberger, 2012).

The resulting ROS have many functions at lower concentrations. ROS function as signaling molecules for certain regulated processes in the process of plant growth and development (Thannickal & Fanburg, 2000). These include processes such as cell elongation (Foreman *et al*., 2003, Tsukagoshi *et al*., 2010), as well as in responses to a variety of environmental stimuli (Dat *et al*., 2000). H_2O_2 has the functional role in algae through interaction with various peroxidase enzymes to produce secondary metabolites of considerable importance (Dring, 2005). In a study conducted on *Fucus* embryos, during hyperosmotic stress, H_2O_2 produced outside of the plasma membrane caused an increase in Ca^{2+} channel activity, resulting in cell wall strengthening (Coelho *et al*., 2002).

An oxidative burst will be released in the apoplast as a response to the presence of oligomeric degradation products of alginate by oligoguluronates which evoked

respiratory (Kupper *et al*., 2001) and inflammatory activities by bacterial lipopolysaccharides (Kupper *et al*., 2006). In this process superoxide anions produced by oxidases with a flavoprotein subunit (NADPH oxidase), are converted into H_2O_2 which easily diffuses into seawater (Küpper *et al*., 2001). This H_2O_2 acts as defence against microbes on the thallus surfaces (Küpper *et al*., 2002; Weinberger & Friedlander, 2000).

2.3 Vanadate binding site in VHPO

Vanadium is a transition metal found in milimolar solution as dihydrogen vanadate (H_2VO_4); the conjugated base of orthovanadic acid H_3VO_4 shares many characters similar to phosphate (Wischang *et al*., 2012). In a condition of pH 8, free vanadate exists in the form of a mixture of mono and dihydrogenvanadate $H_2VO_4^- / HVO_4^{2-} + H^+$; this attains equilibrium state at pKa 7.95 when the mean salt concentration is 0.6 M (Wischang *et al*., 2012).

Vanadate (H_2VO_4) coordinated to a histidine residue in the enzyme's active centre in VHPOs appeared different from the heme based and non- heme peroxidase (Rehder, 2014). During the halogenation, the metal center of V-HPOs was stable with no changes to the oxidation state with the ability to halogenate a range of organic compounds in a regio- and stereo-specific manner (Wang *et al*., 2014). In contrast the change of oxidation states +V and +IV causes catalytic activity in the homogenous and heterogeneous catalysis of common applications (Rehder, 2014). This is because inorganic vanadium (v) compounds are versatile oxidation catalysts. Under non-physiological conditions peroxide also uses basic inorganic vanadium such as $[VO_2(H_2O)_4]^+$ and H_2VO_4 compounds for the oxidation of bromide (Rehder, 2014).

Activity of algal peroxidases depends on the Lewis acidity of the V^{5+} center (Rehder, 2014). Electronic environment surrounding VO_4 moiety influences the catalytic

properties and halide specificities of VCPO and VBPO based on these structure- function studies (Rehder, 2014).

Table 2.2: Similarity and Differences between vanadate of VHPO and phosphatase

VHPO (Vanadate)	Phosphatase
Similarity	
• Active site arrangements • Structural similarity (between tetrahedral anion phosphate and vanadate)	
Differences	
• Redox activity • Potential expansion of the coordination sphere	
• Reduced to oxidovanadium (IV)	• Redox stable
• Vanadium easily adopt coordination number five(as in VHPOs)- inhibitory effect of vanadate towards phosphatases	• Penta-coordinate phosphate restricted to enzymes' transition state
	• Vanadate substituted bacterial acid phosphatases exhibited BPOs as well as sulfide peroxidase activity

2.4 Halides or Sulfides oxidation and function

VHPOs catalyzes the halide substrate, (X^-) at various threshold with the concentrations of 0.55 M for chloride, 0.82 mM bromide, and 0.47 μM iodide from the

seawater (Rehder, 2014). Red algal families have also been reported to utilize halides to provide osmotic balance and supply the basic building blocks for complex cellular activities (Fenical, 1975). Based on thermodynamic and kinetic considerations for the reaction of halides with oxidants in a living system, it was found that iodide was found to be a better antioxidant than bromide and can serve as a chemical defence (Küpper *et al*., 2013, 2008).

In the absence of halides, VHPO catalyzes sulfoxidation by nucleophilic attack to the peroxo oxygen of the hydroperoxido intermediate (Wang *et al*., 2014).The enantioselective sulfides oxidized into sulfoxides at the vanadium-binding site through a direct oxygen transfer from the peroxovanadium intermediate (Wang *et al*., 2014). The potential of VHPO oxidizing sulfides and selection of organic compounds has potential in pharmaceutical application. Meanwhile in industrial application, VCPO oxidized decarboxylation of glutamic acid forming 3-cyanopropanonic acid (key intermediate in the synthesis of acrylonitrile) in the presence of H_2O_2 and bromide (Rehder, 2014).

2.5 Affinity to vanadium and halide substrates

The study of X-ray diffraction and X-ray absorption spectroscopy revealed that first step of catalytic cycle is the binding of hydrogen peroxide to vanadate, known as bi-bi-ping-pong mechanism resulting stable peroxovanadate intermediate (Fournier *et al*., 2014a). In order to provide an active site for bromide oxidation, dihydrogenvanadate has high binding affinity to bromoperoxidase proteins at the s (tele-remote) imidazole nitrogen of a histidine side chain (Wischang *et al*., 2012). The VBPO has bromide in the active center is located in between vanadate and an active site arginine (Arg 387) within hydrogen bonding distances, showing the activation of bromide by arginine (Rehder, 2014). In the help of V-XAS studies was proven that absence of direct binding of bromide to vanadium. Hydrogen peroxide binding and followed bromide oxidation reaction ease

by the vanadate cofactor and proximate amino acid side chains which provide template for locking water molecules into a supramolecular network (Wischang *et al*., 2012).

2.6 Halocarbons and the resulting metabolites

Organobromines are one of the constituents of planetary cycle transporting bromide from ocean water through secondary metabolites and volatile organic compounds to mineral deposits or continental brines (Wischang *et al*., 2012). Marine boundary layer contains free radical releasing hydrocarbon bromofunctionalization. Majority of secondary metabolite formation are due to electrophilic bromination of carbon nucleophiles (Wischang *et al*., 2012).

Hypobromite resulting from the bromide oxidation present as hypobromous acid (HOBr) is released into water with pH 8 (Wang *et al*., 2014). This HOBr in the presence of bromide form bromine and also binds to Br^- resulting into tribromide. In the bromination activity Br_2, an electrophilic substances forms C-Br bond (Wang *et al*., 2014).

$$HOBr + Br^- \rightarrow Br_2 \quad (3a)$$

$$Br_2 + Br^- \rightarrow Br_3^- \quad (3b)$$

Bromofuranone is an example of a natural product formed in the wake of the catalytic activity of haloperoxidases in marine macroalgae. For example VBPO from red alga *Laurencia nipponica* produces compounds from laurediol by cyclisation plus bromination (Wischang *et al*., 2012). In comparison with native species, the mutant form has more effect in the antibacterial properties for external application. This has the potential in the development of disinfection formulation for submerged structures and in medicinal context (Wischang *et al*., 2012).

2.7 Antimicrobial properties of HPOs

The antibacterial and antifouling characteristics of VHPOs in principle, have been exploited for the disinfection of medicinal equipment (Rehder, 2014). The antimicrobial properties of VCIPOs have use in endodontics where they can destroy the dental biofilms produced by *Streptococcus mutants* and *Enterococcus faecalis* (Rehder, 2014). Recently BrCN was detected as one of the products released from this HPOs activity. The marine benthic diatom *Nitzschia pellucida* has the allelopathic activity categorized under antimicrobial characteristics (Rehder, 2014). It releases cyanogen bromide BrCN that has the potential of causing death of other competing diatoms. More novel studies should be done due to the catalytic potentiality of high- valent vanadium compounds and anti-fouling properties of nanoparticulate V_2O_5, reminiscent of the antibacterial and anti-fungal potential of VHPOs present in seaweeds and diatoms (Rehder, 2014).

2.8 Impact on atmospheric chemistry

Coastal and equatorial regions known as bromocarbons hotspots are due to releases in estimation of the global coastal sea- to- air fluxes of about 2.5·109 mol (2·108 kg) of bromine in the form of brominated methanes ($CH_{4-x}Br_x$). Consequently bromine that is released photolytically from $CH_{4-x}Br_x$, x = 2, contributes to about 25% of tropospheric and lower stratospheric ozone depletion per year (Wang *et al*, 2014).

CHAPTER 3: METHODOLOGY

3.1 Collection of macroalgae (seaweeds)

The study site was a fringing coral reef, at Cape Rachado, Port Dickson in the west coast of Peninsular Malaysia (Figure 3.1). It has been reported that the site is dominated by *Sargasssum, Padina* and *Turbinaria* species (Wong & Phang, 2004). The selected tropical species, *Sargassum binderi* were collected at rocky shore sites which are protected from human activities. The sample collections were done at low tidal levels from 0.3m or lower, as the reef flats are exposed and remain for more than three hours to ease the collection process. After collection, the macroalgae (seaweeds) were kept in ice-chests and transported to the laboratory, and cleaned by washing with distilled water. Portions of 100g fresh weight (FW) were measured, and the seaweed chopped into small pieces and immediately frozen in liquid nitrogen and kept at -80°C.

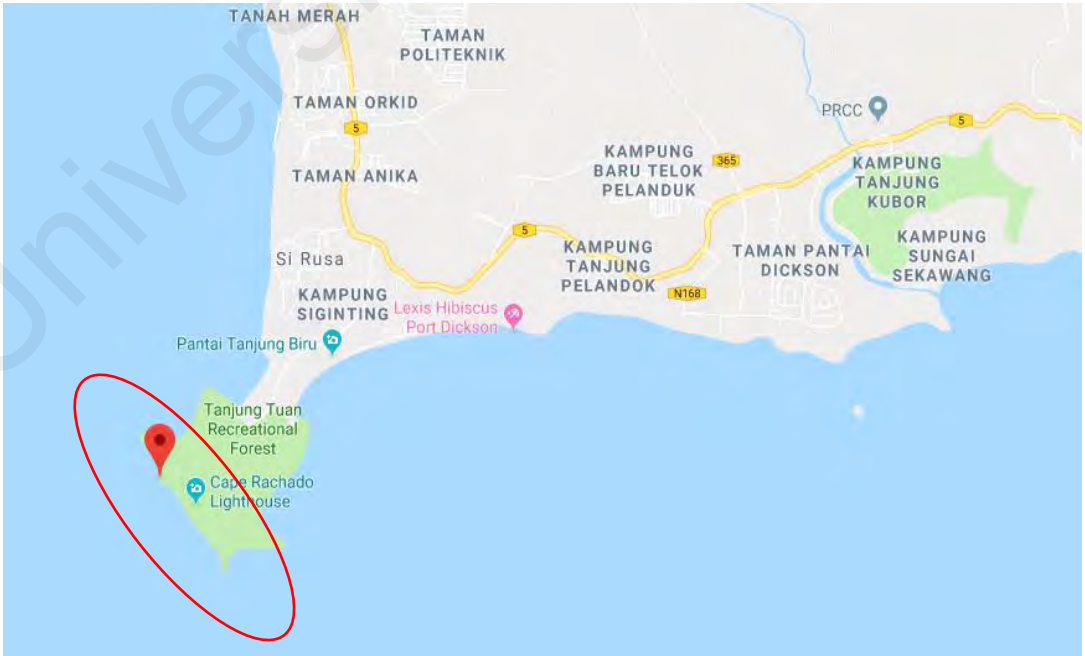
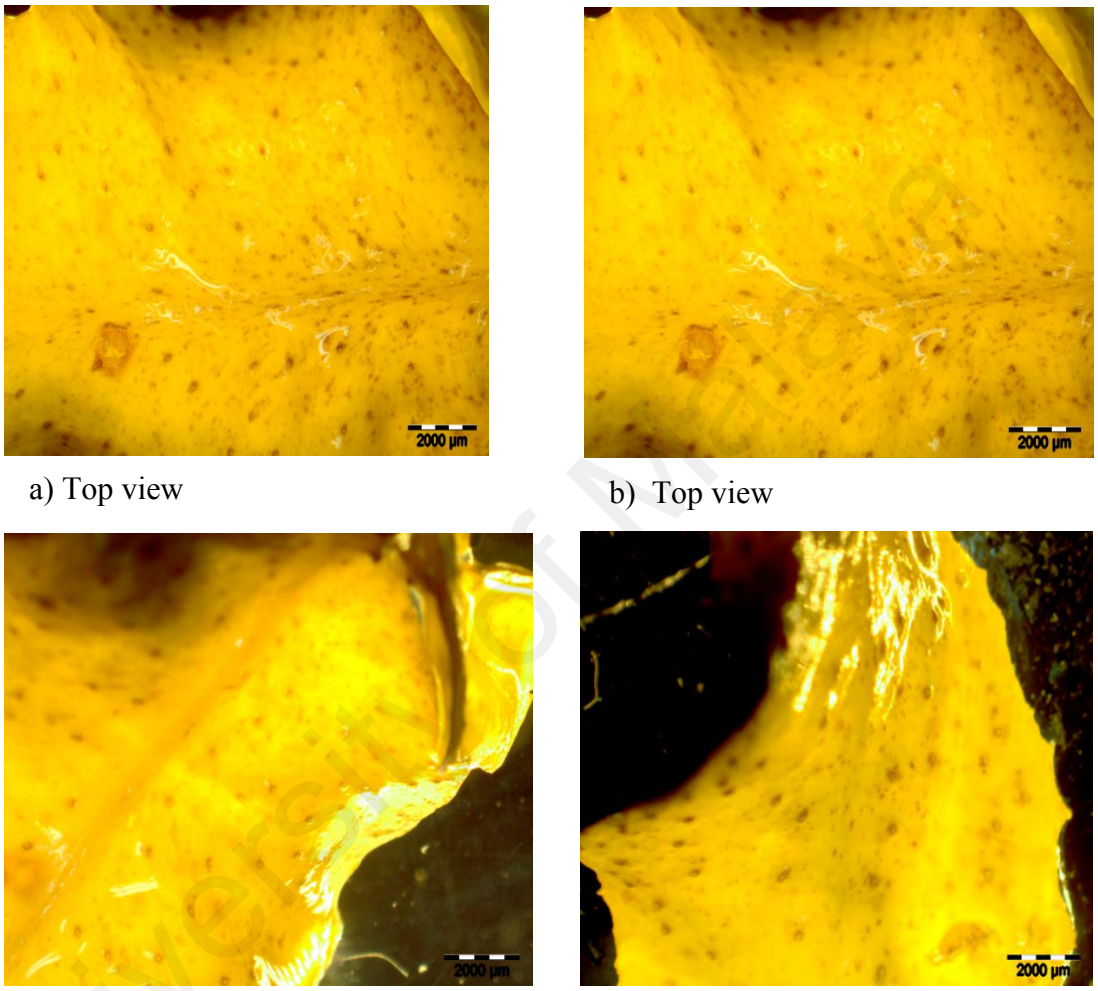


Figure 3.1: Sampling location, Cape Rachado, Port Dickson

(GPS Coordinates: 2.409210, 101.850281)

The collected seaweeds were observed under the compound light microscope (Olympus) to check for absence of epiphytes. (Figure 3.2 a, b, c, d).



a) Top view

b) Top view

c) Bottom view

d) Bottom view

Figure 3.2: *Sargassum binderi* under compound light microscope the top and bottom view (magnifications 40 X)

Voucher specimens of the seaweed was prepared and deposited in the University of Malaya Seaweed and Seagrasses Herbarium.

3.2 General Research Approach

Figure 3.3 gives the Flow-Chart to show the Research Approach.

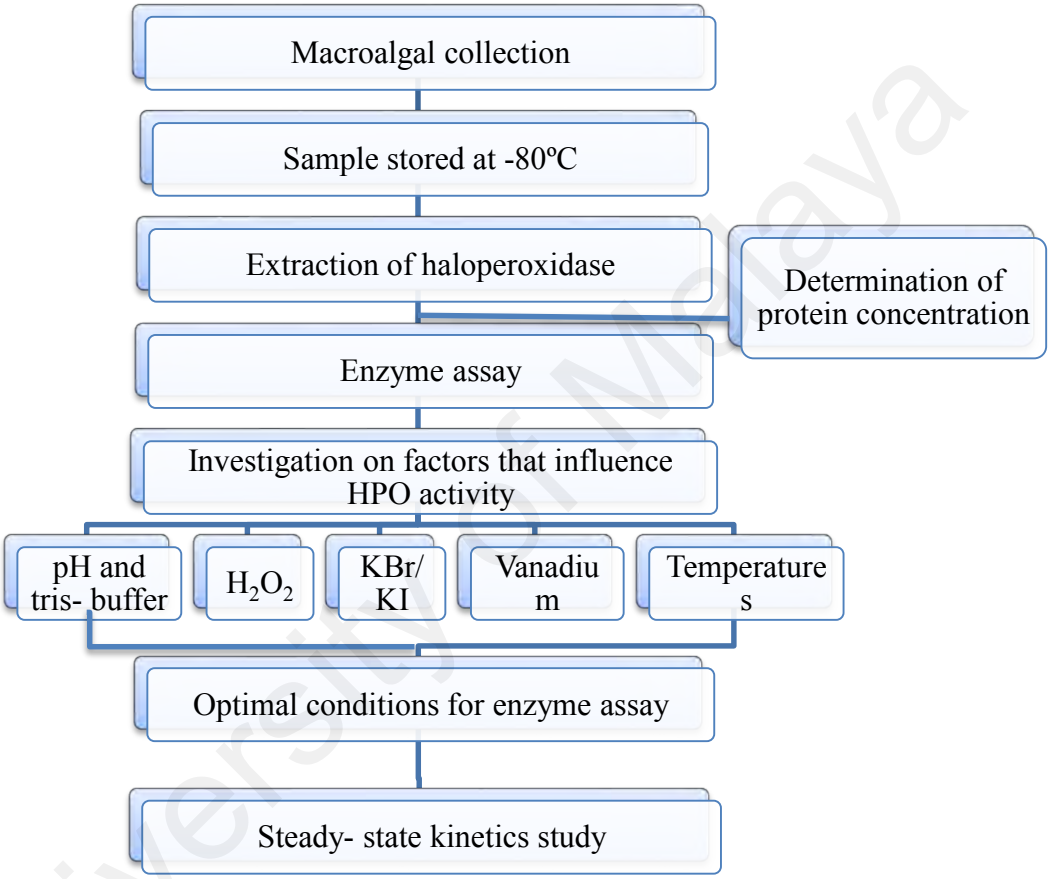


Figure 3.3: Flow Chart of Research Methodology

3.3 Extraction of haloperoxidase from *Sargassum binderi.*

The freeze dried samples of *Sargassum binderi* were finely ground in liquid nitrogen and the HPOs were extracted by a method developed by Vilter (1994), using an aqueous salt/ polymer two phase system. Haloperoxidases are extracted using the salt/polymer two-phase system because brown seaweeds are extremely rich in alginates, anionic polysaccharides and polyphenolic compounds, which will complicate the extraction process. The powdered *S. binderi* was extracted using 20% (w/v) K_2CO_3 and 13% (w/v) polyethylene glycol, (1500Da). The two phase solution was centrifuged at 5 000 x g for 15 minutes at 4°C. This was followed by addition of acetone that was 3 times the volume of the supernatant, and kept at -20°C for 1 hour before centrifuge at 10 000 g for 30 min. The pelleted protein extract was dissolved in 30mM Tris-HCl, pH 8. The same crude enzyme was used for all of the enzymatic assays (Colin *et al*., 2003).

3.4 Determination of protein concentration

Protein concentration was determined by using Bradford commercial (Bio-Rad) coomasie protein assay and bovine serum albumin was used as a standard (Sigma Aldrich) (Colin *et al*., 2003).

3.5 Enzyme activity assay

The enzymatic activity assays were conducted in triplicate at 20 ± 1°C in a clear flat bottomed, 96 well polystyrene microplate (Greiner bio-one). The thymol blue (TB) stock solution was prepared in miliqwater: dimethyl sulfoxide (DMSO) with ratio of 4: 1. The preparation of final DMSO content was ensured not to exceed 2%. The assay mixtures were prepared as follows:

The reaction mixture (180 µl), was prepared with Tris-HCl buffer [40 mM (KBr) and 50 mM (KI), pH 8.0], TB (100 µM), KI or KBr (0.005-0.64 µM), and HPO crude (0.1062 ± 0.0220 mg/µl), followed by addition of 20 µl of H_2O_2 (90 µM final) (Verhaeghe *et al*., 2008).

The absorbance (OD_{595nm}) of the resulting mixture (200 µl) was measured using a microplate reader (Epoch), after incubation for 20 minutes. The OD_{595nm} values were converted to milimolars of diiodothymolsulfonphthalein (TBI_2) using [C] = A_{595} / (40.3 $mM^{-1}cm^{-1}$ x 0.29 cm) and of dibromothymolsulfonphthalein ($TBBr_2$) using [C] = A_{595} / (37.2 $mM^{-1}cm^{-1}$ x 0.29 cm) (Verhaeghe *et al*., 2008).

$$c = \frac{A}{\varepsilon . l}$$

Figure 3.4: Beer- Lambert law mathematical formula that expresses concentration of an analyte dissolved in solution is directly proportional to its absorbance.

The OD_{595nm} values were converted to actual concentrations using the Beer- Lambert law. The equation explain, **C** is the concentration of the absorbing material, **A** is the absorbance measured at 595nm, and ***l*** is the length of the light path. The extinction coefficient is $\boldsymbol{\varepsilon}$. Thus, using this Beer-Lambert equation, absorbance reading converted into an actual amount of product formed (Bisswanger, 2014).

Specific activities are expressed in units per milligram of protein, where one unit of bromoperoxidase/ iodoperoxidase activity is defined as the amount required for brominating/ iodinating 1 µml of thymolsulfonphthalein per min. The differences between the non- enzymatic and the non-enzymatic- plus- enzyme reaction were calculated for each sample (Colin *et al*., 2003).

3.5.1 Thymol Blue as chemical probe

Thymol blue as a substrate and hence a chemical probe, has been used recently in many studies related to haloperoxidase because other substrates have their fallible characters (Suthiphongchai, Boonsiri, & Panijpan, 2008). For instance, using Monochlorodimedone (MCD) precisely produces one product but is fairly detectable in the UV range, whereas phenol red converts substrates into color products but gives more products due to multiple bromination (Suthiphongchai, Boonsiri, & Panijpan, 2008). The first VIPO was detected using triodide assay, which is specific for iodide oxidation but has restriction because of high chemical instability of I_{-3} in solution. In earlier studies, phenol red and *o-dianisidine* were used as the chemical probes for the assay but due to the limitations, are now only used for qualitative studies. Thymolsulfonepthalein, also named thymol blue (TB), was later developed for use with oxidized bromide and iodide species (Suthiphongchai, Boonsiri, & Panijpan, 2008).

3.5.2 Selection of suitable wavelength by spectrum studies

The spectrum analysis using the Epoch microplate reader, showed that the suitable wavelength for the blank without addition of crude extract is 420nm and the suitable wavelength after addition of crude extract is 595nm. The Spectrum curves for colorimetric assay in microplate reader (Epoch), (a) Blank well (b) Well added with crude enzyme; n =3 is given in Appendix 1.

3.6 Properties of enzyme activity

The crude extract containing the enzyme, was prepared from the seaweed samples by two-phase aqueous system. The properties of the enzyme (haloperoxidase) based on response of the enzymatic activity to various parameters such as buffers with different pH and concentrations, various range of hydrogen peroxide, vanadium, temperature were investigated for the stability of enzyme and its reaction. The ranges for each parameters were selected based on preliminary study. The enzymatic reactions were analyzed based on the resultant compound that was determined as absorbance measured using wavelength of 595 nm in a microplate reader (Epoch) after an incubation period of 20 minutes. The results can be used for optimisation of the enzymatic activity of the haloperoxidase in future studies.

3.6.1 pH and concentration of Tris-HCl buffer to be added for crude extracts stability

The objective was to select the suitable pH and concentration of the Tris- HCl buffer for the crude enzyme (pellet) to be dissolved. The buffers ranging from pH 7-9 and concentrations from 20- 50 mM were used to select the most suitable buffer for the crude enzyme to be dissolved in order to retain the enzymatic activity for analysis. The selected range of buffer concentrations were chosen because at concentration higher than 25mM buffering capacity will be attained. In relation to that from the preliminary study the analysis were started with 20mM. Meanwhile the concentration of buffer was increased to 50mM, which was observed from the preliminary study where there is a good activity shown. This is probably because up to this concentration there will be not interference found from good buffers in cell culture experiments proven by Ferguson et al. (1980) ("Biological buffers," 2008).

3.6.2 Hydrogen Peroxide (H_2O_2) concentration

The objective was to select the suitable hydrogen peroxide (H_2O_2) concentration for the enzymatic reaction in the 96 well microplate (Epoch). The concentrations of H_2O_2 ranging from 10 μM to 0.42 mM were used to determine the most suitable concentration for the enzyme activity assay with the crude extract.

3.6.3 pH and concentration of stabilizing buffer for enzymatic assay

The objective was to select the suitable buffer for the enzymatic reaction in the 96 well microplate reader (Epoch). Two different buffers were selected which were 0.1mM phosphate buffer and 20-50 mM Tris-HCl buffer with various pH 7-9 were used to determine the most suitable buffer for the enzyme activity assay. These two buffers were chosen because in the HPOs catalyzing oxidation, where transfer of electrons to and from the molecules will be carried out, requires slightly alkaline conditions which can be provided by Tris buffer in conjunction with hydrochloric acid. Meanwhile phosphate buffers are used to maintain pH as it mimics the biological conditions in most conditions ("Enzymatic Activity and pH buffer", 2017).

3.6.4 Potassium bromide concentration

The objective was to select the suitable potassium bromide concentration for the enzymatic reaction in the 96 well microplate reader (Epoch). The potassium bromide concentration ranging from 0.005 to 0.64 μM was used to determine the most suitable range for the enzyme activity assay.

3.6.5 Potassium iodide concentration

The objective was to select the suitable potassium iodide concentration for the enzymatic reaction in the 96 well microplate reader (Epoch). The potassium iodide

concentration ranging from 0.005 to 0.64 μM was used to determine the most suitable range for the enzyme activity assay.

3.6.6 Vanadium concentration for crude activation

The objective was to select the suitable vanadium concentration to incubate with the crude enzyme. The crude holoenzyme were re-constituted by adding $NaVO_3$ in Tris-HCl pH 8 at 4ºC for 24 hours. The concentrations ranging from 1- 4 mM were used to determine the most suitable concentration for the reactivation of the crude enzyme activity (Suthipongchai *et al*., 2007).

3.6.7 Thermal stability studies

The crude enzymes were incubated in thermostated vessels for 10 min at temperature ranging from 25 to 70 °C. The activity of the sample was determined after the temperature was equilibrated to room temperature.

3.6.8 Steady state kinetics

The kinetic parameters were determined, from the experimental initial velocities, expressed in milimolars of x converted per minute. The initial rates, Vo plotted as a function of bromide or iodide concentration and the apparent kinetic parameters $K_m^{app\ Br-}$ and $K_m^{app\ I-}$ as well as V_{max} were obtained fit to a Michaelis-Menten equation using a non-linear regression program developed in Sigmaplot (Verhaeghe *et al*., 2008).

3.7 Statistical Analysis

One-way ANOVA with Tukey or Dunnet was used to test the significance ($p< 0.05$) of difference physiological factors which influences the enzymatic activity reaction at OD_{595nm}. Statistical analyses were subject to the normality test using the skewness and kurtosis and results indicated that all data were distributed normally.

CHAPTER 4: RESULTS

4.1 Haloperoxidase extracted from *Sargassum binderi*.

The crude extract from *Sargassum binderi* was analysed for protein content using the Bradford Method (Bradford, 1976). The standard curve for protein determination is given in Figure 4.1.

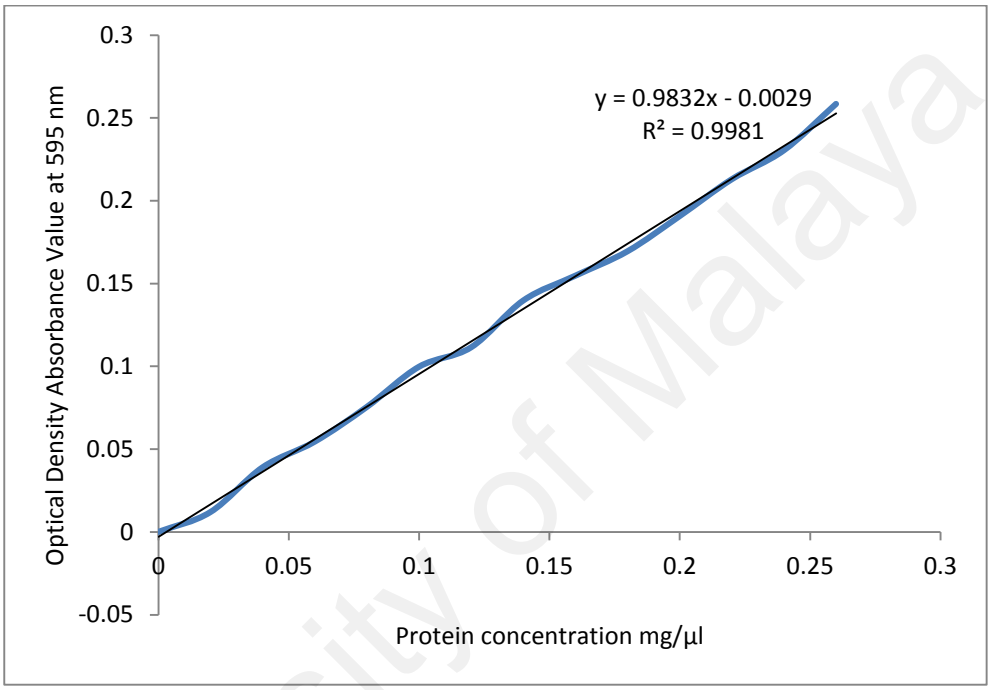


Figure 4.1: Standard curve for protein quantification.

The crude extract was diluted for the protein assay. The diluted protein concentration was 0.1109 ± 0.0220 mg/µl. The concentration of the enzyme was then calculated by substituting into the linear line equation (y= 0.9832x – 0.0029), giving a concentration of 0.1062 ± 0.0220 mg/µl.

4.2 Factors that affect enzyme activity

The investigations were carried out using two different substrate, namely potassium bromide and potassium iodide.

4.2.1 Potassium bromide (KBr) as the substrate

4.2.1.1 Effect of Hydrogen peroxide (H_2O_2) concentration on enzymatic assay

(a) **Product concentration formation of three hydrogen peroxide concentrations using concentration of substrate KBr, from lowest (0.005 µM) to medium (0.04, 0.08 µM) and highest (0.64 µM).**

The concentration of the product was plotted against time for the enzymatic reaction. From the graph (Figure 4.2):

a) 80 µM and 90 µM have higher product formation but are not significantly different between each and 0.1 mM concentration has low product formation and significantly different with the earlier concentrations. All the three concentrations did not produce significantly different product concentrations, by time series because there is no increase in product formation over time.

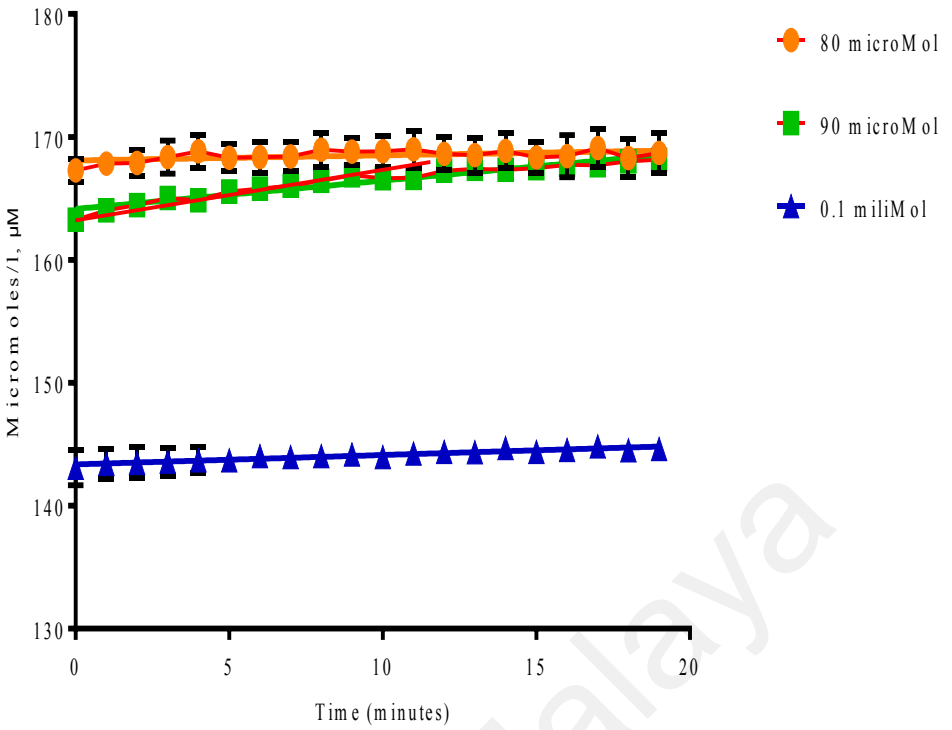
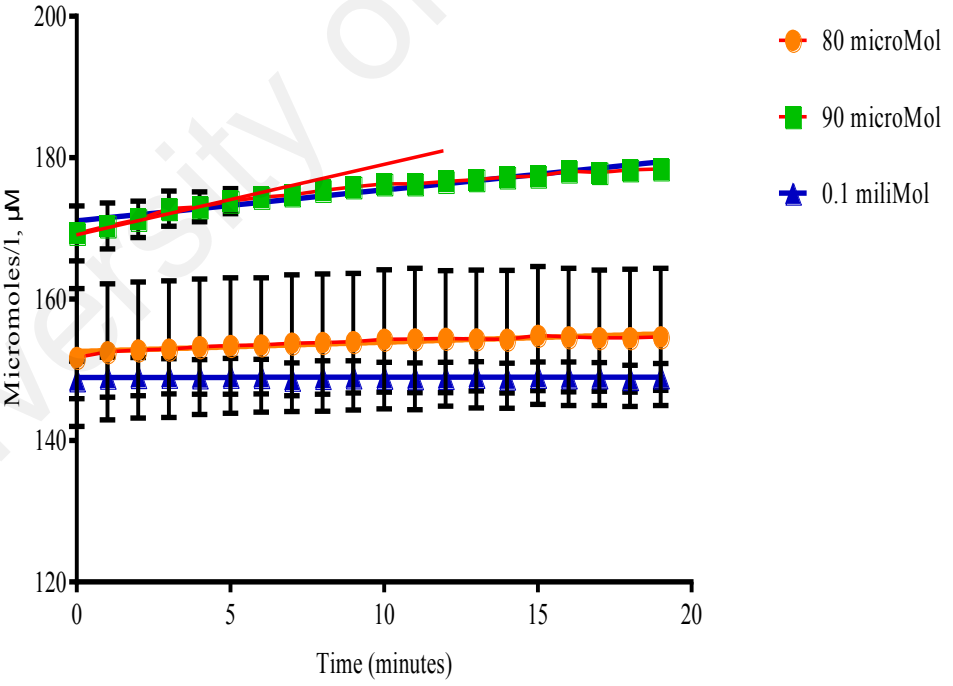
b) 80 µM and 0.1 mM have lower product formation and are not significantly different between each but 90 µM concentration has higher product formation and significantly different from the other two concentrations. At 90 µM H_2O_2 concentration, the product concentration is directly proportional by first order to time series from 0 until point 6 and after reaching the plateau. The other concentrations 80 µM and 0.1 mM, are not significantly different against time series.

c) 80 µM and 0.1 mM have lower product formation and are not significantly different between two. At 90 µM H_2O_2 from time point 0 until 8 minutes are overlapped with the other two concentration and not significantly different.

Meanwhile concentration is directly proportional by first order to time series from point 9 to 20 minutes. The other concentration 80 μM and 0.1 mM, are tend to be not significantly different against time series.

d) 80 μM and 90 μM have higher product formed and were not significantly different between the two and 0.1 mM concentration significantly different although has lower product formation. All the three concentrations are statistically not significant by time series because there is no increasing in product formation by time.

The total product formation for different hydrogen peroxide concentrations are shown at Appendix 2.

(a) 0.005 µM KBr	
(b) 0.04 µM KBr	

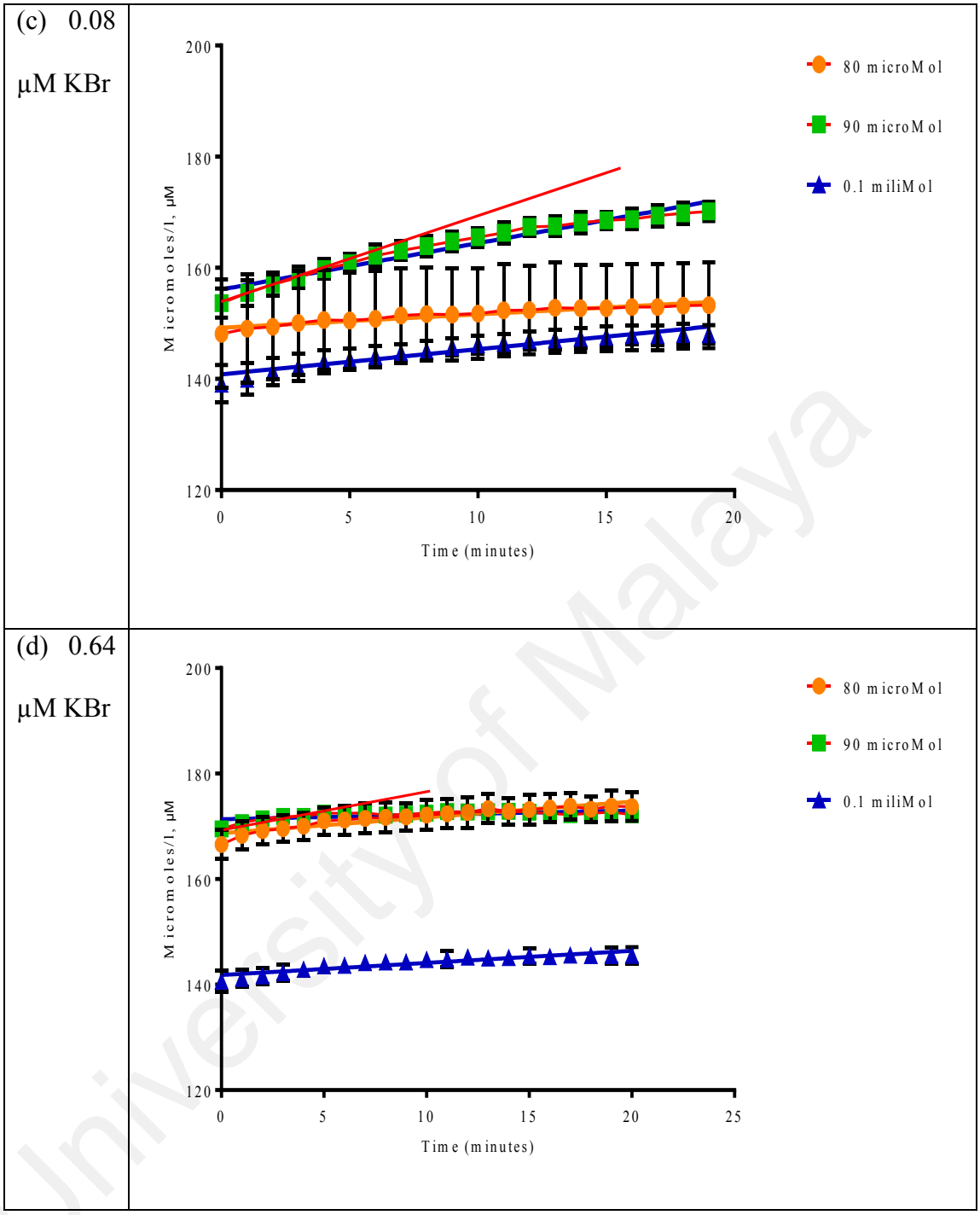


Figure 4.2: Product concentration against time series of enzymatic reaction for three different hydrogen peroxide concentrations ranging from 80 µM, 90 µM and 0.1 mM.

These three different concentrations are treated with selected increasing concentration of substrate potassium bromide (KBr) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

(b) **Comparison of product concentration for different H_2O_2 concentrations at selected substrate (KBr) concentration**

Based on the Figure 4.3, the reaction gives highest product with linear reaction at the concentration of 90 µM especially when the substrate was provided at the suitable concentration compared to the other two concentrations, 80 µM and 0.1mM.

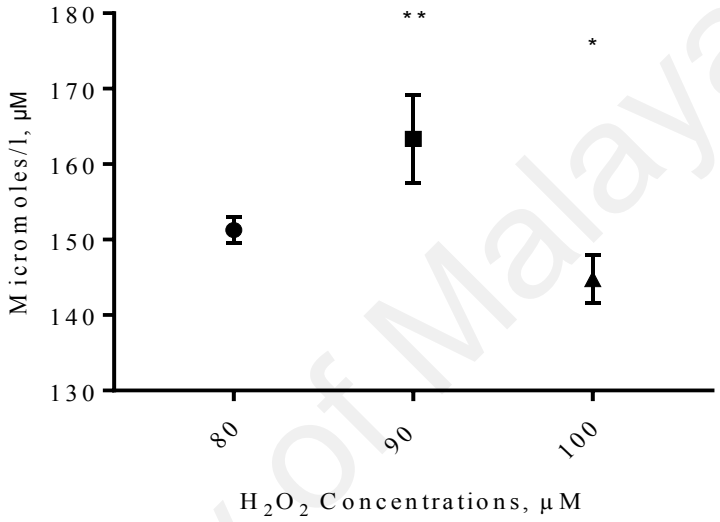


Figure 4.3: Crude extracts added by different hydrogen peroxide concentrations, 80, 90 µM and 0.1 mM at the selected substrate (KBr) concentration, 0.08 µM.

Data are the means of three independent experiments, with standard errors represented by vertical bars. The enzymatic reaction gives slightly highest product when treated with 90 µM H_2O_2 concentration and the other two concentration, 80 µM and 100 µM give low product concentration for 20 minutes reactions.

The graph (Figure 4.4) shows that the product concentration increases as the substrate concentration increases and highest when the addition of the substrate is at the concentration of 0.04 µM. The reaction dropped and reached plateau when the concentration are increased further.

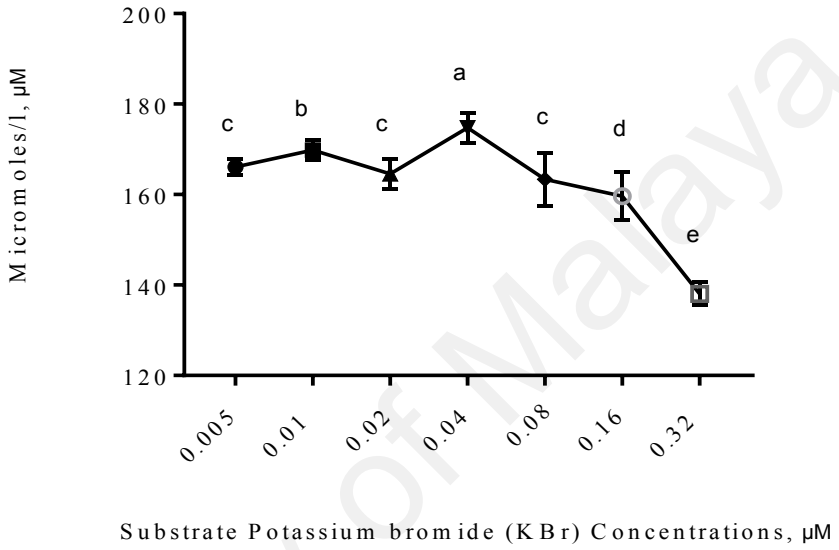


Figure 4.4: Product concentration of optimized H_2O_2 concentration, 90 µM against increasing substrate (KBr) concentration.

Data are the means of three independent experiments, with standard errors represented by vertical bars. Different letters denote a significant difference among the mean values of KBr at different concentrations. n = 3, ANOVA followed by Tukey's test.

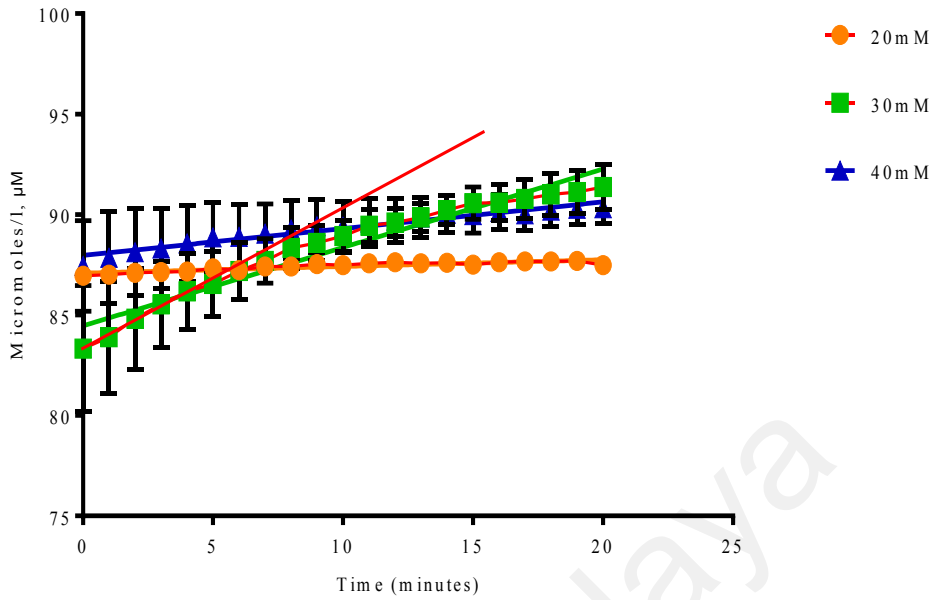
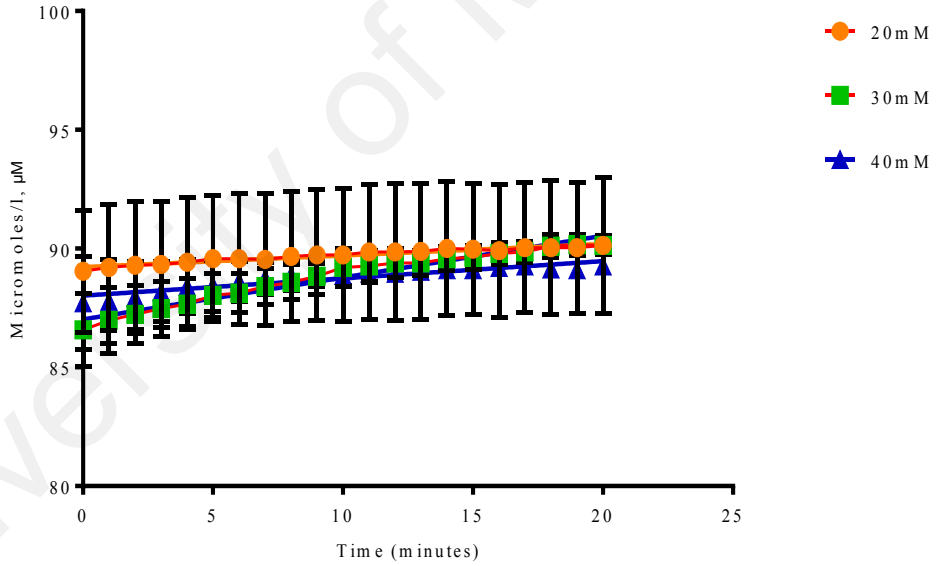
4.2.1.2 Effect of different concentration of Tris-HCl buffer, pH 8 added to the crude extracts for stability

(a) **Product concentration formation by introducing different Tris-HCl concentrations, at pH 8 using concentrations of substrate (KBr) from lowest (0.005 µM) to medium (0.04, 0.08 µM) and highest (0.64 µM).**

The concentration of the product plotted against time for the enzymatic reaction. From the graph (Figure 4.5);

a) 20, 30 and 40 mM have no differences in the product formation because are not significantly different among the concentrations. 30mM is significantly different in comparison with the other two concentrations by time series. At 30mM there are product formation increasingly for a period of time point 0 to 5 and then start to attained saturations.
b) 20, 30 and 40 mM have no differences in the product formation because are not significantly different among the concentrations and also against time series.
c) 20, 30 and 40 mM have no differences in the product formation because are not significantly different among the concentrations and also against time series.
d) 20 and 40 mM have no differences in the product formation except for 30mM although there are no significant differences for the concentration against time series. 30mM has higher product formation compared to the other.

The total product formation for different Tris-HCl buffer concentrations are shown at Appendix 3.

(a)0.005 µM KBr	
(b)0.04 µM KBr	

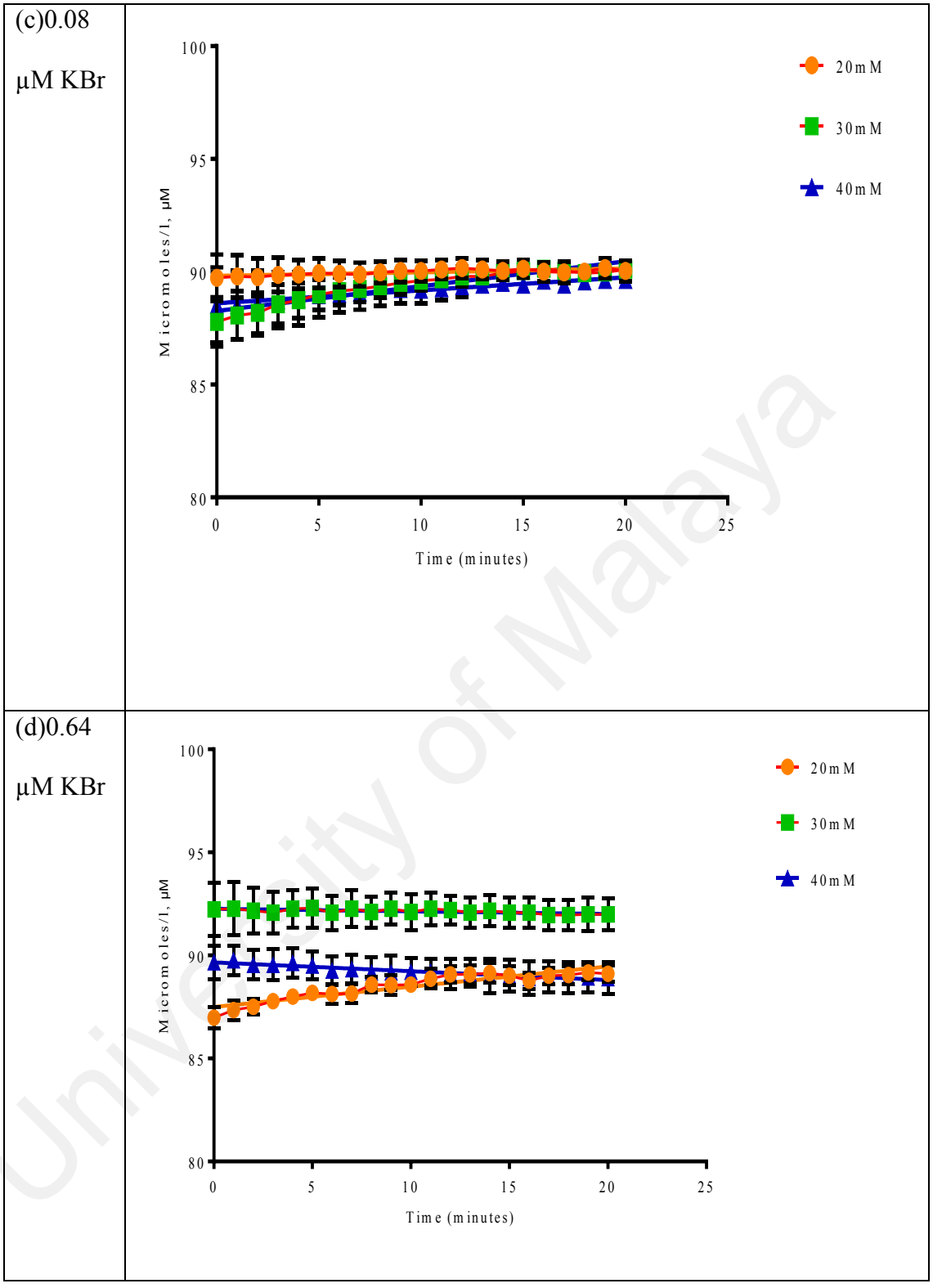


Figure 4.5: Product concentration formation against time series of enzymatic reaction for three different concentration of Tris-HCl buffer ranging from 20, 30 and 40 mM were added to the crude for the stability purpose.

These three different concentration are treated with selected increasing concentration of substrate potassium bromide (KBr) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08

µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

(b) **Comparison of product concentration for three different concentrations of Tris-HCl, at pH 8 at selected substrate concentration**

Based on the Figure 4.6, the reaction gives high product concentration at 30mM especially by introducing selected substrate concentration compared to the other two concentrations, 20 and 40 mM.

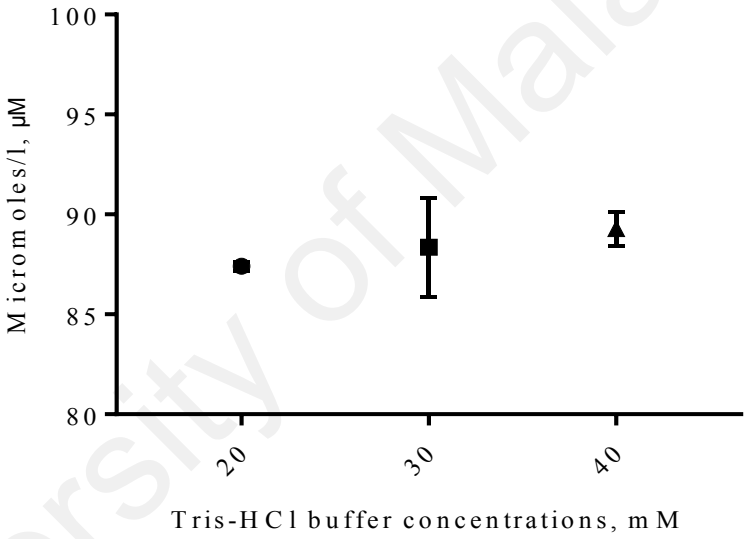


Figure 4.6: The crude extracts added with different Tris-HCl buffer concentrations, 20, 30 and 40 mM at selected substrate (KBr) concentration, 0.005 µM.

Data are the means of three independent experiments, with standard errors represented by vertical bars. Although the reaction shows similar to all the three concentrations but appeared linear, only for 30mM Tris-HCl, pH 8 buffer concentrations at a specified substrate concentration

The line graph (Figure 4.7) shows that the product concentration increases as the substrate concentration increases and highest when the addition of the substrate is at the concentration of 0.16 µM although not significantly different with 0.08 µM. The reaction dropped and reached plateau when the concentration are increased further.

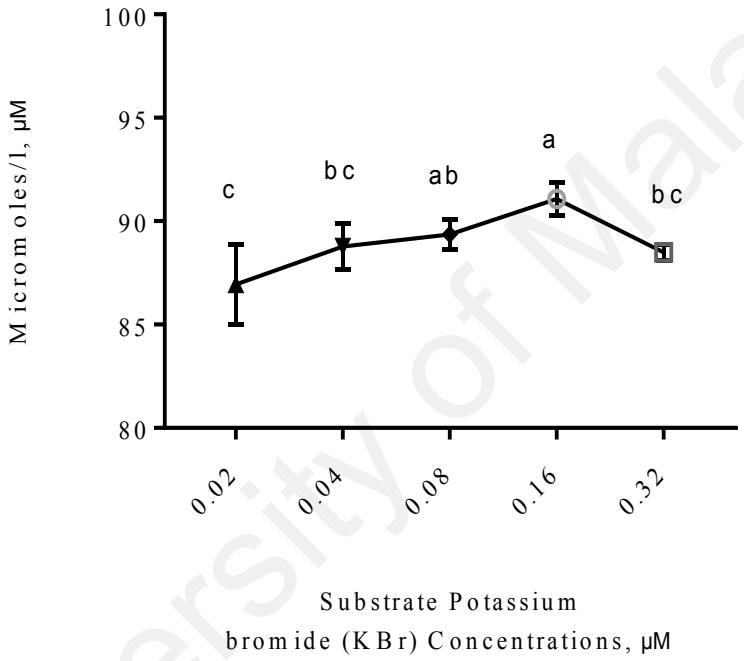


Figure 4.7: Product concentration for optimized 30 mM Tris HCl, pH 8 against increasing substrate concentration.

Data are the means of three independent experiments, with standard errors represented by vertical bars. Different letters denote a significant difference among mean the values of KBr at different concentrations. n = 3, ANOVA followed by Tukey's test.

4.2.1.3 Effect of stabilizing buffer towards reaction mixture during assay

(a) Product concentration formation by introducing two different buffers, Tris-HCl and phosphate buffer for comparison.

Two different buffers with various concentrations and pH were treated to the crude extracts by adding KBr, substrate concentrations increasingly.

The concentration of the product plotted against time for the enzymatic reaction. From the graph (Figure 4.8; 4.9; 4.10):

a) Although 20 and 30 mM have high product formation but are not significantly different between the concentrations. 40 and 50 mM were significantly different in comparison with the other two concentrations and also by time series.

b) 20 and 40 mM have no differences in the product formation but 30 and 50 mM significantly different with the other two concentrations. 30mM has the high product formation. There are no significant differences for all the concentrations against time series.

c) 20, 30 and 40 mM have no differences in the product formation because are not significantly different among the concentrations but 50mM significantly different from the other concentrations with low product formation. All the concentration are not significantly different against time series.

d) 20 and 40 mM although have high products but not significantly different between them. 30 and 50 mM were significantly different with low product formation. All the concentrations were not significantly different against time series.

e) Phosphate buffer with pH 7.8, 7.4, 8.4, 7 by order have high product formation but were not significantly different in comparison with pH 8 has lower product.

f) Phosphate buffer with pH 7.8, 7.6, 7.4, 7 and 8.4 by order have high product formation but were not significantly different in comparison with pH 8 has lower product.

g) Phosphate buffer with pH 7.8, 8.4, 7.4, 7.6, 7 by order have high product formation but were not significantly different in comparison with pH 8 has lower product.

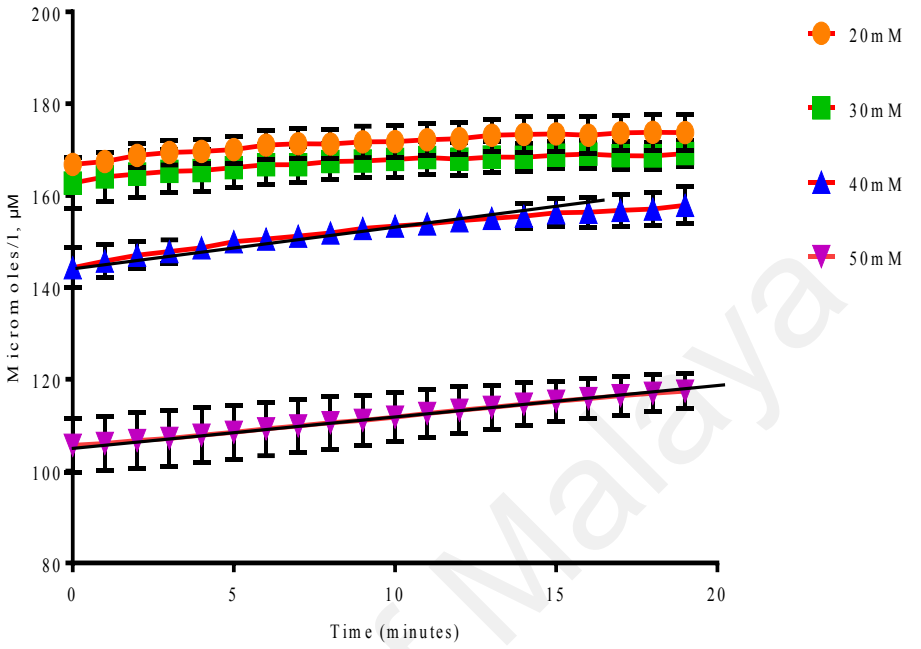
h) Phosphate buffer with pH 7.4, 7.8, 8.4, and 7 by order have high product formation but were not significantly different in comparison with pH 8 has lower product.

i-l) 40mM Tris-HCl and phosphate buffer pH 7.8 were not significantly different from each other. 50mM Tris-HCl buffer concentrations has lower product formation compared to others with significantly different.

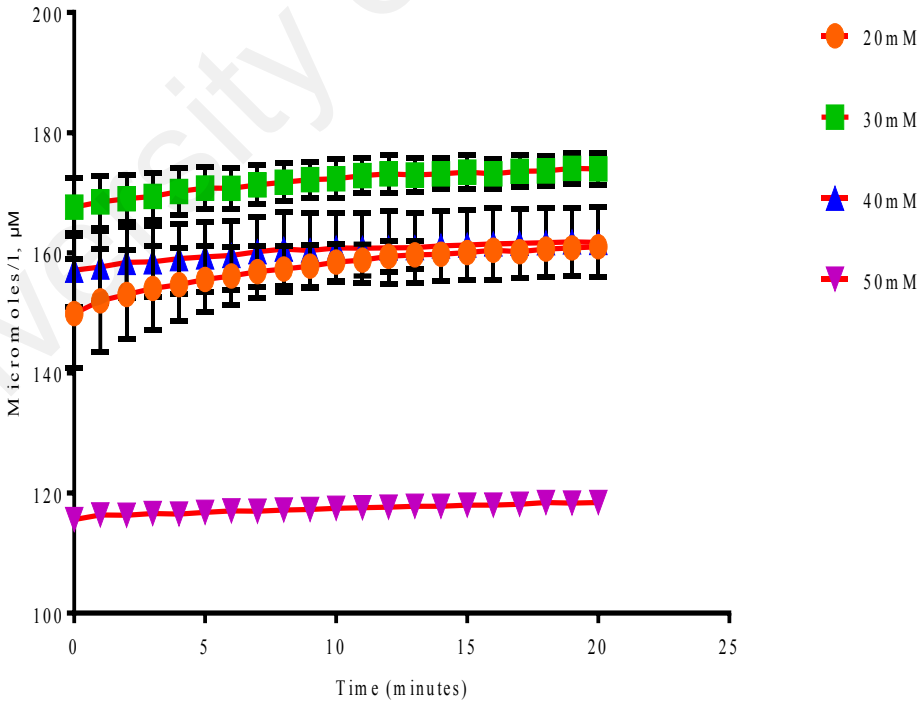
The total product formation for buffers with different concentrations and pH are shown at Appendix 4.

i **Effect of Tris-HCl, pH 8 buffer concentrations**

(a)0.005 µM KBr



(b)0.04 µM KBr



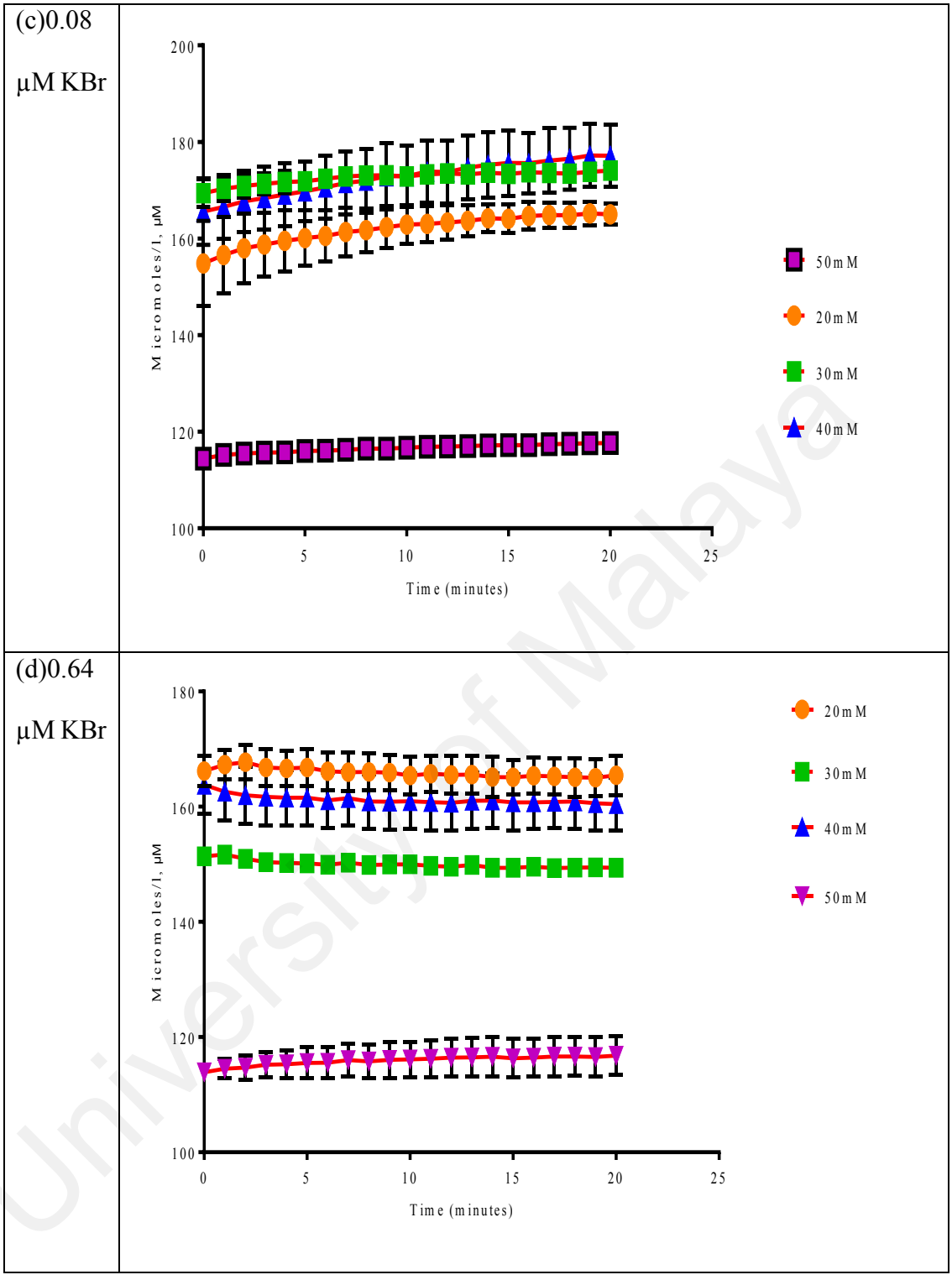
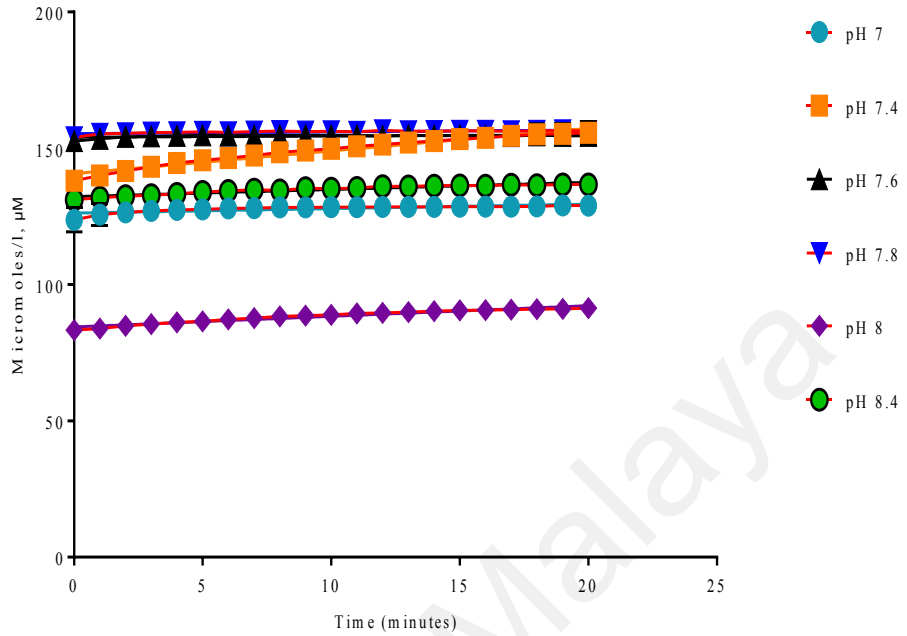
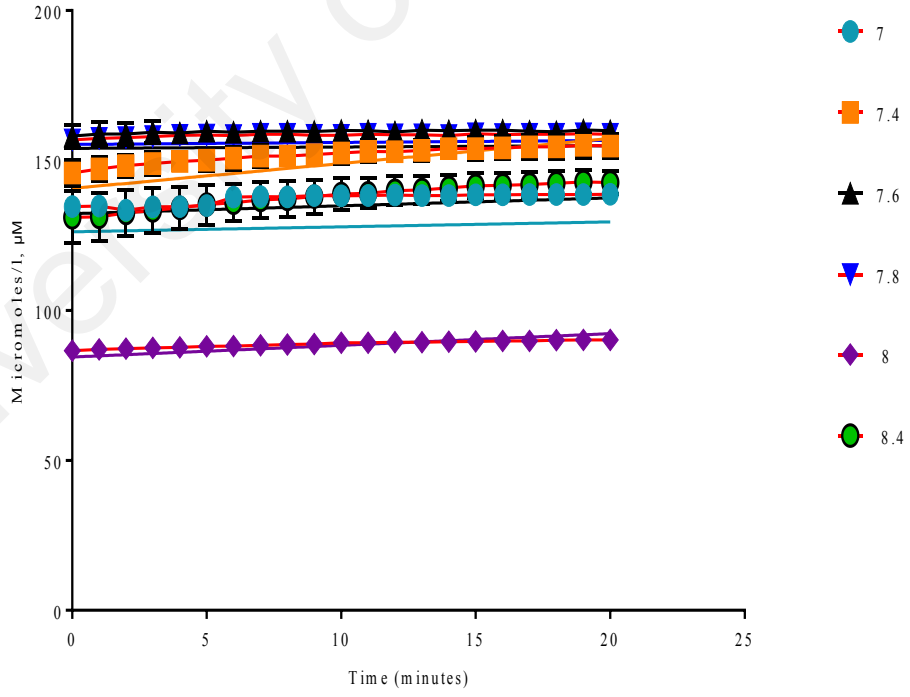


Figure 4.8: Product concentration formation against time series of enzymatic reaction for four different concentration of Tris-HCl, pH 8 buffer ranging from 20, 30, 40 and 50 mM were added to assay for the reaction.

These four different concentration are treated with selected increasing concentration of substrate potassium bromide (KBr) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

ii **Effect of different pH of 0.1mM Phosphate buffer**

(e)0.005 µM KBr	
(f)0.04 µM KBr	

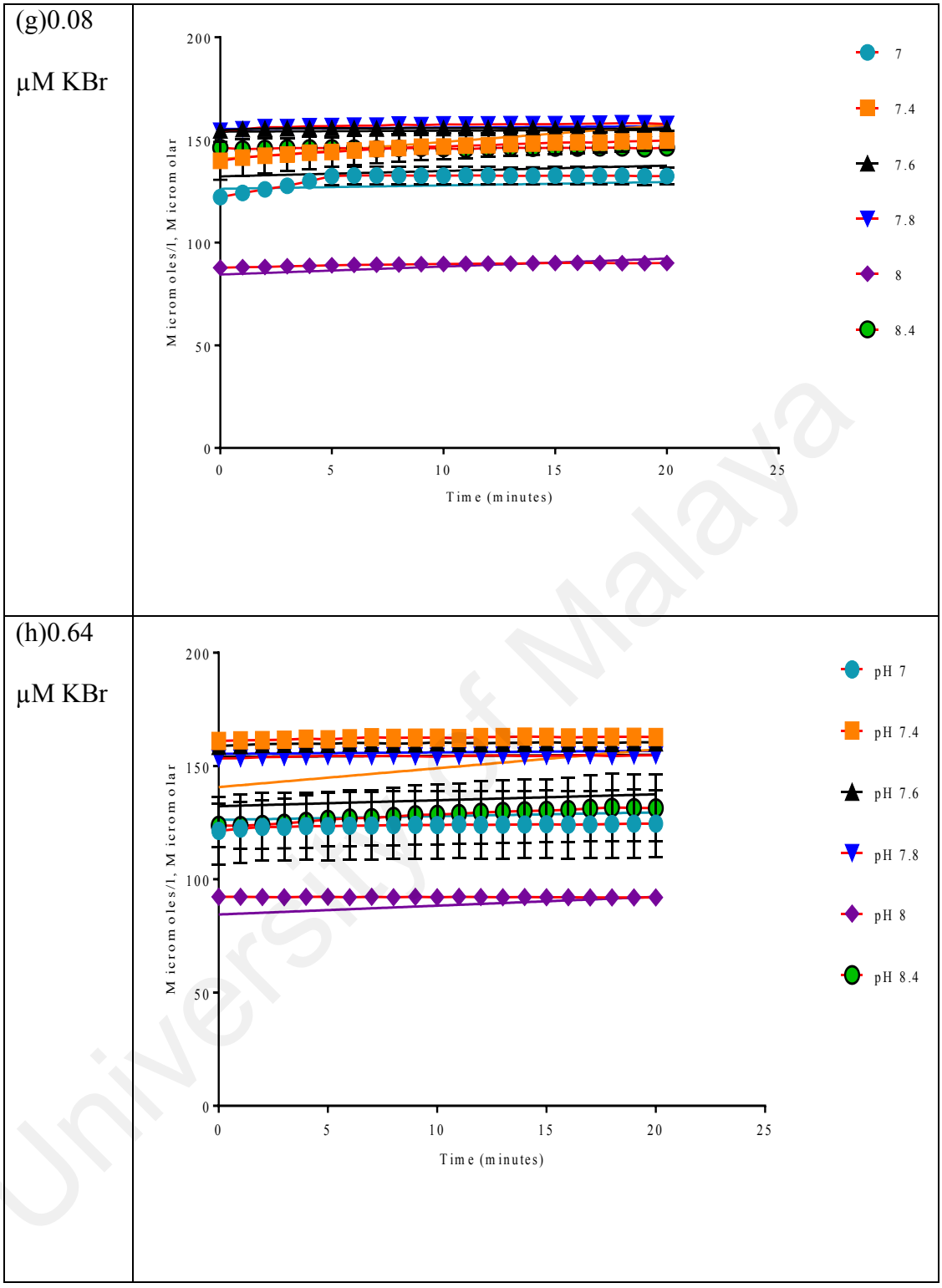


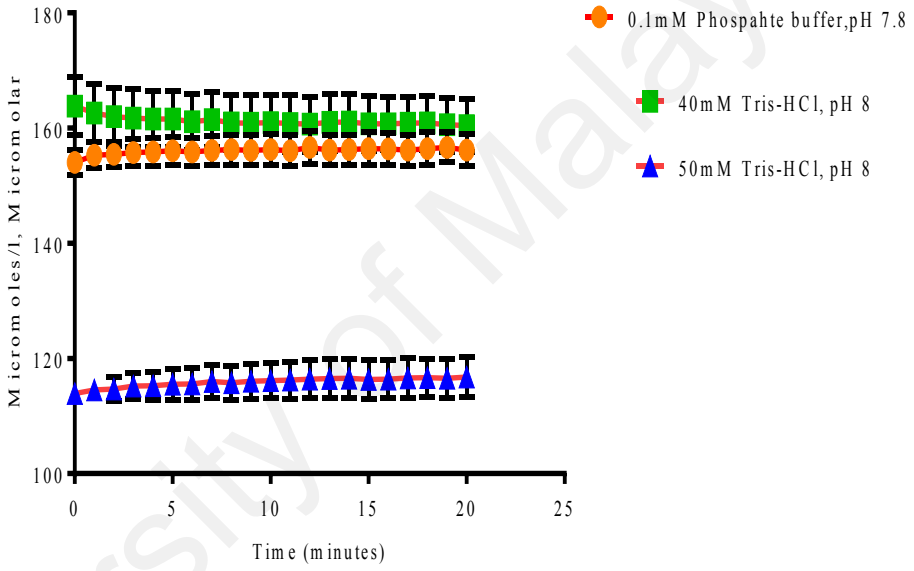
Figure 4.9: Product concentration formation against time series of enzymatic reaction for 0.1mM concentration of phosphate buffer, pH ranging from 7, 7.4, 7.6, 7.8 and 8.0 were added to assay for the reaction.

These different pH value buffers were treated with selected increasing concentration of substrate potassium bromide (KBr) during the assay, (a) 0.005 µM, (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

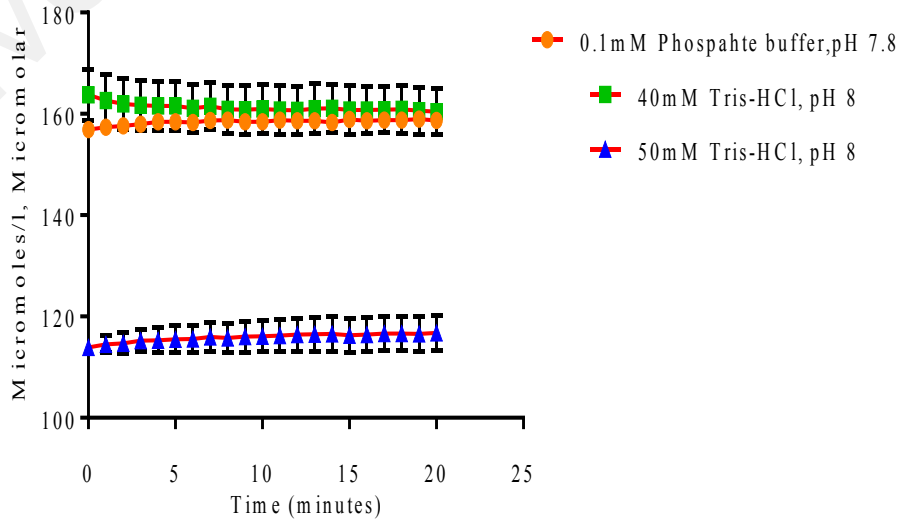
iii Comparison between effects of Trsi-HCl buffer, pH 8 (concentration) and 0.1mM phosphate buffer (pH)

Based on the graph from Figure 4.10, at 40mM concentration of Tris-HCl pH 8 the product concentrations increased and attained saturation compared to the other two concentrations. At 50mM Tris-HCl increasing substrates even at low concentration the product formation become limited or reduced.

(i)0.005 µM KBr



(j)0.04 µM KBr



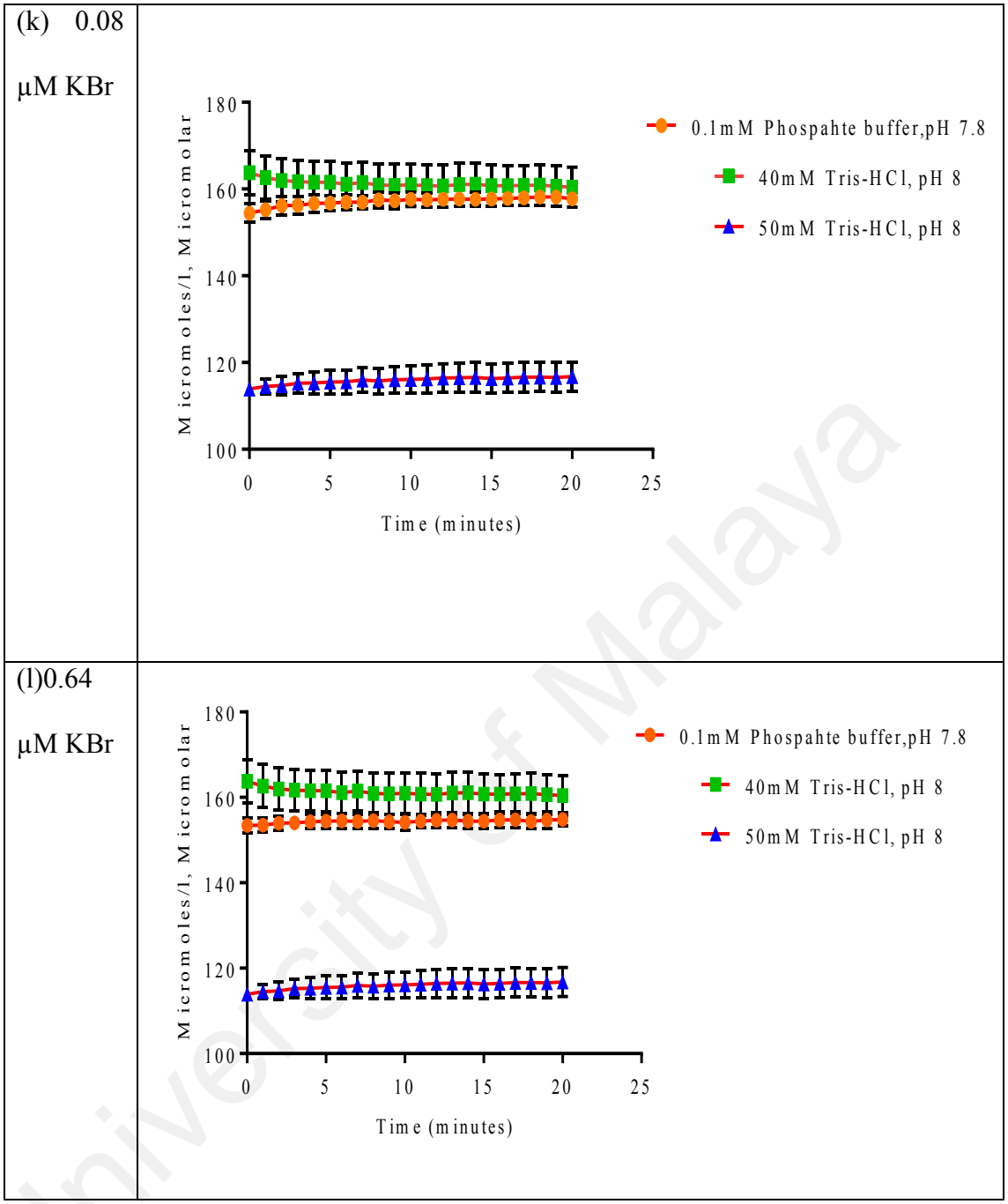


Figure 4.10: Product concentration formation against time series of enzymatic reaction, by comparing among optimized 0.1mM concentration of phosphate buffer pH 7.8 and Tris-HCl with concentrations of 40 and 50mM, pH 8 were added to assay for the reaction.

These buffers were treated with selected increasing concentration of substrate potassium bromide (KBr) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

iv **Different pH of 0.1mM phosphate buffer at selected substrate concentration**

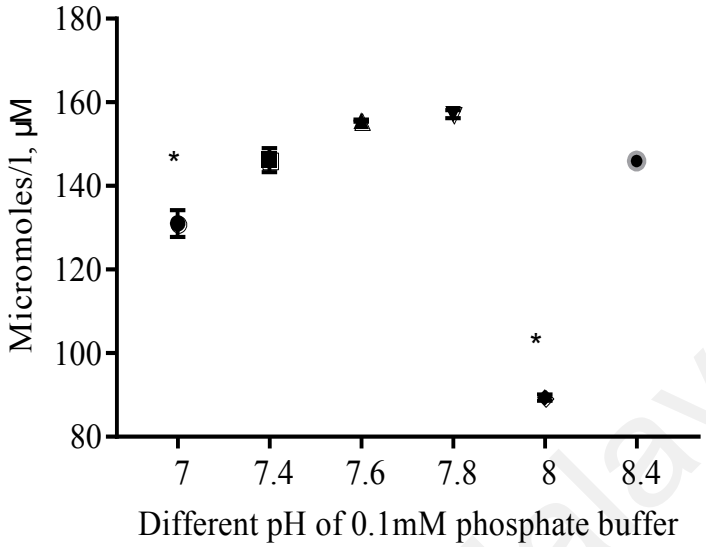


Figure 4.11: Activity assay were added by different pH of 0.1 mM phosphate buffer as the stabilizing buffer for the selected 0.08 µM KBr, substrate concentration.

Data are the means of three independent experiments, with standard errors represented by vertical bars. The reaction gives highest product when 0.1mM phosphate buffer of pH 7.6 and 7.8 were used for 20 minutes reactions. In comparison with all, pH 7 and 8 are significantly different and the rest are not.

v **Different concentrations of Tris-HCl, pH 8 buffer at selected substrate concentration**

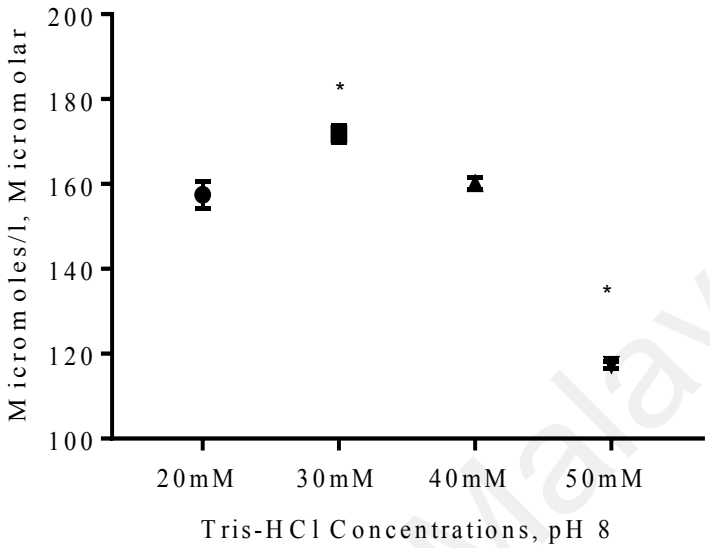


Figure 4.12: Activity assay were added by different concentrations of Tris-HCl, pH 8 as the stabilizing buffer for the selected 0.04 µM KBr, substrate concentration.

Data are the means of three independent experiments, with standard errors represented by vertical bars. The reaction at highest product when 30 and 40mM Tris-HCl pH 8 were used for 20 minutes reactions. Although 30mM has shown high product formation, at 40mM Tris-HCl, pH 8 product formation was increasing slightly linear.

(b) **Comparison between effect of Tris-HCl, pH 8 buffer and 0.1mM phosphate buffer,pH 7.8 at selected substrate concentration**

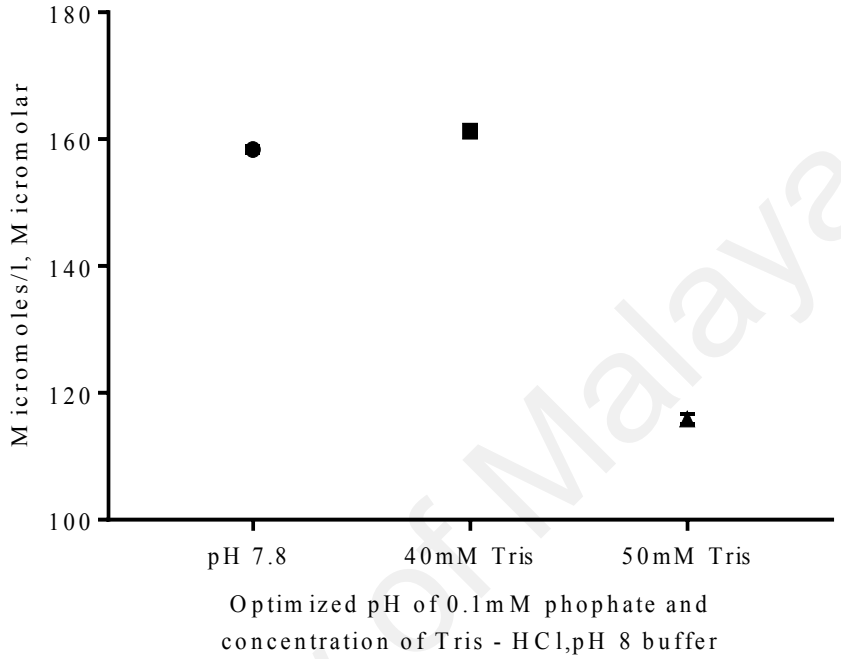


Figure 4.13: Activity assay were added by optimized Tris-HCl, pH 8 buffer and 0.1mM phosphate buffer,pH 7.8 as the stabilizing buffer for comparison.

Selected 0.04 μM KBr, substrate concentration were used to determine which buffer has high product formation. Data are the means of three independent experiments, with standard errors represented by vertical bars. The reaction gives highest product at 40mM Tris-HCl pH 8 for 20 minutes reactions. Data are not significantly different.

The line graph (Figure 4.14) shows that the formation of product concentration increases as the substrate concentration increases and highest when the addition of the substrate is at the concentration of 0.08 µM. The reaction dropped and reached plateau when the concentration are increased further.

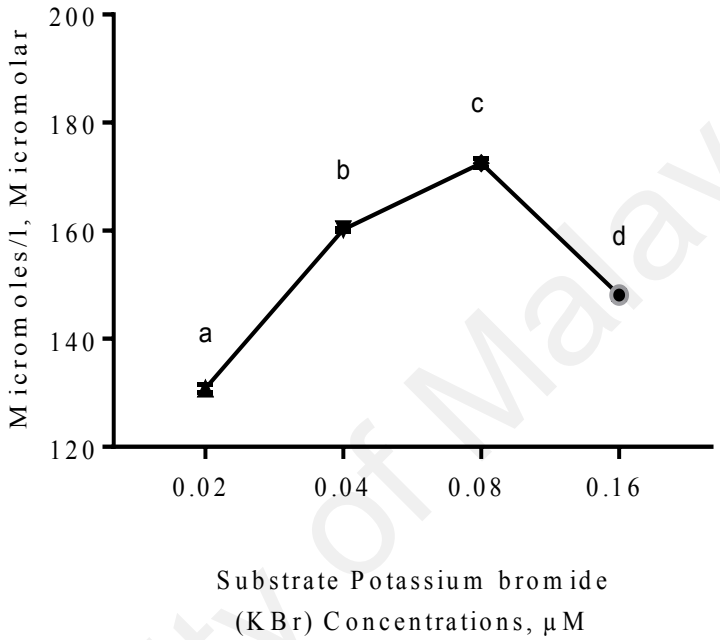


Figure 4.14: Product concentration formation for selected 40mM Tris-HCl, pH 8 buffer against increasing substrate concentration.

Data are the means of three independent experiments, with standard errors represented by vertical error bars. Different letters denote a significant difference between mean values across concentrations. n = 3, ANOVA followed by Tukey's test.

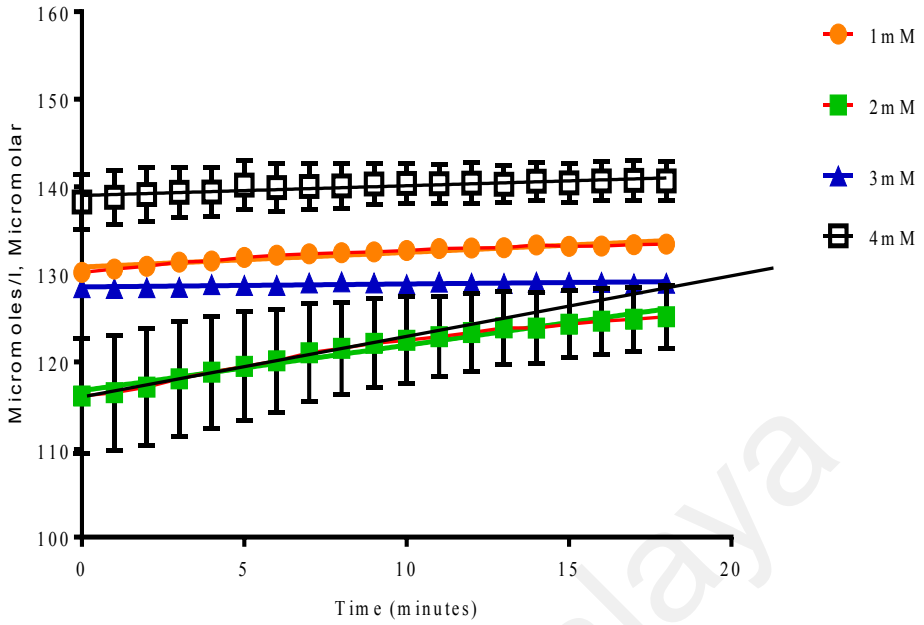
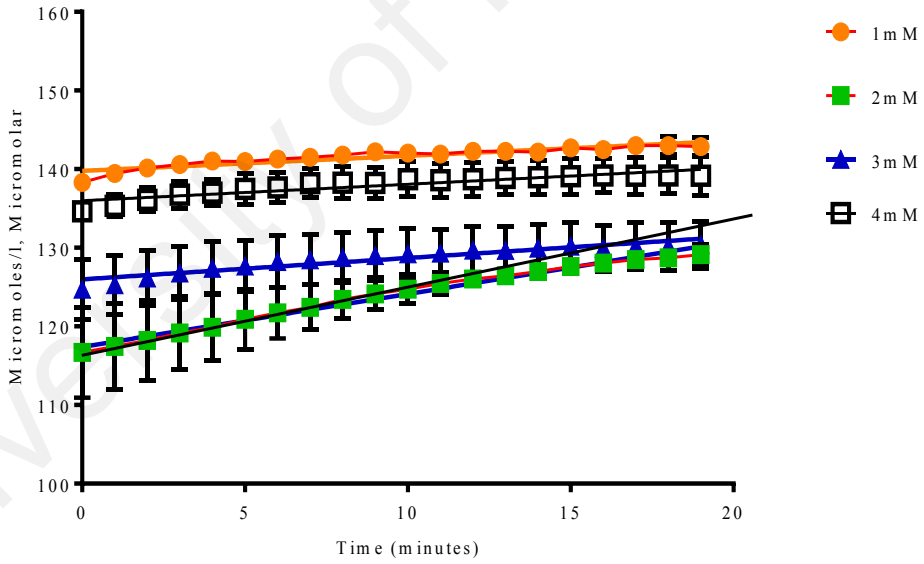
4.2.1.4 Effect of vanadium concentration on crude extracts activation

(a) Product concentration formation at different concentration of substrate KBr to the crude extracts incubated with different concentration of vanadium

The concentration of the product plotted against time for the enzymatic reaction. From the graph (Figure 4.15)

a) 2mM has linear product formation compared to 1, 3 and 4 mM concentration. All the three concentrations are not significantly different by time series except 2mM concentration. There is an increasing product formation across time for the 2mM concentration.
b) All the three concentrations are not significantly different by time series except 2mM concentration. There is an increasing product formation across time for the 2mM concentration.
c) All the three concentrations are not significantly different by time series except 2mM concentration. There is an increasing product formation across time for the 2mM concentration.
d) The 1 and 4 mM concentrations are not significantly different in comparison with 2 and 3 mM yet there are no differences across time being for all the concentrations.

The total product formation for different concentrations of vanadium were calculated in Appendix 5.

(a) 0.005 µM KBr	
(b) 0.04 µM KBr	

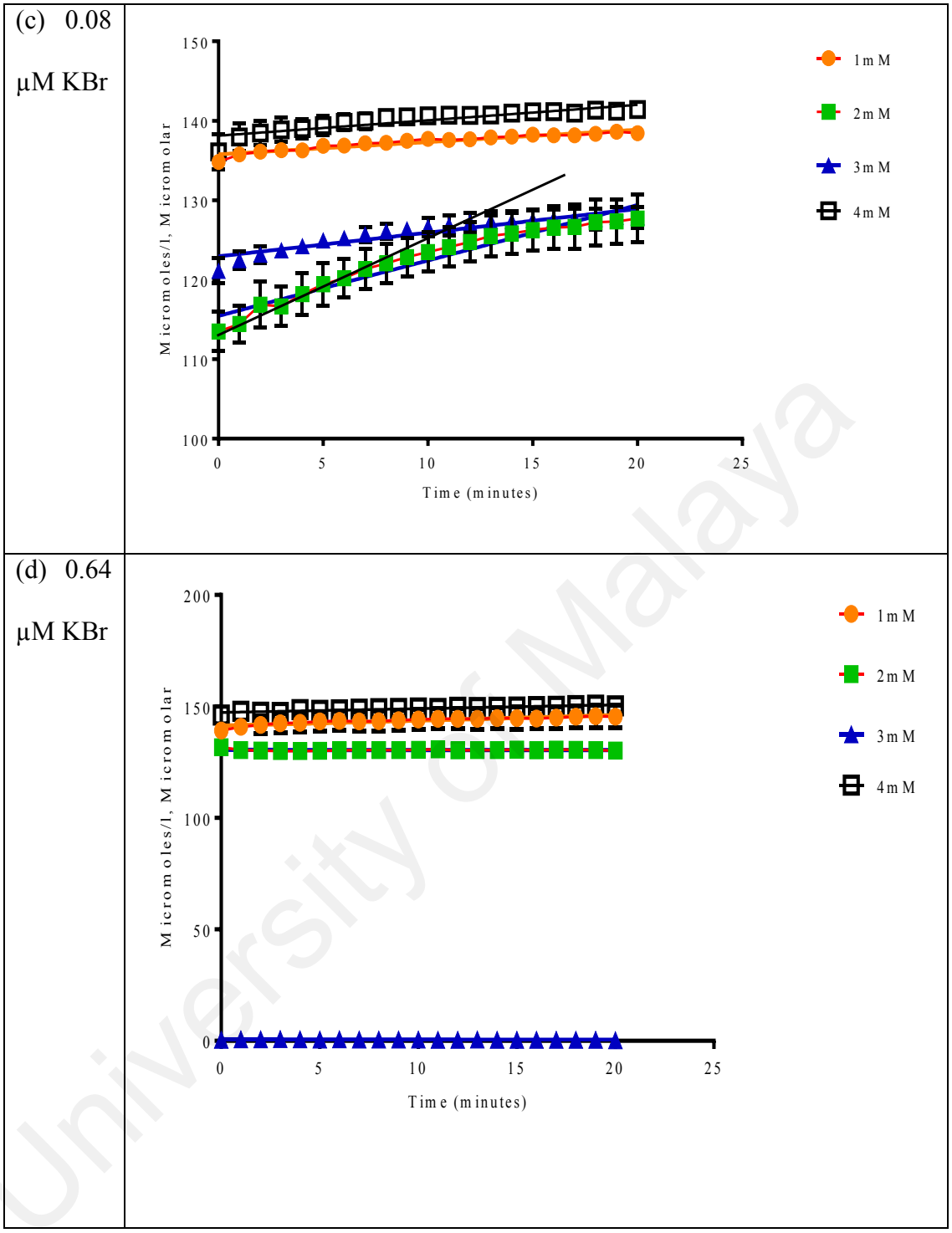


Figure 4.15: Product concentration formation against time series of enzymatic reaction for four different vanadium concentration ranging from 1-4 mM.

These four different concentration are treated with selected increasing concentrations of substrate potassium bromide (KBr) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

(b) **Comparison of product concentration formation for different vanadium concentrations at selected substrate (KBr) concentration**

Based on the Figure 4.16, linear reaction formation at the concentration of 2 mM especially when the added substrate at the suitable concentration to give an increasing activity compared to the other three concentrations 1, 3 and 4 mM.

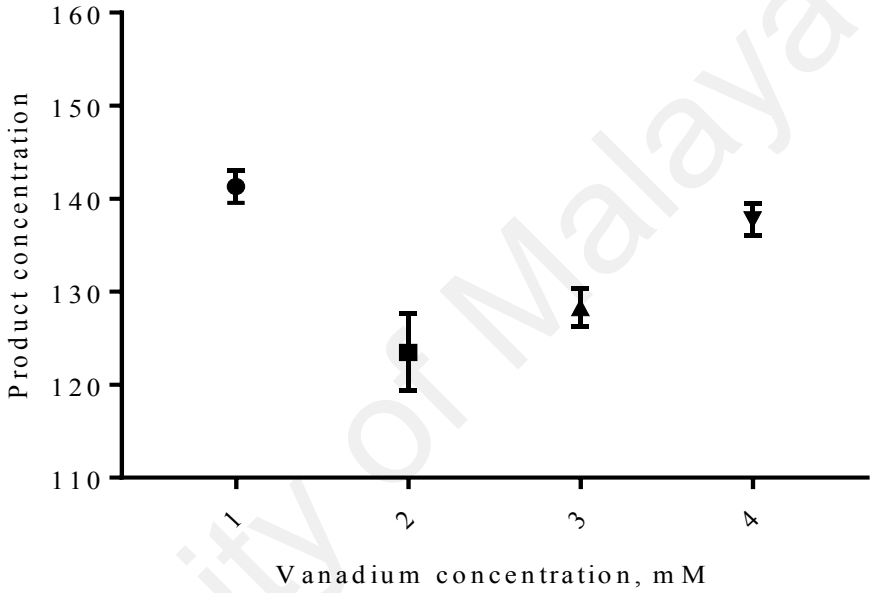


Figure 4.16: The crude extracts added with different vanadium concentrations, 1, 2, 3 and 4 mM were treated with selected substrate (KBr) concentration, 0.08 µM.

Data are the means of three independent experiments, with standard errors represented by vertical bars. The reaction gives highest product for crude activated using 1mM but reaction was not linear. Although 2mM has lowest activity yet provides stable linear reaction throughout 20 minutes reaction.

The line graph (Figure 4.17) shows that the product concentration increased at 0.04 µM concentration and reached plateu when the concentration are increased further after that.

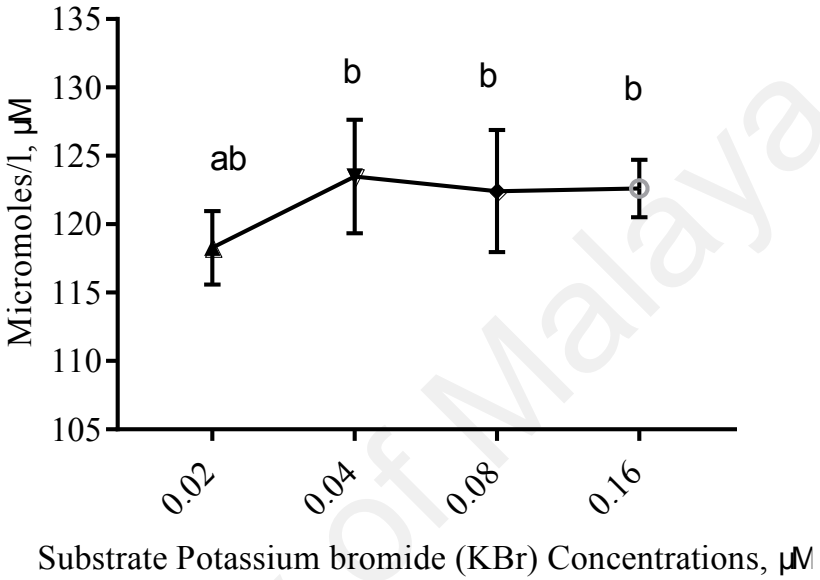


Figure 4.17: Product concentration formation by crude extracts activated using 2mM vanadium with an increasing of substrate (KBr) concentration.

Different letters denote a significant difference between different substrate concentrations. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3, ANOVA followed by Levene's test.

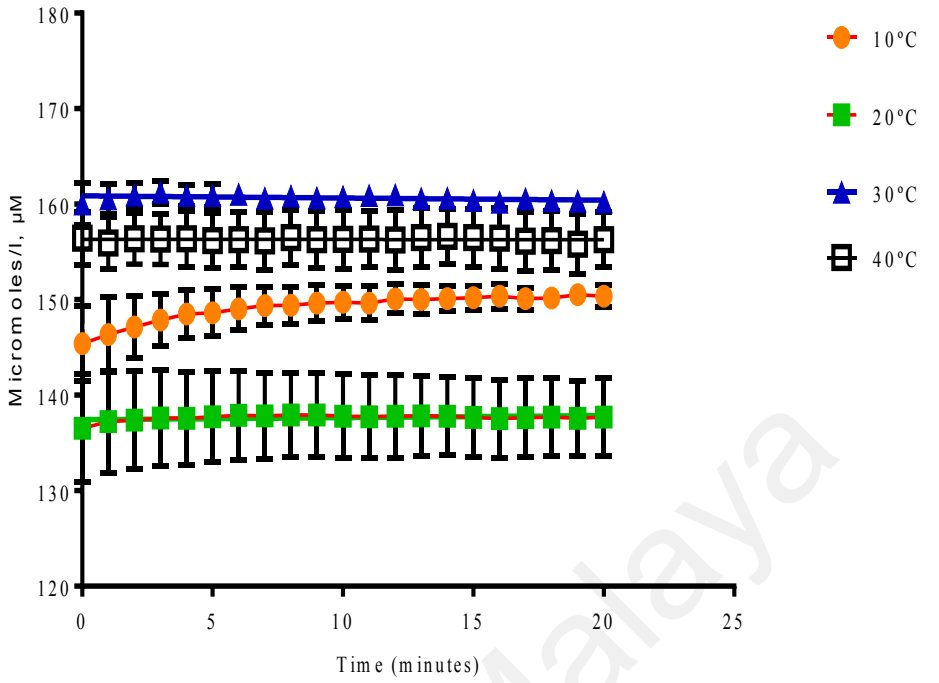
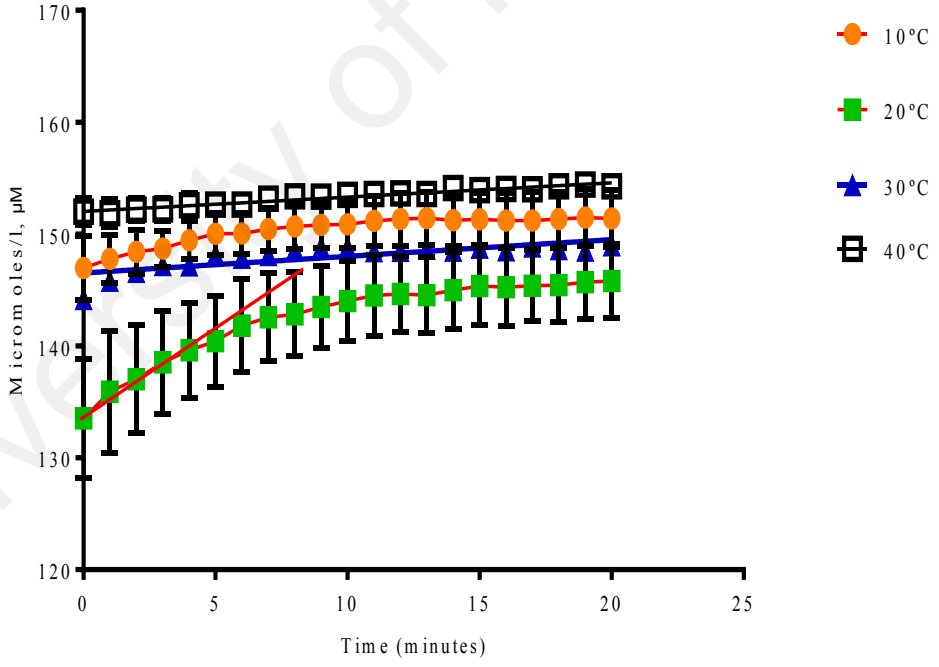
4.2.1.5 Effect of temperatures on enzyme activity.

(a) **Product concentration formation by crude extracts introduced to different temperatures at different substrate (KBr) concentrations.**

The concentration of the product plotted against time for the enzymatic reaction. From the graph (Figure 4.18);

a) 30 and 40 ºC were not significantly different although have higher product formation. Meanwhile 10 and 20 ºC have lower product formation. All the three temperatures 20, 30 and 40 ºC are not significantly different by time series.
b) Product formation at temperature 20 ºC is significantly different than other. Temperature 30 and 40 ºC were not significantly different. The temperature 10, 30 and 40 ºC were not significantly different by time series except 20 ºC. At temperature 20 ºC from 0-6 minutes there are significant differences but after minutes 7 the reaction reached saturations.
c) Product formed at temperature 10 ºC higher and significantly different from the rest but not significantly different across time. Temperature 20 and 40 ºC were not significantly different with lower product formation. All the four temperature were not significantly different by time series.
d) The product concentrations formed for all the four temperature were not significantly different with each other and also by across time being.

The total product formed for crude extracts exposed to different temperature are shown at Appendix 6.

(a)0.005 µM KBr	
(b)0.04 µM KBr	

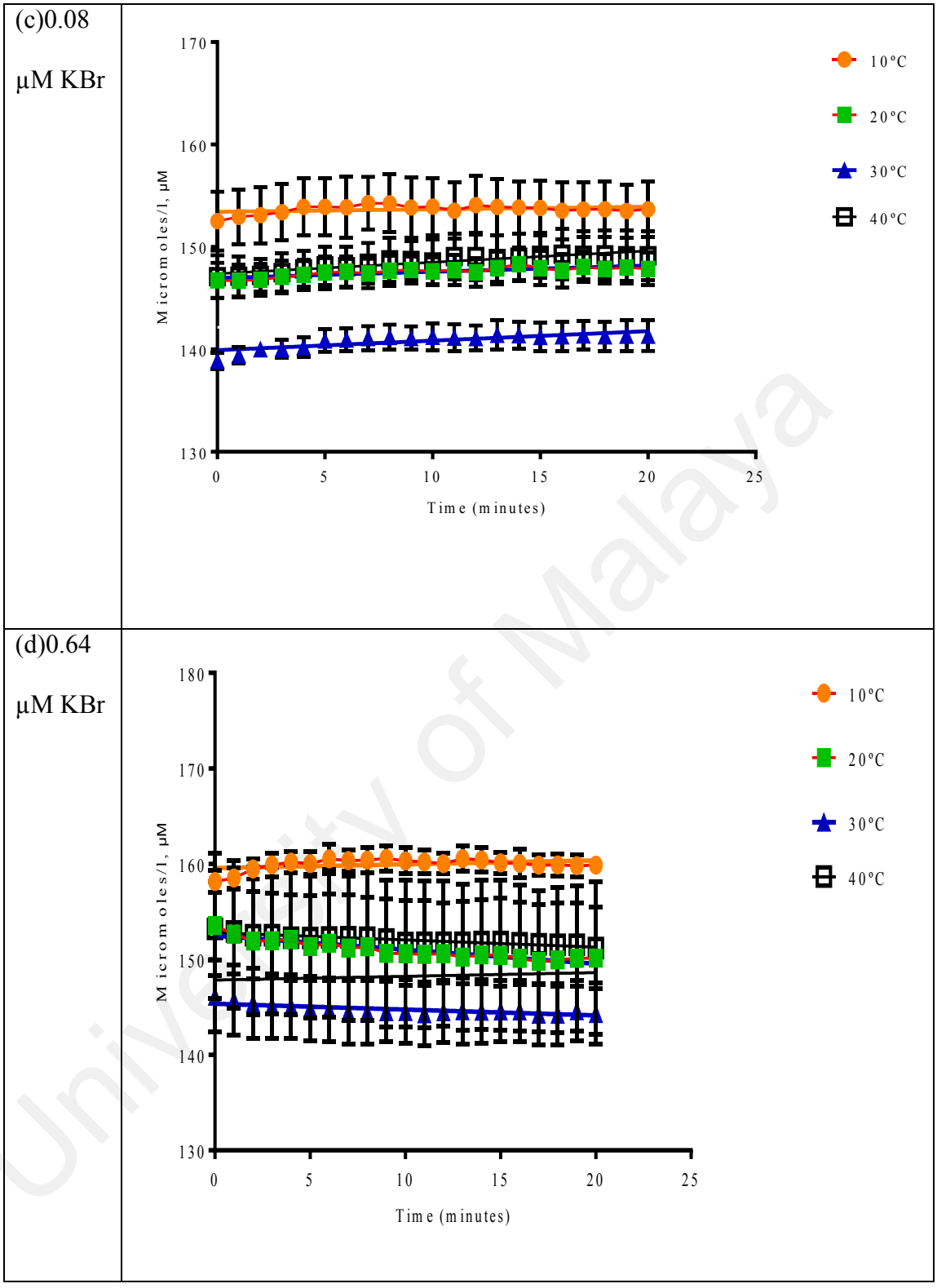


Figure 4.18: Product concentration formation against time series of enzymatic reaction for crude extracts exposed towards different temperatures ranging from 10, 20, 30, and 40 ºC.

These different temperatures exposed crudes were treated with selected increasing concentrations of substrate potassium bromide (KBr) during the assay, (a) 0.005 µM ,

(b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

(b) **Comparison of product concentration formation by crude extracts exposed to different temperatures at selected substrate (KBr) concentration**

Based on the Figure 4.19, the reaction gives linear and higher product formation at temperature 20 ºC especially at a specific substrate compared to the other three temperature output.

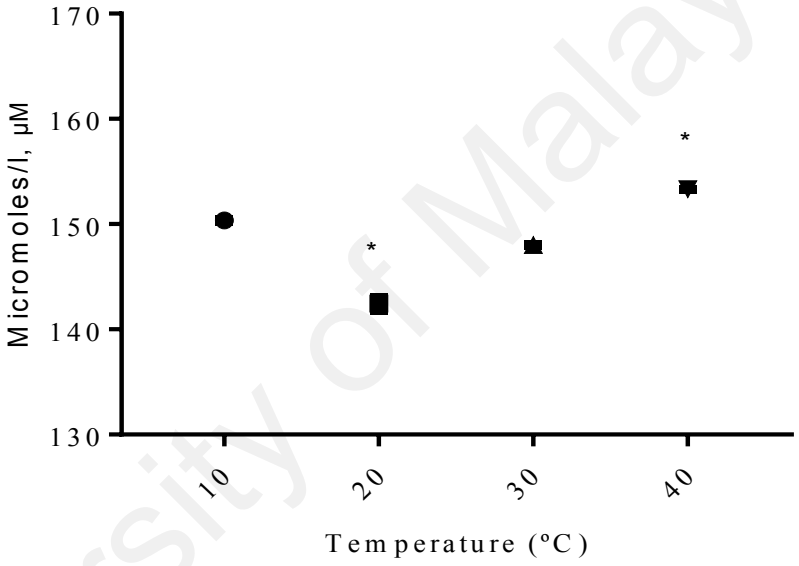


Figure 4.19: The crude extracts were exposed to different temperatures namely, 10, 20, 30 and 40 ºC were treated with selected substrate (KBr) concentration, 0.04 µM.

Data are the means of three independent experiments, with standard errors represented by vertical bars. Crude extracts exposed to 20 ºC has given linear reaction for 20 minutes reactions although have low product formation.

The line graph (Figure 4.20) shows that product concentration formation is highest at 0.04 µM concentration and reached saturation as the substrate concentration increases.

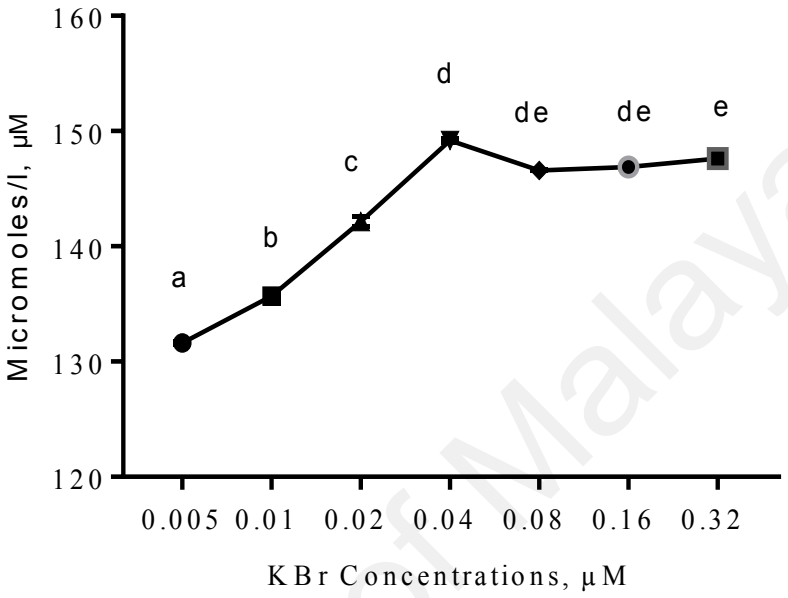


Figure 4.20: The effect of the KBr concentration toward the product concentration at 20 ºC.

Data are the means of three independent experiments, with standard errors represented by vertical bars. n = 3, ANOVA followed by Levene's test.

4.2.2 Potassium iodide (KI) as the substrate

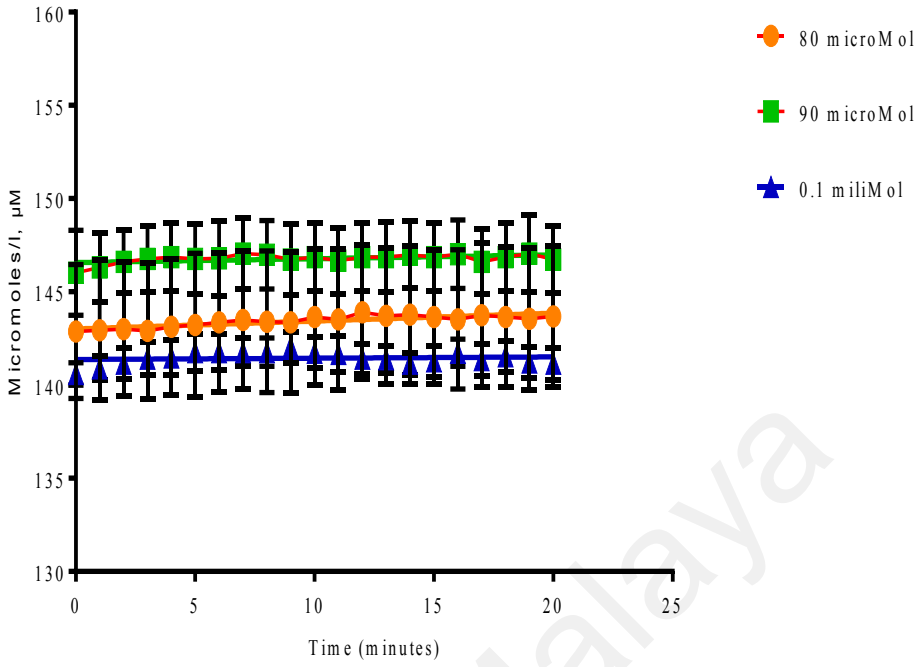
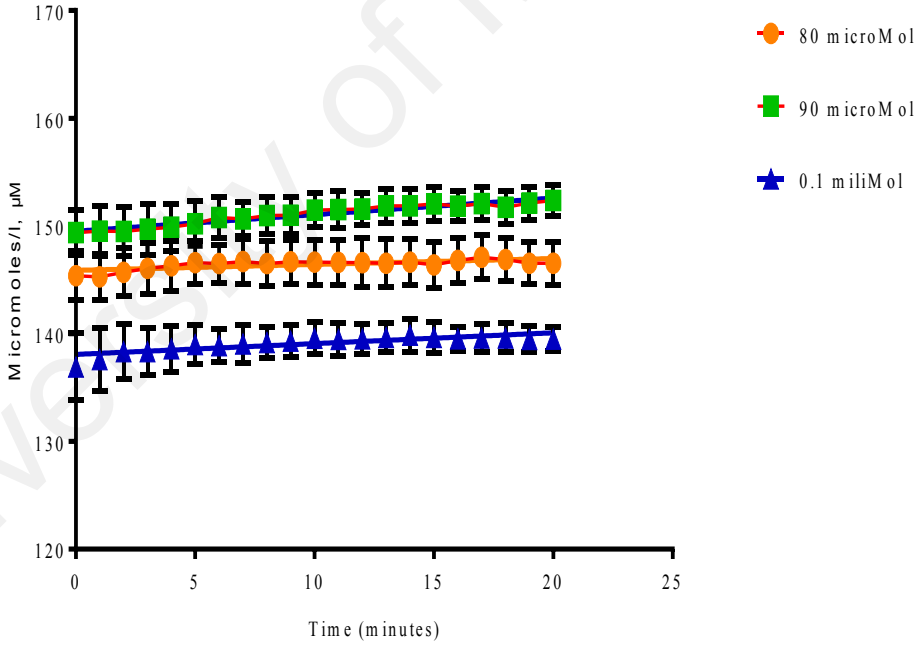
4.2.2.1 Effect of Hydrogen peroxide (H_2O_2) concentration on enzymatic assay

(a) **Product concentration formation by introducing three hydrogen peroxide concentrations using different concentration of substrate potassium iodide, KI from lowest (0.005 µM) to medium (0.04, 0.08 µM) and highest (0.64 µM).**

The concentration of the product plotted against time for the enzymatic reaction. From the graph (Figure 4.21);

a) 80 µM, 90 µM and 0.1mM have similar product formation but are not significantly different. All the three concentrations are also not significantly different by time series because no product formed by time.
b) 80 µM and 0.1 mM have lower product formation and 90 µM concentration has higher product formation and significantly different from the other two concentrations. The 80, 90 µM and 0.1 mM, are not significantly different against time series.
c) 80 µM and 0.1 mM have lower product formation and are significantly different between two. At 90 µM H_2O_2 have slightly higher product formation and significantly different. The concentrations 80, 90 µM and 0.1 mM, are tend to be not significantly different against time series.
d) 90 µM have higher product formation than 80 µM and 0.1 mM and are significantly different among each. All the three concentrations are statistically not significant by time series because no product formation by time.

The total product formation for different hydrogen peroxide concentration are shown at Appendix 7.

(a)0.005 µM KI	
(b)0.04 µM KI	

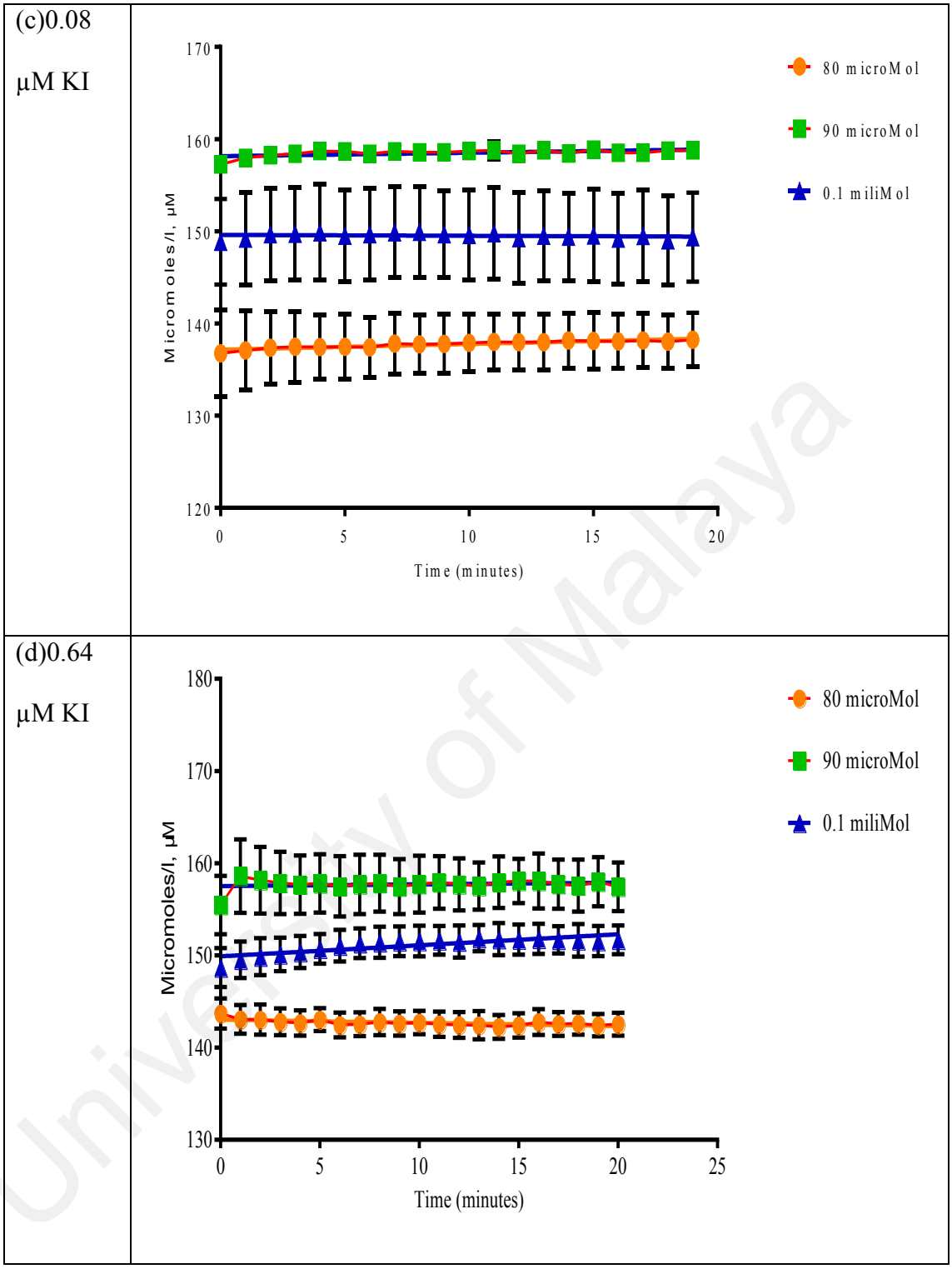


Figure 4.21: Product concentration formation against time series for three different hydrogen peroxide concentrations ranging from 80 µM, 90 µM and 0.1 mM.

These three different concentrations are treated with selected increasing concentration of substrate potassium iodide (KI) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

(b) **Comparison of product concentration for different H_2O_2 concentrations at selected substrate (KI) concentration**

Based on the Figure 4.21, the product concentration increases as the substrate concentration increases for 90 µM compared to the other two concentrations, 80 µM and 0.1 mM.

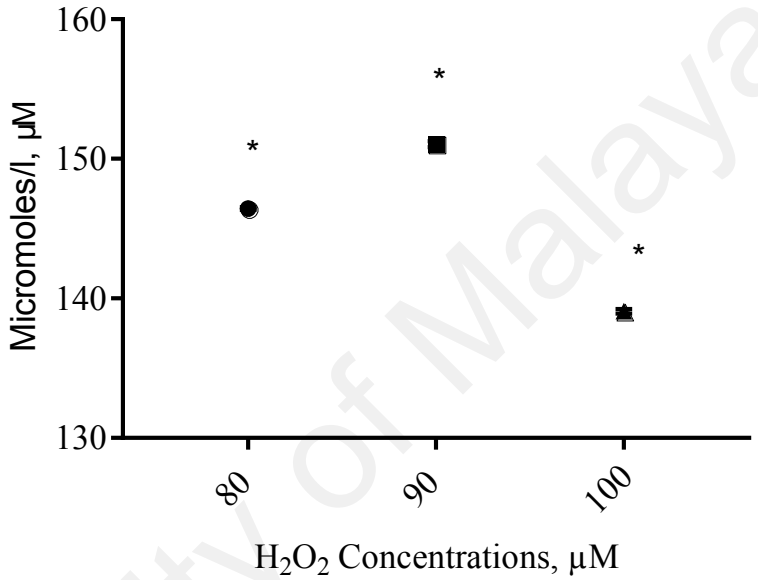


Figure 4.22: Crude extracts added by different hydrogen peroxide concentrations, 80, 90 µM and 0.1 mM at the selected substrate concentration, KI 0.04 µM.

Data are the means of three independent experiments, with standard errors represented by vertical bars. The enzymatic reaction gives highest product when treated with 90 µM H_2O_2 concentration and the other two concentration, 80 µM and 0.1 mM give low product concentration for 20 minutes reactions.

4.2.2.2 Effect of stabilizing buffer for the reaction mixture during assay

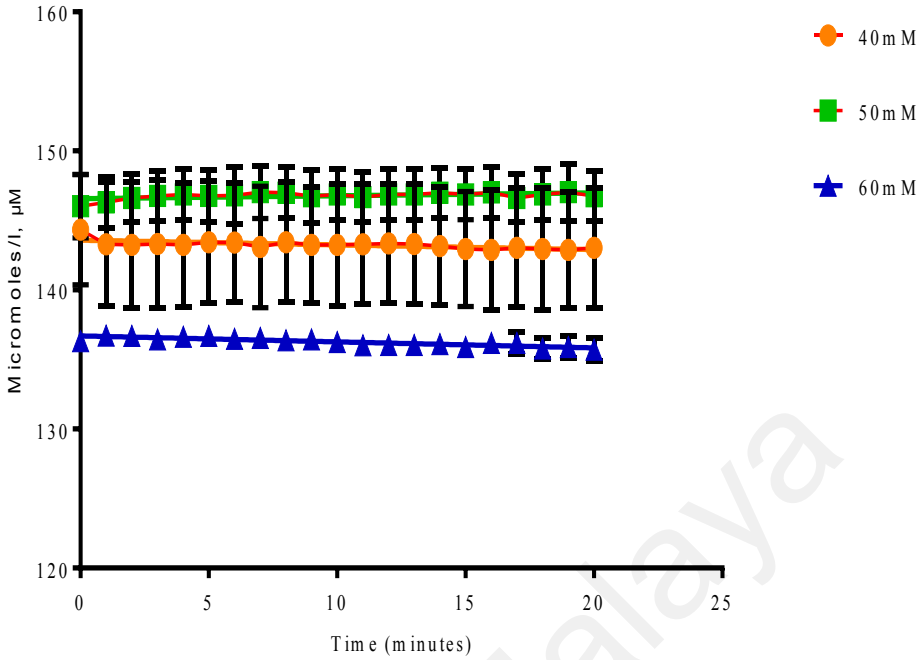
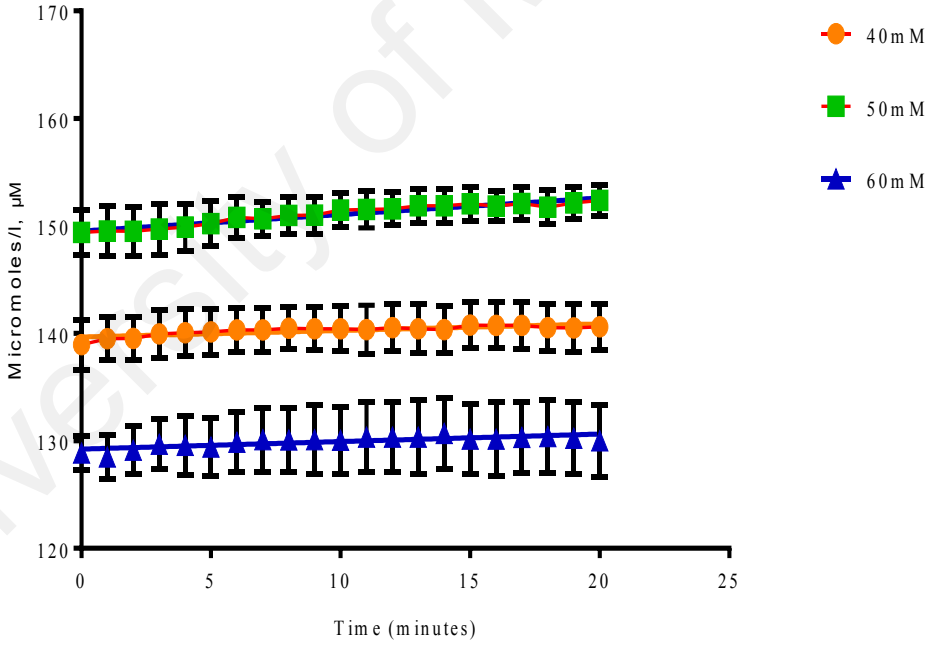
(a) Product concentration formation by introducing different concentration of Tris-HCl, pH 8 at various potassium iodide, KI concentration for optimization.

The concentration of the product plotted against time for the enzymatic reaction. From the graph (Fig 4.22);

a) 40 and 50 mM although have higher product formation but are not significantly different in comparison with 60mM which is significantly different. There are no significant differences for all the concentrations against time series

b-d) 40 and 60 mM have lower product compared to 50mM and are significantly different among each. There are no significant differences for all the concentrations against time series.

The total product formation for stabilizing buffers with different concentrations are shown at Appendix 8.

(a)0.005 µM KI	
(b)0.04 µM KI	

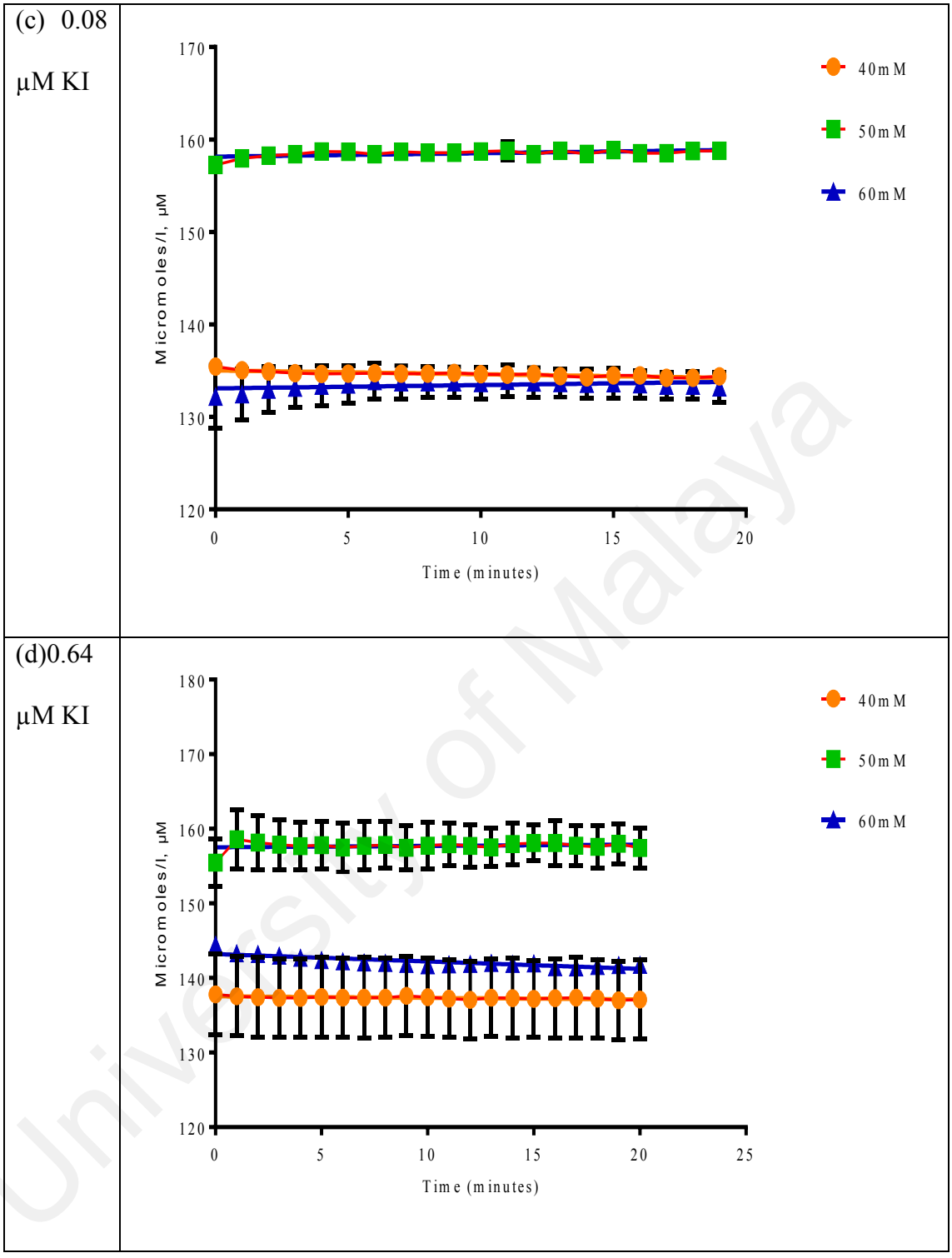


Figure 4.23: Product concentration against time series of enzymatic reaction for three different concentration of Tris-HCl buffer ranging from 40, 50 and 60 mM were added as the stabilizing buffer.

These three different concentrations are treated with selected increasing concentration of substrate potassium iodide (KI) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

(b) **Comparison of product concentration for different Tris-HCl concentrations, pH 8 at selected substrate (KI) concentration**

Based on the Figure 4.24, the reaction gives highest product at 50mM concentration compared with the other concentrations.

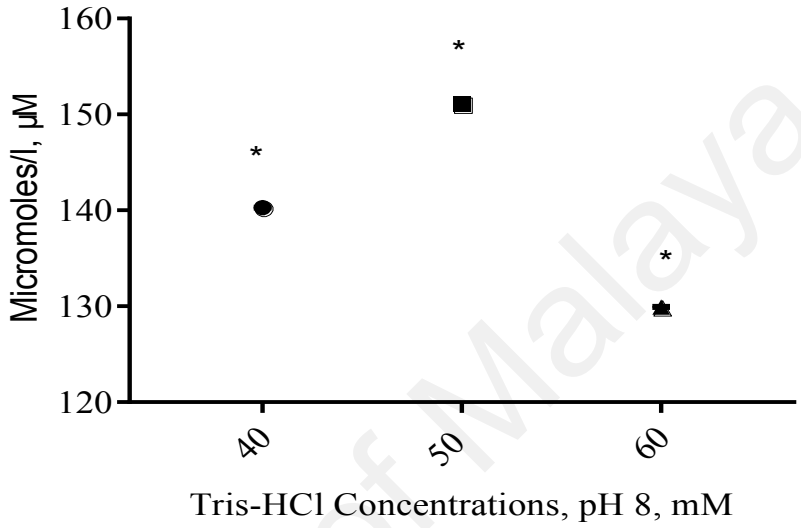


Figure 4.24: Activity assay were added by different concentrations of Tris-HCl, pH 8 buffer as the stabilizing buffer for the selected 0.04 µM KI, substrate concentration.

Data are the means of three independent experiments, with standard errors represented by vertical bars. The reaction at highest product when 50 mM Tris-HCl pH 8 were used for 20 minutes reactions.

The line graph (Figure 4.25) shows that product concentration increases as the substrate concentration increases and highest when the addition of the substrate is at the concentration of 0.32 µM. The reaction dropped and reached plateau when the concentration are increased further.

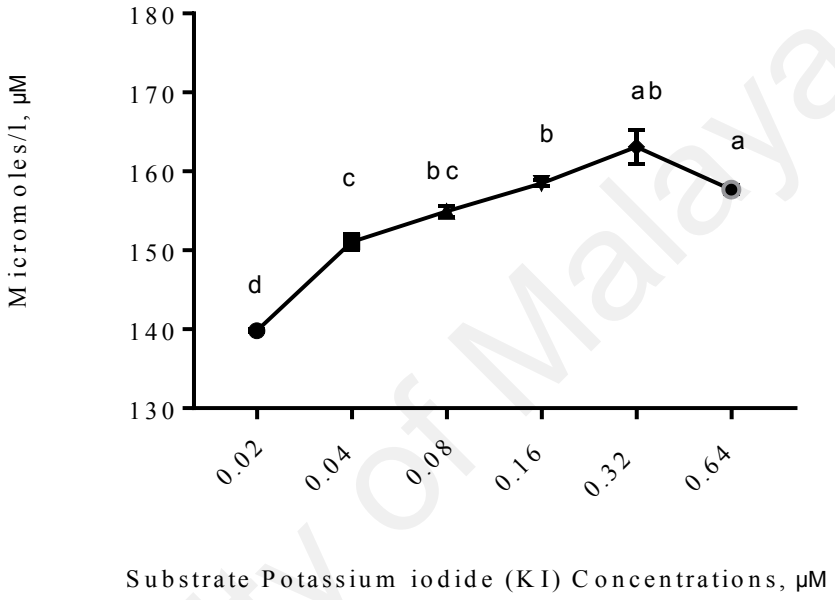


Figure 4.25: Product concentration for selected 50mM Tris-HCl, pH 8 with optimized H_2O_2 concentration, 90 µM against increasing substrate concentration.

Data are the means of three independent experiments, with standard errors represented by vertical error bars. Different letters denote a significant difference between mean values across concentrations. n = 3, ANOVA followed by Tukey's test.

4.2.2.3 Effect of vanadium concentration for crude activation

(a) **Product concentration formation by introducing different concentration of vanadium at different concentration of substrate KI.**

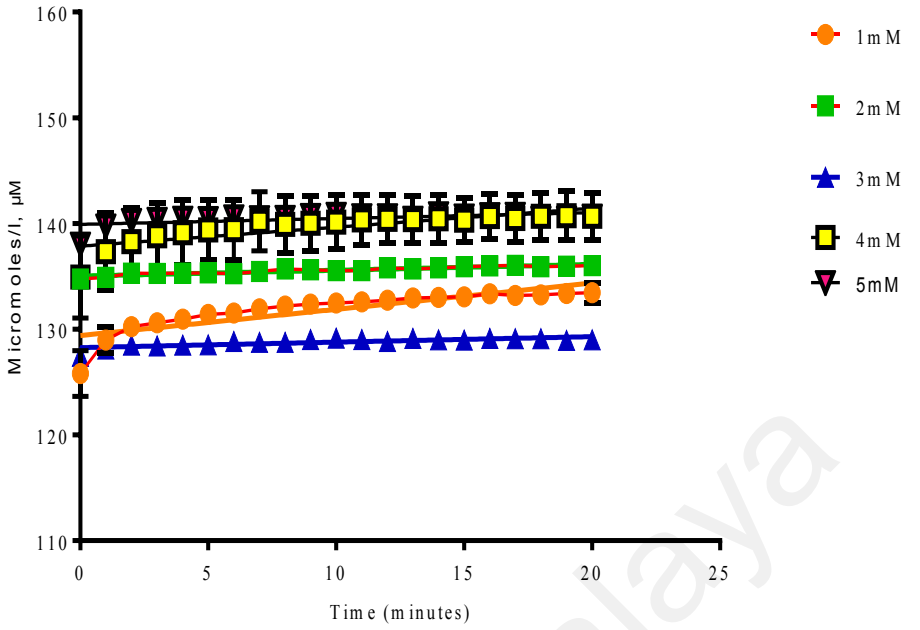
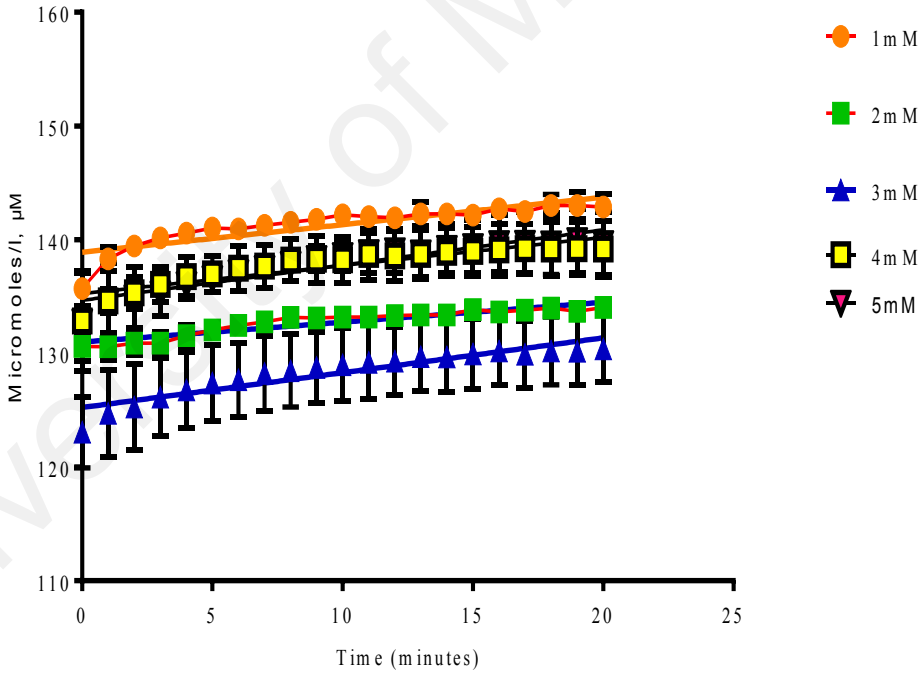
The concentration of the product plotted against time for the enzymatic reaction. From the graph (Figure 4.26);

a) Product concentration formation low at 3, 2, and 1 mM with significantly different. High product formation found at 4 and 5mM. All the concentrations are not significantly different across time.

b) Product concentration formation low at 2 and 3 mM with not significantly different. High product formation at 1, 4 and 5mM also not significantly different. All the concentrations are not significantly different across time.

c-d) Product concentration formed low at 3 mM with significantly different. High product formed at 1, 2, 4 and 5mM. All the concentrations are not significantly different across time.

The total product formation for different concentrations of vanadium are shown at Appendix 9.

(a)0.005 µM KI	
(b)0.04 µM KI	

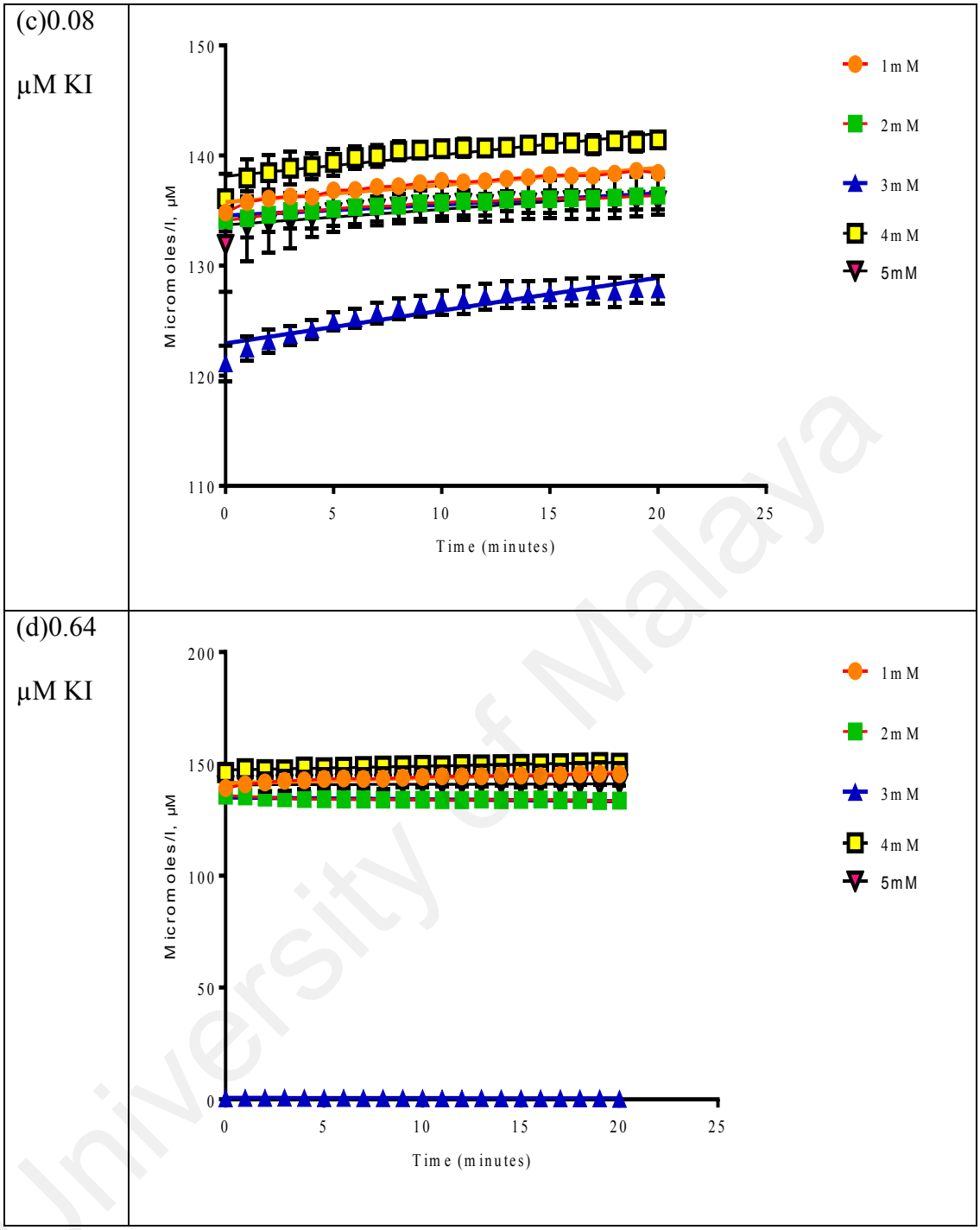


Figure 4.26: Product concentration against time series of enzymatic reaction for four different vanadium concentration ranging from 1-4 mM.

These four different concentration are treated with selected increasing concentrations of substrate potassium bromide (KI) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

(b) **Comparison of product concentration for different vanadium concentrations at selected substrate (KI) concentration**

Based on the Figure 4.27, the reaction gives highest product at the concentration of 1 mM compared to the other three concentrations 2, 3 and 4 mM. The line graph (Figure 4.27) shows that the product concentration increases as the substrate concentration increases and highest when the addition of the substrate is at the concentration of 0.04 µM. The reaction dropped and reached plateau when the concentration are increased further.

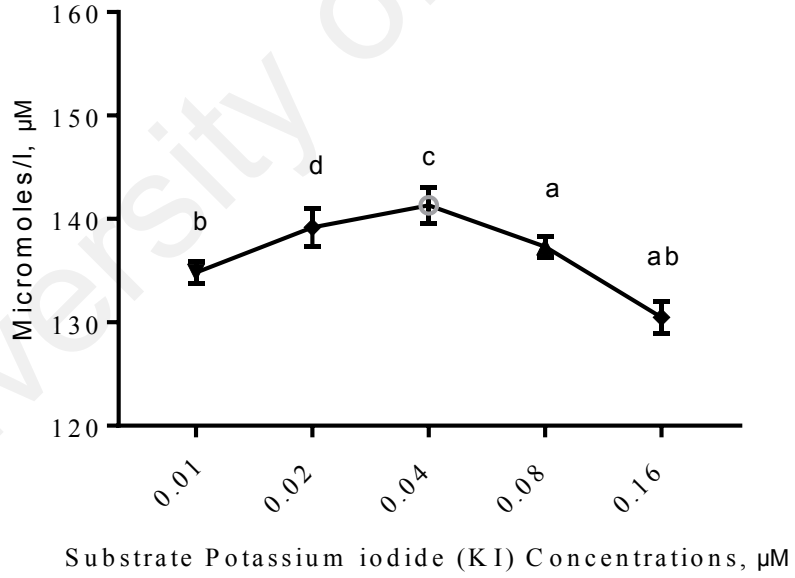


Figure 4.27: Product concentration by crude extracts activated using 1mM vanadium with an increasing of substrate (KI) concentration.

Different letters denote a significant difference between different substrate concentrations. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3, ANOVA followed by Levene's test.

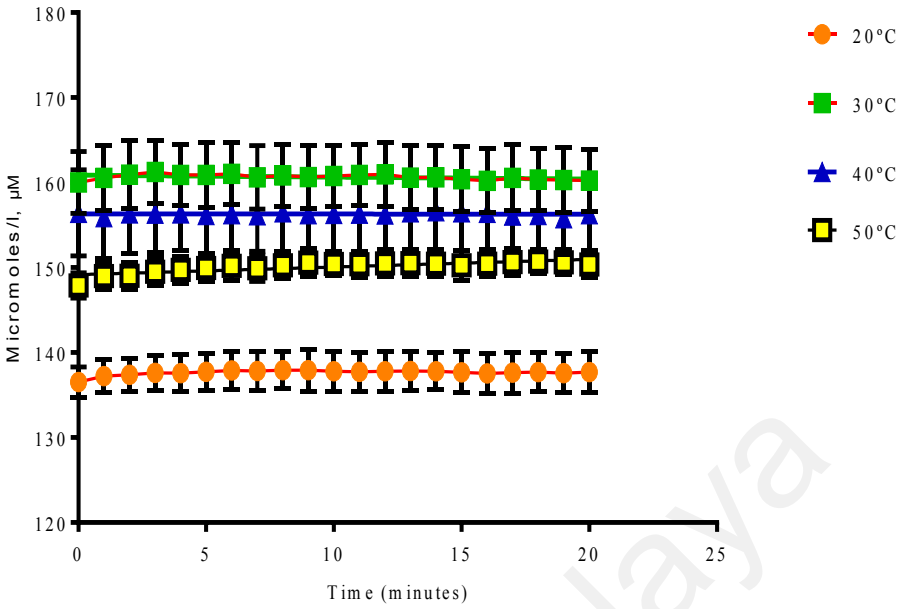
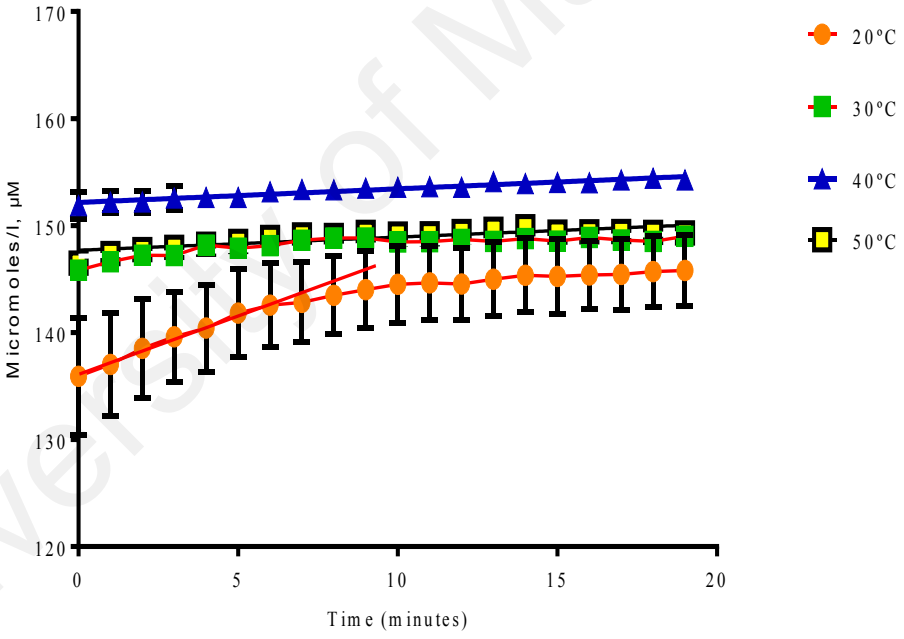
4.2.2.4 Effect of different temperature towards crude extracts stability

(a) **Product concentration formation by crude extracts introduced to different temperatures at different substrate (KI) concentrations.**

The concentration of the product plotted against time for the enzymatic reaction. From the graph (Figure 4.28);

a) 30, 40, 50 ºC were not significantly different although have higher product formation. Meanwhile 20 ºC has low product formation. All the crude extracts exposed to different temperature are not significantly different by time series.
b) Product formation at temperature 20 ºC is significantly different than other although has low product formation. At temperature 30, 40 and 50 ºC were not significantly different. The three concentrations, 30, 40 and 50 ºC were not significantly different by time series except 20 ºC.
c) Product formation at temperature 30 and 50 ºC are significantly different than other. At temperature 20 and 40 ºC were not significantly different. All the crude exposed to these temperatures were not significantly different by time series except.
d) The product concentrations formation for all the four temperature were not significantly different with each other and also by across time being.

The total product formation for crude extracts exposed to different temperature are shown at Appendix 10.

(a)0.005 µM KI	
(b) 0.04 µM KI	

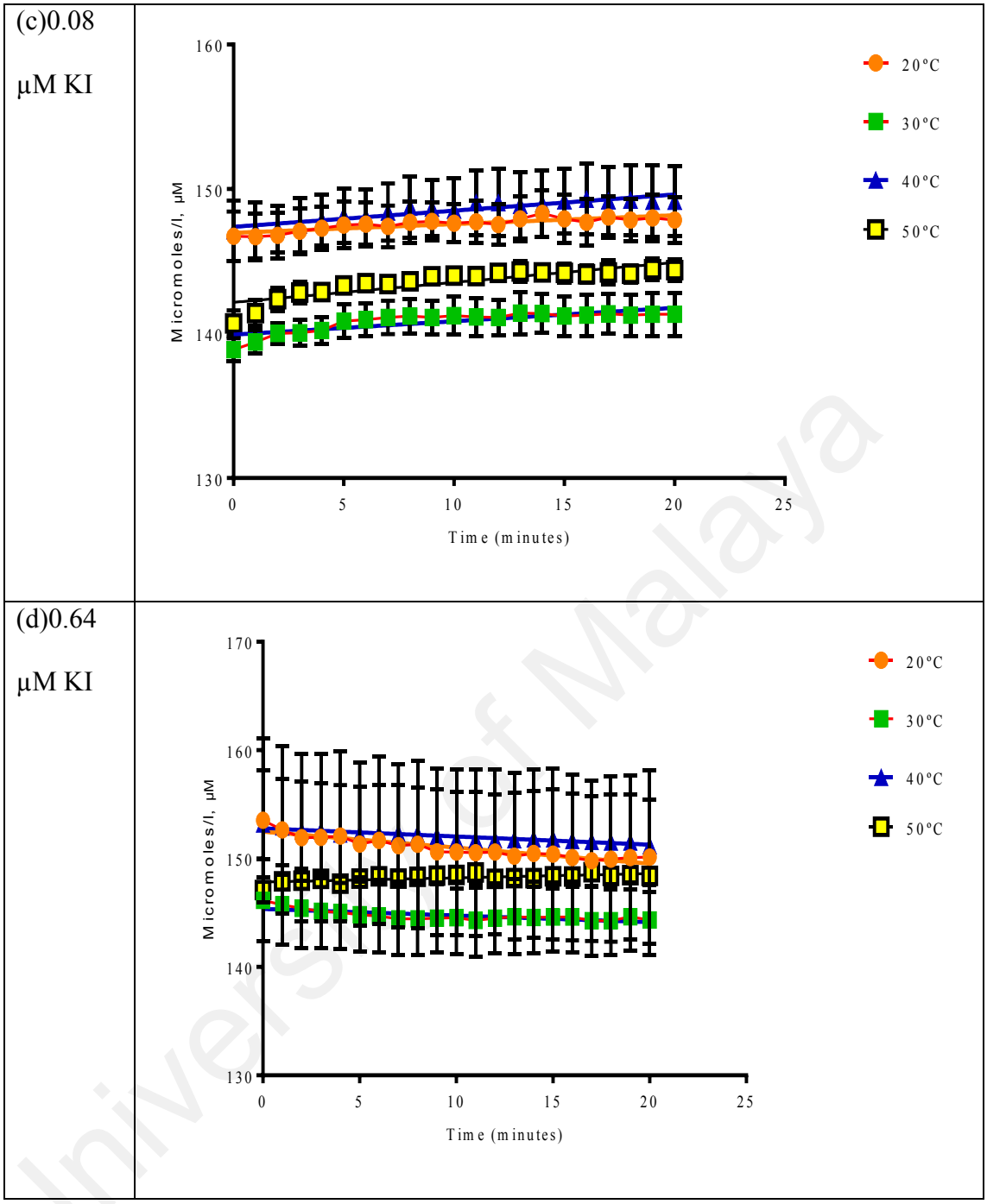


Figure 4.28: Product concentration against time series for crude extracts exposed towards different temperatures ranging from 20, 30, 40 and 50 ºC.

These different temperatures exposed crudes were treated with selected increasing concentrations of substrate potassium bromide (KI) during the assay, (a) 0.005 µM, (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

(b) **Comparison of product concentration for crude extracts exposed to different temperatures at selected substrate (KI) concentration**

Based on the Figure 4.29, the reaction gives partially linear but low product at the temperature 20 ºC especially when the added substrate at the sufficient concentration compared to the other three temperature output.

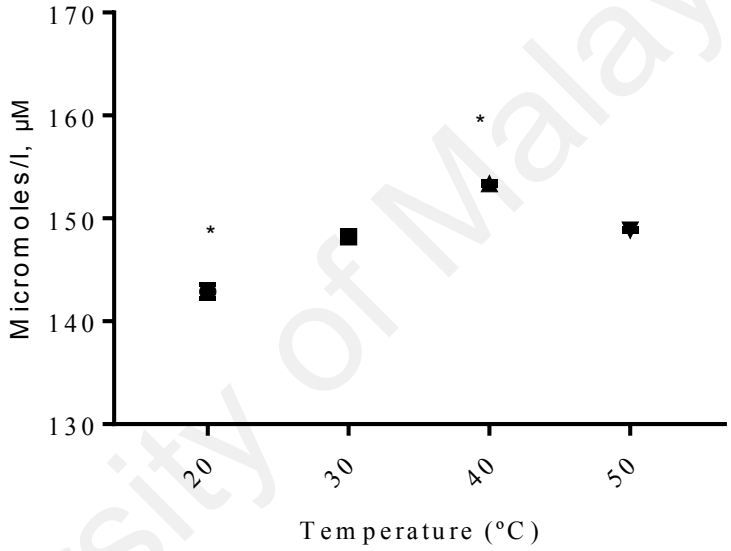


Figure 4.29: The crude extracts were exposed to different temperatures namely, 20, 30, 40 and 50 ºC were treated with selected substrate (KBr) concentration, 0.04 µM.

Data are the means of three independent experiments, with standard errors represented by vertical bars. Although 40 ºC shown highest activity but the reaction being linear at 20 ºC.

The line graph (Figure 4.30) shows that product concentration formation highest at 0.32 µM concentration and dropped as the substrate concentration increases.

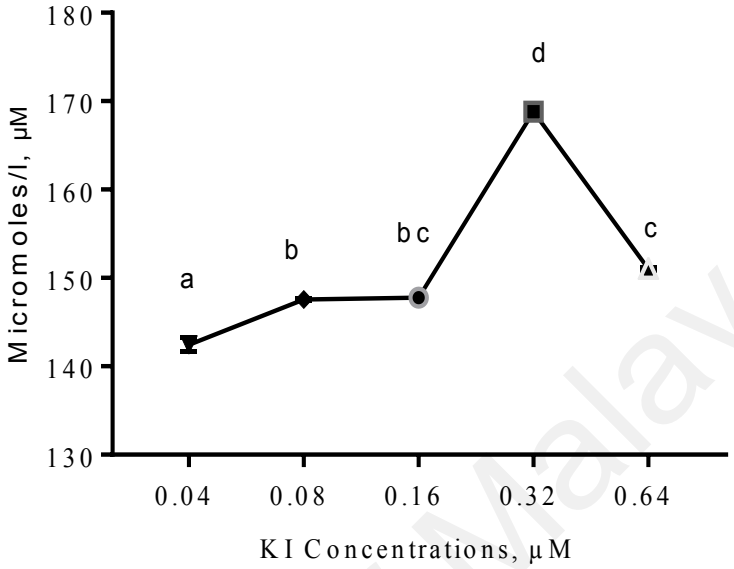


Figure 4.30: Product concentration formation by optimum temperature 20 ºC against increasing substrate concentration.

Data are the means of three independent experiments, with standard errors represented by vertical bars. n = 3, ANOVA followed by Levene's test.

4.3 Use of most suitable conditions for enzymatic activity of the haloperoxidase in the crude extract.

The most suitable conditions obtained from the previous experiments were used for determination of the enzymatic activity of the haloperoxidase extracted from *Sargassum binderi.*

4.3.1 Potassium bromide as the substrate for crude extracts with optimized conditions

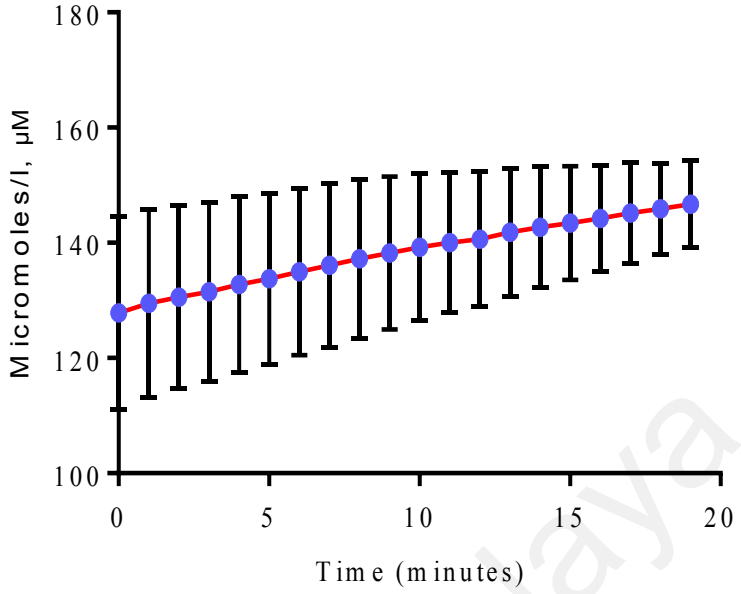
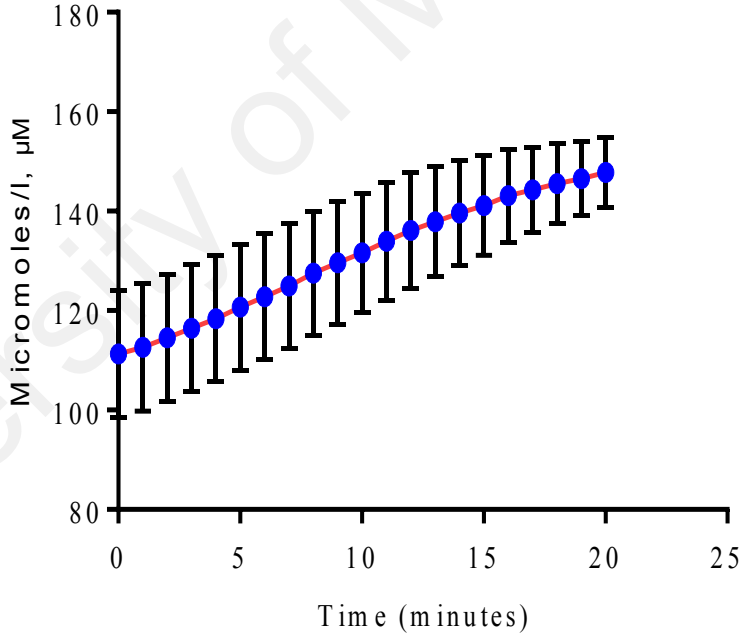
4.3.1.1 Product concentration formation by crude extracts with different concentration of substrate KBr, from lowest, medium and highest.

The concentration of the product plotted against time for the enzymatic reaction. From the graph (Figure 4.31);

a-c) Crude extract gives high product formation and significantly different by time series.

(d) Crude extract gives high product and attained saturation within 12 minutes

Appendix 11 shows Total Product concentration formation for different concentrations of KBr by introducing all the optimized conditions.

(a)0.005 µM KBr	
(b)0.04 µM KBr	

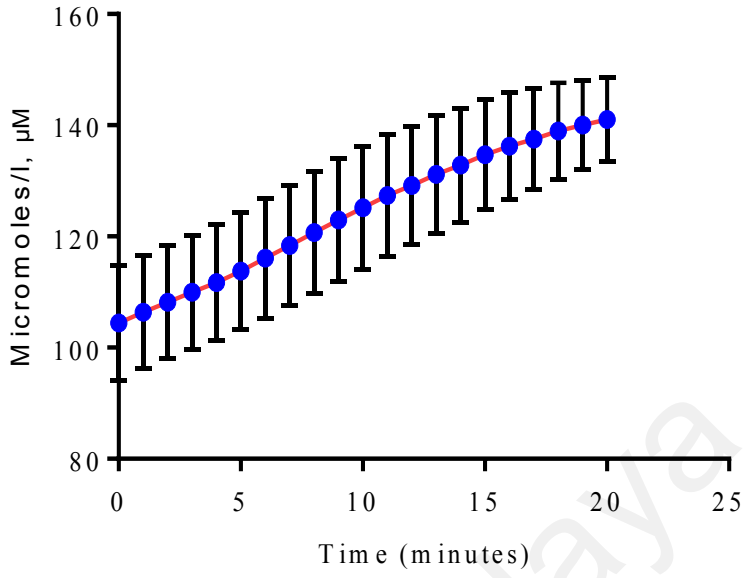
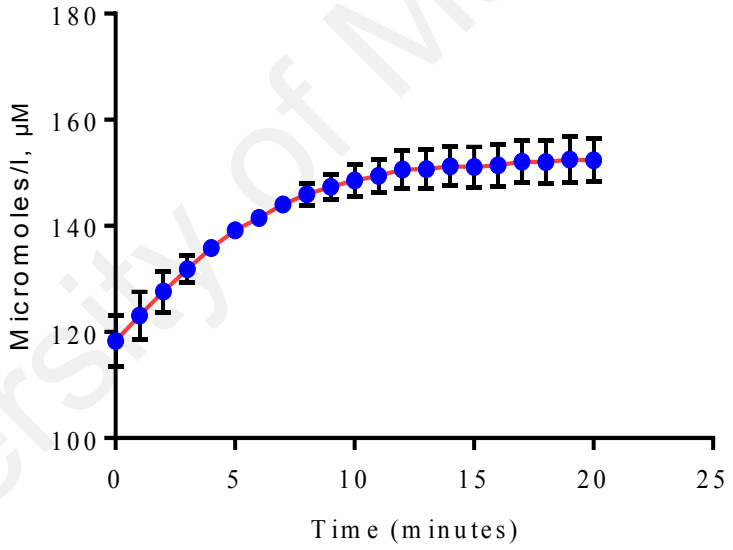
(c)0.08µM KBr	
(d)0.64µM KBr	

Figure 4.31: The enzymatic activity of crude sample against product concentration using different KBr concentration.

The crude sample was treated with selected increasing concentration of substrate potassium bromide (KBr) during the assay, (a) 0.005 µM , (b) 0.04 µM (c) 0.08 µM (d) 0.64 µM. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

The line graph (Figure 4.32) shows that product formation not significantly different as the concentration increases further.

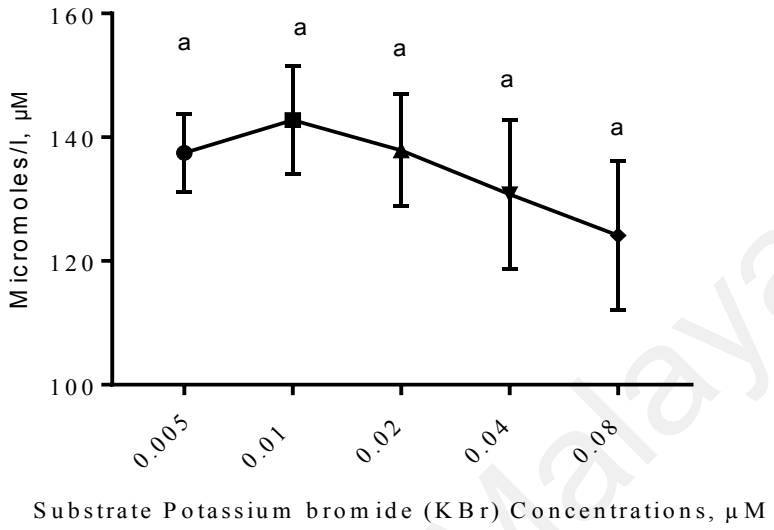


Figure 4.32: Product concentration for extracted crude with increasing substrate concentration.

Data are the means of three independent experiments, with standard errors represented by vertical bars. n = 3, ANOVA followed by Tukey's test.

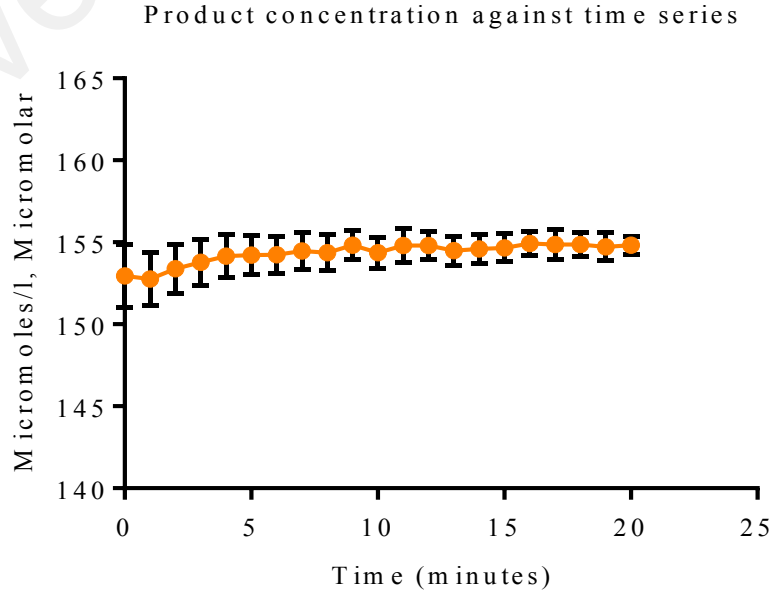
4.3.2 Potassium iodide as the substrate for crude extracts with optimized conditions

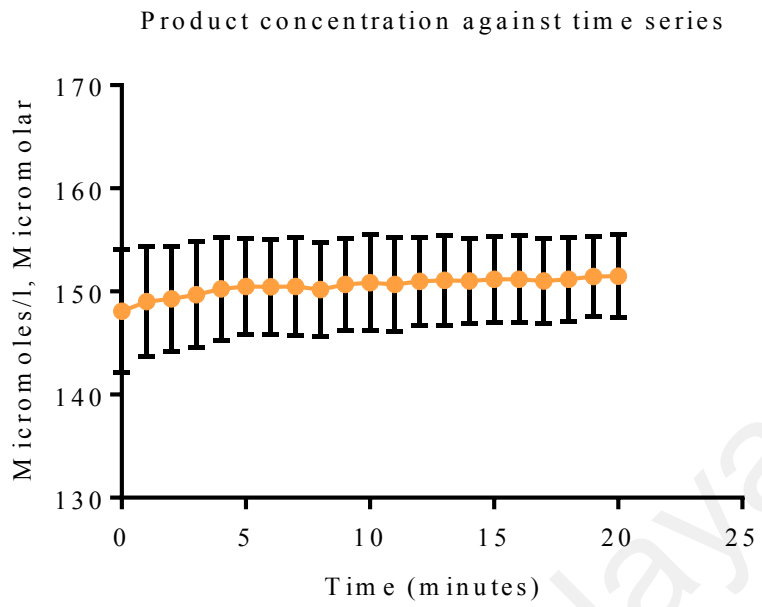
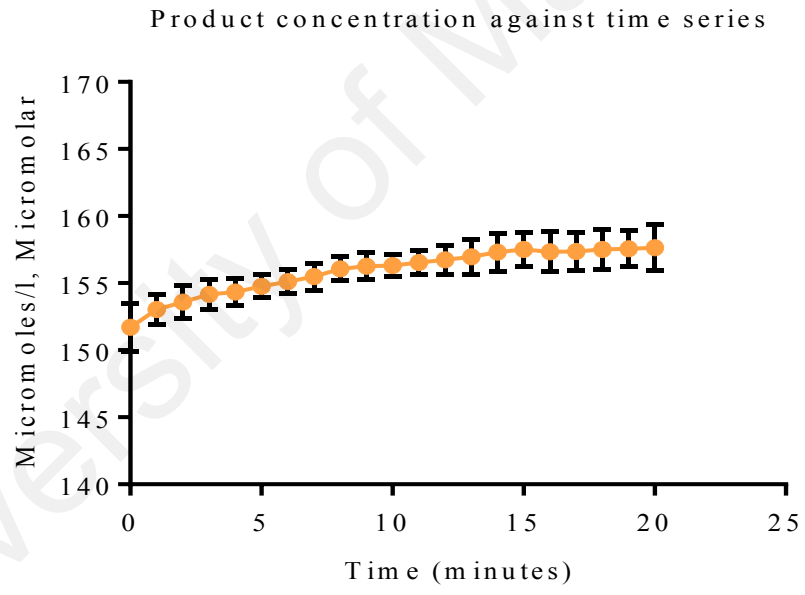
4.3.2.1 Product concentration based on different concentrations of substrate potassium iodide, KI from lowest, to medium and highest.

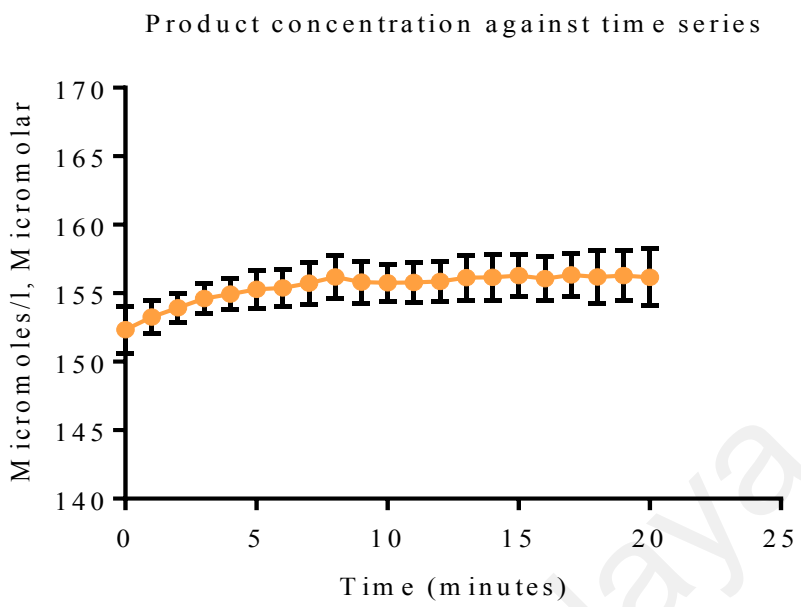
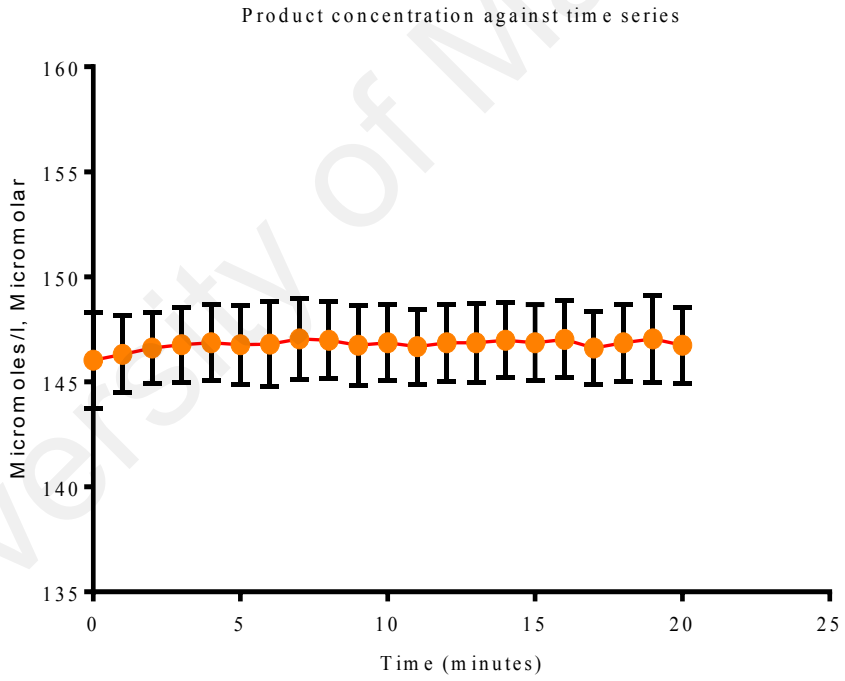
The concentration of the product plotted against time for the enzymatic reaction. Based on Figure 4.33, from the graph (a-b) crude extract does not produce product and were not significantly different by time series. Then after at graph (c-d) gives slightly higher product formation and significantly different by time series. Eventually, at graph (e-h) there isno product formation and it's not significantly different by time series.

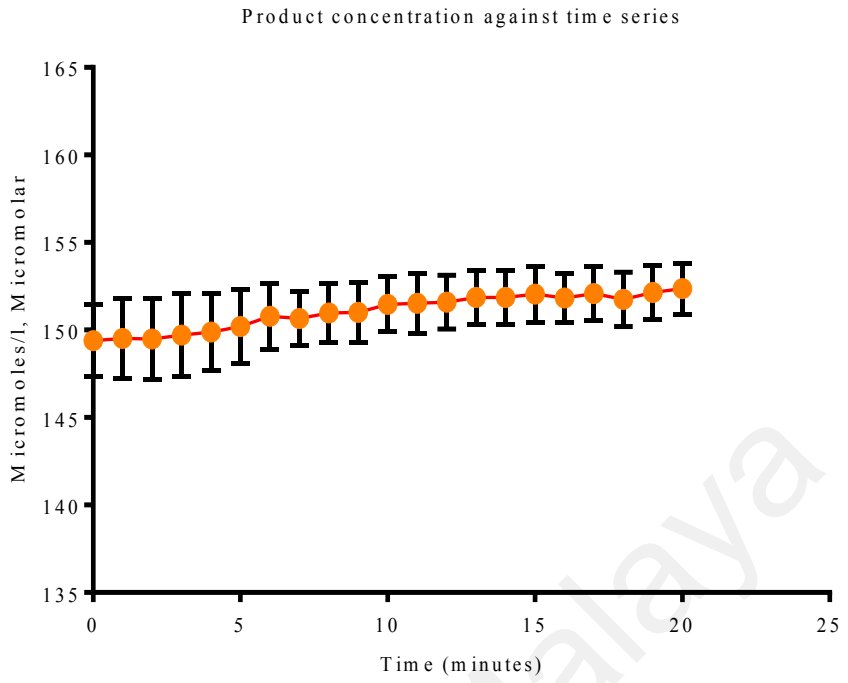
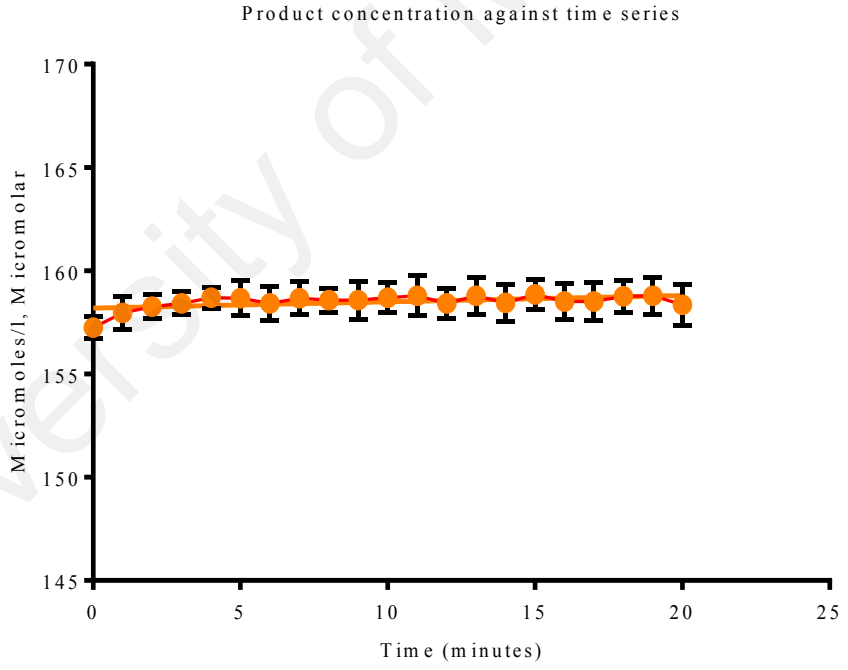
The total product formation for different substrate concentrations shown at Appendix 12. Based on the Figure 4.33, the reaction gives lowest product, and only slightly higher for the added substrate at the sufficient concentration.

(a)0.3125 nM KI



(b)0.625 nM KI	
(c)1.25 nM KI	

(d)2.5 nM KI	
(e)0.005 µM KI	

(f)0.04 µM KI	
(g)0.08 µM KI	

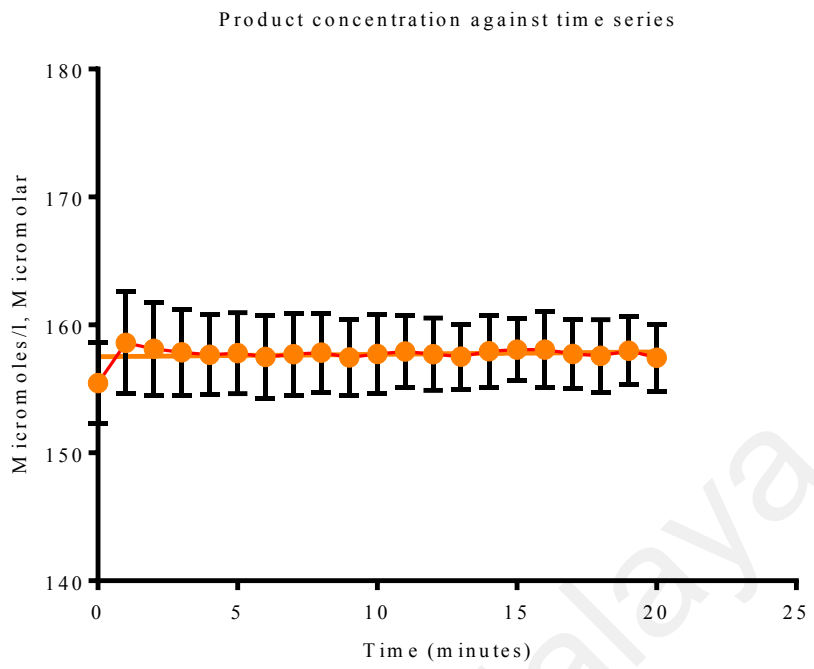
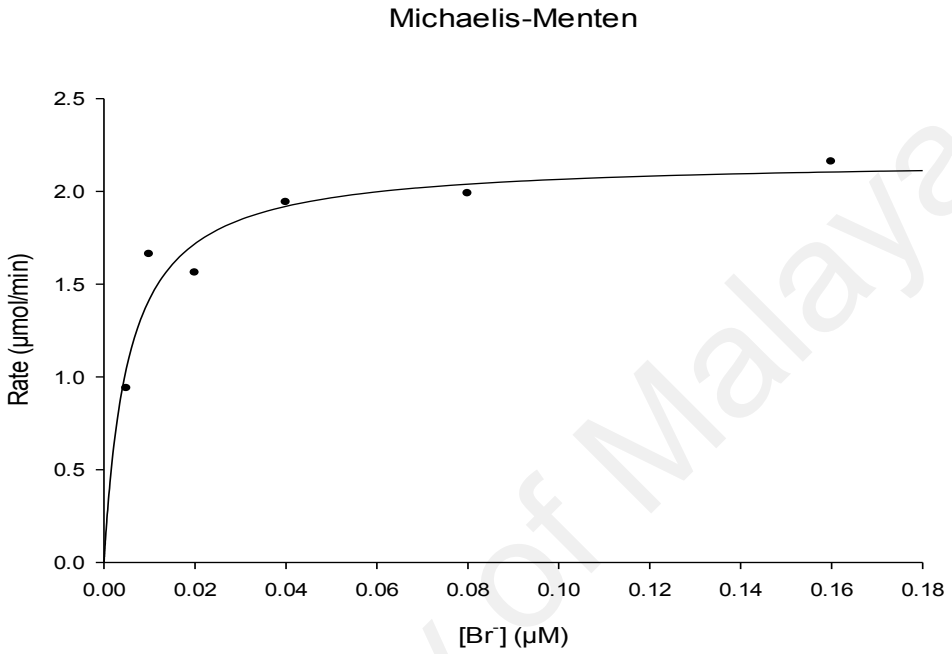
(h)0.64 µM KI	

Figure 4.33: Product concentration formation against time series of enzymatic reaction.

The sample crude was treated with selected low to high concentration of substrate potassium iodide (KI) during the assay. Data are the means of 3 independent experiments, with standard errors represented by vertical error bars. n = 3.

4.4 Steady-state kinetic assay

The assays were performed at 20± 1 ºC in a 96 well microplate. The nonlinear fit of the initial velocities versus [I^-] and [Br^-] resulted in $K_m^{app\ Br-}$: 0.005306 and $K_m^{app\ I-}$: 0.004293 respectively. The Vmax as well for [I^-] and [Br^-] are 2.2 and 53.9 respectively were obtained by fitting to a non- linear regression Michaelis-Menten equation: $1/V = K/Vmax*1/S + 1/Vmax$.



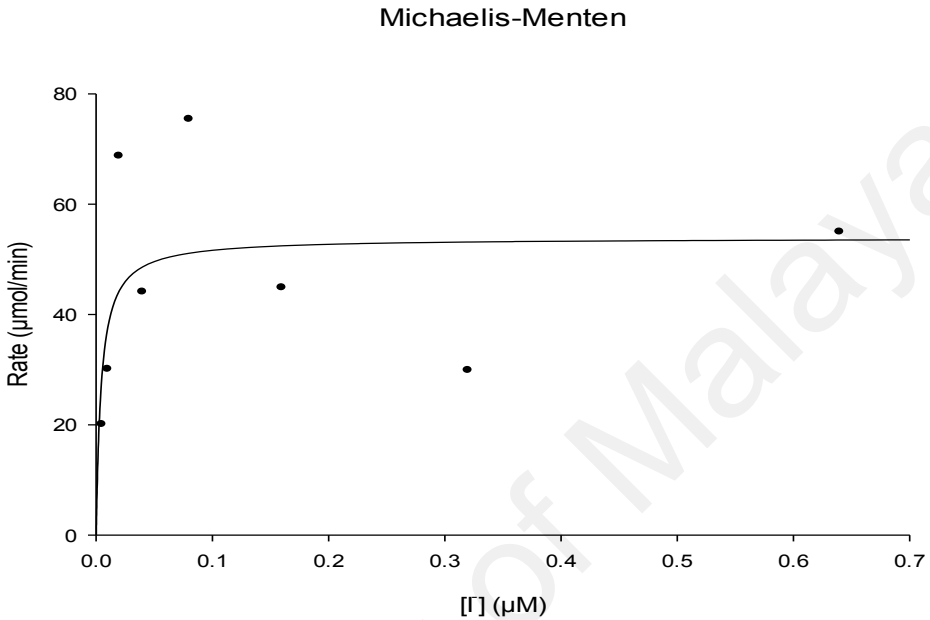


Figure 4.34: Determination of apparent Km and Vmax values of iodide and bromide for vanadium bromoperoxidase from *Sargassum binderi.*

CHAPTER 5: DISCUSSION

5.1 Types of haloperoxidase extracted from *Sargassum binderi*

The enzymatic activity measured using spectrophotometric assays with thymol blue acts as a substrates in the presence of bromide and iodide over wide range of pH showed that the bromide ion was used more than the iodide and weak iodoperoxidase activity was detected in the crude protein extracts from *Sargassum binderi*. This explained that activity is more confined to the bromoperoxidation by bromoperoxidases (BPO). The crude extracts of BPO displayed a specific activity of 0.0009 Units/mg towards bromide at pH 8. BPO remained partially active up to 40 ºC. This result relates to the fact that bromide concentration (3mM) in the seawater is few times higher than the iodide (0.25 µM), which makes the enzyme more adapted for bromide uptake (Leblanc *et al*., 2015).

5.2 Hydrogen peroxide (H_2O_2) optimization using substrate potassium bromide (KBr) and potassium iodide (KI) for enzymatic assay

The flavoenzymes function as oxygenase inadvertently transfer a fraction of electron flux directly to molecular oxygen generating superoxide and hydrogen peroxide into the cell interior near the mitochondria (Elsner et al. 2013).

In the assays using the substrates, potassium bromide and potassium iodide, both assays showed that use of 90 µM of H_2O_2 increases the activity of the enzymatic reaction. In comparison when introducing the lower concentration of H_2O_2, the enzyme reacts slowly with low product formation. With higher concentration such as 0.1mM or more of H_2O_2, the product formation become limited and low. Thus the 90 µM concentration is most suitable of the three concentrations used for the enzyme reaction. This indicates that at medium concentration (90 µM), H_2O_2 was found to be stable and can play important roles in some specific chemical reactions. Hydrogen peroxide acts as an

autocrine (includes intracellular) as well as a paracrine (intercellular) signaling molecule in many cellular processes. Intracellular signals to be signaled within a cell and intercellular signal needs to be transported across membranes (Bienert, Schjoerring, & Jahn, 2006). This range of concentration has been supported by another study which shows having a medium H_2O_2 concentration (0.45 micromol), increases the reaction progressively (Mahajani, Haldar, & Datta, 1973). Meanwhile, increasing above 0.65 micromol leads to reducing the enzyme activity gradually (Mahajani, Haldar, & Datta, 1973).

The higher concentration of H_2O_2 may be toxic to the cell itself probably one of the factors resulting in the necrosis of cell in this assay. The ROS, especially superoxide and hydrogen peroxide, has the ability to damage iron- sulfur cluster of dehydratase (Liu & Imlay, 2013). Consequently, iron atoms are released and intracellular unincorporated iron is overproduced. The elevated pool of iron will react with hydrogen peroxide released by the Fenton reaction, producing hydroxyl radicals. These radicals will directly damage DNA or indirectly oxidize the deoxynucleotide pool, and eventually will be incorporated into the DNA (Liu & Imlay, 2013).

Besides that, presence of excess H_2O_2 also causes carbonylation of proteins and oxidation of methionine residues or thiol groups of cysteines (Bienert, Schjoerring, & Jahn, 2006). The oxidization of sulfhydryl group (-SH) of one cysteine molecule results in sulfenic (-SOH), sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) derivatives (Bienert, Schjoerring, & Jahn, 2006). The intra- and intermolecular disulfide linkages can change binding capacity of a transcription factor or protein structures, and eventually protein function (Bienert, Schjoerring, & Jahn, 2006). Intramolecular disulfides causes changes in the enzyme activity through conformational changes (Bienert, Schjoerring, & Jahn, 2006). Meanwhile intermolecular disulfides causes di- or oligomerization. This

conformational changes modify the reaction centres of enzymes, modify DNA binding motifs or uncover localization signals like in the case of transcriptional factors (Bienert, Schjoerring, & Jahn, 2006). H_2O_2 directly affects other redox systems. For example, Glycerol-dehyde-3-phosphate dehydrogenase, fatty acid synthase, fructose bisphosphatase and Cu-Zn superoxide dismutase are only a few examples of very important metabolic enzymes, which are directly or indirectly modified by H_2O_2 (Bienert, Schjoerring, & Jahn, 2006). Diverse reactions from epimerization to dehydrogenations driven by enzymes and various metabolic pathways tend to expose to H_2O_2 stress. H_2O_2 influences cell proliferation and cell death under developmental control (Mishra & Imlay, 2012). In conjunction with that, introduction of hydrogen peroxide depending on the concentration, either will enhance or inhibit disulfide bonding of selected proteins (Bienert, Schjoerring, & Jahn, 2006).

Sometimes ROS concentration even below dangerous level can lead to cell death (Bienert, Schjoerring, & Jahn, 2006). In most cases many higher organisms produce proteins that have the ability to degrade hydrogen peroxide to oxygen and water (Mishra & Imlay, 2012). Superoxide dismutases and reductases on one side and catalases with peroxidases on another side, will be produced in response to superoxide and hydrogen peroxide in vivo (Mishra & Imlay, 2012). In this brown seaweed *S. binderi*, bromoperoxidase is being expressed with higher possibility to scavenge the H_2O_2.

5.3 The pH and concentration of the buffer

In the assay reaction in the present study, Tris buffer was used, with the pH adjusted with HCl. Tris is inert in many enzymatic systems (no interactions with other components), very freely soluble in water, and has a high buffering capacity (Ugwu & Apte, 2004). Tris buffer has been shown to produce stable reactions of many biological systems (pH 6.0-8.0) (Ugwu & Apte, 2004). The change from the optimum pH can affect

tertiary structures of enzymes which is an irreversible process. The changed process is time-dependent, the more it deviates from the optimum pH in time lapse, the more is the inactivation of the enzyme. The inactivation can occur in a single contact either when the pH is a result of strong acids (<3) or strong base (>11) (with the exception of special enzyme resistant to such conditions, like trypsin). Although HEPES, HEPPS, HEPPSO, and PIPES contain a piperazine ring, and are known as good buffers, in comparison with Tris- buffer, the former are not suitable for redox processes because in the presence of H_2O_2, oxygen radicals, autoxidising iron or under certain electrolytic conditions, they easily form radicals (Ugwu & Apte, 2004).

In the present assay, Tris-HCl, pH 8 buffer with ionic strength of 40 mM for KBr and 50 mM for Kl, used as stabilizing buffer exhibited stable reaction with high product formation. The ionic strength is important for an enzymatic reaction as it is a measure of the ionic milieu. It has influence on the catalytic activity of an enzyme. The same pH 8 but with slightly lower concentration, 30 mM Tris-HCl, was added to the crude for storage purpose. As this will not affect the pH of the assay after addition of aliquot of enzyme into the reaction, the pKa value of the buffer should correspond to the optimum pH of the enzyme under investigation (Ugwu & Apte, 2004).

The ionic composition of the surrounding medium, decides the protonisation and deprotonisation in the reaction set-up, thus affecting the binding and conversion of enzyme substrate by the enzyme. Protonation is a reversible process, but damage of the protein structure is irreversible. Protonation can either promote the catalytic activity or break it down depending on the functional group (Ugwu & Apte, 2004).

When the protonised and deprotonised products are altered under unsuitable physiological conditions, the amino acid residues in the protein interacts with the substrate and the substrate itself will not perform as how it should under suitable physiological conditions (Ellis & Morisson 1982). The buffer has its specific components which help in stabilizing or destabilizing protein structure (Ugwu & Apte, 2004).

5.4 Temperature

The dissociation of compounds and buffer strongly rely on temperature (Ugwu & Apte, 2004). Enzymes at their optimum temperatures exhibit highest activity but deviating from that depending on the degree of deviation, results in a reduction in the activity (Bisswanger, 2014). A slight change in the temperature causes acceptable small decreases in the activity. In normal physiological conditions, 20ºC is used as a standard temperature for buffers (Ugwu & Apte, 2004). In the present assay for the enzymatic reaction, the substrates potassium bromide and potassium iodide, perform better at 20ºC. Meanwhile at temperature lower than that the product formation was lower. The enzymatic reaction in the crude extract showed a decrease in activity when exposed to high temperature (above 50 ºC). At high temperature, the enzyme becomes destabilized and eventually denatured, because three dimensional structure of enzyme is thermo-sensitive (Bisswanger, 2014). The higher the temperature increases, the faster denaturation happens, although it depends on the period of exposure (Bisswanger, 2014). There are some thermophilic enzymes which are stable at high temperatures, while others are unstable (Bisswanger, 2014). The heat inhibits Rubisco activase causing a decrease in Rubisco activation, eventually leading to inhibition of photosynthesis (Ruelland & Zachowski, 2010). Rubisco activase is sensitive even at moderate heat level.

Photostasis process become imbalanced when photosynthetic fixation of CO_2 is limited. This will cause ROS production triggering some ROS scavenging enzymes which are thermolabile (Ruelland & Zachowski, 2010). Electron transport system, the photosystems PSII and PSI are also affected by heat stress (Ruelland & Zachowski, 2010).

The changes in temperature may cause other enzymes to actively take over the role of the enzymes. Thus enzyme activities in cell metabolism are influenced by temperature. The slight deviation from the optimum will produce signaling pathways and enhance heat or cold responses. The stability of enzymes in metabolic cycles, cascades and redox chain may be influenced as the temperature changes (Ruelland & Zachowski, 2010). The output of a metabolic chain is determined by the turnover rate of the slowest link. Temperature changes increase the pool of certain intermediate metabolites and also shrinks others. The commonly used temperature must be indicated for pH specification because pH is altered with temperature changes (Ruelland & Zachowski, 2010).

5.5 Vanadium

Crude extracts incubated with vanadium ions activated bromoperoxidatic activity, showing that there exists some BPO, that are nonheme and needs vanadium as a co-factor, just like some known vanadium-BPOs (Suthiphongchai, Boonsiri, & Panijpan, 2008). In the present study, crude extracts from *S. binderi* incubated with 2mM for KBr and 1mM for KI substrates were activated better than other concentrations, thus showing enhanced activity.

In nature, some enzymes exist in the apoenzyme form, and is activated by vanadium, similar to thiamine pyrophosphate activates blood transketolase. Ocean water contains ~ 42 nM in vanadium. The binding of vanadate (VO_4), prosthetic group of VHPO into the enzyme is essential for the catalytic activity (Leblanc *et al*., 2015). Thus, reconstitution of vanadium with crude extracts can recover the haloperoxidatic activity lost during extraction. Vanadate has stronger affinity than oxyanions, towards VHPO depending on the pH (Leblanc *et al*., 2015).

In VHPO during the production of halogenated compounds, the metal center becomes insensitive to oxidative inactivation during turnover (Suthiphongchai, Boonsiri, & Panijpan, 2008).

5.6 Substrate

During an enzymatic reaction, the disappearance of substrate and the formation of product, is used as a measure of the progress of the reaction (Bisswanger, 2014). In the present study, although the enzyme (haloperoxidase) can catalyze the two substrates (potassium bromide and potassium iodide), difficulty in identifying the products formed, made it necessary to perform the reactions separately. The type of substrate selection depends on the factors such as stability, solubility and the accessibility to a detection method and concentration of the substrate. As the reaction progresses, the amount of substrates decrease and slows down eventually stopping the reaction.

The nonlinear fit of Michaelis-Menten plot resulted in $K_m^{app\ Br-}$: 0.005306 and $K_m^{app\ I-}$: 0.004293 respectively. The Vmax as well for [I^-] and [Br^-] are 2.2 and 53.9 respectively. Since the Km value is low the enzyme has has high affinity towards the substrates. From the kinetic analysis we can deduce that the substrate binding to the enzyme forms a hyperbolic saturation function based on the Michaelis- Menten equation. In the presence of high amount of substrate, a complete saturation was attained. For the assay treated with higher concentration of substrates, the enzyme activity is influenced in a non-specific manner, and directly acts as an inhibitor of the enzyme reaction.

CHAPTER 6: CONCLUSION

This research represents the first project on haloperoxidase on tropical *Sargassum* and will contribute important fundamental information for understanding HPO production and function in brown seaweeds in the tropical region. The research has focused on the extraction of the haloperoxidase as well as investigating properties of HPOs in the crude extracts of *Sargassum binderi*, a brown seaweed.

The following details summarise the findings of this study:

(1) The crude extract exhibits highest possible degree of stability and activity when conditions for the enzymatic reactions are as follows:

   i) Use of 90 μM of H_2O_2 concentration during the reactions.

   ii) 30mM Tris-HCl, pH 8 added to the crude extracts for enhancing its stability.

   iii) 40mM and 50mM Tris-HCl, pH 8 buffer for substrate KBr and KI respectively are added as the stabilizing buffer for the reaction.

   iv) Temperature at 20ºC activates the enzymatic activity of the crude extracts.

   v) Use of vanadium with concentration of 2mM and 1mM for KBr and KI respectively enhances the enzymatic reactions.

(2) The extracted crude from the tropical brown seaweed *Sargassum binderi* Sonder ex J. Agardh exhibits bromoperoxidatic activity.

(3) The factors such as high temperature more than 50ºC reduces the activity and eventually results in denaturation of the enzyme; pH of buffer being acidic or lower than 7.6 does not produce high activity; substrate concentration higher than the specified range inhibits the reactions, and result in limitations that influence the resulting activity of haloperoxidases from the tropical brown seaweed *Sargassum binderi* Sonder ex J. Agardh.

There are many VHPOs that have been shown to possess beneficial characteristics such as halogenated compounds with antibiotic properties. For example, *Laminaria digitata* excretes VBPO that deactivates acylated homoserine lactones (cell-to- cell signaling molecules) (Leblanc *et al*., 2015). Along with this, halogenated compounds control formation of biofilms and growth in Gram-negative bacteria (Leblanc *et al*., 2015). Due to these characteristics, VHPOs have been explored in medical applications; for example, *Curvularia inaequalis* which produces VCPO, has antimicrobial effects on *Enterococcus faecalis* biofilms (Leblanc *et al*., 2015).

Although in the present study, only crude extracts from *S. binderi*, were used for all experiments, we were able to determine the optimal conditions for the enzymatic reactions. The main enzyme present was also shown to be a vanadium bromoperoxidase.

Further studies on optimization of extraction and purification including crystallisation of the enzymes should be carried out in the future. This is especially important to explore the use of haloperoxidases in medical applications. This further purification step for the enzyme present in the crude extract of *Sargassum binderi* is needed to ensure that the number of isoforms present can be tested for specificities with different types of halides. In the present study, there were many challenges in proceeding till the purification step where the enzyme activity tend to decrease drastically or fully has been the major limitations. Thus, the purification steps needs to be improved and optimized thoroughly. The purified enzyme can be used for molecular characterization to allow for the biosynthesis of various types of halogenated compounds. To gather more information, the recombinant active enzymes can be over- expressed in *E.coli,* in yeast system, or in other heterologous systems.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Punitha, T., Phang, S.-M., Juan, J. C., & Beardall, J. (2018). Environmental Control of Vanadium Haloperoxidases and Halocarbon Emissions in Macroalgae. *Marine Biotechnology*, 1-22.