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Cholinesterase from the Liver of *Diodon hystrix* for Detection of Metal Ions

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ABSTRACT

The discharge of industrial effluents into nearby water bodies affects the inhabitants including living organisms. The presence of foreign materials such as heavy metals can be a threat to the ecosystem as they are enormously carcinogenic even though in minute concentration. Hence, an economical and time-efficient preliminary screening test is crucial to be developed for the detection of heavy metals, prior to employment of high technology instruments. In this study, cholinesterase (ChE) from Sabah porcupine fish, *Diodon hystrix* was purified to test for its potential as an alternative biosensor in detecting metal ions. Few enzymatic parameters including specificity of substrate, temperature and pH were applied to determine its optimal enzymatic activity. ChE enzyme was found to be more sensitive towards the presence of substrate, butyrylthiocholine iodide (BTC), in contrast to acetylthiocholine iodide (ATC) and propionylthiocholine iodide (PTC) with the effective coefficient at 7193, 3680.15 and 2965.26 V_{max}/K_m, respectively. Moreover, the extracted ChE enzyme showed the optimum activity at pH 9 of 0.1 M Tris-HCl and at 25°C to 30°C range of temperature. When subjected to heavy metals, ChE enzyme was significantly inhibited as the enzyme activity was reduced in the sequence of Hg > Ag > Cr > Cu > Cd > Pb ≥ Zn > As. As a conclusion, the partially purified ChE enzyme proved its sensitivity towards metal ion exposure and can be used as an alternative method in screening the level of contamination in the environment.

Keywords: Cholinesterase, diodon hystrix, heavy metals, pH, substrates, temperature

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INTRODUCTION

Water covers around 71% of the Earth surface. Water is being utilised by all life forms in various industries including food manufacturing and leisure industries. Yet, the accessibility of excellent water sources is gradually worsened as it is becoming polluted over time (Ahmad et al., 2016a; Gonzalez et al., 2009). The incorporation of noxious wastes such as heavy metals and pesticides into the ecosystem, deriving from multiple agrarian and industrial activities can produce alarming effects (Fatima et al., 2014; Sabullah et al., 2014).

The heavy metals are naturally prevailed elements and no less fivefold larger relative to water molecules. Their toxicity corresponds to exposure level, types of chemical and dosage. Mercury, lead, arsenic and cadmium are among the pre-eminent important materials that could affect the community health (Gupta et al., 2015). Based on the United States Environmental Protection Agency (USEPA), toxicity of heavy metals is discerned as carcinogenic to humans.

A series of observations is implemented as precautionary steps in controlling and reducing contamination level (Wang et al., 2018). The presence of toxicants may cause negative health effects and death. Hence, the preliminary evaluation of water quality is crucial for utilisation in everyday life. Equipped with contemporary automation such as High-Performance Liquid Chromatography (HPLC) and Inductively Coupled Plasma (ICP) in quantifying toxicants level, these inventions are costly, time-consuming and require expertise for handling (Sabullah et al., 2015). Therefore, alternatively, biosensor using enzyme can be used as a preliminary screening to detect contaminants level semi-quantitatively.

Cholinesterase (ChE) is an esterase responsible for lyses of choline esters, most of which act as neurotransmitters in the nervous system. ChE involves in hydrolysing acetylcholine (Ach) into its constituents, acetic acid and choline (Ch) (Čolović et al., 2013). Certain compounds such as carbamates and organophosphates possess high affinity in inhibiting ChE activity (Fukuto, 1990; Johnson & Moore, 2012). Other than that, the metal ions are competent in binding at either ChE active or allosteric sites, impeding its activity.

The inhibition of ChE activity permits the detection of toxicants. From the observation, this scenario can be exploited in detecting metal ions at varying concentrations. Moreover, the process can be minimised as no involvement of the experts is needed. The screening time could also be reduced in which only samples showing enzymatic inhibition are selected for the secondary screening. Thus, numerous researchers are opting in addressing future biosensor advancement in order to satisfy the needs of the emergent world despite many issues arisen in the development of biosensor.

The porcupine fish, *Diodon hystrix* which holds hundred spines on its body is selected in this project. *D. hystrix* exhibits a distinct defence mechanism when it senses athreat, this fish will inflate its body by taking water inside and deflating when it is no longer threatened.

This fish' through its skin can produce toxic or poisonous substances. It is interesting as *D. hystrix* uses many ChE enzymes to properly function for its unique expertise. The objective of the present project was to extract and partially purify the ChE enzyme from the liver tissue of *D. hystrix*. Then, the substrate specificity, optimum temperature and pH of purified ChE were determined and the inhibitor effects on the ChE enzymatic activity were assessed.

MATERIALS AND METHODS

Materials

Five adult porcupine fish, *Diodon hystrix* sized 30-33.5 cm and weighing 800-1000g were obtained from the local Sabah wet market at Kota Kinabalu, Sabah.

Sample Preparation

The liver of *Diodon hystrix* was dissected out and weighed immediately. Using mortar and pestle for extraction, liver tissue was squeezed and subsequently transferred into a beaker containing sodium phosphate buffer (0.1 M, pH 7) in a ratio of 1:4 (w/v) in cold condition. The homogenisation was performed using Ultra-Turrax T-25 homogeniser in which 500 µL homogenate was stored at -20°C for enzyme and protein assay. The centrifugation of homogenate was taken place at 10,000 $\times g$ for 10 minutes at 4°C to eliminate the presence of cell debris. The supernatant was collected and stored at -20°C for further purification.

Ion Exchange Chromatography Purification

The purification of ChE was performed using diethylaminoethanol (DEAE) matrix linked to Sepharose (Peterson & Sober, 1956). A syringe (0.9 cm diameter, 6 cm height) was packed with matrix and let to settle until 3 cm bed height was obtained. The flow rate was calibrated at 0.2 mL/min. The column was washed with 5 batch volumes of washing buffer (25 mM sodium phosphate buffer, pH 7.5) and then eluted with eluting buffer (25 mM sodium phosphate buffer, pH 7.5 at various concentrations of 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M NaCl). The fractions were collected with a volume of 1 mL from washing to eluting stages. The column then washed with five batch volumes of washing buffer and stored in 20% ethanol at 4°C.

Cholinesterase Enzyme and Protein Assay

The enzyme activity of ChE was assayed using modified Ellman method (Ellman et al., 1961). The reaction mixture was prepared containing 200 µL of 0.1 M sodium phosphate buffer, pH 7.5, 20 µL of 0.067 mM 5.5'-dithiobis-2-nitrobenzoic acid (DTNB) and 10 µL of ChE sample. The mixture was loaded into 96 microplates well. The mixture was incubated

for 15 minutes at room temperature before the addition of 20 µL acetylthiocholine iodide (ATC) into the mixture. It was then incubated for additional 10 minutes before absorbance reading was recorded at 405 nm using multimode detector. The ChE sample was substituted with 0.1 M sodium phosphate buffer, pH 7.5 for the control test. The production of yellow colour signified ChE activity with extinction coefficient of 0.0136 $\mu M^{-1}cm^{-1}$. A unit of activity is defined as amount of substrate (µmole) hydrolysed by AChE per minute (U) with 0.0136 $\mu M^{-1}cm^{-1}$ extinction coefficient.

The protein assay was performed using Bradford method (Bradford, 1976). The determination of protein content was quantified using bovine serum albumin as a standard. The reaction mixture containing 200 µL Bradford reagent and 20 µL ChE sample was loaded into the well and absorbance was recorded at 595 nm after 10 minutes incubation at room temperature.

Optimal pH, Temperature and Substrate Specificity

The determination of optimum pH was carried out using an overlapping buffer system; 0.1 M acetic acid (pH ranged 3.0 to 5.5), sodium phosphate (pH ranged 6.0 to 8.0) and Tris-HCl buffers (pH ranged 7.0 to 9.0). The optimal temperature was determined by incubation at 6 different temperature ranged from 15°C to 45°C, followed by addition of substrate.

The substrate specificity test was performed using three substrates, acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC). The assay was performed separately for each substrate with concentration from 0 mM, 0.1 mM, 0.5 mM, 1.0 mM, 1,5 mM, 2.0 mM and 2.5 mM. The determination of preferred substrate was calculated using Michaelis-Menten kinetics (maximal velocity, V_{max} and biomolecular constant, K_m) using GraphPad PRISM version 5 software.

Metal Ions Inhibition Study

The eight types of heavy metals were used which were copper (Cu^{2+}), silver (Ag^{2+}), cadmium (Cd^{2+}), arsenic (As^{5+}), chromium (Cr^{5+}), mercury (Hg^{2+}), lead (Pb^{2+}) and zinc (Zn^{2+}) at concentration of 5 mg/L . The assay was prepared containing 150 µL 0.1 M Tris-HCl buffer, pH 9, 50 µL 5 mg/L metal ions, 20 µL 0.067 mM DTNB and 10 µL ChE enzyme. The reaction mixture was loaded into the well and incubated for 15 minutes at room temperature. BTC with a volume of 20 µL was added into the reaction mixture and incubated for 10 minutes at room temperature. The absorbance was recorded at 405 nm and the control test was conducted by replacing heavy metals with distilled water.

Statistical Analysis

All data obtained were in the form of means ± standard deviation (SE) and analysed using GraphP ad Prism version 5.0. The one-way analysis of variance (ANOVA) with post hoc

analysis by Tukey's test was employed to calculate the comparison between two or more groups of data.

RESULTS AND DISCUSSION

Purification Profile

The purification of ChE from *Diodon hystrix* was successfully performed using DEAE-Sepharose matrix with total of 26 fractions. The highest ChE activity was in fraction of 19 to 26. The fractions 1 to 16 showed low ChE activity indicated no overloading of enzyme inside the column with fraction 27 as a control. Figure 1 shows ChE from liver tissue of *D. hystrix* was purified at 3.65-fold with 45.3% recovery. The low recovery may be due to ligand leakage, absorption of non-specific protein and interaction on the surfaces such as unsuitable temperature and pH (Efremova et al., 2001). Besides, low total ChE recovery may be due to the existence of thermal energy in the system.

The partial purification procedure was summarised in Table 1. The fold was denoted as specific activity after fractionation divides with crude homogenate and applied to estimate

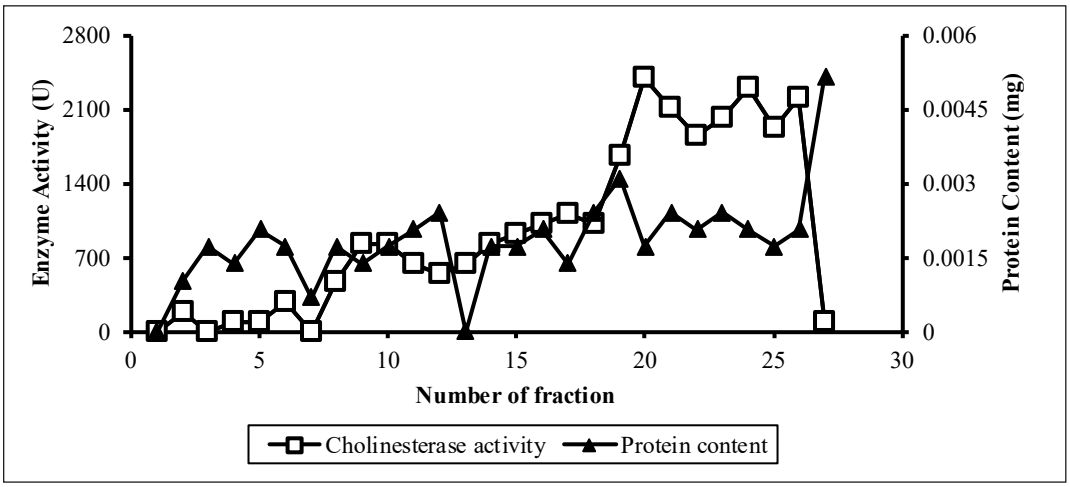

Figure 1. The purification profile of ChE from liver extract of *D. hystrix* using DEAE matrix

Table 1
The comparison of extraction and purification methods of D. hystrix *ChE, in which total ChE activity was expressed in U for each purification step*

	Total recovery		Specific activity (U/mg)	Purification folds (x)	Yield (%)
	Total protein (mg)	Total ChE activity (U)			
Crude homogenate	8.76	7659.375	871.91	1	100
Supernatant	3.97	5269.669	1326.70	1.52	68.8
Purified	0.75	2389.71	3186.28	3.65	45.34

the times of enzyme has purified. Meanwhile, yield was determined using the homogenate as a reference point with 100 % retained enzymatic activity during purification process. From Table 1, it can be noted that the decrement of the number of protein and ChE activity through each step. The removal of unwanted protein such as insoluble fats and inorganic during the elution stage has increased the specific activity of the desired protein.

The low yield percentage indicated the denaturation of purified enzyme during purification process. The partially purified enzyme may denature due to inappropriate surrounding temperature and pH used along the process. Temperature can cause the enzyme to be more active and produce high kinetic energy to facilitate more collision which can alter the structure of enzyme. For pH, the enzyme reacts with hydrogen ion that binds to the active site, hence changing the shape of the enzyme (Efremova et al., 2001; Robinson, 2015).

Substrate Specificity

The cholinesterase (ChE) works in hydrolysis of the predominant choline ester, acetylcholine, Ach. However, Ach was not being utilised as a substrate in this study as the liver could contain acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and propionylcholinesterase (PuChE) (Askar et al., 2011; Garcia-Aylion et al., 2012). Lockridge (2015) stated that BuChE and PuChE were abundant in livers but differed in their functions.

As can be seen in Figure 2, the prompt increase on the steepness of the BTC line was noted, indicating the sensitivity of the purified enzyme towards BTC as compared to the other substrates. However, PTC showed high enzymatic activity indicating the affinity of both synthetic substrates towards the purified ChE enzyme. To double confirm, the kinetic parameters of purified enzyme on substrates were determined using GraphPad Prism software.

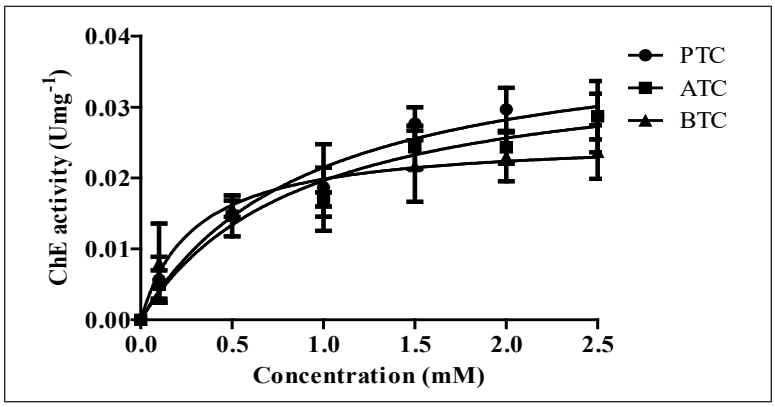

Figure 2. Michaelis-Menten plot of *D. hystrix* ChE incubated in three different synthetic substrates; Acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) at concentration of 0 to 2.5 M

Based on Table 2, the value of K_m for BTC, 0.2909 mM was lower than ATC and PTC. The highest V_{max} value was noted by PTC which was 3355.56 Umg^{-1}, followed by ATC (3054.22 Umg^{-1}) and BTC (2092.44 Umg^{-1}). The data obtained was in line with Figure 2. The preferable synthetic substrate of the enzyme was determined based on the catalytic efficiency (V_{max}/K_m). The BTC was demonstrated as the preferable synthetic substrate with the highest ratio of 7193 U mg^{-1} mM^{-1} as compared to the other two substrates. The result obtained was in line with the previous studies conducted using BTC as a specific substrate for the purified ChE from liver tissue of *Puntius javanicus, Anabas testudineus* and *Clarias gariepinus* (Ahmad et al., 2016b; Padrillah et al., 2017; Sabullah et al., 2014).

Table 2
The comparison of K_m and V_{max} of synthetic substrates; acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) using GraphPad Prism software

	Mean point (95 % confidence intervals)		
	ATC	**BTC**	**PTC**
V_{max} (U/mg)	3054.22	2092.44	3355.56
K_m (mM)	1.030	0.2909	0.9118
Catalytic efficiencies (V_{max}/ K_m)	2965.26	7193	3680.15

pH Profile

The pH profile of the purified enzyme was assessed using three buffer types; acetic acid, sodium phosphate and Tris-HCl buffer. An overlapping buffer system was utilized to cancel the effects of other buffers on the enzymatic activity.

Figure 3 presents the highest pH condition of ChE activity was at pH 9 (the highest studied) in 0.1 M Tris-HCl buffer. The pH can alter the hydrogen and ionic bonding of

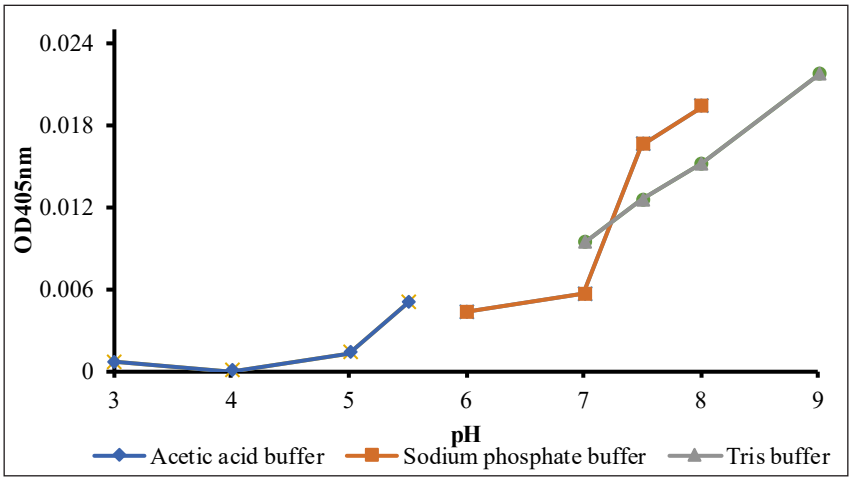

Figure 3. Optimization of pH for *D.hystrix* ChE activity. Data presented in absorbance value at wavelength of 405 nm (Final reading – initial reading)

enzyme and stop the enzyme-substrate complex formation (Reece et al., 2011). At low pH, the presence of excessive hydrogen ion leads to protonation of the imidazole group of histidine at catalytic triad of enzyme, and thus resulting in the loss of catalytic properties of enzyme (Masson et al., 2002; Masson & Lockridge, 2010). At high pH, the alteration of charge of substrate binds to the enzyme causes no enzyme-substrate complex formation. The previous studies on the ChE activity of *Lates calcarifer, Clarias gariepinus* and *Monopterus albus* were also conducted using Tris-HCl buffer system which signified that ChE enzyme could work efficiently in the alkaline environment (Fadzil et al., 2018; Hayat et al., 2015, 2017; Khalidi et al., 2019; Sabullah et al., 2019).

Temperature Profile

The optimisation of the purified enzyme was conducted in different incubation temperatures as shown in Figure 4. The bell-shaped curve showed the optimum temperature of *D. hystrix* ChE at the range of 25°C to 30°C. At optimal temperature, more enzyme-substrate complexes are formed which lead to an elevated number of products produces. Basically, the activity of the purified ChE was retarded at the temperature lower than 25°C, as it did not have sufficient kinetic energy to facilitate the number of effective collisions between enzyme and substrate per unit time. High temperature may denature the enzyme and inactivates the active site. At extreme temperature, the thermal energy can cause more vibration until high enough to alter the bonds supporting the three-dimensions (3D) configuration of the enzyme and lowered the enzymatic activity (Bernabei et al., 1993; Fairbrother et al., 1991; Reece et al., 2011).

Fish was categorised as cold-blooded organisms, hence warmer temperature, beyond 30°C may denature or inactivate ChE (Sabullah et al., 2014). Almost all published studies on bioassay using liver ChE source from various animal types were carried out at temperature

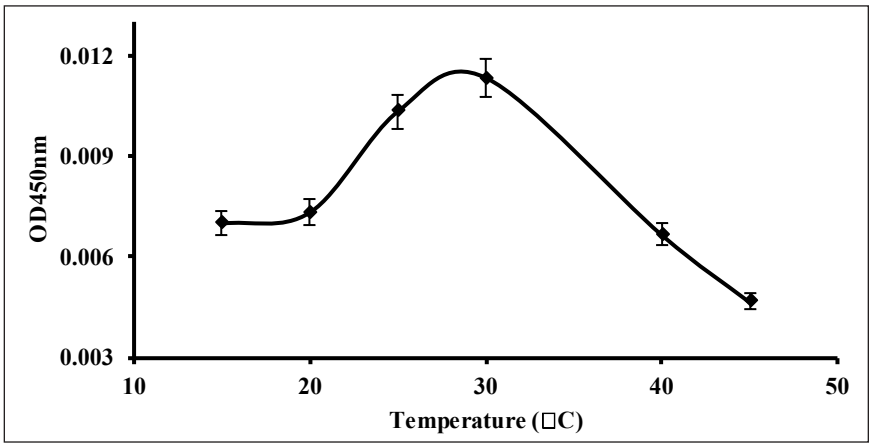

Figure 4. Optimization of temperature profile for *D. hystrix* ChE activity. Data presented in absorbance value at wavelength of 405 nm (Final reading – initial reading)

range of 25 to 30°C (Askar et al., 2011; Sabullah et al., 2014; Sanchez-Hernandez et al., 2011).

Inhibition Study

The effectiveness of bioassay for purified ChE was determined by inhibition study of toxicants. The purified ChE was exposed with incubation of eight metal ions with final concentration of 5 mg/L. From Figure 5, all metal ions were significantly inhibited the purified ChE by lowering the activity to 63.42, 92.68, 80.56, 70.73, 75.37, 2.44, 85.36 and 87.05 % for $Ag^{(2+)}$, $As^{(5+)}$, $Cd^{(2+)}$, $Cr^{(6+)}$, $Cu^{(2+)}$, $Hg^{(2+)}$, $Pb^{(2+)}$ an $Zn^{(2+)}$, respectively. Mercury (Hg^{2+}) showed the highest inhibition of ChE activity, followed by other four metal ions, but the inhibition did not exceed 50% activity. The toxicity level was assigned in decreasing order from $Hg^{2+} > Ag^{2+} > Cr^{6+} > Cu^{2+} > Cd^{2+} > Pb^{2+} \geq Zn^{2+} > As^{5+}$.

Heavy metals involved in the formation of enzyme-substrate complex, although they can bind to enzyme active or allosteric sites and disrupt the formation. The binding of metal ions initiated reaction with functional hydroxyl and sulfhydryl groups, which eventually changes the shape and stops the substrates binding (Frasco et al., 2007; Glusker et al., 1999). The amino acids produce protein attraction on the presence of metal ions. The cation pull from imidazole group of histidine relates to attraction of free heavy metals and nitrogenous substrates (Dvir et al., 2010; Ma & Dougherty, 1997; Sussman & Silman, 1992).

The researches done by Frasco et al. (2007) and Wang et al. (2009) stated that among all metal ions, mercury and copper were the potent inhibitors for ChE, which coincided

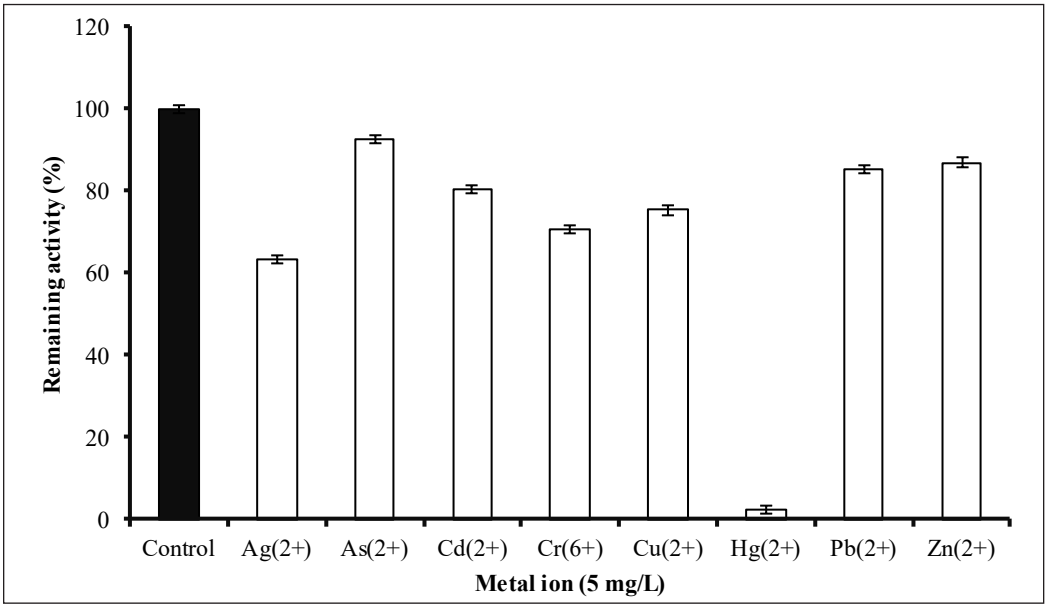

Figure 5. The percentage remaining activity of *D. hystrix* ChE after incubated in 5 mg/L of eight heavy metals. The alphabet denotes as statistically significant differences between each metal ion ($p<0.05$)

with the finding in this study. Ariöz & Wittung-Stafshede (2018) specified that the classic structural of transition metals like Cu permitted illustration on the binding potency of metal ions in inhibiting the ChE activity. Numerous studies conducted using fishes, for example *Lates calcarifer, Puntius javanicus, Anabas testudineus* and *Clarias gariepinus* displayed high Hg inhibition towards ChE activity (Ahmad et al., 2016b; Hayat et al., 2017; Padrillah et al., 2017; Sabullah et al., 2014). Study by Aidil et al. (2013) demonstrated the sensitivity of AChE of *Pangasius hypophthalmus* towards metal ions exposure such as Ag, Hg, Cd, Cu, Zn, Cr and Pb. The inhibition of these metal ions displayed the exponential decay type curve and its IC_{50} values were equal and lower in comparison to the existing biosensor assays like papain, immobilised urease, bromelain, Microtox™, *Daphnia magna* and rainbow trout. This study provides insight on the capability of ChE of *D. hystrix* in the detection of contaminants.

CONCLUSION

The cholinesterase (ChE) enzyme from liver tissue of *D. hystrix* was successfully purified using ion-exchange chromatography using DEAE-Sepharose as the matrix. The enzymatic parameters of ChE were determined, in which the enzyme worked at optimal rate in condition of pH 9, 0.1 M Tris-HCl buffer at temperature range of 25°C to 30°C, alongside with the presence of BTC as the synthetic substrate. The inhibition study concluded that *D. hystrix C*hE was sensitive towards a few metal ions, especially towards Hg^{2+}. Therefore, ChE of *D. hystrix* provides a promising alternative source for biorceptor to substitute the current ChE source in the market. It is recommended to explore the potential of purified *D. hystrix* ChE in detecting other pollutants like detergents, drug, dyes or pesticides.

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