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Potential use of Acetylcholinesterase from *Osteochilus hasselti* in Monitoring Heavy Metal Pollution in Malaysia River

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River pollution gave significant effects by declining the quality of freshwater sources, and cause a negative impact on the aquatic habitat and nearby aquaculture sector. Scheduled river monitoring was implemented to determine and investigate the pollution source in order to minimize the number of waste release into the river. Preliminary screening using biosensor tool considered as an effective method where capable to reduce the cost of implementation, easy to handle as well as rapid analysis. The potential use of acetylcholinesterase (AChE) purified from the brain tissue of *Osteochilus hasselti* as a biosensor tool for heavy metal pollution was investigated. Prior to the study, several water samples were collected from the selected state in Malaysia; Derhaka River (Penang), Perak River (Perak), Kuyuh River (Selangor), Melaka River (Malacca), Peta Waterfall (Endau-Rompin National Park, Johor), followed by filtered then brought to the laboratory immediately. AChE was extracted from the brain tissue of *O. hasselti* followed by affinity purification using procainamide-based chromatography. AChE was tested by incubated separately with the water samples. Based on the semi-quantitative assessment, all the sample from Derhaka River show higher inhibition towards AChE activity compared to the other river. DR01 capable of lowering almost half of the AChE activity to 56.8±2.8 % followed by DR03 (67.96 %) and DR02 (76.74%). Both Melaka river samples; MR1 and MR2 capable to inhibit AChE more than 10% while MR03 around 4 %. Sample from Kuyuh river; KR01, KR02 and KR03 significantly inhibiting the enzyme activity more than 10%. PR (PR01, PR02 and PR03) slightly affecting the AChE activity around 3 to 5 %. However, all the sample from Endau-Rompin National Park considered as unaffected. Secondary screening was done on each river samples using ICP-OEM for quantitative analysis. This can conclude that the inhibition level of AChE corresponds to the concentration of metal ion. From the study proves that *O. hasselti* AChE works as an alternative source of biosensor in monitoring the environmental pollution.

Keywords: acetylcholinesterase, biosensor, *Osteochillus hasselti*, ICP-OES, metal ion

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I. INTRODUCTION

Malaysia encountered with the increasing number of a polluted river from a year to a year. According to the report from DOE (2015) 1,488,848 sources of pollution were recorded and the major contribution came from the sewage treatment plant around 86.69% followed by 12.94 % food services and 0.37% from the others such manufacturing industry, agriculture, pig farming and wet market. However, wastewater released from the manufacturing industry or reached from the agricultural area need to be concerned due to highly persistence and resistance, highly toxic to the environment as well as difficult to be remediated (Hayat *et al.*, 2016; Sabullah *et al.*, 2015a, 2014) Usually, the metal ion is one of the elements that present in the wastewater yet capable of causing contamination at the high concentration level. Moreover, metal ion unable to be degraded by the environmental factor and tend to concentrate into the river sediment. There is no doubt the aquatic life also become threatened as this element able to bioaccumulation at each of the organs and cause abnormality from biochemical to physiological level of the organism (Dixit et al., 2015; Sabullah *et al.*, 2016, 2015c)

Acetylcholinesterase (AChE) is a hydrolase enzyme that plays a role to metabolize the neurotransmitter accumulation at the synaptic cleft (Tham *et al.*, 2009; Mohd Hayat et al., 2015) Several studies were conducted by exploiting AChE as a biosensor tool for the environmental contaminant such pesticides, detergent and heavy metals.(de la Torre *et al.*, 2002; Shukor & Sulaiman, 2013) In this study, AChE from *Osteochillus hasselti* was used for monitoring tool as our previous study prove that this enzyme was sensitive towards carbamate, organophosphate insecticides, and heavy metals (Sabullah, 2011; Sabullah *et al.*, 2013) *Osteochillus hasselti* AChE was tested by incubation with the water samples from a different source of the river to prove their sensitivity and become the future candidates for the development of biosensor kit.

II. MATERIALS AND METHODS

A. Extraction and Purification

Osteochillus hasselti (weight 100-150 gram) were caught alive from Malaysia National Park, Kuala Atok, Pahang, Malaysia. The fishes were brought to the laboratory; Bioremediation Lab, UPM, then acclimatized in an aquarium containing free chlorinated water with fully aerated for two days. Next, the fishes were killed through immersion in a box of ice for 30 minutes. The brain was dissected, weighted followed by homogenization using an Ultra-Turrax T25 homogenizer in 0.1 M of sodium phosphate buffer pH 7.5 at the ratio of 1:5 (w/v). The extractant was then centrifuged at 15 000×g for 10 minutes at 4 ^{o}C. The supernatant was collected and 400 µL was pipette out and load onto the affinity column; procainamide sephacryl S-1000 (0.5 widths

and 3 cm height) then wash with five batches of washing buffer (0.1M sodium phosphate buffer, pH 7.5). Another five batch of eluting buffer (0.1 M sodium phosphate buffer, pH 7.5 containing 1 M NaCl) was then loaded and each 1 mL fractions were collected until the elution process was completed. All the fractions were tested for determination of enzyme activity according to the method of Ellman et al. (1961) with slight modification using 96 well microplate and the absorbance was read at 405 nm. Fractions exhibiting AChE activity were then pooled and concentrated through dialysis using VivaSpin tube spun at 10, 000xg for 10 minutes. Purified AChE was stored in the refrigerated condition.

B. Sampling

The water samples were collected from five different states in Malaysia with three sampling location (Table 1). The sampling processes were conducted at the hot season (from early of April to end of Jun) which the sample was collected around 10.00 a.m. to 12.00 p.m. This river was selected due to their classification as polluted, slightly polluted and unpolluted river (Baskaran et al., 2013; Daud, 2017). Each 250 mL of water sample was placed in sampling bottle containing a drop of 10% of Nitric acid (v/v) followed by filtration using a syringe filter (1.5 cm in diameter, 0.45-micron pore size). The water samples were then stored under refrigerated condition until subsequent use.

Table 1: Sampling location at different state in Malaysia. Numbering at the sample name indicated as sampling location marked with the coordinate.

Location (State)	Sample name	Coordinate	Classification	Remarks
Derhaka River (Penang)	**DR1**	**N 05° 21.111' E 100° 24.780'**	Polluted	The river is black in colour and smelly.
	DR2	**N 05° 20.87' E 100° 24.692'**		
	DR3	**N 05° 21.205' E 100° 24.975'**		
Malacca river (Malacca)	**MR1**	**N 2° 11.567' E 102° 14.762'**	Polluted	The colour of the river is dark brownish color and quite smelly.
	MR2	**N 2° 13.478' E 102° 15.182'**		
	MR3	**N 2° 13.442' E 102° 15.162'**		
Kuyuh River (Selangor)	**KR1**	**N 3° 04'49 " E 101° 42'793"**	Slightly polluted	The river is yellowish colour.
	KR2	**N 3° 00'853" E 101° 42'781"**		
	KR3	**N 3° 00'86.0" E 101° 42'793"**		
Perak River (Perak)	**PR1**	**N 4° 06.851' E 100° 53.251'**	Slightly polluted	The river is yellowish colour. Sometime is clear.
	PR2	**N 4°06.732' E 100° 53.641'**		
	PR3	**N 4° 06.920' E 100° 55.767'**		
Endau-Rompin Waterfall (Johor)	**EW1**	**N 02° 30.674' E 103° 21.387'**	Unpolluted	The water is clean and clear
	EW2	**N 02° 30.802' E 103° 21.086'**		
	EW3	**N 02° 30.784' E 103° 21.02'**		

C. Preliminary Screening

AChE assay was conducted based on an optimal assay condition determined by Sabullah *et al.* (2013). Using a 96 wells microplate, each well containing a mixture of 150 µL of 0.1M sodium phosphate buffer pH 7.5, 50 µL of a water sample, 20 µL of 0.1mM DTNB and 10 µL of O. hasselti AChE then incubated for 30 minutes at room temperature. 20 µL of 1 mM ATC was then added and the absorbance was read at the wavelength of 405nm from 0 and 10 minutes of incubations. For the control assay, the water sample was replaced with distilled water. Tap water was also tested in this study as an example of the sample which is clean and free from the contaminant. Data was generated according to this equation below;

$$\Delta_{405nm} = \text{Final reading}_{10min} - \text{Initial reading}_{0min} \quad [1]$$

$$\% \text{ activity} = \frac{(C\Delta_{405nm} - S\Delta_{405nm})}{C\Delta_{405nm}} \times 100 \quad [2]$$

where,

Δ_{405nm} = Absorbance at the wavelength of 405nm after 10 minutes of incubation (final – initial).

% activity = Percentage inhibition of AChE activity.

C = Distilled water work as the control of the study.

S = Each water sample from different river

D. Stationary Screening

Each water sample from different sources, which exhibited the highest inhibition towards AChE activity was selected for secondary screening by using inductively coupled plasma-optical emission spectrometry; ICP-OES to determine the metal ion that presents in the samples as well as the quantity amount of each element. The metal ion standards such arsenic; As^{5+}, cadmium; Cd^{2+}, copper; Cu^{2+}, cobalt; Co^{2+}, chromium; Cr^{6+}, Molybdenum; Mo^{6+}, lead; Pb^{2+} and zinc; Zn^{2+} were prepared in a series of concentrations for measuring the exact concentration of heavy metals content in every sample.

E. Statistical Analysis

Analysis of variance (ANOVA) followed by Tukey's multiple comparison tests at 5% of the significant level by was performed for each sampling location. $p<0.05$ was considered statistically significant.

III. RESULTS AND DISCUSSIONS

Purified AChE from the brain tissue of *O. hasselti* was exposed with each of different water samples and the result was calculated based on the percentage inhibition of AChE activity (Figure 1). Tap water show no significant effect on AChE activity. The same situation also observed on all EW samples except for EW1 which show only small inhibition at 1.61 % but statically no significant different with the control ($p>0.05$). Slightly polluted river such

Kuyuh River inhibit the activity of AChE more than 10 % compared to the sample from Perak River capable to inhibit AChE activity around three to six percent. AChE show higher inhibition towards sample from Derhaka River where DR1 capable to lowering almost half of AChE activity; 43.2 % inhibition, while DR2 and DR3 inhibits 23.26 and 32.04 %, respectively.

Referring to the table 2, sample DR1, MR2, KR3, PR3 and EW1 were selected for secondary screening using ICP-OES. Here proves a good correlation between enzymatic activities and instruments. Highest inhibition of SJ1 corresponds to the highest concentration of metal ion especially zinc, copper, and lead at 33.15, 21.10 and 3.19 ppm, respectively. Cr^{6+} show the highest concentration of metal ion at 1.81 ppm in MR2 sample while cobalt and lead are not detected or its presents at very low detection level. KR3 and PR3 show highest concentration of Cu^{2+} and Cd^{2+}, respectively. For EW1 sample, only zinc was detected at 0.008 ppm and this metal ion cause slight inhibition towards *O. hasselti* AChE. Previous study prove single effects of Zn2+ caused almost 50% of *Puntius javanicus* ChE activity. (Sabullah *et al.*, 2014)

Several metal ion able to interact with each side chain of amino acids in AChE such negatively charge R group (aspartate and glutamate), hydroxyl group (serine and threonine), aromatic ring (tryptophan, phenylalanine and tyrosine), and sulfur ligands (methionine and cysteine) which blocking the formation of enzyme-substrate complex(Frasco *et al.*, 2008; Petukh & Alexov, 2014). Frasco *et al.* (2007) determined the binding site of mercury through X-ray crystallography at the surface of ChE where the interaction of mercury with amino acids such histidine, methionine, asparagine, threonine, and tryptophan, while DLS assay shows the alteration of ChE structure based on the increasing hydrodynamic radius as the increasing exposure concentration of mercury. AChE also was applied in the determination of toxicity effect of metal ion from the molecular, cellular and physiological level of an organism (Ahmad *et al.*, 2016a, 2016b, 2016c; Aidil *et al.*, 2013; Hayat *et al.*, 2016; Mohd Hayat *et al.*, 2015; Padrilah *et al.*, 2017; Sabullah *et al.*, 2015b)

Manufacturing industry, such production of electronics, mechanicals and constructions equipment highly needs a number of raw materials especially Zn, Cu, Pb, Cd, Co and Mo to make each component. High probability, the contaminated river that contains a high concentration of such elements came from the wastewater that was deliberately released from nearby industrial activities. This situation is reflecting to the contamination of Derhaka River, which congested with light and heavy industry from the upstream to the downstream of the river. Although the appearance of MR2 is similar with DR1 (Table 1), but each concentration of metal ions are significantly lower. Normally, the differences between both samples associate with the frequent release of waste in these rivers. Shukor *et al.* (2013) use two monitoring technique; AChE inhibitive assay and ICP-OES to assess the contamination level in water sample at Prai Industrial Park at

different time interval. Determine that the metal ion concentration suddenly rising at 8.00 a.m. and drastically drop at 10.00 a.m. until sharply increase at 10.00 p.m which perhaps this situation is related to the release of waste without any detection by enforcement agencies. Biomonitoring on Malacca River was conducted where several metal cation such Cu and Cr was detected and significantly inhibiting (>20%) *Periophthalmodon schlosseri* AChE activity which considered as significant toxic to living organism (Sabullah *et al*., 2015c). From our observation, along Kuyuh river has a highest densities and activities of residential and industrial area compared to Sungai Pinji indicating the difference concentration of metal ion in both river. According to the number of multiple element in KR3 exhibiting more synergistic effect toward *O. hasselti* AChE. The present of Zn at low concentration in ER1 sample considered as natural occurrences that came from the weathering reaction on the surface of soil and rock, or from the decomposition of dead plant nutrient.

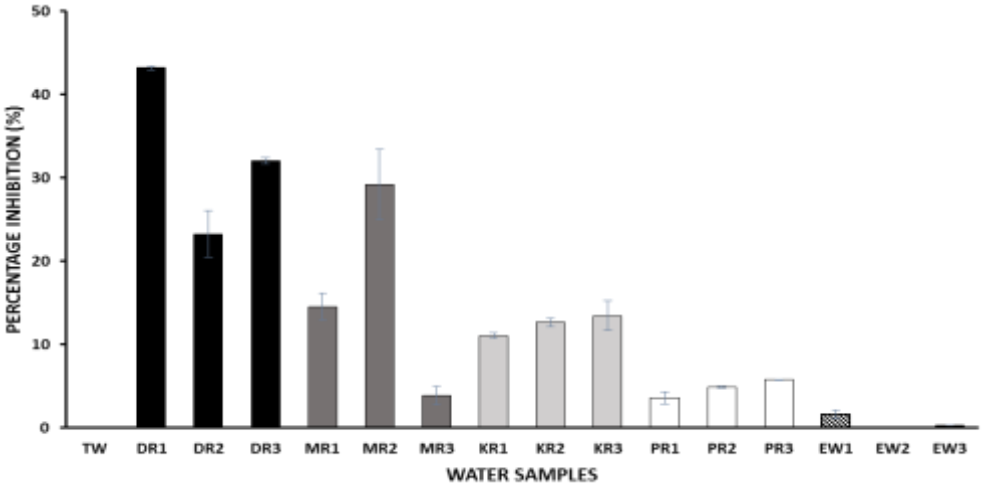

Figure 1. *Osteochilus hasselti* AChE was tested by incubated with different water samples; Derhaka River, Malacca River, Kuyuh River, Perak River and Endau-Rompin Waterfall denoted as DR, MR, KR, PR and EW, respectively. The numbers show different sampling location. All values represent mean ± standard deviation of mean (STDEV), n=3. TW denoted as tap water represents as clean and free contaminant sample

Table 2. Metal ion concentration in each water samples. The alphabet indicate that the same group of no significant different with same metal ion ($p<0.05$), while * show the standard deviation is lower than detection level. nd = not detected

Samples	Metal ion (ppm)							
	As^{2+}	Cd^{2+}	Co^{2+}	Cu^{2+}	Cr^{6+}	Mo^{6+}	Pb^{2+}	Zn^{2+}
DR1	0.063 ± 0.002^{ab}	0.037 ± 0.006^{ab}	0.119 ± 0.010	21.1 ± 0.032^{a}	1.185 ± 0.006^{a}	0.001^{a}*	3.191 ± 0.007^{a}	33.146 ± 0.023^{a}
MR2	0.052 ± 0.005^{b}	0.029 ± 0.001^{b}	n.d.	0.021 ± 0.004^{b}	1.808 ± 0.010^{b}	0.001^{a}*	n.d	0.022 ± 0.005^{b}
KR3	0.071 ± 0.008^{a}	0.004^{c}*	n.d.	1.944 ± 0.007^{c}	0.675 ± 0.005^{c}	0.005^{b}*	1.71 ± 0.003^{b}	0.004^{c}*
PR3	n.d	0.131 ± 0.003^{d}	n.d.	0.119 ± 0.005^{d}	0.129 ± 0.004^{d}	0.078 ± 0.001^{c}	0.055 ± 0.003^{c}	0.161 ± 0.002^{d}
EW1	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.008^{e}*

IV. SUMMARY

The study indicates that the inhibitive assay of *O. hasselti* AChE on collected river water samples proved the sensitivity and capable as an alternative tool for preliminary screening of river monitoring. The data was strengthen by secondary assessment using ICP-OES for quantitative analysis which show the concentration of metal ion correspond to the inhibition level of *O. hasselti* AChE activity.

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