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Heavy Metals Detection from Contaminated River Using Molybdenum Reducing Enzyme

(Pengesanan Logam Berat daripada Sungai Tercemar Menggunakan Enzim Penurun Molybdenum)

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ABSTRACT

Heavy metals contamination in Malaysia River has attracted concern among Malaysians. The issue arises when the Department of Environmental Malaysia in 2015 claimed the number of polluted river has increased from year 2000 to 2015. Therefore, the monitoring method is required in order to screen the polluted area. Current monitoring systems involved analytical methods such as Atomic Absorption Spectrophotometer (AAS) and Inductively Coupled Plasma (ICP) are costly, thus, the development of effective method and cost effective in screening heavy metal contamination is required. Molyhdenum-reducing enzyme has a potential to detect heavy metals contamination in river water samples. The enzyme extracted from locally isolated bacteria labeled as Bacilus sp. strain A.rzi was partially purified based on ion exchange chromatography using High Performance Liquid Chromatography (HPLC). The screening for heavy metals inhibition towards Mo-reducing enzyme was based on colorimetric analysis. Data for IC_{50} determined using statistical analysis (one phase decay plot) showed. Significant inhibition with three heavy metals (copper, lead and mercury) towards Mo-reducing enzyme. Copper showed the highest IC_{50} value of 0.25 mgL^{-1} followed by mercury and lead at 0.35 mgL^{-1} and 0.49 mgL^{-1} respectively. Screening for river water samples showed that Mo-reducing enzyme positively reacted to heavy metals in water samples. The Colorimetric based assay also showed heavy metals present in river water samples had inhibited Mo-reducing enzyme activity. The enzyme could reduce heavy metals to form molybdenum blue and was observed during reaction. Due to this unique feature, Mo-reducing enzyme can be proposed as a bioassay method for detecting heavy metal in river samples.

Keywords: Heavy metals; Bioassay; Molybdenum Blue; Molybdenum reducing enzyme

ABSTRAK

Pencemaran sungai di Malaysia yang berpunca dari logam berat telah menarik perhatian masyarakat di Malaysia. Isu ini menjadi perhatian apabila Jabatan Alam Sekitar Malaysia pada tahun 2015, melaporkan bahawa bilangan sungai yang tercemar semakin meningkat dari tahun 2000 hingga 2015. Oleh yang demikian, satu kaedah pemantauan diperlukan bagi menyaring kawasan-kawasan yang tercemar. Kaedah terkini melibatkan kaedah analitik seperti Atomic Absorption Spectrophotometer (AAS) dan Inductively Coupled Plasma (ICP) memerlukan kos yang tinggi. Bagi mengurangkan kos penyaringan sungai yang tercemar, pembangunan kaedah yang murah dan berkesan bagi mengesan pencemaran logam berat perlu dibangunkan. Enzim penurun molybdenum mempunyai potensi untuk mengesan pencemaran logam berat di dalam sampel air sungai. Enzim yang diekstrak dari bakteria tempatan yang diberi nama sebagai Bacillus sp. strain A.rzi melalui kaedah separa penulenan menggunakan kolum kromatografi penukaran ion dengan bantuan mesin High Performance Liquid Chromatography (HPLC). Pengesanan logam berat adalah melalui kaedah penukaran warna yang berlaku semasa perencatan enzim penurun molybdenum. Melalui analisis statistik, nilai IC_{50} ditentukan dan 3 logam berat (kuprum, plumbum dan merkuri) dikesan mampu merencat aktiviti enzim penurun molybdenum. Kuprum menunjukan bacaan yang paling sensitive dengan nilai IC_{50} sebanyak 0.25 mgL^{-1}diikuti oleh merkuri dan plumbum sebanyak 0.35 mgL^{-1} dan 0.49 mgL^{-1}. Penyaringan menggunakan air sungai yang sebenar menunjukan keputusan yang positif. Berdasarkan keputusan kajian ini, enzim penurun molybdenum boleh digunakan sebagai salah satu kaedah alternatif bagi mengesan logam berat di dalam sampel air sungai.

Kata kunci: Logam berat; Bioassai; Molybdenum biru; Enzim penurun molybdenum

INTRODUCTION

Heavy metal pollution has become an important issue in developing countries like Malaysia. Water quality monitoring of the 140 river basins in Malaysia shows the number of rivers being polluted has increased from years 2000 to 2015 (Environment 2015). River contamination is mainly caused by various factors and heavy metals is one of the major contributor towards this pollution (Shukor et al. 2009a). The source of heavy metals contamination usually come from landfill, open dump area and paddy field (Ismail et al. 2015; Mohamed et al. 2016). Urbanizations activity as well as industrial activities also contribute to heavy metals contamination especially in industrial state like Selangor (Lim et al. 2013; Yuswir et al. 2015; Kamil & Abdul-Talib 2010; Mukhtar et al. 2001). Since the number of polluted river is increasing yearly, more rapid and efficient method is needed to monitor the heavy metals pollution. The use of various microorganisms and their enzymes as a tool for detection of heavy metals were suggested. This technique known as bioassay allows screening of a higher number of samples and could perform frequent sampling. The samples with positive results can be identified and sent for instrumentation analysis. This system is time saving and could enhance monitoring efficiency system for heavy metals detection.

Enzymes can be used in the detection of heavy metals from the environment samples. Most of the enzymes used in bioassay show low sensitivity to heavy metals or high interference with other residue such as Ammonia (Zhang et al. 2016). Thus, more enzymes with high sensitivity and low interference are needed. The use of the Mo-reducing enzyme in detection of copper has been published previously (Shukor et al. 2009a). In this work, the enzyme of metal sensitive bacterium identified as *Bacillus* sp. strain A.rzi (Othman et al. 2013a) was extracted and will be used to detect copper, mercury and lead. An inhibitive concentration (IC_{50}) will be determined to measure it sensitivity towards (copper, mercury and lead). This enzyme will be tested for its sensitivity on river water samples taken from several places in Malaysia.

MATERIALS AND METHODS

CHEMICALS

Heavy metals (copper, mercury and lead) analytical standard was purchased from Merck (M) Sdn Bhd. Sodium molybdate (VII) analytical grade and other chemicals used in the media were obtained from Fisher (Fisher Scientific (M) Sdn. Bhd.).

STATISTICAL ANALYSIS

All data were subjected to one-way analysis of variance (ANOVA). Treatment means were compared using Tukey's multiple comparison tests. Statistic software Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA) was used for all statistical analyses.

PREPARATION OF CRUDE ENZYME

Bacillus sp. isolate A.rzi was grown in one liter medium at the optimum conditions obtained from the characterization studies done by Othman et al. (2013b). The cultivation was carried out at high phosphate molybdate medium to avoid in molybdenum blue complex and affecting the molybdenum reducing enzyme activity (Ghani et al. 1993; Shukor et al. 2008). The following experiments were carried out at 4°C unless stated otherwise. Harvested cells were centrifuged at 10 000 g for 10 minutes. Then, the cells were washed three times with distilled water. The cells were then resuspended and recentrifuged at 10 000 g for 10 minutes. The pellet was resuspended with 10 mL of 50 mM Tris buffer pH 7.0. The cells were then sonicated in ice bath for 20 minutes with 4 minutes interval rest. The sonicated fraction was centrifuged at 10 000 g for 20 minutes and the supernatant consisting crude enzyme was kept for further analysis.

PARTIAL PURIFICATION

Method of ion-exchange chromatography was chosen as it is low cost, able to accommodate high amounts of protein, able to cope with large sample volume and the ability to concentrate protein from dilute solution prior to elution process (Scopes 1994). Due to these properties, ion-exchange is the most popular fractionation method employed after ammonium sulphate fractionation (Scopes 1994). Preliminary studies showed that the Mo-reducing enzyme does not bind to cation-exchanger such as carboxymethyl-cellulose or CM-cellulose and thus an anion-exchanger; strong quaternary ammonium exchanger Mono Q were used. Mono Q (GE healthcare) with a 4 mL bed volume was equilibrated with 200 mL of 10 mM Tris buffer until the eluent reach pH 7.5. The crude fraction (2 mL) was loaded into the column and washed with 30 mL of 10mM Tris buffer. Two millilitres fractions were collected and assayed for Mo-reducing enzyme activity in the unbound fraction. A linear elution gradient of sodium chloride between 0 to 1000 mM NaCl (50 mL) in 10mM Tris buffer were generated using a gradient mixer. Two millilitres of fractions were collected and assayed for Mo-reducing enzyme in the eluted fractions.

Eluted proteins were monitored using a UV flow-through monitor set at 280 nm (Bio-Rad Laboratories, Inc. USA) and collected with a fraction collector (Bio-Rad Laboratories, Inc. USA). Peak profile was automatically recorded on a recorder chart.

EFFECT OF HEAVY METALS ON ENZYME ACTIVITY

Heavy metals such as mercury (II), arsenic (V), cadmium (II), lead (II), copper (II) and silver (II) prepared from Atomic Absorption Spectrometry (AAS) standard solutions from Merck. Heavy metals initially diluted in 0.1 M Tris-HCl buffer pH 7.0 to the final concentration of 20 mgL^{-1} to ensure that the nitric acid from the commercial heavy metals solution are neutralized. Specific volumes of heavy metals (as calculated) were then incubated with 100 µL of enzyme

for 20 minutes at 4°C. The mixture was then added into the assay mixture consisting Lab Prepared Phosphomolybdate (LPPM) and Nicotinamide Adenine Dineuclotide (NADH). The final volume of the reaction mixture was 1 mL. Absorbance was then measured at 865 nm.

DEVELOPMENT OF INHIBITIVE ENZYME ASSAY (IC_{50})

The inhibition study was further studied using various concentrations of the inhibitory heavy metals in order to determine the IC_{50} values. IC_{50} is defined as the toxicant concentration that gives 50% of inhibition towards enzyme activity (Shukor et al. 2006). It is important to determine the toxic concentration of toxicant towards certain enzyme. In all of the cases, one phase exponential decay model was used in determining the IC_{50} value for each of the heavy metals.

FIELD TRIALS

Field trial works were carried out on available heavy metals present in water samples to test the efficacy of the enzyme as prove for bioassay. Polluted water samples were collected from various locations in Malaysia include several streams in places that have been gazette as clean by the Malaysian Department of Environment (DOE) (DOE 2007) such as Ulu Bendul Recreational Jungle (UBRJ), Kuala Pilah, Negeri Sembilan (GPS; N 02°43.767' E 102°04.668') and Gunung Arong Forest Reserve (GAFR) and Mersing Johor, (GPS; N 02°33.197' E 102°45.340'). All water samples were collected in acid-washed Polypropylene bottles containing several drops of 1% (v/v) HNO_3. The samples were filtered with 0.45 µm syringe filter. The clear filtrate (100 µL) was mixed with 30µL Mo-Reducing enzyme and incubated for 30 minutes before being assayed as above. The determination of heavy metals in the samples was carried out using Inductively-Coupled Plasma Optical Emission Spectrophotometer on a Perkin Elmer 7500 ICP-OES. All experiments were performed in triplicates.

RESULTS AND DISCUSSIONS

PARTIAL PURIFICATION OF THE MO-REDUCING ENZYME

Partial purification of Molybdenum reduces enzyme produced by *Bacillus* sp. isolate A.rzi that was conducted using Mono-Q anion exchange column performed by High Performance Liquid Chromatography (HPLC). The separation was based on a linear gradient method using 1 M NaCl in 10 mM Tris buffer as an elution buffer. The fractions contained molybdenum reduced enzyme are shown in Figure 1. Approximately two-fold purification were achieved after anion exchange chromatography (Table 1). This value indicates that the difference for the specific activity between crude and partially purified enzyme. Compared to EC 48 (≈19 fold) (Shukor et al. 2003). The difference of the folding purification value between isolate A.rzi and EC 48 were due to the low in specific enzyme activity. Specific enzyme activity of molybdenum reduces enzyme in isolate A.rzi significantly low to EC 48 and believed due to the dissociation of the enzyme cofactor. Cofactors for molybdenum reducing enzyme can be easily dissociate through purification process (Claassen et al. 1982).

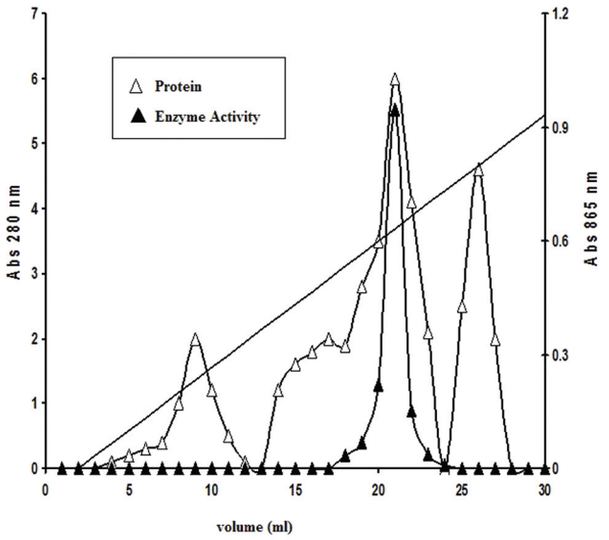

FIGURE 1. Mono Q anion exchange chromatography using HPLC of Mo-Reducing enzyme produced by *Bacillus* sp. isolates A.rzi. Sample was initially added and washed with 100 ml of Tris pH 7.5 at 4°C. The elution was commenced with the same buffer containing 1 M NaCl at a flow rate of ≈ 1 mL min^{-1} per tube and assayed for Mo-reductase activity and protein as described in the text. White triangle represents protein peak while black triangle represents enzyme activity peak throughout the purification process. Tube from 20 mL to 23 mL was collected for assays.

TABLE 1. Purification of Mo-reducing enzyme by *Bacillus* sp. isolate A.rzi.

Fraction	Total protein (mg)	Total activity (U)	Specific activity (Umg^{-1})	Percentage yield (%)	Fold purification
Crude	11.0	316.1	28.7	100	1
Ion-Exchange Chromatography (Mono-Q)	0.4	22.3	55.75	7.1	1.95

EFFECT OF HEAVY METALS ON MOLYBDENUM-REDUCING ENZYME

All heavy metals tested were standardized at 1 mgL^{-1} (ppm). There were three heavy metals showed an inhibition of more than 50%, mercury gave almost 60% of total inhibition. Lead showed an inhibition of up to 80%. Copper was the most potent heavy metal that resulted more than 90% inhibition. Only two heavy metals As and Cd have no inhibition towards the enzyme (Figure 2). With only three heavy metals (Cu, Hg, Pb) show inhibition more than 60%. Among these three heavy metals, copper gave the highest inhibition. Other report also claim that copper can inhibit the Mo-reducing enzyme from *S. marcescens* with IC_{50} value of 0.099 ± 0.013 mg/L

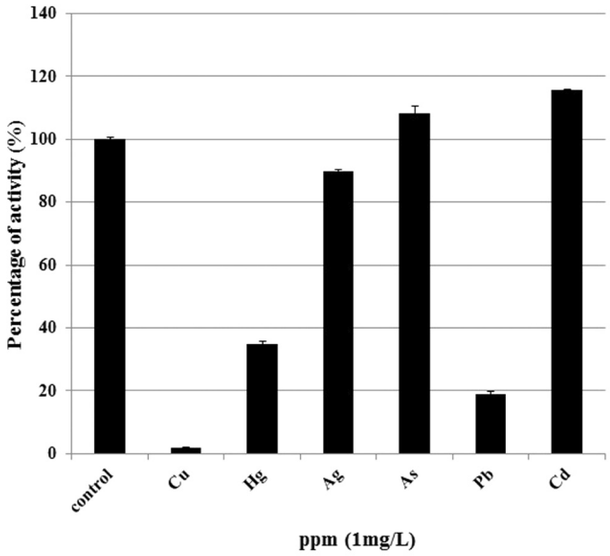

FIGURE 2. Screening of six heavy metals on molybdenum-reducing enzyme activity. Assay for control consist of Mo-reducing enzyme, NADH and LPPM. Enzyme showed high sensitivity at three heavy metals (Cu, Hg, and Pb). Each reading is a mean of 3 replicates and the bar represent standard error. Each reading is mean (±) standard error (n = 3)

(Shukor et al. 2009b). The mechanism of copper inhibition is still unclear while mercury and lead most probably inhibited the sulfhydryl group at the active sites.

INHIBITIVE CONCENTRATION (IC_{50}) FOR COPPER

The IC_{50} value for copper was 0.2467 mgL^{-1} with a regression coefficient of 0.98 (Figure 3). Detection for cooper inhibition gave IC_{50} value at 0.2467 mgL^{-1}. In comparison with enzyme isolated from *Serratia* sp. strain Dr Y5 showed more sensitivity towards copper with IC_{50} value is 0.099 mgL^{-1} (Shukor et al. 2009a). The best enzyme for copper detection is papain with LOQ (limits of quantitation) value of 0.004 mgL^{-1} (Shukor et al. 2006). Despite this, the system could still be used for copper detection in the environment since the maximum permissible limit by the Malaysian Government is 1.0 mgL^{-1} (MOH 2010).

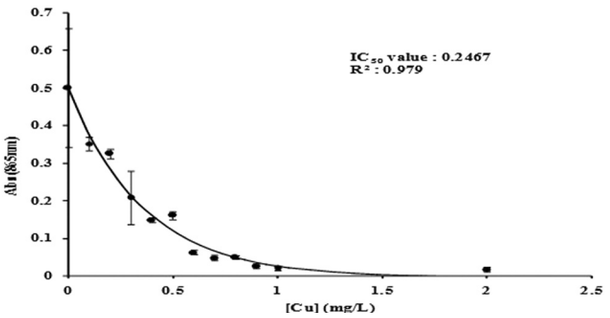

FIGURE 3. Inhibition of partially purified molybdenum-reducing enzyme by copper. Regression plot shows that IC_{50} value for copper is 0.25 mgL^{-1}. Each reading is a mean of 3 replicates and the bar represent standard error. Each reading is mean (±) standard error (n = 3)

INHIBITIVE CONCENTRATION (IC_{50}) FOR LEAD

Based on Figure 4, IC_{50} value for lead exhibited by the studied strain is 0.49 mg/L^{-1}. For lead inhibition assay, the IC_{50} value is low when compared to IC_{50} value from papain, where the value for papain detection was reported at 2.16 mgL^{-1} (Shukor et al. 2006). Even though the detection by analytical methods (AAS and ICP) more sensitive (up to μgL^{-1}) (Zhong, Ren & Zhao 2016), this system could be a promising tool for lead detection in the environment due to the in situ application.

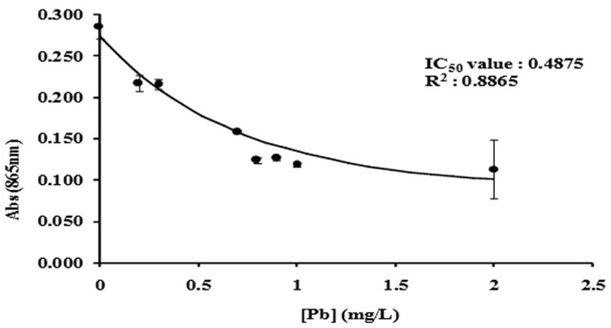

FIGURE 4. Inhibition of partially purified molybdenum-reducing enzyme by lead. Regression plot shows that IC_{50} value for lead is 0.49 mgL^{-1}. Each reading is a mean of 3 replicates and the bar represent standard error. Each reading is mean (±) standard error (n = 3)

INHIBITIVE CONCENTRATION (IC_{50}) FOR MERCURY

Figure 5 shows IC_{50} concentration of mercury detection. The value showed at 0.35 mgL^{-1} mercury inhibits 50% of the enzyme activity. For mercury inhibition activity showed that this enzyme has similar sensitivity towards mercury when compared to immobilized urease (Jung, Bitton & Koopman 1995). However, compared to Mo-reducing enzyme isolated from *Serratia sp*. Dr. Y5, partially purified Mo-reducing enzyme from Isolate A.rzi is more sensitive to mercury (Shukor et al. 2014). This assay is relatively fast with an assay time of 30 min. Other assays require longer period, and some require four days to produce results (Jung, Bitton & Koopman 1995). This system may not be a sensitive method

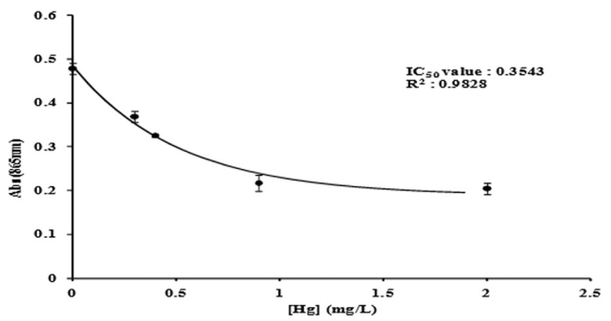

FIGURE 5. Inhibition of partially purified molybdenum-reduces enzyme by mercury. Regression plot shows that IC_{50} value for mercury is 0.35 mgL^{-1}. Each reading is a mean of 3 replicates and the bar represent standard error. Each reading is mean (±) standard error (n = 3)

for mercury detection in the environment since the maximum permissible limit is 0.001 mgL^{-1} (MOH 2010).

FIELD TRIAL OF PARTIALLY PURIFIED MOLYBDENUM-REDUCING ENZYME ACTIVITY

The results for the field analysis indicate that several samples collected from several spots showed positive results for heavy metal screening (Table 2). Based on the inhibition percentage, Sg. Derhaka Juru gave 100% of inhibition enzyme activity followed by water sample from Bukit Jutong, Pahang with 50% of enzyme inhibition. Analytical analysis for both water samples using Inductively Couple Plasma (ICP) and Atomic Absorption Spectrophotometry (AAS) analysis showed that there were presences of heavy metals. Sample from Sg. Derhaka Juru showed the presence of mercury, copper and lead at concentration of 18.46 mgL^{-1}, at 0.13 mgL^{-1} and 0.04 mgL^{-1} respectively. Slightly different for sample from Bukit Jutong, where only mercury and copper were detected at concentration of 0.48 mgL^{-1} and 0.85 mgL^{-1} respectively. The main objective for this experiment was to develop an enzyme based bioassay that can be used as heavy metals detector for environment samples. All seven samples are water samples, six of them collected at different places and tap water were used as the control sample. Various locations were chosen for sampling. One sample was taken from Sg. Derhaka Juru Penang. This site was considered as highly polluted area since it is located in industrial area. Second sample was taken from Bukit Juntong, Pahang. This area is slightly exposed to pollution, since the river that selected for sampling site is near to painting industry and other several industries. Other environmental water samples were mainly taken from the reserve forest and recreational park in Melaka, Negeri Sembilan and Johor. Samples assayed were compared with control assay. Based on absorbance taken, calculation based on percentage (%) value was constructed. The most polluted samples were obtained from Sg. Derhaka Juru, Juru, Penang. Based on the percentage calculation, this sample gave 100% of inhibition towards Mo-reducing enzyme. A hypothesis could be made that the water from Sg. Derhaka Juru was contaminated with heavy metals. Based on the inhibitive assays, heavy metals that probably present in the water from Sg. Derhaka Juru are copper, mercury and lead. To verify the hypothesis, sample from Juru was sent for Inductively Couple Plasma (ICP) and Atomic Absorption Spectrophotometry (AAS) analysis. The ICP and AAS analysis demonstrated a high concentration of mercury (18.46 mgL^{-1}) and copper (0.13 mgL^{-1}) in Sg. Derhaka Juru samples. Samples from Bukit Juntong, Bentong, Pahang exhibited nearly 50% of enzyme activity and was corroborated by Inductively Couple Plasma (ICP) and Atomic Absorption Spectrophotometry (AAS) analysis. ICP and AAS analysis showed that sample from Bukit Juntong contain copper and mercury at 0.848 mgL^{-1} and 0.475 mgL^{-1} respectively. Due to the heavy metals present in Bukit Juntong sample that was not very high, the inhibition towards Mo-reducing activity is lesser when compared to the sample collected from Penang. Another sample; from Kg. Ladang showed small amount of copper but not enough to show inhibition to the enzyme activity. Water from clean areas and tap water showed no inhibition to the enzyme activity.

TABLE 2. Concentrations of lead, mercury and copper in samples collected as determined from various location of peninsular Malaysia using Perkin Elmer ICP-OES 7500 and Atomic Absorption Spectrophotometer (AAS)

Locations	Inhibition of enzyme activity (%)	Concentration of Heavy metal (mg L^{-1})		
		Hg*	Pb*	Cu**
Sg. Derhaka Juru	100	18.46	0.04	0.13
Bukit Juntong, Pahang	50	0.475	n.d.	0.848
Kg Ladang, Gemas	0	n.d.	n.d.	0.080
Sungai Udang Recreational Jungle, Melaka (SURJ)	0	n.d.	n.d.	n.d.
Ulu Bendul Recreational Jungle, Kuala Pilah, Negeri Sembilan (UBRJ)	0	n.d.	n.d.	n.d.
Gunung Arong Forest Reserve, Mersing Johor (GAFR)	0	n.d.	n.d.	n.d.
Tap water	0	n.d.	n.d.	n.d.

*Represent data obtained from ICP-OES
**Represent data obtained from AAS

CONCLUSION

Molybdenum reduced enzyme purified from *Bacillus sp*. isolate A.rzi were proven to be sensitive towards copper, mercury and lead. IC_{50} analysis gave value for copper, mercury and lead at 0.25 mgL^{-1}, 0.35 mgL^{-1} and 0.49 mgL^{-1} respectively. Comparison between enzymatic bioassay and analytical analysis using ICP-OES and AAS showed a positive result towards heavy metals presence in environment. Thus, this finding suggests that molybdenum-reducing enzyme can be used as a bioassay for heavy metals detection in Malaysian river.

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REFERENCES

Claassen, V.P., L.F. Oltmann, J. Van 'T Riet, U.A.Th. Brinkman & A.H. Stouthamer. 1982. Purification of molybdenum cofactor and its fluorescent oxidation products. *FEBS Letters* 142(1): 133-37.

Environment, Department of Malaysia. 2015. Status kualiti air sungai berdasarkan pencemaran utama 2000-2015, Malaysia." Departemnt of Environment, Malaysia.

Ghani, B., Masataka, T., Zul, H.N., Noriaki, K., Mohamed, I.A.K., Tatsuo, T. & Tsuyoshi, S. 1993. Isolation and characterization of a Mo6+-reducing bacterium. *Applied and Environmental Microbiology* 59(4): 1176-80.

Ismail, S., Che, I., Mohd, S., Erneeza, H. & Aini, W. 2015. Soil contamination from non-sanitary waste landfill in langat water catchment area, Malaysia. *Journal of Scientific Research and Reports* 7(6): 480-93.

Jung, K., Bitton, G. & Koopman, B. 1995. Assessment of urease inhibition assays for measuring toxicity of environmental samples. *Water Research* 29(8): 1929-33.

Kamil, N.A.F.M. & Suhaimi, A.T. 2010. Hazards due to polycyclic aromatic hydrocarbons (pahs) and heavy metals at the closed Kubang Badak landfill, Selangor,*Proceeding IEEE (Institute of Electrical and Electronics Engineers: International Conference on Science and Social Research (CSSR 2010).*

Lim, W.Y., Ahmad, Z.A. & Tengku, H.T.I. 2013. Spatial geochemical distribution and sources of heavy metals in the sediment of Langat river, western peninsular Malaysia. *Environmental Forensics* 14(2): 133-45.

Ministry of Health. 2010. Drinking water quality surveillance programme – Ministry of Health. Engineering Service Division, Ministry of Health Malaysia. 2010.

Mohamed, Z., Rika, T., Juwaidah, S. & Golnaz, R. 2016. Determinants of paddy farmer's unsustainability farm practices. *Agriculture and Agricultural Science Procedia* 9, 191-96.

Mukhtar, A., Ahmed, W. & Norazmin, S. 2001. Evaluation of groundwater and soil pollution in a landfill area using electrical resistivity imaging survey. *Environmental Management* 28(5): 655-63.

Othman, A.R., Bakar, N.A., Halmi, M.I.E, Johari, W. L.W., Ahmad, S.A., Jirangon, H., Syed, M.A. & Shukor, M.Y. 2013. Kinetics of molybdenum reduction to molybdenum blue by *Bacillus* sp. strain A.rzi. *BioMed Research International* 2013: 1-9.

Scopes, R.K. 1994. *Protein Purification: Principles and Practice*. Springer-Verlag.

Shukor, M.Y., Habib, S.H.M., Rahman, M.F.A., Jirangon, H., Abdullah, M.P.A., Shamaan, N. A. & Syed, M.A. 2008. Hexavalent molybdenum reduction to molybdenum blue by *S. marcescens* strain dr. Y6. *Applied Biochemistry and Biotechnology* 149(1): 33-43.

Shukor, M.Y., Bakar, N.A., Othman, A.R., Yunus, I., Shamaan, N.A. & Syed, M.A. 2009a. Development of an inhibitive enzyme assay for copper. *Journal of Environmental Biology* 30(1): 39-44.

Shukor, M.Y., Halmi, M.I.E., Rahman, M.F.A. Shamaan, N.A. & Syed, M.A. 2014. Molybdenum reduction to molybdenum blue in *Serratia* sp. strain Dry5 is catalyzed by a novel molybdenum-reducing enzyme. *BioMed Research International* 2014: 1-8.

Shukor, Y., Nor, A.B., Fadhil, A.R., Mohd., P.A., Nor, A.S. & Mohd, A.S. 2006. Development of a heavy metals enzymatic-based assay using papain. *Analytica Chimica Acta* 566(2): 283-89.

Shukor, M.Y.A., Lee, C.H., Omar, I., Karim, M.I.A., Syed, M.A., Shamaan, N.A., Karlin, M.I.A. & Shamaan, A. 2003. Isolation and characterization of a molybdenum reducing enzyme in *Enterobacter cloacae* strain 48. *Pertanika Journal Science & Technology* 11(2): 261-72.

Yuswir, N.S., Sarva, M.P., Ahmad, Z.A., Sharifah, N.S.I., de Burbure, C. & Zailina, H. 2015. Heavy metal contamination in urban surface soil of Klang district (Malaysia). *Soil and Sediment Contamination: An International Journal* 24(8): 865-81.

Zhang, X., Yuqian, T., Yao, S., Nianpeng, H., Xuefa, W., Qiang, Y., Chunyu, Z., Xiaomin, S. & Weiwen, Q. 2016. Responses of soil hydrolytic enzymes, ammonia-oxidizing bacteria and archaea to nitrogen applications in a temperate grassland in inner Mongolia. *Scientific Reports* 6: 32791.

Wen-Si, Z., Ting, R. & Li-Jiao, Z. 2016. Determination of Pb (lead), Cd (cadmium), Cr (chromium), Cu (copper), and Ni (nickel) in chinese tea with high-resolution continuum source graphite furnace atomic absorption spectrometry. *Journal of Food and Drug Analysis* 24(1): 46-55.