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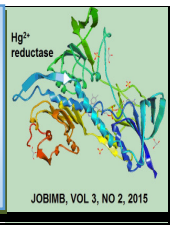

A Rapid Inhibitive Enzyme Assay for Monitoring Heavy Metals Pollution in a River Running Through a UNESCO Heritage Site

Baskaran Gunasekaran[1], Siti Aqlima Ahmad[1] and Mohd. Izuan Effendi Halmi[2]*

[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia
43400 UPM Serdang, Selangor, Malaysia.
[2]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia.

*Corresponding author:
Department of Land Management,
Faculty of Agriculture,
Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia.
Email: m_izuaneffendi@upm.edu.my

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ABSTRACT

The Malacca river runs through the Malacca UNESCO heritage site where a number of historical buildings are located. The river itself runs through several industrial sites that increase the chances of the water being polluted. Water pollution including heavy metals, in the long run, can damage the reputation of the site. Hence monitoring of the water quality needs to be done periodically. As the cost of instrumental monitoring is costly, biomonitoring using enzyme is being intensely developed worldwide. In this study, a rapid inhibitive enzyme assay using the molybdenum-reducing enzyme from the bacterium *Serratia* sp. strain DRY6 sensitive to the heavy metals mercury, copper, silver, and chromium was developed as a method for a rapid monitoring of heavy metals. The IC_{50} values for mercury, copper, silver and chromium were 0.268, 0.352, 0.393 and 0.499 mg L^{-1}, while the LOD values were 0.166, 0.071, 0.033 and 0.064 mg L^{-1}, respectively. The IC_{50} values for these heavy metals are comparable and in several cases, more sensitive than established assays. Water samples from various locations in the Melaka river were tested for the presence of heavy metals using the developed assay. Enzyme activity was found to be inhibited in one sampling location, but the concentration of metal ions on the site was found to be below the Maximum Permissible Limit according to Malaysian Environmental Quality standard. The assay for heavy metals can be completed in less than 10 minutes and can be carried out at ambient temperature. The assay is rapid and simple and can be used as a first screening method or even near real-time method for routine monitoring of heavy metals.

The considerable mining, manufacturing and agricultural activities in Malaysia have triggered the rise of pollution, which include toxicants such as pesticides, organic and inorganic compounds and heavy metals in the surrounding aquatic environment. The Malaysian Department of Environment (DOE) in 2011 has classified that out of 464 rivers, 189 are reported as contaminated or marginally contaminated. This is a worrying issue as heavy metals pose toxicity problems. Toxicants can result in possibly damaging consequences to humans, marine organisms, and food webs due to the fact a number of them are not completely degraded. Toxicants, particularly heavy metals, are dangerous to marine organisms. Consequently, it is essential to regularly monitor toxicants in polluted water bodies.

Instrumental detection of heavy metals is astronomically expensive, takes a long time to executed and is not amenable to near real time detection. A fairly easy and rapid course of action is needed to screen for the existence of toxic substances from industrial effluents, polluted rivers, as well as other polluted locations. Biomonitoring using inhibitive biological-based assays is a crucial alternative and is a breakthrough in mass monitoring of heavy metals in rivers. In addition, some assays especially using enzymes exhibit the ability to produce near real time results that can indicate a temporal variation of heavy metals' level. Inhibitive determination of heavy metals using living organisms and enzymes can be carried out by laymen and schoolchildren due to their simplicity, and allows the screening of thousands of samples per river with near real-time capability; an exercise which is important in forensic analysis of pollutants.

The use of bioassays such as enzymes and whole cells in monitoring exhibits excellent properties such as economic, simple, rapid detect only bioavailable metal ions instead of the water insoluble metal sulphides that are less toxic and not bioavailable. Systems using microorganisms in biomonitoring of heavy metals include luminescence bacteria-based assays such as Xenoassay Light [1], Microtox [2] and MTT-based assays [3,4]. Systems using enzymes include the use of urease [5], proteases [6–11], acetylcholinesterases [12–14] and the molybdenum-reducing enzyme [15–17].

Of the nearly 1800 rivers in Malaysia, nearly 43 are classified as class 5 indicating severe pollution [18]. The Malacca river runs through a UNESCO heritage site. Recently, several thousands of fish died due to unknown causes in the river [19]. The reputation of the heritage site is on stake due to such incidence. Due to this, routine monitoring of this river, especially monthly monitoring is very important as an assessment of the health of this river and remediation and forensic activities can be carried out when necessary. Routine monitoring is economically viable via biomonitoring that is coupled with instrumental validation. Biomonitoring using biological assays can be utilized as a pre-screening methods and only positive samples are sent for validation via instrumental methods such as Atomic Emission Spectrometry (AES) or Flow Injection Mercury System (FIMS).

The molybdenum-reducing enzyme has been utilized for heavy metal detection as the enzyme is only inhibited mainly by heavy metals. The assay is rapid and assay time of less than 5 minutes can be routinely achieved. However, to date only single element detection for a particular molybdenum-reducing enzyme system has been achieved [15–17]. The product of molybdenum (sodium molybdate) reduction is molybdenum blue that is measured at 865 nm, a wavelength that sits near the infrared region.

The Mo-reducing enzyme is a novel enzyme that has been purified [20] but not fully identified. The enzyme helps bacteria to combat the toxicity of soluble molybdenum by converting it to a colloidal form [21–32]. This colloidal form can be contained in membrane such as dialysis tubing, and is a promising future method not only for bioremediation of molybdenum but for the potential recycling of molybdenum from industrial effluents [33,34]. During our work with the molybdate-reducing bacterium *Serratia* sp. strain DRY6 a local isolate [21], we found that the molybdenum-reducing (Mo-reducing) enzyme or activity is strongly inhibited by the heavy metals Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}.

MATERIALS AND METHODS

Source, growth and maintenance of *Serratia* sp. strain DRY6

The bacterium was previously isolated from the state of Perak in 2008. The growth and maintenance of the bacterium was carried out using the low phosphate medium supplemented with molybdenum as outlined previously [21]. The composition (w/v%) of the medium (pH 7.0) is as follows: NaCl (0.5), glucose (1.0), $(NH_4)_2SO_4$ (0.30), Na_2HPO_4 (0.05), $MgSO_4.7H_2O$ (0.05), yeast extract (0.05) and $Na_2MoO_4.2H_2O$ (0.484). The bacterium was maintained on solid media supplemented with 1.5% agar on this medium. The low phosphate media positively maintained the molybdenum-reducing property of this bacterium.

Molybdenum-reducing enzyme assay

The enzyme assay utilized in this work consists of NADH as the electron donor and 12-phosphomolybdate as the electron acceptor. The enzyme quickly reduces 12-phosphomolybdate to molybdenum blue. Briefly, phosphomolybdic acid or 12-phoshomolybdate (sodium phosphomolybdate hydrate, Sigma, St. Louis USA) was prepared as a 50 mM stock solution in 10 mM phosphate buffer pH 5.8 and added to a 1 mL enzyme reaction mixture to a final concentration of 3 mM [35,36]. NADH (30 mM stock) is added to a final concentration of 2.5 mM. Fifty microlitres of crude molybdenum-reducing enzyme fraction (1 mg ml^{-1} final protein) was added to start the reaction. The total reaction mixture was 1 ml.

The absorbance increase after an incubation period of exactly one minute at ambient temperature was read at 865 nm. One unit of molybdenum reductase activity is defined as that amount of enzyme that produce 1 nmole molybdenum blue per minute at room temperature. The specific extinction coefficient for quantifying molybdenum blue is 16.7 $mM.^{-1}.cm^{-1}$ at 865 nm. An increase in OD 865 nm of 1.00 unit absorbance per minute per mg protein would yield 60 nmole of Mo-blue in a 1 ml assay mixture [36].

Preparation of crude enzyme

For large-scale growth, the bacterium was grown in a high phosphate media with the only difference to the low phosphate media is the concentration of the phosphate, which was raised to 100 mM to prevent molybdenum blue formation but still maintaining Mo-reducing activity of the bacterium [36]. Molybdenum blue formation is a problem for cell harvesting, thus the need to increase the phosphate concentration. The media still contained molybdate. The bacterium was grown in several 250 mL conical flasks with the total volume of the growth media was 5 L. Growth was carried out at 30ºC for 48 hours on an orbital shaker set at 120 rpm (Yihder, Taiwan).

Protein was assayed using the Coomassie dye-binding method using crystalline BSA as the standard. The following experiment was carried out at 4 ºC unless stated otherwise. Cells were harvested through centrifugation at 10 000 g for 10 minutes. Cells pellet were briefly washed at least once with 50 mM Tris.Cl buffer pH 7.5 (Tris buffer prepared at 4 ºC), resuspended in a minimal volume of the same buffer and recentrifuged. The pellet was reconstituted with 10 ml of the above Tris.Cl buffer but supplemented with 0.1 mM dithiothreitol. Cells were sonicated for 1 minute on an ice bath with 4 minutes cooling until a total sonication time of at least 20 minutes was achieved. The sonicated fraction was centrifuged at 10 000 g for 20 minutes and the supernatant consisting of the crude enzyme fraction was taken. The enzyme has an optimum pH of 6.0 and a broad maximum activity between 25 and 35 ºC (unpublished results). The Mo-reducing enzyme was freeze dried and was stable for more than one year when stored at -20 ºC.

Preparation of heavy metals solutions

The initial screening for enzyme inhibition was carried out using several heavy metals at the final concentration of 1 mg L^{1}. The heavy metals tested were chromium (VI) ($K_2Cr_2O_7$, BDH), nickel (II) ($NiCl_2$, (Ajax Chemicals), zinc (II) ($ZnSO_4$ anhydrous J.T. Baker). These heavy metals were prepared from their commercial salts. Other heavy metals such mercury (II), arsenic (V), cadmium (II), lead (II), copper (II) and silver (I) were prepared as stoc solutions from Atomic Absorption Spectrometry standard solutions from Merck, and were initially diluted in 50 mM Tris.Cl pH 7.0 to the final concentration of 20

mg l^{-1} to ensure that the nitric acids from the commercial heavy metals solution have been neutralized. Regression curves for the enzyme inhibition were generated using the PRISM (Prism version 5.00 for Windows) non-linear regression analysis software available from GraphPad, (GraphPad Software Inc., San Diego, CA).

Real water samples monitoring works

River water samples were collected from the Malacca river in polycarbonate containers and brought to the lab in chilled containers. The samples were collected in 2012-2013. The pH of the water samples was between 6.5 and 7.5. The water samples were initially passed through Teflon membrane filter (0.45 µm). Suitable volumes of heavy metals or river water samples of up to 50 µl were directly incubated with 50 µl of enzyme for 5 minutes at room temperature. The mixture was then assayed as above at room temperature. The determination of heavy metals in the samples was carried out using Atomic Emission Spectrometry on a Perkin Elmer Optima 3000 ICP-AES. Mercury was determined using a Perkin Elmer Flow Injection Mercury System (FIMS). All experiments were performed in triplicate.

Calculation for percent of inhibition

The percent inhibition was computed according to following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

RESULTS AND DISCUSSION

The Mo-reducing enzyme is a novel enzyme that has been purified but not fully identified. The enzyme helps bacteria to combat the toxicity of soluble molybdenum by converting it to a colloidal form. This colloidal form can be contained in membrane such as dialysis tubing, and is a promising future method not only for bioremediation of molybdenum but for the potential recycling of molybdenum from industrial effluents.

Enzyme inhibition studies

Using the newly developed assay we screened for the inhibitive effects of heavy metals on the assay. Out of the heavy metals tested at the final concentration of 1 mg l^{-1}, mercury, silver, copper and chromium showed more than 50% inhibition (**Fig**. 1). The IC_{50} values for mercury, copper, silver and chromium were 0.268, 0.352, 0.393 and 0.499 mg L^{-1}, while the LOD values were 0.166, 0.071, 0.033 and 0.064 mg L^{-1}, respectively. Two models were found to give the highest correlation coefficient values for modelling the inhibition of heavy metals to the Mo-reducing enzyme from this bacterium. The best model for describing the mode of inhibition of mercury was four-parameter logistics while the one phase exponential decay best describes the mode of inhibition for the other heavy metals (**Table** 1).

Based on the LOD values for all of these heavy metals with the exception of mercury, the developed assay could detect copper, silver and chromium at the Maximum Permissible Limit as outlined by the Malaysian Department of Environment [18]. Reproducibility of the developed assays was assessed by repeated measurement of the enzyme inhibition by the heavy metals The resulting CV (coefficient of variation) of the replicated data was from 7 to 15% suggesting adequate reproducibility. In biological system mercury binds preferably to sulfhydryl groups. The element also binds with lesser affinity to phosphoryl, carboxyl, amide and amine groups. This makes mercury one of the most toxic metal ions. Both silver and chromium (chromate) also bind to sulfhydryl groups of enzymes [37]. Copper inactivates enzyme by binding to cysteine, histidine, and methionine residues of enzymes [38].

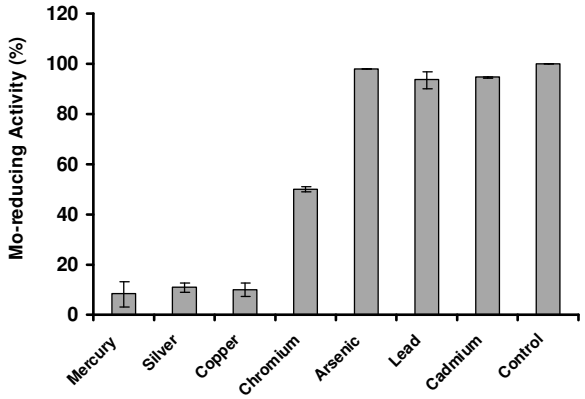

Fig. 1. Screening results for the inhibitory effect of heavy metals on the Mo-reducing enzyme assay. Data is mean± standard error (n=3).

Table 1**.** Correlation coefficient, most effective nonlinear model and Limits of Detection (LOD) of developed assay, and comparison with Maximum Permissible Limit as outlined in the interim national water quality standards for Malaysia [18].

Heavy metals	Regression model	R^2	IC_{50} (mg/L) (95% CI)	LOD (mg/L) (95% CI)	MPL (Class IIA and IIB)
Hg	Four-parameter logistics	0.99	0.268 (0.254-0.283)	0.166 (0.154-0.178)	0.001
Cu	One phase exponential decay	0.99	0.352 (0.295-0.435)	0.071 (0.055-0.086)	0.020
Ag	One phase exponential decay	0.99	0.393 (0.339-0.466)	0.033 (0.018-0.046)	0.050
Cr	One phase exponential decay	0.99	0.499 (0.401-0.658)	0.064 (0.044-0.081)	0.050

The sensitivity of the developed method to other established assays was then compared based on IC_{50}. The process of evaluating whether a developed method is more sensitive than established method is through looking at the confidence interval of the IC_{50}. If the confidence intervals of two IC_{50} values are not overlapping, then we can infer that the two IC_{50} values differ significantly from each other at the $p<0.05$ level. On the other hand, overlapped confidence intervals do not indicate nonsignificant between the two values, but designates that further experimentations are needed to show non-significance [39]. In the developed method, the IC_{50} value for copper shows that it is more sensitive than published for instance achromopeptidase and AChE from *Electrophorus electricus*, while other assays such as AChE from *Electrophorus electricus*, AChE from *Pangasius* sp., papain and 48 hours *Daphnia Magna* are more sensitive than the developed assay for copper.

Overlapped confidence intervals with other assays such as immobilized urease, 15-min Microtox and 96 hours Rainbow trout meant that the assay is comparable in sensitivity to copper although more data is needed to assess nonsignificant between the assay methods. The IC_{50} value for mercury shows that the developed assay is more sensitive than the papain assay, and less sensitive than, AChE from *Electrophorus electricus*,

achromopeptidase, AChE from *Pangasius* sp. and 15-min Microtox assays while other assays such as immobilized urease, *Daphnia magna* and rainbow trout show overlapped confidence intervals. The IC_{50} value for chromium shows that it is more sensitive than achromopeptidase, AChE from *Pangasius* sp., 96-h rainbow trout, 48-h *Daphnia magna*, 15-min Microtox and immobilized urease assays, and shows overlapped confidence interval with AChE from *Electrophorus electricus* while other assays are not sensitive at all to chromium. the IC_{50} value for silver shows that it is more sensitive than the AChE from *Pangasius* sp., papain, 48-h *Daphnia magna* and 96-h rainbow trout assays and other assays are not sensitive at all.

The results (**Table** 2) shows that he IC_{50} value for copper was higher than the papain assay, within the range of the *Daphnia magna* assay and lower than the rest of the assays. The IC_{50} value for lead was lower than the rest of the assays. The IC_{50} value for mercury was within the range of the Microtox™, *Daphnia magna* and rainbow trout assays and lower than the rest of the assays. The IC_{50} value for chromium was within the range of the *Daphnia magna* assays and lower than the rest of the assays.

In general, the Mo-reducing enzyme based assay was better than the rest of the assays. All of the assays using live organism needed precise temperature-controlled water bath, infrastructures for housing and rearing the organisms to get reproducible results [3]. The assays using urease and papain requires several development steps [5,6], and the urease assay takes at least 2 hours to complete with the requirement of a water bath precluding its use for field trial works. This assay is a one-step inhibitive assay, making it an attractive assay to be used in field trials.

Table 2. Comparison of developed assay to various other assays. The range is C.I. (95% Confidence Interval).

	LC_{50} or IC_{50} (mg/l)									
Metal	Urease[a]	15-min. Microtox™ [a, c]	48 hours *Daphnia Magna*[a]	96 hours Rainbow trout[a,c]	Papain[b]	AChE from *Pangasius* sp.[d]	Achromo-peptidase[e]	AChE from *E. electricus*[g]	This study
Cu^{2+}	0.14-0.41	0.076-3.8	0.020-0.093	0.25	0.004	0.065- 0.096	6.65-9.63	0.64-1.690	0.295-0.435
Hg^{2+}	0.021-	0.029-0.05	0.0052-0.21	0.033-0.21	0.24-0.62	0.059-0.088	0.10-0.14	0.084-0.115	0.154-0.178
Cr^{6+}	0.33	13	0.1-1.8	11	n.i.	0.80-0.97	4.65-5.57	0.074-0.29	0.044-0.081
Ag^{+}	2.5-36.1 n.a.	n.i.	1.930	0.05	0.33-0.49	0.082-0.095	n.i.	n.i.	0.018-0.046

Note
[a][5]
[b][6]
[c][2]
[d][7]
[e][40]
[f][11]
[g][13]
n.a. Not available
n.i no inhibition

Evaluation of developed assay

The developed assay was then tested on water samples from Malacca river. None of the water samples tested showed more than 10% of enzyme inhibition (**Table** 3). Heavy metal analysis showed that these water samples did not contained heavy metals at levels above the maximum permissible limit. The results indicate that the Malacca river was free of elevated concentration of toxic heavy metals. Future monitoring of this UNESCO heritage site is important for positive representation of an efficient preservation of the site by the relevant authority.

Table 3. Table of percentage activity (%) the MO-reducing enzyme and the concentration of heavy metals contained in each Malacca River samples as determined by ICP-OES.

Melaka river samples	GPS location	Percentage activity (%)	Concentration of heavy metals presence in the samples (mg/l)			
			Cu^{2+}	Hg^{2+}	Cr^{6+}	Ag^{+}
Malacca 1	N 2^0 12.466' E 102^0 15.096'	99.02	n.d.	n.d.	n.d.	n.d.
Malacca 2	N 2^0 12.414' E 102^0 15.075'	94.15	n.d.	n.d.	n.d.	n.d.
Malacca 3	N 2^0 12.388' E 102^0 15.022'	95.03	n.d.	n.d.	n.d.	n.d.
Malacca 4	N 2^0 12.361' E 102^0 15.056'	94.02	n.d.	n.d.	n.d.	n.d.
Malacca 5	N 2^0 12.360' E 102^0 15.061'	92.19	n.d.	n.d.	n.d.	n.d.
Malacca 6	N 2^0 12.283' E 102^0 15.078'	98.73	n.d.	n.d.	n.d.	n.d.
Malacca 7	N 2^0 12.257' E 102^0 15.084'	99.86	n.d.	n.d.	n.d.	n.d.
Malacca 8	N 2^0 12.214' E 102^0 15.096'	98.21	n.d.	n.d.	n.d.	n.d.

n.d., not detected.

CONCLUSION

A sensitive and rapid assay for heavy metals using the molybdenum-reducing enzyme have been developed. The assay is comparable in sensitivity to many of the currently developed inhibitive assays and shows good reproducibility. The enzyme is sensitive to the heavy metals mercury, silver, copper and chromium and was successfully used to demonstrate and absence of heavy metals in a UNESCO heritage site in Malaysia. The developed assay is economic, rapid and simple and can be used as a biomonitoring tool.

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