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A Rapid Inhibitive Enzyme Assay for Monitoring Heavy Metals Pollution in the Juru Industrial Estate

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ABSTRACT

The volume of contaminated rivers in Malaysia continues to keep rising through the years. The cost of instrumental monitoring is uneconomical and prohibits schedule monitoring of contaminants particularly heavy metals. In this work, a rapid enzyme assay utilizing the molybdenum-reducing enzyme as an inhibitive assay, prepared in crude form from the molybdenum-reducing bacterium *Serratia* sp. strain DRY5 has been developed for monitoring the heavy metals mercury, silver, copper and chromium in contaminated waters in the Juru Industrial Estate. The crude enzyme extract transformed soluble molybdenum (phosphomolybdate) into a deep blue solution, which is inhibited by heavy metals such as mercury, silver, copper and chromium. The IC_{50} and Limits of Detection (LOD) values for mercury, copper, silver and cadmium were 0.245, 0.298, 0.367, 0.326, and 0.124, 0.086, 0.088 and 0.094 mg L^{-1}, respectively. The assay is rapid, and can be carried out in less than 10 minutes. In addition, the assay can be carried out at ambient temperature. The IC_{50} values for these heavy metals are more sensitive than several established assays. Water samples from various locations in the month of November from the Juru Industrial Estate (Penang) were tested for the presence of heavy metals using the developed assay. Enzyme activity was nearly inhibited for water samples from several locations. The presence of heavy metals was confirmed instrumentally using Atomic Emission Spectrometry and a Flow Injection Mercury System. The assay is rapid and simple and can be used as a first screening method for large scale monitoring of heavy metals.

INTRODUCTION

The heavy metals mercury, lead, chromium and cadmium continue to persist in the environment, and also have been discovered to pollute water bodies all across the globe. This has motivated the introduction of a variety of technological innovation to monitor and remediate them. The primary source of these heavy metals pollution is anthropogenic activities [1–

4]. The primary sites of heavy metals absorption into the body of a human is via the digestive tract. Persistent contact with heavy metals can result in numerous health problems including liver and kidney malfunction, learning failures, neuropsychiatric trouble, sterility, tiredness, gout, headache, high blood pressure, and secondary bacterial infections [5]. One of the most notorius cases of metal pollution in Malaysia comes from the abandoned copper mine in Mamut Sabah, where episodic ruptures of metal-carrying pipes have contaminated the surrounding areas [6]. Industrial sites such as in the Juru and Prai Industrial Estates have also been reported to contribute to metal pollutions [7,8].

Current monitoring of heavy metals in Malaysia is a challenge as there are about 180 major river basins in Malaysia. Of these, about 147 basins are being continuously monitored. The rivers are classified from 1 to 5, with class 5 being the most polluted. Currently, only water parameters such as total suspended solid, turbidity, pH, biological- (BOD) and chemical oxygen demands (COD) are reported annually while data on heavy metals content are considered too expensive to be monitored for all rivers. Of the nearly 1800 rivers in Malaysia, nearly 43 are classified as class 5 indicating severe pollution. The waters of these rivers are deemed not suitable for agriculture and drinking [9]. A trace amount of heavy metals can cause a river to be classified as class 5. In the case of mercury, its value must not exceed the maximum permissible limit (MPL) of 0.001 mg/L.

Independent sporadic testing of several of the rivers in Malaysia has indicated that several of these rivers may need reclassification as their heavy metals content was found to exceed the MPL [7,10,11]. Schedule monitoring of such rivers, particularly month-to-month monitoring is vital as an evaluation of the wellbeing of these rivers. In addition, reclassification exercise can be executed if pollution is confirmed. Schedule monitoring is fiscally practical via biomonitoring that is in conjunction with instrumental affirmation. Biomonitoring making use of biological assays works extremely well as pre-screening strategies and just positive samples are routed for validation via instrumental strategies such as Atomic Emission Spectrometry (AES) or Flow Injection Mercury System (FIMS). Present microbial systems employed in biomonitoring of heavy metals involve luminescence bacteria-based assays such as Microtox [12] and Xenoassay Light [13] and MTT-based assays [14,15]. Enzyme-based assay systems include the use of enzymes such as urease [16], proteases [7,8,17–20] and acetylcholinesterases [21–23].

Our studies with molybdenum-reducing bacteria that converts the relatively nontoxic metal to molybdenum blue, we discovered that this process is strongly inhibited by heavy metals. The type of heavy metals which inhibit the process is species-specific and the sensitivity for a particular metal ion also varies between bacterial species [24–27]. This present a vast pool of bioassay reservoir. In this work, we report on the development of an assay for the heavy metals mercury, silver, copper and chromium using the molybdenum-reducing enzyme from another source of the bacterium and succesfully uses this assay to monitor heavy metals pollution in Juru, an industrial complex in Penang.

MATERIALS AND METHODS

Source, growth and maintenance of *Serratia* sp. strain DRY5

The bacterium was previously isolated from the state of Perak [28]. A slightly modified low phosphate medium supplemented with molybdenum was utilized as for the growth and maintenance of this bacterium [28]. The low phosphate media positively maintained the molybdenum-reducing property of this bacterium. The composition (w/v%) of the medium (pH 7.0) was 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.

Molybdenum-reducing enzyme assay

A previously-developed enzyme assay was utilized for this work, and consists of NADH as the electron donor and 12-phosphomolybdate as the electron acceptor [29]. 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 [29,30]. 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) were 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 produces 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 [29].

Preparation of crude enzyme

The bacterium was grown in a high phosphate media for large-scale growth, with the only difference to the low phosphate media is the concentration of the phosphate (100 mM). This hig concentration of phosphate prevents molybdenum blue formation but the Mo-reducing activity of the bacterium is still intact [29]. 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 by 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 between 6.0 and 6.5, and a broad maximum activity of between 28 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 in the presence of 0.1 mM phenylmethanesulfonate fluoride (PMSF) as an inhibitor of proteases.

Preparation of heavy metals solutions

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 stock 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. The initial screening for enzyme inhibition was carried out using several heavy metals at the final concentration of 1 mg L^1. 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 Juru river and rivers surrounding the Juru Industrial estates in polycarbonate containers and brought to the lab in chilled containers. The samples were collected in November 2012. 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 the 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 the 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 [31] but not fully identified. The enzyme helps bacteria to combat the toxicity of soluble molybdenum by converting it to a colloidal form [28,32–42]. This colloidal form can be contained in a membrane such as a dialysis tubing, and is a promising future method not only for bioremediation of molybdenum but for the potential recycling of molybdenum from industrial effluents [43,44].

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}, silver, cadmium, mercury and copper showed 97.22, 94.68, 92.88 and 91.52% inhibition, respectively (**Fig.** 1). The IC_{50} and Limits of Detection (LOD) values for mercury, copper, silver and cadmium were 0.245, 0.298, 0.367, 0.326, and 0.124, 0.086, 0.088 and 0.094 mg L^{-1}, respectively (**Table** 1). 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, the developed assay could not detect these heavy metals at the Maximum Permissible Limit as outlined by the Malaysian Department of Environment [9]. 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 8 to 14% suggesting adequate reproducibility. Mercury is one of the most toxic metal ions. In biological system mercury binds preferably to sulfhydryl groups and lesser affinity to phosphoryl, carboxyl, amide and amine groups. Both silver and cadmium bind to sulfhydryl groups of enzymes [45]. Copper is reported to inactivate enzyme by binding at the cysteine, histidine, and methionine residues of enzymes [46].

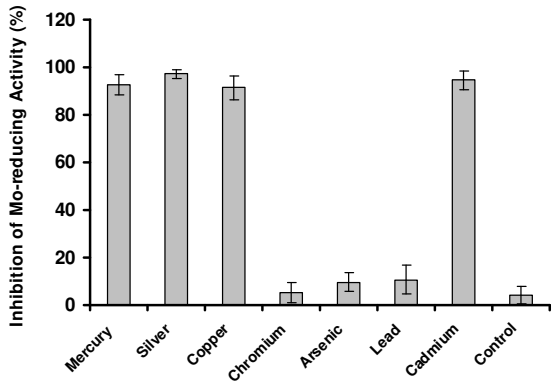

Fig. 1. Screening results for the inhibitory effect of heavy metals on the Mo-reducing enzyme assay. Data is mean± standard error (n=3).

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 [9].

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	0.245 (0.224-0.272)	0.124 (0.103-0.146)	0.001
Cu	One phase exponential decay	0.99	0.298 (0.245-0.384)	0.086 (0.058-0.122)	0.020
Ag	One phase exponential decay	0.99	0.367 (0.323-0.397)	0.088 (0.072-0.127)	0.050
Cd	One phase exponential decay	0.98	0.326 (0.289-0.414)	0.094 (0.078-0.135)	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 the 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 further experimentations are needed to show non-significance [47].

In the developed method, the IC_{50} value for copper is within the range of the bromelain, 96 hours Rainbow trout, 15-min. Microtox and the Mo-reducing enzyme from *Serratia* sp. strain DrY8 assays and less sensitive than the rest of the assays. The IC_{50} value for mercury is within the range of the papain assays and less sensitive than the rest of the assays. The IC_{50} value for cadmium is within the range of the 96 hours Rainbow

trout, 48 hours *Daphnia magna* and the urease assays, more sensitive than the AChE from *Pangasius* sp., bromelain, and the 15-min. Microtox assays while it is less sensitive than the papain assay. The IC_{50} value for silver is within the range of the papain assay, more sensitive than the bromelain, 48 hours *Daphnia magna*, 15-min. Microtox and bromelain assays, while it is less sensitive than the AChE from Pangasius sp., 96 hours Rainbow trout and the Mo-reducing enzyme from Serratia sp. strain DrY8, assays (**Table** 2). All of the assays using live organism needed precise temperature-controlled water bath, infrastructures for housing and rearing the organisms to get reproducible results [14]. The assays using urease and papain requires several development steps [7,16], 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)							
Metals	15-min. Microtox™ [a, c]	48 hours *Daphnia magna*[a]	96 hours Rainbow trout[a c]	Papain[b]	Bromelain[d]	AChE from *Pangasius* sp.	This study	Mo-reducing enzyme from *Serratia* sp. strain DrY8[e]
Cu^{2+}	0.076-3.8	0.020-0.093	0.25	0.004	0.163-0.305	0.065- 0.096	0.245-0.384	0.295-0.435
Hg^{2+}	0.029-0.050	0.005-0.21	0.033-0.21	0.24-0.62	0.13-0.16	0.059-0.088	0.224-0.272	0.154-0.178
Cd^{2+}	19-220	0.041-1.9	0.15-2.5	0.1	n.i.	0.85-0.99	0.289-0.414	n.i.
Ag^{+}	n.i.	1.930	0.05	0.33-0.49	n.i.	0.082-0.095	0.323-0.397	0.108-0.046

Note
[a][27]
[b][7]
[c][12]
[d][8]
[e][21]
n.i no inhibition

Evaluation of developed assay

The developed assay was then tested on water samples from the Juru industrial estate (**Fig**. 2). Several of the water samples tested showed more than 50% enzyme inhibition. Copper appears as the main pollutant with four out of the seven samples contained elevated levels of this heavy metal. Heavy metal analysis showed that these water samples contained heavy metals at levels above the maximum permissible limit.

The MPLs for mercury, silver, copper and chromium for Class IIA (water supply) and Class IIB (recreational use body contact) are 0.001, 0.050, 0.020 and 0.050 mg L^{-1} [9]. This indicates that most of the water samples tested that inhibit the developed assay contained heavy metals above the MPL suggesting that the developed assay successfully detected high concentrations of heavy metals in real water samples. The results also showed that several locations in the Juru industrial estate are polluted with heavy metals, at least at the time of sampling. More frequent sampling can reveal whether the elevated level of heavy metals found in this work is a one-time case of heavy metal pollution or is an indicative of a continual source of pollution from industries nearby.

The Juru industrial estate is one of the oldest industrial sites in Malaysia. Numerous industries including metal works are located in this location. Water bodies from this site are notorious for reports of their pollution including heavy metals. Biomonitoring activities have been carried out in this area in the hope that they would aid in reducing the cost of expensive instrumental-based approach by providing as an early detection system [20].

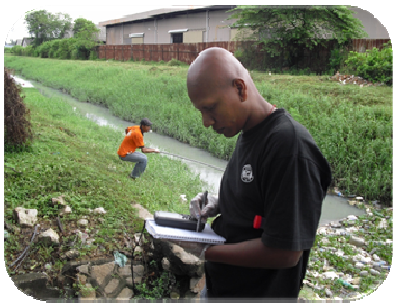

Fig. 2. Water sampling in the Juru Industrial Estate by graduate students.

Currently, rivers with levels of heavy metals above the MPL are automatically assigned as a class 5 river, indicating severe pollution. Unfortunately, the determination of the concentrations of heavy metals in the 180 river basins are done sporadically and done on a need-to-know basis. Independent studies indicate a worrying trend of pollution over the years with no dissipation in sight. Many of these rivers are used as sources of farming and irrigation, and the heavy metal content can cause acute and chronic effects [7,9,13,20,23,48,49].

Table 3. Evaluation of the developed assays on polluted water from the Juru Industrial Estate, Penang on November 2012.

	% Inhibition of Mo-reducing Enzyme Activity1 n=3	Concentrations of Heavy metal (mg/L)			
		Cd	Ag	Cu	Hg
N 05° 20.447 E100° 26.403'	0	n.d	n.d	n.d	n.d.
N05° 20.665' E100° 26.364'	2	n.d	n.d	n.d	n.d.
N05° 20.601' E100° 26.427'	4	n.d	n.d	n.d	n.d.
N05° 20.640' E100° 26.470'	100	n.d	0.33±0.01	0.64±0.01	0.09±0.00
N05° 18.947' E100° 26.348'	100	n.d	0.12±0.023	0.92±0.02	0.11±0.01
N 05° 20.96, E 100° 24.17'	100	n.d	n.d.	6.13±0.09	n.d.
N 05° 20.96, E 100° 17.25'	100	n.d	0.05±0.001	2.34±0.04	0.12±0.001
University Putra Malaysia	0	n.d	n.d.	n.d.	n.d.

[1] 20% Inhibition is considered significant toxicity
[2] n.d. = not detected

CONCLUSION

The molybdenum-reducing enzyme is an excellent assay method for the detection or monitoring of heavy metals in the environment. The assay is rapid, simple and can assay for bioavailable toxic heavy metals. 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 detect heavy metals in polluted waters from the Juru Industrial estate. The presence of heavy metals has been confirmed via instrumental analysis. The developed assay is economic, rapid and simple and can be used as a biomonitoring tool.

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