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River Monitoring of Mercury using a novel Molybdenum-Reducing Enzyme assay

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Abstract

An inhibitive assay for mercury using a molybdenum-reducing enzyme assay system from *Serratia* sp. Strain DRY8 is presented. Mercury showed a sigmoidal inhibition curve with a calculated IC_{50} using the four-parameter logistic model of 2.101 mg l^{-1}. The limit of detection (LOD) and limit of quantitation (LOQ) for mercury were 0.021 and 0.237 mg l^{-1}, respectively. Other heavy metals tested at the final concentrations of 5.0 mg l^{-1} were not inhibitory to the assay. The enzyme requires 12-molybdophosphoric acid as an electron acceptor substrate and NADH as the electron donor substrate. The enzyme in the crude extract converted the yellowish solution into a deep blue solution with a maximum peak at 865 nm and a shoulder at 710 nm. The comparative IC_{50} (concentration causing 50% inhibition) data for lead for different toxicity tests show that the IC_{50} value for mercury was lower than the synthetic activated sludge assay and within the range of the *Spirillum volutans* and the dehydrogenase activity assays. A water sample from the Juru Industrial estate gave positive toxicity results with mercury far exceeding the maximum permissible concentrations allowed by the Malaysian Department of Environment. The waters from tap, a forest reserve, and a recreational area gave negative toxicity for mercury in agreement with ICP-AES results which showed the presence of heavy metals at the non-detectable levels.

INTRODUCTION

The presence of toxic xenobiotics in water bodies such as azo dyes [1], detergents [2], acrylamide [3,4], diesel [5,6], pesticides [7] and heavy metals [8-12] is a global phenomenon that have warranted the development of technologies to detect and remediate these xenobiotics. Of all the xenobiotics, heavy metals are elements and thus they could not be destroyed and pose a great danger to humanity and the ecosystem. In our industrialized society, toxic heavy metals are ever present in our air, water, soil, and food supply such that the level of contamination has reached a certain point that it is no longer a question of whether one has been exposed to heavy metals, but rather the level of exposure [8]. Acute toxicity from heavy metals such as lead, mercury, arsenic, and cadmium are obvious and usually classical signs of poisoning are exhibited by victims. But toxicity from chronic low-level exposure is much more dangerous in the long term. Chronic low-level exposure can lead to a wide variety of problems, ranging from neuropsychiatric disturbances such as aggressiveness, memory loss, depression, irritability, and learning deficits, to physical symptoms such as liver and kidney dysfunction, fatigue, infertility, gout, hypertension, headache, and Candida (yeast) infections [13].

Traditional ways of heavy metals determination for example atomic absorption spectroscopy, inductively coupled plasma optical emission spectrometry, inductively coupled plasma mass spectrometry as well as their conjunction with chromatographic techniques have been in wide use. Electrochemical techniques such as ion selective electrodes, polarography, along with other voltammetry are being widely used. These techniques require advanced instrumentation, skilled personnel, complex sample pre-treatment and quite often an extended measuring interval. Thus quick and easy methods used as screening tests for industrial process water or foodstuffs to point out the existence of toxic heavy metals are specifically crucial [14]. Assays which use microorganisms [15], antibodies [16] and enzymes [11,12,17-19] to detect heavy metals within the natural environment has progressively gained interest because of their excellent qualities to be utilized as preliminary mass monitoring program. In this particular work we documented the development of a novel mercury assay using the molybdenum-reducing enzyme assay originating from a molybdate-reducing bacterium.

MATERIALS AND METHODS

Isolation of Molybdate-Reducing Bacterium

Soils were gathered 15-20 centimetres (cm) underneath the surface from the grounds of a housing area (Muzeum Lane) within the city of Taiping, Perak, Malaysia in December 2004 utilizing sterile spatula and were put into sterile screw-capped vials. The samples were kept at -20°C until delivered to Universiti Putra Malaysia, Serdang, Selangor, Malaysia for more examination. Five grams of the well-mixed soil sample were suspended in 45 ml of 0.9% saline solution. An appropriate serial dilution aliquot (0.1 ml) of soil suspension was spread plated on to an agar of low phosphate (2.9 mM phosphate) media (pH 7.0) that contains glucose (1%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.0.5%), $Na_2MoO_4.2H_2O$ (20 mM) and Na_2HPO_4 (5 mM) [20]. Glucose was autoclaved independently. Growth in liquid media uses exactly the same media as in the solid media previously. Molybdenum blue is produced in this media but is not at high phosphate media (100 mM phosphate). The only real distinction between the high and low phosphate media is the phosphate concentration. Several white and blue colonies appeared after overnight incubation at room temperature. One single colony demonstrating the best blue intensity observable by eye was inoculated into 50 ml of low phosphate media and incubated at room temperature for 24 hours. The production of molybdenum blue from the media was measured at 865 nm.

Recognition at species level was done by using Biolog GN MicroPlate (Biolog, Hayward, CA, USA) in line with the manufacturer's instructions and molecular phylogenetics studies. A pure culture of a bacterium was grown on the Biolog Universal Growth agar plate. The isolate is oxidase and catalase positive. The bacteria were swabbed from the surface of the agar plate, and suspended to a specified density in GN/GP Inoculating Fluid. A hundred fifty µl of a bacterial suspension was pipetted into each well of the MicroPlate. The MicroPlate was incubated at 30° or 35°C dependant on the character of the organism for 4-24 hours in accordance with manufacturer's requirements. The MicroPlate was examined with the Biolog MicroStationTM System and compared to database. Molecular characterization was based on 16S ribosomal DNA (rDNA) sequencing. The BLAST programs from the National Centre for Biotechnology Information server were utilized for similarity searches. The final results extracted from BiologTM Identification system gave very low probability (95%) to the Serratia genus. Genomic DNA extraction, PCR of the 16S rDNA and comparison of the partial sequence obtained (1315 baseS) with the GenBank database while using Blast server at NCBI [21] demonstrated that the sequence to be 99% comparable to several species from the Serratia genus. The 16S rRNA ribosomal gene sequence for this isolate have already been deposited in GenBank under the accession number DQ226209. As of this juncture, the isolate is designated tentatively as Serratia sp. strain DRY8 (unpublished results).

Molybdenum-reducing Enzyme Assay

Molybdenum-reducing enzyme was assayed using molybdate as the electron acceptor and NADH as the electron donor [22]. Briefly, phosphomolybdate was prepared arbitrarily as a 60 mM stock solution in deionized water by combining 600 mM molybdate ($Na_2MoO_4.2H_2O$) with 240 mM phosphate ($Na_2HPO_4.2H_2O$). Modification of the phosphomolybdate solution to pH 5.0 was accomplished utilizing 1 M HCl. Into 1 ml of reaction mixture comprising 15 mM (final concentration) laboratory-prepared electron acceptor substrates in 50 mM citrate-phosphate buffer pH 5.0 at room temperature, 100 µl of NADH (80 mM stock) was included with a final concentration of 8 mM. Fifty microlitres of crude preparation of the Mo-reducing enzyme was added to start the reaction. The increase in absorbance in one minute was read at the wavelength of 865 nm. One unit of Mo-reducing activity is defined as that amount of enzyme that produce 1 nmole molybdenum blue per minute at room temperature. The specific extinction coefficient at 865 nm for the product; molybdenum blue, was determined by means of a standard curve obtained using ascorbate-reduced 12-phoshomolybdate. The specific extinction coefficient at 865 nm is 16.7 $mM.^{-1}.cm^{-1}$ (Shukor et al., 2000). An increase in absorbance at 865 nm of 1.00 unit absorbance per minute per mg protein would yield 60 nmole of 12-phoshomolybdate or 60 units of enzyme activity in a 1 ml assay mixture.

Preparation of Crude Enzyme

Bacteria were grown in one litre of media containing high phosphate at 30°C for 24 hours on an orbital shaker (100 rpm). Although the high phosphate inhibits molybdate reduction to molybdenum blue, the cells contain active enzymes [23]. Growth on low phosphate resulted in a blue sticky culture that complicated the preparation of crude enzyme and enzyme assay. The following experiment was carried out at 4°C unless stated otherwise. Cells were harvested through centrifugation at 10 000 g for 10 minutes. Cells were washed at least once with distilled water, resuspended and recentrifuged. The pellet was reconstituted with 10 ml of 50 mM Tris buffer pH 7.5 (Tris buffer prepared at 4°C). 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.

Preparation of heavy metals solutions

Heavy metals and metals such as chromium (VI) ($K_2Cr_2O_7$, BDH), selenium (VI) (Na_2SeO_4, BDH), nickel (II) ($NiCl_2$, (Ajax Chemicals), zinc (II) ($ZnSO_4$ anhydrous, J.T. Baker), iron (II) ($(NH_4)_2Fe(SO_4)_2.6H_2O$, BDH), tungsten (VI) ($Na_2WO_4.2H_2O$, BDH), tin (II) ($SnCl_2.2H_2O$, BDH), manganese (II) ($MnSO_4.H_2O$, BDH), borate (III) (H_3BO_3, anhydrous BDH), cobalt (II) ($CoCl_2.6H_2O$, J.T. Baker) and aluminium (III) ($Al_2(SO_4)_3$, anhydrous, BHD) were prepared from commercial salts or from Atomic Absorption Spectrometry standard solutions from MERCK such as mercury, arsenic, cadmium, lead, copper and silver. Heavy metals were initially diluted in 0.1 M Tris.Cl buffer pH 7.0 to the final concentration of 20 mg/L to ensure that the nitric acids from the commercial heavy metals solution are neutralized. Suitable volumes of the heavy metals of up to were then incubated with 50 µl of enzyme for 1 hour at 4°C. The mixture was then added into the enzyme reaction mixture as before. Ferrous iron in the samples can reduce the phosphomolybdate reagent to molybdenum blue. Although ferrous iron in environmental samples is usually negligible due to oxidation to ferric irons, some samples which contain high amount of ferric irons do give appreciable ferrous iron especially if nitric acid is added. In order to remove this interference, samples were incubated with the phosphomolybdate reagent for one hour. If blue solution develops then the sample contains ferrous iron. The sample was extensively bubbled with air, the pH adjusted to neutral and manganese (MnO_2) is added to a final concentration of 10 mg/L to oxidise the ferrous iron to ferric iron. Samples which contain excessive ferric irons were diluted and the process repeated until no further formation of molybdenum blue was

observed upon reacting with the reagent. The final volume of the reaction mixture was 1 ml.

Field trials

Water samples were acquired locally from identified polluted sites from several Industrial outlets which are focused within the Prai Industrial estate in Penang (Geopositional Satellite (GPS) location; (1); N 05º 20.96, E 100º 24.17', (2); 'N 05º 21.06, E 100º 24.21' and several streams in places that have been gazetted 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), Mersing Johor, (GPS; N 02°33.197' E 102°45.340'). The Prai Industrial estate, contained by the Juru River, is one of the very first industrial complex integrated the 1970's and it is well known for causing high level of heavy metals concentration in the river [24]. The rivers make up the major collection points for blended effluents originating from electric, electronic and metal foundries industries in the estate. It's not easy to track the composition of the effluents to the actual pollutant-emitting industries considering that the effluents from all of these industries are directed through underground pipes and therefore are further mixed at combining channels before draining their content in to these rivers. All of water samples were collected in acid-washed HDPE bottles containing several drops of 1% v/v HNO_3. The samples were filtered with 0.45 µm syringe filter. Suitable volumes of the clear filtrate was mixed with the enzyme and incubated for 30 minutes before assayed as before. The determination of heavy metals in the samples was carried out using Atomic Emission Spectrometry on a Perkin Elmer Optima 3000 ICP-AES**.** All experiments were performed in triplicate.

Statistical Analysis

Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test [25]. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

The foundation of the assay is the lowering of activity of the still unknown molybdenum-reducing enzyme from bacteria in the existence of heavy metals. Molybdate reduction to molybdenum blue by microbes is surely an old phenomenon. According to Levine [26], the phenomenon was initially reported in *E. coli* [27]. Since then, reports on molybdate reduction by other bacterium trickle in [10, 22, 23, 28-30]. The enzyme is assayed using NADH as an electron donor and phosphomolybdate as an electron acceptor [20, 31] with molybdenum blue as the reduced product. The amount of molybdenum blue produced would be lowered in the presence of heavy metals such as copper [11].

ANOVA analysis showed that out of the 18 metals tested at the final concentration of 10 mg l^{-1}, only mercury showed significant inhibition ($p<0.05$) with more than 50% inhibition (**Fig. 1**). Mercury exhibited sigmoidal inhibition curves (**Fig.** 2). The calculated IC_{50} using the GraphPad software (GraphPad software, Inc., San Diego, CA) the four-parameter logistic model for mercury was 2.109 mg l^{-1} and the regression coefficient was 0.998. The limit of detection (LOD) is the lowest concentration which can be detected with confidence (99% confidence interval) and is usually assigned as three times the standard deviation of the blank for the y-intercept. The LOD for mercury was 0.021 mg l^{-1}. The limit of quantitation (LOQ) is the concentration level above which the concentration can be determined with acceptable precision (usually RSD < 10 to 25%) and accuracy (usually 80-120% recovery) and is usually assigned as ten times the standard deviation of the blank for the y-intercept [25]. The LOQ for mercury was 0.237 mg l^{-1}, respectively. The LOD value was well below the maximum permissible limit for mercury (0.05 mg l^{-1}) outlined by Malaysian Department of Environment [32].

The comparative LC_{50}, EC_{50} and IC_{50} data for mercury in different toxicity tests is shown in Table 1. The results show that the IC_{50} value for mercury was lower than the synthetic activated sludge assay and within the range of the *Spirillum volutans* and the dehydrogenase activity assays. The IC_{50} value of immobilized urease is utilized rather than free urease because the all-pervasive existence of ammonia in environmental samples intervenes the assay, therefore, the need to immobilize the urease [33]. Repeated measurement of the assay for mercury suggests the assay was reproducible with CV (Coefficent of Variation) of the replicated data ranging from 4.5 to 8.4%.

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

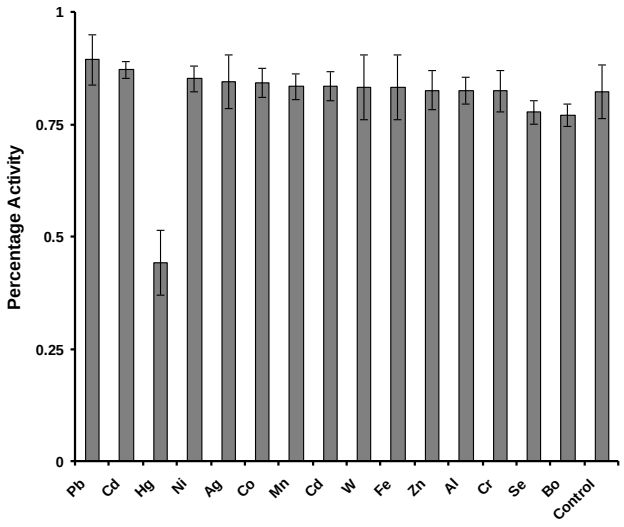


Fig. 2. Inhibition profile by mercury. Goodness of fit value, R^2 for the curves representing mercury inhibitions is 0.999 respectively. Data is mean± standard error (n=3).

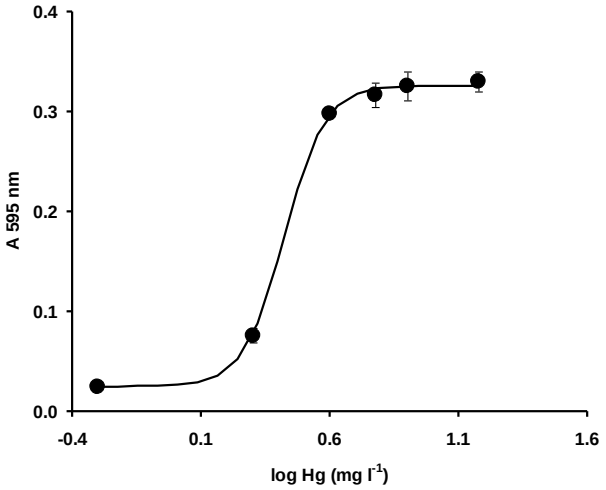


Table 1. Sensitivity of the assay to mercury in comparison to EC_{50}, LC_{50} and/or IC_{50} of several assays.

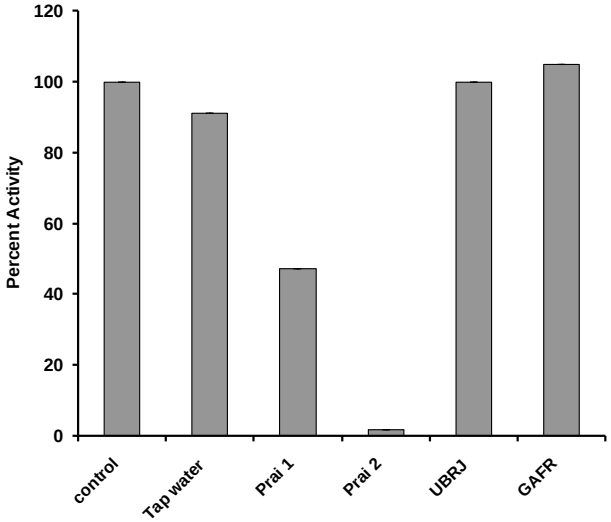
Assays	**IC_{50} , LC_{50} or EC_{50} (mg l^{-1})**
Immobilized Urease [a]	0.33±0.021
Papain [b]	0.24-0.62
15 min Microtox™ [a]	0.003±0.002
96 hours *Daphnia magna* [C]	0.0052-0.21
Rainbow trout [c]	0.033-0.21
R. meliloti [d]	0.0159± 0.004
Baker's Yeast [e]	0.8.± 0.1
Dehydrogenase activity (TTC or INT) [f]	1.5-2.6 (TTC), 0.8 (INT)
In vitro ATPase activity [f]	0.48
Spirillum volutans [f]	0.2-3.7
Aeromonas hydrophilia [f]	0.05
P. fluorescens (18 Hr) [g]	0.03
Synthetic activated sludge (180 min) [g]	4.5
Bromelain[h]	0.13 to 0.16
This Study	2.109

[a] [33]
[b] [18]
[c] [34]
[d] [15]
[e] [35]
[f] [36]
[g] [37]
[h] [19]
*n.d. Not determined

Field trial results.

A field trial was set up in the Juru Industrial estate, a site well known to harbour many metal-related work industries. The results shows that the samples from the Juru Industrial estate gave positive toxicity results with most of the heavy metals especially mercury far exceeding the concentrations allowed by the Malaysian Department of Environmental for Class II (Fishery). However, despite having high concentrations of mercury, the magnitude of inhibition or toxicity effect to the Mo-reducing activity assay is not pronounced as expected and is possibly due to the limited bioavailability or solubility of the mercury form in the samples. The waters from the forest reserve, recreational areas and tap water gave negative toxicity results in agreement with ICP-AES results which showed heavy metals at non-detectable levels. The waters from the Juru Industrial estate flow into the Juru Estuary. The estuary holds among the biggest cockle (Anadara granosa) culture in Malaysia [24], which makes the outcomes obtained in this work highly important for a more thorough field trials in the Juru Industrial Estate. In this work, a Mo-reducing enzyme assay system was utilized to detect mercury in environmental samples. The assay is shown to be simple, reproducible and quick with good level of sensitivity. The assay system was tested with water samples from contaminated areas that contain heavy metals. There are many advantages of testing toxicity of heavy metals using the Mo-reducing activity assay. The bacterium is easy to grow and its crude extract is easily prepared. The rapidity and simplicity of the system would allow development of a simple kit to detect heavy metals from the environment.

Fig. 3. The inhibition studies on water sample from a polluted area using the Mo-reducing activity assay. Data is mean± standard error (n=3).



	Prai 1 N 05° 21.09', E 100° 24.39'	Prai 2 05° 21.06', E 100° 24.21'	Tap water	Ulu Bendul Recreational Jungle, Kuala Pilah, Negeri Sembilan (UBRJ)	Gunung Arong Forest Reserve, Mersing Johor (GAFR)
Mercury	18.460±0.775	16.670±0.224	n.d.	n.d.	n.d.
Copper	0.133±0.001	0.003±0.003	n.d.	n.d.	n.d.
Zinc	0.390±0.015	0.0270.002±	n.d.	n.d.	n.d.
Cadmium	n.d.	n.d.	n.d.	n.d.	n.d.
Silver	n.d.	n.d.	n.d.	n.d.	n.d.
Lead	0.152±0.035	0.152±0.035	n.d.	n.d.	n.d.

* n.d. Not detected

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