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Inhibitive bacterial MTT Assay for River Monitoring of Heavy Metals

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Abstract

An inhibitive assay for metals using a MTT-bacterium assay system is presented. The assay is based on the ability of respiring bacteria to reduce the tetrazolium dye MTT. Out of the 13 metals screened, silver, mercury and copper exhibit strong inhibition to bacterial respiratory activity. The calculated IC_{50}s for silver, mercury and copper were 0.068, 0.092 and 0.517 mg/L, respectively. The IC_{50} values obtained for these heavy metals are comparable and in certain cases lower than existing bioassay methods such as *Daphnia magna*, rainbow trout, Microtox™ and papain assays. Works on real water samples from several rivers in Malaysia using the MTT–bacterium assay showed positive correlation with instrumental method (ICP-OES) in detecting heavy metals. The bacterium was tentatively identified as *S. marcescens* strain Dr.YS8 based on carbon utilization profiles using Biolog GN plates and partial 16s rDNA molecular phylogeny. The system could be used to biomonitor heavy metals extensively in Malaysian waters.

INTRODUCTION

The Department of Environment, Malaysia has revealed that about 5% of the major 180 river basins in Malaysia are considered to be polluted with heavy metals. Some areas of the Straits of Malacca are also reported to have high levels of heavy metal [1]. The Juru river has been reported to contain toxic metal ions like mercury and copper at levels several orders of magnitude higher than the MPL [2]. Since heavy metals ranked as the number one pollutant in major rivers, their monitoring is of utmost importance. The annual DOE report does not include the status of heavy metals in major rivers in Malaysia due to the high cost incurred in monitoring them [1]. Thus, there is a need for simple and fast procedures as a preliminary screening method to determine the presence of toxic heavy metals before embarking upon costlier instrumental methods. The USEPA has recognized biomonitoring as an economical approach in monitoring heavy metals in aquatic bodies.

Both enzyme-based and bacterial based assays are recognized. For example, the enzyme urease and the bacterial system Microtox are two bioassays routine employed [3-5]. Urease assay must be in its immobilized form because ammonia, a key product of the enzyme, is found in abundance in the environment. Immobilized urease, are however, weakly sensitive to heavy metals with IC50 for toxic metal ions higher than 1 ppm [6-8]. Microtox is costly and the whole system costs about MYR 100,000. The system is also not field trial friendly as the bulky instruments are a hindrance. Another bacterial-based assay that has been tested for its biomonitoring potential is *Rhizobium meliloti* using a simple MTT dye reduction as an inhibitive assay system. However, it is also weakly sensitive to heavy metals with IC50 higher than 1 ppm. The MTT dye-reduction system is appealing due to its simplicity, economic and relatively rapid [9-11]. In this work we demonstrate the use of such a system together with a locally-isolated bacterium that shows optimal dye reduction capability at ambient temperature, and using a portable spectrophotometer, we were able to monitor heavy metals at a test site producing almost real time results- an important tool if spatial and temporal status of heavy metal pollution is needed especially in the area of environmental forensics. The IC_{50}s for certain heavy metals are better than existing assay and the system could be used to monitor heavy metals extensively in Malaysian waters.

MATERIALS AND METHODOLOGY

Preparation of Reagents

All reagents were of analytical reagent grade unless otherwise stated. The MTT dye was purchased from Sigma. All the plastics and glassware were cleaned by soaking in dilute HNO_3 (10%) to remove all traces of metal ions and were rinsed with an appropriate amount of deionized water prior to use. MTT dye at 10 mM stock solution was prepared by dissolving 0.2072 g in 50 mL of 10 mM PBS, pH 7.5. The solution was stored at 4 ºC. This

MTT dye was added into the reaction mixture to a final concentration of 1 mM.

Heavy metals such as manganese ($MnSO_4.H_2O$), borate (H_3BO_3), tin ($SnCl_2.6H_2O$), selenium (Na_2SeO_4), zinc ($ZnSO_4$ anhydrous), tungsten ($Na_2WO_4.2H_2O$), caesium ($CsCl_2$) were prepared from commercial salts by dissolving in deionized water with a few drops of concentrated nitric acid to solubilise the heavy metals and stored in acid-washed polypropylene containers. Mercury, arsenic, cadmium, lead, copper, silver, magnesium and calcium are Atomic Absorption Spectrometry 1000 mg l^{-1} standard solutions from MERCK (Merck, Darmstadt, Germany). Working solutions were prepared from these stock solutions by diluting in deionized water to the required concentrations.

Pesticides with chemical purities of >99%, (Ehrenstorfer, Augsburg, Germany and Pestanal®, Riedel de Häen, Germany) such as metolachlor, glyphosate, diazinon, endosulfan sulphate, coumaphos, imidacloprid and dicamba were prepared by dissolving the pesticides in the appropriate solvents or used directly from the liquid solutions. The final concentration of all these pesticides in the reaction mixture was 4 mg l^{-1}. The xenobiotics tested are as follows; acetonitrile (MERCK), ethylene glycol (MERCK), ethyl acetate (MERCK), ethanol (BDH), isopropanol (BDH), methanol (BDH), Triethanolamine, Polyethylene Glycol (PEG) 400, 600 and 1000 (SIGMA), diethylamine (SIGMA), acrylamide (SIGMA), Nonidet-P40 (SIGMA), Triton-X-100 (SIGMA) and SDS (SIGMA). These xenobiotics were prepared as 2% (v/v) solution in deionized water and added into the reaction mixture to a final concentration of 0.4% (v/v). The concentration of pesticides and xenobiotics chosen in this study is generally much higher than normally found in natural water and also limited to the solubility of pesticide and xenobiotics in water.

Isolation of Bacteria

Collected samples were first washed with tap water, and cut into smaller pieces. For soil samples, only stipeses of fruiting bodies and mycelia in soil were used. Cleaned and cut samples were immersed in a commercial solution (5.25 % sodium hypochlorite Clorox ™ in sterile distilled water) for approximately 15 min for disinfection purpose. Samples were gently shaken to remove excess disinfectant solution before being placed on potato Dextrose Agar (PDA). Once white-rot mycelia (whitish or yellow-brown, flat or cotton-like mycelia radiate out of the original sample, no spore-forming structure) were detected, they were cut and subcultured on new PDA plates. This process was repeated until a pure culture (homologous mycelial morphology) was obtained.

Field trials

Soil and water samples were collected from several locations in Peninsular Malaysia from December 2005 to January 2007. The soil samples were placed in sterilized plastic bags and stored on ice during transfer from the sites to the laboratory. Individual bacteria were isolated from the soil samples based on colony morphology on Nutrient agar plates after suitable serial dilutions in sterile distilled water. Bacteria were grown by inoculating in 15 mL of nutrient broth (NB) for 18 hours in a 50 mL conical flask. The bacteria were incubated at room temperature in a rotary incubator shaker at 150 rpm for growth. Bacterial suspensions (1 mL) were centrifuged at 10 000 g for 10 min in an Eppendorf tube at room temperature. The supernatant was discarded while the pellets were washed once with 10 mM phosphate buffer saline (PBS), pH 7.5 and re suspended in the same buffer by mixing vigorously. Preliminary screening based on the MTT assay in the presence of common divalent cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) at the highest final concentration of 25 mg l^{-1} and 50 mg l^{-1} respectively was carried out to select isolates that were not inhibited by these common cations. The final concentration in the reaction mixture for preliminary screening was 10 mg l^{-1} for Ag, Hg, Cd, As and Pb and 5 mg l^{-1} for Cu.

The MTT assay was carried out by combining 50 µL of 10 mM PBS, pH 7.5 at the final concentration in a 250 µL total reaction mixture followed by 75 µL aliquot of 1% (v/v) bacterial suspensions, 25 µL of each two divalent cations, Ca^{2+} and Mg^{2+} and finally 50 µL of tested heavy metals in a flat bottomed 96-well microplate. As a control, the tested heavy metals were replaced with deionized water in the first well. The reaction mixture was pre-incubated for an hour at room temperature by covering the microplate with aluminium foil as a precaution before adding 25 µL of MTT (10 mM stock) to allow the reaction to proceed. Colour development was observed after the incubation period. The absorbance at 550 nm was measured using a microplate reader (Stat Fax® 3200 Microplate Reader, Awareness Technology Inc., USA). All the tests were performed in triplicate. Inhibition (%) was calculated as follows: [(Initial control absorbance-final absorbance)/Initial control absorbance] x 100. Regression curves to determine concentrations of heavy metals causing 50% inhibition (IC_{50}) are generated using the PRISM (Prism version 4.00 for Windows) non-linear regression analysis for one-phase exponential decay models software available from GraphPad, (GraphPad Software Inc., San Diego, CA).

Identification of Bacterium

Identification at species level was performed by using Biolog GN MicroPlate (Biolog, Hayward, CA, USA) according to the manufacturer's instructions and molecular phylogenetics studies. A pure culture of a bacterium was grown on a Biolog Universal Growth agar plate. The bacteria were swabbed from the surface of the agar plate, and suspended to a specified density in GN 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 depending upon the nature of the organism for 4-24 hours according to manufacturer's specification. The MicroPlate was read with the Biolog MicroStation™ System and compared to database.

16s rDNA Gene Sequencing

Genomic DNA was extracted from bacterial colonies by alkaline lysis. PCR amplification was performed using a thermal cycler (Biometra, Gottingen, Germany). The PCR mixture contained 0.5 pM of each primer, 200 µM of each deoxynucleotide triphosphate,10x reaction buffer, 2.5 U of Taq DNA polymerase (Promega) to achieve a final volume of 50 µ1. The 16s rDNA gene from the genomic DNA was amplified by PCR using the following primers; 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3'corresponding to the forward and reverse primers of 16s rDNA respectively [13]. PCR was performed under the following conditions: initial denaturation at 94 °C for 3 min; 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min.

Cycle sequencing was subsequently performed with the Big Dye terminator kit (Perkin-Elmer Applied Biosystems) as recommended by the manufacturer. Sequence data were initially recorded and edited using CHROMAS Version 1.45. The resultant 593 bases were compared with the GenBank database using the Blast server at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The 16s rRNA ribosomal gene sequence for this isolate have been deposited in GenBank under the following accession number EF121822.

Phylogenetic analysis

A multiple alignment of 20 16S rRNA gene sequences closely matches the strain were retrieved from GenBank and were aligned using clustal_W [14] with the PHYLIP output option. A phylogenetic tree was constructed by using PHYLIP, version 3.573 (J. Q. Felsenstein, PHYLIP—phylogeny inference package, version 3.573, Department of Genetics, University of Washington, Seattle,WA.(http://evolution.genetics.washington.edu/phylip.html) with *Bacillus subtilis* as the outgroup in the cladogram. Evolutionary distance matrices for the neighbour-joining/UPGMA method were computed using the DNADIST algorithm program. The model of nucleotide substitution is of Jukes and Cantor [15]. Phylogenetic tree (**Fig. 1**) was inferred by using the neighbour-joining method of Saitou and Nei [16]. With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1000 bootstraps [17] by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed for the topologies found using a family of consensus tree methods called the Ml methods [18] using the CONSENSE program and the tree was viewed using TreeView [19].

RESULTS AND DISCUSSION

Identification of Bacteria.

Strain DR.YS8 is linked to the genus *Enterobacter* and *Serratia* in different sister groups indicating that the phylogenetic relationship of this strain to either Enterobacter genus is weak, and the phylogenetic position can still be further modified in the future **(Fig. 1).** The results obtained from Biolog™ Identification system gave very high probability (99%) to *Serratia marcescens*. For now, strain Dr.YS8 is assigned tentatively as *S. marcescens* strain Dr.YS8. The bacterium grew optimally between 25 and 35 ºC. This broad optimal range is conducive to inhibitive assay at ambient temperature.

Bacterium Inhibition Studies

It has been proposed that the tetrazolium dyes are reduced by the cytochromes [20]. In prokaryotes, the electron transport system is associated with the cytoplasmic membrane. Hydrophobic toxic chemicals will interact with the membrane, affecting cytochrome activity. The dye can also be reduced by a NAD(P)H reductase [21,22]. In the presence of xenobiotics and heavy metals in the electron transport system, the production of NADH will be inhibited and will reduce the respiratory activity of the cells as measured by the MTT assay. The inhibition could be in any enzymes at any stages of NADH production. In the original Botsford's MTT assay, two common nontoxic cations; calcium and magnesium were found to be toxic at 5.6 and 50.8 mg l^{-1},

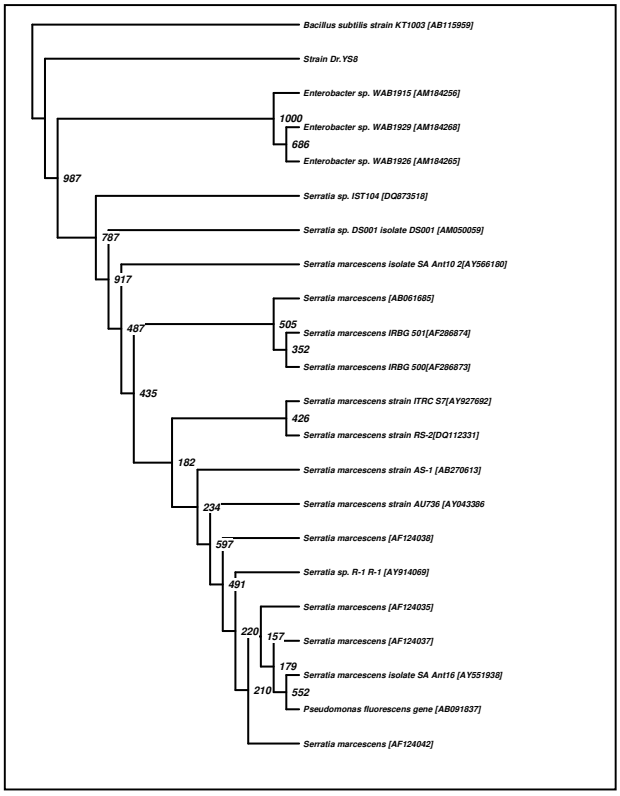

Fig. 1. Phylogenetic tree of newly isolated bacteria for bioindicator of mercury. Neighbour-joining method cladogram showing phylogenetic relationship between Strain Dr.YS8 and other related reference microorganisms based on the 16S rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16S rDNA sequences. The numbers at branching points refer to bootstrap values based on 1000 resamplings. The branch lengths in the cladogram are not to scale. *B. subtilis* is the outgroup.

respectively [3,11]. Both of these cations can be found at much higher concentrations in soil. Botsford added the chelators EDTA (ethylenediamine tetraacetic acid) or EGTA (ethylene glycol-bis-(β-aminoethyl ether) N,N,N'N'-tetraacetic acid) at 0.08 mM to alleviate this problem. The inclusion of EDTA is undesirable as it relieves the inhibition by heavy metals [3]. We purposely included these cations into the screening procedure and we found that there are several bacteria sensitive to these cations. These bacteria were removed from the screening tests.

Out of the 13 metals screened, silver, mercury and copper exhibit strong inhibition to the bacterial respiratory activity at 10 mg l^{-1} **(Fig. 2).** The calculated IC_{50} for silver, mercury and copper is 0.068, 0.092 and 0.517 mg l^{-1} whilst the regression coefficients for the one-phase exponential decay curves are 0.988, 0.998 and 0.962 respectively. The comparative LC_{50}, EC_{50} and IC_{50} data for the metals; presented as 95% Confidence Intervals, in different toxicity tests are shown in **Table 1**. The results from this study were also compared with fish (rainbow trout) assay, Daphnids (*Daphnia magna*), immobilized urease, *R. meliloti*, papain and Microtox™ toxicity data in the same table. The IC_{50} value for mercury is lower than immobilized urease and papain and falls within the range of *Daphnia magna* and rainbow trout assays. The IC_{50} values for silver is lower than papain and *Daphnia magna* but

is higher than Microtox™ and Rainbow trout. The IC_{50} values for copper is lower than *R. meliloti*, falls within the range of the immobilized urease assay and higher than the rest of the assays. The IC_{50} value of immobilized urease is used instead of free urease since the ubiquitous presence of ammonia in environmental samples interferes the assay, hence the need to immobilize the urease [23]. The MTT assay developed by Botsford et al. requires the addition of 0.08 mM of EDTA if samples contain calcium ions but to the detriment of the sensitivity of the entire divalent metals ion tested [3,11]. Thus, the results of the *R. meliloti* MTT assay for heavy metals are valid if the sample contains negligible calcium ions. Repeated measurement of the MTT assay for all of the heavy metals suggests the assay is reproducible with CV (Coefficient of Variation) of the replicated data ranging from 2 to 15%. The sensitivity towards mercury is of special interest as this toxic metal ion is reportedly to exceed the Maximum Permissible Limit in water bodies around Malaysia [1,2] Its detection in the 180 major river basins in Malaysia is reported sporadically by small research groups in Malaysia, and not by enforcement agencies due to the high costs incurred by instrumentals methods.

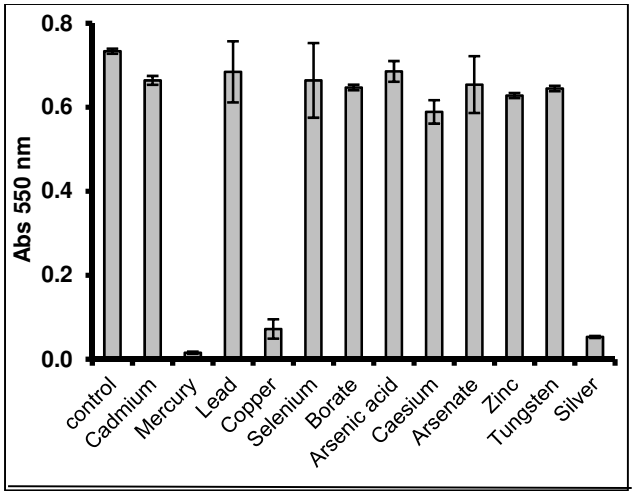

Fig. 2. Screening results for the inhibitory effect of heavy metals on bacterial respiratory activity using the MTT assay. Data is mean ± standard error of the mean (n=3).

Interference Study

The influence of foreign species on the assay was investigated. Of the other 13 xenobiotics tested at the final concentration of 0.4%, only SDS inhibited the assay strongly **(Fig. 3)**. The concentration of xenobiotics tested in this assay at 4000 mg l^{-1} is not normally found in aquatic bodies at this high level. The IC_{50} for SDS is 25 mg l^{-1} (Data not shown). Two of the pesticides tested; metolalchlor, and coumaphos at 4 mg l^{-1} showed appreciable inhibition at 13.8 and 12.3% respectively **(Fig. 4).** The concentration of pesticide chosen in this work is generally much higher than normally found in natural water and also limited to the solubility of these pesticides in water.

Field Trial

The field trial results show that several sites gave positive toxicity results with 100% inhibition of enzyme activity **(Table 2).** Samples from industrial sites; Prai Industrial Estate and Bukit Tengah Industrial Estate, both in Penang, one sample from the Melaka river and several samples from a galvanized metal factory outlet caused 100% inhibition of Mo-reducing enzyme activity. These samples showed the presence of toxic heavy metals at levels above the Maximum Permissible Limit (MPL) allowed by the Malaysia Department of Environment (DOE)[19]. In contrasts, sample waters from major rivers (Perak and Melaka rivers) are almost free of heavy metals with the exception of one sample that contain copper at concentrations below the MPL for Malaysian river water standard while water samples from pristine areas (SURJ, UBRJ and GAFR) including tap water are free from heavy metals. of the rivers using current instrumental practice is astronomical, hence the absence of a detailed report. Many of the rivers classified as pristine or slightly polluted (classes 2 and 3) would have to be revised if the concentrations of heavy metals in them are known. Many of these rivers are used as a source of irrigational waters and heavy metals can be absorbed by plants [4,22] and cause chronic detrimental effects.

Table 1. Sensitivity of the assay to heavy metals in comparison to to LC_{50} and/or IC_{50} of free and immobilised urease, papain Microtox™, *Daphnia Magna,* fish bioassays (Rainbow trout) and *R. meliloti.*

	IC_{50} or EC_{50} (mg l^{-1})						
Metals	Immobilized Urease[a]	Papain[b]	15 min Microtox™ [a]	96 hours *Dapnia magna*[c]	Rainbow trout (95% Confidence Interval of LC_{50})[c]	*R. meliloti* (without 0.08 mM EDTA)[d]	This Study (95% Confidence Interval of LC_{50})
Hg	0.33±0.021	0.24-0.62	0.003±0.002	0.0052-0.21	0.033-0.21	0.0159± 0.004	0.106-0.221
Ag	n.d.*	0.33-0.49	0.008±0.001	1.930[a]	0.05	n.d.	0.115-0.173
Cu	0.41±0.14	0.004	0.076-3.8	0.020-0.093	0.25	0.950±0.181	0.414 to 0.689

[a] [23]
[b] [5]
[c] [24]
[d] [4]
*n.d. Not determined

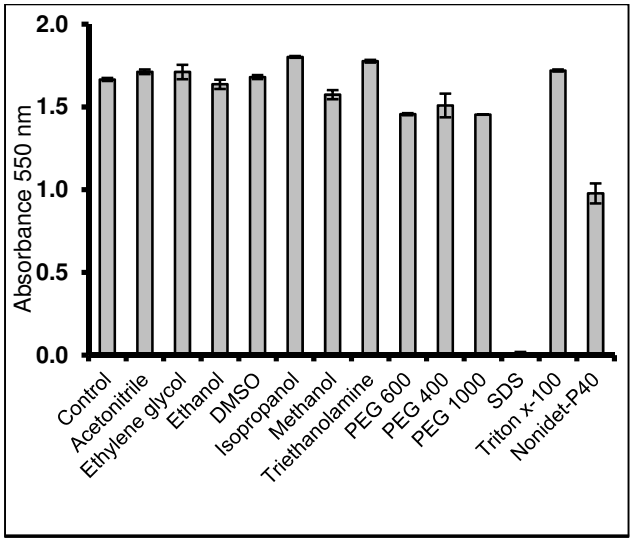

Fig. 3. Effects of the bacterial MTT assay by xenobiotics. Data is mean± standard error of the mean (n=3).

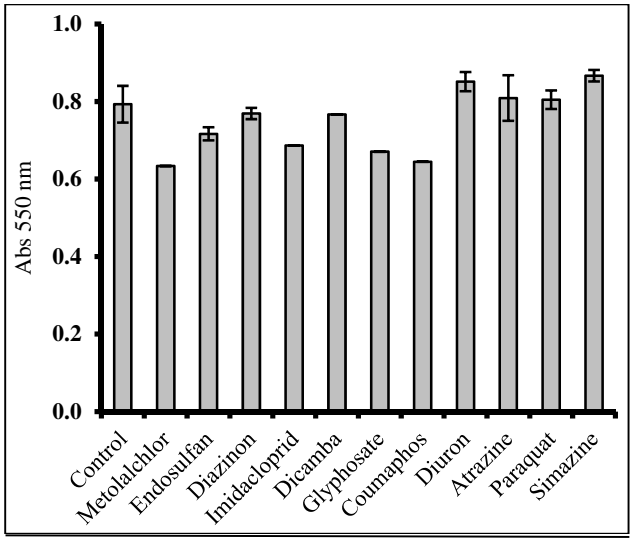

Fig. 4. Effects of the bacterial MTT assay by pesticides. Data is mean± standard error of the mean (n=3).

Table 2. Field trial results. Abbreviations used: PIE-Prai Industrial Estate, Penang; MR-Melaka River, Melaka; PR-Perak River, Perak; BTIA-Bukit Tinggi Industrial Estate, Penang; GMF-Galvanized metal factory, Perak; SURJ-Sungai Udang Recreational Jungle, Melaka; UBRJ-Ulu Bendul Recreational Jungle, Kuala Pilah, Negeri Sembilan; GAFR-Gunung Arong Forest Reserve, Mersing, Johor.

Locations	locations	%Inhibition of MTT-reducing Activity[1]	Concentrations of Heavy metal (mgl^{-1})					
			Cd	Ag	Cu	Pb	Hg	Cr
BTIA 1	N 05º 20.447 E100º 26.403'	0	n.d[2]	n.d	n.d	n.d	n.d.	n.d.
BTIA 2	N05º20.665' E100º26.364'	1.5	n.d	n.d	n.d	n.d	n.d.	n.d.
BTIA 3	N05º20.601' E100º26.427'	3.2	n.d	n.d	n.d	n.d	n.d.	n.d.
BTIA 4	N05º20.640' E100º 26.470'	101	0.04±0.001	0.328±0.009	0.642±0.012	0.03±0.004	0.36±0.00	0.45±0.08
BTIA 5	N05º18.947' E100º26.348'	99	0.03±0.004	0.12±0.023	0.916±0.021	0.102±0.04	1.57±0.02	1.24±0.03
PR 1	N04^0 30.685 E 100^0 55.583	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PR 2	N04^0 30.804 E 100^0 55.555	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PR 3	N04^0 30.901 E 100^0 55.569	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PR 4	N04^0 31.970 E 100^0 55.586	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PR 5	N04^0 31.077 E 100^0 55.637	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MR 1	N02°12.132 E 102°151	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MR 2	N02°12.732 E 102°14.569	99	n.d.	n.d.	0.78±0.03	n.d.	n.d.	n.d.
MR 3	N02°11.572 E 102°14.541	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MR 4	N02°11.513 E 102°14.553	2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

MR 5	N02°11.433 E 102°14.591	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PIEP 1	N 05° 20.96, E 100° 24.17'	103	18.4±1.34	n.d.	6.13±0.09	1.22±0.00	n.d.	n.d.
PIEP 2	N 05° 20.96, E 100° 17.25'	100	0.401±0.02	0.05±0.001	2.341±0.04	0.927±0.03	0.122±0.001	n.d.
GMF 1	N 04° 37.59', E 101° 05.18'	100	0.186±0.001	n.d.	5.342±0.057	37.64±0.069	n.d.	n.d.
GMF 2	N 04° 37.59', E 101° 04.14'	101	0.074±0.02	n.d.	2.342±0.004	2.04±0.021	n.d.	n.d.
SURJ 1	N 02°18.102' E 102°07.837'	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SURJ 2	N 02°18.103' E 102°07.774'	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
UBRJ 1	N 02°43.767' E 102°04.668'	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
UBRJ 1	N 02°43.732' E 102°04.645'	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAFR 1	N 02°33.197' E 102°45.340'	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAFR 1	N 02°33.194' E 102°45.312'	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tap water	University Putra Malaysia	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[1] 20% Inhibition is considered significant toxicity. Data were presented as whole number.
[2] n.d. = not detected

Due to this, biomonitoring of heavy metals using simple and rapid assays is urgently needed. Using our assay system, for instance, only positive samples that show enzyme activity inhibition would be needed to be sent for instrumental validation. This would dramatically reduce monitoring costs. The assay is based on the ability of heavy metals to inhibit the molybdenum-reducing enzyme of a bacterium. The calculated IC_{50} was generally better than existing assays and would be useful for the determination of heavy metals. The assay is simple, reproducible and rapid. There are many advantages of testing toxicity of heavy metals using this assay. The bacterium is easy to grow, and its crude extract is easily prepared.

CONCLUSION

In this work a bacterial respiratory assay was used to detect mercury and silver in environmental samples. The assay has been shown to be simple, reproducible and rapid with good sensitivity. In contrast to a previous bacterial assay using MTT, the bacterium employed in this work is not sensitive to calcium ions. The assay system was tested with water samples from polluted areas containing heavy metals and were found to satisfy the sensitivity level required for routine heavy metals detection in the field. There are many advantages of using microbial bioassay for testing toxicity of heavy metals using the MTT assay. Microorganisms are easy to culture, besides providing rapid results. The generation time of bacteria is very rapid and so their response times to organic enrichment or to toxic substances are likely to be quite rapid. Bacterium can also be maintained under known, controlled conditions in large number.

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