INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–317/2014/16–5–1123–1128
http://www.fspublishers.org

Full Length Article

Development of a Bacterial-based Tetrazolium Dye (MTT) Assay for Monitoring of Heavy Metals

Halmi Wasoh Mohamad Isa[1], Wan Lutfi Wan Johari[2], A. Syahir[1], Mohd Yunus Abd Shukor[1], A.A. Nor Azwady[3], N.A. Shaharuddin[1] and M. Muskhazli [*]

[1]*Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia 43400 UPM Serdang, Selangor, Malaysia*

[2]*Department of Environmental Science, Faculty of Environmental Studies, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

[3]*Department of Biology, Faculty of Science, Universiti Putra Malaysia 43400 UPM Serdang, Selangor, Malaysia*

*For correspondence: muskhazli@science.upm.edu.my

Abstract

An inhibitive assay for metals using a bacterial respiratory assay system is presented. The assay is based on the ability of bacteria to reduce the water soluble tetrazolium dye (MTT). The isolate was tentatively identified as *Bacillus* sp. strain Khayat. The *Bacillus* sp based MTT assay is sensitive towards Hg^{2+}, Cu^{2+}, Ag^{2+}, Cd^{2+} and Zn^{2+} with concentration of toxicant giving 50% inhibition (IC_{50}) values at 0.046, 0.057, 0.044, 0.857 and 1.716 mg/L, respectively. A Limit of Detection (LOD) value was 0.001 mg/L for Hg^{2+} and Cu^{2+} while 0.003, 0.067 and 0.201 mg/L, respectively for Ag^{2+}, Cd^{2+} and Zn^{2+}. This assay is xenobiotics and pesticide tolerance and can be completed within 20 min. Field test on identify polluted water sample from Bukit Tinggi Industrial Estate, Penang and Bukit Tinggi Industrial Estate, Penang proved that *Bacillus* sp-based MTT assay was sensitive in toxic response. © 2014 Friends Science Publishers

Keywords: *Bacillus* sp.; Limit of Detection; IC_{50}; Toxic sensitive; Reduction activity

Introduction

Due to high demand for efficient heavy metal monitoring tool and the high cost of instrumental-based monitoring, the idea to use microorganism's biochemistry activity to develop new monitoring tool has becoming one of the novel approaches. Bio-monitoring assays using microorganisms (Sun *et al.*, 1994), bacteria (Zonneveld, 1983; Chishti and Arshad, 2013), antibodies (Mehraban *et al.*, 1998) and enzymes (Jung *et al.*, 1995; Shukor *et al.*, 2006; 2008) to detect the toxicity of xenobiotics such as heavy metals, pesticides and organic solvents has been reported previously. Microbial bioindicator/bioassay provides a simpler and less expensive method than the classical bioassay since no specialized equipment is required (Botsford *et al.*, 1998). In recent years, bioindicators using bacteria have been commercialized such as the Lux-Fluoro (Baumstark-Khan, 2003), the Polytox™ (Sun *et al.*, 1994), the Deltatox™ and the Microtox® assays (Bullich, 1986).

The use of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) conversion into a blue coloured formazan by mitocondria as a rapid and simple assay tool (Mosmann,1983). Combination of MTT-reduction assay and bacteria for testing heavy metal toxicity has many advantages. 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. Furthermore, they also can be maintained under known, controlled conditions in large numbers.

In 1998, MTT assay using *Rhizobium meliloti* as indicator was reported and it was based on the ability of *R. meliloti* to reduce MTT. The use of MTT allowed fast visual observation and it is cheaper than luminescence-based assays. Unfortunately, this reduction process is inhibited by many toxic chemicals especially heavy metals and non-toxic metal ions such as Mg^{2+} and Ca^{2+} (Botsford *et al.*, 2000).

Hypothetically, the presence of heavy metals will block MTT reduction in bacteria and this will be subjected to degree of bacteria sensitivity toward particular heavy metals. In order to exploit the advantages of the MTT assay for heavy metals without the inherent weakness of inhibition by non-toxic ions, we screen for bacteria that are not inhibited by non-toxic ion, xenobiotics and pesticide. Therefore the aim of this study were set to screen heavy metals-sensitive bacterium with xenobiotics and pesticide tolerance and also to investigate the level of heavy metal sensitivity of bacteria-based MTT assay on polluted water sample.

To cite this paper: Isa, H.W.M., W.L.W. Johari, A. Syahi, M.Y. Abd Shukor, A.A. Nor Azwady, N.A. Shaharuddin and M. Muskhazli, 2014. Development of a bacterial-based tetrazolium dye (MTT) assay for monitoring of heavy metals. *Int. J. Agric. Biol.,* 16: 1123–1128

Materials and Methods

Preparation of Reagents

All reagents were analytical reagent grade unless otherwise stated. To remove trace of metal ion, all the plastics and glassware were cleaned by soaking in 10% HNO_3 and then rinsed with an appropriate amount of deionized water prior to use.

Ten mM MTT dye (Sigma) stock solution was prepared by dissolving 0.2072 g in 50 mL of 10 mM PBS (pH 7.5) and stored at 4ºC. Heavy metals were prepared from either commercial salts or atomic absorption spectrometry standard solutions (1 g/L). Pesticides (Ehrenstorfer and Pestanal®, 99% pure) were dissolved in appropriate solvents or used directly from the liquid solutionsto obtain a final concentration of 4mg/L in the reaction mixture. Testing on xenobiotics were carried out using 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 were added into the reaction mixture to a final concentration of 0.4% (v/v).

Isolation of Bacteria

Isolation of bacteria was carried out form water and soil water samples were collected from several locations in Peninsular Malaysia such as Tanjung Karang, Selangor; Tanjung Blanja, Perak; Taiping Lake Garden, Perak and Congkak River, Selangor. Pure colony of bacteria was isolated based on colony morphology on nutrient agar plates after serial dilutions in sterile distilled water. In addition, bacterial culture collections from the Department of Microbiology and Biochemistry, Universiti Putra Malaysia were also included in the screening of heavy metals-sensitive bacterium.

Screening of Inhibition of Bacterial MTT-reduction by Heavy Metals

Bacteria pellet for screening was prepared by inoculating bacteria in 15 mL of nutrient broth (NB) for 18 h and incubated on rotary incubator shaker (150 rpm; 27 ± 1°C) before 1 mL of bacterial suspensions were centrifuged at 10 000 × g for 10 min in micro-centrifuge tube at room temperature. The pellet was collected and washed once with 10 mM phosphate buffer saline (PBS; pH 7.5) and resuspended in the same buffer by mixing vigorously. The target heavy metal; mercury (Hg) was added to the final concentration of 0.1 mg/L while other heavy metals such as cadmium (Cd), silver (Ag), lead (Pb), copper (Cu), selenium (Se), nickel (Ni), arsenic (As), manganese (Mn), cesium (Cs), cobalt (Co) and zinc (Zn)were added to the final concentration of 1 mg/L.

MTT Assay

The MTT assay was carried our as suggested by Botsford *et al.*, (1998) by combining 100 µL of 10 mM PBS (pH 7.5) with 75 µL of 20% (v/v) bacterial suspensions (OD_{600nm} = 1.0) and 50 µL of tested metals or water samples in a flat bottomed 96-well microplate. Deionized water was used as control. The reaction mixture was pre-incubated for 5 min at 27 ± 1°C before 25 µL of 10 mM MTT was added into the mixture (final volume 250 µL). Color development was measured at 550 nm using a microplate reader. All tests were performed in triplicate.

Inhibition (%) was calculated as follows: [(Initial control absorbance-final absorbance) / (Initial control absorbance)] x 100. While determination of heavy metals concentration that cause 50% inhibition of reduction of the dye (IC_{50}) was calculated form regression curve generated using PRISM (Prism version 4.00 for Windows) non-linear regression analysis for one-phase exponential decay models software (GraphPad Software Inc., San Diego, CA).

Identification of Heavy Metals-sensitive Bacteria

Genomic DNA extraction and PCR of the 16s rRNA were used for bacteria identification. The genomic DNA extraction was extracted using DN easy blood and tissue kit (Qiagen) according to the manufacturer's procedure and used as template in PCR. In order to amplify the 16S region, PCR was performed using 16S universal primer (Weisburg *et al*., 1991). Comparison of the partial sequence obtained with the GenBank database using the Blast server at NCBI (htwww.ncbi.nlm.nih.gov/BLAST/) was carried out.

Field Trials

Water samples were taken from the Bukit Tinggi Industrial Estate, Penang and Prai Industrial Estate. Effluents came out form these estates were coming from electric, electronic and metal foundry industries. Water samples were also taken from a pristinegazetted area near Gunung Arong Forest Reserve, Mersing, Johor as control. All water samples were collected in Polypropylene containers with a drop of concentrated nitric acid prior to the MTT assay. The determination of heavy metals in the samples was carried out using Atomic Emission Spectrometry on a Perkin Elmer ICP-OES (Optima 3700DV, Perkin-Elmer, USA) and a Perkin Elmer Flow Injection Mercury System (FIMS 400). All experiments were performed in triplicate.

Statistical Analysis

All the data of heavy metals concentration and the degree of inhibition of MTT-reducing activity using

Bacillus sp-based MTT assay were was analyzed using one-way ANOVA where the confidence level was set at 95%. Any significance difference ($p<0.05$) was analyzed using Turkey's post hoc test. The data was analyzed using SPSS program version 16.0.

Results

Screening of Heavy Metal-sensitive Bacteria

Preliminary screening for heavy metal-sensitive bacteria showed that a strain from the Department of Microbiology and Biochemistry culture collection was the most sensitive to all the heavy metals and therefore used in further studies. Further comparison of the partial sequence obtained for this isolate with the GenBank database showed 99% similar to several species to the *Bacillus* spp. The 16s rRNA ribosomal gene sequence for this isolate (1455 bases) has been deposited in GenBank (Accession number: HM583795) and the isolate is assigned tentatively as *Bacillus* sp. strain Khayat.

Inhibition of MTT reduction by heavy metal and foreign species

The profile of MTT activity in *Bacillus* sp. strain Khayat sensitivity towards heavy metal is reflected in Fig. 1. Five heavy metals exhibited higher than 50% inhibition of MTT activity. Among the heavy metals, the rank of toxicity at 1 mg/L was Hg > Cu > Ag > Cd > Zn.

During further evaluation on the proposed system, the values for the limit of detection (LOD) and the limit of quantitation (LOQ) were determined based on the inhibition profile which was constructed using a One-phase Exponential decay model (GraphPad) with a correlation coefficient of >0.97 (Fig. 2). The LOD is the lowest concentration which can be detected with 99% confidence interval and is usually assigned as three times the standard deviation of the blank for the y-intercept. The LODs for Hg and Ag were 0.001 mg/L while Cu, Cd and Zn were 0.003, 0.067 and 0.201 mg/L, respectively. Because of the risk of undetectable analyte, which exist for every assay system, LOQ was also determined. The LOQ is the concentration level equal or above the value of LOD with acceptable precision (usually RSD < 10 to 25%) and accuracy (usually 80-120% recovery) and usually assigned ten times the standard deviation of the blank for the y-intercept (Miller and Miller, 2000). The LOQs for Hg, Cu, Ag, Cd and Zn were 0.008, 0.010, 0.006, 0.199 and 0.678 mg/L, respectively. Repeated measurement of the MTT assay for all the heavy metals suggests that the assay is reproducible with CV of the replicated data in 2 to 10% range.

All of the pesticides tested at 4 mg/L showed no effect to the assay relative to the control. In case of xenobiotics, only SDS gave interference of more than 50% activity compared to the control (Fig. 3) even though the concentration of xenobiotics used in this assay (4000 mg/L) was higher than average concentration found in aquatic bodies.

Real Water Samples Application

Despite of promising result in laboratory trial, some irregularities could be identified for several heavy metals. The magnitude of inhibition or toxicity of Hg to the MTT assay was not pronounced as expected even though at high concentrations (Table 2). On the other hand, in spite of lack in sensitivity of suggested *Bacillus* sp.-based MTT assay towards Cd (Table 1), the result showed that MTT-reducing activity was reduced to 100% even though Cd concentrations (BTIA 1, BTIA 2, PIEP 2) were lower than detectable range (Table 1). However, results suggested that *Bacillus* sp.-based MTT assay was similar with quantification made using atomic emission spectrometry analysis.

Discussion

Out of the 12 metals screened on *Bacillus* sp. strain Khayat, Hg, Ag, Cd, Cu and Zn exhibited higher than 50% inhibition of MTT activity.The results showed that formazan production was saturated after 20 minutes, thus indicated the potential of this strain to be used as a part of assay system. Even though, heavy metal sensitivity has been previously reported in several microorganisms such as *Mytilus galloprovincialis* (Beiras *et al*., 2003), *Tetrahymena pyriformis* (Bogaerts *et al.*, 2001) and *Vibrio fisheri* (Hsieh *et al.*, 2004), in this study, heavy metal monitoring system based on MTT reduction in *Bacillus* sp. strain Khayat offered extra edge as complete MTT reduction can be achieved in 20 min at room temperature. A very low LOD concentration especially for Hg and Ag reflects the sensitivity of MTT reduction in *Bacillus* sp. strain Khayat. *Bacillus* sp. has been report for having significant resistance in the presence of toxic material (Gomaa and Azab, 2007), therefore we believe that *Bacillus* sp.-based MTT assay will have some degree of resilience towards heavy metal toxicity and flexibility of microorganism,

The comparisons of the 50% inhibitory concentration (IC_{50}) obtained from this work to rainbow trout assay, daphnid (*Daphnia magna*), immobilized urease, papain, bromelain and Microtox® toxicity data are shown in Table 1. Higher IC_{50}value for Hgcompared to other assays indicated it to be less sensitive towards Hg but overlapped confidence interval of Hg IC_{50} values with *D. magna* and rainbow trout IC_{50} values probably suggest its comparable sensitivity. Sensitivity of MTT assay towards Ag is much less compared to Microtox® but almost ten times higher compared to papain. IC_{50}value for Cd in MTT reduction based *Bacillus* sp is lower than the confidence interval of other assays indicating a higher sensitivity for the MTT assay towards Cd. In general, *Bacillus* sp-based MTT assay is better than *D. magna* but less sensitive towards heavy metal compared to Microtox®.

Table 1: Sensitivity of the assay to heavy metals in comparison to LC_{50} and/or IC_{50} of free and immobilized urease, papain, bromelain, Microtox®, *Daphnia Magna,* Rainbow trout and *R. meliloti*

	IC_{50} or EC_{50} (mg/L)						
Metals	MTT reduction by *Bacillus* sp. strain Khayat[#]	Immobilized Urease[a]	Papain[b]	15-min Microtox®[a]	*Dapniamagna*[c]	Rainbow trout[c]	*R. melilott*[d]
Hg	0.046 (0.041-0.051)	0.33	0.39	0.003	0.0052-0.21	0.033-0.21	0.0159
Ag	0.044 (0.037-0.056)	n.d	0.40	0.008	1.930	0.05	nd
Cu	0.057 (0.049-0.068)	0.41	0.004	0.076-3.8	0.020-0.093	0.25	0.950
Cd	0.857 (0.748-1.004)	1.59	0.1	19-220	0.041-1.9	0.15-2.5	0.791
Zn	1.716(1.496-2.010)	14.6	2.11	0.27-29	0.54-5.1	0.55-2.2	0.84

[#] This Study with 95% Confidence Interval of LC_{50}. [a] Jung *et al.* (1995). [b] Shukor *et al.*, (2006). [c] Rodgers *et al.*, (1997). [d] Botsford *et al.*(1998). *nd, Not determined

Table 2: Field trial results: Comparison of heavy metal concentration and the degree of inhibition of MTT-reducing activity using *Bacillus* sp-based MTT assay. Data is mean± standard error of the mean (n=3).

Locations	Longitude and latitude position	% Inhibition of MTT-reducing Activity[1]	Concentrations of Heavy metal (mg/L)[#]				
			Cd	Ag	Cu	Hg	Cr
BTIA 1	N05º20.640' E100º26.470'	100	0.04±0.001[c]	0.328±0.009[a]	0.642±0.012[c]	0.06±0.00[b]	0.45±0.08[b]
BTIA 2	N05º18.947' E100º26.348'	100	0.03±0.004[c]	0.12±0.023[b]	0.916±0.021[c]	0.24±0.02[a]	1.24±0.03[a]
PIEP 1	N 05º 20.96, E 100º 24.17'	100	18.4±1.34[a]	n.d.	6.13±0.09[a]	n.d.	n.d.
PIEP 2	N 05º 20.96, E 100º 17.25'	100	0.401±0.02[b]	0.05±0.001[c]	2.341±0.04[b]	0.022±0.001[a]	n.d.
GAFR 1	N 02°33.197' E 102°45.340'	0	n.d.	n.d.	n.d.	n.d.	n.d.
GAFR 2	N 02°33.194' E 102°45.312'	0	n.d.	n.d.	n.d.	n.d.	n.d.

Means in each column with same superscript letter are not significantly different amongst themselves when Tukey's post hoc tests were used at 5% significance level; [1] 20% Inhibition is considered significant toxicity. Data were presented as whole number; n.d. = not detected; [#] Quantification using atomic emission spectrometry analysis; BTIE-Bukit Tinggi Industrial Estate, Penang; PIE-Prai Industrial Estate, Penang; GAFR-GunungArong Forest Reserve, Mersing, Johor

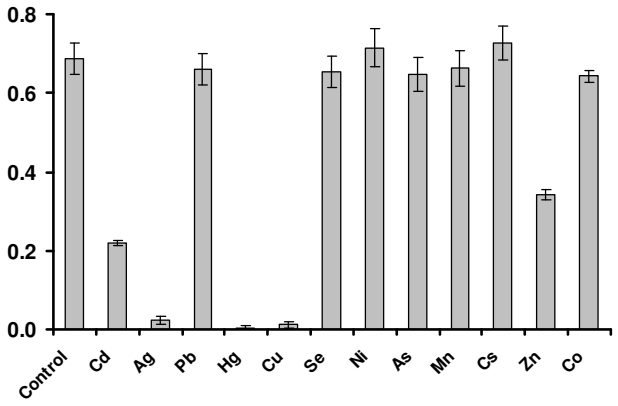

Fig. 1: Screening results for the inhibitory effect of heavy metals on bacterial respiratory activity using the MTT assay. Data is mean ± standard error of the mean (n=3)

In order to investigate the influence of foreign species on MTT assay, series of tests using xenobiotics and pesticide were conducted. Since MTT can be reduced by NAD(P)H reductase (Berridge and Tan, 1993), the presence of xenobiotics and heavy metals in the electron transport system will inhibit NADH production. Therefore, low NADH concentration will reduce the respiratory activity of the cells which is measured by the MTT assay. Recent data suggests that *Bacillus* sp-based MTT assay is only sensitive to heavy metals suggesting specific usage of this assay to the detection of heavy metals in the environment.

In 1996, only 13 rivers in Malaysia has been classified as polluted but within this number has gone up to 111 rivers in 2010 (DoE, 2011) and cases of water pollution have increased over the years. Thus, there is an urgent need for development of simple and fast screening procedures to determine the presence of toxic heavy metals. Industrial parks in the Juru River Basin are long suspected to be anthropogenic sources of elevated heavy metals in the rivers due to the many metal-related work industries within the park and this is one of the most studied areas in heavy metal pollution (Shukor *et al.*, 2006; 2008). Due to the fact that this river basin is one of water source for the surrounding agricultural areas and its waters have deteriorated over the years, heavy metals monitoring of this area is crucial (Al-Shami *et al.* 2011).

The present data (Table 2) allow us to (i) evaluate the ability of the assay to detect heavy metal in non-laboratory prepared effluent and (ii) to establish level of sensitivity of *Bacillus* sp.-based MTT assay as near real time monitoring tool for heavy metal. In theory, the use of MTT-reduction activity in *Bacillus* sp. will provide a simple, less expensive and will produce result within 20 min. The results showed that all four sampling sites showed an overwhelmingly high concentration of heavy metals above the maximum permissible limit allowed by the Department of Environment, Malaysia with copper ubiquitously found to exceed the limit in positive samples as tested with the *Bacillus* sp.-based MTT assay. In the case of Hg, the results of the MTT assay are not as expected, possibly due to the limited bioavailability or solubility of the

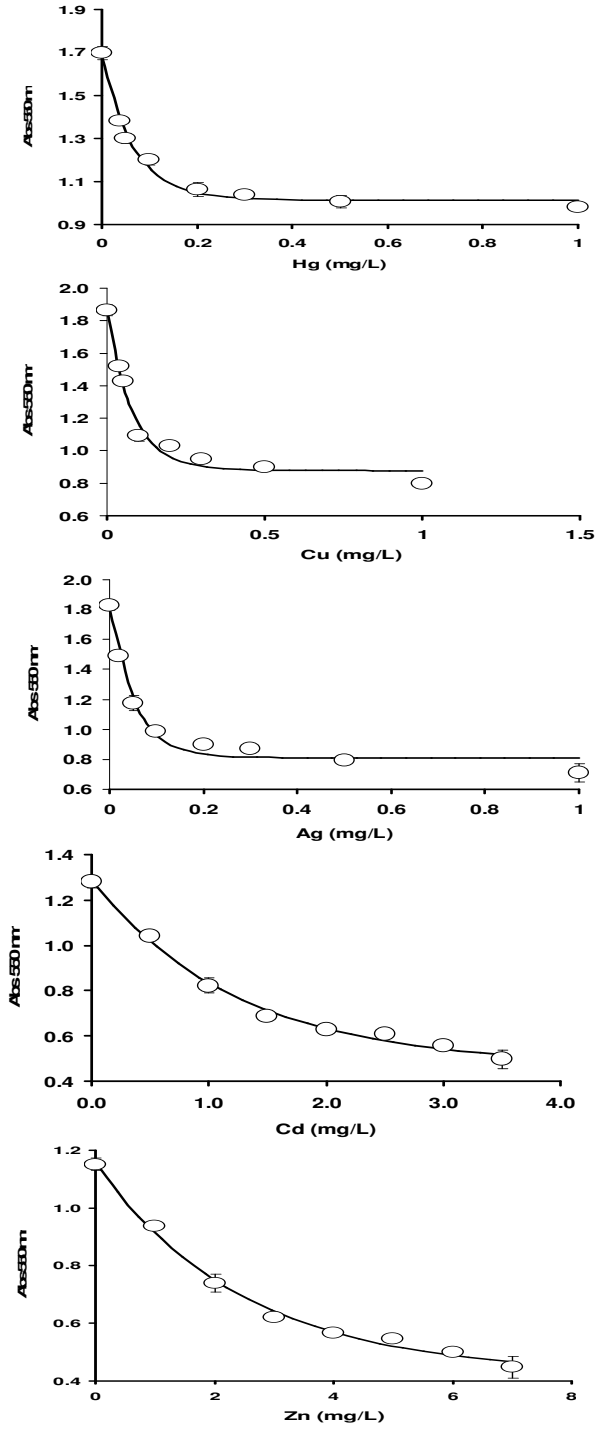

Fig. 2: Inhibition profile mercury (A), copper (B), silver (C), cadmium (D) and zinc (E) using the MTT assay. Goodness of fit value, R^2 for the curves representing the heavy metals inhibitions is higher than 0.97. Data is mean± standard error of the mean (n=3)

mercury form in the samples. Increases in the sensitivity of *Bacillus* sp.-based MTT assay especially for Cd

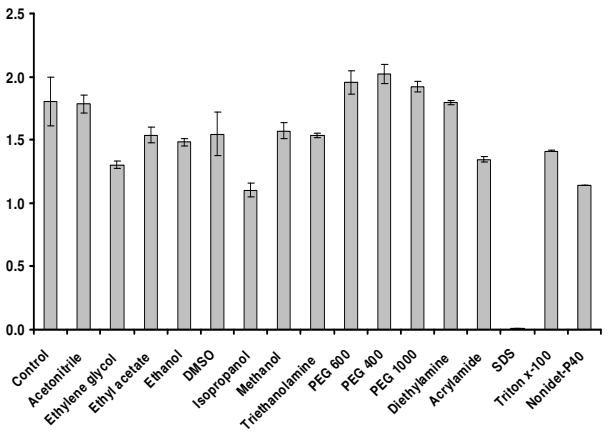

Fig. 3: Effects of bacterial MTT assay by xenobiotics. Data is mean± standard error of the mean (n=3)

suggested no resistance to toxicants by bacteria. Cd sensitivity of *Basillus* sp. in water source is a plus point for MTT assay since there is a chance of this water for irrigation use. In long term, any contamination of Cd in water will be transferred to seed and into human via food chain (Hussain *et al.* 2010). Similar pattern can also be seen for Ag. As mentioned above, it was difficult to confirm the IC_{50} values or detection range obtained under laboratory condition in the field trial due to factors such as exposure time and sample condition (Fulladosa *et al.,* 2004).

In conclusion, a novel assay using MTT-reduction activity in *Bacillus* sp was developed for heavy metal monitoring. *Bacillus* sp has several advantages in terms of culture and generation time of bacteria which their response times to organic enrichment or to toxic substances are likely to be quite rapid, thus able to provide rapid result. This *Bacillus* sp-based MTT assay was is not affected by foreign species such as xenobiotics and pesticide, and therefore has a potential to be used as monitoring tools.

References

Al-Shami, S.A., C.S. MdRawi, A.H. Ahmad, S. Abdul Hamid and S.A. Mohd Nor, 2011. Influence of agricultural, industrial, and anthropogenic stresses on the distribution and diversity of macro invertebrates in Juru River Basin, Penang, Malaysia. *Ecotoxicol. Environ. Saf.,* 74: 1195–1202

Baumstark-Khan, C., R.A. Khan, P. Rettberg and G. Horneck, 2003.Bacterial Lux–Fluoro test for biological assessment of pollutants in water samples from urban and rural origin. *Anal. Chim. Acta,* 487: 51–60

Beiras, R., N. Fernández, J. Bellas, V. Besada, A. González–Quijano and T. Nunes, 2003. Integrative assessment of marine pollution in Galician estuaries using sediment chemistry, mussel bioaccumulation and embryo–larval toxicity bioassay. *Chemosphere*, 52: 1209–1224

Berridge, M.V. and A.S. Tan, 1993. Characterization of the cellular reduction of MTT. Subcellular localization, substrate dependence and involvement of mithochondrial electron transport in MTT reduction. *Arch. Biochem. Biophys.,* 303: 474–482

Bogaerts P., J. Bohatier and F. Bonnemoy, 2001. Use of the ciliated protozoan *Tetrahymen apyriformis* for the assessment of toxicity and quantitative structure–activity relationships of xenobiotics: comparison with the Microtox test. *Ecotoxicol. Environ. Saf.,* 49: 293–301

Botsford, J.L., 1998. A simple assay for toxic chemical using a bacterial indicator. *World J. Microbiol. Biotechnol.,* 41: 369–376

Botsford, J.L., 2000. Role of EDTA in the simple method determining toxicity using a bacterial indicator organism. *World J. Microbiol. Biotechnol.,* 16: 353–359

Bullich, A.A., 1986. Bioluminescene assays. *In*: *Toxicity Testing Using Microorganism*. pp: 57–72. Bitton, G. and B.J. Dutka (eds). CRC Press Inc, Boca Raton, Florida, USA

Chishti, Z. and M. Arshad, 2012. Growth linked biodegradation of chlorpyrifos by *Agrobacterium* and *Enterobacter* spp. *Int. J. Agric. Biol*., 15: 19–26

DoE., 2011. *Environmental Quality Report 2010.* Department of Environment, Ministry of Science, Technology and the Environment, Malaysia

Fulladosa, E., J.C. Murat, M. Martínez and I. Villaescusa, 2004. Effect of pH on arsenate and arsenite toxicity to luminescent bacteria (*Vibrio fischeri*). Arch. *Environ. Contam. Toxicol.*, 46: 176–182

Gomaa, O.M. and K.S.H. Azab, 2007. Resistance to hydrogen peroxide in textile waste water *Bacillus sp*. *Int. J. Agric. Biol.*, 9: 247–251

Jung, K., G. Bitton and B. Koopman, 1995. Assessment of urease inhibition assays for measuring toxicity of environmental samples. *Water Res.,* 29: 1929–1933

Hussain, A., G. Murtaza, A. Ghafoor, S.M.A. Basra, M. Qadir and M. Sabir, 2010. Cadmium contamination of soils and crops by long term use of raw effluent, ground and canal waters in agricultural lands. *Int. J. Agric. Biol.*, 12: 851–856

Hsieh, C.–Y., M.–H.Tsai, D.K. Ryan and O.O. Pancorbo, 2004. Toxicity of the 13 priority pollutant metals to *Vibrio fisheri* in the Microtox® chronic toxicity test. *Sci. Total Environ.*, 320: 37–50

Mehraban, K., R.P. Andrey, C.F. George and A.B. Diane, 1998. Detection of heavy metals by immunoassay: Optimization and validation of a rapid, portable assay for ionic cadmium. *Environ. Sci. Technol.,* 32: 137–142

Miller, J.N. and J.C. Miller, 2000. *Statistics and chemometrics for analytical chemistry*. 4th ed. pp: 232–235. Prentice Hall, England

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65: 55–63

Rodgers, J.H.J., E. Deaver, B.C. Suedel and P.L. Rogers, 1997. Comparative aqueous toxicity of silver compounds: laboratory studies with freshwater species. *Bull. Environ. Contam. Toxicol.,* 58: 851–858

Shukor, M.Y., N.A. Baharom, F.A. Rahman, M.P. Abdullah, N.A. Shamaan, M.A. Syed, 2006. Development of a heavy metals enzymatic–based assay using papain. *Anal. Chim. Acta,* 566: 283–289

Shukor, M.Y.,N. Masdor, N.A. Baharom, J.A. Jamal, M.P.A. Abdullah, N.A. Shamaan and M.A. Syed, 2008. An inhibitive determination method for heavy metals using bromelain, a cysteine protease. *Appl. Biochem. Biotechnol.,*144: 283–291

Sun, B., N. Nimalakhandan, E. Hall, X.H. Wang, J. Prakash and R. Maynes, 1994. Estimating toxicity of organic chemicals to activated–sludge micro–organisms. *J. Environ. Eng.,* 120: 1459–1469

Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane, 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.,* 173: 697–703

Zonneveld, I.S., 1983. Principles of bioindication. *Environ. Monit. Assess.,* 3: 207–217

(Received 08 March 2013; Accepted 23 September 2013)