ASIAN JOURNAL OF PLANT BIOLOGY
Website: http://journal.hibiscuspublisher.com

Use of microorganisms, enzymes and plant proteases for heavy metals biomonitoring- a mini review

Mustafa Kaira, Mohd Izuan Effendi Halmi and Mohd Yunus Shukor

Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

Corresponding Author: Mohd. Yunus Abd. Shukor; Email: mohdyunus@upm.edu.my

HISTORY
Received: 1 October 2013
Received in revised form: 30 November 2013
Accepted: 13 Disember 2013
Available online: 25 Disember 2013

KEYWORD
Heavy metals;
Microtox;
Bacteria;
Enymes;
Protease

ABSTRACT

Heavy metals are toxic elements that are beneficial in industrial process but are toxic to organisms at certain levels once they enter the environment. Hence their monitoring are urgently important. Instrumental analyses are the current accepted method but are cumbersome, time-consuming, expensive and required highly-skilled technical personnel to operate them. Assays using microorganism and their products such as enzymes are beginning to emerge as plausible methods for preliminary screening followed by instrumental methods. This approach cut costs and time but the challenge is to find a robust enzyme system that could withstand variation in environmental conditions and sampling. Various enzymes such as urease, acetylcholinesterase, invertase and glucose oxidase have been used but their activity is strongly affected by extreme variation of pHs and temperature often associated with real samples. The plant proteases such as papain and bromelain have been proven to be robust enzymes for the detection of heavy metals and are the subject of this mini review.

DEFINITION OF HEAVY METALS

First of all, metals are defined according to the Encyclopaedia Britannica as "any of a class of substances characterized by high electrical and thermal conductivity as well as by malleability, ductility, and high reflectivity of light". Out of these metals are metals that of special biological and hence environmental effect referred to as heavy metals. They have been defined in many different ways based on their chemical properties, toxicity, atomic weight or atomic number. In general, heavy metals are defined as metals with densities greater than 5 g cm-3.

The term "heavy metal" even though it is widely used in many different fields, is considered to be a "misinterpretation" because it lacks a "coherent scientific basis" by an IUPAC technical report [1].

HEAVY METAL POLLUTION

In our industrialized society, heavy metal pollution is becoming a bigger problem day by day. Heavy metal pollution is wide spread mainly because of its industrialised applications. As we know, heavy metals are very toxic to living systems and inhibit most enzymes at even low concentrations. This knowledge is applied in industry to develop insecticides, pesticides, fungicides and many other biocides. Use of these biocides in agriculture and house hold finds its way to the underground water. Another reason for heavy metal's large usage is its catalytic properties due to its unique physio-chemical nature. Heavy metals are used in some industrial processes to catalyse certain reactions. Effluents from such industries which sometimes contain heavy metals are poured directly into the sea. At first this was not perceived as a problem because it was assumed that the heavy metal will be diluted to low sub-lethal concentrations, but the discovery of bioaccumulation and biomagnification in marine life proved otherwise. In Malaysia here, a survey report by the Malaysian Department of Environment reveals that 10.4% of the 420,000 tonnes of scheduled wastes contain heavy metals [2].

THE MINAMATA BAY INCIDENT

One of the most famous cases of heavy metal pollution, the Minamata Bay Incident, took place in the 1950's in a small town in Japan. It was rooted to an acetaldehyde-producing company called Chisso Minamata. This company used methyl sulphate as a catalyst in their production of acetaldehyde. The highly toxic compound, after being used, was released into the Minamata Bay resulting to the bioaccumulation of the mercury in fish which was the main source of food for the local inhabitants. Estimates by scientists reveal that the biomagnification could have been as great as a million fold. This caused a disease called Minamata

Disease, a neurological syndrome. Symptoms include numbness, lack of muscular coordination in the limbs, muscle weakness, narrowing of the field of vision and even auditory and speech constraints [3]. It was reported that in extreme cases it can cause insanity, coma paralysis and ultimately result to death within weeks of the manifestations of the symptoms. Symptoms persist way after cessation of exposure [4]. Minamata disease has a very high mortality rate as it was reported that approximately 80 % of the victims ultimately died (Ministry of the Environment Government of Japan, 2001).

DETECTION OF HEAVY METALS

Heavy metal can be detected in many different ways. Either by using electronic appliances, which is by far the most accurate but expensive means of detection; by using animals -this is now obsolete because of its slowness and animal rights issues, and also by using biological systems such as enzymes, micro-organisms or even antibodies.

Table 1. Summary of heavy metals data for the West Coast of Peninsular Malaysia.

Metals	State with samples above standard	Highest reported level	Lowest reported level	No. of samples above standard
Arsenic (As) 0.05mg/L	Johore (entire state)	1.83	0.037	2
	Perak	0.38	below detection level	31
Lead (Pb) 0.09mg/L	Perak	0.33	0.14	43
		2.01	0.2	23
Mercury (Hg) 0.001mg/L	Johor	0.05	below detection level	66
	Pulau Pinang	0.08	below detection level 0	20
	Perak	0.04	0.003	14
	Kedah	0.018	0	6
	Melaka	0.004	0	16
	Negeri Sembilan	0.2	below detection level 0	2
	Pulau Pinang	0.07		4
Cadmium (Cd) 0.005mg/L	Johor	0.27	below detection level below detection level	1
	Selangor	0.01		6
	Kedah	0.02		33
	Perak			

CONVENTIONAL METHOD

Conventional methods of detecting heavy metals involve the detection of heavy metals by using mechanical instruments. They are the most of accurate type of detection technique hence they are always used as a standard to test the sensitivity and accuracy of other detection techniques. Conventional detection techniques are very specific to the heavy metal they detect since they detect the metal ion base on features that are very unique to the metal. This category of detection technique is also known to be very precise and sensitive, meaning that it can detect heavy metals at extremely low concentrations such as one part per trillion using the Inductively coupled plasma mass spectrometry (ICP-MS).

ATOMIC ABSORPTION SPECTROMETRY AND INDUCED COUPLED PLASMA

Among the two, Atomic Absorption Spectrometry (AAS) is said to be the most widely used and the most conventional. AAS makes use of absorption spectrometry of samples to assess the concentration of the analyte because the absorption varies directly and consistently with the concentration of the analyte (atoms), it

relies on the Beer-Lambert Law. The nature of the absorbed light is unique to each element, this confers specificity to the AAS. AAS can detect atoms in as little as parts per billion.

The Induced Coupled Plasma- Mass Spectrometry (ICP-MS) on the other hand is based on coupling together inductively coupled plasma to produce ions with a mass spectrometer as a means of isolating and detecting the ions. ICP-MS a wide spectrum of metals and many non-metals as well at concentrations as low as a part in a trillion. It has a number of advantages over AAS in that it is faster, more precise and it is more sensitive.

The drawbacks of these type of detection techniques is that they are expensive to procure and the repair and maintenance costs are expensive too. Another thing is that these techniques are very complicated and require trained personnel to operate. They cannot be used in the field site because of their size and heavy electricity requirements which eliminates the possibility to use batteries. Hence simple, fast and cheap means of detection are needed especially for routine analysis of samples.

BIOASSAY

Bioassay is define as the use of living things or their products such as enzymes and antibodies to detect and quantify toxicants. Bioindicators involve the use of living organisms that are naturally found in a sampling site for indication of toxicity or the general health condition of an environment or an ecosystem.

More advance bioassay have been developed that produce electrical signals called biosensors Biosensors combine the biological component with an electrochemical detector component which together produces an electrical signal in response to analyte concentration. It is generally more user friendly since it displays the results in a screen or a meter reader like we have in our cars, no standard curves are needed here [5].

MICROORGANISM-BASED BIOASSAYS

Polytox™

Polytox is a commercialised consortium of 12 bacteria that is used to test for the presence of toxic compounds in waste water or even biological organisms. It is one of the most widely used forms of bioassays in the market. The kit is designed to indicate the presence of toxicants. In the presence of toxicants, respiration of the blend of bacteria is inhibited. The extent to which the respiration, i.e. the uptake of oxygen, is retarded upon exposure to toxic compound is directly proportional to the concentration of toxicants in the sample being tested. The drawback of this assay is that it is difficult to maintain once activated and that it needs a computer program to analyse the data. Also, this assay requires a trained personnel to run and it is not selective, it does not distinguish between toxicants. Another problem is that it requires expensive and fragile equipment such as the oxygen electrode [6].

Microtox ™

Microtox ™ developed by Beckman Instruments Inc. It is also like Polytox™, it is a wide spectrum non-selective bioassay that detects a wide variety of toxicants but uses only a biolumiscent bacteria *Photobacterium phosphoruem*. The most important edge this bioassay has over other forms of bioassay is its convenience to monitor a large spectrum of toxicants. It can be used to test both liquid and material and semi-solid material which makes it very useful for bioremediation sites [7].

The main problem with this assay is its cost and its sophistication, hence requiring a trained personnel to operate properly in order to avoid miss-use and damage to the equipment. Besides that, the assay is difficult to maintain as it must be run under cold temperatures which is not convenient for on-site use especially in our part of the world which is always hot.

The problems highlighted above further emphasis the need for cheaper, faster and easy-to-operate methods of bioassays to monitor environmental toxicity. These assay a very sensitive and reproducible as well but they sometimes require expensive equipment and require trained personnel to operate either because they are too complicated or because the instruments are too expensive and hence cannot be trusted with amateurs [8]. This takes us to the next form of bioassay which is the modern of bioassay that does not employ much instrumentation.

The MTT assay

The MTT (3-(4,5-dimethyl-thiazol-2,5-diphenyltetrazolium bromide) is a bioassay that has been developed using *Phizobium weliloti* as the indicator by its reduction of the MTT-fromazan dye [8]. Reduction of this dye results in a colour change from colourless to purple-blue. Hence, the more the inhibition from a toxic compound, the less the reduction of the MTT-formazan and so the lower the colour intensity. This relationship is the basis by which the assay works. Unlike the bioassays above, it is quite fast, cheap, very simple to carry out, and does not require much instrumentation to run, a simple calculator can be used to analyse the results yet the sensitivity is comparable to that of Microtox and Polytox.

Enzyme inhibition-based bioassays

Because of the nature of enzymes and the effect of certain chemicals on their activity, enzymes have been employed in a lot of bioassays to detect toxicants. In fact, most bioassays are enzyme based, directly or indirectly. Even the microorganism-based assays are in actual sense enzyme based and a lot of anti-body based bioassays are linked with enzymes. But it this section we would take a look at selected bioassays that are based on enzymes explicitly.

Proteases papain trypsin and bromelain

Papain a protease extracted from papaya, was used to develop a bioassay [9]. The assay was developed based on the inhibitive nature of heavy metals towards the enzyme. It was assayed using the Casein-coomassie-dye-binding assay. This means that the enzyme activity was determined by using Bradford reagent to determine the amount of casein hydrolysed within a given period of time. The higher the concentration of heavy metal, the lower the enzyme activity and hence the less casein substrate is hydrolysed by the enzyme and vice versa. The amount is casein was detected by the addition of Bradord reagent. This relationship was exploited in the detection of heavy metal by the enzyme. This is the very principle by which this work goes by. The assay was found to be sensitive to Hg2+, Ag2+, Pb2, Zn2+ with IC_{50} values of 0.39, 0.40, 2.16, 2.11 ppm respectively. Papain was later used to develop a simple bioassay in the form of a kit called XenoAssay™ that was very easy to operate even by secondary school sudents, without the need for complicated electronic equipment. This makes the detection of heavy metals in the environment very easy as even teenagers can biomonitor their neighbourhood and report to the relevant authorities. If the work is left to authorities only it might not be as effective.

Modified trypsin [10] and the typical trypsin-based bioassay [11] were also developed, under the same principle by which the papain-based bioassay works. In the presence of heavy metals, casein is not hydrolysed and hence stains blue while in the absence of inhibitors the casein is hydrolysed hence the Bradford reagent [12] cannot bind to the small polypeptide fragments as a result remaining brown. Trypsin was most sensitive to zinc with an IC_{50} value of 5.78 ppm.

Bromelain-based bioassay under the same principle as trypsin and papain was also developed [13]. It also employs Coomassie dye to track the hydrolysis of casein by the enzyme. It was reported to be most sensitive to Hg2+ and Cu2+ exhibiting a dose response curve with an IC_{50} of 0.15 ppm for Hg2+ and a one-phase binding curve with an IC_{50} of 0.23 ppm for Cu2+. It showed lower IC_{50} values than immobilised urease [14] although it suffers from interference [15] and papain for Hg2+ but higher values than 15-min Microtox, and rainbow trout for Cu2+. Later on bromelain was further purified [16] to study the effect of partial purification of the enzyme on its heavy metal sensitivity. The same thing we are trying to do with ficin in this work. After partial purification, the enzyme showed lower IC_{50} than before partial purification for all heavy metals ficin was sensitive to. The table below shows the comparison of the IC_{50} values.

Table 2. Comparison of IC_{50} (ppm) of crude and partially purified bromelain (95% Confidence interval) [16].

Heavy Metal	Regression model	Crude Bromelain	Partially purified Bromelain
Cu	One phase binding	0.172 to 0.322	0.07 to 0.112
Hg	Four parameter logistic	0.132 to 0.164	0.09 to 0.115

CONCLUSION

Emerging technologies for heavy metals monitoring are limited by the source of biological materials suitable for heavy metal detection. The use of novel materials such as enzymes could pave ways for novel methods of heavy metals monitoring.

Microbiological methods such as Microtox and Polytox are here to stay due to their ability to detect a multitude of toxicants whilst enzyme-based methods are more specific and fast allowing for near-real-time potential.

REFFERENCE

[1] Duffus, J. H. (2002). "Heavy metals" a meaningless term? (IUPAC Technical Report). Pure and Applied Chemistry, 74(5), 793–807. doi:10.1351/pac200274050793

[2] DOE, Malaysia Environmental Quality Report. Department of Environment, Ministry of Natural Resources and Environment Malaysia, ISSN 0127-6433, 2011.

[3] Gilbert, Steven G. A Small Dose of Toxicology. CRC Press, 2004.

[4] Ekino, S., Susa, M., Ninomiya, T., Imamura, K., & Kitamura, T. (2007). Minamata disease revisited: An update on the acute and chronic manifestations of methyl mercury poisoning. Journal of the Neurological Sciences, 262(1–2), 131–144. doi:10.1016/j.jns.2007.06.036

[5] Sakti, S. P., Lucklum, R., Hauptmann, P., Bühling, F., & Ansorge, S. (2001). Disposable TSM-biosensor based on viscosity changes of the contacting medium. Biosensors & Bioelectronics, 16(9-12), 1101–1108.

[6] Nirmalakhandan, N., Arulgnanendran, V., Mohsin, M., Sun, B., & Cadena, F. (1994). Toxicity of mixtures of organic chemicals to microorganisms. Water Research, 28(3), 543–551. doi:10.1016/0043-1354(94)90005-1

[7] Sun, B., Nirmalakhandan, N., Hall, E., Wang, X. H., Prakash, J., & Maynes, R. (1994). Estimating Toxicity of Organic Chemicals to Activated-Sludge Microorganisms. Journal of Environmental Engineering, 120(6), 1459–1469. doi:10.1061/(ASCE)0733-9372(1994)120:6(1459)

[8] Botsford, J. L. (1997). A simple, rapid, inexpensive assay for toxic chemicals using a bacterial indicator. Global Environmental Biotechnology Proceedings of the Third Biennial Meeting of the International Society for Environmental Biotechnology (Vol. Volume 66, pp. 429–443). Elsevier. Retrieved from http://www.sciencedirect.com/science/article/pii/S0166111697800611

[9] Shukor, Y., Baharom, N. A., Rahman, F. A., Abdullah, M. P., Shamaan, N. A., & Syed, M. A. (2006). Development of a heavy metals enzymatic-based assay using papain. Analytica Chimica Acta, 566(2), 283–289. doi:10.1016/j.aca.2006.03.001

[10] Šafařík, I., Ptáčková, L., Koneracká, M., Šafaříková, M., Timko, M., & Kopčanský, P. (2002). Determination of selected xenobiotics with ferrofluid-modified trypsin. Biotechnology Letters, 24(5), 355–358. doi:10.1023/A:1014521021795

[11] Shukor, M. Y., Baharom, N. A., Masdor, N. A., Abdullah, M. P. A., Shamaan, N. A., Jamal, J. A., & Syed, M. A. (2009). The development of an inhibitive determination method for zinc using a serine protease. Journal of Environmental Biology / Academy of Environmental Biology, India, 30(1), 17–22.

[12] Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248–254.

[13] Shukor, M. Y., Masdor, N., Baharom, N. A., Jamal, J. A., Abdullah, M. P. A., Shamaan, N. A., & Syed, M. A. (2008). An inhibitive determination method for heavy metals using bromelain, a cysteine protease. Applied Biochemistry and Biotechnology, 144(3), 283–291.

[14] Rodriguez, B. B., Bolbot, J. A., & Tothill, I. E. (2004). Urease–glutamic dehydrogenase biosensor for screening heavy metals in water and soil samples. Analytical and Bioanalytical Chemistry, 380(2), 284–292. doi:10.1007/s00216-004-2704-0

[15] Krawczyński vel Krawczyk, T., Moszczyńska, M., & Trojanowicz, M. (2000). Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. Biosensors and Bioelectronics, 15(11–12), 681–691. doi:10.1016/S0956-5663(00)00085-3

[16] Masdor, N.A., Said, N.A.M. 2011. Partial purification of crude stem bromelain improves it sensitivity as a protease inhibitive assay for heavy metals. Australian Journal of Basic and Applied Sciences 5 (10) , pp. 1295-1298