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Near Real-time Biomonitoring of Copper from an Industrial Complex Effluent Discharge Site Using a Plant Protease Inhibitive Assay

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ABSTRACT

In this work, a temporal monitoring work for heavy metals from an effluent discharge point in the Juru Industrial Estate was carried out using the protease extracted from garlic (*Allium sativum*) as the principal bioassay system. casein-Coomassie-dye binding assay method has utilized this purpose. The periodic sampling results for one day of a location in the Juru Industrial Estate showed temporal variation of copper concentration coinciding with garlic protease inhibition with the highest concentrations of copper occurring between 12.00 and 16.00 hours of between 3 and 3.5 mg/L copper. The crude proteases extracted from *Allium sativum* successfully detect temporal variation of copper form this location. In conclusion, this assay method has the potential to be a rapid, sensitive, and economic inhibitive assay for the large-scale biomonitoring works for the heavy metal copper from this area.

INTRODUCTION

Heavy metals have common industrial usage and are found in escalating levels as pollutants in every part of the world [1–6]. Soil and water bodies near industrial places including the Juru river in Penang, Langat river in Selangor and Linggi river in Negeri Sembilan are contaminated with organic and inorganic toxicants including heavy metals with levels exceeding the Maximum Permissible Limit outlined by the Department of Environment (DOE) [7,8]. Therefore, quick and economic solutions to monitor the existence of heavy metals in the environment are essential [9–11]. Current analytical methods based on instruments, for example, atomic absorption and emission spectrometry are extremely sensitive but at the same time expensive and is time-consuming as samples need to be brought to the lab. In addition, these methods are affected strongly by sample matrix and further processing time to remove these interferences will prolong analysis times [5,12]. The single utilization of these instruments for heavy metal detection is incredibly costly, requires a competent individual to operate it and are not receptive to near real-time investigation where an assay for toxicants needs be completed in a short time period instead of days [13]. To overcome these problems, the dominant circumstance for routine biomonitoring of heavy metals is to form a composite between instrument- and bioassay methods [14–17]. One of the most important outcomes in the use of biomonitoring-based systems is the possible development of an economical assay methods with near real-time capability [18–20].

Presently, the United States environmental agency or USEPA has recommended the use of whole cell-based bioassays for the detection of heavy metals, and these include systems such as Polytox™ and Microtox™ [21,22]. The high cost of these systems is prohibitive to be used as a rapid assay for large-scale monitoring. The temporal and spatial concentrations of heavy metals in running waters monitored in near real-time is very useful in environmental forensics to pinpoint the source of heavy metals [23–31]. Currently, there several enzyme-based inhibitive assays for toxicants have been

developed, and they have demonstrated exceptional qualities such as being rapid, fast and simple [19,20,32,33]. As large-scale monitoring involves the use of a lot of enzymes, a cheaper source, preferably that can be sourced locally is needed. In one of our previous works, we demonstrated a near-real time capability of a bacterial protease; achromopeptidase in detecting heavy metal pollution in the Juru Industrial complex [20]. One of the problems in using this protease inhibitive assay is the source is expensive and difficult to be obtained readily.

As previously we have developed a garlic-based assay for heavy metals and show that it is sensitive to the heavy metal copper [34], we utilize this system in detecting heavy metal pollution in a similar site under near real-time operation.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA), Fisher (Malaysia) and Merck (Darmstadt, Germany).

Preparation of the Coomassie Brilliant Blue G-250-based Bradford reagent

About 0.1 g of Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St. Louis, USA) was dissolved in a mixture of 100 mL of phosphoric acid (85%) and 50 mL of 95% ethanol. The solution was brought to 1 L with deionized water and then stirred overnight. After filtration through a filter paper (Whatman Filter Paper No. 1), the filtrate was stored in a dark bottle.

Preparation of casein solution

A casein stock solution (10.0 mg/mL) was prepared by dissolving two grams of casein (Sigma-Aldrich, St. Louis, USA) in alkaline solution at pH 8.0 and incubated with stirring at 60ºC overnight. The solution was filtered through several layers of cheesecloth and then centrifuged for 15 min at 10,000×g until a clear supernatant was obtained. The concentration of the casein was measured using the Bradford dye-binding assay with crystalline BSA (Sigma-Aldrich, St. Louis, USA) as the standard. Casein working solutions were prepared fresh daily at the final concentration of 0.3 mg mL^{-1} [12].

Preparation of proteases from *Allium sativum*

Allium sativum crude protease was prepared as before [34]. Several cloves of *Allium sativum* were weighed and soaked overnight at 4ºC in deionized water in a 1:2 ratio (w/v). Then, an ice-cold steel blender was utilized to homogenize the garlic preparation. The crude preparation was then centrifuged at 15,000×g for 10 min at 4°C. The supernatant was further filtered using a syringe filter (0.45 µm). The concentration of the garlic protein was measured using the Bradford dye-binding assay with crystalline BSA (Sigma-Aldrich, St. Louis, USA) as the standard.

Temporal Field trial

Water samples were collected from the Prai Industrial estate (N 05° 21.599, E 100° 24.282) on the 1st of December 2013. This site was chosen as the location of the near-real-time biomonitoring using the tomato crude plant protease inhibitive assay as our previous study [34] shows a very high concentration of copper here (21.04 mg/L). Water samples were collected periodically in acid-washed HDPE bottles containing several drops of 1% (v/v) HNO_3. The samples were filtered with 0.45 µm syringe filter. Twenty microliters of the clear filtrate were mixed with 120 µl of 50 mM phosphate buffer pH 6.5 followed by the addition of 50 µl of the crude enzyme in an Eppendorf tube and again mixed thoroughly. The mixture was incubated for 20 min at ambient temperature. After the incubation period with a water sample, 50µl of casein from a stock solution of 0.3 mg ml^{-1} was added and mixed thoroughly, and the absorbance was measured using a portable spectrophotometer. The calculation for the percentage of inhibition was as before [20]. The validation of heavy metals in the water samples was carried out using Inductive Couple Plasma spectrophotometer on a Perkin Elmer Optima 3000 ICP-OES. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

The assay system utilized in this study originates from the protease Coomassie dye-binding assay originally developed by Shukor et al. [12] using the plant protease papain to detect heavy metals. Plant proteases are generally heated stable and have rapid enzyme activity with broad temperature and pH stability. In addition, plant proteases are easy to prepare and are very cheap. The garlic-based inhibitive assay utilizes in this study has been demonstrated previously to be sensitive to heavy metals. The IC_{50} values for the heavy metals mercury, copper, cadmium, nickel and chromium were 0.0590 mg/L, 0.6398 mg/L, 1.291 mg/L, 0.9865 mg/L and 1.871 mg/L, respectively, with the calculated LOD values for mercury, copper, cadmium, nickel and chromium were 0.0002 mg/L, 0.006 mg/L, 0.05 mg/L, 0.02mg/L and 0.1 mg/L, respectively [34]. These very low LOD values meant that the garlic-based inhibitive assay could be a sensitive assay for detecting heavy metals.

The periodic sampling results for one day of a location in the Juru Industrial Estate showed temporal variation of copper concentration coinciding with garlic protease inhibition with the highest concentrations of copper occurring between 12.00 and 16.00 hours of between 3 and 3.5 mg/L copper (**Fig. 1**). In a previous similar study from another location nearby, from an industrial drain in the Juru Industrial Park that flows into the Juru River Basin, a temporal variation in copper concentration was observed using a local luminescent bacterium, which closely correlates with the commercial luminescent Microtox® assays.

It was discovered that an elevated copper concentration occurred approximately between 10.00 and 18.00 hours. Furthermore, in a similar study using achromopeptidase as the principal proteolytic enzyme, it was discovered that copper exceeded the DOE Maximum Permissibility Level between 18.00 and 20.00 pm [20]. Copper analysis at the site showed that the highest copper concentration was 2.17 mg/L which occurred at the 14.00 hour [31]. Copper is one of the heavy metals detected in this area as early as 2006 [12]. The fluctuation concentration of copper over the day probably coincides with industrial activity in the morning throughout the evening. More data will be taken in the future to check for temporal variation over a period of several weeks to ascertain whether the trend observed in this study really indicates a trend of copper discharge from the industrial complex.

The garlic protease assay demonstrated near-real-time ability of the system. It was found that copper concentration exceeded the Maximum Permissible Limit (MPL) outlined by the Department of Environment of Malaysia at 0.02 mg/L for class II water [35]. The errors of the inhibition measurement

(coefficient of variation) were less than 10% (n=5) suggesting good reproducibility.

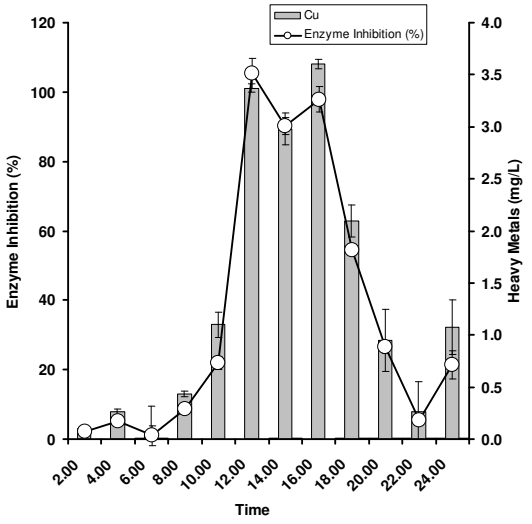

Fig. 1. Inhibition of garlic protease activity and copper concentration in water samples taken periodically on an hourly basis. Data are mean ± standard error of the mean (n=3).

Considering that metal-related industrial sectors are available in great quantity in this region, it can be assumed these particular forms of industrial sectors are accountable for the raised quantities of heavy metals and copper in particular. Temporal variation in the concentration of copper observed in this study illustrates the problem in heavy metals monitoring in flowing water bodies such as rivers and seas. This deviation is most likely brought on by the secret discharge of waste materials that contain heavy metal pollution into rivers to avert discovery by enforcement agencies [25].

Throughout the world, implementing real-time or near real-time biomonitoring of heavy metals including copper is practically not reported or no carried out given that the use of instruments and the current biological-based assays call for cumbersome instrument or take too much time to perform [36]. Presently, water samples must be transported to the research laboratory, and therefore these kinds of monitoring methods are regarded as a batch system monitoring (Shukor et al., 2006). Near-real-time biomonitoring is a thrilling current pattern as realtime, or near-real-time biomonitoring of heavy metals will allow forensic technique in the direction of apprehending ecological offenders [37].

CONCLUSION

Bioassay using in vitro systems has the potential to be a rapid and more economical than other classical toxicity bioassays for preliminary screening. A garlic-based inhibitive assay that leverage on the simple and rapid Coomassie dye binding assay was utilized in this study and showed a great promise as a simple, rapid and cheap preliminary large-scale monitoring assay system. A field trial was carried out in an effluent site coming from an industrial site that channels its waters directly into the public and agricultural areas in this area demonstrated a temporal variation of the heavy metal copper and also reflects the functionality of this system for water quality biomonitoring.

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