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SHORT COMMUNICATION

Monitoring of Heavy Metals Level in Fish Using the Microtox® assay

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Abstract

Luminescence-based assays for toxicants such as Microtox®, ToxAlert™ and Biotox™ have been used extensively worldwide. However, the use of these assays in near real-time conditions is limited due to non-optimal assay temperature for the tropical climate. An aerobic isolate that exhibits a high luminescence activity in a broad range of temperatures was successfully isolated from the mackerel, *Rastrelliger kanagurta*. This isolate was tentatively identified as *Photobacterium* sp. strain MIE, based on partial 16S rDNA molecular phylogeny. Optimum conditions that support high bioluminescence activity occurred between 24 and 30 ºC, pH 5.5 and 7.5, 10 and 20 g/L of sodium chloride, 30 and 50 g/L of tryptone, and 4 g/L of glycerol as the carbon source. Assessment of near real-time capability of this bacterium for heavy metals in a contaminated river running through the Juru River Basin that fed a large agriculture area has shown near real-time capability with assaying time of less than 30 min per samples. Thus, this strain is suitable for near real-time monitoring of toxicants especially in the tropics.

INTRODUCTION

The extensive industrial and agricultural activities in Malaysia have resulted in the increase of pollutants such as heavy metals, pesticides, organic and inorganic solvents in the environment. Malaysian Department of Environment (DOE) in 2011 has categorized 189 out of 464 rivers as polluted or slightly polluted [1], which is an issue of concern due to the toxicity, carcinogenicity and mutagenicity of the pollutants. Toxicants can cause potentially harmful effects to human beings, aquatic organisms and food webs because some of them cannot be fully degraded [2]. Toxicants, especially heavy metals are toxic to aquatic organisms. Therefore, it is important to periodically monitor toxicants in the environment [3]. A simple and fast procedure is required to screen for the presence of toxic substances from industrial effluents, polluted rivers and other polluted locations [4, 5].

Bioluminescence-based systems are suitable for preliminary screening of toxicants in the environment. This system is sensitive to many toxicants and utilizes a rapid and simple operation [6]. Despite its simplicity, it represents the real impact of all chemicals present in a given sample or ecosystems [7]. The system involves bioluminescent bacteria, which are widely distributed in marine, freshwater and terrestrial environment [8]. Bioluminescence is an aerobic oxidation process and the enzyme involved in the production of luminescence is luciferase. The enzyme catalyzes the oxidation of its substrate, luciferin, and is mediated by a reduced coenzyme, flavin mononucleotide. The interactions of toxicants with the bioluminescent bacteria cause the inhibition of the luminescence production [7]. The effect of toxicants on the microorganisms can be determined within 30 min or less depending on the types of toxicants [9]. An example of a commercially produced bioassay using bioluminescent bacterial cultures is the Microtox® system [10].

This system uses the bacterium *Vibrio fischeri* and has an optimal assay temperature of 15 °C [11, 12]. Deviations of a few degrees Celsius from this temperature can dramatically affect the luminescence production [13]. The system requires a refrigerated water bath, which is not practical, expensive to maintain and instrument-dependent for field applications in the tropical regions [14]. A tropical country such as Malaysia can exhibit a wide variation of daily temperatures ranging from 23 at night to 34 ºC in the afternoon [15]. Other commercial bioluminescence-based

systems such as ToxAlert™ also requires an exact assay temperature at 15 ºC and Biotox™ as well as Toxi-Screening Kit™ are stable between 15 and 25 ºC [13]. Thus, a tropical bioluminescent bacterium that could cover this broad tropical temperature range is urgently needed.

In this work, we isolated *Photobacterium* sp. strain MIE from mackerel (*Rastrelliger kanagurta*) from the local market. The tropical isolate is capable of high luminescent activity within a broad range of temperature from 24 to 30 ºC. This study aims to characterize the optimum conditions for growth and the environmental factors that are essential for bioluminescence production. A field trial has been conducted on Malaysian water to demonstrate the near real-time application of this bacterium.

MATERIALS AND METHODOLOGY

Measurement of luminescence

Luminescence was measured using a Beckman Counter DTX 800 multimode detector and reported as Relative Luminescence Unit (RLU). 200 µL of samples were collected in DTX microplate 96 wells before the readings were taken. The measurements were taken in triplicate.

Statistical Analysis

All data were analyzed using Graphpad Prism version 5.0. Values are means ± standard errors. The comparison between groups was performed using a Student's t-test or a one-way analysis of variance with post-hoc analysis by Tukey's test. $P < 0.05$ was considered statistically significant.

Digestion of fish tissue

Digestion of fish tissues were carried out by adding 5 ml of 65% nitric acid to 0.5 g fish tissues and the mixture was stored at room temperature for 1 day. After that, the samples were heated to 60-80 ºC for 3 h for digestion. After digestion, the samples were allowed to cool and filtered. Then, elutions were added with 25 ml deionized water. For this bioassay study, the pH of the fish tissue mixtures was adjusted to pH 7 before being assayed.

Toxicity test organism, mediums and cuvettes

The Microtox® chronic toxicity test uses the marine bioluminescent bacteria, *V. fisheri*, as the test organism. The Microtox® chronic test reagent was purchased from Strategic Diagnostic Inc. (Newark, DE, USA) as a freeze-dried bacterial form. The reagent was stored at −20 to −25 °C to preserve microbial activity. To obtain more reproducible results, the reagent was used within 2 h of reconstitution. The Microtox® chronic test medium, reconstitution solution, activation solution and test cuvettes used in the toxicity test were also purchased from Strategic Diagnostic Inc. Microtox® chronic test medium. Reconstitution solution is an ultrapure water used to reconstitute the chronic test medium. Activation solution is a prepared 3.5% sodium chloride solution used to rehydrate the Microtox® chronic test reagent at the appropriate osmotic pressure for the test organism. Test cuvettes used to contain the samples were made of clean borosilicate glass. The cuvettes were disposed of after use to eliminate potential carry-over of cleaning reagent to subsequent samples.

Toxicity test procedure

Individual metal samples were analyzed by the Microtox® bioassay according to the procedure in the Microtox® system operating manual. The test is based on the reduction of bioluminescence of the marine bacterium, *V. fisheri*, following direct exposure of the sample to the bacterial suspension. The vial containing the fresh culture was maintained at 5 °C between analyses using a sensitive Beckman Counter DTX 800 multimode detector. Each test consisted of five different concentrations (for sample and positive control, 0.5 dilution factor) were obtained by serial dilution from the prepared stock solution. A reference toxicant, copper sulfate (as Cu), was used as positive control. The positive control was performed concurrently with the sample as a quality control. Test results were discarded if the result of the reference toxicant fell outside of the acceptability limits (i.e. LOEC values for copper from 12 to 100 µg/l). All tests were performed in three replicate sets.

Measurement of luminescence

Luminescence was measured using a Beckman Counter DTX 800 multimode detector. 20 + 180 incubate for 15 min. 200 µL of samples were pipetted into 96 wells DTX microplates before the readings were taken. The unit used for luminescence was Relative Luminescence Unit (RLU) [16]. All the experiments were repeated at least three times. Percentage luminescence and percentage of inhibition can be calculated according the formula below:

$$\text{Luminescence (\%)} = \frac{\text{Luminescence of sample after 15 min}}{\text{Luminescence of control after 15 min}} \times 100$$

$$\text{Inhibition (\%)} = 100 - \frac{\text{Luminescence of sample after 15 min}}{\text{Luminescence of control after 15 min}} \times 100$$

The determination of heavy metals in the samples was carried out using Atomic Emission Spectrometry on an ICP-OES (Optima 3700DV, Perkin-Elmer, USA). 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.

RESULTS AND DISCUSSION

Fish is one of the main sources of protein other than meat and poultry products and contributes to a large percentage of dietary protein globally. In most Asian countries, especially in Southeast Asia like in Thailand, Indonesia and Malaysia, fish is taken as the main dish of their diet. However, fish may also contain heavy metals that could give negative effects to health. Svensson et al. (1991) mentioned that diet and food of animal's origin are the most predominant sources (>90%) of heavy metals and other chemical contaminants to humans [17]. Basically, marine organisms accumulate contaminants such as metals from the environment and this phenomenon have been extensively used in marine pollution monitoring programmes [18, 19].

In this study, the presences of heavy metals in fish tissues were monitored using *Photobacterium* sp. strain MIE. Figure 1 shows that these samples have no inhibitory effect on the luminescence produced by *Photobacterium* sp. strain MIE as compared to the control. This is validated by heavy metals analysis of the samples using ICP-OES and FIMS done by Alina et al. (2012) that showed that they contain very low concentrations of heavy metals **[20].** Table 1 shows the type and concentration of heavy metals that inhibited the Microtox assay. Microtox is most sensitive against mercury and is excellent for the determination of mercury from mercury-contaminated fish.

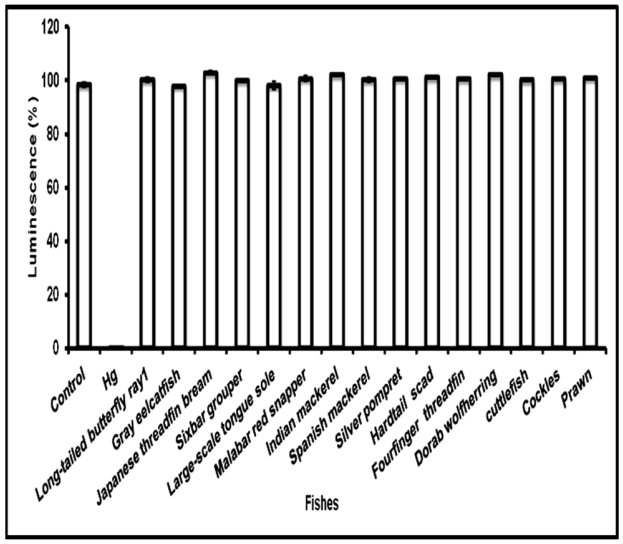

Fig. 1. The inhibitory effects of the fish tissue mixture on luminescence produced byPhotobacterium sp. strain MIE. The bacterium was incubated with samples for 15 min as described in Section 3.2.7.3. All values represent mean ± standard error mean (SEM), (n=3).

Table 1. Summary of the IC_{50} value on different bioassay of selected metals.

Metals	*Vibrio fischeri* [a] Microtox®
	IC_{50}-min 15 (mg/L)
mercury	0.065
copper	8
chromium	500-600
nickel	3.64
zinc	17

Note: n.d-not detected, N/A-not available [9]

CONCLUSION

Heavy metals in fish especially mercury is a great concern. Normal methods in detecting mercury are cumbersome, expensive and time consuming. The luminescence-based detection of mercury developed in this work is suitable for large scale biomonitoring works. The result of this study was in agreement with conventional analysis indicating the robustness of the method.

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