The Potential of Luminescent Bacteria '*Photobacterium leiognathi*' as a Biosensor for the Detection of Aquatic Toxicity

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Abstract

Evaluation of environmental aquatic toxicity is a convenient way for environmental pollution management before any detailed chemical pollutant analysis is performed. In this study, the luminescent bacteria *Photobacterium leiognathi* was used to evaluate the toxicity polychlorinated biphenyl (PCB) and lead (Pb). PCB at 2 ppm to 10 ppm and Pb, 0.001 ppm to 100 ppm were exposed to bacteria and the resulting bioluminescence was measured spectrophotomatically at 484.74 nm. There were consistent decreases in bioluminescence with increased toxicity of Pb. However, the bioluminescence was inhibited and did not show consistent decrease with increasing concentration of PCB. *Photobacterium leiognathi* appear to be selectively responding to toxicants at certain level of concentration. *Photobacterium leiognathi* bacterium is potentially useful as biosensors especially for Pb but not for the PCB. However, more work needed to be carried out to determine the detail responses pattern and the threshold value for *Photobacterium leiognathi* in respond to Pb

Key words: Bacteria/ Bioluminescence/ Luminescent/ *Photobacterium leiognathi*/ Toxicity

1. Introduction

Environmental monitoring of pollutants is becoming increasingly important to the general public. This is particularly true for compounds that pose a potential risk to human health or to the environment (Sung et al., 2000). In recent years, bioassay studies on toxicants using bacteria as a sensing organism have been promoted due to their sensitivity, ease to use, cost-effective and fast responses for detection and evaluation of environmental toxicity (Froehner et al., 1999; Kudrysheva, 2005; Tencaliec et al., 2005; Girotti et al., 2008). Bioassay measures changes in physiology or behavior of living organisms resulting from stresses included by biological or chemical toxic compounds, which can cause disruption of the metabolism (Girotti et al., 2008). In aquatic environments, bacteria play an important role in metabolite activities and nutrients cycles (Gellert et al., 1998). A living microbe is a package containing all the substances necessary for complex chemical reaction without the needs of any external substances which makes a living microbe a sensitive detector to its surroundings (Tauriainen' et al., 2000).

The bioluminescence or chemo luminescence is an aerobic bio-oxidation process alternative to normal bacterial respiration (Perego et al., 2002). It is a characteristic shown by a number of marine organisms and land animals including bacteria (Rees et al., 1998; Girotti et al., 2008) of about 666 genus

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from 13 phyla (Girotti et al., 2008). Besides bacteria, bioluminescence is also exhibited from phyla as low as dinoflagellate to marine vertebrates, not higher than fish (Girotti et al., 2008).

Bioluminescent assay generally shows good correlation with other toxicity bioassays such as algae, crustacean, and fish (Girotti et al., 2008). The most thoroughly studied bioluminescent bacterium used in toxicity testing is *Photobacterium phosphoreum* (also known as *Vibrio fischeri*), a marine bacterial strain (Frymier and Ren, 2002). A commonly used test method that employs *P. phosporeum* is the Microtox test (Frymier and Ren, 2002). Bioassay based on bacteria is a gaining popularity as useful method for an early warning system for environmental monitoring and pollution management due to its sensitivity to toxic chemical substances and the fast results that can be obtained from such assay. (Girotti et al., 2008).

Luminescent bacteria are from Vibrionaceae family (Tai, 2004; Medvedeva et al., 2005) and there are 3 genera namely Photobacterium, Vibrio, and Alteromonas (Tai, 2004; Frischer, 2005). A few examples of luminescent bacteria are *Vibrio fischeri*, *Vibrio harveyi*, *Vibrio logei*, *Photobacterium phosphoreum*, *Photobacterium leiognathi*, and *Alteromonas hanedai* (Ainul, 2002). Luminescence in *Photobacterium leiognathi* and other luminuous bacteria is the product of bacterial luciferase, a mixed function oxidase that uses oxygen, reduced flavin mononucleotide, and a long-chain fatty aldehyde as substrates to produce blue-green luminescence (Ast et al., 2007).

The bioluminescent enzyme system contains oxidoreductase NAD (P): FMN, and component of luciferase which emits lights in the presence of FMN (flavin), NAD (P) H, a long chain aldehyde (RCHO) and oxygen molecules (Kudrysheva, 2005; Hernando et al., 2007; Girotti et al., 2008). Bacteria's luciferase is very specific to $FMNH_2$ (mononucleotide flavin), and at the same time shows a weak activity to the others flavins (Girotti et al., 2008).The metabolise energy produced in this pathway is changed to chemical energy through the electron transport system to visible light (Hernando et al., 2007) as in equation (1) and (2) (Girotti et al., 2008). In equation (2), with the presence of luciferase, a species of heterogen enzyme, light is produced as a by products in the catalysis reaction (Tauriainen' et al., 2000).

$$NAD(P)H + FMN + H \xrightarrow{\text{oxidoreductase}} NAD(P)^{+} + FMNH_2 \quad (1)$$

$$FMNH2 + RCHO + O2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H2O + Light \quad (2)$$

In this work, the species of bacteria *P. leiognathi* was investigated as a potential biosensor for environmental toxicant, PCB and Pb is investigated. Toxicity test is based on inhibition of light production by the luminescent bacteria (El-Alawi et al., 2000).

2. Material Studied

The luminescent bacteria, *P. leiognathi* was isolated from squid and supplied by School of Biosciences and Biotechnology of Universiti Kebangsaan Malaysia (PPBsBt, UKM). *P. leiognathi* is a marine bioluminescent bacterium that is widely distributed in tropical and temperate coastal environments, and it is also known as a light

organ symbiont of fish species belonging to the families Leiognathidae, Acropomatidae, and Apogonidae (Orndorff and Colwell, 1980; Wada et al., 2006). *P. leiognathi* is chosen due to its strong luminescence which can be detected easily.

3. Methods and Techniques

3.1 Growth Medium

Medium for growth of bacteria (OXOID) was prepared by adding 28 g of nutrient agar and 20g (2%) of sodium chloride (NaCl) to 1000 ml of distilled water and heated until completely dissolved. The nutrient agar contained Lab-Lemco flour (1g L^{-1}), yeast extract (2 g L^{-1}), peptone (5 g L^{-1}), NaCl) (5 g L^{-1}) and agar (15 g L^{-1}). It was autoclaved at 121^0C for 15 minutes. The solution was poured into Petri dishes and stored in cool room.

3.2 Cell Culture

All manipulations were done in aseptic conditions. Individual colonies were transferred into the Petri dish. The bacterium was incubated for 18 hours at 30^oC (Girotti et al., 2008).

3.3 Growth of Bacterial Suspension

Saline solution was used in this study to reduce noise in spectro-photometer. *P.leiognathi*, a marine species need 2% NaCl (saline solution) for suspension (Zhang et al., 2008). Bacteria from Petri dish were rinsed twice with 30 ml of saline solution into a beaker to produce bacterial suspension and was transferred into a conical flask and shaken well (250 rpm) at room temperature.

3.4 Determination of Optimal Bio-luminescence Measurement

3.4.1 Wavelength

Bacterial suspension of 3.0 ml was pipetted into a 4 ml of 4 clear sides cuvette and their luminescence scanned at wavelength from 200 nm to 800 nm by using a fluorescence spectrophotometer (slit opening: 5 nm; scan speed: 150 nm/minute) (Perkin Elmer LS55). Excitation light source was fixed at 0 to eliminate any background fluorescence. The data was processed by FL Winlab software.

3.4.2 Optimal Time for using bacterial suspension

The optimum time for culturing luminescent bacteria is 18 hours (Girotti et al., 2008). The bioluminescent intensity was measured every 30 minutes for 6 hours to test its intensity stability. The first bioluminescent intensity was measured half hour after the optimum cultured time

3.4.3 Determination of Cells Density

A serial of bacterial suspensions (3:0; 2:1; 1:1; 0.5:1; 0.5:5; 0:3)v/v in saline solution were prepared in triplicates. A serial mixture of 0.9 µL was dropped between a Weber hemacytometer and a slide. The number of bacterial cells was counted with BX51 microscope (Olympus, USA) equipped with 100x magnification. The light intensity of the mixture was then measured by fluorescence spectrophotometer.

3.5 Toxicants Preparation

Polychlorinated biphenyls solutions (PCB No. 52) (Dr. Ehrenstorfer) were prepared by dissolving with 1% methanol (J.T Baker) before diluting to 2, 4, 6, 8, and 10 ppm using distilled water. The solutions were stored at 4^oC until use and is stable for 3

months. Lead (Pb) solutions (0.001, 0.1, 1, 5, 10, 30, 50, 80, and 100 ppm) were prepared from standard lead nitrate ABN, Australia solution (Pb in 0.5% of nitric acid) and are stable for 1 year.

3.6 Toxicity Test

A total of 1.5 ml of toxicants (PCB and Pb) was added to 1.5 ml of bacteria suspension in a cuvette containing 266 ± 20 of cells bacteria. The mixture was incubated for 30 minutes and the cuvette was covered with parafilm to prevent contamination from surroundings. For control, 1.5 ml of saline solution was added to 1.5 ml of bacteria suspension.

The bioluminescence inhibition was calculated based as the equation below (Wong et al., 2008).

$$\% \text{ of Inhibition} = \frac{Io - It}{Io} \times 100 \qquad (3)$$

where, Io: saline solution with bacterial suspension

It: toxicants with bacterial suspension

4. Results and Discussion

4.1 Peak Emission Wavelength of Photobacterium leiogntahi

P.leiognathi was shown to emit light at 484.74 nm (Figure 1) maximally and this wavelength was used throughout this study. The wavelength has been used consistently in this study. This emission is slightly lower than another bioluminescent that has been used in many bioassay studies, *V.fischeri* with the reported wavelength of 490 nm (Frymier and Ren, 2002; Nagata and Zhou, 2005; Girotti et al., 2008). Different species has been showed to emit bioluminescent at different wavelength (Haddock et al., 2010).

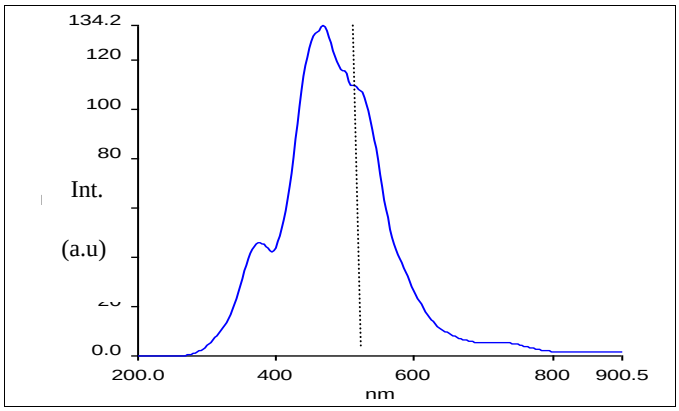

Figure 1: The wavelength of luminescent bacteria

4.2 Optimal Time

Within 6 hours the experiment was undertaken, bioluminescence intensity remained relatively constant with only small variation of ±8 units. In this study, fresh luminescent bacteria assay was used within the optimum time range and conditions where bioluminescence shows the highest intensity that is within the 6 hours after the optimum culturing time to ensure good signal of emission.

4.3 Density of Cells

Figure 2 shows the relation of bioluminescence intensity at different numbers number of bacterial cells.

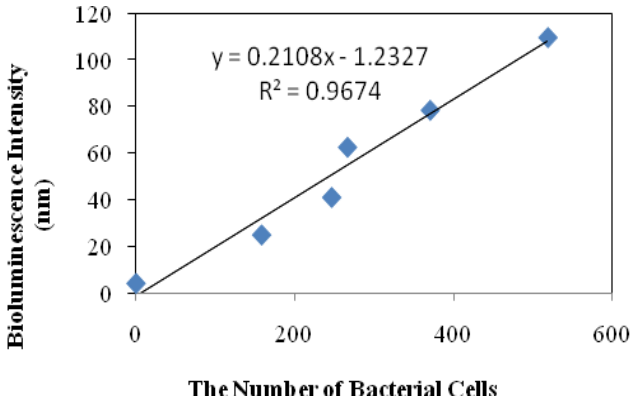

Figure 2: Bioluminescence intensity and number of bacterial cells

Bioluminescence intensity showed a linear relationship to the bacterial cells (Pearson correlation $p<0.01$, $R^2=0.9674$). The bacteria bioluminescent are controlled by autoinducer, a signal which is accumulated during cell growth and detected by receptor which in turn can excite the luciferase enzyme. It will cause the luminescent reaction when the concentration achieves threshold value (Katznelson and Ulitzur, 1977; Strauss, 1997; Tai, 2004). Coordinating mechanism function and accumulation of auto-inducer also known as quorum sensing, depend on cell population density (Strauss, 1997; Holloway, 2004; Tai, 2004; Stolper et al., 2008).

4.4 Toxicity Testing

Figure 3 showed bacterial response to Pb. This experiment was repeated and the results showed increase bioluminescence inhibition pattern with increase toxicity. The readings for bioluminescence inhibition were in the range of 4.59% to 58.03%.

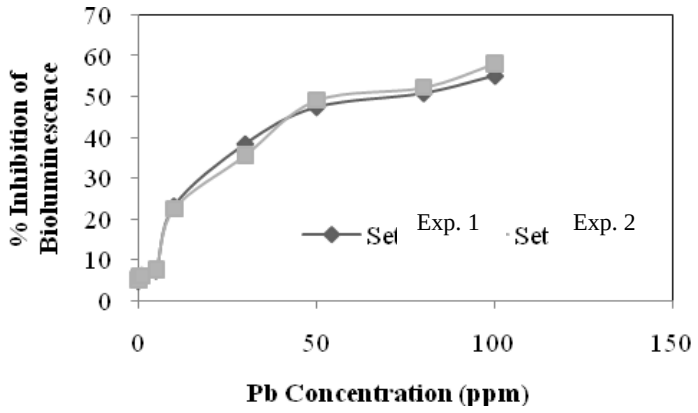

Figure 3: Bioluminescence inhibition pattern cause by exposure to Pb.

The response of the *P.leiognathi* to an inorganic pollutant, Pb is shown at Figure 4. Two ways Pearson Correlation test shows the significant correlation ($p<0.01$) between bioluminescence inhibition and increased Pb toxicity. Girotti et al. (2002),

reports that as the toxicants concentration increase, the light emitted by bioluminescent bacteria will decreased. This indicates that the cell energy status has been disturbed or cell structure was damaged (Backhaus et al., 1997) after the bacterial cells are incubated with toxicants. Although there is no study has been conducted on *P.leiognathi* before, Tsiridis et al. (2005) reported the almost same inhibition profile by Pb with *V. fisheri.*

Figure 4 shows bacterial response with PCB. The experiment was repeated and both sets of data shows regular pattern but did not showed continuous bioluminescence inhibition. The results showed bio-luminescence inhibition at 2, 4, 8 and 10 ppm. However, the results showed decrease in bioluminescence inhibition at 6 ppm for both sets of data. The bioluminescence inhibited in the range of 36.69% to 57.06%. At the concentration tested, bioluminescence was inhibited moderately 2 to 10 ppm. It is not clear why concentration at 6 ppm was lower in the bioluminescence inhibition.

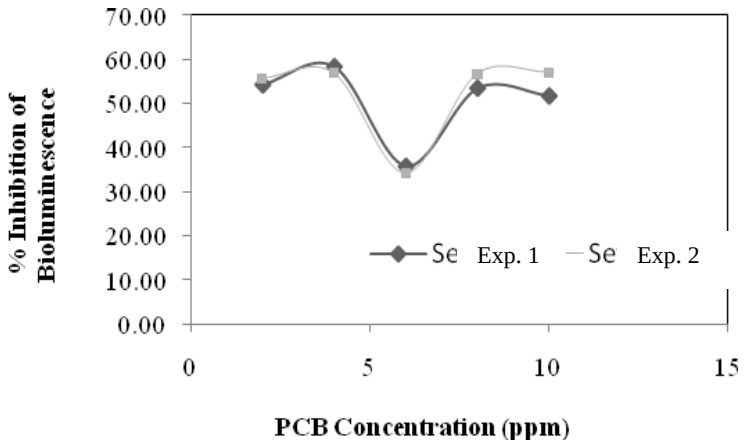

Figure 4: Bioluminescence inhibition pattern cause by exposure to PCB

Steevens et al. (1999) reported no significant bioluminescence inhibition for concentrations higher than 24 ppm and below 1.6 ppm for anthracene and benzo[a]pyrene because both toxicants are relatively insoluble in water (<45 ppb). Therefore bioavailability of the 2 PAHs may significantly influence the exposure and effect on bioluminescence inhibition. Farré et al. (2000) showed that chlorfenvinphos at 0.2 ppb and 0.3 ppb and on endosulfan between 0.007 ppb and 0.12 ppb does not show any toxicity effect to *V.fischeri* although pesticide concentration exists at ppb level.

Not all organic toxicants can inhibit bioluminescence by bacteria. From previous studies, nalidixic acid shows no effect on inhibition though the incubation period with the bioluminescent cell was increased (Backhaus, 1997). For Choi and Gu (2001), their studies do not show obvious bioluminescent respond for two out of five tested toxicants, which included 2, 4, 5-trichlorophenol (2, 4, 5-TCP) and pentachlorophenol (PCP).

Bacterial cell sensitivity to various toxicants has a threshold value. The range and threshold value vary with different toxic substances or sample. It is reported at certain concentration, many microorganisms are able to modify a toxic substance and try to adapt through various processes such as oxidation, reduction, bioremediation, transformation, bio-absorbtion, bioaugmentation, and bio-degradation (Tamar and Schaefer, 2001).

The presence of ions such as the saline (2% NaCl) as used in this study may disturb the reaction mechanism by decreasing response through interaction with bacteria target receptors (Hernando et al., 2006). However since *P. leiognathi* is a marine luminescent bacterium, therefore 2% of saline solution was needed to be used in this study.

5. Conclusion

P.leiognathi has been tested with Pb and PCB to find out its potential to be developed as a biosensor for aquatic toxicity detection. *P.leiognathi* was potentially useful as bioassay especially for Pb as shown in this study. However, more work needed to be carried out to determine the detail respond pattern and the threshold value for *P. leiognathi* in the respond with Pb.

6. Acknowledgement

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