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The Behavior of Immobilized Cyanobacteria *Anabaena torulosa* as an Electrochemical Toxicity Biosensor

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Abstract: The cyanobacteria *Anabaena torulosa* was immobilized onto an oxygen electrode using a poly (2-hydroxylethyl methacrylate) matrix. The behavior of the organism towards some toxicants was investigated via inhibition of its photosynthetic activity, which could be monitored by the changes of photosynthetic oxygen release. Using lead and 2, 4-dichlorophenoxyacetic acid (2, 4-D) as the toxicants, it was shown that the cyanobacteria response was not affected by cell age or phase of cell growth. But repetitive exposures to a toxicant such as Pb altered the inhibition behavior of the cyanobacteria ($p<0.05$). The 50% inhibition of the cyanobacteria by Pb occurred at a concentration of 0.4 mg L^{-1} Pb whilst for the herbicide 2, 4-D at 0.1 mg L^{-1}. The results showed that the immobilized organism can be used as a toxicity biosensor for the assessment of Pb toxicity in river water samples.

Key words: Electrochemical biosensor, lead toxicity, photosynthetic activity, 2, 4-D toxicity

INTRODUCTION

The cyanobacteria, *Anabaena torulosa* as been reported sensitive to metal toxicity (Heng *et al*., 2004; Jusoh *et al*., 2003). Immobilization of cyanobacteria cells in poly(2-hydroxyl ethyl methacrylate) (pHEMA) offers several advantages, e.g., stability as well as reproducible cell response (Naessans and Canh, 1998). Moreover, the closely knitted structure of pHEMA prevents entry of other bacteria and thus protects the cells from bacterial degradation (Trevan and Mal, 1988). The polymerization procedure is suitable for entrapment of cells and the small pore sizes of the polymer prevent leaching of the cells to the outside environment (Guisan, 2006).

Immobilised cyanobacteria are useful as a biosensor for the detection of water toxicity because of its varied and versatile metabolism characteristics such as photosynthetic activity, respiration, fermentation and nitrogen fixation, all of which can generate responses against many environmental factors (D'Souza, 2001) Other advantages of using immobilised *Anabaena torulosa* are they grow rapidly on simple media, generally self-sufficient and do not require additional enzymes, co-factors or co-enzymes Like all cyanobacteria, *Anabaena torulosa* is tolerant to microenvironments such as temperature and pH changes. Furthermore, the cells can easily be harvested for immobilization to be interfaced with a transducer, thus requiring no prior purification compared to the use of enzymes or organelles.

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In a biosensor format, the toxicity measurements can be used to determine quantitatively the level of toxicants but biosensor can also be used as a screening tool of aquatic toxicity. Lead is widely distributed naturally but the greatest risks to the environment and humans normally arise from the emissions associated with human use of the metal and its derivatives. Lead affects microorganisms by retarding the heterotrophic breakdown of organic matter. 2, 4-dichlorophenoxyacetic acid (2, 4-D) is widely used in agriculture for protection of crops against weeds. The toxicity of these compounds is well documented (Chiu *et al*., 1998; Hasan *et al*., 2000; Okay and Gaines, 1996). The assay of toxicity of these compounds normal involved the various procedures suggested by the Organisation for Economic and Co-operation Development (OECD). However, a more rapid method of toxicity assay can be performed by designing a biosensor containing a selected microorganism. In the case of Pb and 2, 4-D, conventional methods for the analysis are well established but sophisticated instrumentations and trained personnel are required (Meulenberg and Stoks, 1995). Instrumental analysis requires transportation of samples to the laboratory and extensive pretreatment of samples before analysis (Skladal and Kalab, 1995). Therefore, on site detection using biosensors is desirable for rapid results (Rogers, 1995).

In this study, the behavior of the cyanobacteria *Anabaena torulosa* immobilized in a poly (2-hydroxyl ethyl methacrylate) (pHEMA) was examined using two toxic substances, i.e., Pb and 2, 4-D by monitoring the inhibition of its photosynthetic activity. The inhibitory behavior of the immobilized cyanobacteria was applied as a biosensor for the determination Pb toxicity in river water.

MATERIALS AND METHODS

Reagents

Reagents and biological materials used in the experiment were: lead (II) nitrate, $Pb(NO_3)_2$ (R and M Chemicals, UK) and 2, 4-D 98.5% (GmbH, Germany), Bold Basic Media for culturing cyanobacteria, axenic cultures of *Anabaena torulosa* (Carolina Biological Supply Co, USA), poly (hydroxyl ethyl methacrylate, M_w = 30,000 (Sigma, UK), 1, 4-dioxane (Fisher, UK). All reagents were prepared in distilled water.

This study was conducted in 2006 at the School of Environmental Science and Natural Resources, Faculty of Science and Technology.

Anabaena Torulosa Culture

Anabaena torulosa was cultured in Bold's Basic Medium at 18.5°C under a 3350 lumen cold white fluorescent illumination, with light and darkness maintained at 16 and 8 h intervals, respectively in a culture chamber (GC-500, Protech). Aeration was carried out by manual shaking about two or three times daily to avoid cells clumping. Concentration of suspended cell was determined at 700 nm using a Perkin Elmer UV/VIS spectrophotometer. Cell density was determined by a Microscope BX51 (Olympus, USA) and a Weber haemocytometer. The optical density, OD, at 700 nm was used to estimate the cell density for immobilization because the two quantities were closely correlated according to the relation $y = 10^7x$ ($r^2 = 0.989$). The OD at 700 nm is also the absorption peak of chlorophyll a that present in the cyanobacteria.

Immobilization of Cyanobacteria and Biosensor Construction

Poly(hydroxyl ethyl methacrylate) (pHEMA) solution was prepared by dissolving the polymer in a mixture of water:dioxane. Typically 50 μL this pHEMA solution containing 70 μL (2.15×10^7 cell) of *Anabaena torulosa* cells was deposited on a teflon gas-permeable membrane. The cyanobacteria

entrapped in the pHEMA film (~ 9 mm diameter) were left to dry at 18°C for 4-5 h. The sensor membrane was then attached onto the tip of the Orion oxygen electrode and secured using a rubber O-ring. The photosynthetic activity of the immobilized cells in the coated membrane was determined by measuring oxygen changes when illuminated by a 1200 lumen illumination with reference to non-illuminated condition. The change in oxygen level was recorded continuously throughout the experiment by a DO sensorlink PCM 800 computer system (Thermo Orion, USA).

Evaluation of toxicity of lead and 2, 4-D with biosensor. The initial activity of the immobilized cells of the biosensor was established by measuring the evolution of oxygen under illumination after immersion in 300 mL of oxygen-saturated distilled water (pH = 6-7). The biosensor was then incubated in 10 mL of toxicant solution (0.05, 0.1, 0.3, 0.5, 0.75, 1.0, 2.0 and 5.0 mg L^{-1} Pb and 0.05, 0.1, 0.2, 0.3, 0.5 and 1.5 mg L^{-1} 2, 4-D, pH = 7) for 15 min at room temperature. After the incubation period, the biosensor was transferred to distilled water again and the photosynthetic activity of the cells was measured under illumination condition. The reduction in the oxygen concentration after exposure indicated the photosynthetic inhibition of the immobilized cyanobacteria by Pb or 2, 4-D. The percentage of photosynthetic inhibition is calculated as followed:

$$I(\%) = \frac{(I_0 - I)}{I_0} \times 100\%$$

Where:

I_0 = amount of oxygen evolved before exposure to the toxicants (Pb or 2, 4-D)

I = amount of oxygen evolved after exposure to the toxicants (Pb or 2, 4-D)

The effect of cell culture age on the toxicity response was evaluated using cell age at 3 and 9 days (lag and exponential growth phase respectively) and the effect of repetitive and non-repetitive exposure of the cells to Pb was also assessed using cell of age 9 days. In repetitive exposure, the same biosensor was subjected to increasing concentrations of Pb ions (several exposures). While for non-repetitive exposure, a different biosensor was used to test one concentration of Pb (one exposure only).

Application of Biosensor to River Water Samples

The application of the biosensor to determination Pb in Langat River, Malaysia was studied. Langat River was chosen as the sampling site because it is an important river where the Department of Environment, Malaysia has many water quality-monitoring stations to monitor pollution under the Water Quality Improvement Program for Malaysian Rivers. Six water samples were collected along the river and the location of each sampling point is shown in Table 1.

The water samples from the Langat River were used directly for toxicity evaluation without any pretreatment. To determine the level of lead in the water sample, an atomic absorption spectrophotometer (Perkin Elmer) was used to quantify the lead content after the water samples were filtered.

Table 1: The location of water sampling points at the Langat River, Malaysia

Water sampling station	Location
1	02°59.434'U 101° 47.261'T
2	03°09.954'U 101° 50.921'T
3	02°59.599'U 101° 47.132'T
4	02°57.931'U 101° 47.117'T
5	02°48.934'U 101° 30.776'T
6	02°52.258'U 101° 26.997'T

RESULTS AND DISCUSSION

Effects of the growth phase of cells on biosensor response. Cyanobacteria at different growth phases may show various metabolic activities and sensitivity towards toxicants and hence the response of the biosensor can be affected. Therefore, the effect of the growth phases on the biosensor response was investigated. Figure 1 shows the profile of inhibition of A. torulosa photosynthetic activity by various concentrations of Pb for cells at the exponential growth phase (9th day) and the lag growth phase (3rd day). Both the profiles showed that almost complete inhibition of the cell photosynthetic activity occurred at Pb concentrations greater than 2 mg L^{-1}. The 50% inhibition by Pb occurred at 0.4 mg L^{-1} for the exponential phase compared to 0.6 mg L^{-1} at the lag phase (Table 2). However, statistical analysis showed that these two values are not significantly different ($p>0.05$) and hence the cells at the two growth phases do not affect the toxicity behaviour of Pb towards *A. torulosa*.

The IC_{50} value for Pb reported here is lower than the LD_{50} -96 h reported for the species *Anabaena flos-aquae*, which is 0.99 mg L^{-1} (Heng *et al*., 2004) and this indicates a more sensitive nature of the biosensor towards Pb toxicity when compared with conventional toxicity assay of Pb on *Anabaena* sp. by Heng *et al*. (2004).

For the herbicide 2, 4-D, the complete inhibition of photosynthetic activity of the *A. torulosa* cells was obtained at greater than 0.6 mg L^{-1} for both growth phases (Fig. 2). There is no difference between the 50% inhibition values for both growth phases (Table 2) and thus this again indicates that the cells at different growth phases show similar vulnerability to the toxic effect of 2, 4-D.

Nevertheless, the response of the biosensors with immobilized cells at the exponential phase showed better reproducibility when compared to the lag phase. The exponential phase seems to be the best growth phase to harvest cells for biosensor applications. Pandard *et al*. (1993) has utilized cells of *Scenedesmus subspicatus* and *Chlorella vulgaris* from the exponential growth phase for optimum biosensor response. Rouillon *et al*. (1999) also found that the cyanobacteria *Synechococcus* sp. yielded

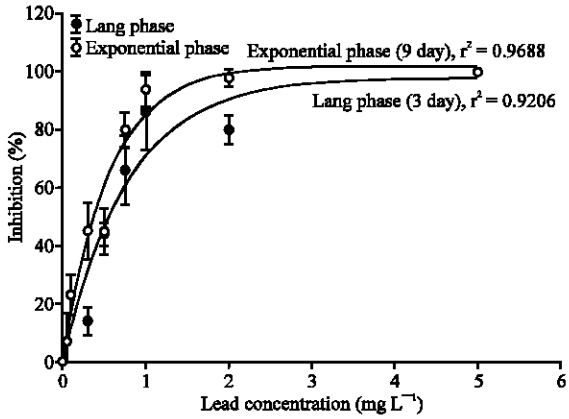

Fig. 1: The response of immobilized cyanobacteria *A. torulosa* of different growth phases to Pb inhibition. $p>0.05$

Table 2: The 50% inhibition (IC_{50}) of biosensor at different cell growth phase by Pb and 2,-D

Toxicant	Growth phase	IC_{50} (mg L^{-1})	Replicates
Pb	Lag	0.60±0.11	5
	Exponential	0.40±0.00	3
2,4-D	Lag	0.10±0.05	5
	Exponential	0.10±0.00	3

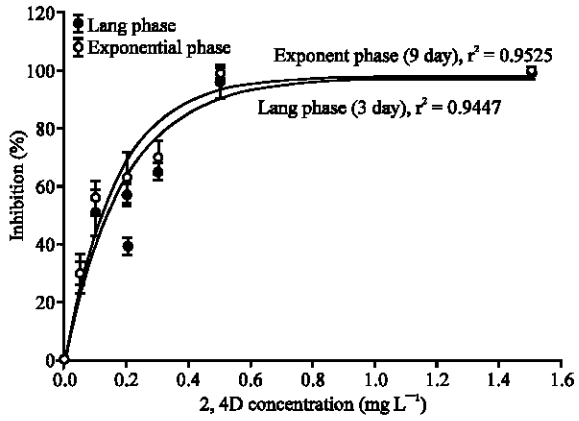

Fig. 2: The response of immobilized cyanobacteria *A. torulosa* of different growth phases to 2,4-D inhibition. $p>0.05$

Table 3: The linearity of the inhibitory response of *A. torulosa* with concentrations of the toxicants Pb and 2, 4-D (non-repetitive exposure)

Toxicant	Linear relationship	Range (mg L^{-1})	R^2	n
Pb (Exponential phase)	*Y = 65.3 Log *C + 88.4	0.05-1.00	0.9762	5
2, 4-D (Exponential phase)	Y = 48.8 Log C + 97.8	0.05-0.31	0.9199	4
2,4-D (Lag phase)	Y = 42.4 Log C + 88.1	0.05-0.31	0.9410	4

*Y: Inhibition (%), *C: Concentration (mg L^{-1})

best biosensor response at the exponential growth phase. Tatber *et al.* (2001) reported the same observation where cells at exponential phase showed optimum responses to acid toxicity. Another study that demonstrated optimal response at the exponential phase was reported for a biosensor for the detection of benzene and toluene using cells from bacteria with recombinant luminescence gene (Gil *et al.*, 2002).

The inhibitory response of the cyanobacteria towards Pb demonstrated a good linearity with respect to the logarithmic concentration of Pb. But for 2, 4-D, the linearity of the inhibitory responses with concentrations were somewhat poorer when compared to that of Pb (Table 3).

Comparison of Repetitive and Non-Repetitive Exposures for Biosensor to Pb

Studies on repetitive exposure of the biosensor to toxicants are important because they allow the reusability of the biosensor to be assessed. The study was carried out for Pb toxicity on immobilized *A. torulosa* cells at the exponential phase. The profiles of response for both repetitive and non-repetitive exposures to Pb (Fig. 3) indicate that the cells became more sensitive to the effect of Pb during repetitive exposures. For example, for repetitive exposure at 100% inhibition, the Pb concentration was approximately 1 mg L^{-1} whilst for non-repetitive exposure, 100% inhibition was approximately 5 mg L^{-1} Pb. At 50% inhibition, the Pb concentrations recorded were 0.3 and 0.4 mg L^{-1} Pb for repetitive and non-repetitive exposures, respectively. These results were significantly different statistically ($p<0.05$). Therefore, the application of the biosensor for toxicity evaluation will be affected by the repeated use of the device.

Application of Biosensor River Water Analysis

To examine whether the toxicity biosensor can be used to assess water toxicity for real samples, water samples from the Langat River were used. The inhibition of the biosensor by the water samples are shown in Table 4.

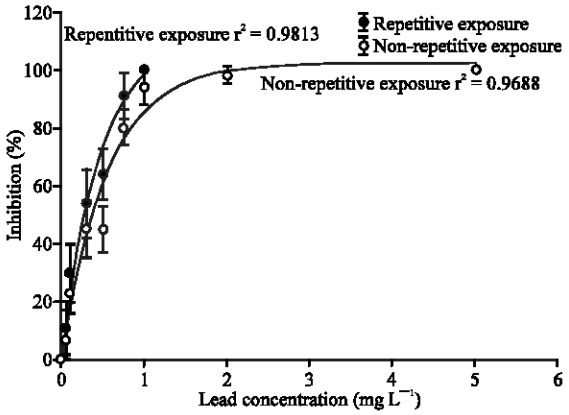

Fig. 3: The response of the toxicity biosensor constructed from immobilized *A. torulosa* by repetitive and non-repetitive exposure to various concentrations of Pb. $p<0.05$

Table 4: The toxicity level of river samples from the Langat River as determined by the toxicity biosensor using *A. torulosa* and the content of Pb analysed by an atomic absorption spectrophotometer

Sample	Maximum inhibition recorded (%) (n =3)	Pb by AAS (mg L^{-1})
Exponent phase		
S1	8	0.012
S2	0	0.005
S3	0	0.014
S4	15	0.023
S5	9	0.047
S6	0	0.030
Lag phase		
S1	8	
S2	0	
S3	0	
S4	16	
S5	2	
S6	1	

The biosensor demonstrated that some water samples from the Langat river showed low value of toxicity of less than 15% inhibition of *A. torulosa*. The toxicity exhibited by the biosensor is unlikely to be caused by Pb since atomic absorption analysis of Pb shows value of Pb less than 0.05 mg L^{-1}. Based on the equation for Pb in Table 3, the levels of Pb in the river water can cause a maximum level of inhibition of not more than 2%. Thus, the higher inhibition level observed may be attributed to other toxicants that present in the water, which may exert the same toxic effects as that of Pb. Nevertheless, the study showed that the application of the biosensor to evaluate water toxicity is possible.

CONCLUSION

The immobilized of cyanobacteria *A. torulosa* demonstrated responses to toxicants, which was not affected by cell age or phase of cell growth. But repetitive exposures to a toxicant such as Pb could alter its inhibition behaviour ($p<0.05$). The results showed that the immobilized microorganism can be used as a toxicity biosensor in rapid evaluation of water toxicity, especially the toxicity caused by 2, 4-D and Pb. In addition, the developed biosensor could be used for broad range of toxicity detection within minutes without or with minimum sample pretreatment. It is recommended that the biosensor is best used as a screening tool of pollution by some toxicants.

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