Asian Journal of Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 3 (3): 162-168, 2008
ISSN 1815-9923

Toxicity Biosensor for the Evaluation of Cadmium Toxicity Based on Photosynthetic Behavior of Cyanobacteria *Anabaena torulosa*

Wong Ling Shing, Salmijah Surif and Lee Yook Heng
Faculty of Science and Technology, University Kebangsaan Malaysia,
43600 Bangi, Selangor, Malaysia

Abstract: A biosensor based on electrochemical transduction using an oxygen probe has been developed for the measurement of Cd^{2+} toxicity by using cyanobacteria (*Anabaena torulosa*), where the cells were immobilized on the surface of an oxygen probe. The biosensor responded to the changes in photosynthetic oxygen release under illumination by a light source. Exposure to Cd^{2+} at concentrations below approximately 8 mg L^{-1} did not demonstrate any inhibition but stimulation of oxygen production was observed. Inhibition occurred only when concentration of Cd^{2+} was above 8 mg L^{-1}. Using the same concentration range of Cd^{2+} but increasing the exposure time of Cd^{2+} from 15 to 30 min, inhibition of oxygen release was mainly observed. This pattern of inhibition of *A. torulosa* by Cd^{2+} was different from that of Cu^{2+}, which was also measured by the same toxicity biosensor, where no stimulation in oxygen release was observed. The use of toxicity biosensor has enabled the detail toxicity behaviour of *A. torulosa* towards Cd^{2+} to be evaluated.

Key words: Anabaena torulosa, toxicity mechanism, photosynthetic oxygen, whole cell biosensor, metal

INTRODUCTION

Whole cell based biosensors can respond to a wide range of changes in their environment and suitable for use in toxicity test and environmental monitoring where the sources and nature of the toxicant cannot be predicted (Bentley *et al*., 2001). The biosensors response to real physiological impact of active compounds present in the samples and act as broad band monitoring for toxins, which might not necessary discriminate between the different types of toxicants (Giardi *et al*., 2001; Mattiasson 1997; Roger, 1995; Carpentier *et al*., 1991; Evans *et al*., 1986). Biosensors can be configured to be sensitive and inexpensive to manufacture and can be significant in terms of reducing cost and increase the efficiency of certain environmental monitoring applications (Roger, 1995).

Anabaena torulosa is a type of filametous cyanobacteria (cyanophyceae) from Nostocaceae family, which formally known as blue-green algae (Linda, 2000). The organism contains chlorophyll a and undergoes photosynthesis and the physiology changes can be observed by oxygen evolution. Based on the research by Chay *et al*. (2005), the organism has shown potential as a good biosensor to copper (Cu), lead (Pb) and 2,4-Dichlorophenoxyacetic acid (2,4-D). However, the previous studies had not investigated the usefulness of cyanobacteria *A. torulosa* as a biosensor for Cd toxicity evaluation. In view of Cd is a toxicant, which is non-essential, non-beneficial and possesses high toxic potential (Giardi *et al*., 2001; Lockwood, 1976) and a common pollutant in water resources (Heever and Grobbelaar, 1998), the focus of this study is to assess the usefulness of *A. torulosa* as a biosensor for rapid Cd toxicity evaluation.

Corresponding Author: Lee Yook Heng, Faculty of Science and Technology, University Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

MATERIALS AND METHODS

Reagents

Copper (2) nitrate, $Cu(NO_3)_2$, Cadmium (2) nitrate, $Cd(NO_3)_2$ (Merck, Germany), poli-hydroxylethyl methacrylate, (pHEMA) (Sigma UK), 1,4-dioxane (Fisher, UK), cyanobacteria *Anabaena torulosa* (Carolina Biological Supply Co., US), Bold's Basic Medium (James, 1979) as culture medium for *Anabaena torulosa*. All reagents were prepared in distilled water.

A. torulosa Culture

A. torulosa was cultured in Bold's Basic Medium at 18.5°C, 1000 Watt $unit^{-1}$ area white fluorescent illumination, with light and dark period maintained at 16 and 8 h, respectively in culture chamber (GC-500, Protech). Each liter of Bold's Basic Medium prepared contains 10 mL of several macronutrients stocks and 1 mL of trace elements stocks respectively (Table 1). Aeration was carried out by manual shaking three times daily to avoid cells clumping.

Determination of Cells Growth

The number of cells was determined by using a Microscope BX51 (Olympus, USA) and a Weber haemocytometer. Concentration of suspended cell can be estimated by using UV/VIS spectrophotometer (Perkin Elmer). The optical density at 700 nm can be used to estimate the cell density for immobilization as the two quantities were closely related. OD at 700 nm is the absorption peak of chlorophyll a in *A. torulosa* (Desikachary, 1959). The growth of the cyanobacteria was determined every 2 days for 14 days to establish the growth profile and cells at the most active phase of the growth were selected for toxicity biosensor studies (Chay *et al*., 2005).

Table 1: The preparation of stocks for Bold's Basic Medium

Macronutrient	Concentration
$NaNO_3$	10 g in 400 mL distilled water
$MgSO_4.7H_2O$	2 g in 400 mL distilled water
K_2HPO_4	4 g in 400 mL distilled water
KH_2PO_4	6 g in 400 mL distilled water
$CaCl_2$	1 g in 400 mL distilled water
NaCl	1 g in 400 mL distilled water
Trace elements	
EDTA Stock	
EDTA	50 g
KOH (85%)	31 g
Distilled Water	1 L
H-Fe Stock	
$FeSO_4.7H_2O$	4.98 g
Acidified water	1 L
H-H5 Stock	
$ZnSO_4.7H_2O$	15.7 g
MoO_3	0.71 g
$Co(NO_3)_2.6H_2O$	0.49 g
$MnCl_2.2H_2O$	1.85 g
$CuSO_4.5H_2O$	1.57 g
Acidified water	1 L
Boron stock	
H_3BO_3	11.42 g
Distilled water	1 L
Acidified water	
H_2SO_4	1 mL
Distilled water	999 mL

Cyanobacteria Immobilization

The pHEMA solution was prepared by dissolving the polymer in a mixture of water and dioxane with the ratio 4:1 and 1 mL of solution contained 60 μg of pHEMA. By using 50 μL of pHEMA solution, a certain amount of *A. torulosa* cells were coated on a Teflon gas permeable membrane. The cyanobacteria entrapped in the pHEMA film were left to dry at 18.5°C for 4-5 h. The membrane was then assembled onto the tip of an oxygen electrode (Orion, USA) by using a rubber O-ring. The oxygen evaluation can be measured by a change of dissolved oxygen when the immobilized cells were illuminated by a 100 W lamp (Phillip Classictone, Holland) compared to non-illuminated condition. The change of oxygen level was recorded using a PC (DO sensorlink PCM 800, Thermo Orion, USA).

Exposure of *A. torulosa* to Various Concentrations of Cadmium Ions

Cd toxicity tests were conducted for both immobilized and free cells of *A. torulosa*. The amount of pHEMA used for immobilization, cell density and cell culture age were optimized. Each biosensor was used only once for toxicity evaluation at each concentration of Cd. The experiment for immobilized cyanobacteria was performed in pH 7 and at room temperature, as stated by Chay *et al.* (2005). The biosensor was left in 10 mL of distilled water for 15 min until a stable baseline was obtained. The photosynthetic oxygen release of the immobilized *A. torulosa* cells was determined continuously for 5 min after light illumination began. The biosensor probe was then incubated in 10 mL of various concentrations of Cd^{2+} (0.1, 0.25, 0.5, 1.0, 5.0, 10.0, 20.0 mg L^{-1}) solutions for 15 min at room temperature. After the period, the probe was then transferred to 10 mL of distilled water again and illuminated to allow photosynthetic activity for another 5 min. Any inhibition of the immobilized cells on the oxygen probe can be calculated as follows:

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

where,

Io = Highest amount of oxygen evolved before exposure to the Cd ions within a fixed duration.

I = Highest amount of oxygen evolved after exposure to the Cd ions within a fixed duration.

The highest amount of oxygen = The highest oxygen value observed within a fixed duration - the baseline oxygen value

In free cell (i.e., non-immobilized cells) toxicity evaluation for *A. torulosa*, 10 mL *A. torulosa* cells with an absorbance unit of 1.0 Abs at 700 nm was left for 15 min until a stable baseline was obtained. Further illumination for 5 to 10 min was performed to oxygen evolution. Various amount (<0.1 mL) of 100 μg L^{-1} of Cd solution was the added to the free cell solution to make the total Cd concentration range from 0.1-5.0 μg L^{-1}. It was then incubated for 15 min. Illumination was conducted once again at 5 to 10 min to determine the oxygen evaluation. The percentage of photosynthetic inhibition was then calculated.

RESULTS AND DISCUSSION

Immobilization techniques in biosensor offer advantages in terms of controlling the number of cells, better cell stability, more reproducible response, enabling continuous monitoring and greater mechanical strength (Mattiasson, 1997; Trevan and Mak, 1998). The usage of a thin layer of gel to immobilize the cells provides a fast diffusion of gases and short analysis time (Philp *et al*., 2003).

Cd toxicity tests on immobilized *A. torulosa* used in the biosensor construction showed that at lower concentrations of Cd, i.e., below approximately 8 mg L^{-1} of Cd there was no inhibition on photosynthetic oxygen release. Beyond this value, the inhibition of oxygen production occurred (Fig. 1). In fact, stimulation of oxygen release was observed at low concentration of Cd and the response is varied as the oxygen release can increase by up to 100% in one instance and only < 20% in another.

In photosynthetic organisms, Cd^{2+} can affect several metabolic activities in different ways. Some examples are photosynthesis and growth inhibition, binding onto chlorophylls, chlorosis and decrease in nutrient and water uptake (Clijsters and Assche, 1985; Krupa, 1999; Nies, 1999; Prasad and Strzalka, 1999, Clemens *et al*., 2002). Several studies in isolated chloroplasts have confirmed that Cd^{2+} affected photosynthesis in both the PS 1 and PS 2 (Atal *et al*., 1991; Siedlecka and Krupa, 1996; Pagliano *et al*., 2006).

A. torulosa is a photosynthetic organism. Although Cd can inhibit the activities of PS 1 and PS 2, results from this study showed that *A. torulosa* has mechanism to mediate the effect of Cd^{2+} especially at low concentration and after a short period of exposure. The mechanism appeared to accelerate the photosynthetic rate and produced more oxygen. The damage caused by low level of Cd^{2+} might initiate some biochemical processes that are related to the self-recovering mechanism from damage. The cyanobacteria cells used in the experiments were from the most active phase of growth and hence they may be capable of influence the Cd^{2+} transformation process in the cells (Krishnan *et al*., 2007).

However, the inhibition of photosynthesis began as soon as the Cd^{2+} concentration increased beyond the threshold value where the recovering mechanism no longer operational. Similar behaviour was also observed for the non-immobilized cells but in this case inhibition was observed until 1 mg L^{-1} of Cd after which stimulation occurred. At 5 mg L^{-1}, the cells demonstrated inhibition again (Fig. 2). The more susceptible of the non-immobilized cells to inhibition by Cd when compared with the immobilized cells is presumably attributed to the immobilized cells being protected from the pHEMA matrix that used to immobilize the organisms. This enables the organism to launch its self-recovery mechanism when Cd^{2+} is present.

The exposure duration of Cd^{2+} can have different effects on the toxicity behaviour. At short duration of exposure to Cd^{2+}, an organism can adapt to higher Cd^{2+} concentrations by employing resistance mechanisms, such as expression of Cd^{2+}-sequestering compounds (e.g., phytochelatins) or exporters capable of transporting Cd^{2+} out of cell (Nies, 1999). It has been observed that the cyanobacteria *Trichodesmium erythraeum* bloom when the Cd^{2+} concentration increased to

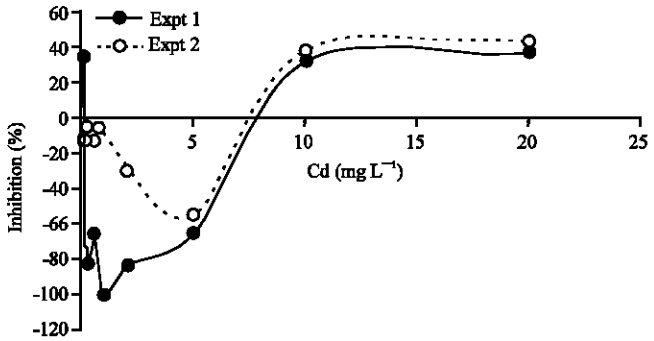

Fig. 1: The response of the toxicity biosensor containing immobilized *A. torulosa* when exposed to various concentrations of Cd^{2+}. Expt 1 and Expt 2 are duplicate experiments

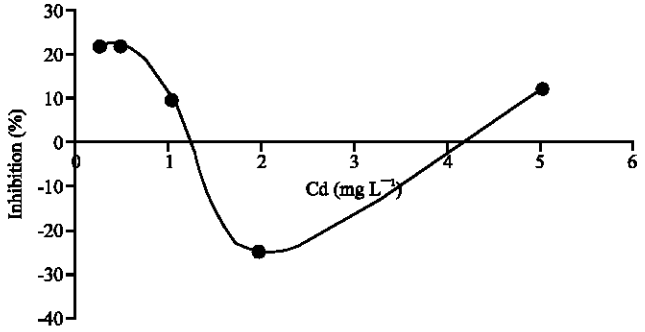

Fig. 2: The response of non-immobilized (free) *A. torulosa* cells to various concentrations of Cd^{2+} demonstrate a response similar to the immobilized *A. torulosa*

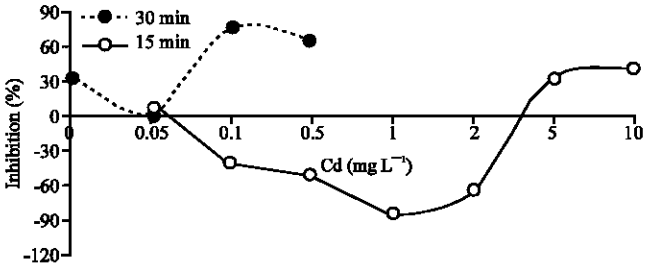

Fig. 3: The effect of the length of exposure time on the inhibition behaviour of *A. torulosa* by Cd^{2+}

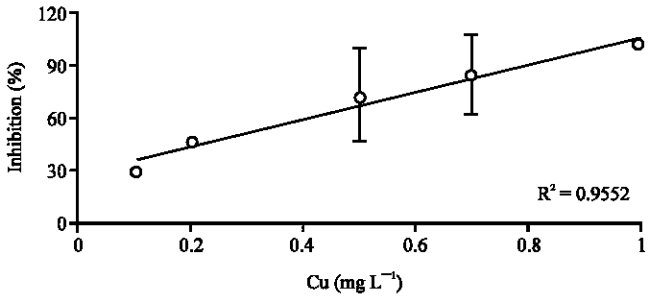

Fig. 4: The inhibition pattern from various concentrations of Cu^{2+} on the toxicity biosensor containing immobilized *A. torulosa*. The reproducibility of the biosensor to Cu is indicated by the errors on the data

0.31 mg L^{-1} (Krishnan *et al*., 2007). For *A. torulosa* studied here, The IC_{50} for 30 min of exposure is 0.9 mg L^{-1}. However, the IC_{50} for 15 min of exposure was not reached even with very high concentration of Cd^{2+} (Fig. 3). The inhibition of oxygen observed throughout the Cd^{2+} concentrations used still demonstrated the cells attempted to recover after an initial inhibition by Cd^{2+} during the prolonged exposure to Cd^{2+} to 30 min.

The peculiar behaviour of Cd^{2+} toxicity on *A. torulosa* did not exhibited by other metal such as copper (Cu). Exposure of the biosensor to Cu^{2+} using the present experimental setup showed no increase of oxygen production by the cyanobacteria (Fig. 4). In fact inhibition of oxygen production was observed throughout when exposed to Cu^{2+} and the response of the biosensor was linear with the

concentrations of Cu^{2+}. This is in agreement with previous finding by Chay *et al*. (2005) who used a same experimental setup to study Cu toxicity of *A. torulosa*.

In other species of algae or cyanobacteria, the presence of Cd^{2+} induced a significant decrease in activities of both PS 1 and PS 2, which led to inhibition of oxygen production. The level of inhibition on oxygen release yielded IC_{50} value of 5-10 mg L^{-1} in *Chlamydomonas* sp. and for *Anabaena inaequalis*, 1 mg L^{-1} of Cd^{2+} significantly inhibited the photosynthetic rate (Nagel and Voigt, 1995; Stratton and Corke, 1979). For *Anabaena flos-aquae*, the LD_{50} 96 h for Cd^{2+} was reported to be 0.14 mg L^{-1} (Heng *et al*., 2004). Thus, even Anabaena species demonstrated different toxicity response to Cd^{2+}. Based on the results observed here, some further studies may be useful to understand how the behaviour of this cyanobacteria species when under Cd^{2+} exposure.

CONCLUSION

The toxicity biosensor for the evaluation of Cd^{2+} toxicity on *A. torulosa* demonstrated that the Cd^{2+} toxicity behaviour of the cyanobacteria is peculiar where at lower concentration of Cd^{2+} (<8 mg L^{-1}), stimulation of the photosynthetic activities occurred. This is in contrast to metal such Cu^{2+} toxicity on the same cyanobacteria where the stimulation phase was not observed even at much lower concentrations Cu^{2+} when compared to that of Cd^{2+}. However, when the exposure time of the organism to Cd^{2+} was increased to 30 min, the biosensor registered a diminished effect of the stimulation phase and the inhibition effect became dominant.

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