Phycocyanin Fluorescence in Whole Cyanobacterial Cells as Bioindicators for the Screening of Cu^{2+} and Pb^{2+} in Water

Wan Nurul Aisyah Wan Jusoh[1], Ling Shing Wong[2]*, Mee Kin Chai[1]

1 College of Engineering, Universiti Tenaga Nasional (UNITEN), Jalan IKRAM-UNITEN, 43000 Kajang, Selangor, MALAYSIA
2 Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, 71800 Nilai, Negeri Sembilan, MALAYSIA
*Corresponding author. E-Mail: lingshing79@yahoo.sg; Tel: +06-798 2000 (Ext. 2372)

ABSTRACT: Heavy metals such as Cu^{2+} and Pb^{2+} are commonly used as coats for pipelines and various kitchen appliances; they are also used for alloys and stainless steels. In this study, cyanobacteria, A. *cylindrica* was chosen as bioindicator. The study focused on phycocyanin of the cyanobacteria, one of the major pigments that is located on the thylakoid membrane that absorbs light to drive photosynthesis. It is also able to emit fluorescence naturally, hence it could be exploited to screen for Cu^{2+} and Pb^{2+} in water. Our present study indicated that immobilized A. *cylindrica* displayed high responsiveness upon exposure to Cu^{2+} and Pb^{2+}. A. *cylindrica* was also able to give out responses at concentrations as low as 0.01 mg/L, hence proving it to be sensitive and making it a great candidate for biosensors in detecting heavy metals in drinking water.

KEYWORDS: Bioindicators; cyanobacteria; fluorescence; heavy metals; phycocyanin

Received 31 August 2017 **Revised** 26 September 2016 **Accepted** 3 October 2016 **In press** 26 February 2017 **Online** 20 March 2017

INTRODUCTION

Presence of heavy metal even in traces is toxic and detrimental to both flora and fauna (Wu *et al.*, 2004). Microalgae account for most of the biologically sequestered trace metals in aquatic environments. They possess the ability to adsorb and utilize metals due to their large surface: volume ratios, metal binding groups on their cell surfaces, high-affinity presence and efficient uptake of metal and storage system (Rajamani *et al.*, 2007). In recent years, heavy metal pollution has become one of the most serious environmental pollution. The use of biological materials in general, and microalgae including cyanobacteria in particular, has received considerable attention during recent decades for the detection of heavy metals as the environment friendly alternative technology (Teo & Wong, 2014). As opposed to conventional techniques such as atomic absorption spectrometry (AAS), inductively coupled plasma mass spectrometry (ICP-MS) and high performance liquid chromatography (HPLC), the utilization of cyanobacteria provides wider range of advantages. Since cyanobacteria are the earliest organism to evolve, this provides better adaptability and stability with the change of their surroundings. Cyanobacteria are also able to detoxify metals through metal-binding proteins, non-toxic nature and simple nutrient requirements. In cyanobacteria, major pigments involved in light-capturing for photosynthesis are chlorophyll, together with their accessory pigments such as phycocyanin and carotenoid (Dmitriy *et al.*, 2013). In this case, the fluorescence emitted by phycocyanin of *A. cylindrica* is of interest in this study. No studies had been previously recorded in the utilization of phycocyanin of whole cyanobacterial cells as bioindicators.

Phycocyanin is located on the surface of the thylakoid membrane and is directly involved in photosynthesis, where these pigments absorbed photons in the thylakoid membrane (Checchetto *et al.*, 2013). The photon absorbed by phycocyanin can undergo one of three fates: (i) to drive photosynthesis, (ii) to dissipate as heat or (iii) or re-emitted as fluorescence (Shevela & Shevela, 2011). These three fates of light energy occur in competition, where any increase in the efficiency of one will result in a decrease in the yield of the other two. These changes can be measured

qualitatively, hence making them applicable as bioindicators for the screening of pollutants such as heavy metals in the water. Therefore, the purpose of this study is to determine the fluorometric response of phycocyanin in cyanobacteria, *A. cylindrica* and to determine applicability of this cyanobacterial cells as bioindicators for the screening of Cu^{2+} and Pb^{2+} in water.

MATERIALS AND METHODS

Cyanobacterial Cultures Conditions and Cell Growth Determination

The unicellular and filamentous cyanobacteria, *Anabaena cylindrica* was obtained from Culture Collection of Algae and Protozoa (CCAP), U.K. The culture of *A. cylindrica* was aseptically inoculated into several 250 mL Erlenmeyer flasks containing approximately 100 mL of liquid Jaworski's medium, as described by Tompkins *et al.* (1995). The medium consisted of the following components (per 200 mL of distilled water): 4g $Na_2HPO_4{\cdot}12H_2O$, 16 g $NaNO_3$, 2.48 g KH_2PO_4, 4g $Ca(NO_3)_2{\cdot}4H_2O$, 10 g $MgSO_4{\cdot}7H_2O$, 3.18 g $NaHCO_3$, 0.45 g EDTAFeNa, 0.45 g EDTANa2, 0.496 g H_3BO_3, 0.2 g $MnCl_2{\cdot}4H_2O$, 0.2 g $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 0.008 g cyanocobalamin (B12), 0.008 g thiamin HCl (B1) and 0.008 g biotin. The flasks were exposed to white fluorescent illumination at room temperature 24 ± 2 ˚C. Cultures were continuously homogenized on a rotary shaker at 95 rotates per min (rpm) until they reached sufficient density of cyanobacteria for inoculation in further experiments. Light and dark periods remained at 16 hours and 8 hours respectively (Hemlata, 2009; Wong *et al.*, 2012). The cell growth was determined through manual cell count with a Neubauer cell counting chamber (Marienfeld) and a light microscope (Eclipse E100, Nikon). The count was made every two days for two weeks following a standard protocol written by Willén (1976).

Experimental Design

A volume of 0.5 mL of 7-day old *A. cylindrica* cells was mixed and immobilized with 0.5 mL of 1% agarose solution (w/v) in a methacrylate cuvette. This mixture was left to solidify for 15 minutes on the bench prior to exposure. By using a spectrofluorometer (Glomax, United States), the fluorescence emitted by phycocyanin of *A. cylindrica* could be measured using customized filter that was designed specifically for phycocyanin with excitation and emission wavelengths of 560 nm and 620 nm respectively.

Optimization of Cell Number and Time of Exposure

This study was conducted in a batch where a culture medium containing *A. cylindrica* suspensions with 1.64×10^6 cells/mL, 4.58×10^6 cells/mL, 8.65×10^6 cells/mL and 13.4×10^6 cells/mL. The cells suspension were immobilized and exposed to a volume of toxicant containing 1 mg/L of Cu for a period of 1 hour with measurement of fluorescence at 10 minutes interval. The change in fluorescence intensity with cells at 4.58×10^6 cells/mL, with optimum time of exposure of 10 minutes yielded the highest fluorometric response, thus they were selected as optimized conditions for this study.

Fluorometric Response of Phycocyanin to Different pH

The pH of copper solution was adjusted with the addition of 1 N of NaOH or 1 N of HCl to give pH values of 6.0, 6.5, 7.0, 7.5 and 8.5. The measurement of pH was conducted using a portable pH meter (Ohaus, India). For the optimization, a volume of 2 mL of Cu^{2+} with concentration of 1 mg/L at pH 6.0 was added to the immobilized cells and the fluorescent readings were taken before and after exposure under optimum conditions. These steps were repeated for pH 6.5, 7.0, 7.5 and 8.5 (Kunasakaran *et al.*, 2014).

Fluorometric Response of Phycocyanin to Cu^{2+} and Pb^{2+}

Immobilized *A. cylindrica*, cells were exposed to 2 mL of Cu^{2+} at different concentrations – 0.01 mg/L, 0.1 mg/L, 1 mg/L and 10 mg/L under optimized conditions. The same procedures were repeated for Pb^{2+} with the same ranges of concentrations. The fluorescence intensity was measured before and after exposure.

DATA ANALYSIS

All experiments were conducted in triplicates mean ± Standard Deviation (SD) and were shown graphically. The average and ANOVA tests were calculated out using Microsoft Excel 2015. The fluorescent readings were averaged and the percentage of change in fluorescent intensity was calculated using the following formula: [(F'- F) / F] × 100%; where, F'= fluorescent reading after exposure to toxicants; F = fluorescent reading before exposure to toxicants.

RESULT AND DISCUSSION

Cyanobacteria Cell Growth Determination

A typical growth curve for bacteria and other microorganisms is usually consists of four phases, which are lag phase or acceleration phase, exponential phase, stationary phase and death phase (Gonçalves *et al.*, 2016). As seen in Figure 1, only three growth phases were determined in *A. cylindrica*, namely the lag phase, exponential phase and stationary phase. Death phase was not observed in this study. This pattern is very common in cyanobacteria and green algae (Dang *et al.*, 2012; Hori *et al.*, 2002; Page *et al.*, 2012). The reason for the absence of death phase was perhaps due to the ability of *A. cylindrica* to re-germinate from akinetes under certain conditions (Kaplan-Levy *et al.*, 2010). In this case, it was the depletion of the nutrients and increment of wastes such as organic acids that triggered the secondary growth of cyanobacteria (Parma, 2003).

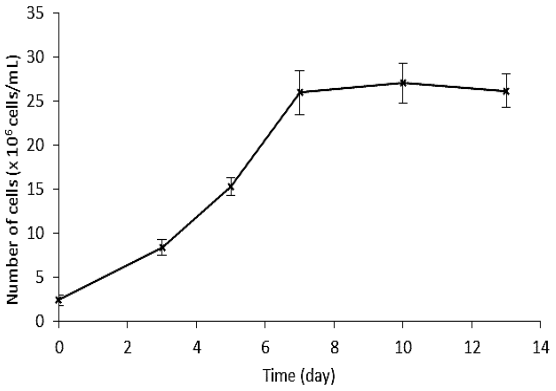

Figure 1. Growth curve of *A. cylindrica*

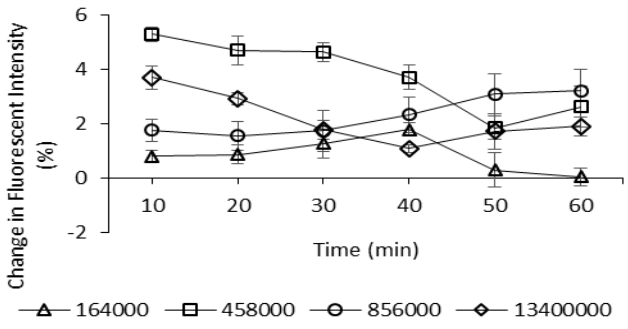

Figure 2. The optimization of cell density

Optimization of Cell Number and Time of Exposure

Cell number is an important parameter that must be taken account into because many authors had indicated that the sensitivity of toxicity tests increases with a decrease in initial cell number (Rodrigues *et al.*, 2011; Sutton, 2011). In the point of view of ecotoxicology, standard guidelines of growth inhibition of algae utilized high cell densities, approximately 10^4 to 10^6 cells/mL (Debelius *et al.*, 2009). Immobilized *A. cylindrica* was found to be the most responsive with cell number of 4.58×10^6 cells/mL (see Figure 2). ANOVA tests also revealed $p<0.05$, which indicated the responses obtained at 4.58×10^6 cells/mL were significant, hence it was chosen as the optimum cell number in the study. Wong *et al.* (2012) had tested on chlorophyll fluorescence of *A. torulosa* and it was found that the optimum cell number was also within the same range which was thought to be quite close to the current results obtained in this study. On the same note, Cu^{2+} was chosen as the initial toxicant

for the optimizations due to its mild toxicity effect on the cells. For cyanobacteria, at concentration below 0.03 mg/L of Cu is also a micronutrient which is essential for cells growth (ENR, 1988). The exposure time is significant especially in determining the optimum response of these immobilized *A. cylindrica* to Cu (Farghali *et al.*, 2013). As seen in Figure 3, phycocyanin optimized the fluorometric response in just 10 minutes of exposure.

Fluorometric Response of Phycocyanin to Different pH

In general, pH is an important parameter to consider especially in the screening of heavy metals using immobilized cells (Das *et al.*, 2008). It affects the chemistry of the toxicants, functional groups activity in the cells and competition of metallic ions (Das *et al.*, 2008). This affects the availability of the binding sites on the surface of the cyanobacterial cells hence affecting the whole photosynthetic electron transport and osmotic potential of the cytoplasm (Farghali *et al.*, 2013; Vishwanath & Rajashekhar, 2014). From Figure 4, a gradual increase in the fluorometric response was observed with increasing pH from 6.0 to 7.0, where it reached its optimal pH, yielding the maximum change in fluorescence yield in *A. cylindrica*. A noticeable decrease was observed as the pH increased from 7.5 to 8.5. The observed pH optimum value is consistent with the values reported by Vedrine *et al.* (2003). At low pH, protonation of the cell wall of *A. cylindrica* adversely affected the capacity for the metal binding but its effect becomes minor with increasing pH in its environment (Wu *et al.*, 2004). Higher pH also causes metal speciation where OH^- ions are widely distributed in the solution (Giraldez-Ruiz *et al.*, 1997), hence creating a competitive environment for the carboxylate group on the surface of the agarose and the cell wall. Latter results in the reduction of fluorescence yield. At neutral pH, carboxylate groups of the agarose and the cell wall of *A. cylindrica* are free, hence enabling higher uptake of metal from the environment. Thus the pH of the solution was maintained at neutral pH for the whole experiments.

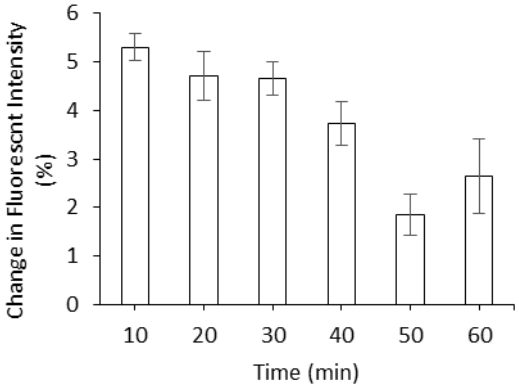

Figure 3. The optimization of time of exposure

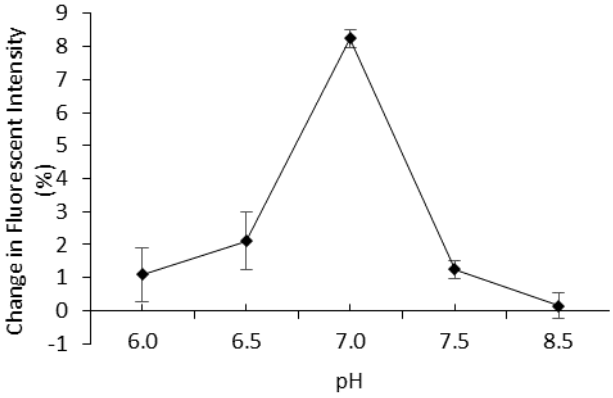

Figure 4. Response of *A. cylindrica* to different pH

Fluorometric Response of Phycocyanin to Cu^{2+} and Pb^{2+}

Naturally, cyanobacteria have the ability to incorporate metals into their readily-present polyphosphate granules, which also acts as detoxicants as reported by Arunakumara & Zhang (2009). The accumulation of heavy metals in the cells will damage the structure of various photosynthetic pigments and result in inhibition of enzymes required for biosynthesis of phycocyanin (Krause & Weis, 1991). It will also disrupt the whole electronic transport chain at cellular level, hence resulting in cell death (El-Sheekh *et al.*, 2003; Ozaki & Sonoike, 2009). Our present study indicated that the function of phycocyanin was more affected by Cu^{2+} than Pb^{2+} as shown in Fig. 5. The highest change in fluorescence intensity in the presence of Cu was found to be 1.0 mg/L while for Pb it was at 0.1 mg/L. Upon reaching the peak in the change in fluorescence intensity for Cu, Pb and Ni, a noticeable decrease in fluorescence yield was observed. As the heavy

metal concentration increased, reactive oxygen species (ROS) might have formed due to leakage of electrons and respiration electron transport chain under stress condition (Mrinamala & Mehta, 2014). The ROS accumulation causes retardation of growth, respiration and loss of membrane integrity as seen in microalgae *Pseudokirchneriella subcapitata, Chlorella vulgaris* and *Chlamydomonas reinhardtii* (Machado *et al.*, 2015). The response to of *A. cylindrica* to Pb^{2+} was relatively low compared to Cu^{2+} as Pb2+ has no known biological function and is toxic to any living cells (Dao & Beardall, 2016). Yet the fluctuation of fluorescence as seen in Figure 5 could be due to rapid synthesis of phycocyanin to sequester Pb^{2+}. This is in agreement with Rzymski *et al.* (2014) whose study showed the same hike in chlorophyll fluorescence in *Microcystis aeruginosa*. Phycocyanin is also located on the surface of thylakoid membrane, which makes it more susceptible to damage by Pb^{2+}. As seen in *Synchocystic* sp., Pb^{2+} had definitely resulted in an apparent structural damage upon exposure to higher concentration of Pb^{2+} (Arunakumara & Zhang, 2009). This was proven to be common in the response of cyanobacteria against heavy metals.

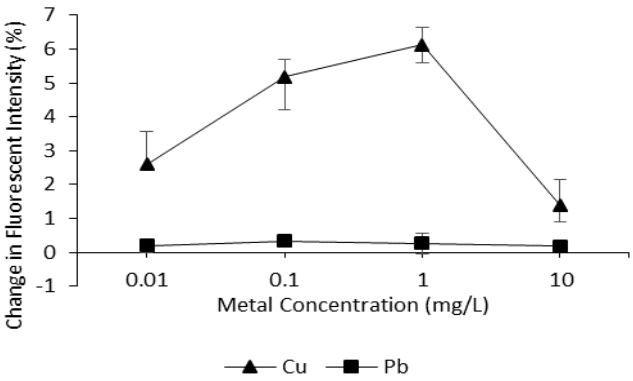

Figure 5. Fluorometric response of phycocyanin of *A .cylindrica* to Cu^{2+} and Pb^{2+}

CONCLUSION

The results obtained from this study confirmed that *A. cylindrica* responded well to the short-term exposure to Cu^{2+} and Pb^{2+} under optimized conditions (cell number: 4.58×10^6 cells/mL; t = 10 minutes; pH = 7.0). The cyanobacteria was also able to emit detectable fluorescence signal to as low as 0.01 mg/L of Cu^{2+} and Pb^{2+}. These immobilized cyanobacteria had indicated higher sensitivity to Cu^{2+} compare to Pb^{2+}. However, the high sensitivity of the cyanobacteria to Cu makes it a promising candidate to be applied as bioindicators for drinking water.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education Malaysia (FRGS/1/2014/SG03/INTI/02/1).

REFERENCES

[1] Arunakumara, K. & Zhang, X. (2009). Effects of heavy metals (Pb2+ and Cd2+) on the ultrastructure, growth and pigment contents of the unicellular cyanobacterium Synechocystis sp. PCC 6803. *Chinese Journal of Oceanology and Limnology,* **27**(2), 383-388.

[2] Checchetto, V., Teardo, E., Carraretto, L., Formentin, E., Bergantino, E., Giacometti, G. & Szabo, I. (2013). Regulation of photosynthesis by ion channels in cyanobacteria and higher plants. *Biophysical Chemistry,* **182,** 51-57.

[3] Dang, T., Fujii, M., Rose, A., Bligh, M. & Waite, T. (2012). Characteristics of the Freshwater Cyanobacterium Microcystis aeruginosa Grown in Iron-Limited Continuous Culture. *Applied and Environmental Microbiology,* **78**(5), 1574-1583.

[4] Dao, L. & Beardall, J. (2016). Effects of lead on two green microalgae Chlorella and Scenedesmus: photosystem II activity and heterogeneity. *Algal Research,* **16**, 150-159.

[5] Das, N., Vimala, R. & Karthika, P. (2008). Biosorption of heavy metals - An overview. *Indian Journal of Biotechnology,* **7,** 159-169.

[6] Debelius, B., Forja, J., DelValls, Á. & Lubián, L. (2009). Toxicity and bioaccumulation of copper and lead in five marine microalgae. *Ecotoxicology and Environmental Safety,* **72**(5), 1503-1513.

[7] Dmitriy, S., Pishchalnikov, R. & Eichacker, L. (2013). *Oxygenic photosynthesis in cyanobacteria*: CRC Press/Taylor & Francis Group.

[8] El-Sheekh, M., El-Naggar, A., Osman, M. & Mazaly, E. (2003). Effect of cobalt on growth, pigments and the photosynthetic electron transport in Monoraphodium minutum and Nitzchia perminuta. *Brazilian Journal of Plant Physiology,* **15(**3), 159-166.

[9] ENR. (1988). *Illinois Departmnet of Energy and Natural Resources: Guidelines for applying copper sulfate as an algicide: Lake Loami Field Study* (ILENR/RE-WR-88/19). Retrieved from Illinois: http://www.isws.illinois.edu/pubdoc/CR/ISWSCR-450.pdf

[10] Farghali, A., Bahgat, M., Enaiet Allah, A., & Khedr, M. (2013). Adsorption of Pb(II) ions from aqueous solutions using copper oxide nanostructures. *Beni-Suef University Journal of Basic and Applied Sciences,* **2**(2), 61-71.

[11] Giraldez-Ruiz, N., Mateo, P., Bonilla, I. & Fernandez, P. (1997). The Relationship between Intracellular pH, Growth Characteristics and Calcium in the Cyanobacterium Anabaena sp. Strain PCC7120 Exposed to Low pH. *The New Phytologist,* **137**(4), 599-605.

[12] Gonçalves, A., Pires, J. & Simões, M. (2016). Biotechnological potential of Synechocystis salina co-cultures with selected microalgae and cyanobacteria: Nutrients removal, biomass and lipid production. *Bioresource Technology,* **200,** 279-286.

[13] Hemlata, T. (2009). Screening of Cyanobacteria for Phycobiliproteins and Effect of Different Environmental Stress on Its Yield. *Bulletin of Environmental Contamination and Toxicology,* **83**(4), 509-515.

[14] Hori, K., Ishii, S., Ikeda, G., Okamoto, J., Tanji, Y., Weeraphasphong, C. & Unno, H. (2002). Behavior of filamentous cyanobacterium Anabaena spp. in water column and its cellular charactersitics. *Biochemical Engineering Journal,* **10,** 217-225.

[15] Kaplan-Levy, R., Hadas, O., Summers, M., Rucker, J. & Sukenik, A. (2010). Akinetes: Dormant cells of cyanobacteria *Dormancy and Resistance in Harsh Environments*.

[16] Krause, G. H. & Weis, E. (1991). Chlrophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Physiology and Plant Molecular Biology,* **42**(1), 313-349.

[17] Kunasakaran, R., Vijayan, S., Wong, L. S. & Chai, M. K. (2014). The effects of pH and cell density to the responses of immobilized cyanobacteria for copper detection. *Journal of Life Sciences and Technologies,* **2**(2), 78-81.

[18] Machado, M. D., Lopes, A. R. & Soares, E. V. (2015). Responses of the alga Pseudokirchneriella subcapitata to long-term exposure to metal stress. *Journal of Hazardous Materials,* **296,** 82-92.

[19] Mrinamala, D. & Mehta, S. K. (2014). Changes in antioxidative enzymes of cyanobacterium Nostoc muscorum under copper (Cu2+) stress. *Science Vision,* **14**(4), 207-214.

[20] Ozaki, H. & Sonoike, K. (2009). Quantitative analysis of the relationship between induction kinetics of chlorophyll fluorescence and function of genes in the cyanobacterium Synechocystis sp. PCC 6803. *Photosynth Res,* **101(**1), 47-58.

[21] Page, L. E., Liberton, M. & Pakrasi, H. B. (2012). Reduction of Photoautotrophic Productivity in the Cyanobacterium Synechocystis sp. Strain PCC 6803 by Phycobilisome Antenna Truncation. *Applied and Environmental Microbiology,* **78**(17), 6349-6351.

[22] Parma, N. (2003). *Bacterial growth.* Retrieved from http://research.omicsgroup.org/index.php/Bacterial_growth

[23] Rajamani, R., Siripornadulsil, S., Falcao, F., Torres, M., Colepicolo, P. & Sayre, R. (2007). *Phycoremediation of heavy metals using transgenic microalgae* (R. Leon, A. Galvan, & E. Fernandez Eds.). New York: Springer.