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The Responses of Carotenoids in *Anabaena cylindrica* to Single and Combined Metals of Nickel, Aluminum and Lithium

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ABSTRACT

In the real polluted environment, the metals usually do not present in single pure form, but occur in mixture. Cyanobacteria are the most commonly available aquatic organisms in these environment, thus, there is a need to study the responses of carotenoids, a kind of antioxidant in cyanobacteria towards combined heavy and light metals. In this paper, *Anabaena cylindrica* was immobilized using agarose and exposed to the different concentration of single and combined Ni, Al and Li for 2 hours. The responses of carotenoids were determined using spectrophotometer at $\lambda = 450$ nm. The result showed that immobilized *A. cylindrica* responded differently to the exposure of single and combined metals. The cells were sensitive to single and combined Ni, Al and Li within the detection range of 0.001 mg/L to 10.000 mg/L. The value of R^2 indicated the immobilized *A. cylindrica* responded in correlation to the concentration of the metals. Thus, *A. cylindrica* has the potential to be used as bioindicator for detection of single and combined metals.

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INTRODUCTION

With rapid development of industrialization and urbanization, amount of polluted metals released into the environment has grown enormously. Some metals such as nickel (Ni), aluminum (Al) and lithium (Li) are found to be harmful to human [1-4].

Ni is one of the essential micronutrient for plant growth and cyanobacteria metabolism. In plant, Ni is required for formation of urease, an enzyme that converts urea to carbon dioxide and ammonia [5]. In cyanobacteria, low concentration of Ni stimulates the nitrogenase activity [6] and hydrogenase activities [7], but it is toxic to photosynthetic organisms in high concentration [8]. Lithium and aluminium are not required for plant and cyanobacteria growth and the accumulation of the metals is toxic to these organisms [9; 10].

Carotenoids are naturally occurring pigments in cyanobacteria. Carotenoid pigments serve two major functions- as accessory pigments for light harvesting and in prevention of photo-oxidative damage [11; 12]. Carotenoids protect photosynthetic apparatus by quenching the oxidizing species and triplet state of the chlorophyll, and other excited molecules in the other pigments, to prevent damage to cellular components and disruption to metabolisms [13]. In addition to that, some studies suggested that carotenoid play a role against metal oxidative damage [14; 15]. Thus, the presence of toxic metals will trigger responses of carotenoids in cyanobacteria.

In the real polluted environment, several types of metals may present in one sample at the same time. Sacan *et al*. [16] showed that the combination of lead and aluminum synergistically caused cell membrane to lyse in marine algae *Dunaliella tertiolecta*. The synergistic toxicity effect of combined copper and cadmium was observed in algae by Qian *et al*. [17], while Al-Mousawi [18] reported that the antagonistic effect was found on cyanobacteria *Chroococcus* sp. at most concentration of Ni and Cu. However, the toxicity effects of the combination of Ni, Al, and Li in cyanobacteria *A. cylindrica* have not been reported before. As the effects of combination toxicity of metals might be varied from the effects individual metals, the response of combination toxicity was therefore important to be studied. In this paper, the responses of immobilized *A. cylindrica* toward

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single and combination of three light metals- Ni, Al and Li are reported. The potential utilization of the cyanobacteria to be used as bioindicator is evaluated as well.

MATERIALS AND METHODS

A.cylindrica culture and determination of growth phase:

A. cylindrica was cultured in Jarwoski medium [19]. The cultured was maintained in room temperature and illuminated with 16 hours of cool-white fluorescence light followed by 8 hours of dark period. Aeration was carried out with orbital shaker (A3446, Smith) at 90 rpm to avoid clumping of cells. Subculture was carried out every 15 days.

Growth phases of the cells were determined by cell count using hemocytometer (Marienfeld-Superior, Neubauer) light microscope (Eclipse E-100 LED, Nikon). The number of cells was then correlated to the OD reading at λ = 700 nm. The OD was determined using spectrophotometer (GeneQuant 1300, GE Healthcare Life Sciences).

Immobilization of cyanobacteria:

Immobilization was carried out using 1% (w/v) agarose prepared by mixing 1g of agarose powder with 100 mL deionized water. 0.5 mL of cells and 0.5 mL of agarose medium were added into the cuvette at 45 °C. Then, the cuvette was sealed with plastic paraffin film (Parafilm M, Pechiney Plastic Packaging) and left in room temperature until the mixture solidified [20].

Cell density for optimized response:

Cells with different densities (OD700nm = 0.5, 1.0 and 1.5 A) taken from exponential phase were prepared. The cells were then immobilized and exposed to 2 mL of 0.1 mg/L Ni for 0 hour, 1 hour, 2 hour, 6 hour and 24 hour in room temperature. The immobilized cells exposed to 2 mL of deionized water were used as the negative control. All exposure tests were conducted with n = 3 and measured at λ = 450 nm using the spectrophotometer.

Exposure to single metal:

Different concentration of Ni, Al and Li solution was prepared using $Ni(NO_3)_2 \cdot 6H_2O$, $KAl(SO_4)_2 \cdot 12H_2O$ and $LiNO_3$ purchased from Sigma-Aldrich. The immobilized cell were exposed to 0.001, mg/L, 0.010 mg/L, 0.100 mg/L, 1.000 mg/L, and 10.000 mg/L of single Ni, Al and Li under optimized condition.

Exposure to combined metal:

Al and Li were mixed in 1:1 ratio (v/v) in different concentrations. The combination of Ni + Li, Li + Al, and the combination of all three metals were prepared using the similar method. The percentage of change in absorbance (%OD) was calculated using equation 1.

$$\% OD = [(OD_1 - OD_0) / OD_0] \times 100\% \quad \text{(Eq. 1)}$$

Where,

OD_1 = absorbance after the exposure to metal

OD_0 = absorbance before the exposure

RESULTS AND DISCUSSION

The growth phase was be determined based on the cell count and OD measurement at λ = 700 nm. The results showed the cells grew slowly from day 0 to day 2 on lag phase, entered the exponential phase from Day 3 to Day 7, and followed by the stationary phase after day 8. Figure 1 shows the correlation between cell density and OD = 700 nm of cells. The correlation (R^2) value = 0.9638 indicated the cell density and OD = 700 nm were strongly correlated [21]. Compared to cell count, OD measurement was more convenient and faster way to determine cell density [22], thus was adopted in the experiment. Microbes at their exponential phase was used for toxicity tests as they had been proven to give optimized performance [23; 24]. Therefore, the cells from day 5 to day 7 culture were used for the determination of optimized condition.

The average cell densities for 0.5 A, 1.0 A and 1.5 A were 6.1 $\times 10^5$ cell/mL, 1.07 $\times 10^6$ cell/mL and 1.92 $\times 10^6$ cell/mL respectively. The highest responses of three concentrations of *A. cylindrica* taken from Day 5, 6 and 7 cultures after exposed to 0.1 mg/L Ni were measured. As day 5 culture density = 0.5 A produced significantly high response (OD changes) in two hours of exposure, thus the condition was selected for the exposure tests. The results showed that different cell densities would respond differently, which the higher cell concentration or density not necessarily produced the best response [25; 26].

The response profile for different concentration of single Ni, Al and Li after 2 hours exposure are shown in Figure 2, Figure 3, Figure 4 and Figure 5. For Ni, the absorbance increased from 0.001 mg/L to 0.100 mg/L, and decreased thereafter. The results was in agreement with Shukla *et al*. [27] which the carotenoids content

increased in the low concentration of Ni and decreased in high concentration of Ni. The increase in carotenoids can be explained as a mechanism to reduce Ni toxicity through quenching free oxygen radicals. Decrease of carotenoids content in higher concentration of Ni might due to the net degradation of the pigments.

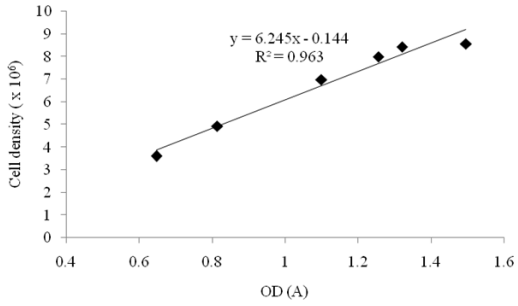

Fig. 1: Correlation between cell density and OD at λ = 700 nm.

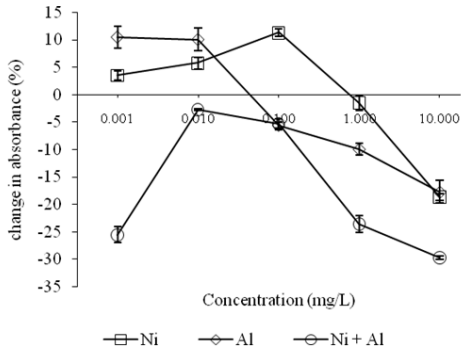

Fig. 2: Response of *A. cylindrica* to Ni, Al and Ni + Al (1:1).

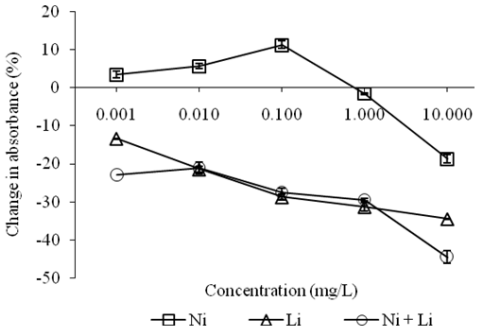

Fig. 3: Response of *A. cylindrica* to Ni, Li, and Ni + Li (1:1).

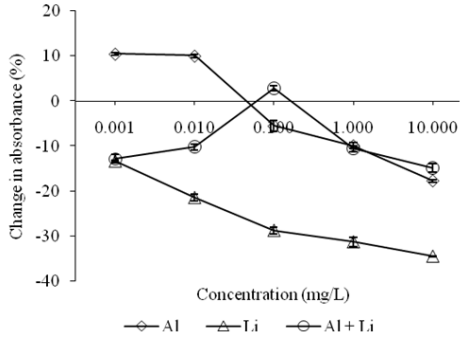

Fig. 4: Response of *A. cylindrica* to Al, Li, and Al + Li (1:1).

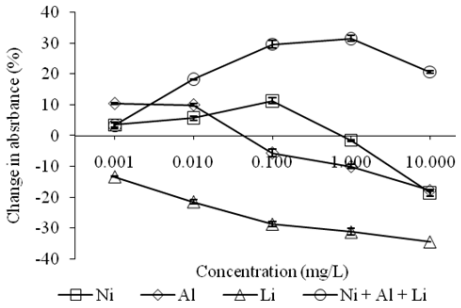

Fig. 5: Response of *A. cylindrica* to Ni, Al, Li, and Ni + Al + Li solution (1:1:1).

The content of carotenoids decreased with the increase of concentration of Al. Pettersson *et al*. [28] explained that Al toxicity to *A. cylindrica* was due to intracellular accumulation of the metal through passive diffusion. The high affinity of Al to phosphate group caused accumulation of Al in both cell wall and polyphosphate granules. The accumulation of Al disturbed the synthesis and metabolism of important energy-rich phosphate compounds such as ATP inside the cell, which later inhibited the growth and reduced the pigments in the cyanobacteria.

Carotenoids content decreased in all the concentrations of Li. Few studies have investigated the effect of Li to the metabolic activity of cyanobacteria or algae. Porter & Berno [29] reported that an exponential decline in microbial respiration occurred in the range between 0 and 0.150 mg/L of Li. Respiration is vital to microbial function. Decline in respiration will cause the death of cell and thereby inhibit carotenoids production. Another possible reason might be substation of Mg ion with Li in plant cells [30].

The toxicity effects of combined metals on cyanobacteria can be additive, synergistic or antagonistic [31]. Figure 2, Figure 3, Figure 4, and Figure 5 depicts the response of *A. cylindrica* to the combined Ni + Al and Ni + Li respectively. The toxicity effects of single metals were inserted as references. The response of the cellsto the combined Ni + Al, Ni + Li, Al + Li showed predominantly antagonistic effect. The response of *A.* cellsto Ni + Al + Li showed different trend as the absorbance of the combined toxicity increased while the absorbance of single metals decreases.

Summary of linear detection ranges, R^2 values and slope for carotenoids exposed to single and combined Ni, Al and Li for 2 hour exposure is showed in Table 2. The slope values which obtained from the linear equation indicated the sensitivity of *A. cylindrica* toward different concentration of metals [24]. The value of the slope of linear detection range showed the sensitivity (regardless the concentration range) of the *A. cylindrica* increased with Li < Ni + Al + Li <Ni + Li < Ni + Al< Ni < Al + Li< Al.

The response of *A. cylindrica* toward Ni, Al, Ni + Al, Ni + Li, Al + Li and Ni + Li + Al produced overall higher values of R^2 (> 0.9) for all the tests conducted, showing a good correlation between the carotenoids changes to the concentration of the metals. This indicated that *A. cylindrica* has the potential not only used for detection of single metal, but also detection of combined metals.

Table 1. Linear detection ranges, R^2 values and slope for carotenoid exposed to single and combined Ni, Al and Li.

Toxicants	Linear detection range (mg/L)	R^2	Value of |Slope|
Ni	0.001 – 0.100	0.960	72.07
Al	0.001 – 0.100	0.997	166.52
Li	0.100 – 10.000	0.866	0.49
Ni + Al	0.010 – 1.000	0.999	20.65
Ni + Li	0.001 – 10.000	0.909	2.03
Li + Al	0.001 – 0.100	0.994	152.60
Ni + Al + Li	0.001 – 0.100	0.941	1.03

Conclusion:

The responses of immobilized *A. cylindrica* towards the exposure of single and combined Ni, Al and Li was determined at λ = 450 nm, with the cells from day 5 culture, cell density of 0.5 A (measure at λ = 700 nm), and the exposure time set at 2 hours. The studies confirmed the cyanobacteria *A.cylindrica* responded differently to Ni, Al, and Li, as well as to the combined toxicity of these three metals. Although the biological responses of the cells are determined, the real biochemical reactions that lead to the responses are yet to be studied. *A. cylindrica* showed good sensitivity toward single and combined Ni, Al and Li, with the linear detection ranges varied from each other. The high R^2 value indicated good correlation between the metal concentrations to the

response of the cells as well. As conclusion, the cyanobacteria posed good potential as whole cell bioindicator for the detection of single and combined metals.

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Authors' contributions:

Ling Shing Wong was responsible in designing the experiment, Yeong Hwang Tan were responsible in laboratory works, while Nurul Afiqah Kishamuddin, and Mee Kin Chai shared equal responsible for the data analysis and reconfirmation.

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