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Synechococcus sp. AND *Pseudanabaena* sp. CELL CULTURE BIOGENESIS TOWARDS pH, PERIOD OF TIME AND BIO-CONCENTRATION FACTOR (BCF)

Rashidi Othman[1+]
Ruhul 'Izzati Shaharuddin[2]
Wan Syibrah Hanisah Wan Sulaiman[3]
Nur Hanie Mohd Latiff[4]

[1,2,3,4]*International Institute for Halal Research and Training, Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University Malaysia, Jalan Gombak, Kuala Lumpur, Malaysia.*
[1]*Email: rashidi@iium.edu.my Tel: +6012-6644772*
[2]*Email: ruhulizzatiium@gmail.com Tel: +60172726465*
[3]*Email: syibrahanis@iium.edu.my Tel: +601114275534*
[4]*Email: nurhanielatiff@iium.edu.my Tel: +60176526796*


(+ Corresponding author)

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ABSTRACT

Water pollution and water scarcity due to the discharge of untreated domestic and industrial wastewater into aquatic bodies need serious attention. A number of physical, chemical, and biological methods have been developed for wastewater treatment; among these, the use of cyanobacteria is considered as sustainable ways of averting the consequences. The dual application of microalgae for phycoremediation and biomass production for value products like biofuels and fertilizers is a feasible and eco-friendly alternative. This research aimed to investigate variables controlling heavy metal sequestration mechanism by assessing the effects of environmental variables from the mixtures of artificial wastewater samples using *Synechococcus* sp., and *Pseudanabaena* sp. Six heavy metals (Pb, Fe, Cr, Cd, Al, and Cu) treatments were tested and analyzed by Inductively Coupled Plasma (ICP) at three different periods of time (week 1 until week 3). The results indicated that both cyanobacteria species have equal potential to sequester heavy metals. However, *Pseudanabaena* sp. is the best phycoremediator agent due to substantial high Bio-concentration factor (BCF) compared to *Synechococcus* sp. On top of that, a regulatory step for the cyanobacteria cell growth and production mechanism is mediated by pH.

Contribution/ Originality: This study is one of the very few studies which have investigated key factors in controlling heavy metal sequestration mechanism by assessing the effects of environmental variables such as pH, the period of time, and type of heavy metals using Synechococcus sp., and Pseudanabaena sp.

1. INTRODUCTION

Anthropogenic pollution of freshwater ecosystems by the addition of heavy metals and nutrients an increasing phenomenon that affects many lakes and rivers worldwide, and threatens the health of animals and human beings via the food chain [1]. Many heavy metals and metalloids are toxic and can cause undesirable effects and severe problems even at very low concentrations [2]. The most severe is that metals cannot be degradable and dangerous because they tend to bioaccumulate in living things and stored faster than they are excreted [3]. Besides, the liquid anthropogenic wastes discharged such as Hg, Mn, Ni, Pb, and Cu ions affect the water quality from various standard

parameters [4]. On top of that, the presence of multiple heavy metal ions in the algal growth media imparts major physiological and biochemical consequence [5, 6]. Phycoremediation of heavy metals possess excellent potential [7] and several studies have proven that use of algae is a cost-effective method for heavy metal sequestration from various type of water, and such treated water can be reused without additional treatment [7]. Phycoremediation is a process to treat pollutants in environment either water or soil by employing algae. Algae in general categorized into two groups of macroalgae and microalgae. Category of microalgae consist of four groups which are Cynophyceacea, Chlorophycae, Bacillariophyceae, and Chrysophyceae. Cynophyceacea refers to oxygenic photosynthetic bacteria, namely cyanobacteria or previously called blue-green algae. Cyanobacteria is a great water treatment agent in different management towards sustainability environment. The cyanobacteria are equipped with ability to decompose the organic wastes and residues [8] detoxify heavy metals [7] pesticides [9] crude oil [10-12] and other xenobiotics, declorize effluent [13-15]. Besides, cyanobacteria also fix atmospheric N_2, catalyze the nutrient cycling, suppress growth of pathogenic microorganisms in soil and water, and also produce some bioactive compounds such as vitamins, hormones, and enzymes which contribute to plant growth [16]. In contrary, the conventional physicochemical techniques for removing heavy metals from wastes are generally very expensive to include chemical precipitation, ion exchange, membrane processing and adsorption [17]. Hence, a great deal of interest has recently been generated in using algae as they represent the best biological treatment for wastewater [16]. Thus, in this study, the artificial waste water which contains a mixture of six heavy metals were tested in different concentration by cyanobacteria (blue-green algae).

2. MATERIALS AND METHODS

2.1. Cell Culture

The sterilized *Synechococcus* and *Pseudanabaena* strain were subcultured on modified Bold's Basal Medium (BBM). The pH was altered to 7.8 and autoclaved. Algae strain was cultivated indoors in growth chambers. 10 percent algae stock was transferred by sterilized pipette tips into media in sterile 2 liter Schott bottle. The culture was kept under Philips fluorescent light TL-D 36W/54-765.3.5.3 under 12:12 of the dark/light cycle, at a temperature of 24±1°C day and night. The carbon dioxide and air were bubbled through the medium constantly.

2.2. pH Optimization

The stock cultures of Synechococcus and *Pseudanabaena* strain were transferred and cultured in modification of Bold's Basal Medium (BBM) recipe which contained macronutrient, trace metals and vitamins. The pH was adjusted with various range of 3.8, 5.8, 6.8 and 7.8 to identify the ideal pH by determine fast growth of algae via cell count. The pH of solution was adjusted by potassium hydroxide (KOH) or hydrochloric acid (HCl). The heavy metal solutions were then autoclaved for sterilization. All strains were cultivated indoors in growth chambers. 2ml of each algae OD750~0.5 stock was transferred by sterilized pipette tips into 250mL media in sterilized conical flask and tightly closed with sterile cotton plug. The culture was kept under Philips fluorescent light TL-D 36W/54-765.3.5.3 at a temperature of 24±1°C under 12:12 dark/light cycle. The flasks were hand shaken twice a day to prevent adherence of the algal cells to the bottom of the flasks. The cell count was taken every 4 days in two weeks using a microscope Carl Zeiss GmbH with the aid of haemocytometer as described by Pancha, et al. [18] and Guillard and Sieracki [19].

2.3. Preparation of Model System

Firstly, analytical grade of heavy metals salt were weighed according to desired concentration of heavy metals which were 1 mg/L, 2 mg/L and 3 mg/L. Then, salt was poured into the beaker contained of 3.6L deionized water and mixed using magnetic stirrer until completely dissolved. Next, the pH of solution was adjusted to 6.8 and the heavy metal solutions were then autoclaved for sterilization. For the model system, 3.6L of heavy metals solution

was transferred into Schott bottle replicates of three and added to $2x10^4$ cells per ml of cyanobacteria in 400ml and three controls of heavy metals solution without micro-algae. The treatments were conducted over three different periods of time which were week 1 until week 3 and were placed in the growth room. The culture was kept under Philips fluorescent light TL-D 36W/54-765.3.5.3 and set to 12h light/ 12h dark cycle as per ideal result at a temperature of 24±1°C day and night. Carbon dioxide and air were bubbled through the treatment constantly.

2.4 Heavy metals analysis: The solutions were then swirled gently to homogenise all the mixtures. The rate of temperature was adjusted to ± 170°C, and 1,200 Watt of power was set for 30 minutes. The microwave was run until completion, and the digested samples were left to cool down until reaching room temperature before the samples can be transferred inside 50ml centrifuge tube. Additional 18MΩ deionised (DI) water was then added into each of the 50ml tubes until the final volume of the samples reaches 50ml. Six heavy metals which are Pb, Fe, Cr, Cd, Al, and Cu included in the development of the model system were analysed by using ICP-MS (Perkin Elmer NexION 300X) as detailed in US EPA 6020B. Each of the digested samples will have three replicates for ICP-MS analysis. To prepare samples for ICP-MS analysis, 1ml of each of the samples was pipetted into a 15ml centrifuge tube. Standard calibration solutions were prepared by using Multi-element Calibration Standard 3 whereas, internal standard solutions were prepared by using Internal Standard Mix. Both of the solutions were provided in the Environmental Standard Kit for ICP-MS (PerkinElmer Part No. N9307111). 0.5ml of 10mg/l Standard 3was diluted into 50ml of 1%HNO_3 in 18 MΩ deionized (DI) water to produce concentration of 100 ppb as stock solution before preparation of the standard calibration. Five calibration concentrations ranging from 0.5 ppb, 1.0 ppb, 2.0 ppb, 5.0 ppb and 10 ppb and one quality control (QC) solution with concentration of 7.0 ppb were prepared from the stock solution. 20 ppb of internal standard solution was prepared by pipetting 1 ml of 10mg/l Internal Standard diluted into 500 ml of 1%HNO3 in 18 MΩ deionized (DI) water. The samples then ready for heavy metals analysis by ICP-MS.

2.4. Data Analysis

All the experiments were carried out in triplicates and data presented as mean values of three independent replicates. Data were further analyzed using analysis of variance (ANOVA). The efficiency of phycoremediation was quantified by calculating bioconcentration factor. Bioconcentration factor specifies the efficiency of algae species in accumulating a metal into its tissues from the surrounding environment [20]. It is calculated as detailed by Zhuang et al. (2007). BCF = Heavy metal concentration in algae (mg/kg) / Initial heavy metal concentration in water (mg/L).

3. RESULTS AND DISCUSSIONS

3.1. Effects of Different pH of Culture Media on Cyanobacteria

Analysis of variance showed that there was a highly significant difference (P<0.0001) in cell growth and production of *Synechococcus* sp. and *Pseudanabaena* sp. in response to the different level of pH treatment and period of time Figure 1. Both cyanobacteria species exhibited an increased cell growth from week 1 until week 3 and from acidic to alkaline condition. They were grown best at pH 7.8 with *Synechococcus* sp. detected to have the highest cell count measured compared to *Pseudanabaena* sp. of 120 and 32 x 10^4 ml^{-1} respectively. This increase reflected a substantially higher amount of cell growth in correlation with a period of time, pH level as well as genotype. Different pH media was found to have a prominent effect on the inhibition growth of algae. The ideal growth conditions for microalgal cultures and the biomass productivity depends upon many factors. These include abiotic factors like temperature, minerals, carbon dioxide, pH, water quality, light cycle and intensity [21]. It is also reported that pH is the third most important factor after temperature and light, for growing algae [22]. These findings were in agreement with other studies where microalgae were grown best at pH 7.5 for *Phaeodactylum tricornutum* [23] 7.10 and 8 for *Spirogyra* sp. and *Oedogonium* sp. [24]. Therefore, in the next heavy metal

sequestration rate assessment is investigated in cyanobacteria as a potential model system by providing a high level of pH at 7.8.

3.2. Assessment of Heavy Metal Sequestration Rate and BCF of Pseudanabaena Sp. and Synechococcus Sp. at Different Period of Time

Six heavy metals were analysed as indicated in Table 1 and Figure 2 from two cyanobacteria species of *Synechococcus* sp. and *Pseudanabaena* sp. to investigate the stability of their sequestration rate and BCF. Analysis of variance exhibiting highly significant differences ($P < 0.0001$) between the two species, heavy metals sequestration rate as well as a period of time. The data revealed that the removal efficiency differed according to the types of heavy metal and genotype used. Overall when the period of time increased from week 1 until week 3, BCF value decreased. However, when the period of time decreased to week 1, BCF value increase.

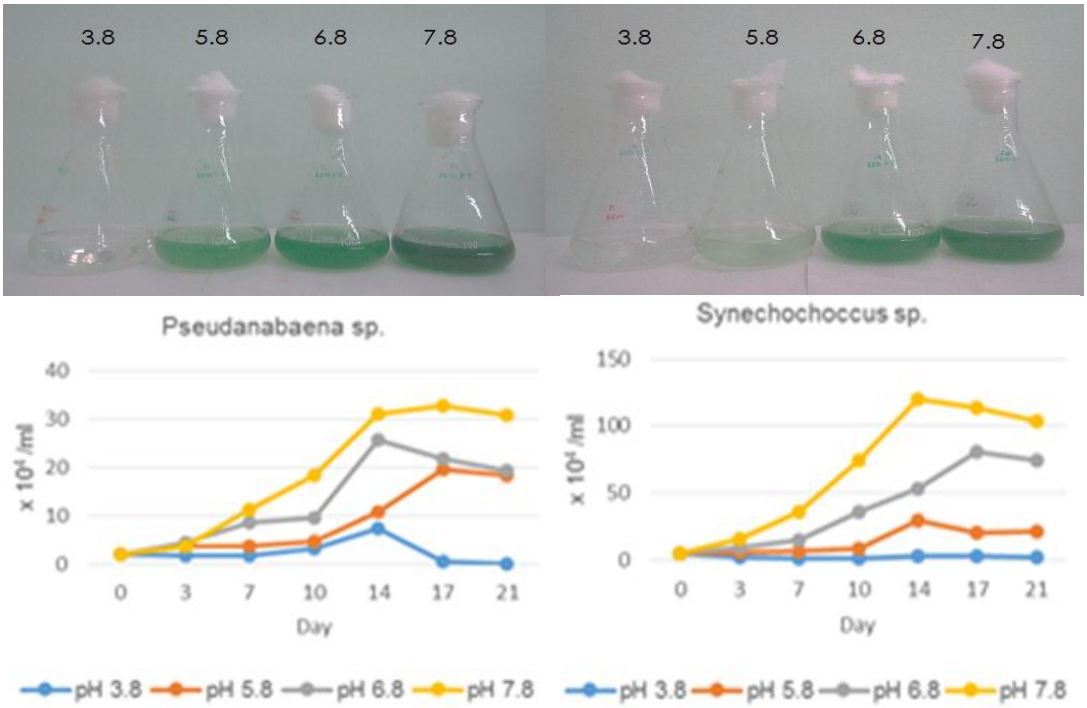

Figure-1. The growth of *Pseudanabaena* sp. (left) and *Synechococcus* sp. (right) at different pH and period of time.

Table-1. BCF of *Synechococcus* sp. and *Pseudanabaena* sp. heavy metals sequestration rate at different period of time.

Cyanobacteria Species	Week	Heavy Metal: Aluminium (Al)	Chromium (Cr)	Iron (Fe)	Copper (Cu)	Cadmium (Cd)	Lead (Pb)
Synechococcus sp.	1	4	1	5	5	3	7
	2	2	0	2	0	2	4
	3	0	0	1	1	0	1
Pseudanabaena sp.	1	4	7	4	1	1	7
	2	3	6	4	1	1	5
	3	2	3	2	0	0	3

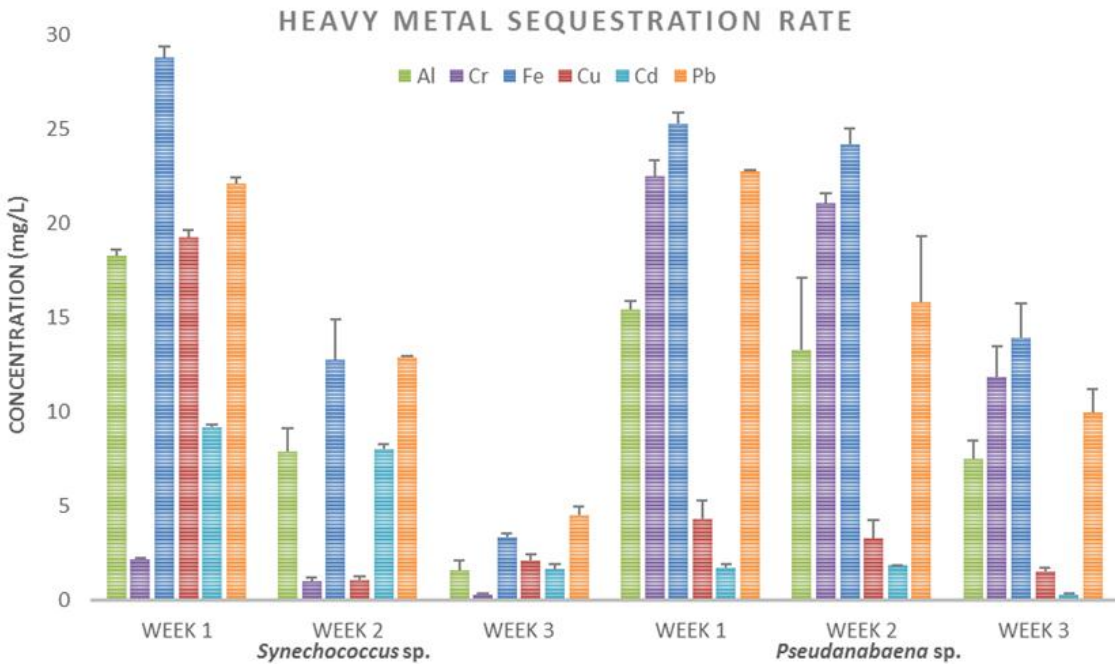

Figure-2. Heavy metals sequestration rate of *Pseudanabaena* sp. and *Synechococcus* sp. at different period of time.

As referred to Table 1 and Figure 2, *Pseudanabaena* sp. is the best bioaccumulator agent of six heavy metals that have been tested compared to *Synechococcus* sp. The highest level of BCF value detected in *Synechococcus* sp. was in lead treatment whereas in *Pseudanabaena* sp. was detected in lead and chromium at week 1. Bioconcentration factor specifies the efficiency of algae species in accumulating a metal into its tissues from the surrounding environment. Therefore, BCF greater than 1 have the potential as a phycoremediation agent [25, 26].

4. CONCLUSION

The development of cyanobacteria model system has proved to be an effective tool for investigating the environmental factor specifically different pH level involved in regulating cell growth and production of 2 cyanobacteria species as well as to sequester 6 types of heavy metal contaminant. In conclusion, *Pseudanabaena* sp. has a better potential to sequester heavy metals which can be recommended for large-scale water treatment. The future recommendation of exploring cyanobacteria should be extended which can provide the novelty of green technology at the forefront of sustainable development.

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