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Biosorption combined with lipid production and growth inhibition of copper on the microalgal *Pediastrum* sp.

Khả năng hấp thu kết hợp với tích lũy sản phẩm lipid và ức chế sinh trưởng của đồng trên vi tảo lục Pediastrum *sp.*

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The contamination of heavy metals in surface waters is an environmental concern due to their persistence and non-degradation that poses a risk to the ecosystem and human health. Microalgae have been known for their ability to remove metals from wastewater and to produce biodiesel. In this study, the copper (Cu) stress on the growth and lipid contents of the green microalgal *Pediastrum* sp. were evaluated along with the removal capacity. The green microalga was grown in a culture medium with the presence of copper at concentrations of 0, 0.1, 0.5, 2, 5 and 15 mg/L for one week. Results indicated that the growing tolerance levels of *Pediastrum* sp. in the presence of copper up to 2 mg/L and Cu inhibited the algal growth with the 96h-EC50 value of 6.67 mg/L. However, the *Pediastrum* sp. showed a promising metal removal efficiency. Cu removal was from 83 to 95% by *Pediastrum* sp. with an initial concentration of Cu less than 2 mg/L. The presence of a low level of Cu increased the lipid yield up to 18%, but a high concentration of Cu has resulted in low removal efficiencies and decreasing lipid accumulation. The present study suggested the potential of employing green microalgae for wastewater treatment and biodiesel.

Ô nhiễm kim loại nặng trong nước mặt đang là vấn nạn môi trường ở nhiều quốc gia bởi vì kim loại nặng tồn tại lâu, khó có khả năng phân hủy và gây ảnh hưởng đến sức khỏe con người và hệ sinh thái. Vi tảo từ lâu được biết đến vì có khả năng loại bỏ kim loại nặng trong nước thải đồng thời tích lũy lipid cho sản xuất nhiên liệu sinh học. Trong nghiên cứu này khảo sát các tác động của đồng (Cu) như ức chế tăng trưởng, ảnh hưởng lên hàm lượng lipid tích lũy, khả năng loại bỏ kim loại cũng như hàm lượng kim loại tích lũy trong nội bào lên vi tảo lục Pediastrum *sp. Vi tảo lục được phơi nhiễm với Cu ở các nồng độ 0, 0,1, 0,5, 2, 5 và 15 mg/L trong thời gian 1 tuần ở điều kiện phòng thí nghiệm. Kết quả cho thấy vi tảo lục* Pediastrum *sp. có khả năng chống chịu với Cu ở ngưỡng nồng độ nhỏ hơn 2 mg/L. Nồng độ gây ức chế sinh trưởng 50% sau 96h (96h-EC50) là 6,67 mg/L.* Pediastrum *sp. cho thấy có khả năng loại bỏ hiệu quả Cu ở ngưỡng nồng độ thấp hơn 2 mg/L với khả năng loại bỏ Cu lên đến 95%. Ở nồng độ Cu 0,1 và 0,5 mg/L cũng cho thấy gia tăng hàm lượng lipid 18% so với lô đối chứng, tuy nhiên khả năng loại bỏ kim loại Cu và hàm lượng lipid tích lũy giảm đi đáng kể khi Cu đồng ở ngưỡng 5 mg/L và 15 mg/L. Nghiên cứu này cho thấy có thể sử dụng vi tảo* Pediastrum *sp. để xử lý ô nhiễm Cu ở nồng độ thấp và thu hồi lipid cho sản xuất nhiên liệu sinh học.*

Keywords: wastewater treatment; biodiesel; heavy metal; bioremediation

1. Introduction

Human activities including agriculture and industrialization have resulted in metal discharges into aquatic ecosystems. Copper (Cu) is one of the most commonly used metal in the world. Subsequently, the level of Cu in aquatic environments has been increasing in recent decades. In a recent study, Hamed et al. (2017) [1] reported that the annual discharge of Cu into the ocean is 9×10^6 t per year. Although, Cu is an essential micronutrient required by photosynthetic species; however, it may be very toxic when present at high concentrations [2, 3]. Cu has caused in

decrease growth rate and reduction of pigment contents in microalgae; exposure to Cu has resulted in increased reactive oxygen species (ROS) in many autotroph organisms [1, 4, 5]. On the other hand, bioaccumulation of heavy metal in aquatic ecosystems and food chains can cause human health risk via trophic transfer. Therefore, many techniques such as reverse osmosis, electrophoresis, ultra-ion exchange, chemical precipitation, phytoremediation have been developed to remove heavy metals from contaminated water. However, all these methods have shown disadvantages such as high cost and energy requirements, or incomplete metal removal [6].

Microalgae have been considered a promising tool for their ability to remove various heavy metals from wastewater [6, 7], and their great potential in producing biodiesel [8]. Therefore, the coupling deals of advanced wastewater treatment and biofuel production based on microalgae is a promising solution. Many green algae such as *Scenedesmus* spp., *Chlorella* spp. have been employed as for removing heavy metals from wastewater and to produce biodiesel [9]. A combination of wastewater treatment and biofuel production is an environmentally friendly approach for stable development in the next century.

Previous studies have indicated that both living and non-living biomass of microgreen algae are effective removal heavy metals from wastewater [10-12]. Several algae species have been found to be very effective in adsorption heavy metals from aqueous solutions. The ability to remove copper from water by living and non-living biomass of the microgreen algae species has been reported [12, 13]. These authors suggested that biological treatment of heavy metal contaminated water based on *Scenesmus abundans* is possible and that is adequately at high algae concentrations. In addition, Ouyang et al. (2012) [13] reported that several green algae such as *Chlorella* spp. and *Scenedesmus* spp. were effective in removing zinc and copper from aqueous solutions, with the highest removal efficiency being near 100% [10, 12]. However, *Scenesesmus* sp. and *Chlorella* sp. are commonly used for the purpose. Other microgreen algae species including *Pediastrum* spp., *Ankistrodesmus* spp., *Staurastrum* spp. are commonly present in water ponds but little known about their ability to remove heavy metals from contaminated waters. There are many unexplored algae species with high ability to remove toxic metal in the natural environment. Therefore, in this study, the green algal Pediastrum sp. was isolated from the Tri An Reservoir, a drinking water supply reservoir near Ho Chi Minh city, and used to examine the effective removal of Cu ion and investigate lipids accumulation. The biosorption and bioaccumulation of Cu from aqueous solution were investigated.

2. Materials and methods

2.1 Alga isolation and cultivation

The microgreen algal species (*Pediastrum* sp.) (Figure 1) was collected from the Tri An reservoir, Dong Nai province, Vietnam. The species was isolated under a microscope and grown in 500 Erlenmeyer flasks containing COMBO medium. All cultures were grown under laboratory conditions at 28±1°C under a 12 h : 12 h period light-dark cycle with a light intensity of 50 μmol photons/m^2/s. The autoclaved medium was renewed every month to maintain the culture under laboratory conditions.
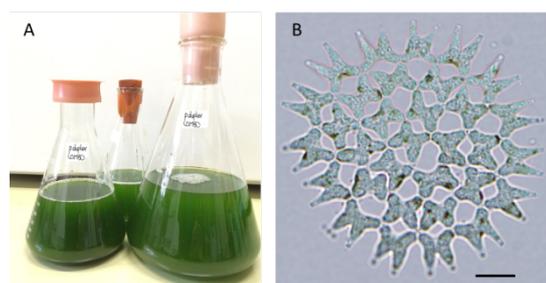

Figure 1. Stock culture (A), and morphology of *Pediastrum* sp. under a microscope (B). Scale bar: 20 μm.

2.2 Biosorption and growth inhibition experiment

The stock solution 1000 mg/L of copper Cu(NO$_3$)$_2$ (Titrisol, Merck, Germany) was used to prepared experimental solutions with concentration of 0, 0.1, 0.5, 2, 5 and 15 mg/L. The living cell of *Pediastrum* sp. was exposed to copper with design concentration in Erlenmeyer flasks (300 mL) containing 200 mL culture medium. The initial cell density of *Pediastrum* sp. was 5×10^4 cell/mL. About 5 mL of samples were taken every day for a period of 7 days. The density of cells was measured by using Rafter counting chamber under an Olympus light microscope at a magnification of 100X. After 7 days, algal biomass was collected on GF/C filters (Whatman, Kent, England), dried completely and stored at –20°C until analysis. 5 mL of filtrate was used for Cu analysis. Erlenmeyer flasks with Pediastrum sp. but without Cu were used as controls. All treatment was prepared in triplicate.

The Cu concentration that resulted in 50% inhibition of alga growth after 96h (EC50-96h) was determined as following equation:

$$\mu_i - j = \frac{\ln Cj - \ln Ci}{t_j - t_i}$$

where $\mu_i - j$ is the average specific growth rate from the time i to time j, t_i is the initial time of the exposure period, t_j is the final time of exposure, Ci is the concentration of cells at the time i and Cj is the concentration of cells at time j.
Percentage inhibition of growth was calculated as:

$$\%Ir = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

where %Ir is the percent inhibition in average specific growth rate; μ_C is the mean value for the average specific growth rate (μ) in the control group and μ_T is the average specific growth rate for the treatment replicate.

2.3 Measurement of total lipid content

For total lipid extraction and measurement, the method of Bligh and Dyer's method [14] was applied. Briefly, about 50 mg dry biomass (M1) was digested with 4 mL HCl 1 M at

80°C for 30 min in 50 mL centrifuge tube (M0), after centrifugation (4000 rpm, 15 min), the liquid supernatant was discarded. Lipid was then extracted with 3 mL methanol: chloroform (2:1 v/v). After 3 h, the chloroform layer was pipetted to a new 15 mL tube (M2), dried completely and the tube was then re-weighed (M3). Total lipid content (LC) was evaluated as follow: LC (%) = (M3−M2)/(M1−M0).

2.4 Heavy metal extraction and measurement

The content of Cu in algal biomass was extracted with 5 mL concentrated nitric acid (70%) for 12 h at 80°C. The samples were then centrifuged at 4000 rpm for 10 min under room temperature. The supernatant contained metals were preserved at –20°C until analysis. Cu content was measured by using an inductively coupled plasma optical emission spectrometer (ICP OES). (VISTA PRO, Varian, Mulgrave, Australia). Briefly, an ICP OES equipped with a solid state detector, a cyclonic spray chamber, and a concentric nebulizer was used for Cu detection. The ICP OES condition used as follows: RF power: 1.3 kW; gas: argon; plasma flow: 15 L/min; auxiliary flow: 1.5 L/min; nebulizer flow: 0.75 L/min; instrument stabilization delay: 15 s; pump rate: 15 rpm; sample uptake delay: 70 s; number of replicates: 3; read time: 5 s; read: peak height; rinse time: 30 s. The data are presented in mg/g DW or mg/L as appropriate. All samples were run in triplicate.

The removal rate Q(%) and the biosorption capacity q (mg/g) was calculated using the following formula:

$$Q = \frac{C_0 - C}{C_0} \times 100\%$$

$$q = \frac{C_0 - C}{M} \times V$$

where, C_0 and C are the initial and final concentrations of Cu(II) (mg/L), respectively. The V and M are the volume of solution (mL) and the mass of dry algal biomass (g), respectively.

2.5 Statistical analysis

One-way analysis of variance (ANOVA) was employed to find out the differences between exposure groups and control groups. When the ANOVAs were significant, pairwise comparison with the Tukey's honestly significant difference (HSD) Post-hoc test was applied to detect significant differences between the exposure concentrations and the control. Data were transformed by log(X+1) to normalize the distribution before analysis. Cu concentration was presented as the mean ± SD. The p-values less than 0.05 were considered statistically significant.

3. Results and discussions

3.1 Algal growth

Growth curve and growth inhibition of microalga *Pediastrum* sp. exposure to different Cu concentrations were shown in Figure 2a, b. *Pediastrum* sp. grew well in COMBO medium and reached maximal density after 6 days (Figure 2a). All treatment reached the stationary growth phase after 5 or 6 days. Cells density in the control treatments increased from 5×10^4 to 4×10^6 cells/mL after 6 days of culture. Results also indicated that different concentration of Cu resulted in different effects on algal growth. Cu at a low concentration from 0.1 to 0.5 did not cause a significant effect on algal growth, but at 2 mg/L or higher, Cu caused a significant decline in the cells concentration of Pediastrum sp.

The algal growth inhibition was increased and dose dependence of Cu. The EC50 values of Cu for inhibition of 50% of algal growth after 96 h was 6.67 mg/L. Cu at the concentration of 2 mg/L or higher caused significant effects and dose-dependent increases on the growth of *Pediastrum* sp. Cu at 15 mg/L inhibited completely the growth of *Pediastrum* sp. (Figure 2b).
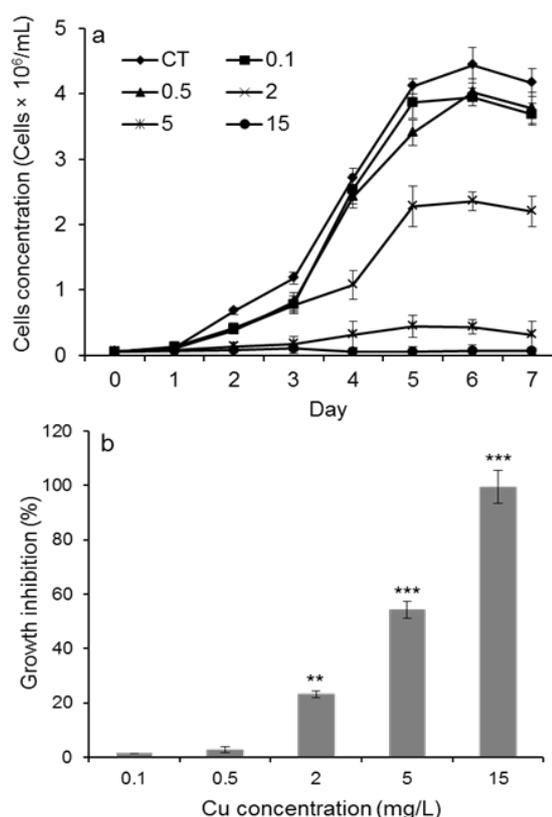
Figure 2. Growth curve (a) and growth inhibition (b) of *Pediastrum* **sp. exposure to different Cu concentrations. Anova test (**, p<0.01; ***, p<0.001).**

Previous studies have reported that Cu is an essential nutrient to nearly all species from animal to algae [15, 16]. However, high concentration of Cu in water environment

may result in toxicity to aquatic organisms [17]. In addition, Cu can generate various toxic effects on different aquatic species [18]. Levy et al. (2017) [19] reported that the effective concentrations of copper on the inhibition of the growth on the marine diatom Minutocellus polymorphous and the green algal *Dunaliella tertiolecta* at 96 h (96 h EC50) were 0.53, 0.68 mg/L, respectively. Some green microalgae such as *Pediastrum*, *Pseudokirchneriella*, and *Chlorella* could exhibit tolerance to heavy metals [20, 21]. And the toxicity of heavy metals to green algae may depend on algal species. Schamphelaere et al. (2014) [22] reported that the microgreen alga *Pseudokirchneriella subcapitata* was 4-fold more sensitive to Cu than *Chlorella kesslerii*.

Heavy metals such as Cu, Cd, Cr, Pb,...may alter the photosynthetic apparatus, decrease photosystem II energetic connectivity [23, 24]. In the present study, Cu at the concentration of 2 mg/L or higher caused significant inhibition on the growth of the microalgae *Pediastrum* sp. The results are in line with the previous observation that the low initial bioaccumulation of Cu by microgreen algae was found to be responsible for its tolerance to Cu [18]. However, the tested species exhibited a tolerance response to Cu with 96h-EC50 higher than other species such as *Dunaliella* sp., *Minutocellus* sp. and *Nannochloropsis* sp. [25].

3.2 Lipid accumulation

Total lipid content of *Pediastrum* sp. exposure to different Cu concentrations was showed in Figure 3. Results indicated that different concentration of Cu caused different effects on lipid accumulation. Cu at concentration of 0.1 and 0.5 mg/L led to a significant increase in total lipid production. However, Cu at the concentration of 2 mg/L did not influence lipid production. Further increasing of Cu (5 and 15 mg/L) caused decreased greatly in total lipid production in *Pediastrum* sp. (Figure 3). The total lipid level in *Pediastrum* sp. is little lower than the previous reports in other green algae such as the *Scenedesmus* sp. [26] and the *Monoraphidium* sp. [27].
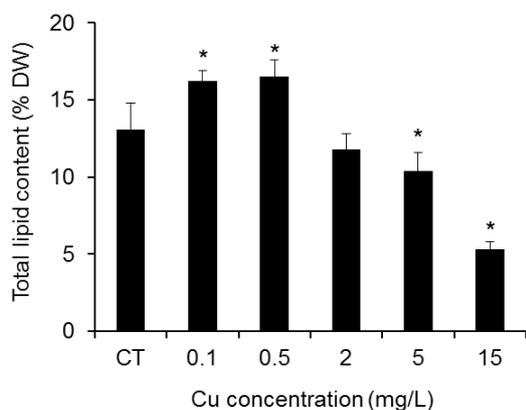

Figure 3. Total lipid content of *Pediastrum* sp. under different concentrations of Cu.

Previous studies have indicated that heavy metals like cadmium, copper, and zinc are known to increase the total

lipid content in the flagellate eukaryotes *Euglena gracilis* or in green alga *Chlorella* sp. [28]. Total lipid content of green algae *C. minutissima* significantly increased by 21% and 94%, respectively with the addition of low concentration of cadmium and copper [28], but an excessively high concentration of Pb^{2+} in the culture medium had an inhibitory effect on the growth and lipid production of *Scenedesmus* sp. Liu et al. (2008) [29] also reported the effect of iron on C. vulgaris and the total lipid content was raised up to 56.6%. The present study results are in line with previous observations that low concentrations of Cu were beneficial for biomass production and lipid accumulation but higher Cu concentration resulted in toxic to green algae [27].

3.3 Copper removal and bioaccumulation

The removal capacity and accumulation of Cu by *Pediastrum* sp. were showed in Figure 4. When Cu concentration was lower than 2 mg/L, more than 80% of Cu was removed. However, a significant decrease of Cu removal capacity was observed when exposed *Pediastrum* sp. to Cu at 5 and 15 mg/L (Figure 4a). Probably, the inhibition on the growth of *Pediastrum* sp. has resulted in a significant reduction of Cu removal capacity in these treatments. The level of Cu accumulated in the living cell of *Pediastrum* sp. was shown in Figure 4b. Higher initial metal concentration resulted in greater Cu accumulation. The lowest Cu concentration (0.07 mg/g DW) was observed in the treatment with 0.1 mg/L and the highest Cu concentration (5.2 mg/g DW) was recorded in the treatment with 15 mg/L (Figure 4b).
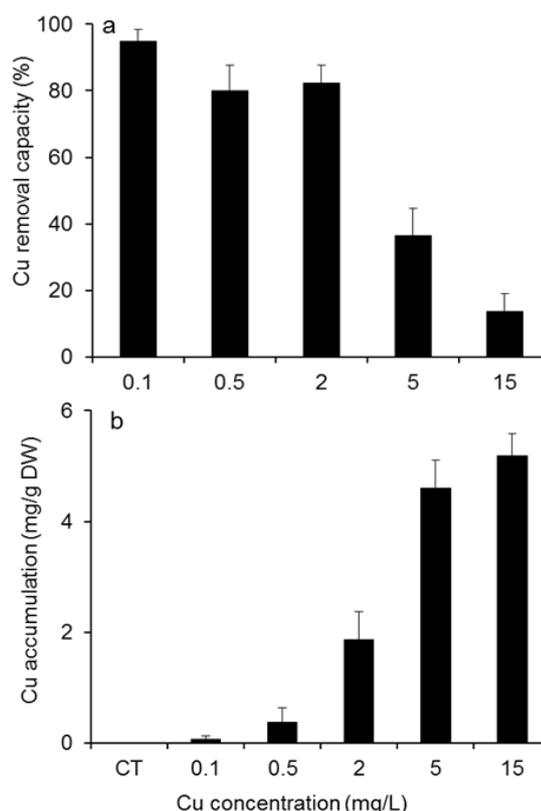

Figure 4. Cu removal capacity (a) and accumulation by *Pediastrum* sp. (b).

Previous studies have indicated that microgreen algae are potential in the removal of heavy metal from aqueous solution [18, 25]. Microgreen algae such as *Scenedesmus* and *Chlorella* are often used as a promising tool for heavy metal remediation [7]. The ability to remove heavy metal up to 90% from aqueous solution has been observed in *Scenedesmus* sp. and *Chlorella* sp. [7]. However, the removal rate of the metal ion in microalgae may depend on several variables such as initial concentration, exposure duration, and target species. Yan and Pan (2002) [18] demonstrated that the bioaccumulation of Cu by the microgreen algae *Closterium lunula* was directly proportional to the initial Cu concentration. In general, higher initial Cu concentration results in greater uptake ratio [7]. Microalgae uptake metal via the two main mechanisms: adsorption on to the cell surface, and (a slower) active uptake into the cytoplasm. However, Flouty and Estephane (2012) [30] found that synergistic and antagonistic effects between Cu and Pb were observed in binary metal systems which imply that the bioaccumulation process is much more dynamic. The present study indicated that the microgreen algae *Pediastrum* sp. has ability to uptake and remove Cu from aqueous solution at a concentration up to 2 mg/L. Higher Cu concentration caused an adverse effect on the cell growth and consequently decreasing the removal capacity. Further investigation is needed to better understand the uptake mechanism of Cu in microalgae.

4. Conclusions

This study indicated that living biomass of *Pediastrum* sp. exhibited the ability to biosorb and bioaccumulate Cu, and has the potential for lipid production. Both initial Cu concentration and algae biomass had influential effects on Cu uptake and removal as well as total lipid accumulation. At a suitable concentration of Cu in water may exhibit high removal efficiency and enhanced total lipid content in the *Pediastrum* sp. However, Cu at high concentration could cause toxic to the green algae and consequently decreasing the removal rate as well as reducing total lipid production. The present results demonstrated that microalgae such as *Pediastrum* sp. is a promising tool for heavy metal remediation as well as a biomaterial for biofuel production.

5. References

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