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Removal and bioaccumulation of copper by the freshwater green alga *Scenedesmus* sp.

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Abstract:

Human activities generate vast amounts of wastewater, which contains various toxic metals. Microalgae are able to remove heavy metals from wastewater and accumulate lipid to produce biodiesel. In this study, the abilities to remove copper (Cu) and accumulate lipid of the green algal species *Scenedesmus* sp. were examined. The microalga *Scenedesmus* sp. was exposed to Cu concentrations of 0, 0.5, 1, 2, 5, and 10 mg/l under laboratory conditions. The results indicated that Cu inhibited the growth of *Scenedesmus* sp. at a 96h-EC_{50} of 7.54 mg/l. Furthermore, the highest removal rate was 89.5%. Lipid accumulation was increased significantly to 23.6% with the addition of Cu at 5 mg/l. The present study indicated that the green alga *Scenedesmus* sp. possesses the ability to remove Cu from aqueous media and accumulate lipid in its cells. Our results suggested that this species could be applied in wastewater treatment technology and biodiesel production.

Keywords: **bioremediation, green algae, heavy metal, lipid accumulation, water treatment.**

Classification number: **3.4**

Introduction

Agricultural and industrial activities generate vast amounts of wastewater that is frequently discharged into bodies of water without prior treatment. Wastewater contains high concentrations of toxic heavy metals, which might be persistent in nature. The presence of heavy metal ions-even at low concentrations-can be toxic to aquatic organisms [1]. Copper (Cu) is one of the most common metals in the world in terms of usage. Cu pollution has large adverse effects on the environment because of its persistency and bioaccumulation potential in living organisms. By transforming and being transported through the food web, heavy metals may result in severe and toxic effects on human health and aquatic life. Acute copper poisoning may cause intravascular haemolytic anaemia, acute liver and renal failure, shock, coma, and death, whereas symptoms of mild Cu poisoning include vomiting, nausea, and diarrhoea [1]. Chronic toxicity of Cu mainly occurs in the liver because it is the first site of deposition after Cu enters the blood [2]. Toxic effects may include the development of liver cirrhosis with episodes of haemolysis and damage to renal tubules, the brain, and other organs. Symptoms can progress to coma, hepatic necrosis, vascular collapse, and death [2]. Therefore, the treatment of wastewater to remove Cu is critical.

Many physical and chemical methods have been developed to remove heavy metals from contaminated water, such as reverse osmosis, electrophoresis, ultra-ion exchange, chemical precipitation, and phytoremediation [3]. However, all have exhibited disadvantages, such as requiring large amounts of reagents, high costs and energy requirements, and incomplete metal removal [3]. By contrast, biological methods such as using microalgae and aquatic plants are the most commonly used for heavy metal removal in wastewater because of their comparatively low construction and maintenance costs. Aquatic plants

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and microorganisms can remove heavy metals from water through the processes of uptake and metabolism-dependent bioaccumulation. Many studies have been performed on metal uptake by microalgae, using both living and nonliving biomass [1, 3, 4]. This method is a promising tool for the treatment of aqueous solutions polluted with heavy metals. It is characterised by its low cost, high metal binding capacity, and high removal efficiency of metals [5-7]. Although dry algae biomass has been successfully utilised in heavy metal adsorption experiments, living cells may be more advantageous because of their metabolic uptake and continuous growth.

Several algal species have been proven to be effective at adsorbing heavy metals from aqueous solutions. The ability of the green alga *Scenedesmus abundans*, both living and nonliving to remove cadmium (Cd) and Cu from water has been reported [4, 8]. These studies have suggested that the biological treatment of heavy metal-contaminated water based on *S. abundans* is possible. Furthermore, both Cu and Cd were sufficiently removed at high algal concentrations. In addition, Ouyang, et al. [8] reported that several green algae such as *Chlorella* spp. and *Scenedesmus* spp. are effective at removing zinc (Zn) and Cu from aqueous solutions, with the highest removal efficiency being near 100%. Other microalgae, including cyanobacteria such as *Spirulina* and *Phormidium* and diatoms such as *Phaeodactylum*, *Nitzschia,* and *Skeletonema* have also been reported as potential solutions for the phytoremediation of heavy metals from contaminated water and soil. Many unexplored algal species with the great ability to remove toxic metals from natural environment remain to be explored.

In Vietnam, heavy metal pollution is becoming a critical problem in environmental management. Studies have shown that in estuarine aquaculture, agricultural soil and surface water are contaminated with heavy metals [9-11]. Heavy metals in aquatic environments may threaten human health through food-web transformation. In cyanobacteria, very high levels of removal efficiency (up to 92%) for Cu and lead (Pb) by *Spirulina platensis* was reported [12]. Furthermore, the use of local green algae for heavy metal removal was studied [13]. Lam Ngoc Tuan (2008) [13] isolated more than 30 strains of *Chlorella* from Vietnamese waters and examined them for Cd removal ability, and reported Cd removal ability up to 95% by several strains. However, information about the removal of Cu by the green alga *Scenedesmus* is scant. In addition, the accumulation of lipid in tested algae remains unknown. Copper contamination in water, soil, and agriculture crops are considered serious problems [9-11]. Furthermore, the removal of copper from contaminated sources in Vietnam remains a challenge. Thus, the present study aimed to isolate *Scenedesmus* strains and use them to examine the effective removal of Cu ions and accumulation of lipid. The biosorption and bioaccumulation of Cu from aqueous solutions were investigated under laboratory conditions.

Materials and methods

Alga isolation and cultivation

The freshwater green alga *Scenedesmus* sp. (Fig. 1) was isolated from the Nhieu Loc-Thi Nghe canal, a polluted waterway in Ho Chi Minh City, and maintained as a pure unialgal culture in COMBO medium under laboratory conditions. All cultures were grown on a 12-h light/dark cycle at a temperature of 28±1^{0}C under a light intensity of 50 μmol photons/m^{2}s provided by cool white fluorescent tubes.

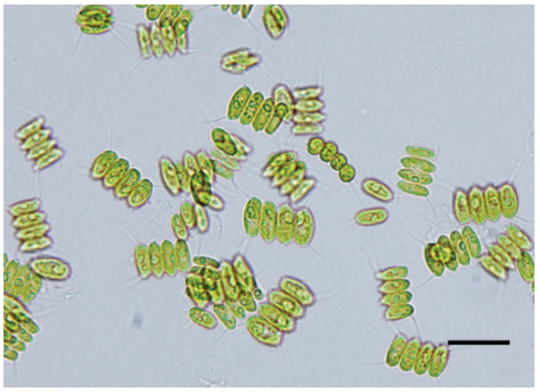

Fig. 1. Morphology of *Scenedesmus* sp. under a microscope. Scale bar: 20 μm.

Biosorption and bioaccumulation experiment

A stock solution of $Cu(NO_3)_2$ (Titrisol, Merck, Germany) with a concentration of 1,000 mg/l was diluted to concentrations of 0, 0.5, 1, 2, 5, and 10 mg/l, which were used in the biosorption and bioaccumulation experiments. Copper was spiked with design concentrations in Erlenmeyer flasks (500 ml) containing 300 ml of culture medium, and the living stock of *Scenedesmus* sp. was added to the initial concentration of 5×10^3 cell/ml. Samples were taken at 1 day intervals for a period of 7 days. Algal density was estimated directly using a Speirs-Levy eosinophil counting slide under an Olympus light microscope. Algal biomass was harvested at the end of the experiment by filtering onto GF/C glass fibre filters (Whatman, Kent, United Kingdom), dried at 80^{0}C overnight, and maintained at -20^{0}C for further processing. Erlenmeyer flasks with *Scenedesmus* sp. but without Cu were used as controls. All treatments were prepared in triplicate.

Growth inhibition test

The concentration of Cu that inhibited algal growth rate by 50% over 96 h (EC_{50}-96h) was determined based on the relative inhibition of growth rate as a function of $Cu(NO_3)_2$ concentration (mg/l). The average of the specific growth rate (ASGR) was obtained as the biomass increase after 96h using the following equation:

$$ASGR = \frac{\ln C_j - \ln C_i}{t_j - t_i}$$

where ASGR is the average specific growth rate from time *i* to time *j*; t_i is the initial time of the exposure period; t_j is the final time of exposure; C_i is the algal biomass at time *i*; and C_j is the algal biomass 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.

Total lipid analysis

The total lipid accumulated in the algal biomass was extracted according to the Bligh and Dyer method [14] and analysed using gravimetric quantification methods. In brief, a 50-ml centrifuge tube was washed and weighed after drying (W0), and approximately 50 mg dry weight (DW) of alga biomass (W1) was digested with 5 ml HCl 1 M at 80^0C for 30 min. The liquid supernatant was discarded after centrifugation. Lipid was then extracted with 5 ml of methanol:chloroform (2:1 v/v). After 3h, the chloroform layer was transferred to a culture dish that had been preweighed (W2). The dish was then dried completely and reweighed (W3). Lipid content (LC) was calculated according to the following formula:

LC (%) = (W3−W2)/(W1−W0)

Heavy metal analysis

The bioaccumulation of Cu in the dry biomass of *Scenedesmus* was homogenised in 5 ml of concentrated nitric acid (70%). After sonication for 3 min, the samples were completely digested for 12h at 80°C. All samples were then centrifuged at 4,000 rpm for 10 min at room temperature to remove cell debris. The supernatant was collected and maintained at 4°C prior to analysis. Cu content was detected using an inductively coupled plasma optical emission spectrometer (ICP OES). The ICP OES system with an axially viewed configuration (VISTA PRO; Varian, Mulgrave, Australia) equipped with a solid state detector, a cyclonic spray chamber, and a concentric nebuliser was used for metal detection. The ICP OES conditions were as follows: RF power: 1.3 kW; gas: argon; plasma flow: 15 l/min; auxiliary flow: 1.5 l/min; nebuliser flow: 0.75 l/min; instrument stabilisation delay: 15s; pump rate: 15 rpm; sample uptake delay: 70s; number of replicates: 3; read time: 5s; read: peak height; and rinse time: 30s. The data were presented in µg/g DW and all analyses were performed in triplicate.

Finally, the removal rate *Q* (%) and the adsorption capacity *q* (mg/g) were 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). The *V* and *M* are the volume of solution (ml) and the mass of dry alga (g), respectively.

Statistical analyses

All data were presented as the mean ± standard deviation. The differences between exposure groups and control groups were tested for significance using a one-way analysis of variance (ANOVA). When the ANOVA was significant, pairwise comparison was applied using Tukey's HSD post-hoc test to detect significant differences between the treatment and control groups; *p*-values less than 0.05 were considered statistically significant.

Results

Algal growth under Cu exposure

The results showed that *Scenedesmus* sp. grew well in the controls and reached a maximal concentration after 6 or 7 days of incubation (Fig. 2A). All treatments reached the stationary growth phase at approximately the same time (after 6 days). Cell density in the control (CT) treatment increased from 5×10^3 to 3.2×10^6 after 1 week of culture. Cu resulted in differences in the algal growth. Cu at low concentrations (up to 2 mg/l) did not influence the growth of *Scenedesmus* sp., but at 5 mg/l or higher Cu caused a significant decrease in its growth. In addition, a further increase of Cu^{2+} concentration up to 10 mg/l or higher resulted in a sharp decrease in biomass concentration (Fig. 2A).

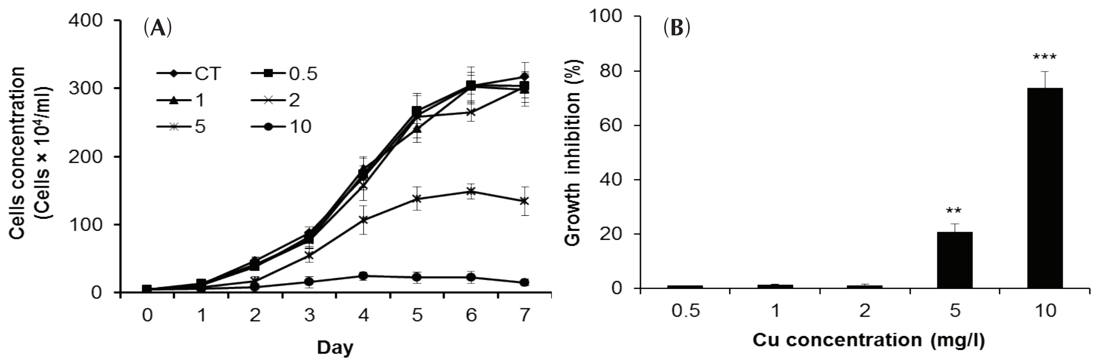

Fig. 2. Growth curves (A) and growth inhibition (B) of *Scenedesmus* sp. exposed to different Cu concentrations. Asterisks indicate significant differences. ANOVA test (*, p<0.05; **, p<0.01; ***, p<0.001). CT: control treatment.

The 96h-EC_{50} value of Cu for the growth inhibition of *Scenedesmus* sp. was 7.54 mg/l. Growth inhibition increased as Cu concentration increased to 1 mg/l or higher. Cu caused significant effects and dose-dependent increases on the growth of *Scenedesmus* sp. Significant differences from the control growth rates were detected at a concentration of 1 mg/l or higher in *Scenedesmus* sp. Cu at a concentration of 10 mg/l almost completely inhibited the growth of *Scenedesmus* sp. (Fig. 2B).

Total LC

Cu metal ion had a small positive influence on the total lipid production in *Scenedesmus* sp. Cu at 1 mg/l did not influence lipid production, but a further increase to 2 mg/l led to a significant increase in total lipid production (Fig. 3). Total LC ranged from 15.1 to 19.4%. The maximum total LC of 19.4% was obtained at a Cu concentration of 5 mg/l. Different concentrations of Cu increased total lipid production by 20.3-23.9% in *Scenedesmus* sp.

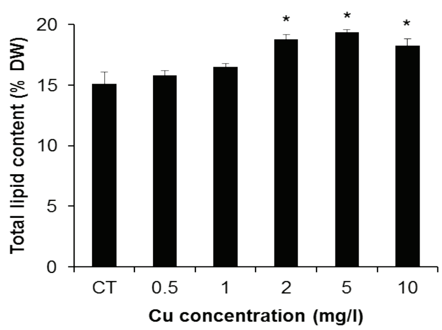

Fig. 3. Total LC of *Scenedesmus* sp. under exposure to different Cu concentrations. CT: control treatment; DW: dry weight.

Removal efficiency and Cu accumulation

The Cu removal efficiency and bioaccumulation of Cu in *Scenedesmus* sp. were investigated at different initial metal concentrations for 7 days (Fig. 4). Results showed that the metal removal rate became higher as metal concentrations increased to 2 mg/l. A further increase in Cu concentration did not result in higher removal rates. The highest removal rate of Cu was 89.5% at 2 mg/l. The highest concentration of Cu (10 mg/l) resulted in a reduction in Cu removal capacity (Fig. 4A).

Figure 4B shows the accumulation of Cu by *Scenedesmus* sp. The Cu concentration in *Scenedesmus* sp. ranged from 0.2 to 4.59 mg/g DW. Furthermore, the accumulation of Cu was dose-dependent. Higher initial Cu concentrations resulted in a larger amount of Cu being accumulated in dry *Scenedesmus* sp. biomass. The accumulation was 0.2 mg/g DW (40%) and 5.59 mg/g DW for 0.5 mg/l and 10 mg/l of Cu, respectively.

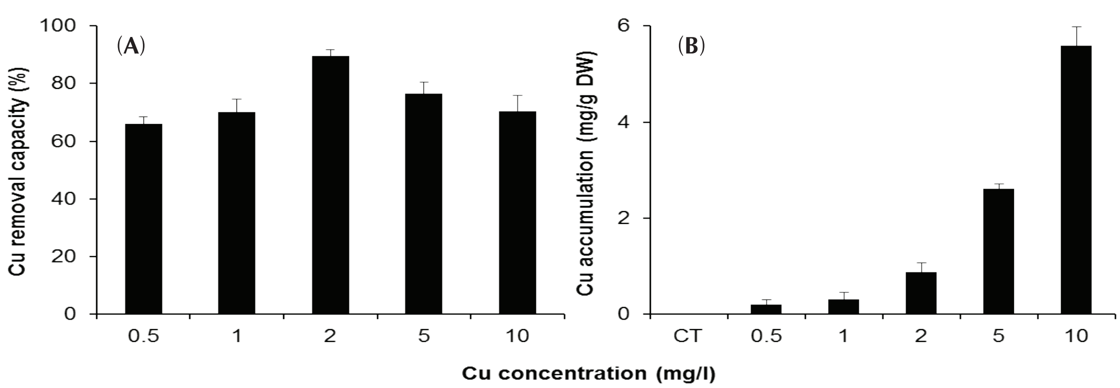

Fig. 4. Removal rate (A) and bioaccumulation of Cu by *Scenedesmus* sp. (B). CT: control treatment.

Discussion

In this study, experiments were performed to characterise the adverse effects and biosorption of Cu from water using the freshwater green algae *Scenedesmus* sp.. Cell density was monitored to determine the effect of Cu on algal growth. Studies have demonstrated that Cu is necessary for algal growth or respiration [15, 16]; however, excessive concentrations have caused adverse effects on the growth of green algae [15, 16]. In addition, Schamphelaere, et al. [17] found that the toxicity of heavy metals toward green algae may depend on the algal species and exposure time. When the green alga *S. abundans* was exposed to different concentrations of Cu, Terry and Stone [18] reported that its growth was inhibited at Cu concentrations up to 15 mg/l. Photosystems of algae can be damaged by excessive amounts of heavy metals, resulting in a reduction in photosynthetic pigments such as chlorophyll-*a*. In addition, high Cd concentrations reduced cells' size and caused a decrease in growth rate [18]. The results of the present study are in agreement with the observations of Ouyang, et al. [8], who reported that some heavy metals, including Cu, Cr, Zn, Cd, and Pb, significantly inhibited the growth of green algae. The effects of these five metals on the growth of green algae were dependent on both concentration and exposure time. The results of the present study indicated that the green algae *Scenedesmus* sp. is sensitive to Cu; hence, contaminated water entering a treatment pond would have to be diluted to maintain algal growth.

In addition, studies have demonstrated that lipid production from algae increased significantly under heavy metal stress conditions. The lipid productivity of the green alga *Scenedesmus* sp. increased in the presence of iron, magnesium, and calcium with the addition of EDTA during cultivation [19, 20]. Che, et al. [21] reported that the effect of iron on the green alga *Monoraphidium* sp. and the biomass and lipid productivity of microalgae exhibited an increasing tendency with the concentration of iron ions being augmented. An appropriate concentration of iron ions in an aqueous solution might result in benefits for biomass production and lipid accumulation [21]. Liu, et al. [22] also reported that the total LC in *C. vulgaris* increased by 3-7-fold when the alga was reinoculated into new media supplemented with an iron concentration of 1.2×10^{-5} mol/l $FeCl_3$. Heavy metals such as Cu and Zn are known to increase the total LC of the flagellate eukaryote *Euglena gracilis* and green alga *Chlorella* sp. [23]. The total LC of the microalga *C. minutissima* significantly increased by 21% and 94% with the addition of Cd and Cu, respectively [23]. In the present study, the lipid production of *Scenedesmus* sp. was enhanced under Cu exposure. Algae use lipid production as a means of energy storage when their growth is depressed by environmental stresses, such as the presence of heavy metals. Under stress conditions, the photosynthetically fixed carbon supply possibly exceeds the ability of the cells to multiply, causing the build-up of carbon in storage molecules [24]. The mechanism formation, pathways, and composition of different lipid types within algae have been well-documented [25, 26]. The main reason for increased lipid production under stress conditions in green algae is the production of major chloroplast fatty acid in cells, which are favourable for triacylglycerol (TAG) production, and thus, appear to be advantageous for higher neutral lipid production [27].

Algae have the ability to remove heavy metals from aqueous solutions; however, the diverse results in terms of toxicity and metal removal ability reported in the literature have indicated that various forms of aquatic organisms possess different responses to heavy metal exposure [1, 6, 18, 28]. Therefore, it is necessary to characterise the effects of metal concentrations on each species considered. Many species of green algae (e.g., *Chlorella* spp. and *Scenedesmus* spp.) have been investigated for their for heavy metal and nutrient removal as well as lipid production in wastewater [29-32]. Moreover, studies have shown that *Scenedesmus* spp. and *Chlorella* spp. possess the ability to remove Pb up to 89% from aqueous solution [6, 33]. Terry and Stone [18] reported that living *S. abundans* had the ability to remove Cu up to 99% from aqueous solution. Chen, et al. [33] invoked a feedback mechanism involving multiple transporters in the presence of hardness cations or other metal ions such as Cu and Ni to explain the increasing Pb bioaccumulation they observed in the green alga *C. reinhardtii*. In the present study, the isolated *Scenedesmus* sp. removed up to 89% of Cu from the solution. However, excessively high concentrations of Cu in water may inhibit the growth of algae. Inhibition of the growth of *Scenedesmus* sp. resulted in a reduction in Cu removal capacity at high concentrations. The results of the present study were in line with relevant studies that have reported *Scenedesmus* sp. as being able to remove and accumulate Cu to some extent depending on the concentration of the metal and duration of contact between the phytoplankton and metal [18, 34]. Further studies are required to better understand the removal and bioaccumulation mechanisms of Cu in tropical microalgae.

Conclusions

The present study indicated that the microalgae *Scenedesmus* sp. exhibited Cu biosorption and bioaccumulation abilities. High concentrations of Cu caused growth inhibition of the green algae. The removal efficiency and accumulation of Cu were most dependent on the initial metal concentrations. The total lipid production in *Scenedesmus* sp. was enhanced under exposure to Cu in concentration range of 2-10 mg/l. The results indicated the potential of *Scenedesmus* sp. in wastewater treatment and biofuel production.

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The author declares that there is no conflict of interest regarding the publication of this article.

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