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Original Research

Lipid Production Combined with Removal and Bioaccumulation of Pb by *Scenedesmus* sp. Green Alga

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Abstract

Microgreen algae have high potential to remove heavy metals from wastewater and to produce biodiesel. In this study, the green algal species *Scenesesmus* sp. was used to examine the metal uptake and lipids accumulation. The microalga *Scenedesmus* sp. was grown under continuous regime in the presence of lead (Pb) at concentrations of 0.05, 0.5, 1, 2 and 10 mg/L in a laboratory scale system. All treatments were conducted using autoclaved aqueous solution. Results indicated that Pb inhibited the algal growth with 96h-EC_{50} of 4.76 mg/L. However, the green alga *Scenedesmus* sp. could efficiently remove Pb at low concentrations. Lipid accumulation was significantly increased by up to 31% with the addition of Pb at up to 1 mg/L. In contrast, lower heavy metal removal efficiencies and decreasing lipid accumulation were observed in the treatment with the highest concentrations of Pb (10 mg/L).

Results demonstrate that Pb concentrations at above 2 mg/L inhibits the algal growth and subsequently reduces lipid content accumulation in the cell. The present study indicates that the green alga *Scenedesmus* sp. has the ability to remove Pb from aqueous media and accumulate lipid content, which could be applied in wastewater treatment technology and biodiesel production

Keywords: heavy metal, bioremediation, lead contamination, lipid accumulation.

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Introduction

Agriculture and industrial activities has greatly increased the input of heavy metal into bodies of water [1]. Even at low concentrations heavy metal ions such as lead, copper and cadmium can be toxic to humans. Lead (Pb) is one of the most commonly used metals in the world. It is highly toxic and causes intellectual and behavioral deficits, impaired hand-eye coordination, and lowered performance in intelligence tests in children.

Recent studies have reported other effects of lead exposure, such as hypertension, cardiovascular problems and renal disease. Chronic exposure to Lead in adults has led to decreased fertility, cataracts, nerve disorders, muscle and joint pain, and memory or concentration problems [2]. Bioaccumulation of heavy metal in aquatic systems and food chains can transfer to higher trophic levels and cause major health problems. Many techniques such as reverse osmosis, electrophoresis, ultra-ion exchange, chemical precipitation, and phytoremediation have been developed to remove heavy metals from contaminated water. However, all these methods have shown disadvantages such as incomplete metal removal, cost and high energy requirements [3].

In addition, the energy crisis has created pressing issues of the 21st century [4]. Fossil fuel reserves have been predicted to be depleted within 60 years [5]. There is requirement for innovative methods of wastewater treatment and exploitation of novel energy forms. Microalgae have attracted considerable attention due to their ability to remove various heavy metals from wastewater [1, 6] and their great potential in producing biodiesel [7]. Therefore, the coupling deals of advanced wastewater treatment and biofuel production based on microalgae is a promising solution [4]. The green alga *Scenedesmus* sp. has proved to be one of the most promising tools to both remove inorganic nutrients from wastewater and to produce biodiesel [8].

Previous studies have been performed on metal removal by microalgae – both living and nonliving [3, 9]. Several species have been found to be very effective in adsorbing heavy metals from aqueous solutions. The ability to remove cadmium and copper from water by living and nonliving green alga *Scenedesmus abundans* has been reported by Terry and Stone (2002) [9]. These authors suggest that adequate biological treatment of heavy metal-contaminated water based on *S. abundans* is possible at high algae concentrations. In addition, Zhou et al. (2012) [10] report 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%. Other microalgae including cyanobacteria such as *Spirulina* and *Phormidium,* diatom such as *Phaeodactylum*, *Nitzschia* and *Skeletonema* have also reported to be potentially effective for phytoremediation of heavy metals from contaminated water and soil [11]. Therefore, there are likely to be many uninvestigated algae species with high ability to remove toxic metal from the natural environment.

In Vietnam, there is severe lead contamination in air, water, soil and crops, with the highest levels of more than 1,000 times that deemed safe being found in the surface [12]. However, removing lead from contaminated sources remains challenging. In this study, the green alga *Scenesesmus* was isolated from the Nhieu Loc-Thi Nghe Canal, a polluted waterway in Ho Chi Minh City. It was then used to study lipid accumulation and the biosorption and bioaccumulation of Pb from an aqueous solution.

Experimental

Alga Isolation and Cultivation

The green alga species *Scenedesmus* sp. (Fig. 1) was morphologically identified under a light microscope (Olympus, Tokyo, Japan). The alga was isolated from the Nhieu Loc-Thi Nghe Canal in Ho Chi Minh City and maintained as pure unialgal in COMBO medium [13]. Erlenmeyer flasks (500 mL) containing 300 mL medium were used as reactors for cultivation. All cultures were grown on a 12 h:12 h light:dark cycle at 28±1ºC under light intensity of 50 μmol photons/m^2s provided by cool white fluorescent tubes.

Biosorption and Bioaccumulation Experiment

Stock solutions 1000 mg/L of lead $Pb(NO_3)_2$ (Titrisol, Merck, Germany) were diluted to concentrations of 0, 0.05, 0.5, 1, 2 and 10 mg/L, which were used in the biosorption and bioaccumulation experiments. Lead was spiked with required concentrations in Erlenmeyer flasks (500 mL) containing 300 mL culture medium, and living stock of *Scenedemus* sp. was added to the initial

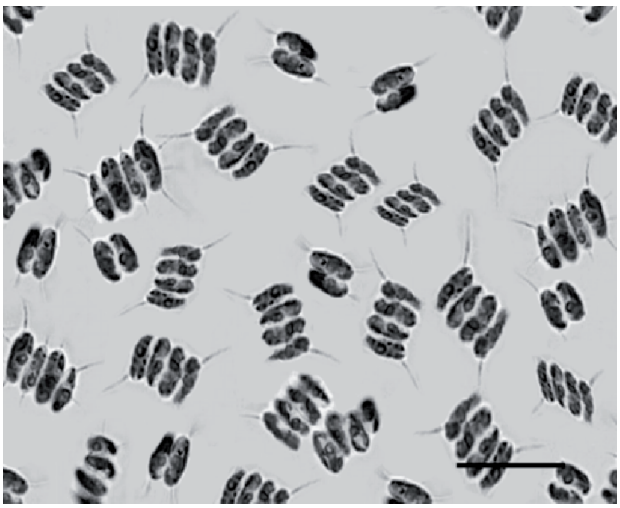

Fig. 1. Morphology of Scenedemsus sp. under microscope; scale bar: 20 μm.

concentration of 5 × 10^3 cell/mL. Samples were taken at one-day intervals for a period of 7 days. Cell density was estimated directly with a Speirs-Levy Eosinophil counting slide under an Olympus light microscope. At the end of the experiment cells were harvested by filtering onto GF/C glass fibre filters (Whatman, Kent, England), dried at 80ºC overnight and kept at -20ºC before analysis. Erlenmeyer flasks with *Scenedesmus* sp. but without Pb were used as controls. All treatment was prepared in triplicate.

The Pb concentration that inhibits algal growth rate by 50% over 96 h (EC_{50}-96 h) was determined based on the relative inhibition of growth rate as a function of the AgNP concentration (mg/L). The average of the specific growth rate for each period was obtained as the biomass increase after 96 h, by the following equation:

$$\mu_i - j = \frac{\ln Cj - \ln Ci}{t_j - t_i} \quad (1)$$

…where μ_{i-j} 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 concentration of cells at time *i* and C_j is the concentration of cells at time *j*.

Percentage inhibition of growth was calculated as:

$$\%Ir = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad (2)$$

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

Total Lipid Fraction Extraction and Measurement

The total lipid fraction in the algal biomass was extracted according to Bligh and Dyer's method [14] and analyzed using gravimetric quantification methods according to the procedure of Han et al. (2011) [15]. Briefly, a 50-mL centrifuge tube (M_0) was washed and weighed after drying, 30 mg dry weight (DW) of alga biomass (M_1) was digested with 3 mL HCl 1 M at 80ºC for 30 min, and liquid supernatant was discarded after centrifugation. The lipid was then extracted with 3 mL methanol: chloroform (2:1 v/v). After 3 h, the chloroform layer was transferred to a culture dish that had been pre-weighed (M_2). The dish was then dried completely and re-weighed (M_3). Lipid content (LC) was calculated according to the following formula:

$$LC\ (\%) = \frac{M_3 - M_2}{M_1 - M_0} \quad (3)$$

Heavy Metal Extraction and Measurement

Lead content in the algal biomass was homogenized in 5 mL of concentrated nitric acid (70%). After sonication for 3 min, the samples were completely digested for 12 h at 80ºC. The digested samples were then centrifuged at 4000 rpm for 10 min under room temperature. The supernatant contained metals were kept at -20ºC period to analysis. Lead concentration was measured according to the method of dos Santos et al. (2014) [16] with minor modifications. Briefly, an inductively coupled plasma optical emission spectrometer (ICP OES) with axially viewed configuration (VISTA PRO, Varian, Mulgrave, Australia) equipped with a solid state detector, a cyclonic spray chamber, and a concentric nebulizer was used for lead detection. The ICP OES condition used the following: 1.3 kW RF power; argon gas; 15 L/min plasma flow; 1.5 L/min auxiliary flow; 0.75 L/min nebulizer flow; 15 s instrument stabilization delay; 15 rpm pump rate; 70 s sample uptake delay; 3replicates; 5 s read time; peak height read; and 30 s rinse time. The data are presented in μg/g DW. All analyses were performed in triplicate.

Finally, removal rate *Q* (%) and adsorption capacity *q* (mg/g) were calculated using the following formula:

$$Q = \frac{(C0-C)}{C0} \times 100\% \quad (4)$$

$$q = \frac{(C0-C)}{M} \times V \quad (5)$$

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

Statistical Analyses

The concentrations of heavy metal in tested treatments were presented as the mean ±SD. The differences between exposure and control groups were tested for significance using one-way analysis of variance (ANOVA). When the ANOVAs were significant, pairwise comparison with the Tukey's honestly significant difference. A post-hoc test was then applied to detect significant differences between the exposure concentrations and the control. The *p*-values less than 0.05 were considered to be statistically significant.

Results and Discussion

Algae Growth

The results showed that *Scenedesmus* sp. grew well in COMBO medium and reached maximal concentration after seven days of incubation. The corresponding biomass growth pattern of *Scenedesmus* sp. is shown in

Fig. 2a). All tests reached the stationary growth phase at about the same time (seven days). Cell density in the CT treatment increased from 5×10^3 to 4×10^6 after one-week culture. Pb induced differently on algal growth. Pb at low concentration from 0.05 to 0.5 mg/L did not induce the growth of *Scenedesmus* sp., but at the concentration of 1 mg/L or higher Pb, it caused a significant decrease on the cell concentration of *Scenedesmus* sp. When the Pb^{2+} concentration increased from 0 to 0.5 mg/L, the biomass is constant (Fig. 2a). However, a further increase of Pb^{2+} concentration to 10 mg/L resulted in a sharp decrease of biomass concentration.

Growth inhibition increased as the Pb concentration rose to 1 mg/L or higher. The EC_{50} values of lead for the growth inhibition of *Scenedesmus* sp. (after 1 week) were 4.76 mg/L. Pb and caused significant effects and dose-dependent increases on the growth of *Scenedesmus* sp. Significant differences from the control growth rates were detected at the concentration of 1 mg/L or higher in *Scenedesmus* sp. Lead at the concentration of 10 mg/L inhibited completely the growth of *Scenedesmus* sp. (Fig. 2b).

Previous studies have demonstrated that Pb is not necessary for algae growth or respiration. Pb is easily accumulated by green algae and thus becomes a toxic influence [17, 18]. When the green alga *Stichococcus bacillaris* is exposed to different concentrations of Pb, Pawlik-Skowrońska (2002) [17] reported that the growth of *S. bacillaris was* inhibited at a Pb concentration of 25 µmol/L. In addition, De Schamphelaere et al. (2014) [19] found that the toxicity of Pb to green alga was dependent on algal species, and *Pseudokirchneriella subcapitata* was 4 times more sensitive to Pb than *Chlorella kesslerii*, with *Chlamydomonas reinhardtii* in-between. The results of this study agree well with previous observations by Ouyang et al. (2012) [18], 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 both on concentration and exposure time.

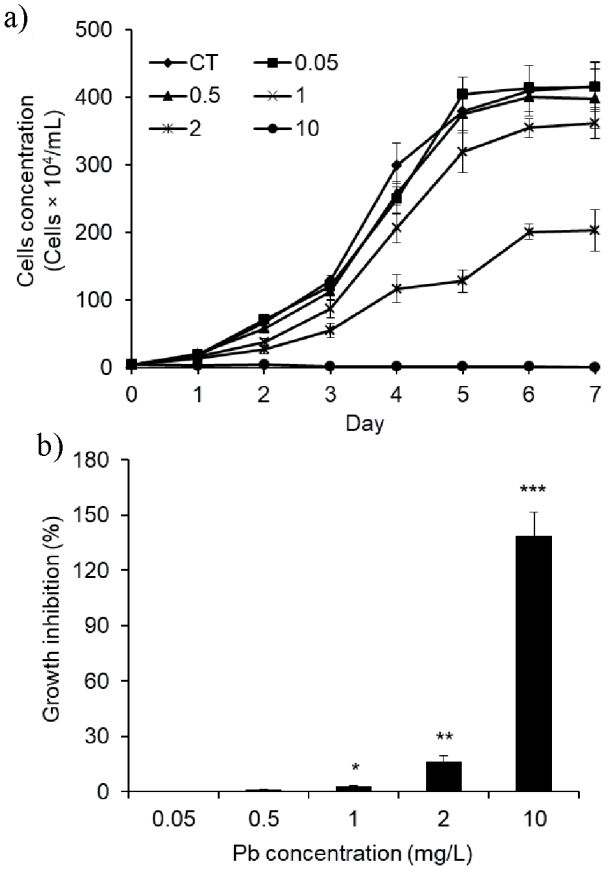

Fig. 2. Growth pattern a) and growth inhibition b) of *Scenedesmus* sp. exposure to different Pb concentrations; asterisks indicate significant differences; Anova test (*, $P< 0.05$; **, $P<0.01$; ***, $P< 0.001$).

Lipid Accumulation

Pb metal ion had great influence on algal growth and lipid production of *Scenedesmus* sp. Pb at the concentration of 0.05 mg/L did not influence lipid production. A further increase of Pb (0.5 and 1 mg/L) led to a significant increase in total lipid production. However, Pb at the concentration of 10 mg/L drastic decreased in total lipid production (Fig. 3). The maximum total lipid content of 31.1% and 30.8% were both obtained at Pb concentrations of 0.5 and 1 mg/L, respectively. The total lipid content of the tested alga species in this study are comparable with the previous reported in other green algae such as *Scenedesmus* sp. [20] and *Monoraphidium* sp. [21].

Previous studies have demonstrated that lipid production from algae has increased significantly under heavy metal stress conditions. The total lipid content and lipid productivity of the green alga *Scenedesmus* sp. increased 28% and 30%, respectively, in the presence of iron, magnesium and calcium with the addition of EDTA during cultivation [20]. Che et al. (2015) [21] reported

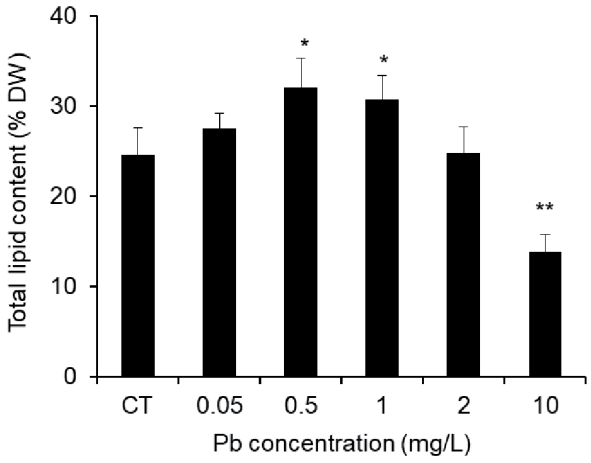

Fig. 3. Total lipid content of *Scenedesmus* sp. exposure to different Pb concentrations.

that the effect of iron on green alga *Monoraphidium* sp. FXY-10, and the biomass and lipid productivity of microalgae exhibited an increasing tendency with the concentrations of iron ion augmenting. Liu et al. (2008) [22] also reported the effect of iron on *C. vulgaris* and the total lipid content was raised up to 56.6%. Heavy metals like cadmium, copper and zinc are known to increase the total lipid content of the flagellate eukaryotes *Euglena gracilis* or in green alga *Chlorella* sp. [23, 24]. Yang et al. (2015) [23] found that the total lipid content of microalgae *C. minutissima* significantly increased by 21% and 94%, respectively, with the addition of cadmium and copper. In this work, appropriate Pb^{2+} concentrations have enhanced lipid production, but an excessively high Pb^{2+} in the culture medium had an inhibitory effect on the growth and lipid production of *Scenedesmus* sp. These results are in line with previous observations that appropriate concentrations of metal ion were beneficial for biomass production and lipid accumulation, but higher concentrations may be toxic to green algae [21].

Heavy Metal Removal and Accumulation

The Pb removal capacity and intracellular accumulation of Pb in living *Scenedesmus* sp. were investigated at different initial metal concentrations for a period of 7 days (Fig. 4). Results showed that metal removal rate was higher at higher initial metal concentrations up to 2 mg/L. *Scenedesmus* sp. attained a Pb removal rate of 70% at the lowest initial metal concentration tested (0.05 mg/L); the maximum removal rates (83.5-84.2%) were observed in the treatment with 1 and 2 mg/L of Pb. When exposed to the highest concentration (10 mg/L), a significant decrease of Pb removal capacity was observed (Fig. 4a). It is probable that the inhibition on growth of *Scenedesmus* sp. has resulted in a significant reduction of removal capacity of Pb in the treatment with 10 mg/L.

The accumulation of Pb in living cells of *Scenedesmus* sp. after seven days' exposure to different concentrations of Pb is shown in Fig. 4b). Results showed that intracellular Pb concentration had a positive correlation with the initial metal concentration. The lowest Pb concentration (0.93 mg/g DW) was observed in the treatment with 0.05 mg/L and the highest Pb concentration (13.6 mg/g DW) was recorded in the treatment with 10 mg/L.

A previous study has shown that *Scenedesmus* spp. and *Chlorella* spp. have the ability to remove Pb up to 89% from aqueous solution [25]. However, the removal rate of metal ions by microalgae depend on variables such as initial concentration, exposure duration and target species. Algae uptake metals both passively and actively; some metals such as Pb and strontium (Sr) may be passively adsorbed by charged polysaccharides in cell wall and intracellular matrix, whereas others (e.g., Zn, Cd) are taken up actively against a large intracellular concentration of gradients [26]. Chen et al. (2010) [27] invoked a feedback mechanism involving multiple transporters as the presence of hardness cation or other metal ions such as copper (Cu) and Ni to explain their observations of increasing Pb bioaccumulation in the green alga *C. reinhardtii*. In general, algae uptake metal via the two main mechanisms: adsorption on to the cell surface, and (a slower) active uptake into the cytoplasm [28]. However, Flouty and Estephane (2012) [29] found that synergistic and antagonistic effects between Cu and Pb were observed in binary metal systems, which implies that the bioaccumulation process is much more dynamic. Pb^{2+} uptake in green alga is thought to occur via Ca^{2+} pathway. Since Pb can mimic Ca, a reasonable explanation for the increased Pb^{2+} uptake might be that Pb^{2+} could block the Ca^{2+}-dependent Pb^{2+} efflux system [30]. The present study indicated that *Scenedesmus* sp. could uptake and remove Pb efficiently from aqueous solution at low concentrations. Higher Pb concentrations caused an adverse effect on cell growth and consequently decreased the removal capacity. The results of the present study were in line with previous reports that *Scenedesmus* sp. is able to accumulate metals to some extend depending on the concentration of the metal and on the time of contact of algae with the metal [28]. Further studies are needed to better

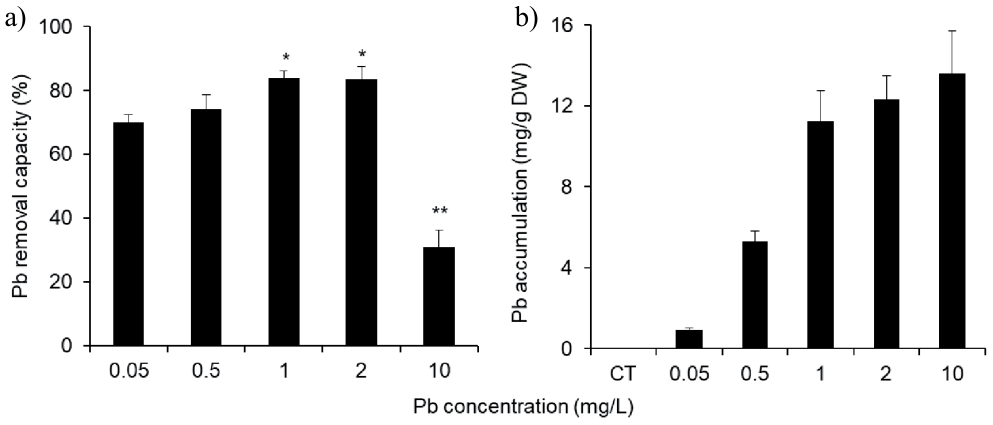

Fig. 4. Pb removal capacity a) and accumulation by *Scenedesmus* sp. b).

understand the bioaccumulation of mechanisms of Pb in microalgae.

Conclusions

The present study indicated that living biomass of *Scenedesmus* sp. exhibited the ability to biosorb and bioaccumulate Pb, and has the potential for lipid production. Initial metal ion and biomass concentrations had an influential effect on Pb uptake and removal. Pb at high concentration could prove to be toxic to the green algae and consequently decrease removal rate as well as reduce total lipid production. However, at a suitable concentration of Pb in water, *Scenedesmus* sp. exhibited a high removal efficiency and enhanced total lipid content. These results are very promising for a potential application of these microorganisms as an efficient and economic biomaterial for wastewater treatment and biofuel production based on tropical microalgae.

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Conflict of Interest

The authors declare no conflict of interest.

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