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Response of *Chlorococcum* sp. AZHB to Copper and Cadmius Stress

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Rapid development of industrial economies has been accompanied by the worsening of the quality of rivers and waterways in the world. The discharge of untreated industrial wastewater has added significantly the concentrations of heavy metals, such as mercury, cadmium, lead, copper, zinc, chromium, nickel and others, to pollute waterways and upset ecosystems. Many field studies have suggested that excessive heavy metal concentrations are toxic to aquatic organisms. Algae are autotrophic organisms that incorporate inorganic materials in their role as primary producers in the food chain. Through the chain, increased heavy metal concentrations are passed to higher aquatic organisms and finally to man, resulting in variety of diseases (Bake et al. 1983). With a high capacity for bioaccumulation of many heavy metals, microalgae can be used to treat polluted water containing such pollutants. Thus, Many researchers have paid attention to selecting heavy metal tolerant algal species in their studies (Travieso et al. 1999; Terry and Stone 2002; Hashin and Chu 2004).

In the present study, *Chlorococcum* sp. AZHB, a unicellular green alga, was utilized as a heavy metal tolerant algal to investigate the following issues: **1.**Effects of Cu^{2+} and Cd^{2+} on the growth of *Chlorococcum* sp. AZHB. **2.** Influences of Cu^{2+} and Cd^{2+} on the physiological characteristics of *Chlorococcum* sp. AZHB. **3.** Effects of the two heavy metals on algal cell structure. **4.** Removal rates of the two heavy metals by *Chlorococcum* sp. AZHB. The final purpose is to evaluate the tolerance of *Chlorococcum* sp. AZHB to Cu^{2+} and Cd^{2+} and to utilize it in the treatment of heavy metal sewage.

MATERIALS AND METHODS

Chlorococcum sp. AZHB was collected from a sewage plant in Phoenix, AZ, USA, and isolated as a unialgal strain. The strain was maintained in BG11 medium at 25±1℃ and 35 ~ 40 $\mu mol \cdot m^{-2} \cdot S^{-1}$ provided by daylight fluorescent lamps.

Different concentrations of the heavy metal ions were prepared by adding $CuSO_4 \cdot 5H_2O$ (AR) and $CdCl_2 \cdot 2.5H_2O$ (AR) to the BG11 medium as 0, 0.01, 0.1, 1, 10, 50, 100, 200mg/L for Cu^{2+} and 0, 0.1, 1, 5, 10, 50, 100, 200 mg/L for

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Cd^{2+} respectively. In experimental cultures, exponentially growing cells were inoculated and grown under 14:10 LD cycle with a photo flux of 35 ~ 40 μmol·m^{-2}·S^{-1} provided by daylight fluorescent lamps at 25±1℃. The cultures were grown in 250 ml flasks containing 150 mL medium. All treatments were carried out in triplicates, and the culture in BG11 medium was employed as the control.

Growth of cultures was observed by measuring optical densities (OD) at a wavelength of 660nm at intervals of 48h for ten days. Cell densities were calculated using the linear regressive equation based our previous experiments:

$$C=0.02776 + 16.76748\ X \quad (R^2=0.998)$$

Where C represents the cell density and X represents the OD_{660}.

In order to evaluate the removal rates of Cu^{2+} and Cd^{2+}, the concentrations of Cu^{2+} and Cd^{2+} after cultivation of 6 days were analyzed with Perkin Elmer Analyst 800 atomic absorption apparatus (Perkin Elmer, USA). Chlorophyll contents of algal cells were determined with the method described by Tang (1999). Net photosynthetic rate and respiration rate after cultivation of 8 days were measured by the Oxygen Electrode (Rank Brothers, England).

After cultivation of 10 days, the cellular structure was examined by using the DM5000 microscope (Leica, Germany).

ANOVA and LSD Post Hoc Comparisons were used to check the significances of the research indexes.

RESULTS AND DISCUSSION

The effects of Cu^{2+} and Cd^{2+} on the growth of *Chlorococcum* sp. AZHB were shown in Figure 1 and Figure 2. When the concentrations of Cu^{2+} and Cd^{2+} were 0.01-0.1 mg/L and 0.1-1 mg/L respectively, the growth of *Chlorococcum* sp. AZHB exhibited no obvious difference from that of the control. As their concentrations rose to 1 –10 mg/L and 5 – 10 mg/L, respectively, *Chlorococcum* sp. AZHB maintained a reduced growth rate. When the concentrations of Cu^{2+} and Cd^{2+} were higher than 50 mg/L, the growth of *Chlorococcum* sp. AZHB was markedly inhibited (ANOVA, $P<0.05$; $n=7$), and furthermore, it was deduced that some cells might die as shown in the following results.

It was shown that the contents of Chl *a* + Chl *b* and Chl *a* after 6 days of cultivation, decreased with increasing concentrations of Cu^{2+} and Cd^{2+}($P<0.05$; $n=7$), suggesting that Cu^{2+} and Cd^{2+} inhibited the synthesis of chlorophyll (Table 1 and Table 2).

Increasing Cu^{2+} concentrations resulted in decrease of the net photosynthesis rates of *Chlorococcum* sp. AZHB after 8 days of cultivation ($P<0.05$; $n=7$) (Table 3). However the respiration rates of *Chlorococcum* sp. AZHB got raised along increasing Cu^{2+} when the concentration of Cu^{2+} was less than 10 mg/L, and showed the tendency to decrease when the concentration of Cu^{2+} was higher than 10 mg/L

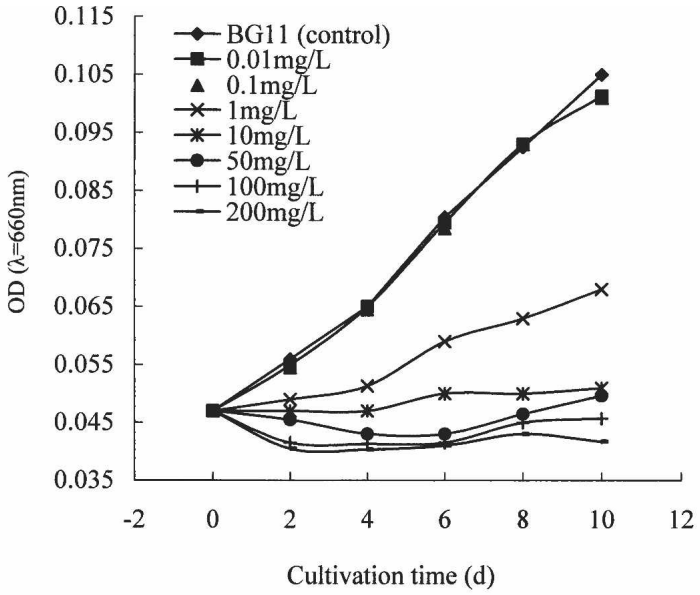

Figure 1. Effect of Cu^{2+} on the growth of *Chlorococcum* AZHB

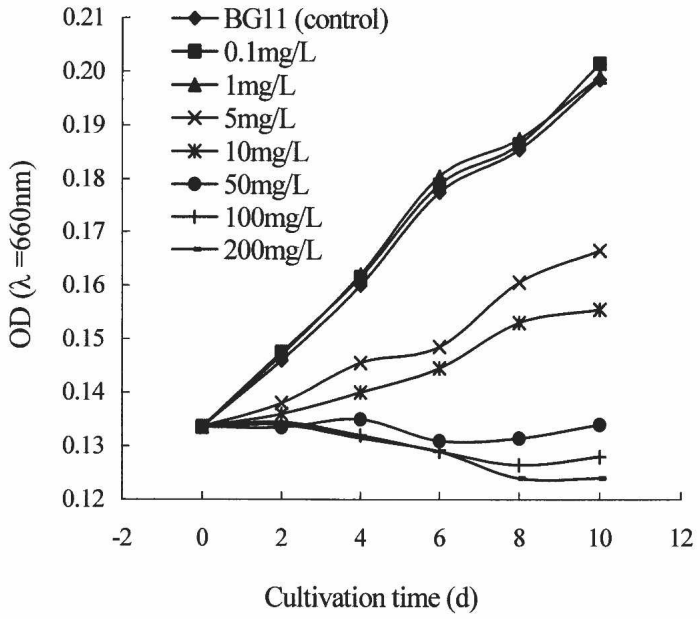

Figure 2. Effect of Cd^{2+} on the growth of *Chlorococcum* AZHB

($P<0.05$; n=7) (Table 3). At the case of Cd^{2+}, the respiration rates of *Chlorococcum* sp. AZHB decreased with increasing Cd^{2+} concentration ($P<0.05$; n=7) (Table 4).

Table 1. The effect of Cu^{2+} on the chlorophyll contents in *Chlorococcum* AZHB （6 days of cultivation）

Cu^{2+}concenration (mg/L)	BG11 (control)	0.01	0.1	1	10	50	100	200
Chl*a*+Chl*b* (pg/cell)	2.12	1.99	2.12	1.73	1.36	1.32	1.31	1.27
Chl*a* (pg/cell)	1.28	1.08	1.14	0.82	0.72	0.68	0.61	0.53

Table 2. The effect of Cd^{2+} on the chlorophyll contents in *Chlorococcum* AZHB （6 days of cultivation）

Cd^{2+}concentration (mg/L)	BG11 (control)	0.1	1	5	10	50	100	200
Chl*a*+Chl*b* (pg/cell)	1.66	1.49	1.46	1.10	0.88	0.64	0.56	0.47
Chl*a* (pg/cell)	0.70	0.66	0.62	0.47	0.39	0.32	0.25	0.17

Nevertheless, the net photosynthesis rates of the algae increased when Cd^{2+} concentration rose from 0.1 to 1 mg/L, and then decreased with increasing Cd^{2+} concentration up to 50 mg/L ($P<0.05$; $n=7$) (Table 4). When Cd^{2+} concentration was more than 50 mg/L, the net photosynthesis rate was not detectable (Table 4). It was generally accepted that high concentrations of metal ions led to photosynthetic depression (Greger and Ogren, 1991; Yruela et al. 2000; Xia et al. 2004). The results in the present study demonstrated that low Cd^{2+} concentration increased the net photosynthetic rate due to decrease of the respiration rate at the same time.

The effects of Cu^{2+} and Cd^{2+} on the cellular structure of *Chlorococcum* sp. AZHB were shown in Figure 3 and Figure 4. When Cu^{2+} concentration was less than 1 mg/L and Cd^{2+} concentration was less than 5 mg/L, thickness of cell wall and cellular color showed no significant changes, but the number of pyrenoids increased largely. When both metal concentrations rose, the cell wall became thicker at higher Cd^{2+} concentration and was thicken as multilayers at higher Cu^{2+} concentrations; the pigment decreased and the number of pyrenoids was reduced to one or disappeared.

The removal rates of Cu^{2+} and Cd^{2+} by *Chlorococcum* sp. AZHB were examined after 6 days of cultivation. Under respective concentrations for both metals described above, the removal rates for Cu^{2+} were 35%, 35%, 80.5%, 88.5%, 51.2%, 39.6% and 22.6%, and those for Cd^{2+} were 6%, 50.5%, 88.4%, 88.1%, 50.3%, 36.4% and 8.0%.

When the concentrations of Cu^{2+} and Cd^{2+} were 2-10μM and 100-200μM, respectively, the growth of G. *lemaneiformis* was inhibited (Xia et al. 2004). When the concentration of cadmium was over 2mg/L, *Scenedesmus acutus* and *Chlorella vulgaris* presented morphological problems and their reproduction was affected (Travieso et al. 1999). The effective concentrations of copper on the inhibition of the growth of *Scenedesmus obliquus*, *Chlorella pyrenoidosa* and *Cloterium lunula* at 96h (96h EC_{50}) were 50, 68 and 200 μg/L, respectively (Yan and Pan, 2002).

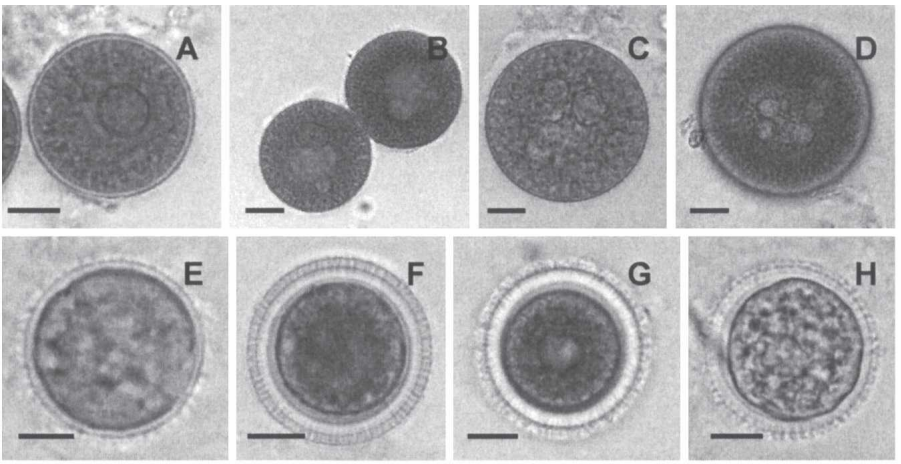

Figure 3. The effect of Cu^{2+} on the microstructure of *Chlorococcum* AZHB A: BG11 (control), B: 0.01mg/L Cu^{2+} C: 0.1 mg/L Cu^{2+} D: 1 mg/L Cu^{2+} E: 10 mg/L Cu^{2+} F: 50 mg/L Cu^{2+} G: 100 mg/L Cu^{2+} H: 200 mg/L Cu^{2+} (Bar=10 μm)

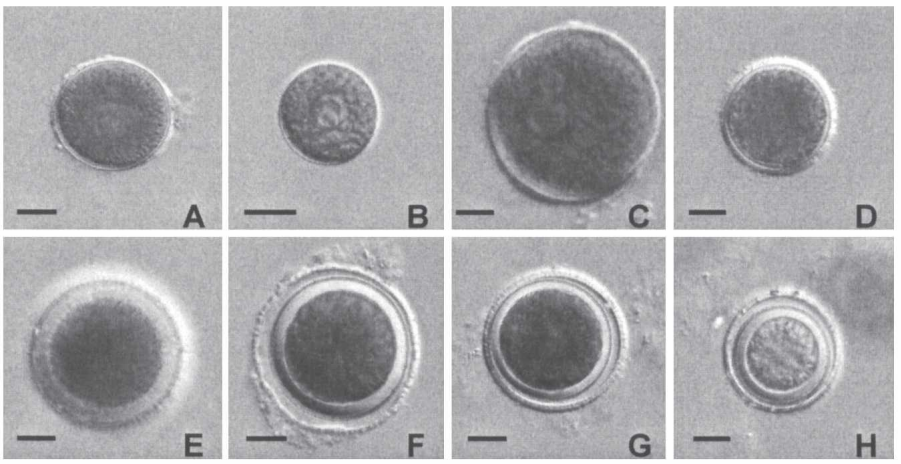

Figure 4. Effect of Cd^{2+} on the microstructure of *Chlorococcum* AZHB A: BG11 (control), B: 0.1mg/L Cd^{2+}, C: 1 mg/L Cd^{2+} ,D: 5 mg/L Cd^{2+},E: 10 mg/L Cd^{2+}, F: 50 mg/L Cd^{2+}, G: 100 mg/L Cd^{2+},H: 200 mg/L Cd^{2+}, (Bar=10 μm)

Compared with the above results, *Chlorococcum* sp. AZHB could endure the higher concentrations of Cu^{2+} and Cd^{2+}. Tolerance of *Chlorococcum* sp. AZHB to high concentrations of Cu^{2+} and Cd^{2+} could be explained as the following reasons: **1.** The *Chlorococcum* sp. AZHB formed a large number of pyrenoids to conserve energy that was expended due to increased respiration. **2.** The functional groups in amylose and protein molecules such as $-NH_2$, -OH, -COOH, -NH, -SH, et al. in the cell wall may have bound to heavy metal ions (Sicko, 1982), which ensured that heavy metal ions were mostly adsorbed on the cell walls when metals were transported to the cell at lower concentrations. *Chlorococcum* sp. AZHB

Table 3. Effect of Cu^{2+} on photosynthesis and respiration of *Chlorococcum* AZHB （8 days of cultivation）

Cu^{2+} concentration (mg/L)	BG11 (control)	0.01	0.1	1	10	50	100	200
Net photosynthetic rate ($\mu molO_2/h \cdot cell*10^{10}$)	6840	5640	4038	3678	3504	3198	2946	1356
Respiration rate ($\mu molO_2/h \cdot cell*10^{10}$)	810	1038	1116	1728	3012	2298	2040	1518

Table 4. Effect of Cd^{2+} on photosynthesis and respiration of *Chlorococuum* AZHB （8 days of cultivation）

Cd^{2+} concentration (mg/L)	BG11 (control)	0.1	1	5	10	50	100	200
Net photosynthetic rate ($\mu molO_2/h \cdot cell*10^{10}$)	7290	8618	8190	1080	507	-787	-801	-814
Respiration rate ($\mu molO_2/h \cdot cell*10^{10}$)	3447	3025	2664	2370	1548	1559	878	637

formed thicker cell walls to improve their adaptation to the stresses caused by high concentrations of Cu^{2+} and Cd^{2+}. **3.** Alterations of antioxidant enzyme levels and isozyme pattern changes in cadmium-treated have been reported in *Nannochloropsi oculata* (Lee and Shin, 2003), and which supposing to be also likely that Cu^{2+} and Cd^{2+} induced the antioxidant enzyme levels and isozyme pattern changes in *Chlorococcum* sp. AZHB so as to increase the endurance of the algae to Cu^{2+} and Cd^{2+}. **4.** Cd^{2+}stress induced cyanobacterium *Synechococcus cedroum* to form a low molecular weight protein that made the algal detoxify from heavy metal cadmium (Dai et al. 1998). Pistocchi et al. (2000) indicated that copper and cadmium ions were able to increase the productivity of extra- and intracellular metal-ligands in phytoplankton. Thus, it is probable that stresses from Cu^{2+} and Cd^{2+} induced the *Chlorococcum* sp. AZHB to synthesize these substances enhancing its resistance to both metal ions. Evidences for reasons of the above 3 and 4 are still needed to be confirmed in *Chlorococcum* sp. AZHB in our future studies.

We determined that *Chlorococcum* sp. AZHB could endure certain concentrations of Cu^{2+} and Cd^{2+}, and exhibited a high removal rate of these heavy metal ions. Hence, *Chlorococcum* sp. AZHB has a high potential to be used in the treatment of wastewater with high concentrations of these heavy metal ions.

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