Volume 50(1-2):19-23, 2006
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Effect of Chromium(VI) on growth, element and photosynthetic pigment composition of *Chlorella pyrenoidosa*

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ABSTRACT The effects of Cr(VI) were investigated on the growth rate, element, photosynthetic pigment and amino acid composition of *Chlorella pyrenoidosa*. Cr(VI) is toxic to Chlorella pyrenoidosa. The influence of chromium on cell density and cell number followed very similar trends, indicating that these growth responses might be correlated. The EC_{50} value for Cr(VI) were 2.0 mg dm^{-3}, the lethal concentration of chromium appears to be approximately 20 mg dm^{-3} for *Chlorella pyrenoidosa*. We have investigated the concentrations of chromium, calcium, magnesium and iron. The cells were fractionated into three fractions: cell wall fraction, membrane fraction, soluble fraction after the cells were disrupted. The amount of metals in whole cells and in each cell fraction was determined. Chromium uptake at each concentration was high within 3 days. *Chlorella pyrenoidosa* can accumulate chromium mainly (approximately 70%) in the cell wall. The concentrations of chromium and calcium show parallel changes with each other. A higher calcium concentration can be observed along with an increasing chromium concentration, both in the cell wall system and in the whole cells. Iron and magnesium concentration show a decreasing tendency. Cr(VI) caused a changes both in free amino acids and proline content. Both free amino acids and proline content increase with the increasing concentration of chromium. Chlorophyll a and b content show a decreasing , while OH-chlorophylls show increasing tendency. Rate of carotene β and α change grows with the increasing chromium concentration. The toxic properties of Cr(VI) can arise from the possibly free diffusion across cell membranes and strong oxidative potential. The toxicological impact of Cr(VI) originates from the action of this form itself as an oxidizing agent, as well as from the formation of free radicals during the reduction of Cr(VI) to Cr(III) occuring inside the cell. **Acta Biol Szeged 50(1-2):19-23 (2006)**

KEY WORDS

Chlorella pyrenoidosa
chromium
element composition
pigment pattern

Chlorella pyrenoidosa is an unicellular green alga, which is found in both fresh and marine waters. Its physiology, biochemistry and photosynthetic apparatus are similar to higher plants but its growth is very quick. For these reasons *Chlorella* is often studied in various metabolic and stress investigations (Rachlin and Grosso 1993; Lustigman et al. 1995).

When the concentration of a metal ions in the environment rises above a specific threshold heavy metal ions inhibit a variety of metabolic activities and prove toxic to most organisms. Interest in chromium originates from widespread use of this metal in various industries, such as metallurgical (steel, ferro and nonferrous alloys) and chemical (pigments, electroplating, tanning, others). Due to industrial run-off, process, large quantities of Cr compounds are discharged in liquid, solid and gaseous wastes into the environment, resulting in significant adverse biological and ecological effects (Kabata-Pendias and Pendias 2001). Chromium can exist in several chemical forms, displaying oxidation numbers from 0 to VI. Only trivalent and hexavalent chromium, are stable enough to occur in the environment. Cr(IV) and (V) form unstable intermediates in reactions of trivalent and hexavalent oxidation states with oxidizing and reducing agents respectively (Ball and Nordstrom 1998; Shriver et al. 2001). Cr(III) is the best known form displaying stability at neutral pH, if the complexation can be neglected. Under redox and pH conditions normally found in natural systems, chromium is removed from the solution as $Cr(OH)_3$, or in the presence of Fe(III) in the form of $(Cr_xFe_{1-x})(OH)_3$ where the x is the mole fraction of chromium (Sass and Rai 1987). Cr(III) generally has a lower toxicity than Cr(VI) compounds.

The Cr(III) is known to be essential for men and other mammals through its important function in glucose and lipid metabolism (Mertz 1975; Anderson 1989). Cr(VI) forms several oxygen associated species, the relative proportions of which depend on both pH and total chromium (VI) concentration. Within the normal pH range in natural waters CrO_4^{2-},

Accepted May 5, 2006
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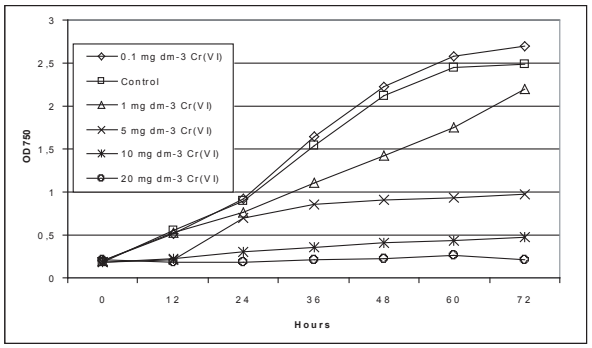

Figure 1. Effects of Cr(VI) on cell density of *Chlorella pyrenoidosa* in synthetic medium.

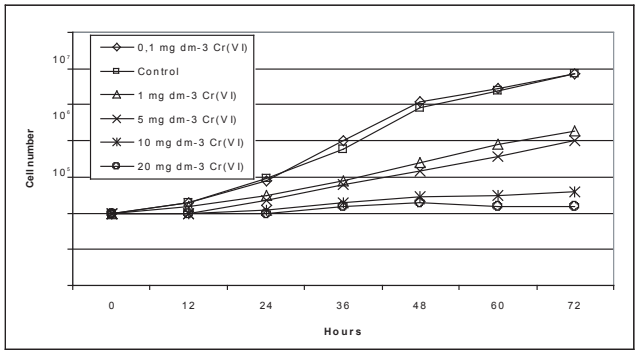

Figure 2. Effects of Cr(VI) on cell number of *Chlorella pyrenoidosa* in synthetic medium.

$HCrO_4^-$ and $Cr_2O_7^{2-}$ ions are the forms generally found and constitute many of the chromium (VI) compounds. They are quite soluble and mobile in the environment (Nieboer and Jusys 1988). Several laboratory studies have dealt with toxic effects of chromium in higher plants and algae (Wong and Chang 1991; Bishnoi et al. 1993).

The effect of Cr(VI) on the growth of *Chlorella pyrenoidosa* at different concentrations was studied under laboratory conditions. We also studied the element and photosynthetic pigment composition of algae, intracellular distribution of chromium and the levels of free amino acids and proline.

Materials and Methods

The algae used in this study were *Chlorella pyrenoidosa* (strain *IAM-C128*) obtained from the collection of the Institute of Applied Microbiology, University of Tokyo (Japan). Chemicals were purchased from Sigma Chem. Co. (St. Louis, USA), and Serva Fine Biochem. Gmbh. (Heidelberg, Germany).

Algae were maintained on agar. Cells were grown in sterile tubes containing synthetic media modified C-30. Cultures were aerated by filtered air bubbled with 5% CO_2, which served as a carbon source and stabilized the algal suspension homogeneity and pH at 7.2. Cells were permanently illuminated with white fluorescent light (18 Wm^{-2}) and were kept at 25°C during the growing period. The cultures were grown for 3 in some case 4 days. When cultures reached approximately $1x10^5$ cells/ml in the nutrient medium, algae were treated with 0.1 - 50 mg dm^{-3} of chromium (VI). We used $K_2Cr_2O_7$ as hexavalent chromium. For each experiment a control was also prepared of untreated *Chlorella pyrenoidosa* cells kept at the same conditions. Algae were autotrophically propagated for 72 hours after chromium treatments. The growth rate of algae cultures was followed by indirect turbidometric assay, and direct count using hemocytometer. Cells were collected by centrifuging (5000g x 10 min), and were washed 2 times with deionized water.

For algal cell fractionation cells were harvested by centrifugation at 5000g x 10 min, were washed twice with deionized water and centrifuged, and the weight of the cells was determined. This type of sample was considered as whole cells (sample 1). These prepared cells were disrupted using French press at 1500 kgf/cm^2 three times. French press is the most useful tool for disrupting *Chlorella* (Takeda and Hirokawa 1984). The homogenate was centrifuged at 3000g x 20 min and the pellet was allowed to stand in 1 ml of 0,5% sodium n-dodecyl sulphate (SDS) for 30 min. The homogenate with SDS was centrifuged at 10000g x 20 min to remove the soluble component, and the pellet was boiled in 80% ethanol for 20 min. The cell wall fraction was prepared by centrifuging the ethanol boiled pellet at 10000g x 20 min (sample 2). The miscellaneous fraction was prepared as the mixed supernatant after the treatment with SDS and ethanol (sample 3). The homogenate was centrifuged 15000g x 45 min after disruption of cells. The pellet was the membrane fraction (sample 4) and the supernatant was the soluble (cytoplasmic; sample 5; Okamura and Aoyama 1994).

The water of the supernatants was evaporated before digestion. Samples were digested for a day in concentrated HNO_3 and 30% H_2O_2 mixture (6:1 v/v rate), and dried. The mineralized residue was redissolved in 5 ml of 2N HNO_3 solution. The element composition was measured by a Spectroflame-type inductively coupled plasma atomic emission spectrophotometer (ICP-AES; Spectro Gmbh Kleve, Germany) with the following parameters: plasma gas 1.6 dm^3min^{-1}, nebulizer gas 0.6 dm^3min^{-1}, coolant gas 15 dm^3min^{-1}, excitation 27 MHz, 1.05 kW cross flow nebulizer.

When individual photosynthetic pigments were separated by HPLC technique algae pigments were extracted with a mixture of chloroform – acetone – isopropyl alcohol (2:1:1 v/v) at 4°C, the analytical procedure was detailed formerly (Simon et al. 1989). To determine free amino acid and proline content 200 mg fresh algae were shaken in 2 ml of 7% trichloroacetic acid for two hours, then they were filtered through paper filter and membrane filter (0.45 μm). The analysis was carried out using Biotronik LC 3000 amino acid analyzer

Table 1. Element composition of algal cell and cell fractions grown without and in the presence of 1, 5 or 10 mg dm^{-3} of Cr(VI) (the values are given in mg dm^{-3}).

	Control				1 mg dm^{-3} Cr(VI)			
	Cr	Ca	Mg	Fe	Cr	Ca	Mg	Fe
Whole alga cell	0,2	38±1	1423±54	148±4	17±1.8	64±5	1116±82	102±4.8
Cell wall fraction	0,2	45±1.4	1512±105	114±7.5	14±2.6	60±7.5	972±58	114±7
Membrane fraction	Nd	4.8±0.4	111±14	32±2.6	2.5±0.8	1.4±0.6	98±21	26±3.6
Soluble fraction	Nd	3.4±0.4	102±7	41±4.9	2.1±0.7	1.7±0.4	68±19	10.5±2.8
	5 mg dm^{-3} Cr(VI)				10 mg dm^{-3} Cr(VI)			
	Cr	Ca	Mg	Fe	Cr	Ca	Mg	Fe
Whole alga cell	28±3.6	74±4.2	981±104	78±8	24±3.8	79.5±4.2	981±81	62±5.1
Cell wall fraction	28.8±1.5	77±4.9	932±71	82±3.8	31±4.2	81.2±3.7	712±88	79±4.2
Membrane fraction	4.1±0.9	6.4±1.1	144±22	11±2.1	2.1±0.9	7.5±1.1	94±7.6	10.2±1.4
Soluble fraction	3.4±1.2	2.8±1	49±11	2.5±0.9	2.8±0.4	3.2±1.2	51±4.9	3.2±1.3

Nd: not detectable.
Values represent the mean ± SD of one experiment in triplicate.

(Galiba et al. 1992). The results are the means of 3-5 replications for each treatment. Three independent repetitions were performed for each experiment. The data were statistically evaluated calculating the standard deviation, and by statistical analysis using Tukey's *b*-test.

Results

The effects of chromium (VI) on *Chlorella pyrenoidosa* was investigated using increasing concentration of chromium from 0 to 50 mg dm^{-3}. As seen in Figures 1 and 2, except for a slight increase in growth of the algal cells at 0.1 mg dm^{-3} in comparison to the control, an increase in chromium concentration caused a significant decrease in the cell density and cell number. The influence of chromium on cell density and cell number followed very similar trends, indicating that these growth responses might be correlated. The EC_{50} value for chromium (VI) based on inhibition of cell growth was 2.0 mg dm^{-3}. The cell cultures treated with Cr(VI) from 20-50 mg dm^{-3} showed chlorotic symptoms, and the cells did not grown when we transferred them to fresh medium without chromium. This suggests, that the lethal concentration of Cr(VI) appears to be approximately 20 mg dm^{-3} for *Chlorella pyrenoidosa.*

The concentrations of 21 elements (Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Sr, Ti, V, Zn) were determined in algae cells, and the concentrations of chromium, calcium, magnesium, iron are presented in Table 1. The cells were fractionated after disruption to cell wall fraction, membrane fraction, and soluble fraction. The concentrations of metals in whole cells and in each cell fractions were determined. Within 3 days chromium uptake at each concentration was high indicating that algal cells are able to bind and accumulate chromium. Generally 70% of the chromium is localized in the cell wall region, while the amount of accumulated chromium was almost the same in the membrane and the soluble fraction.

Comparison the data of the control and treated cells demonstrates the high rate of accumulation of chromium in the cells (Table 1). The chromium content of control cells is 0.2 mg dm^{-3}, while the values of treated cells are a hundred times higher. Intracellular distribution of chromium is heterogenous in different fractions. The concentrations of chromium and calcium show similar changes. An increasing chromium concentration causes higher calcium concentration, both in the cell wall system and in the whole cells. The increased Ca concentration is surprising, because an ion exchange would be expected. Both iron and magnesium concentrations show de creased concentrations with increased chromium concentrations.

All concentrations of chromium produced changes in both free amino acids and in proline concentrations as seen Figure 3 and 4. Increasing the concentration of chromium to 10 mg dm^{-3} increased the concentration of free amino acids and proline. At higher concentration of chromium the amount of amino acid concentration decreased. Proline concentration is known to increase in different type of stresses such as salinity, drought, low and high temperature, and heavy metals (Ibarra-Caballero et al. 1988; Kavi Kishor et al. 1995). The toxic properties of Cr(VI) may arise from free diffusion across cell membranes and strong oxidative potential.

The toxicological impact of Cr(VI) originates from its action as an oxidizing agent, as well as from the formation of free radicals during the reduction of Cr(VI) to Cr(III) occuring inside the cell (Nieboer and Jusys 1988). Decreasing levels of free amino acids and proline at higher concentration of chromium maybe due to the degradation of the cells.

Table 2. Photosynthetic pigments' concentration of algal cells growth with and without Cr(VI).

	Control	Cr(VI) 1 mg dm^{-3}	5 mg dm^{-3}	10 mg dm^{-3}	20 mg dm^{-3}
chlorophylls					
chlorophyllid b	0,028	0,024	0,024	0,020	0,018
chlorophyllid a	0,350	0,250	0,256	0,284	0,126
chlorophyllid a'	0,122	0,086	0,074	0,078	0,082
OH-chlorophyll b-1	0,052	0,070	0,164	0,232	0,258
OH-chlorophyll b-2	0,046	0,082	0,144	0,208	0,220
chlorophyll b	0,842	0,872	0,878	1,352	0,734
chlorophyll b'	0,246	0,272	0,220	0,248	0,182
OH-chlorophyll a-1	0,116	0,158	0,238	0,268	0,276
OH-chlorophyll a-2	0,084	0,202	0,308	0,316	0,304
chlorophyll a	4,338	4,574	4,052	3,808	3,404
chlorophyll a'	0,616	0,756	0,510	0,522	0,580
pheophytin a	0,036	0,090	0,138	0,170	0,228
carotenoids					
violaxanthin	0,160	0,192	0,176	0,202	0,138
anteroxanthin	0,490	0,540	0,458	0,584	0,420
lutein	1,324	1,372	1,390	1,830	1,960
xantophylls*	0,202	0,248	0,344	0,402	0,304
α-criptoxanthin	0,098	0,136	0,130	0,088	0,036
β-criptoxanthin	0,048	0,048	0,034	0,018	0,016
α-carotene	0,198	0,154	0,158	0,150	0,176
β-carotene	0,344	0,274	0,348	0,368	0,462

xantophylls* : unidentified xantophylls

The effects of chromium on concentration of photosynthetic pigments and chlorophyll derivatives are seen in the Table 2. The amounts of both chlorophyll-a and chlorophyll-b decrease with increasing chromium concentration, although the decrease in chlorophyll-a is larger than in chlorophyll-b. Conversely, the amounts of both OH-chlorophyll-a and OH-chlorophyll b (oxidative products of the chlorophylls) increased with the increasing concentration of chromium.

When Chlorella green algae were grown under similar conditions to this experiment, addition of metal ions (namely titanium, gallium and zirconium) to growth medium also caused changes in photosynthetic pigment concentration and composition. Appearance of new chlorophyll derivatives was observed in titanium, gallium and zirconium treated Chlorella cultures (Simon et al. 1988; Simon et al. 1989; Simon et al. 2001). Decomposition of chlorophylls and forming of chlorophyll derivatives could be attributed to in vivo action of enzymes like peroxidase (Kato and Shimizu 1985; Simon et al. 1989). Since peroxidase is located mainly in the vacuoles, and chlorophyll is compartmentized in the chloroplast, presumably peroxidase decomposes chlorophyll in vivo in the processes where membranes are dis-integrated (Kato and Shimizu 1985). We suppose that higher concentration of Cr(VI) acts indirectly to chlorophyll metabolism (*i.e.* by stimulation the dis-integration of membranes, and enhancing the activity of peroxidase), and causes appearance of chlorophyll derivatives as OH-chlorophyll-a and OH-chlorophyll-b.

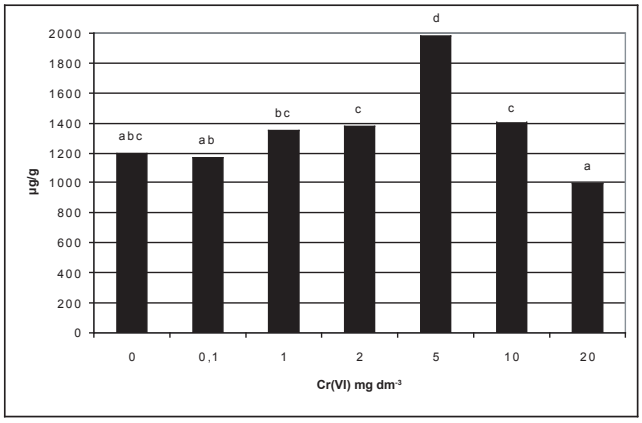

Figure 3. Total amino acid content in *Chlorella pyrenoidosa* grown in synthetic medium treated with Cr(VI). Statistical analysis was done by Tukey's b-test. Data are means of 3 replications. Bars of means signed by the same letter are not statistically significant at P=0.05.

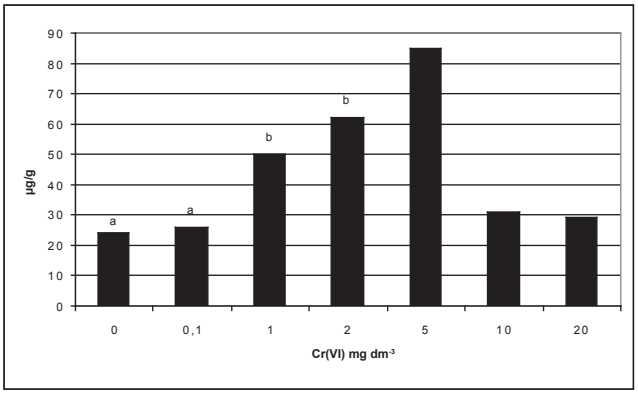

Figure 4. Proline content in *Chlorella pyrenoidosa* grown in synthetic medium treated with Cr(VI). Statistical analysis was done by Tukey's b-test. Data are means of 3 replications. Bars of means signed by the same letter are not statistically significant at P=0.05.

There were also changes in carotenoid levels. In the control cells the ratio of β - α carotene is about 1.5, but the rate became higher with the increasing chromium concentration. This phenomenon could be a protecting process against heavy metal induced oxidative stress (Salguero et al. 2003).

Discussion

Cr(VI) is toxic to *Chlorella pyrenoidosa. Chlorella* green algae can accumulate chromium mainly in the cell wall. Both free amino acids and proline content increased with the increasing concentration of chromium in the growth medium. Chlorophyll a and b decreased, while OH-chlorophylls increased in cells. Ratio of carotene β - α increased with the

increasing chromium concentration. These data suggest that the toxic effects of chromium on green algae originates from the action of Cr(VI) as an oxidizing agent as well as from the formation of free radicals during the reduction of Cr(VI) to Cr(III) occurring inside the cell. The toxic properties of Cr(VI) may arise from the free diffusion across the cell membranes and strong oxidative potential.

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