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Characterization of the Cadmium-Binding Capacity of *Chlorella vulgaris*

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Algae and other aquatic plants possess the capacity to take up toxic trace metals from their environment, resulting in an internal concentration greater than those of the surrounding waters (Whitton 1984; Ahner *et al.* 1995). This property has been exploited as a mean for treating industrial effluent containing metals before they are discharged, and to recover the bioavailable fraction of the metal (Kaplan *et al.* 1995). The advantages of using algae for such purpose are that algae are tolerant to elevated metal levels in their growth media. They grow autotrophically and have a large surface to volume ratio and the potential for genetic manupulation (Cai *et al.* 1995).

Chlorella vulgaris is an unicellular Chlorophyceaen. Similar to other algae, *Chlorella* sp. has been demonstrated to develop tolerance to cadmium (Cd) polluted environment by synthesizing metal-binding proteins (Nagano *et al.* 1984). In order to exploit the use of *C. vulgaris* as a tool for Cd-removal and recovery, it is necessary to characterize its metal-tolerance and its metal-binding capacity. In this study, the tolerance of the cells to elevated Cd in the environment, the biochemical basis of tolerance, as well as the organism's metal-binding capacity was examined.

MATERIALS AND METHODS

C. vulgaris obtained from Carolina Biological Supply Company (Cat # P7-15 2075) was cultured in Bristol solution (PH 6.6) at 25 ± 2°C under light intensity of 2000-3000 lux using cool fluorescent light, with a 16/8 h light/dark cycle. Large conical flasks (1000 ml) used for subculturing were connected to aeration pumps fitted with sterile filters attached to aeration tubings. Small conical flasks (100 and 250 ml) used for experiments were placed on a gyratory shaker set at 100 rpm. All procedures were carried out with cells in the logarithmic growth phase. When studying the effect of Cd on cell growth, incubation periods varied according to the duration and requirements of each particular experiment .

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C. vulgaris was incubated in media containing various concentrations (0, 2.5, 5, 10, 20, 25, 30, 35, 40, 60, 80 and 160 µg/ml) of $CdCl_2$. All concentrations referred to in this study are norminal concentrations. The degree of growth inhibition at each concentration was measured at 48h. The IC_{50} (Cd concentration which inhibits 50% cell growth) was determined from the percentages of growth inhibition at different Cd concentrations by probit analysis, using the SAS statistical software program on PC. The results were used as a basis to determine exposure doses of Cd for subsequent experiments.

In order to observe the effect of Cd on the growth of *C. vulgaris*, the cells were cultured in Bristol solution containing 0, 9.5 and 12 µg/ml of Cd. The cell number was determined with a haemocytometer every 48 h for 12 days, and the suppression of cell growth was determined by comparing the cell number with cultures maintained in Cd-free medium.

Algal cells were cultured in Bristol solution containing 9.5 µg/ml of Cd for 5 days to examine whether *C. vulgaris* can develop tolerance to Cd after previous exposure to the metal. The cells were then inoculated into solution containing 14 µg/ml of Cd. The percentage of growth inhibition was compared with controls that were either maintained in 9.5 µg/ml Cd, or had never been exposed to Cd prior to being challenged with 14 µg Cd/ml.

Cells exposed to 0, 11 and 13 µg/ml Cd for 4 days were harvested by centrifugation at 30,000 x g (Beckman Avanti-J25-1) for 25 min. The cell pellets were washed with Cd-free culture medium, and twice with double distilled water to remove unbound Cd. The washed pellets were then resuspended in 3 ml of double distilled water and stored at -70°C. The frozen cells were disrupted by vortexing in glass beads (0.4-0.5 mm) for 30 min. After removing the glass beads, the disrupted cells were put into 10 mM Tris HCl buffer, at pH 7.4. The samples were ultracentrifuged at 100,000 x g for 60 min (Hitachi Preparative Ultracentrifuge 70P-72). Both the clear supernatant, which contained the cytosolic fraction, and the pellet, which contained the cell wall, were stored for subsequent analysis.

The cell wall was again washed with double distilled water, lyophilized, and digested in nitric acid (70%) in a block digestor (approximately 120°C). The digestate was boiled to dryness and reconstituted in 10 ml of 0.5% nitric acid (diluted with double-distilled water). It was then filtered through a Whatman filter paper (5C) and stored in acid-washed polycarbonate containers. The Cd content of the filtrate and the cytosolic fraction was analysed using flame atomic absorption spectrometry (Allen, 1989) on a Varian Spectra AA20.

The cytosolic fraction obtained from ultracentrifugation was heated at 90°C for 5 min and centrifuged at 9000 rpm for 10 min. The supernatant, which contained the heat-stable proteins, was collected and analysed by differential pulse polarography

using a voltammeter (Metrohm, Switzerland). The polarographic current was used to calculate the amount of thiolic protein (Thompson and Cosson 1984). The amount of total heat-stable thiolic proteins was determined against a standard curve constructed with purified rabbit metallothionein (Comeau *et al.* 1992).

Heat-treated cytosolic fraction was applied to a Sephadex G-75 column (26 x 70 mm), and the proteins were eluted with 10mM Tris HCl pH 7.4. Seven-ml fractions were collected at a flow rate of 0.5 ml/min. The UV absorbance of each fraction was analysed at 254 nm, and the Cd content was measured by flame atomic absorption spectrometry.

Large volumes of *C. vulgaris* were cultured in Bristol solution. Cells were harvested and washed with double-distilled water. They were distributed, in amounts of 5.6 x 10^8, 1.12 x 10^9 and 2.24 x 10^9 cells into 20-ml Bristol solutions containing various concentrations (5, 20, 40 µg/ml) of Cd. Control solutions with same concentrations of Cd were maintained under the same experimental conditions, without the addition of any cells. After being kept on a shaker for 22 h at 25°C the cells were removed by filtering through a Whatman glass fibre filter (GF/C 4.7cm). Cadmium concentration in the filtrate was determined using flame AAS. The percentage of Cd removed was calculated against the controls.

A statistical software program (SAS) was used to conduct the probit analysis on the data obtained from toxicity tests, and the analysis of variance for determining significant difference between treatments. The level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

A typical growth curve (Figure 1) of *C. vulgaris* in Bristol solution had a 4-day lag phase before entering the logrithmic phase during which the doubling time of *C. vulgaris* was approximately 1.5 days. Cadmium inhibited algal growth. During the logrithmic phase, the doubling time increased from 1.5 days to 2 days when cells were grown in media containing 9.5 µg/ml Cd, and to 6 days when grown in 12.5 µg/ml Cd. The cell number at day 12 was reduced by 35% in 9.5 µg/ml Cd, and by 73% in 12.5 µg/ml Cd. The IC_{50} of Cd to algal growth was studied by applying various concentrations of Cd to the Bristol solution and measuring the density after 48h (Figure 2). The 48-h IC_{50} was 15.72 µg/ml, according to the probit analysis using the SAS statistical software.

Growth differed between cells with and without prior exposure to sublethal dose (9.5 µg/ml) of Cd (Figure 3). Cells previously exposed to sublethal dose of Cd withstand a subsequently higher dose (14 µg/ml) of Cd by exhibiting less growth inhibition. One of the mechanisms of the induced metal tolerance had been ttributed to the induction of intracellular metal-binding proteins which chelate the toxic metal and prevent it from damaging cytoplasmic organelles (Steffens 1990; Ahner *et al.* 1995).

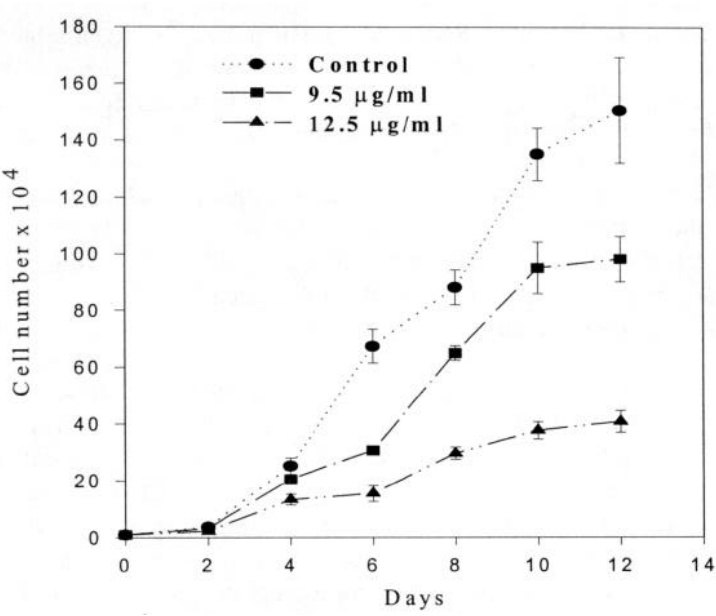

Figure 1. Growth curve of *C. vulgaris* in various growth media. Each point represents the mean of three replicates, and the error bar represents the SD.

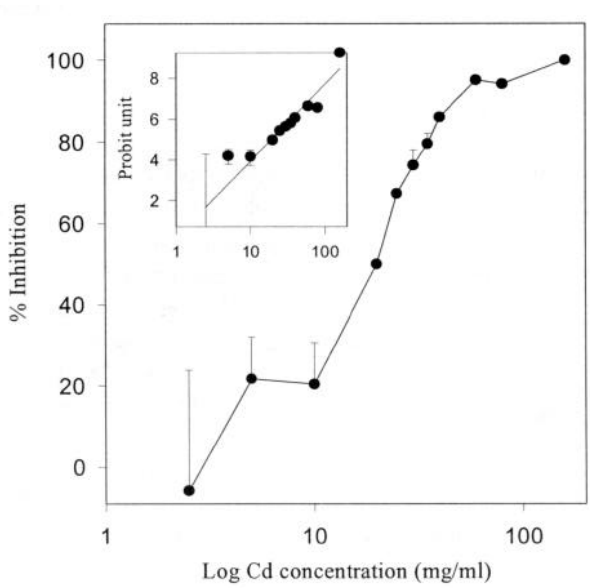

Figure 2. A 48-h toxicity test for Cd. Each point represents the mean and SD of three replicates. Insert represents the probit analsis of the same data.

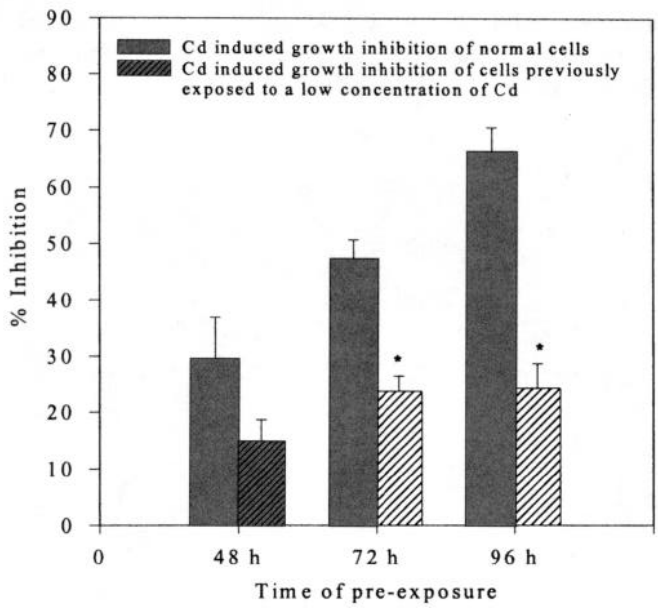

Figure 3. *C. vulgaris* previously exposed to Cd shows less inhibition when challenged with higher concentrations of Cd. Error bars represent SD. * indicates significant difference at $p<0.05$ between cells previously not exposed to Cd and those previously exposed to Cd for 96 h.

Table 1. Levels of total heat-stable thiolic proteins in cytosol

Cd added	Cell number x 10^8	Thiolic proteins (g x 10^{-14} /cell)
0 µg/ml	6.63 ± 0.67	3.66 ± 1.09
11 µg/ml	7.58 ± 0.47	5.14 ± 0.57
13 µg/ml	3.30 ± 0.21	6.81 ± 0.40*

The results were obtained from polarographic analysis of heat-treated cell cytosol for the presence of thiolic proteins. The values represented the mean ± SEM of three replicates. * indicates significantly ($p<0.05$) different from the controls that were treated with 0 µg/ml Cd.

Characteristically, metal - binding proteins are heat-stable proteins and contain a large percentage of cysteine. Differential pulse polarographic measurement is a technique which takes advantge of the reverse suppression of cobaltic current by the sulfur molecules to quantify the levels of thiolic protein present in the cytosol (Olafson and Sim 1979; Olafson and Olsson 1991). The polarographic response was significantly higher ($p<0.05$) for cells treated with 13 µg/ml of Cd

when compared to control cells (Table 1). Upon separation of the heat stable cytosolic proteins on Sephadex G-75 gel column, Cd was found associated with the lower-molecular weight proteins (Figure 4).

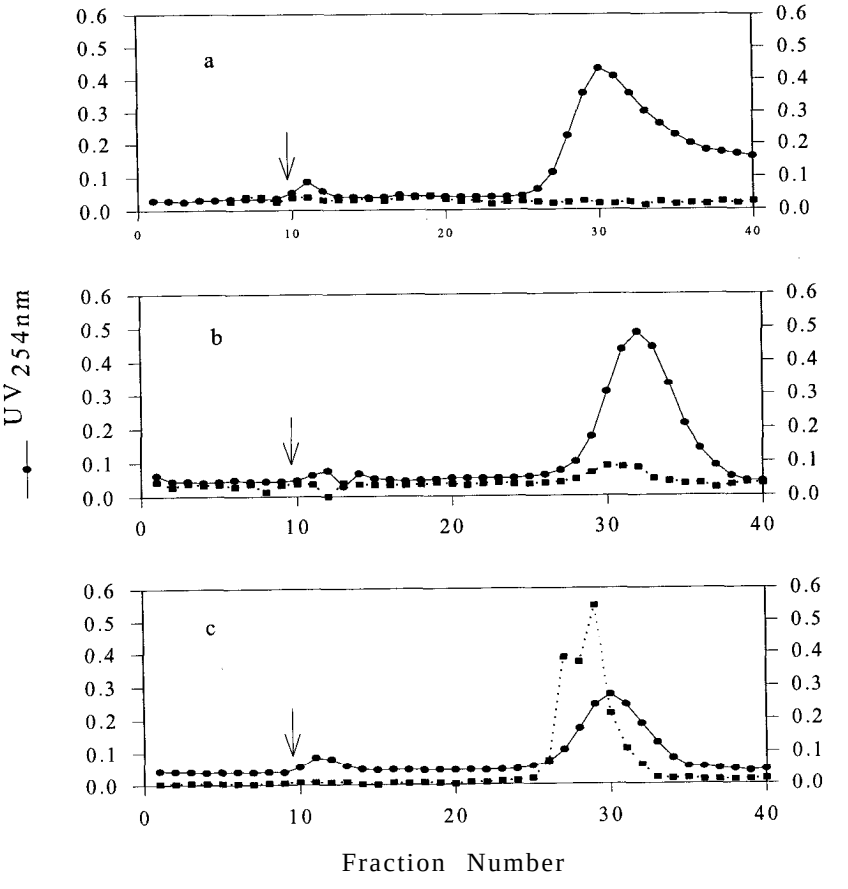

Figure 4. UV absorbance and Cd contents in different fractions of heat-stable proteins from cells after separated by Sephadex G-75 chromatography. Cells were grown in a) Bristol solution, b) Bristol solution with 11 µg/ml Cd and c) Bristol solution with 13 µg/ml Cd. Arrows indicate the void volume.

Some organisms such as *Chlamydomonas,* a motile unicellular green alga was also found to accumulate Cd in the cell wall region (Cai *et al.* 1995). The Cd contents in both the cell cytosol and cell wall fractions of *C. vulgaris* after exposure to 11 µg/ml Cd was significantly higher ($p<0.05$) in Cd bound to the cytosolic fraction than that to the cell wall fraction (Table 2). However, when cells were incubated in 13 µg/ml Cd, the metal was distributed evenly between the two fractions. Thus, *C. vulgaris* also possesses 2 sites for metal binding - the cell wall

and the cytosol. The cell wall may act as an additional line of defense protecting the intracellular environment of the cell by sequestering Cd.

Table 2. Binding of Cd to the cytosol and to cell wall

	Cell number x 10^8	Cadmium content (µg/ml) Cytosolic	Cell wall
0 µg/ml	6.63 ± 0.39	0.008 ± 0.002	0.012 ± 0.001
11 µg/ml	7.58 ± 0.27	0.331± 0.055*	0.103 ± 0.020
13 µg/ml	3.3 ± 0.12	1.690 ± 0.280	1.310 ± 0.100

The table shows distribution of Cd in the cytosol and the cell wall fraction of *C. vulgaris.* The values represent the mean ± SEM of three replicates. * indicates that only with 11 µg/ml treatment was Cd concentration in the cytosolic fraction differn signfiicantly ($p<0.05$) from that in the cell wall fraction.

It should be noted that cell number, after incubation in 13 µg/ml Cd, was only 55% of cells present in the control. The amount of thiolic protein was, however, significantly higher in these cells. Kaplan *et al.,* (1995) isolated a Cd-tolerant *Chlorella* sp. by subjecting the culture to progressively higher doses of Cd. The isolated line had an LD_{50} ten times higher than the original strain from which it was isolated. The results in this study also indicate that Cd may act as a force for selecting a population of cells which have a higher Cd-binding capacity.

Table 3. Assessment of the capacity of *C. vulgaris* to remove cadmium

Initial concentration	% of Cd removal 5 µgm1	20 µg/ml	40 µg/ml
Cell density			
5.56 x 10^8	60.3 ± 5.7	26.8 ± 2.8	13.5 ± 0.8
1.12 x 10^8	73.5 ± 2.4	35.2 ± 0.8	19.2 ± 1.0
2.24 x 10^8	86.6 ± 1.0	57.5 ± 0.5	38.8 ± 2.3

The values represent mean ± SEM of three replicates.

C. vulgaris was able to remove up to 86% of Cd from the medium (Table 3). Increasing the cell number increased the efficiency of metal removal. The efficiency was the greatest when 2.24 X 10^9 (the highest cell density used) was incubated with the lowest dose of Cd (5 µg/ml). Selection of algal strains that are tolerant to the toxic effects of heavy metals, and analysis of the mechanisms that contribute to metal tolerance, may help to improve the efficiency of waste water processing (Kaplan *et al.* 1995). The results in this study show that it is possible to induce Cd tolerance in *C. vulgaris* by exposing it to a low level of the metal. An interspecies comparative study of the capacity and efficiency of various kinds of microalgae to take up heavy metal would probably be useful to determine other candidates that can be used for treatment of Cd polluted water. It will also be of

interest to study whether the Cd-binding capacity can be improved with other algae that have proviously been cultured in elevated Cd concentration. Finally, experiments using actual field conditions would allow for a realistic estimate of how efficient this method could be used.

Acknowledgments

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