Subcellular Localization of Cadmium in *Chlorella vulgaris* Beijerinck Strain Bt-09

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ABSTRACT

Growth response curves of *Chlorella vulgaris* Beijerinck strain Bt-09 to sublethal concentrations of cadmium were evaluated. The growth responses of this microalgal isolate was determined through analysis of chlorophyll *a* levels. Cadmium was effectively taken up by the cells as determined by Flame Atomic Absorption Spectrophotometry (F-AAS). Subcellular fractionation was undertaken to locate sites that accumulate cadmium.

Keywords: Chlorella vulgaris, cadmium uptake, subcellular localization

INTRODUCTION

The heavy metal, cadmium, is found in the environment as inorganic cations or as complexed species. Cadmium serves no known biological roles and is a powerful toxicant even when present at low concentrations (Babich & Stotzky, 1981). As an environmental contaminant, cadmium ions may act on a number of cellular and biochemical processes essential to growth and reproduction of microalgae. Among the deleterious effects of cadmium on photosynthetic organisms are inefficient photosynthesis, unproductive nucleic acid, protein and lipid biosynthesis, ineffective nitrogen fixation, decreased uptake of inorganic nutrients, and eventual death (Boyle, 1984).

Cadmium enters the aquatic environment through various anthropogenic activities, which include mining, smelting, and electroplating. This heavy metal is then released into the waterways where it is readily assimilated and concentrated by microorganisms in the primary trophic levels. Cadmium is incorporated and biomagnified through the food chain. As essential components of aquatic ecosystems, microalgae represent a major proportion of primary producers in the food chain. These organisms are known to bioaccumulate heavy metals, and as such play important roles in the redistribution and bioconcentration of heavy metals.

As survivors in a metal contaminated environment, microalgae have evolved a number of metabolism-dependent and -independent processes for the uptake and accumulation of heavy metals. These processes include exclusion, chelation, binding to cell surfaces, and compartmentalization of the metal ions (Cobbett, 2000). A general response mechanism for heavy metal detoxification is the expression of stress biomolecules, e.g., metallothioneins and phytochelatins.

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Chlorella vulgaris, as well as many species of microalgae, have been observed to bioaccumulate significant amounts of cadmium ions from the medium. Carr et al. (1998) and Matsunaga et al. (1999) observed the ability of *C. vulgaris* to withstand deleterious concentrations of cadmium. The capacity of *C. vulgaris* for taking up cadmium ions makes it a suitable organism for remediation of aqueous surface environments. The cells can be used either as live or inanimate bioscrubbers. The removal of harmful heavy metals from effluents and wastewaters provides an additional or alternative process of metal recovery for environmental protection or economic reasons. The actual biochemical mechanism responsible for the tolerance of *C. vulgaris* to cadmium has not been fully elucidated. Furthermore, the distribution of cadmium ions inside the cells has not been thoroughly investigated. This study reports on the cadmium-binding ability of *Chlorella vulgaris* Beijerinck strain Bt-09 in culture and on the subcellular localization of cadmium. This report comprises the first part of the investigation into the physiological mechanism behind the ability of *C. vulgaris* strain Bt-09 to tolerate cadmium in its growth medium.

MATERIALS AND METHODS

Microalgal isolate, culture conditions, and treatment

Chlorella vulgaris Beijerinck strain Bt-09 cells were collected in Bataan and the cultures acclimatized in BG-11 (pH 7.4) at 28°C ± 2, under constant illumination (60 mE m^{-2} s^{-1}). The composition of the basal medium (in mM) was as follows: macronutrients, viz., 17.65 $NaNO_3$, 0.18 K_2HPO_4, 0.3 $MgSO_4$, 0.25 $CaCl_2$, 0.19 Na_2CO_3, 0.0003 EDTA, 0.029 citric acid, 0.03 ferric ammonium citrate; micronutrients, viz., 0.46 B, 0.17 Co, 0.32 Cu, 9.2 Mn, 1.6 Mo, and 0.77 Zn. All glassware were acid washed. Variable concentrations of cadmium were added to the basal medium to come up with the following concentrations: 0.05 ppm, 0.5 ppm, and 5.0 ppm $CdCl_2$. These were each inoculated with *C. vulgaris* strain Bt-09 at its mid-log growth phase. Cultures without cadmium served as control.

Growth analysis

The growth response was assessed spectrophotometrically using chlorophyll *a* (chl *a*) content, monitored at various time intervals for 30 days adopting the modified combined methods of MacKinney (1941), Butterwick et al. (1982), and Sartory & Grobelaar (1984). Cells from the cultures in test tubes were harvested by centrifugation (13,000 x *g*, 4°C, 5 min). The cell pellet was recovered, rinsed with fresh BG-11 medium plus nitrogen, taken up in 5 mL of 2:1 methanol-petroleum ether, sonicated (20 kHz, 2 min), and subsequently incubated in a water bath (50°C, 4 min). The mixture was then cooled and passed through a coarse filter paper, with the filtrate read at 663 nm.

The generation time (g) and specific growth rate (μ) of the cadmium-exposed, as well as unexposed, *C. vulgaris* cultures were calculated using the formulae shown below (Brock,1979), where A_0 and A_1 are the absorbances at the initial time (t_0) and the final time (t_1), respectively.

$$\mu = \frac{\ln A_1 - \ln A_0}{t_1 - t_0} \qquad g = \frac{\ln 2}{\mu}$$

Cadmium binding capacity of *Chlorella vulgaris* cells

Cells grown in medium with or without cadmium were harvested by centrifugation (10,000 x *g* , 4°C, 10 min). After which, they were subjected to a series of water and 0.1 M EDTA washes. BG-11 medium augmented with cadmium served as positive control, while BG-11 medium without cadmium served as the negative control.

The harvested cell pellet was rinsed thrice with ice cold ultrapure water. The recovered cell pellet was then subjected to triple washings with room temp ultrapure water and recovered each time by high speed centrifugation (10,000 x *g*, 5 min). The supernatants from each centrifugation step were pooled and set aside for AAS analysis. Each of the resulting cell pellet was washed thrice with 0.1 M EDTA solution, the EDTA

washes were likewise pooled and set aside for AAS analysis. The final pellet was recovered, weighed, and set aside also for AAS analysis.

Subcellular localization of Cadmium

Microsomes and cytosols

Cells exposed to 0.5 ppm cadmium for 2 days were harvested by centrifugation (10,000 x *g*, 4°C, 10 min). The resulting cell pellet was resuspended in ice cold ultrapure water and the resulting mixture was sonicated (20 kHz, 6 min). The resulting suspension was centrifuged (1,000 x *g*, 4°C, 7 min) and the resulting pellet (mostly cell walls and cell debris) was washed twice with ice cold ultrapure water and submitted for AAS analysis. One mL aliquot of the supernatant resulting from the first centrifugation was submitted for AAS analysis, with the remaining supernatant further centrifuged (15,000 x *g*, 4°C, 15 min). The pellet (containing intact mitochondria and chloroplast) was washed twice with room temperature ultrapure water and submitted for GF-AAS analysis. Likewise, 1 mL aliquot of the supernatant resulting from 15,000 x *g*-centrifugation was submitted for GF-AAS. The remaining supernatant was finally centrifuged (100,000 x *g*, 4°C, 30 min). The resulting pellet (representing microsomal fraction) and supernatant were also submitted for GF-AAS.

Plasma membrane

To determine the amount of cadmium bound to cell membranes, membranes were isolated following the procedure described by Hodges & Mills (1988). Harvested cadmium-exposed cells were disrupted in 5 mL cold grinding medium [0.25 M sucrose, 3 mM EDTA, 2.5 mM DTT, and 25 mM Tris-MES (pH 7.2)]. The suspension was centrifuged (13,000 x *g*, 4°C, 5 min). The pellet (presumably containing chloroplast, nuclei, and mitochondria) was collected and set aside for further fractionation, while the supernatant was centrifuged (80,000 x *g*, 4°C, 1 h) and the resulting supernatant was submitted for AAS analysis. The recovered pellet was resuspended in 4 mL of 0.25 M sucrose and 5 mM potassium phosphate buffer (pH 7.8). The suspension was layered onto a sucrose step gradient (i.e., 20%, 30%, 34%, and 45%) containing 1 mM $MgSO_4$, 2.5 mM DTT, and 1 mM Tris-MES (pH 7.2). The resulting mixture was centrifuged (95,000 x *g*, 4°C, 2 h). The plasma membrane and microsomes (concentrated at the 34%-45% interface) were pipetted out and submitted for GF-AAS analysis.

Chloroplast and nuclei

To confirm the presence of cadmium in the chloroplast and nuclei, the fraction obtained from the 13,000 x *g* centrifugation was further fractionated following the procedure described by Orozco et al. (1988). The pellet was resuspended in 4 mL (SHEMNa) buffer (330 mM Sorbitol, 50 mM Hepes-KOH (pH 8.0), 2 mM EDTA, 1 mM $MgCl_2$, and 5 mM NaOAc. The resulting mixture was layered onto a 40%-85% Percoll step gradient and was centrifuged (6,650 x *g*, 4°C, 6 min), concentrating broken chloroplasts and intact mitochondria at the 0%-40% interface. The interface was pipetted out and set aside for subsequent isolation of the mitochondria. The intact chloroplasts, which were concentrated at the 40%-85% interface, were similarly taken out. The remaining white pellets presumably contain the nuclei. The fractions containing the intact chloroplasts and nuclei were submitted for GF-AAS analysis.

Mitochondria

Cadmium in the mitochondria was likewise determined following the method of Hanson et al. (1988). The fraction containing the crude mitochondria and broken chloroplasts was resuspended in 2 mL MTESB buffer [0.3 M mannitol, 50 mM Tris-HCl (pH 8.0), 3 mM EDTA, 0.1% BSA, 20 mM 2-mercaptoethanol, and 10 mM $MgCl_2$]. The resulting suspension was mixed with 10 mL of Sucrose-Buffered Solution (SBS) [0.3 M sucrose, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.1% BSA]. The mixture was centrifuged (15,000 x *g*, 4°C, 15 min) and the recovered pellet was resuspended in 4 mL SBS specified above. This suspension was then layered onto a sucrose step gradient [1.6 M, 1.2 M, and 0.6 M sucrose, with 50 mM Tris-HCl, 20 mM

EDTA, and 0.1 % BSA, all at pH 8]. This layered mixture was centrifuged (15,000 x *g* at 4°C for 1 h) and the resulting cream colored bands at the interfaces were collected for GF-AAS analysis.

RESULTS AND DISCUSSION

Growth analysis

The chlorophyll *a* levels of the control, 0.05, 0.5 and 5 ppm $CdCl_2$-treated cultures increased as the cultures aged (Fig. 1). Based on chl *a* levels, the log phase of the treated and untreated cultures occurred from days 1-18, followed by the stationary phase at day 19. Five ppm cadmium adversely affected the normal growth of *C. vulgaris* strain Bt-09 cultures. Cultures grown at 5 ppm $CdCl_2$ showed reduced chl *a* content, decreased specific growth rate and increased generation time (Table 1).

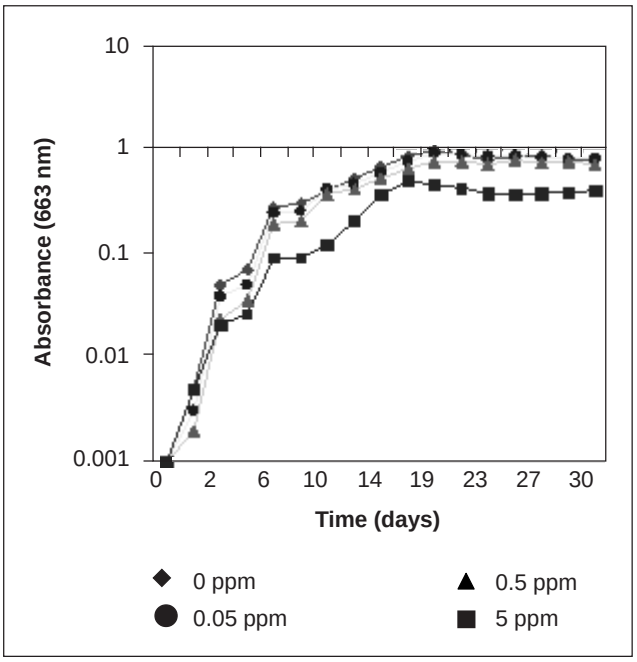

Fig. 1. Chlorophyll *a* profile of *C. vulgaris* strain Bt-09 exposed to varying levels of $CdCl_2$ for 30 days.

Other studies have also reported that cadmium indeed decreases the growth rates of several species of microalgae. Payne & Price (1999) observed that *Chlamydomonas reinhardtii* cells were sensitive to cadmium, showing an inhibition for growth at 3×10^{-8} M (0.005 ppm). Other microalgae, including our *C. vulgaris* strain Bt-09, are resilient to cadmium, not showing any marked growth inhibition when the cadmium concentration is below 5 ppm. In another study, a decrease in the number of *Anacystis nidulans* cells was noted when the cells were exposed to 5 ppm $CdCl_2$ (Lee et al., 1992).

Exposure of cells to lower cadmium concentrations (i.e., 0.05 and 0.5 ppm) did not significantly affect chl *a* levels even when the respective chl *a* content for these cultures were lower than that of the control. On the otherhand, the chl *a* levels of the cells were 54% lower in cultures exposed to 5.0 ppm $CdCl_2$. Lee et al. (1992) also observed a decrease in the chl *a* content of *Anacystis nidulans* when cells were grown in 5 ppm $CdCl_2$. Such a decrease was attributed to the disruption of thylakoid membranes by cadmium ions, resulting to the degradation of pigments. In a study conducted by Masojidek et al. (2000), a decrease in the total chlorophyll content of algal cells was observed when cells were grown in culture media containing either Cd, Cu, Hg, Ni, Zn, or Pb. The total chlorophyll deterioration was attributed to the abstraction and replacement of Mg^{2+} from chlorophyll molecules by the heavy metal atoms, leading to a change in the

Table 1. Effects of cadmium on growth rate and generation time of *C. vulgaris* strain Bt-09.

Cadmium concentration (ppm)	Cell density		% Inhibition	Chorophyll *a* profile		% Inhibition
	Specific growth rate (μ/day)	Generation time (g/day)		Specific growth rate (μ/day)	Generation time (g/day)	
Control	0.5270	1.3152	------	0.6562	1.056	------
0.05	0.5274	1.3142	0	0.6901	1.004	0
0.5	0.4999	1.3865	5.14	0.6678	1.037	0
5.0	0.3923	1.7668	25.56	0.4122	1.682	37.18

functional characteristic of the specific chlorophyll molecules.

Uptake and subcellular localization of Cadmium

Approximately 64% of the original cadmium concentration in the culture medium were associated with the *C. vulgaris* cells, as evidenced by the presence of the cadmium ions in their cytosol and cell wall fractions (Table 2). The cell wall of *C. vulgaris* strain Bt-09 played a major role in metal uptake as was demonstrated by the 5-fold higher cadmium content in the cell wall as compared to that in the cytosol. The same observation was noted by Mapoy et al. (2001) in their study on *Chlorella vulgaris* strain Zs-22. They reported that cadmium associated with the cell walls of *C. vulgaris* strain Zs-22 was 21 times higher than the cadmium associated with the cytoplasm. Our result does not agree with that of Carr et al. (1998) who found that more cadmium ions were found in the cytosol than in the cell wall when cells were treated with 11 ppm Cd. Our result, however, agrees with the observation of Sandau et al. (1996) on the metal binding capacity of *Chlorella vulgaris* and *Spirulina platenesis.* Both live and dead cells demonstrated their capacities to bind metal ions. The dead cells of *C. vulgaris* and *S. platenesis* were observed to adsorb heavy metal cations much more quickly and more effectively than the live cells (Sandau et al., 1996). In our study, no measure was taken to separate the dead and live cells in the harvested cultures. Thus, it is possible that the cell walls are derived from both live and dead cells. The cell wall of dead cells could have contributed to the number of binding sites of our cellular fractions.

A substantial amount of cadmium ions was associated with EDTA washes. The cadmium ions that were stripped off by the EDTA washings correspond to the cadmium which was more intimately bound to the cell wall. These cadmium ions were probably held by electrostatic attractions to divalent functional groups (e.g., sulfates and sulfites) of polysaccharide components of the cell wall. The cadmium ions which were not dissociated by the EDTA chelating agent represented the metal ions very tightly bound to the cell walls. These cadmium ions are bound irreversibly, perhaps by covalent bonding, to the cell wall matrix.

Cadmium content was found to be unevenly distributed among the five subcellular fractions of *C. vulgaris* strain Bt-09 (Fig. 2). The cytosolic fraction contained the highest amount of cadmium. This amount, 198 µg/L Cd, represents 66% of the total cadmium that had either passively or actively entered the interior of cells. It must be noted that our cell fractionation methods do not always preserve the structural integrity of subcellular organelles. And also, there was no attempt to check the purity of the cytosolic fractions. Thus, our cytosolic fraction can be a composite of soluble proteins from mitochondria, chloroplast, and/or vacuoles that have been broken down by our cell fractionation procedures.

Table 2. Uptake of cadmium in *C. vulgaris* strain Bt-09[1] cells.

Fraction	µg/L cadmium associated	% cadmium associated
Total cadmium associated with cell wall	217	52.61
Loosely associated (dissociated with water washes)	6.86	1.67
Tightly bound (dissociated with 100 mM EDTA washes)	172.00	41.72
Non-dissociable (residual cadmium in cell wall)	38.00	9.22
Cadmium associated with cytoplasm	45.10	10.95
Total cadmium recovered from cell fraction	262.10	63.58
Total cadmium used for treatment	372.5 µg	

[1]One L of late log phase cells were grown for 2 days in BG-11 medium with 0.5 ppm $CdCl_2$.

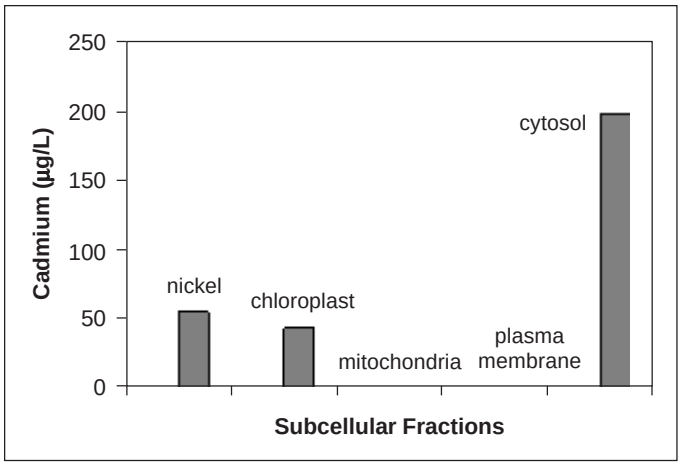

Fig. 2. Subcellular distribution of Cd in *C. vulgaris* strain Bt-09 exposed to 0.5 ppm $CdCl_2$ for 2 days.

In higher plants, the cytosol is generally the site for important metabolic enzyme activities and is the prime cellular compartment where metal-binding complexes are located (Clemens, 2001). The study of Carr et al. (1998) indicates that this situation can also be true for *C. vulgaris*, where they reported that when cells were treated with 11 ppm Cd, most of the cadmium were associated with the cytosolic fraction rather than with the cell wall fraction. Part of their results revealed that cadmium was associated with a low molecular weight polypeptide, presumed to be the metal-binding phytochelatin. The cytosolic enzymes of tolerant and non-tolerant plants are generally metal-sensitive (Ernst et al., 1992; Clemens, 2001). This situation may also be true for microalgae. The presence of cadmium ions in the cytosolic fraction of healthy cells could activate biochemical mechanisms that can alleviate cadmium toxicity. Removal of excess metal ions from the cytosol can be achieved by efflux or by compartmentalization.

A substantial proportion of cadmium (44 µg) was also detected in the chloroplast of *C. vulgaris* strain Bt-09 cells. In higher plants, apart from the cytosol, chloroplasts contain glutathione. Klapheck et al. (1987 & 1988) observed the localization and distribution of glutathione synthetase, glutathione and homoglutathione in plastids, and cytoplasm of *Pisum sativum* and *Phaseolus coccineus* leaves. Glutathione serves to keep plastids in reduced state apart from its protective role against heavy metal toxicity by complexing with the metals. Glutathione-metal binding has been observed in algae although this was not localized in the chloroplast (Gekeler et al., 1988). It is possible that in our case, cadmium may be complexed with glutathione.

The amount of cadmium detected in the mitochondrial fraction of *C. vulgaris* strain Bt-09 was low (1.53 µg). Nevertheless, our results are in agreement with the data reported by Silverberg (1976) who studied the effect of three levels of cadmium (0.03, 0.05, and 0.1 ppm $CdCl_2$) on the mitochondria of *Chlorella pyrenoidosa*. He observed Cd-containing granular inclusions attached to the cristae, detecting these inclusions by energy dispersive x-ray.

Still smaller amounts of cadmium (0.91 µg) were also detected in the plasma membrane fraction of our strain. Since one of the functions of plasma membrane is transport, numerous carrier and channel proteins are embedded in the membrane. The cadmium ions detected in the plasma membrane fraction are probably entrapped metals in transit. One of the protein families important for heavy metal transport is the metal-inorganic transport system which is found ubiquitously in eukaryotic organisms (Nies, 1999). Among the principal structural components of membranes are the phospholipids. It is also possible that the cadmium ions were bound to the phosphate and other charged group of the phospholipids.

Our strain accumulated more cadmium through its cell wall or cell wall-associated biomolecules as compared to its cytosolic fraction. The observed subcellular distribution of cadmium would indicate the presence of more than one mechanism to alleviate heavy metal stress.

Matsunaga et al. (1999) screened 191 strains of microalgae for potential organisms that could be used for on-site removal of heavy metals from the marine environment. Of the 191 strains studied, 24 strains showed high capacity for cadmium uptake. *Chlorella* sp. NKG16014 exhibited the highest cadmium uptake when exposed to 50 mM Cd, taking up 65% of the metal from the medium and was deemed most promising for sequestering cadmium from the marine environment. When exposed to 91.6 mM, *C. vulgaris*

strain Bt-09 cells were able to take up 63.58%. Thus, *C. vulgaris* strain Bt-09 can be likewise classified as a potential bioscrubber, either as whole cells or by using their non-living cell walls.

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