Cadmium Uptake in *Synechococcus aquatilis* (Reynaud) Strain SY01

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ABSTRACT

Cadmium uptake in *Synechococcus aquatilis* (Reynaud) Strain SY01 showed a biphasic behavior, with an initial rapid passive cell wall interaction phase and a later slow intracellular cation uptake process. The cell wall uptake process followed Michaelis-Menten kinetics. The apparent K_m of the uptake system was 38.89 μM, a relatively high affinity K_m value compared to other metal uptake systems. Fitting of experimental data to the Langmuir and Freundlich adsorption isotherms showed that binding of cadmium to the cell surface was monolayer and/or multilayer, although the monolayer adsorption was more probable, as exemplified by a high correlation coefficient. This could be explained by the presence of a strong primary binding site which was responsible for a monolayer adsorption and a weak secondary binding site which could be responsible for a multilayer adsorption. The uptake process was found to be relatively specific for cadmium. Equimolar amounts (200 μM) of cobalt, nickel, magnesium, and copper did not significantly affect cadmium uptake. *Synechococcus aquatilis* Strain SY01 cells also showed an efflux mechanism for cadmium, although the amount of excluded cadmium was insignificant compared with the amount of adsorbed cadmium in the cell wall.

Key words: kinetics, adsorption, cadmium, *Synechococcus*, cell wall

INTRODUCTION

The interaction of heavy metals with microorganisms such as phytoplanktonic algae has become an increasing global interest because of its potential as a biotechnological method in removing heavy metals from polluted aqueous systems. The possibility of separating metal-saturated algae from its medium may provide an economic method for removing heavy metals from wastewaters.

Synechococcus aquatilis, like any other cyanobacteria, belongs to the great subclass of gram-negative prokaryotes as defined by the special structure and chemical composition of the cell wall. The major component of the cell wall is murein, which contains diaminopimelic acid, muramic acid, and N-acetyl glucosamine, with a sheath composed of a matrix of pectic acids and mucopolysaccharides (Garnham and Green, 1995). The presence of a number of charged functional groups in the above mentioned cell wall components and the possibility of an active process of accumulating heavy-metals make *Synechococcus aquatilis* a probable substrate for metal binding experiments. The mechanism of cadmium accumulation in the cell wall of *Synechococcus aquatilis* (Reynaud)

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Strain SY01 was determined. The possibility of intracellular uptake was also examined.

MATERIALS AND METHODS

Organism and preparation of cultures

Synechococcus aquatilis (Reynaud) Strain SY01 was obtained from the Soil Microbiology Laboratory of the International Rice Research Institute, Los Baños, Laguna. The organism was grown in BG-11 medium (Rippka, 1988) and was maintained at 29°C under continuous illumination using a 100-watt fluorescent lamp.

An eight hundred-milliliter culture of 13-day old *Synechococcus aquatilis* Strain SY01 was inoculated into a two-liter Biostat Bioreactor and was maintained at pH 7.4 using automatic alkaline and acid dosing delta pumps. The culture was continually aerated with 5% air and stirring was maintained at 250 rpm. Cell growth was determined turbidimetrically at 680 nm in a Beckmann DU-65 spectrophotometer. The biomass was harvested via centrifugation after 13 days.

Samples were harvested and then transferred to 50-ml centrifuge tubes. The samples were pelleted at 10,000 rpm for 25 minutes. Each pellet was transferred to a microcentrifuge tube and was again centrifuged at 10,000 rpm in an Eppendorf microfuge TM for 5 minutes to remove more water. The wet biomass was collected and weighed for cadmium uptake experiments. glasswares were washed with 5% nitric acid and autoclaved.

Preparation of liquid samples for metal analysis

After the reaction period of cadmium inoculation, the collected supernatant was placed in a beaker on a hot plate and was evaporated to about 15-20 ml, making sure that the sample did not boil. The solution was cooled and 3 ml concentrated nitric acid was added. The sample was again heated to a gentle reflux and evaporated to near dryness, without baking. The addition of 3 ml concentrated nitric acid was repeated until the digest was light in color or did not change in appearance with continued refluxing. The sample was again evaporated to near dryness and 2.5 ml of 1:1 nitric acid and warm distilled deionized water was added to dissolve any precipitate or residue resulting from evaporation. The beaker walls and the watch glass covering were washed down with distilled deionized water and filtered through a Whatman #42 filter paper to remove silicates and other insoluble materials. The final volume was adjusted to 15 ml and this was analyzed by flame atomic absorption spectrophotometry.

Time course of cadmium uptake

Thirteen-day-old *Synechococcus aquatilis* Strain SY01 cells were harvested and then washed with distilled deionized water. Wet biomass weighing 0.2 grams was added to 100-ml BG-11 medium containing 0.54 μM Cd (II) in its chloride salt. The culture was maintained at pH 7.4 and was agitated at 125 rpm under lighted conditions (100-watt fluorescent lamps). Temperature was maintained at 29°C. The culture was then allowed to equilibrate for two hours. Ten-milliliter sampling was done after 35, 70, 105, 145, and 210 minutes. Samples were spun at 10,000 rpm for 25 minutes. The pellet was resuspended and twice washed with distilled deionized water. The supernatant was decanted and the pellet washed with 0.01 M EDTA. The supernatant was collected and the pellet was digested with concentrated nitric acid. The acid digest was centrifuged for 25 minutes at 10,000 rpm and the supernatant was mixed with the EDTA-cadmium supernatant obtained earlier. Samples were prepared for atomic absorption spectrophotometry. The cadmium ion level was determined using a flame atomic absorption spectrophotometer. Data represent the sum of the EDTA bound cell wall adsorbed cadmium and nitric acid lysed cells which intracellularly contain the cadmium.

Kinetics of cadmium uptake

Thirteen-day old *Synechococcus aquatilis* Strain SY01 cells were harvested and then washed with deionized water. Wet biomass weighing approximately 0.12 grams was added separately to 25-ml BG-11 medium

containing 1.7, 25, 40, 60, 100, 150, 300 μM Cd(II) in the form $CdCl_2$. Samples were maintained at 29°C, pH 7.4, and agitated at 125 rpm for 20 minutes. After the reaction period, samples were transferred to 50-ml centrifuge tubes and spun at 10,000 rpm for 25 minutes. The supernatant was decanted and the pellet washed with distilled deionized water to remove all unbound cadmium ions in the cell. After washing, each pellet was suspended in 0.01 M EDTA to remove the cell wall bound cadmium (Silverio, 1996). The suspension was then centrifuged for 25 minutes at 10,000 rpm. The supernatant was collected for cadmium ion analysis. Samples were prepared for metal analysis. Assays were performed in duplicates using flame atomic absorption spectrophotometry. Data represent a passive cadmium binding (adsorption in the cell walls). K_m and V_{max} values were derived from the Michaelis-Menten and Lineweaver-Burk plots.

Each pellet was digested with concentrated nitric acid and the supernatant was separately collected after centrifugation for 25 minutes at 10,000 rpm. The samples were prepared for metal analysis. Data after atomic absorption spectrophotometry represent an intracellular uptake of cadmium. The passive and active uptake was graphed against cadmium concentration.

Mode of adsorption

Six 25-ml BG-11 media containing 25, 40, 60, 100, 150, and 300 μM Cd(II) were inoculated with 0.12 grams wet biomass of *Synechococcus aquatilis* Strain SY01. Samples were maintained at pH 7.4, 29°C, 125 rpm, under lighted conditions for 20 minutes. Samples were transferred to centrifuge tubes and spun for 25 minutes at 10,000 rpm. The supernatant was decanted and the pellet was resuspended twice with distilled deionized water and resuspended again with 0.01M EDTA. The EDTA-cadmium supernatant was collected and prepared for cadmium analysis via atomic absorption spectrophotometry. Samples were prepared in duplicates. The data collected represent adsorbed cadmium ions on the cell wall. Data were fitted to both Langmuir and Freundlich isotherms.

Specificity of cadmium uptake

Twenty milliliters of BG-11 medium were separately dosed with equimolar amounts (200μM) of cadmium and the test metal ion. Four cations, namely, Co(II)(AR, $Co(NO_3)_2.6H_2O$), Ni(II)(AR, $Ni(NO_3)_2.6H_2O$), Cu(II)(AR, $Cu(NO_3)_2.6H_2O$), and Mg(II)(AR, $MgCl_2.6H_2O$) were used. Wet biomass of 0.12 grams was reacted at 29°C, 125 rpm, pH 7.4, under lighted conditions (100-watt fluorescent lamp). After two hours, the samples were collected from each flask and centrifuged at 10,000 rpm for 25 minutes. The pellets were resuspended twice in distilled deionized water and then resuspended again in 0.01 M EDTA. The EDTA-cadmium supernatant was collected and prepared for cadmium ion analysis. The metal ions in the cell wall was determined from the prepared supernatant liquid using flame atomic absorption spectrophotometry. Samples were prepared in duplicates.

Elucidation of an efflux mechanism

Samples of forty milliliters which were left after the course of cadmium uptake experiments were collected after one day of incubation. They were later centrifuged for 25 minutes at 10,000 rpm. The samples were resuspended twice with 0.01 M EDTA and the pellet resuspended in 20 ml distilled deionized water. Each sample was incubated for one day under light conditions and was centrifuged at 10,000 rpm for 25 minutes. The supernatant representing excluded intracellular cadmium was collected and prepared for cadmium ion analysis. Cadmium concentration was determined using flame atomic absorption spectrophotometry.

RESULTS AND DISCUSSION

Like most microorganisms, *Synechococcus aquatilis* Strain SY01 exhibited a typical growth curve beginning with an initial slow phase of growth (lag phase), followed by a period of exponential growth (logarithmic phase), a period of stationary growth, and finally, a slow decline in growth (death). Preliminary experiments (data not shown) established the log phase of *Synechococcus aquatilis* Strain SY01 at 14 days after initial inoculation.

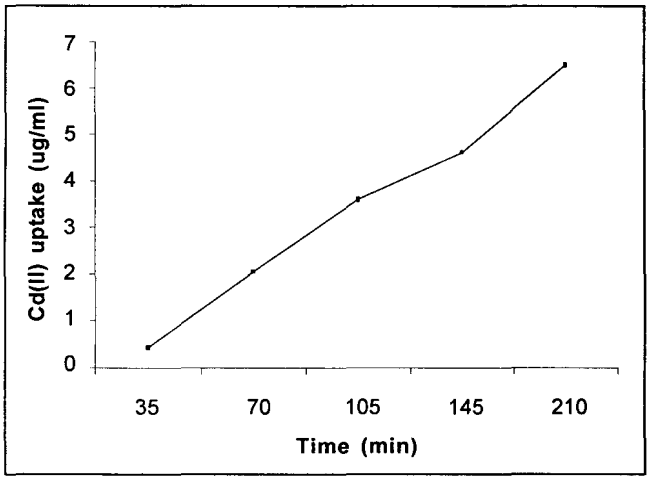

Fig. 1. Time course profile of cadmium uptake in *Synechococcus aquatilis* (Reynaud) Strain 01. Cd(II) uptake (ug/ml) was measured over a 210-minute observation by flame atomic absorption spectroscopy. Data represent both intracellular uptake and cell wall adsorption.

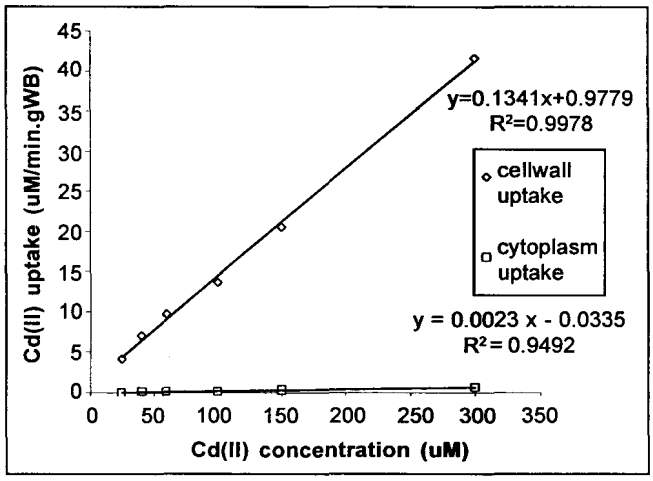

Fig. 2. Effect of cadmium concentration on cadmium uptake

Cells were thus harvested at this phase of growth to get the maximum amount of samples for the treatments.

Time course of cadmium uptake

Cadmium uptake was relatively linear over the 210-minute period of the transport assay (Fig. 1). During the first 70-minute interval, cadmium uptake increased by about 490% of the initial value. This rate of increase was maintained throughout the course of the experiment, indicating that the saturation point of cadmium-binding was not reached. This continuous accumulation of cadmium suggests the presence of a passive uptake process in the cell wall. This metabolism-independent passive uptake process may be explained by the presence of anionic binding sites and other charged groups in the cyanobacterial cell wall (Darnal and Greene, 1988).

Cadmium uptake in the cell wall and in the cytoplasm

Cadmium uptake in the cell wall and across the plasma membrane was observed to be linear against cadmium concentration (Fig. 2). The difference in the gradient of the slope of the two uptake mechanisms shows two distinct strategies of cadmium uptake in *Synechococcus aquatilis* Strain SY01. The first process is a rapid adsorption, indicating a passive surface reaction like physical (electrostatic) or chemical (surface complexation) adsorption onto the surface. The other process is slow and possibly involves active metabolic reaction. This mode of heavy metal uptake was also observed in zinc accumulation by *Candida utilis* (Failla et al., 1976) and *Chlorella vulgaris* (Garnham et al., 1992). The results document the presence of an intracellular uptake mechanism in *Synechococcus aquatilis* Strain SY01 cells.

Kinetics of cadmium uptake

The K_m and V_{max} values of cadmium uptake in the cell wall of *Synechococcus aquatilis* were derived from the Michaelis-Menten and Lineweaver-Burk plot transformation (Fig. 2). The Michaelis-Menten Equation is

$$V_o = \frac{V_{max}[S]}{(K_m+[S])} \quad (1)$$

Rearrangement gives the Lineweaver-Burk Equation:

$$\frac{1}{V_o} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \quad (2)$$

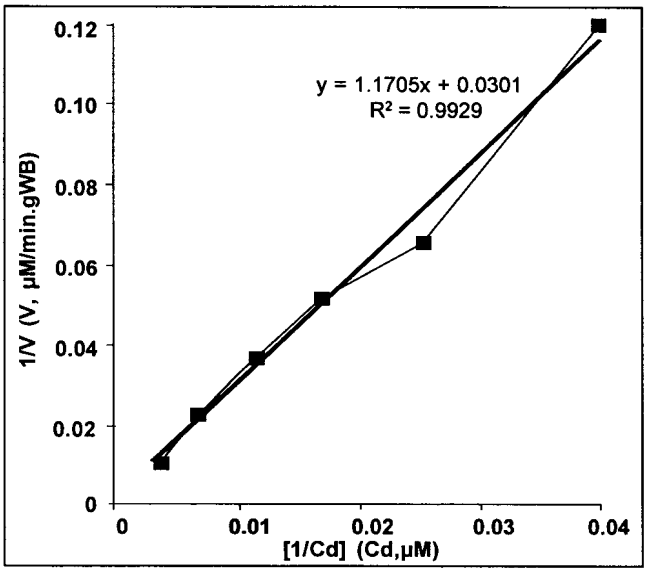

Fig. 3. Lineweaver-Burk plot of cadmium adsorption by *Synechococcus aquatilis* Strain SY01. 1/V is the reciprocal of the rate of cadmium uptake measured in µM/min. Approximately 0.12 g of wet biomass was added to BG-11 media containing cadmium in concentrations ranging from 1.7 to 300 µM. Cultures were incubated at 29°C at pH 7.4 and under agitated and light conditions. Cultures were allowed to equilibrate for two hours before sampling. Data represent both intracellular uptake and cell wall adsorption measured by atomic absorption spectrophotometry.

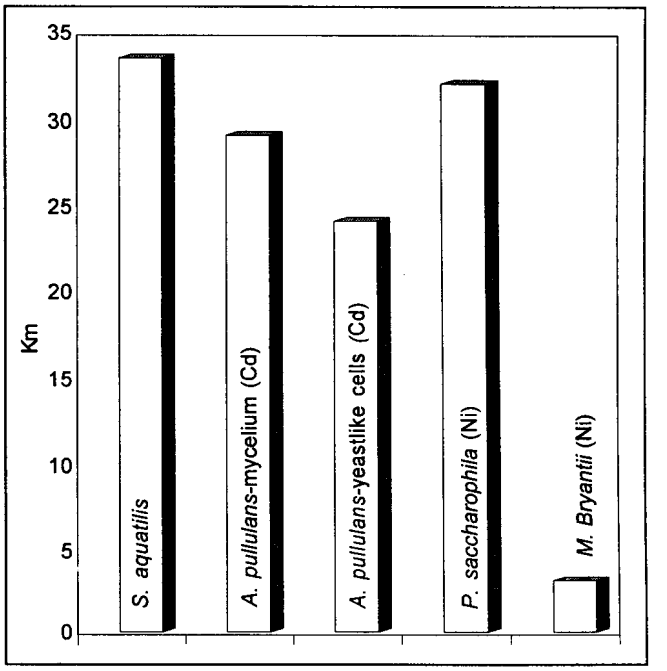

Fig. 4. K_m values of other metal binding systems. The given K_m values of *Synechococcus aquatilis* (Gupta et al., 1992) and *Aureobasidium pullulans* (Gadd and Mowll, 1984) are for Cd(II) uptake while the K_m values of *Plectonema saccharophila* (Jardim and Pearson, 1984), and *Methanobacterium bryantii* (Jarrell and Sprott, 1982) are for Ni(II) uptake.

where

[S] - is the initial substrate concentration,
V_o - is the initial velocity,
V_{max} - is the maximum velocity, and
K_m - is the Michaelis-Menten constant.

If an adsorption process follows the Michaelis-Menten type of kinetics, a plot of $1/V_o$ against 1/[S] will give a straight line graph (Fig. 3). From this plot, the K_m and V_{max} values of cadmium uptake were determined by linear regression analysis. From the slope and the y-intercept, V_{max} and K_m values of 33.2 µM/min.g and 38.89 µM were obtained, respectively. The degree of binding metal ions by organisms usually vary due to the different affinities that they exhibit during uptake. A measure of this is the difference in the K_m values obtained from their kinetics. The higher the K_m value, the lower the affinity for metal binding (Silverio, 1996).

Synechococcus aquatilis Strain SY01 has a relatively high affinity for cadmium relative to other metal-uptake systems (Fig. 4).

Monolayer and multilayer adsorption

The fitting of adsorption isotherm equations to experimental data was conducted to determine whether cadmium adsorption was monolayered and/or multilayered. In a monolayer situation, the Langmuir adsorption isotherm applies:

$$\frac{C_e}{C_{ads}} = \frac{1}{C_{sat}} + \frac{C_e}{(B)\,C_{sat}} \qquad (3)$$

where

C_e - is the equilibrium concentration of metal ion in solution,

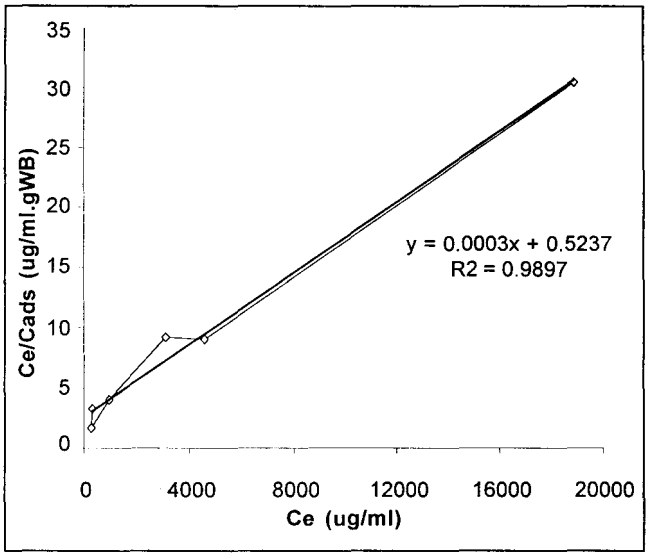

Fig. 5. Langmuir adsorption isotherm for cadmium by *Synechococcus aquatilis* Strain SY01. C_e is the equilibrium concentration of metal ion in solution, while C_{ads} is the equilibrium concentration of the metal ion adsorbed by the cell wall.

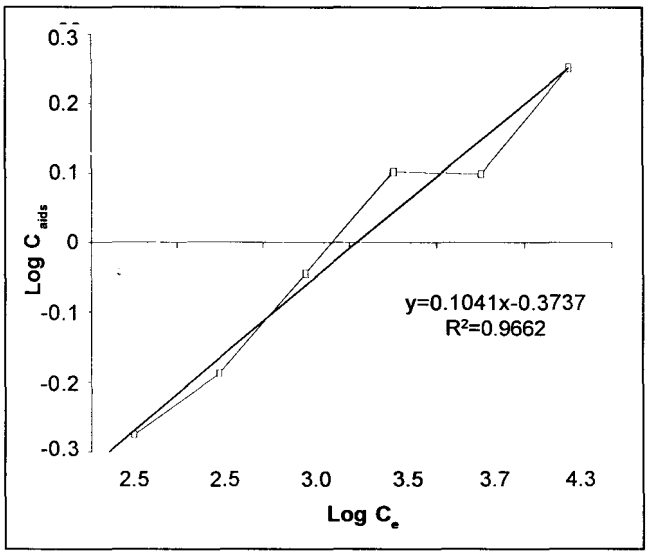

Fig. 6. Freundlich adsorption isotherm for cadmium uptake by *Synechococcus aquatilis* Strain SY01. C_{ads} is the equilibrium concentration of the metal ion adsorbed by the cell wall, while C_e is the equilibrium concentration of metal ion in solution.

C_{ads} - is the equilibrium concentration of metal ion adsorbed, and

B - is the intensity of adsorption.

The model assumes that the specific binding occurs by forming a monolayer. Such concept implies, in a simplified way, that the surface consists of a uniform polymeric network of repeating, energetically equivalent, surface functional groups (Cho et al., 1994). A linear relationship with a relatively high correlation coefficient (r^2=0.9897) was obtained in the C_e/C_{ads} vs. C_e graph (Fig. 5) indicating the possibility of a monolayer mode of adsorption. *Synechococcus aquatilis* Strain SY01 may contain polygalacturonic acid, an important constituent of bacterial cells that was found to complex/ bind with cadmium (Jellinek and Sangal, 1972), as well as with other metal ions like nickel, copper, and manganese.

In a multilayer situation, the Freundlich isotherm applies:

$$\log C_{ads} = \log A + \frac{1}{n} \log C_e \quad (4)$$

where

1/n - is the steepness of the slope, and

A - is the maximum sorption capacity.

A plot of log C_{ads} against log C_e (Fig. 6) yielded a relatively straight line-graph (r^2=0.9662) indicating the possibility of a multilayer mode of adsorption (Silverio, 1996). Similar results were observed in *Synechococcus* sp. PCC6301 and *Anabaena variabilis* (Garnham and Green, 1994). However, this correlation may not be conclusive of the presence of possible multilayer adsorption, as the strains used are different and the metal ion used in their study (chromium) has a very different chemistry from cadmium. The presence of a monolayer and possibly a multilayer mechanism of cadmium adsorption in the cell wall indicates the high affinity of the cell surface to cadmium. A monolayer type of adsorption appears to be more favored compared to that of a multilayer mode of adsorption as shown by the difference in their correlation coefficients.

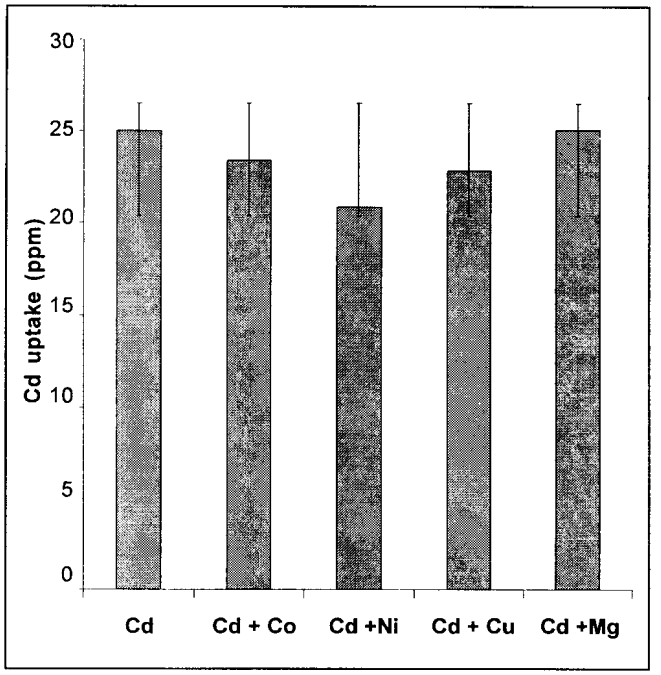

Fig. 7. Effect of other cations in the uptake of cadmium in *Synechococcus aquatilis* Strain SY01. Two hundred μM each of cadmium and a test metal ion were dosed on BG-11 media with 0.12 g wet biomass. Reactions were carried out at 29°C at pH 7.4 under agitated and lighted conditions for two hours. EDTA-cadmium washings of pelleted biomass were analyzed using flame atomic absorption spectrophotometry. Error bars represent standard deviation of 1.75 ppm Cd(II).

Specificity of cadmium uptake

Mg(II), Cu(II), Ni(II), and Co(II) ions did not significantly affect cadmium uptake in *Synechococcus aquatilis* Strain SY01 at equimolar amounts (Fig. 7). The average cadmium uptake was also found to be practically the same for all systems as they fall within the standard deviation of 1.75 ppm. These results suggest the existence of a distinct binding site for cadmium in the cell surface.

Elucidation of an efflux mechanism

The efflux of toxic cations is an important mechanism of bacterial metal ion resistance. *Synechococcus aquatilis* Strain SY01 was found to be able to efflux 6.885 μg of cadmium ions after incubation with distilled deionized water.

Zn(II), Cd(II), Co(II), and Ni(II) resistance in *Alcacigens eutrophus* (Nies and Silver, 1989; Nies, 1992; Silver and Walderhaug, 1992), Cd(II) resistance in *Staphylococcus aureaus* (Tynecka et al., 1981) and arsenic resistance in *E. coli* are well known examples of efflux (Silver and Walderhaug, 1992). Most of these efflux mechanisms are thought to be plasmid encoded — thus it is possible that the cadmium efflux of *Synechococcus aquatilis* Strain SY01 has a plasmid encoded energy-dependent efflux mechanism.

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