Bull. Environ. Contam. Toxicol. (2002) 69:632–637

DOI: 10.1007/s00128-002-0107-5

Bulletin of Environmental Contamination and Toxicology

Application of Mutant Strains of Cyanobacteria for Cd^{2+} Removal

V. Raveender, J. Scaria, S. K. Verma

Biological Sciences Group, Birla Institute of Technology and Science, Pilani, Rajasthan 333 031 India

Received: 26 December 2001/Accepted: 5 August 2002

The current practice for the removal of soluble metal ions present in the industrial effluents includes simple chemical precipitation, ion exchange resins, solvent extraction and electro-winning. Low bulk metal concentration, ion specificity, narrow range of pH and poor settling colloidal properties may limit the effectiveness of these conventional processes (Kratochvil, Volesky 1998). In the last decade, considerable research has been carried out on the materials of biological origin for metal removal (Ting et al. 1991, Volesky, Prasetyo 1994, Uberoi, Shadman 1991). The phenomenon of retention of cations from aqueous solutions by biological material has been termed as 'biosorption' and the material as 'biosorbent'. The superiority of biosorbent over conventional material is well established, owing to their wide working pH range and non-specific metal binding. The mechanism of biosorption is little understood and the information available is largely qualitative, fragmented and rather limited. Removal of metal ions, in general, by microbes includes cellular adsorption and carrier mediated cellular uptake (Verma, Singh 1990, 1991). Both these processes contribute significantly in removal of metal ions from aqueous solutions. Wild type strains being sensitive towards elevated metal concentrations, may not be utilizing the potentials of later process as their metal-sensitive proteins are inactivated under such conditions. These limitations of metal toxicity to living cells provide the need to develop and use genetically modified microbes as biosorbents, which can function efficiently under field conditions. In this regard we have developed and characterized single and multiple metal resistant cyanobacterial strains (Verma, Singh 1991, 1995)

In the present paper we report our results on the cadmium biosorption efficiency of metal resistant strains of the cyanobacterium *Nostoc calcicola*.

MATERIALS AND METHODS

The strains used in the present study were filamentous cyanobacterium *Nostoc calcicola* and it's two mutant strains Met-R1, Met-R2 that were developed and characterized in our laboratory (Verma, Singh 1991, 1995). Cultures were gown in Allen Arnon's medium at 25 ± 1°C under continuous illumination of 75-80 $\mu E/m^2$ as described earlier (Allen, Arnon 1955). For obtaining dead biomass, the

Correspondence to: S. K. Verma

seven-day old cells of different strains were harvested by centrifuging at 3,000 rpm for 10 min before oven-drying at 80^0C for 12hrs. Such oven-dried cells were used in the present study as dead biomass.

The metal sensitivity of parent and two mutant strains of the cyanobacterium *N.calcicola* were determined by exposing the cells to the various concentrations of Cd^{2+} (1-5 m*M*). The growth medium was supplemented with desired level of $CdCl_2$ before inoculation. The flasks were incubated under culture room condition, as mentioned earlier for 200 hr. A 3.0 mL of sample was withdrawn at a regular interval of 20 hr and the sample dry weight/unit volume was used as the growth parameter. Growth of these strains has been expressed as specific growth rate constant (k) as described by Pant et al. 1992.

The cadmium removal experiment involving dead and viable biomass was conducted by inoculating seven-day old viable cells or the dead biomass obtained by such cells, to the growth medium supplemented with 2 m*M* of Cd^{2+}. The biomass density (live or dead) in the solution was maintained at 0.5 mg/mL. A 5.0 mL samples were withdrawn after 200 hr of incubation, centrifuged and the supernatant was subjected to cadmium estimation. The cadmium concentration was measured by using an Atomic Absorption spectrophotometer (Perkin Elmer, *A Analyst 300*). The Cd^{2+} removal is expressed as the amount depleted from the medium/ unit biomass.

The exponential phase cyanobacterial cells obtained by centrifugation and repeated washings, were suspended in 5% (w/v) solution of alginic acid (Sodium salt, Fluka) prepared in the growth medium as above. The mixture was pumped out drop wise in 0.2*M* $CaCl_2$ solution aseptically in a Laminar flow cabinet. The beads thus formed of cyanobacterial cells were subsequently harvested, washed several times with sterile triple glass distilled water and resuspended in a 20 mL volume of growth medium contained in a 250 mL cotton stoppered culture flasks. The beads thus formed were used in subsequent experiment for comparing the Cd^{2+} removal by free and immobilized cells. Such immobilized beads or free cells with the final biomass density of 0.5 mg/mL were inoculated in the growth medium supplemented with 2.0 m*M* of Cd^{2+} for 200 hr. The cell-free alginate beads were also subjected to the similar conditions and were used as control. The supernatant obtained after centrifugation was subjected to Cd^{2+} estimation as mentioned earlier. The difference between initial and final concentration is expressed as the amount of Cd^{2+} removed/unit free or immobilized biomass.

For the estimation of exopeptide secretion by immobilized strains of cyanobacteria, beads were remobilized by treating with sodium citrate (0.1%) and the supernatant obtained after centrifugation (3000 rpm; 10 min) was utilized for the quantification of exopeptides by following the method of Ellman 1959.

The metal partitioning in Cd^{2+} fed cells was studied by centrifuging the cells and the supernatant obtained was further concentrated in Vacuum Centrifuge (*Heto*, Germany). Such supernatant was subjected to Cd^{2+} estimation and the amount of

Cd^{2+} found in the supernatant was the reflection of extracellular chelation by the secreted exopeptides. The cells obtained in the previous step were subjected to repeated washing with EDTA (10 μM) and centrifuged. The amount of Cd^{2+} present in solution (EDTA washable fraction) was indicator of Cd^{2+} adsorption at the cell surface and is expressed as μg Cd^{2+} adsorbed / mg dry weight of the biomass. The biomass pellet obtained in the previous step was digested in HNO_3 : $HClO_4$ mixture (10:1 v/v) and centrifuged. The Cd^{2+} concentration found in the supernatant was the reflection of cellular accumulation of metal.

RESULTS AND DISCUSSION

The removal of metal ions from aqueous solution is a biphasic phenomenon involving an energy-independent bivalent metal ion exchange with counter ions present in cell wall (Hoyle, Beveridge 1984) and energy - dependent active uptake (Ting et al. 1991). The relative contribution of these two processes in metal removal can be demonstrated by comparing the sorption of metal ion by living and dead biomass as only viable cells can demonstrate energy - dependent phenomenon. The growth pattern of parent and two mutant strains of *Nostoc calcicola,* Met-R1 & Met-R2 shows that a 2.0 mM concentration of Cd^{2+} was completely lethal for parent whereas both the mutants could grow normally under the similar conditions (Fig. 1). A comparison of dead and viable cells of all the three strains exposed separately to 2.0 mM Cd^{2+} for 200 hr showed a clear superiority of viable biomass over the dead ones (Fig. 2). The two mutant strains with equal metal tolerance (2.0 mM) showed different behavior of metal removal; Met-R2 showing more than two fold higher cadmium removal than the Met-R1 strain. These data clearly indicate the presence of different mechanisms of metal resistance in the two mutant strains as described earlier by our group (Verma, Singh 1991, Verma, Singh 1995).

The immobilization of cyanobacterial cells is known to enhance various metabolic processes like photosynthetic O_2 evolution (Park et al. 1991), Nitrogen fixation activity, production of H_2 and NH_3 (Vincenzini et al. 1981) and Cu uptake and accumulation (Singh et al. 1989). A comparison of free cells and immobilized beads prepared from Met R2 strain and exposed over a period of 200 hr to the 2.0 mM Cd^{2+} solution showed almost 1.5 fold increase in Cd^{2+} removal by immobilized beads (Fig.3) thus, clearly establishing the efficiency of immobilization in Cd^{2+} removal. Such immobilized beads were used in further studies of Cd^{2+} removal.

The various mechanisms suggested for metal tolerance in microbes include cellular exclusion of metals by the secretion of exopeptides (Olafson 1984) or metal transport across the membrane (Verma, Singh 1995), extrusion of metal (Verma, Singh 1995) and immobilization at cell interior (Shehata, Whitton 1982). The Met-R1 strain falling in first category, may not allow sufficient Cd accumulation due to retarded Cd transport. The microbes are known to release small peptides and proteins extracellularly, when subjected to metal stress (Reddy, Prasad 1990). These peptides can bind the metal in the solution thus

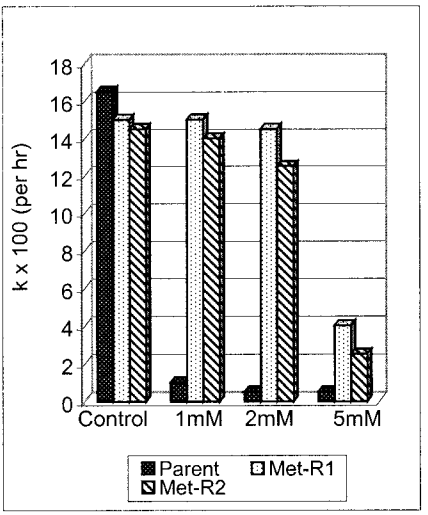

Figure 1. Growth pattern of parent, Met-R1 and Met-R2 of *Nostoc calcicola* exposed to different concentration of Cd^{2+} for 200 hr

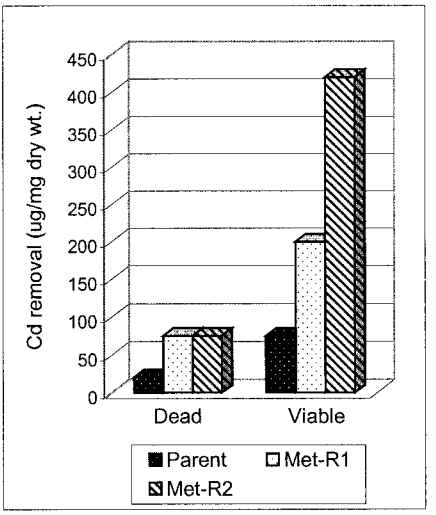

Figure 2. Cd^{2+} removal by dead and viable biomass of parent; Met-R1, Met-R2 strains of *Nostoc calcicola* exposed to 2.0 m*M* Cd^{2+} solution for 200 hr

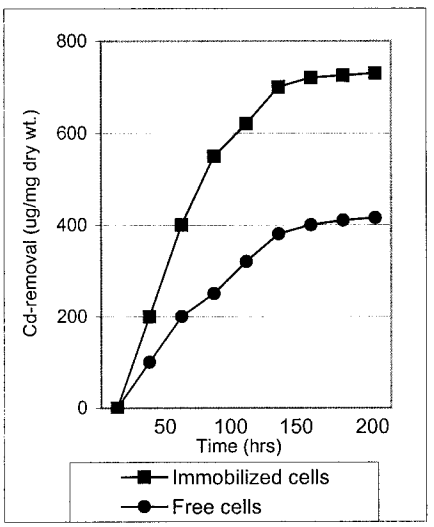

Figure 3. Cadmium removal by free and immobilized cells of Met-R2 strain of *Nostoc calcicola* exposed to 2.0 m*M* of Cd^{2+} for 200 hr

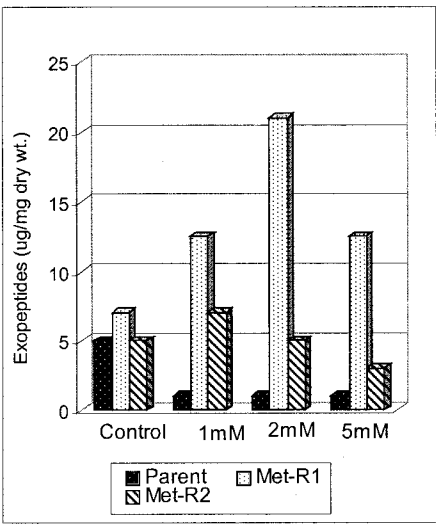

Figure 4.Exopeptide secretion by immobilized cells of parent and two mutants of *Nostoc calcicola* exposed to different concentration of Cd^{2+} for 200 hr

reducing their bioavailability/ toxicity (Olafson 1984). A comparison of exopeptide secretion by experimental strains with increasing Cd^{2+} concentration showed an enhancement in case of Met-R1 strain as compared to other two strains, which did not show a significant increase under similar conditions (Fig.4). Thus, the increased Cd^{2+} removal by Met-R1 may be partly attributed to the enhanced secretion of exopeptides. A study involving cellular partitioning was also conducted to confirm the role of various processes of Cd^{2+} removal by both the mutant strains.

The metal-ion specific cellular adsorption of Cd^{2+} was found to be maximum in case of Met- R2 (250 μg Cd^{2+}/mg dry wt.) contributing about 30% of the total Cd^{2+}-removed from the solution after 200 hr exposure (Table 1). The same process contributed only 16% in case of Met-R1 strain metal removal process.

Table 1. Partitioning of cadmium removal by different strains of cyanobacteria under immobilized conditions.

	Cadmium Removal (μg/mg dry weight)		
	Parent	*Met-R1*	*Met-R2*
Extracellular Chelation	20	175	20
Cellular adsorption	40	20	240
Cellular Accumulation	30	10	490
Total	90	205	750

The apparent superiority of immobilized cells of Met-R2 strain in Cd^{2+} removal over the parent or Met-R1 cells suggests that such system could be successfully applied to scavenge Cd^{2+} in repeated cycles without loss of cells.

Acknowledgments. We thank the Director, BITS for providing laboratory facilities, and to the Board of Research in Nuclear Sciences (Sanction No. 99/36/4/BRNS/1005), Mumbai for financial assistance.

REFERENCES

Allen MB, Arnon DI (1955) Studies on nitrogen-fixing blue green algae. I. Growth and niotrogen-fixation by *Anabeana cylindrica*. Plant Physiol 30: 366-372

Ellman GL (1959) Quantitative determination of peptides. Arch Biochem Biophys 82:70-77

Hoyle BD, Beveridge TJ (1984) Metal binding by the peptidoglycan sacculus of *E.coli* K-12. Canadian J Microbiol 30: 204-210

Kratochvil D, Volesky B (1998) Advances in the biosorption of heavy metals. Trends Biotechnol 16: 291-300

Olafson RW (1984) Prokaryotic metallothionein, Int J Pept Prot Res 24: 303-308

Pant A, Srivastava SC, Singh SP (1992) Methyl mercury uptake by free and immobilized cyanobacterium. Biol Metals 5: 229-234

Park IH, Rao KK, Hall DO (1991) Photoproduction of hydrogen, hydrogen

peroxide and ammonia using immobilized cyanobacteria. Int J Hydrogen Energy 16: 313-318

Reddy GN, Prasad MNV (1990) Heavy metal – binding proteins, peptodes: Occurrence, structure, synthesis and functions – A review Environ Exp Bot 30: 251-264

Shehata FHA, Whitton BA (1982) Zinc tolerence in in strains of blue-green algae *Anacystis nidulans*. British Phycol J 17: 5-12

Singh SP, Verma SK, Singh RK, Pandey PK (1989) Copper uptake by free and immobilized cyanobacterium. FEMS Microbiol Lett 60: 193-196

Ting YP, Lowson F, Prince IG (1991) Uptake of Cadmium and Zinc by Alga *Chlorela vulgaris*: Multi ion situation. Biotech Bioeng 37: 445-455.

Uberoi M, Shadman F (1991) High temperate removal of cadmium compounds using solid sorbents. Environ Sci Technol 25: 1285-1291

Verma SK, Singh SP (1990) Factors regulationg copper uptake in a cyanobacterium. Cur Microbiol 21: 33-37

Verma SK, Singh HN (1991) Evidence for Energy-dependent copper efflux as a mechanism of Cu^{+2} resistance in the cyanobacterium *Nostoc calcicola.* FEMS Microbiol Lett 84: 291-294

Verma SK, Singh SP (1995) Multiple metal resistanec in the cyanobacterium *Nostoc muscorum*. Bull Environ Contam Toxicol 54: 614-619

Vincenzini M, Balloni W, Mannelli D, Florenzano G (1981) A bioreactor for continuous treatment of wastewater with immobilized cells of photosynthetic bacteria. Experientia 37: 710-712

Volesky B, Prasetyo I (1994) Cadmium removal in a biosorption column. Biotech Bioeng 43: 1010-1015