Metallomic Analysis of *Nostoc calcicola* and its Applications in Bioremediation Technology

THESIS

Submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

By
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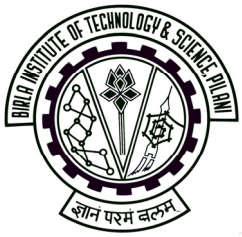
Under the Supervision of
Prof. S.K.Verma



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CERTIFICATE

This is to certify that the thesis entitled **"Metallomic Analysis of *Nostoc calcicola* and its Applications in Bioremediation Technology"** submitted by **S. Ramachandran, ID No. 2000PH29483** for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

Signature in full of the Supervisor

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Abstract

The present work was planned towards exploring the metal induced cellular responses, localization of metals in the cells and to identify stress proteins to understand metal-microbe interactions. The *Nostoc calcicola,* which displays multiple phenotypic characteristics, large genome size and is amenable to genetic manipulation, makes an excellent model organism to study metal inducible protein expression and genetic engineering for bioremediation technology. *N. calcicola* was studied in detail for metal localization and stress regulation using electron microscopy, DNA and protein profiles. Superoxide dismutase and malate dehydrogenase enzymes were overexpressed under Zn and Cr stress. Zn tolerant *N. calcicola* cells showed reduced metal uptake and energy dependent metal efflux as a mechanism to tolerate cobalt, zinc and cadmium (*czc*). Cr(VI) and Zn(II) induced proteins were subjected to trypsin digestion and identification by MALDI-TOF/MS. The protein were identified as Malate Dehydrogenase (MDH), a Methyl-Accepting Chemotaxis Protein (MCP) and Ribulose-1,5-bisphosphate carboxylase/ oxygenase (RubisCO). The mouse metallothionein gene (MT-I) was cloned in frame with the strong promoter *psbA*. The recombinant plasmid pRSKV12 expressing metal sequestering protein metallothionein was used to transform *N. calcicola* cells. The genetically engineered strain showed resistance to metals and enhanced metal accumulation. Such cells were encapsulated on the newly developed silica coated calcium alginate and mesoporous silica gel. The immobilized cells of recombinant *N. calcicola* were superior in terms of metal accumulation and their repeated use for metal removal and recovery. An up-flow columnar bioreactor packed with immobilized *N. calcicola* could treat *ca.* 32 L of simulated copper containing industrial effluent. Recombinant *N. calcicola* has potential applications in bioremediation technology.

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List of Abbreviations

AAS-Atomic Absorption Spectrophotometer
BCA-Bicinchoninic Acid
CAG-Calcium Alginate Gel
CBB-Coomassie Brilliant Blue R-250
DCCD- N,N'-dicyclohexylcarbodiimide
DCMU-3-(3,4-dichlorophenyl)-1,1-dimethylurea
DPC- Diphenyl carbazide
DTT-Dithiothreitol
FCCP- Carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone
IPTG- Isopropyl-ß -D-thiogalactopyranoside
IPTG-Isopropyl-beta-D-thiogalactopyranoside
LB-Luria Bertani Broth
MALDI-TOF-Matrix Assisted Laser Desorption Ionization-Time of Flight
MCS-Multiple cloning site
NBT-Nitroblue tetrazolium chloride
pCMB- p-Chloromercuricbenzoate
PMSF-Phenylmethanesulfonyl fluoride
ROS-Reactive oxygen species
SDS-PAGE-Sodium Dodecyl Sulphate Polyaccrylamide Gel Electrophoresis
SEM-Scanning electron microscopy
Si-CAG-Silica Coated Calcium Alginate Gel
TEM-Transmission electron microscopy

Chapter I

General Introduction

Chapter I
GENERAL INTRODUCTION

The heavy metals and radionuclides pollution from nuclear power plants, mining industries, electroplating industries and agricultural runoffs is a major cause of concern to public health, animals and ecosystem (Rehman et al. 2007 and www.epa.gov). Thus discharge of industrial wastes containing heavy metals in the environment results in bioaccumulation in animals and human beings through food chain. The metal containing liquid wastes are amenable to treatment before being discharged into the environment. For treating such metal laden effluents techniques like reverse osmosis, ion exchange, precipitation, electrochemical treatment or evaporation has been routinely employed (Kuyucak, 1997). These conventional technologies are very expensive for removal of metal ions from solutions, particularly when metal ions are present at very low concentrations and in large volumes (Holan et al. 1993). Most environmental regulations on industrial wastewater treatment have established quality requirements for their disposal (http://envfor.nic.in/; www.epa.gov). To meet the water quality standards consistent with environmental protection laws, industries require systems that can simultaneously remove many contaminating metals and radionuclides from the wastewater (Ashley and Roach, 1990; Malik, 2004).

The processes using materials of biological origin to remediate environmental pollutants is described as “bioremediation”. The bioremediation of environmental pollutants are comparatively less expensive to achieve remediation of the target metals. Microorganisms and plants were studied extensively for their possible applications in treating metal laden wastes and these methods have their own merits and demerits for implementation (Valls and Victor de Lorenzo, 2002). Microorganisms are usually selected based on their capability to sequester, degrade and/or transform the pollutant. Microbes take up metal ions and entrap them in the cell structure; the phenomena of microbial metal accumulation occur by initial binding onto the cell wall through the thiol, hydroxyl, amino, phosphoryl and sulphate groups exposed on the cell surface. This method of uptake is independent of the biological metabolic cycle and is known as

"biosorption" or "passive uptake". Metals can also pass into the cell across the cell membrane by "active uptake". The metal uptake by both active and passive modes is termed as "bioaccumulation". Most of the studies dealing with microbial metal remediation with live cells describe the biphasic uptake of metals, i.e., initial rapid phase of biosorption followed by slower, metabolism dependent active uptake of metals (Verma and Singh, 1991). The metal bioremediation technology involving mobilization and immobilization has been demonstrated to work at low metal concentrations (≤ 5 mg L^{-1}) (Gadd, 2000). Earlier reports involving wild type or transgenic bacterial strains, free living or under immobilized condition (Barkay and Schaefer, 2001) and algae have shown to concentrate metal species several folds (Ting et al. 1989; Ting et al. 1991). They not only accumulate potentially toxic metal ions from the solutions (Al-Saraj et al. 1999) but also offer possible metal recovery (Khoshmanesh et al. 1996; Rollemberg and Goncalves, 2000; Lodi et al. 1998). This has led to the development of genetic engineering approaches to optimize potential enzymes and metabolic pathways relevant to bioremediation technology.

The application of microorganisms to detoxify metals has been tested in a number of systems, but the cell viability and metabolic activity of cells pose as the major limiting factor affecting the detoxification efficiency of cellular biomass and enzymes involved. Thus, microorganisms destined for environmental applications need to be characterized not only by their characteristic genome and proteome, but also by the distribution of metals in a cell, "metallome" (Szpunar, 2004). The metallome refers to the entirety of individual metal species in a cell and encompass the inorganic (free and complexed) element content and the ensemble of its complexes with biomolecules, and especially with proteins, "metalloproteome". Thus, deciphering a metallome can explore distributions of the metal, the biomolecules involved in incorporation or complexation and the concentrations of the metals present. Recently, Haraguchi (2003) suggested the term "metallomics" to denote metal-assisted function biochemistry and postulated it to be considered at the same level of scientific significance as genomics or proteomics. The metallomic information comprises the identities of the individual metal species and their concentrations in a cell (Szpunar, 2004). The species of interest for metallomics will

include complexes of trace elements with endogenous or bio-induced biomolecules such as organic acids, proteins, sugars or DNA fragments (Szpunar, 2004).

In order to gain an insight into the scientific field of ‘‘metallomics’’, a schematic model of the biological system is illustrated in Fig. 1. Haraguchi, (2003) reported the integration of various scientific fields related to biometals that have been independently established so far, under the term ‘‘metallomics’’ with emphasis on the identification of biomolecules bound with metal ions as well as on their functions in the biological systems. The bacterial metallome consists of metals required in appreciable quantities as well as those metals required in trace amounts (Barton et al. 2007; Silver and Phung, 1996). Recently, Bhargava et al. (2006) reported the “cuprome”, the cellular response to copper in the cyanobacterium *Anabaena doliolum*. The metallome of anaerobic bacterium *Desulfovibrio desulfuricans* was reported by Barton et al. (2007). The metallomics approach has assisted in finding the novel gene nicotianamine synthase responsible for metal transport in a hyperaccumulating plant and this gene was overexpressed in yeast and the metabolite, nicotianamine as was identified earlier in vivo by a metabolomics approach, provided not only resistance to heavy metals but also intracellular bioaccumulation (Szpunar, 2004). Thus, metallomic analysis include deciphering of information related to metal binding, distribution and its incorporation in the cells by means of ultrastructural studies, proteins and biomolecules which have direct or indirect role in the metal accumulation and normal functioning of the cells including under metal stress conditions.

Algae including cyanobacteria were considered as biomonitors of heavy metal pollution (Whitton, 1984) and play a major role in metal detoxification through metal binding proteins (Hill et al. 1994a). They possess intricate mechanisms that ensure their survival in harsh conditions on which they grow and ability to tolerate at toxic concentrations of heavy metals (Verma and Singh, 1990; Potts, 2001). Cyanobacterial heavy metal resistance and metal accumulation properties have been reviewed by Singh (1993) and Ehrlich (1997). Recently, Baptista and Vasconcelos (2006) reviewed the cyanobacteria-metal interactions and possible environmental applications.

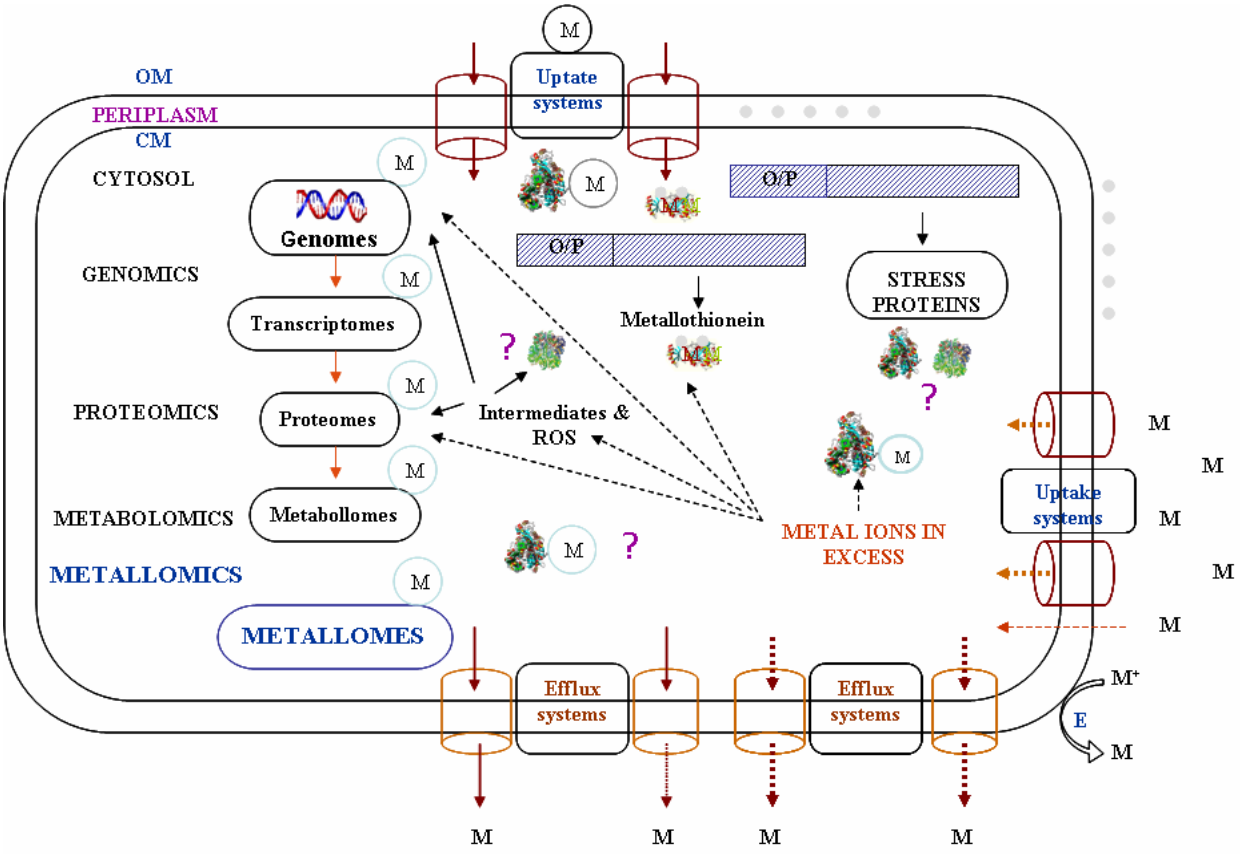


Fig 1. The schematic diagram shows metal-microbe interactions and the metallome. Trace metals are essential for normal growth. Metal ions from the extracellular environment enter the cell via specific and non-specific transporters. They are incorporated into metalloproteins and excess metal ions are transported out by the highly efficient efflux systems. The cysteine rich low molecular weight protein "metallothioneins" sequester essential metal ions to maintain total metal content of the cell. Metallothioneins are overexpressed under metal stress conditions to overcome metal toxicity. Generally, the metal stress in a cell leads to formation of reactive oxygen species (ROS) and other intermediates which disrupt normal cellular processes via interaction with the genome and proteome. The "stress proteins" are expressed in response to excess metals and ROS which play a major role in protein folding and detoxification mechanisms. The cell wall proteins, polysaccharides and fatty acids offer charged functional groups like phosphate, sulfate, amino, hydroxyl groups that favour adsorption of metals (biosorption) at the cell wall. Extracellular or intracellular enzymes are also involved in the reduction of highly toxic metal ions to less toxic forms (biotransformation). M- metal ions; E- enzyme; OM- outer membrane; CM- cytoplasmic membrane; O/P- operator-promoter.

In the aquatic environment, cyanobacteria act as biological sorbents because of their ability to bind dissolved metals, thus playing an important role in metal sequestration and affecting metal speciation (Cavet et al. 2003; Prasad and Pandey 2000; Dittrich and Sibler 2005; Kretschmer et al. 2004; Yee et al. 2004; Phoenix et al. 2002).The cyanobacterial cell wall possess carboxyl, phosphoryl, hydroxyl and amine functional groups that have been shown to deprotonate and bind metal ions to form metal-ligand surface complexes (Yee et al. 2004). In mucilage producing cyanobacteria it has been suggested that the binding of metals take place through the polysaccharides (Phoenix et al. 2002). Studies with *Gloeothece magna*, from which the extracellular polysaccharides (EPS) and envelope polysaccharides were extracted, have shown the ability of these polymers to bind Cd^{2+} and Mg^{2+} (Mohamed, 2001). The EPS produced by *Anabaena spiroides* have affinity to bind Mn^{2+}, Cu^{2+}, Pb^{2+} and Hg^{2+} (Freire-Nordi et al. 2005). The possible applications of cyanobacterial EPS have been thoroughly described by De Philippis and Vicenzini (1998) including applications in metal bioremediation. Analysis of *Anabaena flos-aquae* fractions of peptidoglycan has indicated that Cu^{2+} is coordinated by amine and carboxyl ligands, while in whole cell samples is coordinated by phosphate, carboxyl and amine ligands (Kretschmer et al. 2004). Studies with the *Calothrix* sp. KC97 combining Cu^{2+}, Cd^{2+} and Pb^{2+} sorption experiments, have shown that the reactive sites on surfaces are heterogeneously distributed between the exopolymer sheath and cell wall (Yee et al. 2004 and Phoenix et al. 2002). The carboxyl groups on the cyanobacterial cell wall are the dominant reactive sites and represent the most important sink for metal ions at near neutral pH (Yee et al. 2004). For example, *Spirulina platensis*, a cyanobacterium, was determined to contain detectable levels of mercury and lead when grown under contaminated conditions (Slotton et al. 1989), implying that this cyanobacterium was taking up the toxic metal ions from its environment. Further studies confirmed that these cyanobacteria both absorb and take up metal ions (Bender et al. 1994). Other reports also indicate carboxyl groups on algal cell biomass as being responsible for binding to various metal ions (Gardea-Torresdey et al. 1990). The intracellular polyphosphates are responsible for metal sequestration as well as algal extracellular polysaccharides serving to chelate or bind metal ions (Zhang and Majidi, 1994; Kaplan et al. 1987; Van Eykelenburg, 1978). Strains of *Synechocystis* have been proven to develop a thickened

calyx when exposed to copper-stressed growth conditions to bind the excess copper onto the cell wall (Gardea-Torresdey et al. 1996). The role of cyanobacterial exudates which are released upon exposure to metals and they also shown to have a role in metal detoxification mechanisms (Gouvea et al. 2005; Freire-Nordi et al. 2005; Singh et al. 1999; Miao et al. 2005; Heng et al. 2004; Garcia-Meza et al. 2005).

The availability of genomic sequences accelerate the identification and characterization of cyanobacterial genes. The completed genome sequence of *Anabaena* PCC 7120 suggested a putative Zn exporter similar to the one described for *Synechocystis* sp. PCC 6803 (Cavet et al. 2003; Liu et al. 2005). For *Synechocystis* PCC 6803, it has been suggested that Co^{2+} may also be a substrate of Zn-CPx-type ATPases. Examples of these can be found for the cyanobacteria *Synechocystis* sp., and *Nostoc* sp. (Rutherford et al. 1999). Energy-driven efflux pumps (ATPases) have been shown to be the most common (Silver and Nakamura, 1994) and members of this group were characterized in a variety of organisms, including *Synechocystis* PCC 6803 and *Oscillatoria brevis* (Arnesano et al. 2002). In the *Synechocystis* PCC 6803 a gene encoding a polypeptide, with sequence features of metal transporting P-type ATPases, whose transcription is induced by Zn alone, has been confirmed (Thelwell et al. 1998). A gene cluster involved in Ni^{2+}, Co^{2+}, and Zn^{2+} sensing and tolerance in the *Synechocystis* PCC 6803 has been identified (Garcia-Dominguez et al. 2000; Tong et al. 2002). Physiological studies have indicated that *Synechocystis* sp. PCC 6803 (Tottey et al. 2001) and *Synechococcus* PCC 7942 (Kanamaru et al. 1994) posseses two CPx-type ATPases that can serve both in the extrusion and uptake of Cu (Lloyd, 2003; Barkay et al. 2003). The cation diffusion facilitator (CDF) proteins are widely distributed among bacterial kingdom, e.g., Archaea to bacteria and cyanobacterial species like *Synechocystis* sp., *Nostoc* sp., and they are involved in the transport of Zn^{2++}, Co^{2+}, Ni^{2+}, Cd^{2+} and Fe^{2+}. The metal transport across the membranes by CDF is driven by concentration or chemiosmotic gradient and CDFs are primarily involved in Zn^{++} resistance.

Zn metallothionein SmtA is found in *Synechococcus* sp. PCC 7942 and it is highly expressed in response to elevated concentrations of Zn, but also to Cd and Cu (Robinson,

1989; Turner, et al. 1995). Zn metallothionein-like sequence has been shown in the genome of *Anabaena* PCC 7120 and *Synechocystis* PCC 6803 (Turner and Robinson, 1995; Blindauer et al. 2002; Robinson et al. 2001). *Synechocystis* PCC 6803 and *Synecchocystis* PCC 7942 have related Zn-responsive repressor proteins ZiaR and SmtB. In elevated cytosolic Zn concentrations these proteins dissociate from the O/P regions of their target gene ZiaA and SmtA and trigger expression of structurally different proteins with distinct consequences for the cellular distribution of Zn i.e., export into the periplasm or intracellular sequestration (Thelwell et al. 1998). *Nostoc linckia* has seemed to be tolerant to Zn and Cd because, among other mechanisms, these metals are sequestered via metal binding proteins (El-Enany and Issa 2000). *Anabaena doliolum* synthesizes a low molecular weight Cd-induced protein that confers co-tolerance to metals and provides multiple-tolerance to environmental stresses such as heat and cold shocks (Mallick and Rai, 1998). Genomic sequence data provide an opportunity for global monitoring of changes in genetic expression at transcriptional and translational levels in response to variations in environmental conditions. However, a comprehensive understanding of bacterial mechanisms of heavy metal toxicity and resistance has yet to be achieved (Eoin et al. 2005; Chourey et al. 2006; Latmani et al. 2005). The whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus* (Eoin et al. 2005; Chourey et al. (2006), *Shewanella oneidensis* MR1 (Latmani et al. 2005; Ackerley et al. 2006) and in *Escherichia coli* K-12 has shown up- or down regulation of several genes.

The proteome analysis of microbes represent a powerful tool for the study of acclimation to environmental stress conditions because the newly synthesized or enhanced expression of certain proteins called “stress proteins” can be identified (Frausto da Silva and Williams, 2001; Barton et al. 2007). In particular, the proteomic data is vital, as the observed phenotype is a direct result of the action of the proteins rather than the genome sequence (Bhurja et al. 2003). The majority of the cyanobacterial proteomics work carried out so far has been directed towards *Synechocystis* PCC 6803, *Nostoc commune* DRH1, *Nostoc punctiforme* PCC 73102, *Nostoc* PCC 7120 and *Microcystis aeruginosa* PCC 7806 (refer the review of Bhurja et al. 2003). The preliminary analysis of

"cuprome" of *Anabaena doliolum* using two- dimensional gel electrophoresis reported 26 up and 36 down regulated proteins upon exposure to copper at 20 μM (Bhargava et al. 2006). The results of these studies have demonstrated the importance of applying multiple methodologies toward fuller understanding of cellular response to heavy metal exposure. The complexity of cyanobacterial signal perception under environmental stimuli is best reviewed by Meeks and Elhai (2002). *Nostoc punctiforme* ATCC 29133 (http://www.jgi.doe.gov), a symbiotic cyanobacterium possess a 10 Mb genome with 7432 predicted ORFs (Meeks et al. 2001) and it is considered to be the representative cyanobacteria to possess most of the genes which are common to all cyanobacterial species. The availability of genomic information paves the way for use in proteomics as a powerful tool for large-scale comparison of *Nostoc* proteins for the identification of similar proteins from other *Nostoc* sp., (Hunsucker et al. 2004; Klint et al. 2006).

The present work is aimed at understanding the *Nostoc calcicola*-metal interactions, metal localization in the cell, identification of metal inducible proteins and to develop a recombinant strain which can be used for heavy metal removal and recovery from industrial effluents.

The thesis has been organized into five chapters. Apart from general introduction, second chapter describes metal inducible proteins and metal resistance in *Nostoc calcicola*. Third chapter presents the development of recombinant *N. calcicola* for metal bioremediation. The industrial applications of recombinant *N. calcicola* are described in chapter four whereas chapter five summarizes the overall results of the present investigation.

Chapter II

Metal Inducible Proteins and Metal Resistance in N. calcicola

Chapter II

METAL INDUCIBLE PROTEINS AND METAL RESISTANCE IN *NOSTOC CALCICOLA*

2.1 Introduction

Cyanobacteria require trace metals for their normal growth; they play a major role in catalysis, protein structure and modulate the cell signaling events through metal regulated protein expression. Intracellular metal concentrations and its incorporation into metalloproteins are controlled by membrane transporters and chaperones (Robinson et al. 2001; Silver and Phung, 1996). Metals at high concentrations bind to thiol groups, which may lead to the cell toxicity. Several bacterial species grow in the presence of toxic metals exerting considerable stress on metal homeostasis as they mimic the structure of essential metal ions (Nies, 1999; Hughes and Poole, 1998).

To overcome the prolonged metal toxicity, microorganisms overexpress existing metal transporters incorporate modifications in cell wall structure and/ or ameliorate through metal binding proteins (Robinson, 1989). The accumulated metals are transported into different cellular compartments (Mohamed, 2001; Yee et al. 2004; Phoenix et al. 2002), periplasm (Kretschmer et al. 2004), cytoplasm (Kretschmer et al. 2004), polyphosphate bodies (Kretschmer et al. 2004) and metal binding protein (Turner et al. 1995). In addition to these mechanisms, they express stress proteins. These proteins assist cellular functions by repairing the damaged cellular components, proteins, cell membrane, cell wall and the genetic material (Cavet et al. 2003; Mergeay et al. 2003).

Understanding of toxicological and physiological responses of a cell to different metals is important when attempting to genetically engineer them for any biotechnological applications (Latmani et al. 2005). Several bacteria including *E. coli* (Ackerley et al. 2006), *Shewanella oneidensis* (Eoin et al. 2005), *Pseudomonas* (Eoin et al. 2003) and *Bacillus* (Chourey et al. 2006) have been studied for their responses to metals by highthroughput analysis that have led to catalog stress specific gene expression (Latmani

et al. 2005; Ackerley et al. 2006; Eoin et al. 2005; Chourey et al. 2006; Latmani et al. 2005). These data sets highlight the significance of total cellular components under external influence. However, a comprehensive understanding of bacterial mechanisms of heavy metal toxicity and resistance has yet to be achieved (Eoin et al. 2005; Chourey et al. 2006; Latmani et al. 2005).

Proteome analysis represents a powerful tool for the study of acclimation to environmental stress conditions because newly synthesized or enhanced expression of certain proteins called stress proteins can be identified. The phenotype is a direct result of the action of the proteins rather than the genome sequence alone (Bhargava et al. 2006; Hunsucker et al. 2004; Klint et al. 2006; Bhurja et al. 2003) and provides the basis for improved understanding on metal utilization and its environmental impact (Frausto da Silva and Williams, 2001; Barton et al. 2007). Surveys performed on complete annotated genomes of prokaryotes revealed the differences in total number of genes and the complexity of eco-physiology of the bacterium (Meeks and Elhai, 2002; Ashby, 2004). The total number of all signal transduction proteins increases for most bacteria as a square of the genome size (Galperin, 2005).

Genome sequences of the cyanobacteria have revealed that they possess large genome size (*ca.* 10 Mb) and a variety of two-component proteins to regulate responses to the environment stress conditions (http://www.jgi.doe.gov; Meeks et al. 2001; Dufresne et al. 2003; Mizuno et al. 1996; Ohmori et al. 2001; Sazuka, 2003; Bhargava et al. 2006; Mallick and Rai, 1998). In order to best understand cyanonobacteria-metal interactions, compartmentalization of metals at elevated concentrations and to study metal inducible proteins, the "metallome" of *Nostoc calcicola* was investigated.

2.2 Materials and Methods

2.2.1 *Nostoc calcicola* and culture conditions

Wild type strain of *N. calcicola* obtained from the National Centre for conservation and Utilization of Blue-Green Algae, IARI, New Delhi was grown in Allen and Arnon's (AA) medium (Allen and Arnon, 1955) free from any nitrogenous compounds and was

maintained in the culture room illuminated with cool day light fluorescent tubes (14.4 W m^{-2}) at 25±2 ^{o}C. Exponentially grown *N. calcicola* cells were harvested by centrifugation at 6000 rpm at RT for 10 min for sub-culturing. Stock cultures of axenic *N. calcicola* on 1.5% agar slants were preserved under culture room conditions.

2.2.2 Growth

The growth of *N. calcicola* was monitored in terms of total protein or Chl *a* content.

2.2.2.1 Determination of proteins

Protein content of the cultures was determined by incubating *N. calcicola* onto fresh AA medium; 1 ml culture was withdrawn for every 24 h and used for protein determination. The protein content was estimated by the method adopted by Lowry et al. (1951) and modified by Herbert et al. (1971). 1 ml of the culture was taken in a clean test tube, 1 ml of 1N NaOH was added and placed in a boiling water bath for 5 min. The tubes were cooled in a running tap water and 5 ml of alkaline copper tartrate solution was added and incubated for 10 min at RT. 1 ml of Folin's reagent was added and the intensity of blue color formed was measured after 20 min at 650 nm (Jasco V570 Spectrophotometer). The concentration of the protein was estimated from a calibration curve prepared using lysozyme as standard.

2.2.2.2 Determination of chlorophyll *a*

Total chlorophyll *a* (Chl *a*) content of *N. calcicola* was determined by the method described by McKinney (1941). 1 ml culture was withdrawn, the cells were centrifuged in 1.5 ml micro centrifuge tube; cell free supernatant was discarded leaving ca. 100 µl along with cell pellet. 900 µl of 100% methanol was added, vortexed and incubated in dark at 4 ^{o}C for 12 h. The contents were briefly spun at 10000 rpm (Eppendorf Centrifuge 5415D) to remove cell debris. Chl a in the supernatant was measured at OD_{665} and the readings were multiplied with a factor 12.7 which gave concentration of chl a in µg ml^{-1} culture.

2.2.3 Determination of protein content for electrophoretic separation

Protein concentrations for electrophoretic separation and protein sequencing were determined by using Bicinchoninic acid (BCA) method using QuantiPro BCA assay kit,

Sigma. 1 ml of cell lysate was mixed with 1 part of a protein sample with 1 part of the QuantiPro working reagent. The working reagent was prepared by mixing 25 parts of QA (solution containing sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.2 M NaOH, pH 11.25) with 25 parts of QB (4% bicinchoninic acid, pH 8.5); after a brief incubation at RT, 1 part of reagent QC (4% copper(II) sulfate) was added and mixed well until it was uniform in color. Protein standards (stock standard 1 mg^{-1}, bovine serum albumin, BSA) were prepared with a range between 5 – 30 $\mu g\ ml^{-1}$ and made upto 1 ml using MilliQ water. The unknown samples of the cell lysate were made in the similar way. 1 ml of QuantiPro working reagent was added to 1 ml of each protein standard, blank (water), and unknown samples. The contents were mixed well and incubated at 60 ^{o}C water bath for 1 h. The absorbance was measured at 562 nm after cooling the tubes at RT. The net absorbance of the samples was found by subtracting absorbance of the reagent blank. Concentration of the protein was estimated from a calibration curve prepared using known concentrations of BSA.

2.2.4 Metal salts and stock solutions

Metal stock solutions were prepared using analytical grade metal salts in the form of chlorides or sulphates of Cd, Co, Cr, Cu, Mn, Ni, Sr, and Zn dissolved in MilliQ water; U in the form of uranyl nitrate (BDH, England) was used. If required, metal solutions were acidified with 1 – 2% nitric acid and stored in dark conditions. Metal solutions and working standards were prepared freshly from the stock. Atomic spectral grade standards were procured from National Physical Laboratory (NPL), New Delhi.

2.2.5 Metal ion analysis

2.2.5.1 Atomic absorption spectrometry (AAS)

Metal ion concentrations were determined in a Perkin Elmer (AAnalyst 300) atomic absorption spectrophotometer using air-acetylene or nitrous oxide-acetylene flame. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. AAS was first calibrated with standard metal solutions in the range covering concentrations of metals likely to be found in samples. Samples were analyzed in triplicates to maintain reproducibility. The peak wavelength (nm) for determination of

metals by AAS: Cd 288.8; Co 240.7; Cr 357.9; Cu 324.8; Mn 279.5; Ni 232.0; Sr 460.7 and Zn 213.9 nm.

2.2.5.2 Determination of Cr^{6+} by DPC method

Hexavalent chromium was determined by diphenylcarbazide (DPC) method as described by Eaton et al. (1995). The reaction mixture was set up in an eppendorf tube containing the following: 200 µl of sample or standard Cr(VI) solution, 400 µl of 20 mM MOPS buffer (pH 7.0), 33 µl of 3 M H_2SO_4, 40 µl of 0.25% DPC and 32.7 µl of water. The absorbance maxima for the color developed was 540 nm.

2.2.5.3 Determination of $(UO_2)^{2+}$

Uranium in the form of $(UO_2)^{2+}$ was determined by spectrophotometric method (Genç Ö et al. 2003). A measured volume of solution containing U was mixed with 10% sodium salicylate solution. The color developed was measured spectrophotometrically at 468 nm. Concentration of U in the samples was estimated from a calibration curve prepared using known concentrations of uranyl nitrate standards.

2.2.6 Determination of metal resistance in *N. calcicola*

2.2.6.1 Metal toxicity

Sensitivity of *N. calcicola* towards metals was determined by plating on nutrient agar plates supplemented with respective metals at various concentrations. The exponentially grown cultures were harvested, washed to remove culture medium components, briefly sonicated and allowed to recover in fresh culture medium for 24 h. Such cells were centrifuged and cell density was adjusted to *ca.* 5 µg ml^{-1} chlorophyll concentrations (1µg Chl *a* corresponds to approximately 3 x 10^6 cells), a 50 µl was plated on 1.5% AA agar plates containing different concentrations of metals; after 15 days of incubation under culture room conditions, the number of colonies formed was counted. It was repeated in liquid culture medium and cell viability was tested with 1% TTC solution. Growth of *N. calcicola* on metal supplemented AA medium was monitored regularly in terms of protein and chl *a*.

2.2.6.2 Antibiotic resistance

Antibiotic resistance in the *N. calcicola* was tested by spread plating on 1.5% AA agar plates supplemented with respective antibiotics (kanamycin, chlorampenicol, Neomycin

and Streptomycin) in concentrations ranging from 0.5 – 50 μg ml^{-1}. The plates were incubated under culture room conditions as described in 2.2.1 for 15 days.

2.2.6.3 Plasmid and genomic DNA isolation

Plasmid DNA isolation: A 30 ml culture of exponential phase *N. calcicola* was harvested, washed with sterile Millipore water, centrifuged, and the cell pellet was again spun to remove traces of water. It was resuspended in 3 ml of B1 (200 mM sucrose, 50 mM Tris-Cl, 1 mM EDTA, pH 8.0). The mixture was incubated at 37 ^{o}C for 10 min followed by the addition of 200 μl lysozyme (100 mg ml^{-1}), 50 μl of 1% hemicellulase extract (Sigma), 10 μl of ribonuclease (20 mg ml^{-1}) and incubated at 37 ^{o}C for 2 h. The progress of lysis of the filaments was observed under a light microscope. 200 μl of B2 (250 mM EDTA and 50 mM Tris-Cl, pH 8.0) was added and allowed to stand at RT for 5 min, 90 μl of B3 (20% SDS, 50 mM Tris-Cl, 1 mM EDTA, pH 8.0) was added and incubated at RT for 30 min to achieve complete cell lysis. 75 μl of 3 N NaOH was added and mixed by gentle inversion followed by incubation at RT for 5 min. Immediately, 150 μl of B4 (2 M Tris-Cl, pH 7.0) and 900 μl of ice cold B5 solution (3 M potassium acetate, pH 5.2) was added, mixed well and incubated on ice for 20 min. The precipitate was dislodged by inverting the tube before centrifugation at 10000 rpm at 4 ^{o}C for 20 min. Supernatant was transferred to a fresh tube and centrifugation was once repeated. The clear cell lysate was either loaded onto a Qiagen column (section 3.2.4.1) or proceeded to phenol:chloroform extraction. Plasmid DNA from the supernatant was precipitated by addition of 1 volume of propanol and centrifugation at 10000 rpm at 4 ^{o}C for 20 min. DNA pellet was washed with ice-cold 70% ethanol, air dried and resuspended in 50 – 100 μl of 10 mM Tris-Cl (pH 8.0), aliquots were stored at -20 ^{o}C. Plasmid DNA was electrophoresed (3.2.4.2) on 1% agarose gel; results were documented using Syngene gel documentation system.

Genomic DNA isolation: *N. calcicola* cultures were harvested, washed once with 5 M NaCl, and the cell pellet was resuspended in P1 (100 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8.0); incubated at RT for 10 min. Sarkosyl was added to a final concentrations of 0.1%, mixed gently and placed on a shaker at the lowest setting for 30 min. The contents were centrifuged, the cell pellet was mixed with P2 (50 mM Tris, 5 mM EDTA, 50 mM NaCl, pH 8.0), lysozyme was added to 1 mg ml^{-1} and incubated at 37 ^{o}C water

bath for 30 min. SDS was added to the mixture at 1% and gently inverted to ensure complete lysis of the filaments and kept in the water bath for 10 min, proteinase K was added to the cell lysate at 100 µg ml^{-1} and samples were incubated at 37 ^{o}C for 1 h. The contents in the tubes were extracted with phenol:chloroform:isoamyl alcohol and an additional chloroform:isoamyl alcohol extraction. Genomic DNA from the supernatant was precipitated by the addition of 2/3 volume of 5 M NaCl and 1 volume of isopropanol. The clumps of genomic DNA were transferred carefully into fresh sterile tube, washed with 70% ethanol, air dried and dissolved in 10 mM Tris-Cl (pH. 8.0) and stored at -20 ^{o}C. Purity of the genomic DNA was quantified spectrophotometrically at 260/280 nm.

2.2.6.4 Restriction enzyme digestion

The purified plasmid DNA was used for digestion with methylation sensitive and insensitive restriction enzymes (MBI Fermentas, USA). 1 µg of DNA was used for each reaction after 1 h of incubation at 37 ^{o}C; restriction enzyme digested DNA samples were electrophoresed on 1% agarose gel and the results were documented.

2.2.7 Metal induced cellular responses

2.2.7.1 Metal induced tolerance

Nostoc calcicola grown on AA medium containing 500 µM $ZnCl_2$ was subcultured for several generations under metal stress conditions. Zn(II) stress adapted cells were distinct from wild type *N. calcicola* in terms of their growth pattern, protein, chlorophyll content, and metal accumulation properties. Such trained cells were used for studies on metal accumulation, metal efflux, and identification Zn-stress induced proteins to determine the mechanism of adaptation to environmental stress.

2.2.7.2 Metal uptake

The exponentially grown wild type and Zn-adapted *N. calcicola* were used to evaluate the efficiency of Cu^{2+}, Cd^{2+}, Cr^{3+}, Cr^{6+}, Co^{2+}, Mn^{2+}, Sr^{2+}, $(UO_2)^{2+}$ and Ni^{2+} bioaccumulation at pH 7.0 (Verma and Singh, 1990). 50 ml of 10 mM phosphate buffer (pH 7.0) containing respective metal and metal less control was inoculated with an equivalent of 400 µg protein ml^{-1} in conical flasks which were placed in an orbital shaker at 150 rpm at 25±2 ^{o}C for 2 h in the culture room conditions. Samples were withdrawn after

equilibration and centrifuged; the cell free supernatants were used for metal ion analysis by AAS.

2.2.7.3 Metal efflux

The exponentially growing wild type and Zn-adapted *N. calcicola* were used to evaluate the efficiency of metal efflux at pH 7.0. 50 ml of 10 mM phosphate buffer (pH 7.0) was inoculated with 400 µg protein ml^{-1} of cells (equilibrated in the presence of metals at 50 mg L^{-1} for 1 h) in conical flasks which were placed in an orbital shaker at 150 rpm at 25±2 ^{o}C in the culture room conditions. Samples were withdrawn after 1 h of equilibration and centrifuged. Total metal content in the cell and in the medium was determined by AAS.

2.2.7.4 Metabolic inhibition

The mechanisms of adaptation to metal stress and resistance to other metals by Zn tolerant *N. calcicola* was determined by metabolic inhibition. The uncoupler, FCCP (carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone); inhibitor of ATPases, DCCD (N,N'-dicyclohexylcarbodiimide); inhibitor of PSII, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and –SH inhibitor, pCMB (p-chloromercuricbenzoate) were obtained from Sigma. FCCP, DCCD and DCMU were dissolved in absolute ethanol (Merk) and pCMB was dissolved in 0.1 N NaOH (Verma and Singh, 1990). The aliquots of metabolic inhibitors were stored in dark at -20 ^{o}C. Experimental final concentrations used were 100 µM DCCD; 10 µM of FCCP, DCMU and pCMB.

2.2.7.5 EDTA washable fraction of metals

Metal deposited at the cell wall of *N. calcicola* and Zn-adapted cells were removed by treating with 10 µM of EDTA. First the cells were washed once with distilled water and then treated with EDTA for 10 min and centrifuged at 4000 rpm for 10 min at RT (REMI, R-8C DX Laboratory centrifuge). Care was taken to ensure that no cell loss during each washing. Cell wall bound metal content, in the form of EDTA washable fractions were determined by AAS.

2.2.7.6 Cell digestion of *N. calcicola*

Intracellular bioaccumulation of metals by wild type and Zn-adapted *N. calcicola* was determined by harvesting a 10 ml of cells equilibrated with respective metals for 1 h. The cell pellet was washed once with distilled water and an equal volume of 10 µM EDTA;

centrifuged to remove traces of any liquid and it was dried at 80 ^{o}C for 12 h. 1 ml of acid mixture (10:1 ratio of conc HNO_3:$HClO_4$) was added, and kept in a boiling water bath for 30 min. The tubes were cooled in a running tap water, and total volume was adjusted to 5 ml with Millipore water. Amount of metals present in acid digested contents of the cell mass was determined by AAS.

2.2.8 Metal Induced oxidative stress

2.2.8.1 Preparation of whole cell lysate for determination of enzymes

Metal stress induced enzymes in *N. calcicola* was determined from the cell lysate prepared by mechanical disruption. *N. calcicola* cultures were centrifuged, washed once with wash buffer (20 mM Tris-Cl, 150 mM NaCl, 0.1 mM EDTA, pH 7.0), cell pellet was resuspended in lysis buffer (20 mM Tris-Cl, 1 mM EDTA, 5 mM NaCl, 0.1 mM DTT, pH 7.5) and 1 mg ml^{-1} of lysozyme was added to the cell suspension. Protease inhibitors 1 µM leupeptin, 0.2 mM phenyl methyl sulphonyl fluoride (PMSF) and 1 µM pepstatin were added prior to cell lysis. Such mixture was sonicated 2 times on an ice-bath with 5 sec each pulse at 3 W output power from the sonicator (Microson XL2000 Ultrasonic Cell disruptor). The cell homogenate was centrifuged at 10000 rpm at 4 ^{o}C for 30 min; the supernatant was transferred to fresh sterile tubes. Concentration of the protein content was determined by BCA method and aliquots of cell free supernatants were stored at -70 ^{o}C.

2.2.8.2 Determination of superoxide dismutase (SOD)

The principle of SOD activity assay was based on superoxide radical generation by photo reduction of riboflavin was allowed to react with hydroxylamine hydrochloride to produce nitrite (Regelsberger et al. 2004). The nitrite in turn reacts with sulphanilic acid to produce a diazonium compound which subsequently reacts with napthylamine to produce a red azo compound whose absorbance is measured at 543 nm. 100 µl of cell lysate was added to the reaction mixture containing 1.11 ml, 50 mM phosphate buffer; 75µl, 20 µM L-methionine; 40 µl, 1 % triton X-100; 75 µl, 10mM hydroxylamine HCl; 100µl, 50 mM EDTA) and incubated for 5 min at 30 ^{o}C. 80 µl of 50 mM riboflavin was added to the contents and exposed to light (200 W output from PHILIPS, CFL) for 10 min. 1 ml Griess reagent was added. The color developed was read at 543 nm.

2.2.8.3 In-gel detection of SOD

To identify different isoforms of SOD (Regelsberger et al. 2004) expressed in *N. calcicola* under stress conditions, the proteins were separated on a 12% non-denaturing PAGE (method described in section 2.2.10.2, except SDS in the buffer components**)** at 80/120 V, once the run was completed, the gel was washed twice with Millipore water on shaker at 75 rpm to remove buffer components. The gel was soaked in 50 ml of 1.28 µM NBT and incubated at 30 ^{o}C for 15 min on a shaker. Gel was rinsed with potassium phosphate buffer (100 mM, pH 7.0) followed by the addition of a 100 ml 0.1 M phosphate buffer containing 28 µM TEMED (N,N,N',N'-Tetramethylethylenediamine), 28 µM riboflavin; and continued shaking for another 15 min. The excess reagents from the gel was removed by a brief rinsing with phosphate buffer and immediately exposed to light for 10 - 20 sec. SOD bands were visible as a transparent region on gel in the form of bands (Huang et al. 2002). To detect isoforms of SOD, the gel was soaked separately in 10 mM sodium azide (NaN_3), 10 mM hydrogen peroxide (H_2O_2) solutions and control without any SOD inhibitors preceding NBT treatment . Results were digitized using HP Scanjet Scanner.

2.2.8.4 Determination of malate dehydrogenase (MDH)

Malate dehydrogenase activity was determined by following the decrease in absorption at OD_{340} of NADH (Park et al. 1995). The reaction mixture contains: 0.5 ml, 10 mM phosphate buffer; 50 µl, 4 mM NADH; 50 µl, 1 mM oxal acetic acid; water 0.95 ml. Contents in the tube was incubated at 25± 2 ^{o}C for 5 min, immediately 50 µl of cell lysate was added, after 45 sec, the decrease in OD_{340} was followed for 10 min using JascoV570 UV-Visible spectro-photometer. MDH activity was expressed in U mg^{-1} protein.

2.2.9 Ultra structural studies on *N. calcicola* treated with metals

2.2.9.1 Light microscopy

The nature of *N. calcicola* treated with metals was observed under light microscope (Olympus CKX41). Image was captured using Olympus C-4000 camera and recorded.

2.2.9.2 Scanning electron microscopy (SEM)

N. calcicola was washed several times with 0.1 M phosphate buffer (pH 7.4) and fixed by submerging in 2.5% glutaraldehyde in phosphate buffer for 6 h at 4 ^{o}C. Samples were

washed repeatedly with the same buffer, dehydrated in a graded ethanol (50 - 100% v/v) and acetone to critical point drying. These were then placed on a carbon coated aluminium stub and gold coated at 0.06 – 0.08 mbar for 45 sec and viewed under a scanning electron microscope (PHILIPS, LEO 435 VP).

2.2.9.3 Transmission electron microscopy (TEM)

For TEM studies, the cells were washed with 0.1 M phosphate buffer (pH 7.4). Primary fixation of the sample was done by submerging in 2.5% glutaraldehyde in phosphate buffer for 6 h at 4 ^{o}C followed by post fixation with 1.0% OsO_4 in 0.1 M phosphate buffer for 2 h at 4 ^{o}C. The samples were washed repeatedly with the same buffer, dehydrated in a graded ethanol (50 – 100% v/v) and acetone to critical point drying. It was embedded on araldite and thin sections of 60 – 70 nm were cut with an ultramicrotome (Reichart Ultracut E, Germany) with diamond knives, which were then mounted on copper grids, contrasted with uranyl acetate and lead citrate and viewed under transmission electron microscope at 80kV (PHILIPS Morgagni 268).

2.2.10 Analysis of metal induced proteome of *N. calcicola*

2.2.10.1 Sample preparation

For denaturing polyacrylamide gel electrophoresis, the samples were prepared as described in section 2.2.8.1. The protease inhibitors 1 µM leupeptin, 0.2 mM phenyl methyl sulphonyl fluoride (PMSF) and 1 µM pepstatin were added to cell suspension prior to lysis.

2.2.10.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as per the standard protocol (Sambrook and Russel, 2001). For routine screening, 10 – 50 µg of total protein was electrophoresed on 10, 12 or 15% of 0.75 mm thick gel using BioRad Mini Protean gel electrophoresis apparatus at 80/100 V.

2.2.10.3 Two dimensional gel electrophoresis (2D PAGE)

Protein extracts containing 1 mg total protein was loaded onto rehydrated 18 cm immobiline gel strips (pH 3 – 10 and 4 – 7) by cup loading. The isoelectric focusing of proteins was performed on Protean IEF Cell; the focusing time was adjusted to a total of 80000 V h. The gel strips were equilibrated in the equilibration buffer for 30 min and

then positioned on top of an SDS–polyacrylamide gel (10% polyacrylamide) and sealed with 0.5% (w/v) agarose. The second dimension was carried out in a Protean II xi 2-D cell (BioRad) at 20 mA for 20 min and 40 mA for 6 h.

2.2.10.4 Staining and gel documentation

The proteins separated on polyacrylamide gel were visualized by Coomassie Brilliant Blue R-250 (CBB) or Silver staining method. For CBB staining, the gel was first rinsed with Millipore water for 10 min to remove residual SDS before soaking in staining solution (0.25 % CBB in methanol:water:glacial acetic acid; 45:45:10) for 6 – 8 h. The staining solution was decanted and rinsed once with destaining solution (staining solution devoid of CBB) and placed in sufficient volumes in a gel rocker until clear bands were observed. For analytical purposes, the gel was treated with fixing solution (12% TCA and 3.5% sulfosalicylic acid, Sigma) prior to CBB staining.

Silver staining was performed with ProteoSilver Silver stain kit (Sigma); all the steps were carried out at RT, on an orbital shaker at 50 rpm. After electrophoresis of the proteins, the polyacrylamide gel was placed in a clean tray containing fixing solution for 45 min. The gel was washed once with 30 % ethanol for 10 min followed by a water wash, 10 min; sensitizer solution, 10 min; water wash, 10 min; silver equilibration, 10 min and water wash, 1 min. Gel was placed in sufficient volumes of developer solution and allowed to develop desired intensity of the silver stained protein bands were achieved. The color development was stopped by the addition of ProteoSilver stop solution and continued shaking until bubbles stopped from the gel/solution. Finally, the gel was rinsed with excess water and the gel picture was taken using Syngene gel documentation system.

2.2.11 Protein identification and characterization

2.2.11.1 In-Gel digestion

The desired protein band from SDS-PAGE gel was cut into 1 mm^3 pieces and transferred into a sterile micro centrifuge tube. 500 µl of wash solution (50% acetonitrile, 50 mM ammonium bicarbonate) was added and incubated at RT for 15 min. Gel pieces were washed two more times with 500 µl of wash solution until the CBB dye has been

completely removed. Gel pieces were dehydrated by the addition of 100% acetonitrile and completely dried at RT for 10 – 20 min in a centrifugal evaporator. 150 µl reduction solution (10 mM DTT, 100 mM ammonium bicarbonate) was added and incubated for 30 min at 56 ^{o}C. The supernatant was discarded and gel pieces were mixed with 100 µl alkylation solution (50 mM iodoacetamide, 100 mM ammonium bicarbonate) and allowed to stand for 30 min in the dark at RT. After removing the alkylation solution with a pipette, 500 µl of wash solution was added and incubated at RT for 15 min with gentle agitation. Wash solution from the gel was discarded, and treated with 100 µl 100% acetonitrile for 5 min and the gel was dried in a centrifugal evaporator. Gel containing the desired protein was rehydrated with 20 µl of protease digestion solution (sequencing grade trypsin, Promega). The protein was digested overnight at 37 ^{o}C for 12 h. Supernatant containing tryptic peptides was transferred to a sterile centrifuge tube and gel pieces were extracted with 50 µl of extraction solution (60% acetonitrile, 1% TFA) and added to tube containing peptide mixture. The peptide solution was stored at -20 ^{o}C.

2.2.11.2 MALDI-TOF/MS

Extracted peptide mixture was dried in a centrifugal evaporator to near dryness. 5 µl of resuspension solution (50% acetonitrile, 0.1% TFA) was added to each tube and sonicated in a water bath at lowest setting. It was briefly spun and 0.5 µl was spotted on MALDI plate followed by 0.5 µl of alpha-cyano-4-hydroxycinnamic acid matrix (10 mg ml^{-1} in 50% acetonitrile, 0.1% TFA). Spots were allowed to dry completely and loaded onto Ultraflex MALDI TOF/TOF (Bruker Daltonics)**.** The proteins were identified by comparing peptide mass fingerprints to the NCBInr database using the Mascot search engine (http://www.matrixscience.com). The search parameters allowed for oxidation of methionines, carbamidomethylation of cysteines, one mis-cleavage of trypsin, and 30 ppm mass accuracy. The proteins were successfully identified, based on the first ranking result and Mascot scores >74, which indicates that the hits were significant. Some hits with Mascot scores <74, for which more than half of the peptides used in the search matched, and hits with the correct Mr and pI, were also accepted. The hydropathy profile (Kyte-Doolittle, 1982) and characterization of transmembrane helices and intervening loop regions (TMHMM) in identified proteins was performed using Molecular biology

workbench. To determine the number of conserved proteins from cyanobacteria, plants or green algae were compared and analyzed by CLUSTALW (Lindahl and Florencio, 2003).

2.2.12 Protein structure prediction

Structure of the metal stress induced proteins of *N. calcicola* was determined by molecular modeling using Modeller 9v2 (Sali and Blundell, 1993). The best homologues and template structures for this were downloaded from NCBI, swissprot and pdb database. Pair-wise alignment was used to find gaps/missing aminoacids. Bioedit, PyMOL and DeepView Swiss pdb-viewer was used for documentation.

2.3 Results and Discussion

2.3.1 Metal resistance in *N. calcicola*

Nostoc calcicola is a filamentous nitrogen fixing cyanobacterium, which differentiate into hormogonia, heterocyst and mature vegetative cells (Fig 2A – C). In order to test the metal resistance of *N. calcicola*, the cells were cultured on AA medium supplemented with respective metals at varying concentrations. Growth was measured in terms of total protein content. The minimum inhibitory concentrations (MIC) for the tested metals were shown in Table 1. *N. calcicola* was most sensitive to Cu and Ni followed by Cd, Co and Cr. Zn, Mn and U required high concentrations in the liquid medium for complete inhibition of the growth of *N. calcicola*. This may be attributed to metal availability at neutral pH as these metals get precipitated at this pH range.

Surprisingly, *N. calcicola* grown on liquid medium containing Zn^{2+} at 750 µM and 1000 µM showed an initial complete inhibition of growth and regained its lag phase after 7 and 10 days of incubation. Such effects on the growth of *N. calcicola* were not observed with other metals. Further subculturing of such Zn^{2+}adapted cells showed normal growth in AA medium supplemented with Zn. This could be due to the adaptation of *N. calcicola* to the metal containing environment and can be termed as “metal tolerance”. Moreover, the Zn-tolerant cells when tested for their metal sensitivity showed a marked increase in the MIC for Co, Cd, Cu, Mn in addition to Zn whereas there were no changes in the MIC of Ni, Sr and Cr (Table 1).

Similar reports on cyanobacterial responses to heavy metals (Singh et al. 1999) and the metal tolerance were reported earlier for *N. muscorum* (Verma and Singh, 1995) and *Anabaena doliolum* (Bhargava et al. 2006). In order to test whether the metal tolerance of *N. calcicola* has led to antibiotic resistance, the cells were plated on AA agar plates containing respective antibiotics. Ampicillin and Tetracyclines were excluded as they are light sensitive antibiotics. The MIC of antibiotics for both the wild type and the Zn-tolerant *N. calcicola* remains unchanged (Table 2).

2.3.2 Plasmid DNA in *N. calcicola*

Plasmids are self replicating extrachromosomal DNA present in most of the microorganisms which often carry the genes that confer resistance to metals and antibiotics (Endo et al. 2002). Filamentous cyanobacteria are known to possess plasmids (Boumard and de Marsac, 1988). Thus, *N. calcicola* was used to isolate putative plasmids to determine plasmid encoded functions. Several different protocols have been reported earlier for the isolation of plasmid DNA from filamentous cyanobacteria (Gendel, 1988; Lambert and Carr, 1982). Therefore, three protocols described for plasmid DNA isolation from *Nostoc* sp. and other bacteria were used (Gendel, 1988, Anderson and McKay, 1983); modifications were introduced to optimize plasmid DNA isolation from *N. calcicola* (2.2.6.3). It was found that the culture age was an important factor for successful plasmid isolation and its reproducibility. A minimum volume of 30 ml was used that gave a consistent plasmid DNA with a discernible band equivalent to a supercoiled plasmid DNA (Fig. 3A). The plasmid DNA was compared with other covalently closed circular plasmids of different sizes, ranging 3.3 – 14.1 kb (Fig. 3B). Bacterial plasmids (pBxMT, pRL623, pKT210) have shown multiple forms of plasmids like open circular, linear and supercoiled; the *N. calcicola* gave a prominent single band and a minor supercoiled plasmid (Fig. 3A – B).

The plasmid DNA was treated with restriction enzymes for further confirmation. It was found that the isolated DNA were able to digest with methylation sensitive (PstI and PvuII) and insensitive (BamHI, BglII, EcoRI, HindIII and SmaI) restriction enzymes (Fig. 4A – C). A complete digestion of the DNA into smaller fragments was observed in

the order HindIII, EcoRI and BglII followed by other enzymes at varying levels. All the results have shown that a mixture of several different bands appeared as a smear on the gel. It was indeed a clear indication of the presence of a larger plasmid of more than 100 kb containing multiple restriction sites on the DNA. It is usual that cyanobacteria possess plasmids of size between 1.1 – 400 kb (Boumard and de Marsac, 1988). Thus, such mixtures of DNA fragments arising out of mega plasmids could only be resolved by pulsed field gel electrophoresis (PFGE) system to separate into distinct bands. *Nostoc* sp. PCC 7120, a filamentous cyanobacterium which possesses 5 plasmids with sizes 4.9, 49.3, 63, 110 kb whereas the *Nostoc* sp PCC 8009 has 7.9, 30, 38, 40, 225 and 400 kb were separated on PFGE (Boumard and de Marsac, 1988; Bancroft et al. 1989).

The total DNA of *N. calcicola* was isolated from all metal treated cells and digested with restriction enzymes. Among the different metal treatment, genomic DNA of the Cr(VI) treated cells have shown on the gel blazing bands, an indication of contaminating protein associated with DNA preparation (Fig. 5). Such DNA were shown to have varying levels of protein binding. The DNA from Cr(VI) induced mutants, although having no such protein contamination, showed a faster migration pattern in comparison with the wild type or the Cr treated DNA. There are several reports on Cr(VI) induced -protein cross linking to DNA molecules (Kim et al. 1991; Arakawa et al. 2000; Kortenkamp et al. 1992; Zhitkovich et al. 1996). Such DNA were resistant to restriction enzyme digestion and the protective functions of the bound proteins to DNA remain unclear (Bridgewater et al. 1998; Mattagajasingh et al. 1999; Borges and Wetterhahn, 1991).

2.3.3 Metal induced cellular responses

N. calcicola grown in presence of Cr(VI) at varying concentrations were tested for its total intracellular metal content and its bio-transformations. It was found that Cr(VI) was slowly converted into Cr(III) intracellularly from the reduction of Cr(VI) (Table 3). There were no detectable Cr(VI) present and the Cr(III) at neutral pH are highly reactive as they tend to bind the proteins and nucleic acids. The growth profile as determined by the total protein and Chl *a* have shown that *N. calcicola* was inhibited on a concentration dependent

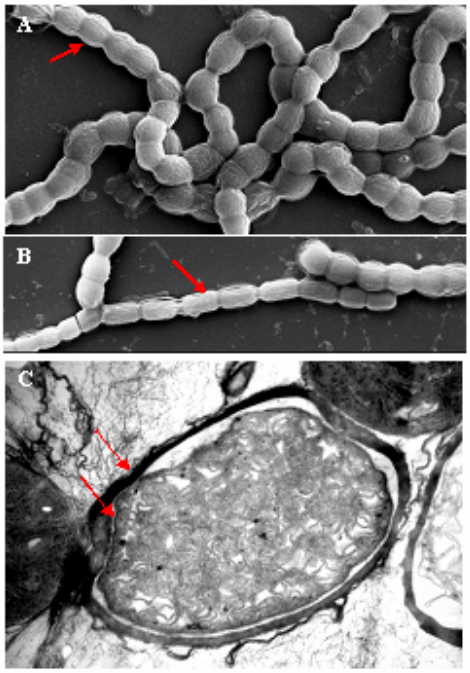


Fig 2. Electron microscopy of *N. calcicola*: Morphology and cellular differentiation. (A) vegetative cells, (B) hormogonia and (C) heterocyst, arrow shows a thick glycolipid containing rigid cell wall.

Table 1. The minimum inhibitory concentrations (MIC) of metals for *N. calcicola*. Cells were grown on AA medium supplemented with different concentrations of respective metals and incubated in the culture room conditions for 15 days. Growth was determined by total protein content.

S. No	Metals	MIC (µM)	
		***N. calcicola* wild type**	**Zn-tolerant *N. calcicola***
1	Cobalt(II)	35	50
2	Cadmium(II)	20	50
3	Zinc(II)	750	1000
4	Copper(II)	10	12.5
5	Nickel(II)	10	10
6	Strontium(II)	70	70
7	Uranium(VI)	500	500
8	Manganese(II)	750	1000
9	Chromium(VI)	200	200

Table 2. The minimum inhibitory concentrations (MIC) of antibiotics for *N. calcicola*. Cells were grown on 1.5% AA agar plates supplemented with different concentrations of respective antibiotics and incubated in the culture room conditions for 15 days.

S. No	Antibiotics	MIC (µg ml^{-1})	
		***N. calcicola* wild type**	**Zn-tolerant *N. calcicola***
1	Kanamycin	2.5	2.5
2	Streptomycin	1	1
3	Neomycin	2	2
4	Chloramphenicol	0.5	0.5

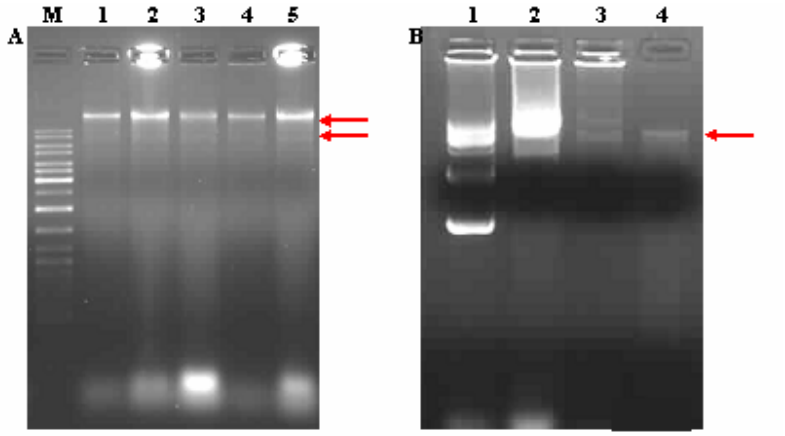


Fig 3. Plasmid DNA of *N. calcicola*. (A) lane 1 – 5, plasmid DNA isolated from *N. calcicola*, arrows show supercoiled plasmids; (B) comparison with other plasmids, lane 1, pBxMT (3.3 kb); lane 2, pRL623 (12 kb); lane 3, pKT210 (14.1 kb) and lane 4, *N. calcicola* plasmid. M*, DNA marker (*MBI Fermentas, SM0331, 1 kb ladder). The DNA samples were separated on a 1.0% agarose gel.

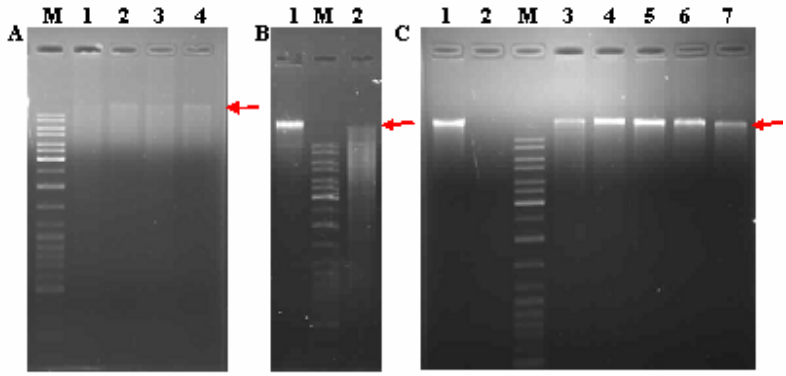


Fig 4. Restriction profile of the *N. calcicola* plasmid DNA. (A) extrachromosomal DNA was digested with HindIII, lanes 1 – 4; (B) lane 1, PstI; lane 2, HindIII; and (C) lane 1, plasmid DNA (uncut); lane 2, HindIII; lane 3, EcoRI; lane 4, PvuII; lane 5 BamHI; lane 6, SmaI; and lane 7, BglII. M*, DNA marker (*MBI Fermentas, SM0331, 1 kb ladder). The DNA samples were separated on a 1.0% agarose gel.

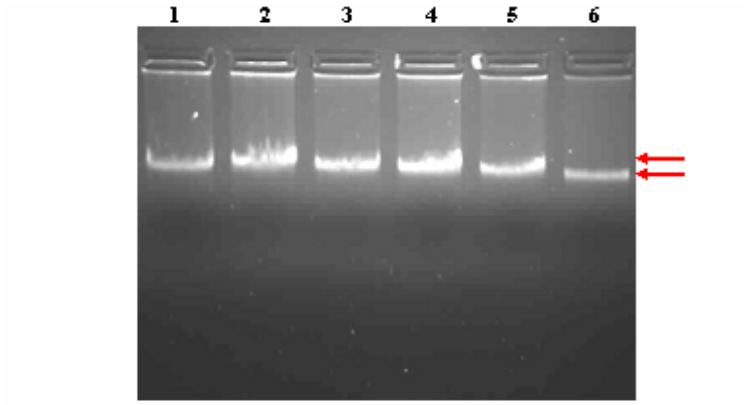


Fig 5. Genomic DNA of Cr(VI) treated *N. calcicola*. Chromosomal DNA isolated from the cells grown in AA medium supplemented with different concentrations of Cr(VI). Lane 1, genomic DNA from wild type cells; Lane 2 – 5, *N. calcicola* treated with 25, 50, 75 and 100 µM of Cr(VI); Lane 6, DNA isolated from Cr(VI) induced mutants, arrow shows the different migration pattern. Gel electrophoresis was performed on 0.8% agarose gel, 1 h at 80 V.

Table 3. Chromium(VI) biotransformation by *N. calcicola*. The cells were grown in AA medium supplemented with different concentrations of Cr(VI) and incubated in the culture room conditions for 15 days. Growth was determined by total protein content. Total Cr was measured by AAS and the Cr(VI) was determined spectrophotometrically.

Chromium in the culture medium (µM)	**5th Day**				**15th Day**			
	Chromium (culture medium) (µM)		**Total intracellular bioaccumulation (µM mg^{-1} protein)**		**Chromium concentrations (culture medium) (µM)**		**Total intracellular bioaccumulation (µM mg^{-1} protein)**	
	Cr(III)	Cr(VI)	Cr(III)	Cr(VI)	Cr(III)	Cr(VI)	Cr(III)	Cr(VI)
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32.94	0.00	29.92	1.50	0.00	0.00	11.34	42.72	0.00
62.94	0.00	59.96	3.02	0.00	0.00	33.86	66.58	0.00
89.56	0.00	84.84	5.98	0.00	0.00	48.48	92.54	0.00
101.62	0.00	98.70	5.46	0.00	0.00	59.60	105.24	0.00
152.22	0.00	145.26	10.90	0.00	0.00	91.58	138.92	0.00

manner. Several reports on the reduction of Cr(VI) by cyanobacteria (Rai and Raizada, 1988; Kotrba et al. 1999).

Metal uptake and efflux studies were conducted to discern the mechanism that confers the Zn tolerant *N. calcicola* resistant to Zn, Cd and Co. The wild type and Zn-tolerant cells were incubated with 750 µM of Zn, after 1 h of equilibration, intracellular bioaccumulation of Zn by the tolerant cells was found to be 73 µM mg^{-1} proteins whereas the wild type could accumulate 0.97 µM mg^{-1} (Table 4 – 5). The metabolic inhibitors with uptake studies showed overall reduction in Zn accumulation. Metal equilibrated tolerant cells when suspended in 10 mM phosphate buffer, it could efflux more than 93% of the metal accumulated by the cells. The effect of inhibitors reduced the efflux of Zn from the tolerant cells and among the inhibitors tested, pCMB showed profound effect on uptake and efflux of Zn. In the case of Cd (Table 6 – 7) and Co (Table 8 – 9) uptake and efflux both the wild type and the tolerant cells showed similar trend. The metabolic inhibitors have reduced the total metal transport across the membrane. From the metal uptake experiments with Zn-tolerant and wild type cells, it was observed that Cd and Co saturate with cells and faster efflux. Similar results were observed with mutant strains of *N. calcicola* (Verma and Singh, 1991)

2.3.4 Metal induced oxidative stress

Superoxide dismutase (SOD) activity in microorganisms often correlates with the severity of environmental stress (Bowler et al. 1992) especially with the metal stress (Pinto et al. 2003). *N. calcicola* exposed to Cr(VI) and Zn(II) increased SOD enzyme levels (Fig. 6 and 7). In comparison with Zn(II), the cells exposed to Cr(VI) at 30 – 40 µM have shown highest SOD activity. Thus, in an attempt to differentiate between the isoforms of SOD overexpressed upon Cr(VI) exposure, the total cellular proteins were separated on a non-denaturing polyacrylamide gel. The SOD activity was visualized using nitro blue tetrazolium (NBT) staining and specific inhibition of Fe-SOD and Mn-SOD with inhibitors (Regelsberger et al. 2004); hydrogen peroxide (H_2O_2) and sodium azide (NaN_3). The results obtained were shown in Figure 8A – C. The enzyme inhibition assays have demonstrated the overexpression of cytosolic Fe-SOD activity in response to

Table 4. Zn^{2+} uptake by *N. calcicola*. The wild type and the Zn tolerant cells at 400 µg ml^{-1} was incubated in 10 mM phosphate buffer containing 750 µM of Zn^{2+} for 1 h; 100 rpm at culture room conditions. Metal accumulation at the cell wall and intracellular bioaccumulation were determined by AAS.

Inhibitors	EDTA washable fraction (Zn content µM)		Total intracellular bioaccumulation (µM mg^{-1} protein)	
	WT	ZnT	WT	ZnT
Control	1.27	9.06	0.97	73.5
PCMB (10 µM)	1.89	7.13	0.19	21.84
FCCP (10 µM)	1.15	7.12	0.21	56.8
DCMU (10 µM)	0.92	9.6	0.37	40.13
DCCD (100 µM)	1.33	9.22	0.2	30.6

* WT- wild type and ZnT-Zn tolerant *N. calcicola*

Table 5. Zn^{2+} efflux by *N. calcicola*. The wild type and Zn tolerant cells at 400 µg ml^{-1} was equilibrated in 10 mM phosphate buffer containing 750 µM of Zn^{2+} for 1 h; 100 rpm at culture room conditions. Cells were washed with 10 µM EDTA and suspended in 10 mM phosphate buffer. Zn efflux was determined from total metal present in the medium after 1 h of incubation; 100 rpm at culture room conditions. Zn content was determined by AAS.

Inhibitors	Zn content in the supernatant (µM)		Zn content (µM mg^{-1} protein)	
	WT	ZnT	WT	ZnT
Control	5.088	45.93	0.707	10.4
PCMB (10 µM)	0.37	7.32	0.19	40.1
FCCP (10 µM)	0.63	23.8.	0.21	59.2
DCMU (10 µM)	0.6	21.6	0.37	51.0
DCCD (100 µM)	0.41	11.54	0.2	44.9

* WT- wild type and ZnT-Zn tolerant *N. calcicola*

Table 6. Cd^{2+} uptake by *N. calcicola*. The wild type and the Zn tolerant cells at 400 µg ml^{-1} was incubated in 10 mM phosphate buffer containing 500 µM of Cd^{2+} for 1 h; 100 rpm at culture room conditions. Metal accumulation at the cell wall and intracellular bioaccumulation were determined by AAS.

Inhibitors	EDTA washable fraction (Cd content µM)		Total intracellular bioaccumulation (µM mg^{-1} protein)	
	WT	ZnT	WT	ZnT
Control	9.442	4.28	97.95	91.55
PCMB (10 µM)	4.602	4.52	89.485	61.70
FCCP (10 µM)	7.04	4.68	87.08	67.34
DCMU (10 µM)	6.575	5.42	90.09	62.86
DCCD (100 µM)	4.505	5.64	86.34	57.18

* WT- wild type and ZnT-Zn tolerant *N. calcicola*

Table 7. Cd^{2+} efflux by *N. calcicola*. The wild type and Zn tolerant cells at 400 µg ml^{-1} was equilibrated in 10 mM phosphate buffer containing 500 µM of Cd^{2+} for 1 h; 100 rpm at culture room conditions. Cells were washed with 10 µM EDTA and suspended in 10 mM phosphate buffer. Cd efflux was determined from total metal present in the medium after 1 h of incubation; 100 rpm at culture room conditions. Cd content was determined by AAS.

Inhibitors	Cd content in the supernatant (µM)		Cd content (µM mg^{-1} protein)	
	WT	ZnT	WT	ZnT
Control	2.22	13.46	34.24	64.2
PCMB (10 µM)	3.20	5.25	58.05	59.02
FCCP (10 µM)	5.34	3.95	59.41	51.02
DCMU (10 µM)	4.85	5.20	57.58	51.02
DCCD (100 µM)	7.08	3.69	53.37	45.48

* WT- wild type and ZnT-Zn tolerant *N. calcicola*

Table 8. Co^{2+} uptake by *N. calcicola*. The wild type and the Zn tolerant cells at 400 µg ml^{-1} was incubated in 10 mM phosphate buffer containing 750 µM of Co^{2+} for 1 h; 100 rpm at culture room conditions. Metal accumulation at the cell wall and intracellular bioaccumulation were determined by AAS.

Inhibitors	EDTA washable fraction (Cd content µM)		Total intracellular bioaccumulation (µM mg^{-1} protein)	
	WT	ZnT	WT	ZnT
Control	4.26	1.65	36.5	39.68
PCMB (10 µM)	1.32	1.51	33.4	43.7
FCCP (10 µM)	2.22	1.72	34.3	42.77
DCMU (10 µM)	2.69	2.20	36.0	40.27
DCCD (100 µM)	1.61	2.64	32.9	40.0

* WT- wild type and ZnT-Zn tolerant *N. calcicola*

Table 9. Co^{2+} efflux by *N. calcicola*. The wild type and Zn tolerant cells at 400 µg ml^{-1} was equilibrated in 10 mM phosphate buffer containing 750 µM of Co^{2+} for 1 h; 100 rpm at culture room conditions. Cells were washed with 10 µM EDTA and suspended in 10 mM phosphate buffer. Co^{2+} efflux was determined from total metal present in the medium after 1 h of incubation; 100 rpm at culture room conditions. Co content was determined by AAS.

Inhibitors	Cd content in the supernatant (µM)		Cd content (µM mg^{-1} protein)	
	WT	ZnT	WT	ZnT
Control	0.028	2.331	12.9	24.3
PCMB (10 µM)	0.019	0.210	22.7	27.7
FCCP (10 µM)	0.36	0.414	24.7	28.9
DCMU (10 µM)	0.317	0.430	22.2	23.9
DCCD (100 µM)	0.412	0.390	21.3	24.32

* WT- wild type and ZnT-Zn tolerant *N. calcicola*

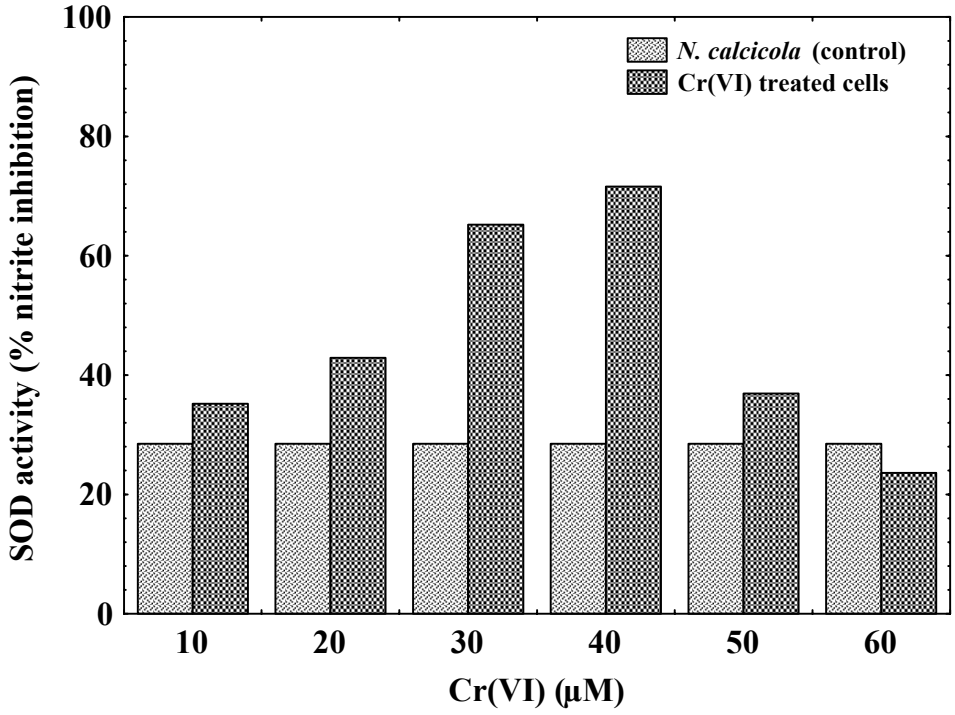


Fig 6. Superoxide dismutase (SOD) activity in Cr(VI) exposed cells. The *N. calcicola* was treated with 10 – 60 µM of Cr(VI) and assayed for SOD expression. SOD activity represents % inhibition of nitrite formation by photoreduction of riboflavin.

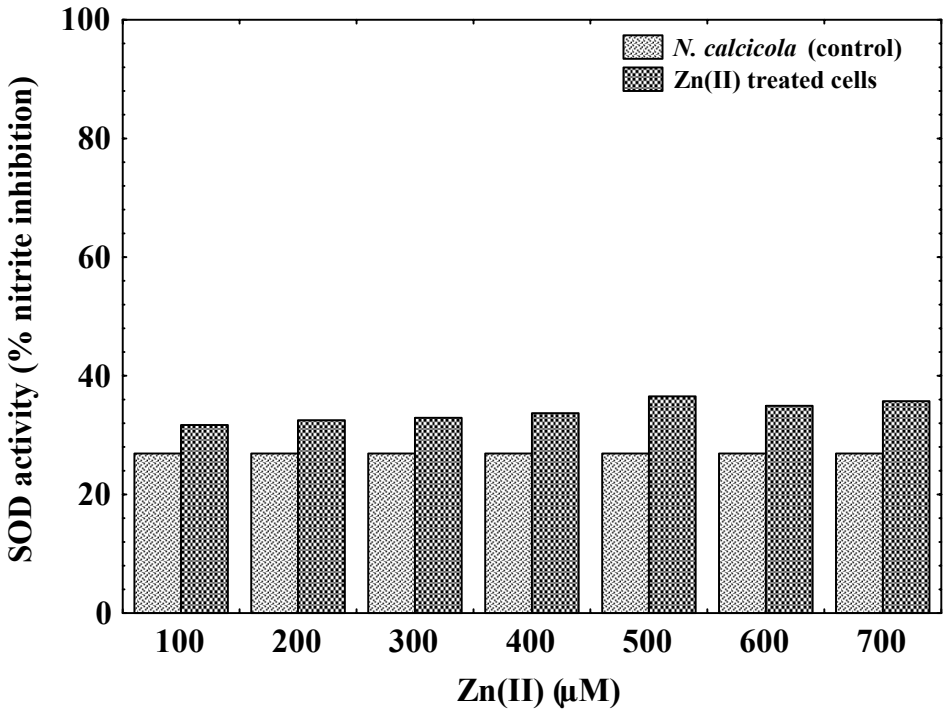


Fig 7. Superoxide dismutase (SOD) activity in Zn(II) exposed cells. The *N. calcicola* was treated with 100 – 700 μM of Zn(II) and assayed for SOD expression. SOD activity represents % inhibition of nitrite formation by photoreduction of riboflavin.

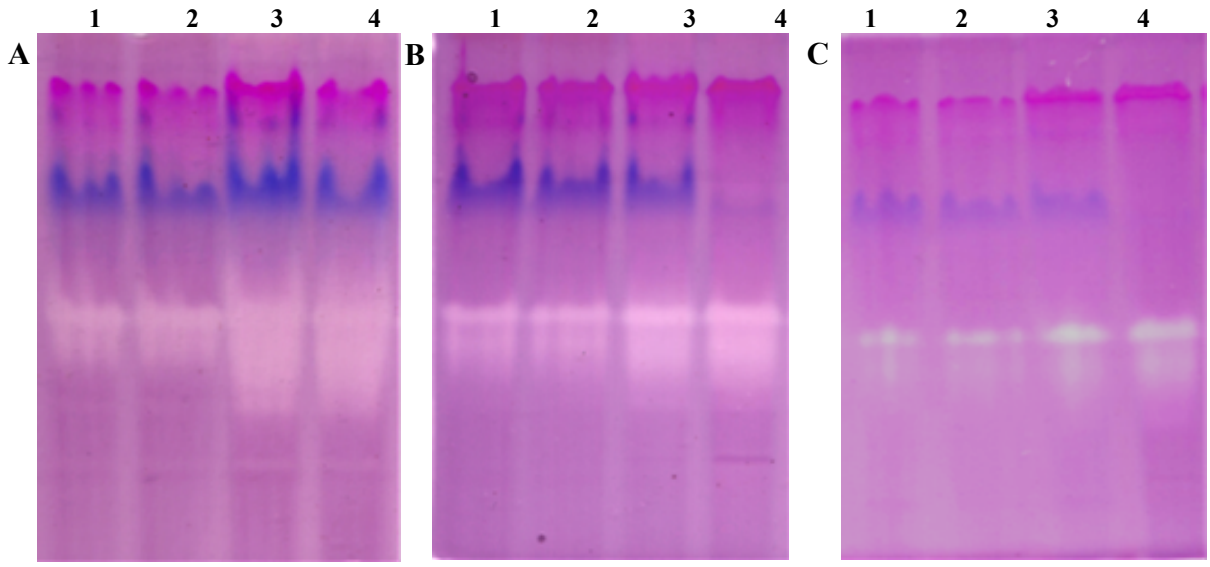


Fig 8. In-gel detection of the superoxide dismutase (SOD). The protein samples from *Nostoc calcicola* were separated on a 12% native PAGE and stained for SOD activity by NBT method. (A) lane 1, *N. calcicola* (wild type) control; lane 2 – 4, the cells treated with Cr(VI) at 20, 30, 40 µM of Cr(VI); and (B) the gel treated with 10 mM NaN_3 and (C) 10 mM H_2O_2.

Cr(VI) (Fig. 8C) as compared to the membrane bound Mn-SOD (Fig. 8B). Thus, iron superoxide dismutase has a greater role in Cr(VI) induced oxidative stress regulation. Few reports on the physiological role of cyanobacterial SOD suggest that these enzymes protect cellular components during oxidative stress (Shirkey et al. 2000). In *Nostoc commune* a significant role of Fe-SOD in response to desiccation has been identified (Shirkey et al. 2000). Similarly, a mutant strain of *Synechococcus* sp. strain PCC 7942 lacking detectable Fe-SOD activity was shown to be much more sensitive to reactive oxygen species (ROS) (Herbert et al. 1992) and chilling stress (Thomas et al. 1999). In *E. coli*, Fe-SOD is synthesized constitutively to protect cytoplasmic enzymes from oxidative damage (Prieto-Alamo et al. 1993) whereas its Mn-SOD only induced upon specific stress conditions i.e., exposure to redox cycling agents, heat shock, DNA binding drugs, high salt and metal stress.

In addition to SOD, the Malate dehydrogenases are considered a key indicator of cellular stress. MDH activity of the *N. calcicola* exposed to Cr(VI) and Zn(II) were shown in Table 10 and 11. The cells exposed to Cr(VI) have shown a concentration dependent increase and a sharp decline in the activity whereas the Zn decreased the MDH levels. The Cr(VI) induced overexpression of MDH could be partially due to the depletion of intracellular OAA content (Oh, 2002) as substantial quantity could be involved in the scavenging of free radicals generated by Cr exposure.

2.3.5 Ultra structural studies on *N. calcicola* treated with metals

Metals tend to bind to the cell surface and to specific proteins rich in thiol groups (Suroz and Palinska, 2000). Microbial cells also accumulate metals either in periplasm or the cytosol (Fernandez-Pinas et al. 1995). Such details can be explored by using electron microscope by which the deposition of metals could be observed as electron dense aggregates. Such studies not only identify the site of metal accumulation but also cellular alterations undergone during metal exposure (Rangsayatorn et al. 2002).

The effect of Cd, Zn and Cr(VI) on *N. calcicola* was studied in detail using transmission electron microscope (TEM). The cross-section of a typical control cell of *N. calcicola* is

Table 10. Malate dehydrogenase activity in *N. calcicola* exposed to Cr(VI). The *N. calcicola* was treated with 10 – 60 µM of Cr(VI) and assayed for MDH activity. Exponentially grown cells were harvested, lysed by sonication and the cell free lysate was used for enzyme assay.

S. No.	Cr(VI) (µM)	MDH activity (mU mg^{-1} protein)
1.	Wild type cells (control)	2.1
2.	10	4.0
3.	20	3.76
4.	30	3.2
5.	40	2.91
6.	50	2.2
7.	60	2.2

Table 11. Malate dehydrogenase activity in *N. calcicola* exposed to Zn(II). *N. calcicola* was treated with 250 – 750 µM of Zn(II) and assayed for MDH activity. Exponentially grown cells were harvested, lysed by sonication and the cell free lysate was used for enzyme assay.

S. No.	Zn(II) (µM)	MDH activity (mU mg^{-1} protein)
1.	Wild type cells (control)	2.11
2.	250	1.86
3.	500	1.69
4.	750	0.74

shown in Figure 9A. Characteristic arrangement of thylakoids was clearly visible and they were found to be located in whole region of protoplast. The ultrastructural characteristics of vegetative cells changed markedly when exposed to cadmium (Fig. 9B) with its bio-accumulation throughout the cell. A reduced bioaccumulation of Zn was observed with Zn(II) stressed cells (Fig. 9C). The responses of *N. calcicola* to Cr(VI) was found to be very distinct when compared to other tested metals. Analysis of the fine structure of Cr(VI) exposed cells exhibited discernible changes in the membranous system and formation of homogenous unique granular structures throughout the cell (Fig. 9D – E) which were confined to the intrathylakoidal space (Fig. 9F). In addition, the cell wall was swollen upon Cr(VI) exposure (Fig. 10A). Cells which were treated with Cr(VI) have developed random intracellular patches (Fig. 10B), under higher magnification they were found to be spherical structures of unknown functions (Fig. 10C). The intra-cellularly reduced Cr(VI) in the form of Cr(III) bioaccumulation was seen as electron-opaque spots (Fig. 10C). Moreover, such cells had intracellular precipitate material spread across the cell (Fig. 10D) and also had localized necrosis (10E), an indication of metal induced protein denaturation and cell lysis. Cr(VI) induced mutants which have lost the filamentous shape are shown in the Figure 10F.

It was evident from TEM studies that the *N. calcicola* alters its intracellular organization in response to metals and is able to survive under such stress conditions. The influence of heavy metals on morphology and ultrastructure has been described for several genera of cyanobacteria eg., *Anabaena* and *Plectonema* (Rangsayatorn et al. 2002; Lazinsky and Sicko-Goad, 1983), *Nostoc* sp., (Fernandez-Pinas et al. 1995), and *Phormidium* (Suroz and Palinska, 2000). Studies on the effect of copper and cadmium on unicellular cyanobacterium *Anabaena flos-aquae* have shown intracellular metal bioaccumulation, large intra-thylakoidal spaces, formation of cyanophycin and polyphosphate bodies (Surosz and Palinska, 2004). These studies have shown overall changes in the fine structure of the tested strains, but no studies have reported likely morphological and ultrastructural modifications which were observed on the effect of Cr(VI) in *N. calcicola*.

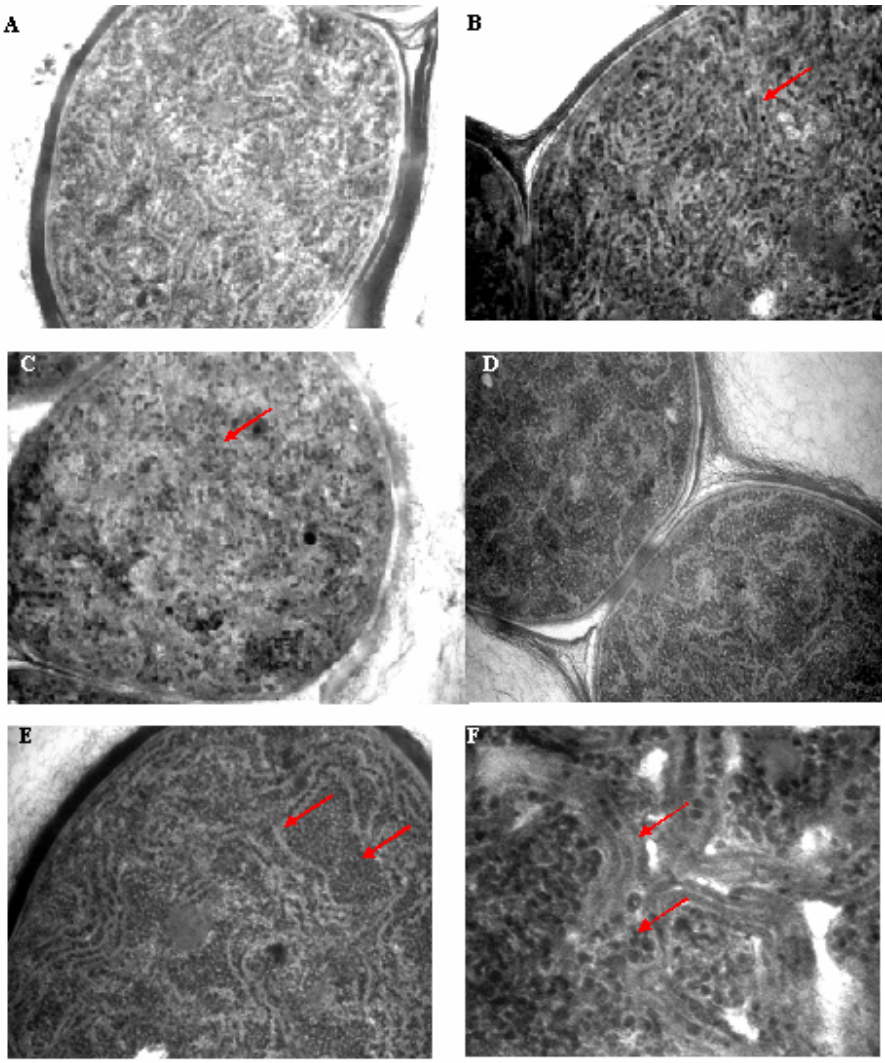


Fig 9. Transmission electron microscopy (TEM) of *N. calcicola*. (A) The intracellular organization, thylakoid membranes and cell wall of wild type cells, 4400x; (B) bioaccumulation of Cd as seen by the electron dense metal aggregates, 5600x; (C) reduced accumulation of Zn on Zn(II) tolerant *N. calcicola*, 5400x; (D) Cr(VI) induced macromolecular structures, 5600x; (E), 8900x and (F) a closer view of thylakoid membranes and compartmentalized macromolecular structures, 11000x.

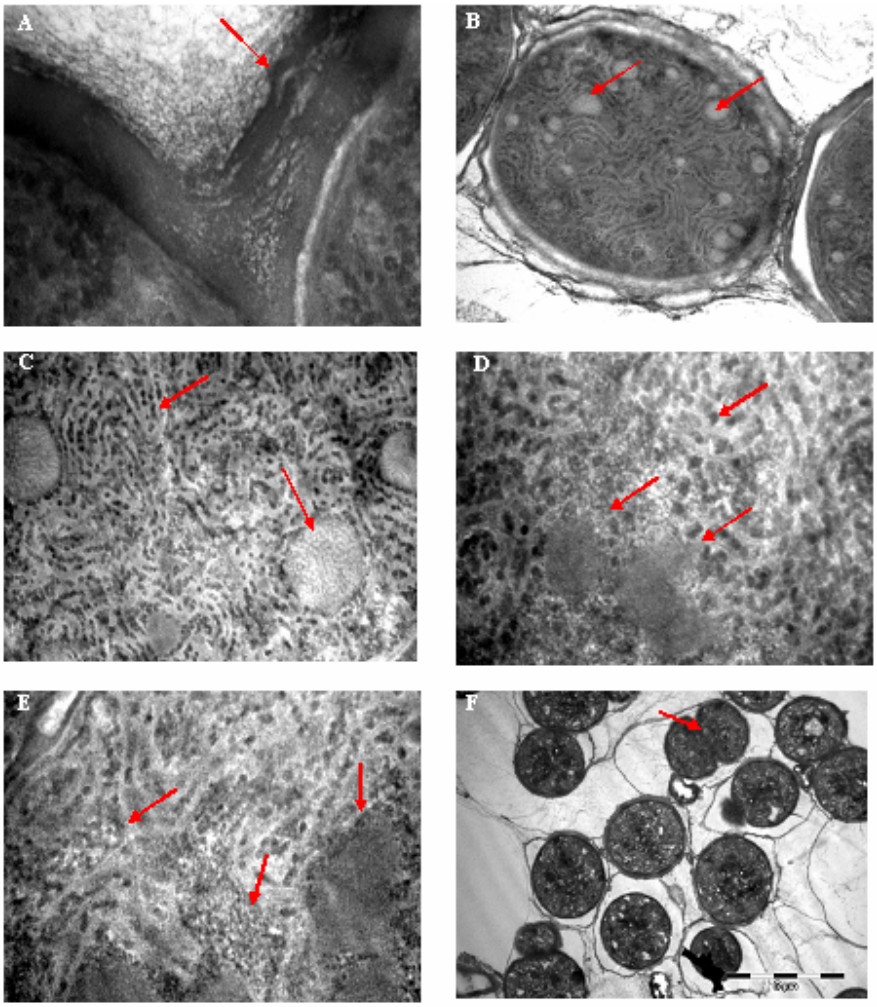


Fig 10. TEM of Cr(VI) induced cellular damage in *N. calcicola*. (A) The swollen cell wall, 14000x; (B) highly dispersed specialized assembly in the cell, 3500x; (C) intracellular Cr(III) accumulation, as a result of Cr(VI) reduction, and specialized assembly, 7100x; (D) protein precipitates, 11000x; (E) highly localized necrosis, 14000x; and (F) Cr(VI) induced mutants of *N. calcicola*, 1800x

2.3.6 Analysis of metal induced proteome of *N. calcicola*

In order to gain an insight into the changes in protein expression upon exposure to metals in *N. calcicola*, the total proteins were separated on 1D and 2D SDS-PAGE. *N. calcicola* upon exposure to different metals have shown up- or down regulation of several proteins. Specifically, Cr(VI) has shown to be inhibitory to most of the cellular proteins in comparison with other metal ions (Fig. 11A). In particular, some of the protein bands were highly overexpressed (Fig. 11B – E). Such changes in cellular proteins are common with other cyanobacteria exposed to heavy metals (Bhargava et al. 2006; Fulda and Debatin, 2005) as they bind to proteins non-specifically depending on the external concentration thereby inhibiting DNA and protein synthesis.

The two-dimensional electrophoresis of protein involves, first the isoelectrofocussing (IEF) followed by the denaturing polyacrylamide gel electrophoresis (O'Farell, 1975). IEF plays a key role in the effective separation of most of the proteins into different zones on the pH gradient strip. Such gradients are routinely available with different range from pH 3 – 11. Initial separation of *N. calcicola* proteins showed most of them were centered on pH 4 – 7 (Fig. 12). Further, samples were separated over pH 4 – 7 (Fig. 13); the PD Quest analysis of the 2D gel of the wild type *N. calcicola* has identified *ca.* 200 protein spots. Although, the total number of proteins spots identified was smaller in number, the metal stress inducible proteins can be identified and characterized. The protein sample preparation and separation of *N. calcicola* proteins were standardized.

2.3.7 Protein identification and characterization

To identify the role of Zn(II) and Cr(VI) induced proteins in *N. calcicola*, the prominent bands (Fig. 11B, C and D) with Mr 33 kD; 90 kD, 43 kD (abbreviated as RPV1, RPV2 and RPV3) were picked from the 1D SDS-PAGE. The trypsin digested peptides were subjected to MALDI-TOF/MS. The results of the MALDI-TOF/MS of the RPV1, RPV2 and RPV3 were shown in Figures 14 – 16. MASCOT search on NCBInr database results have shown that the RPV1, Malate Dehydrogenase (33 kD); RPV2, Histidine kinase (70 kD) and RPV3, Ribulose-1,5-bisphosphate carboxylase/oxygenase (44 kD). The percentage sequence coverage, the number of matched peptides, the NCBI accession

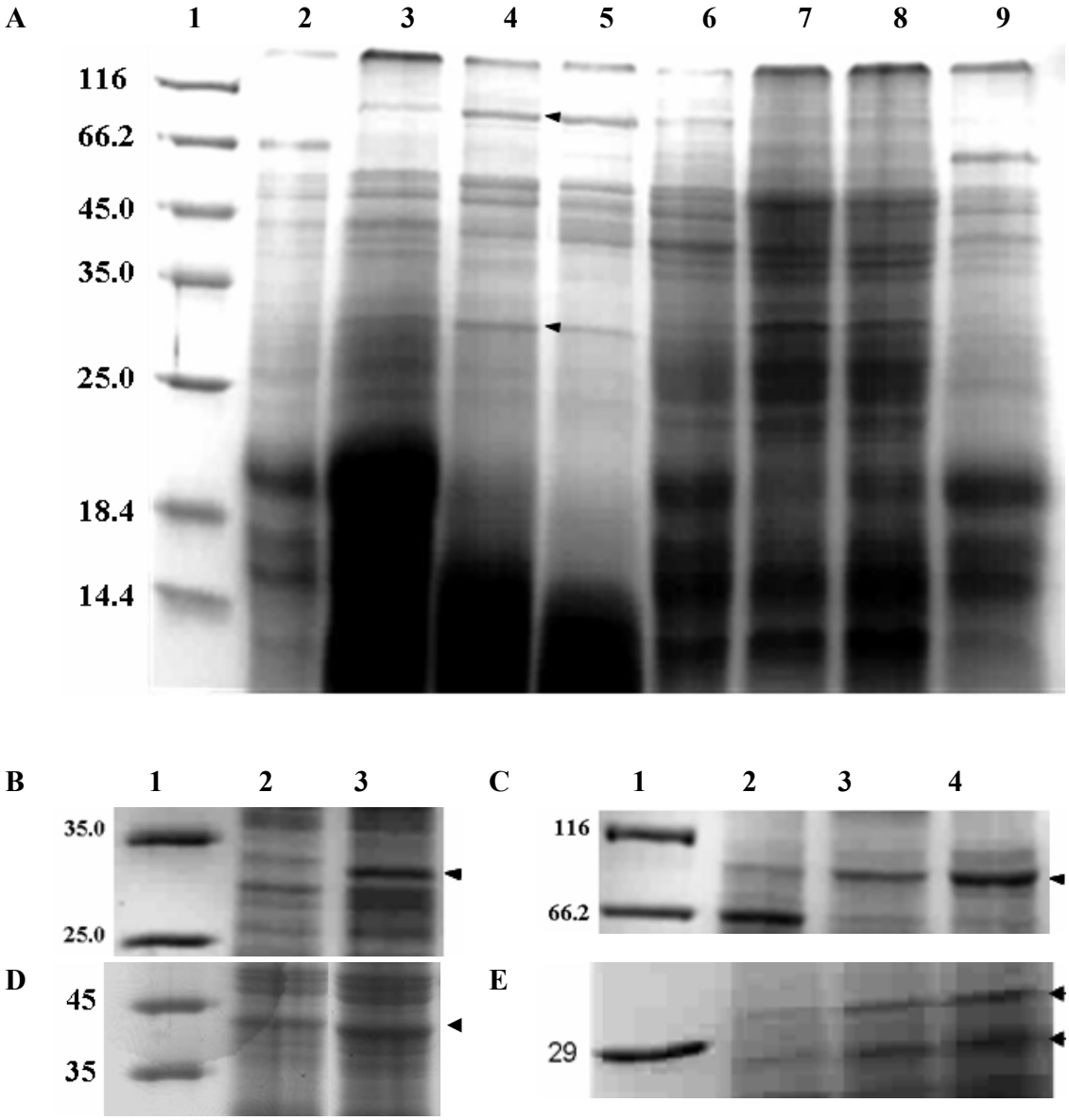


Fig 11. SDS-PAGE of total cellular *N. calcicola* proteins. (A) lane 1, protein molecular weight marker; lane 2, wild type cells; lanes 3 – 5, cells treated with Cr(VI) at 20, 40, 60 µM; lane 6, Mn(II) at 500 µM; lane 7 - 8, Zn(II) at 250, 500 µM; lane 9, wild type cells. (B – C), Cr(VI) and (D – E), Zn(II) induced proteins. Samples were electrophoresed on a 12% polyacrylamide gel and stained with Commassie blue.

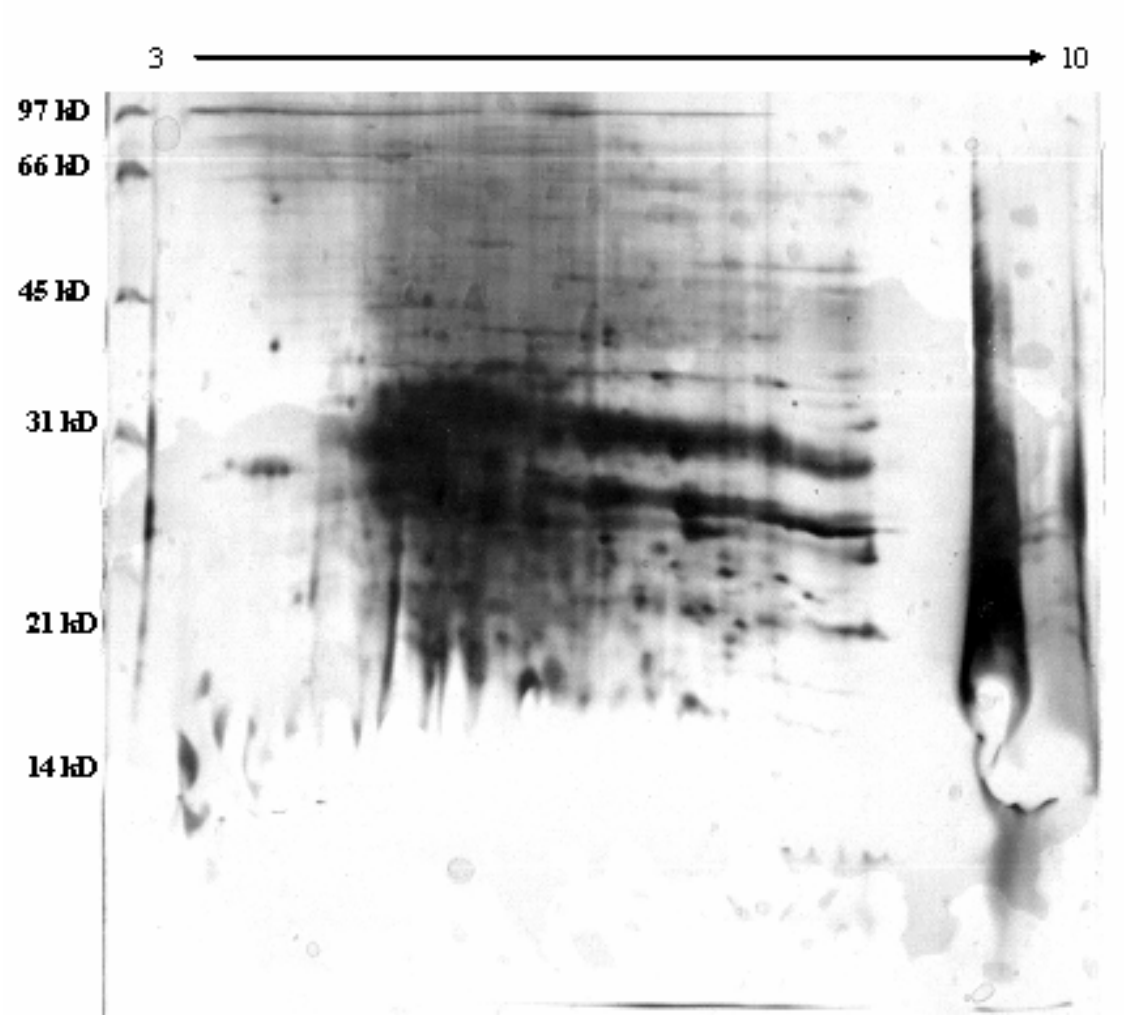


Fig 12. 2D-PAGE of total cellular *N. calcicola* proteins. The total proteins separated on a pH 3 – 10 linear immobilized pH gradient, the second dimension on a 12% SDS–PAGE and silver stained. Protein molecular weight marker is shown on left.

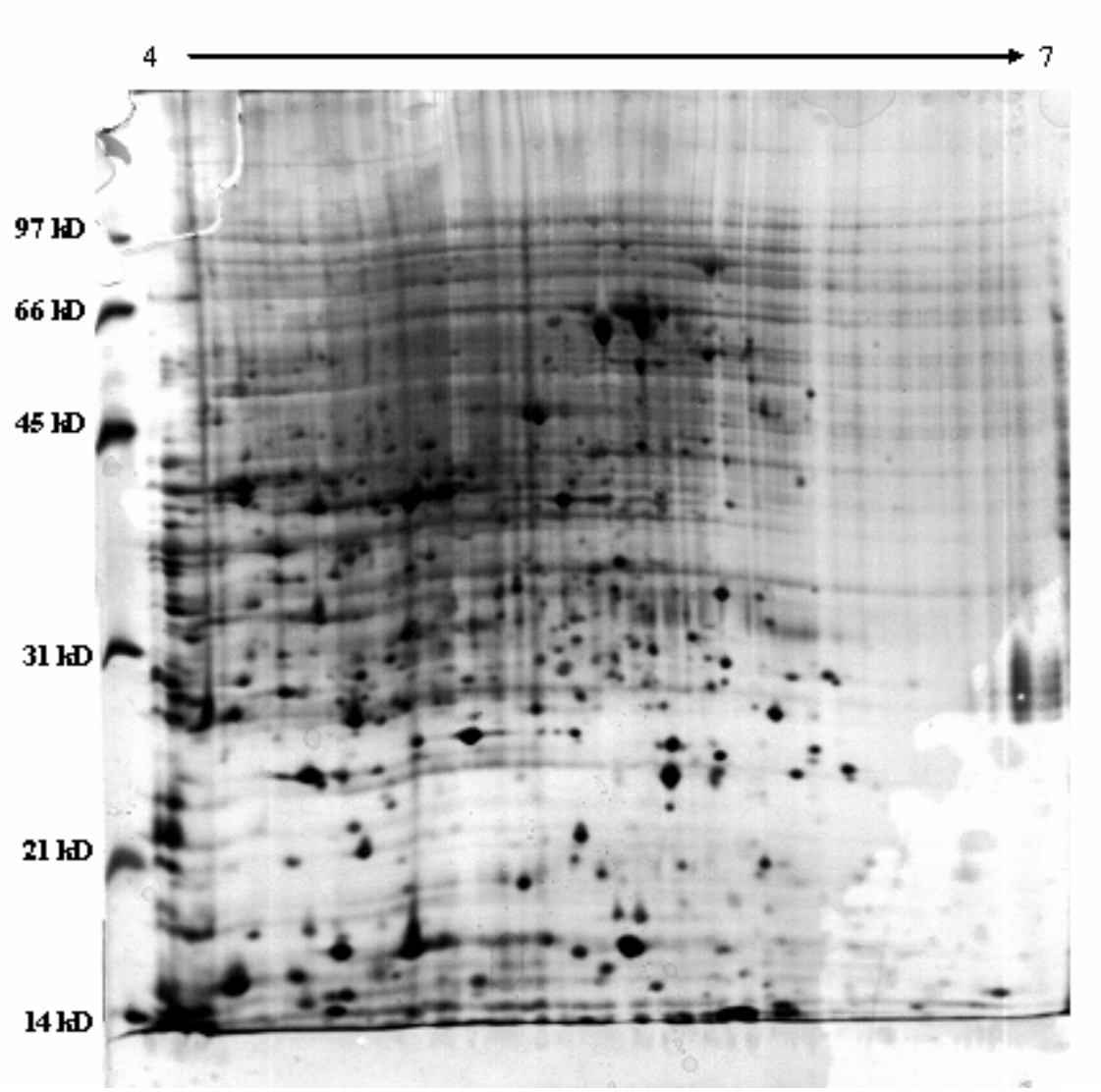


Fig 13 . 2D-PAGE of total cellular *N. calcicola* proteins. The total proteins separated on a pH 4 – 7 linear immobilized pH gradient, the second dimension on a 12% SDS–PAGE and silver stained. Protein molecular weight marker is shown on left.

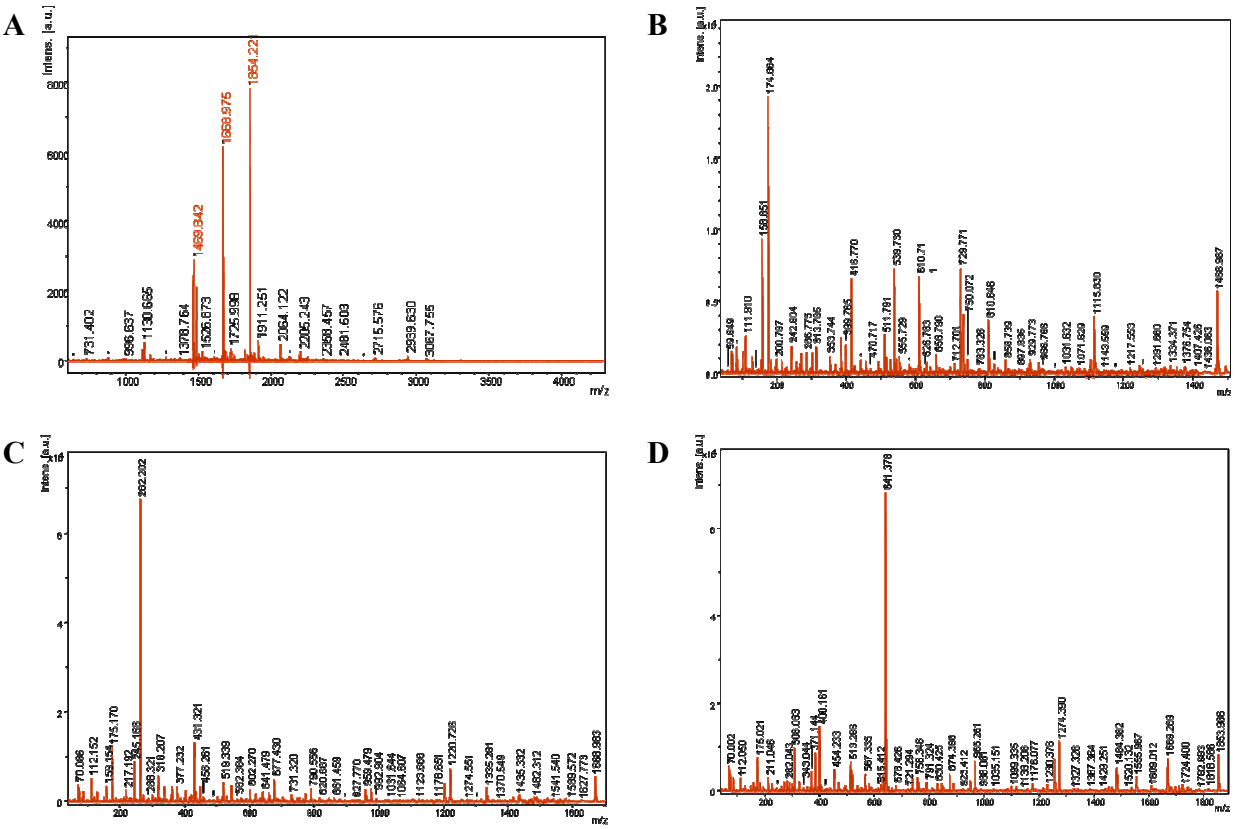


Fig 14. MALDI-TOF/MS of RPV1. **A**. Peptide mass fingerprinting (PMF) of the protein RPV2, **B – D**, post source decay (MS/MS) of the prominent peaks from PMF. The protein was identified from the NCBInr using MASCOT as NAD^+-dependent Malate dehydrogenase, a 33 kD cytosolic protein.

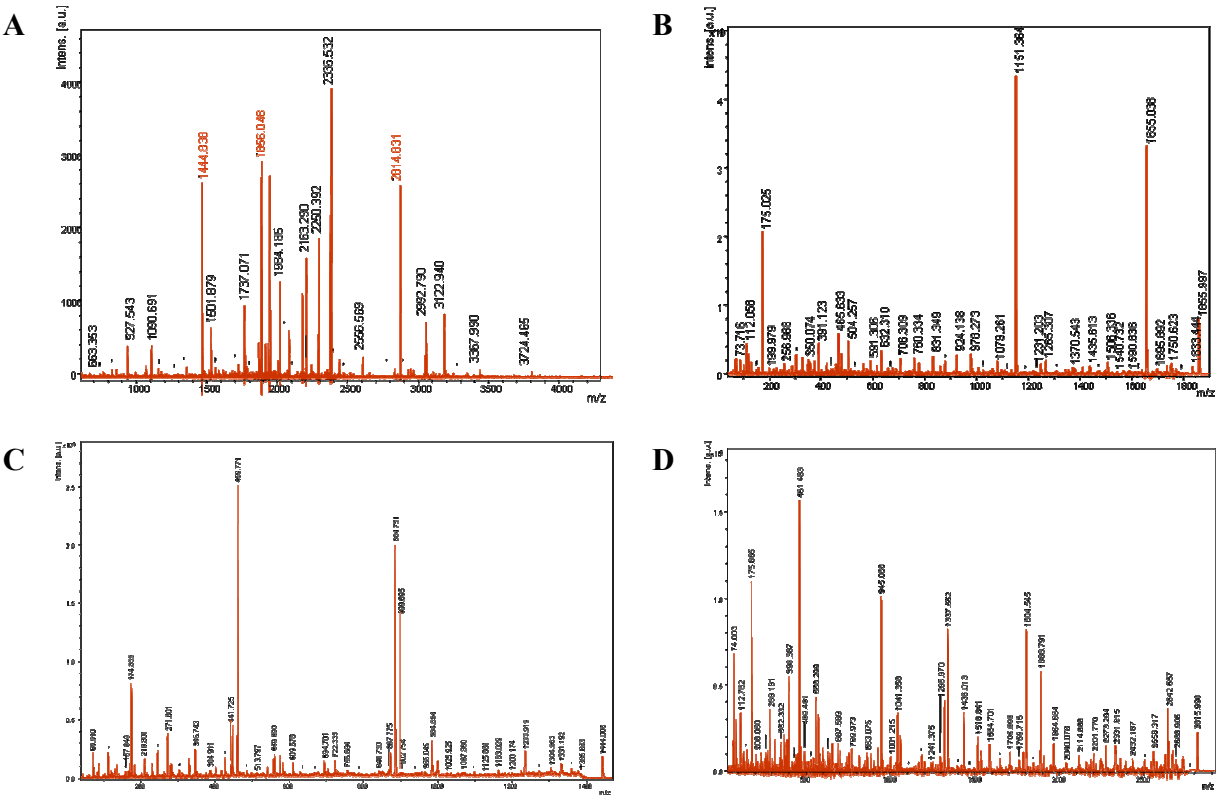


Fig 15. MALDI-TOF/MS of RPV2. **A**. Peptide mass fingerprinting (PMF) of the protein RPV3, **B – D**, post source decay (MS/MS) of the prominent peaks from PMF. The protein was identified from the NCBInr using MASCOT as a histidine kinase, a 72 kD membrane bound protein. It possesses a signal peptide, the HAMP and Tar domain, a unique feature to the Methyl-accepting chemotaxis proteins. MCPs are involved in cell motility and secretion/signal transduction mechanisms of the chemotaxis sensory transducer.

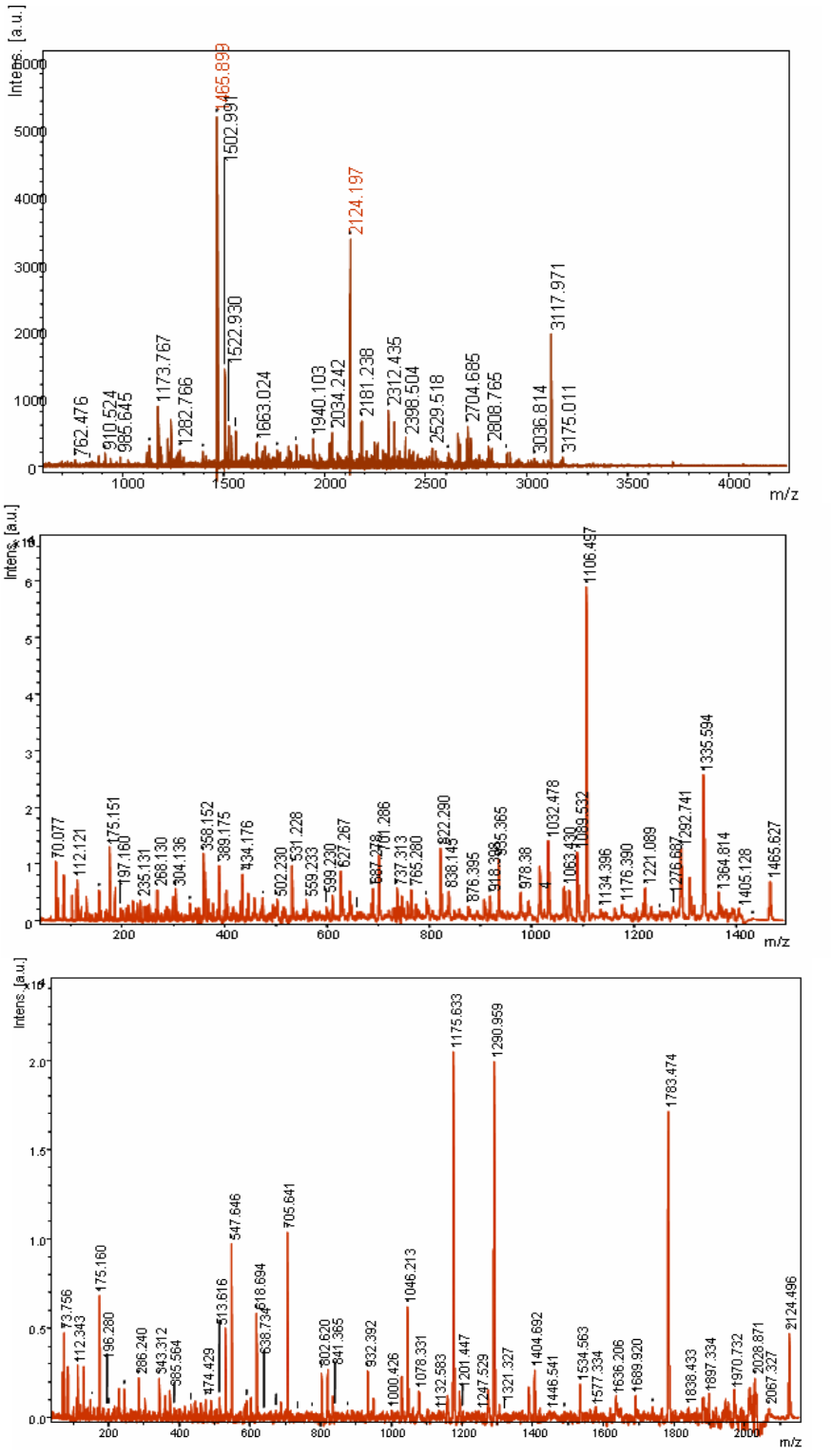


Fig 16. MALDI-TOF/MS of RPV3. **A**. Peptide mass fingerprinting (PMF) of the protein RPV1, **B – C**, post source decay (MS/MS) of the prominent peaks from PMF. The protein was identified from the NCBInr using MASCOT as ribulose-1,5-bisphophate carboxylase/ oxygenase (RubisCO), a 44 kD cytosolic protein.

numbers and probability Mowse score are shown in Figures 17 – 20. The multiple sequence alignment of the proteins using CLUSTALW (Lindahl and Florencio, 2003) among cyanobacterial proteins have shown that they are highly conserved within the unicellular and filamentous cyanobacterial strains (Annexure. III – V). The hydropathy profile (Kyte and Doolittle, 1982) and transmembrane helices and intervening loop regions (TMHMM) of the proteins has identified RPV2 as the membrane protein with 2 membrane anchoring regions. These proteins may have a role in oxidative stress regulation thereby its survival at extreme metal stress conditions. Moreover, overexpression of these proteins not only increases the cell viability but also assist the cells in the metal bioaccumulation processes.

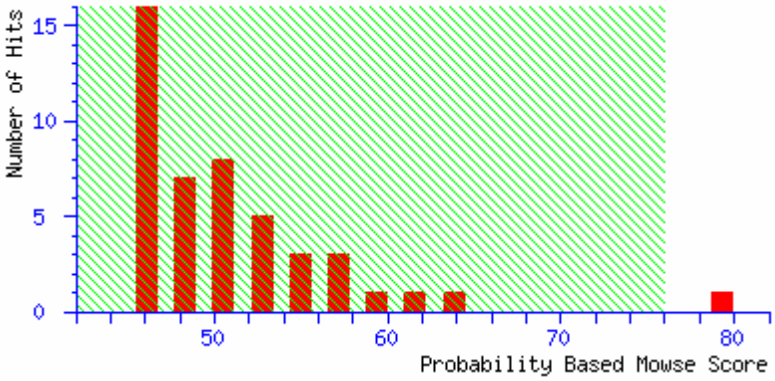
2.3.8 Protein structure prediction

The deduced amino acid sequences of *Nostoc* sp., from cyanobase (cyanobacterial genome database) was used for finding the homologous proteins in the PDB database for each protein. The protein structures were determined by using Modeller 9v2 (Sali and Blundell, 1993). The best homologues and template structures for this were downloaded from NCBI, swissprot and PDB database. Pair-wise alignment was used to find gaps/missing amino acids using Swiss pdb-viewer. PyMOL and DeepView were used for documentation. The modeled structures of the protein Malate dehydrogenase, Ribulose-1,5-bisphosphate carboxylase/oxygenase and Methyl accepting chemotaxis proteins were shown in the Figure 21A – C.

Type of search : **Peptide Mass Fingerprint**
Search title : **RPV1**
Database : **NCBInr 20070324 (4759802 sequences; 1642270362 residues)**
Taxonomy : **Bacteria (Eubacteria) (2291388 sequences)**
Top Score : **79 for gi|69935175, Malate dehydrogenase, NAD-dependent [Paracoccus denitrificans PD1222]**

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 76 are significant (p<0.05).



1. gi|69935175 Mass: **33740** Score: **79** Expect: **0.027** Queries matched: **11**
Malate dehydrogenase, NAD-dependent [Paracoccus denitrificans PD1222]
2. gi|113951449 **Mass: 16126** Score: **60** Expect: **2.3** Queries matched: **7**
conserved hypothetical protein [Shewanella baltica OS195]

Match to: **gi|69935175** Score: **79** Expect: **0.027**
Malate dehydrogenase, NAD-dependent [Paracoccus denitrificans PD1222]
NCBI BLAST search of gi|69935175 against nr; Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M); Cleavage by Trypsin: cuts C-term side of KR unless next residue is P; Number of mass values searched: **91**; Number of mass values matched: **11**
Sequence Coverage: **52%**; Nominal mass (M_r): **33740**; Calculated pI value: **5.44**

Matched peptides shown in **Bold Red**

```
     1 MARPKIALIG AGQIGGTLAH LAAMKELGDV VLFDIAEGTP QGKALDIAQS
    51 GPSEGFDAVM KGANDYADIA GADVCIVTAG VPRKPGMSRD DLLGINLKVM
   101 KSVGEGIKQH APNAFVICIT NPLDAMVWAL REFSGLPHEK VVGMAGVLDS
   151 ARFRHFLSLE FGVSMRDVTA FVLGGHGDTM VPLTRYSTVG GIPLPDLVKM
   201 GWTTQEKLDA IVQRTRDGGA EIVGLLKTGS AFYAPATSAI EMAEAYLKDQ
   251 KRVLPCAAYV KGAYGLDGLY VGVPTVIGAG GIERVIDITL DKDEQAMFDK
   301 SVDAVKGLVT ACKGIDGTLA
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
2 - 25	2358.4570	2357.4497	2357.3729	0.0768	1	M.ARPKIALIGAGQIGGTLAHLAAMK.E
62 - 83	2205.2430	2204.2357	2204.0532	0.1825	0	K.GANDYADIAGADVCIVTAGVPR.K
84 - 98	1672.9660	1671.9587	1671.8978	0.0610	1	R.KPGMSRDDLLGINLK.V*
132 - 140	1043.6220	1042.6147	1042.5083	0.1064	0	R.EFSGLPHEK.V
141 - 152	1190.6980	1189.6907	1189.6125	0.0782	0	K.VVGMAGVLDSAR.F*
153 - 166	1725.9980	1724.9907	1724.8821	0.1086	1	R.FRHFLSLEFGVSMR.D
200 - 207	996.6370	995.6297	995.4382	0.1915	0	K.MGWTTQEK.L*
208 - 216	1071.6240	1070.6167	1070.6196	-0.0029	1	K.LDAIVQRTR.D
228 - 248	2192.2510	2191.2437	2191.0507	0.1930	0	K.TGSAFYAPATSAIEMAEAYLK.D
252 - 261	1176.6960	1175.6887	1175.6485	0.0402	1	K.RVLPCAAYVK.G
262 - 284	2234.2940	2233.2867	2233.1743	0.1124	0	K.GAYGLDGLYVGVPTVIGAGGIER.V

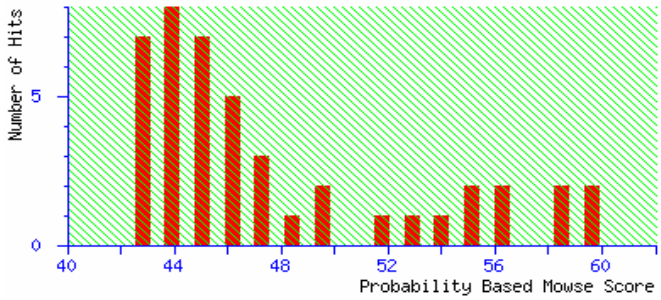
* Oxidation (M)

Fig 17. Peptide Mass Fingerprint of RPV1

Type of search: Peptide Mass Fingerprint
Search title : RPV2
Database : NCBInr 20070504 (4900652 sequences; 1692193060 residues)
Taxonomy : Bacteria (Eubacteria) (2353538 sequences)
Top Score : 53 for gi|76797054, Histidine kinase, HAMP region:Cache:Bacterial chemotaxis sensory transducer [Thermoanaerobacter ethanolicus ATCC 33223]

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 76 are significant (p<0.05).



1. gi|15807545 **Mass:** 6886 **Score:** 60 **Expect:** 2.6 **Queries matched:** 6
hypothetical protein DR2561 [Deinococcus radiodurans R1]
2. gi|89100497 **Mass:** 30599 **Score:** 59 **Expect:** 2.9 **Queries matched:** 11
Dihydropteroate synthase [Bacillus sp. NRRL B-14911]
2. gi|76797054 **Mass:** 71970 **Score:** 53 **Expect:** 13 **Queries matched:** 14
Histidine kinase, HAMP region:Cache:Bacterial chemotaxis sensory transducer [Thermoanaerobacter ethanolicus ATCC 33223]

Match to: **gi|76797054** Score: **53** Expect: **13**
Histidine kinase, HAMP region:Cache:Bacterial chemotaxis sensory transducer [Thermoanaerobacter ethanolicus ATCC 33223]; NCBI BLAST search of gi|76797054 against nr; Fixed modifications: Carbamidomethyl (C); Variable modifications: Oxidation (M); Cleavage by Trypsin: cuts C-term side of KR unless next residue is P; Number of mass values searched: **100**; Number of mass values matched: **14**;
Sequence Coverage: **22%**; Nominal mass (M_r): **71970**; Calculated pI value: **4.96**

Matched peptides shown in **Bold Red**

```
     1 MKSIRTKLLV FILLFILIPL VITGYFSTNI AESVLKTKIN DSNQAALSVL
    51 NKYINSIKQD TESMLEMLAE SNTIMNYDGS SASDEAVLKK LEEVKKSMPN
   101 AMNVYFATPD KKMILYPPQK LENYDPTERP WYKDAEKAGG NIVWTDPYED
   151 FNTKAPEITV TKAITGSSGK LVGILGIDIS LEQLSKNISS VKLGKTGYIY
   201 LVTKDGTVIT HPDTTKLFTS IKKYDFGEKL LSLNNATIQY SSNNVYKFAS
   251 VRNLDSFGWK AVVTMGNSEL TKDVTKIRDF VITVSIIVLL IGFLIAYFFA
   301 NSISSGIKKV VTAMSAAAKG DITVKANVKA KDEVGILANS FNTMIEGIKR
   351 LVFDIRSVSE SVNHSAENMA VASEQAAQAT QDVAKAIEEI AQGASSQARE
   401 AEESTNATVL LGQLIDQSLK NADEINQEVA NVSMVSNEGL VIIDDLIKKT
   451 ELTVEANNNV KESTNYLLEK STEISKIVET ITSIADQTNL LSLNAAIEAA
   501 RAGEAGRGFA VVADEVRKLA EQSSQAARNI ANLISEIQNT INDTYKTVED
   551 STKSIEEQSN VVNTTKDVFE GILHAVNFIV EKIDNLNKSL KEIEEHKNKI
   601 VDSIQNIAAV SEESAASAEE VSATSQEQSA IVEEMASTAN ELKNYANTLI
   651 EAIKQFKVE
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
53 - 58	736.4280	736.4280	736.4119	0.0161	0	K.YINSIK.Q
91 - 95	616.3410	616.3410	616.3432	-0.0022	0	K.LEEVK.K
155 - 170	1558.8990	1558.8990	1558.8566	0.0424	1	K.APEITVTKAITGSSGK.L
224 - 229	757.4510	757.4510	757.3283	0.1227	0	K.YDFGEK.L
230 - 247	2041.2200	2041.2200	2041.0480	0.1720	0	K.LLSLNNATIQYSSNNVYK.F
230 - 252	2601.5080	2601.5080	2601.3551	0.1529	1	K.LLSLNNATIQYSSNNVYKFASVR.N
253 - 272	2196.2610	2196.2610	2196.0885	0.1725	1	R.NLDSFGWKAVVTMGNSELTK.D
309 - 319	1091.6740	1091.6740	1091.6009	0.0731	1	K.KVVTAMSAAAK.G*
310 - 319	947.5890	947.5890	947.5110	0.0780	0	K.VVTAMSAAAK.G
330 - 349	2165.2830	2165.2830	2165.1038	0.1792	1	K.AKDEVGILANSFNTMIEGIK.R*
449 - 461	1458.7520	1458.7520	1458.7678	-0.0158	1	K.KTELTVEANNNVK.E
462 - 476	1741.0440	1741.0440	1740.8781	0.1659	1	K.ESTNYLLEKSTEISK.I
471 - 476	663.3530	663.3530	663.3439	0.0091	0	K.STEISK.I
644 - 657	1651.9720	1651.9720	1651.8933	0.0787	1	K.NYANTLIEAIKQFK.V

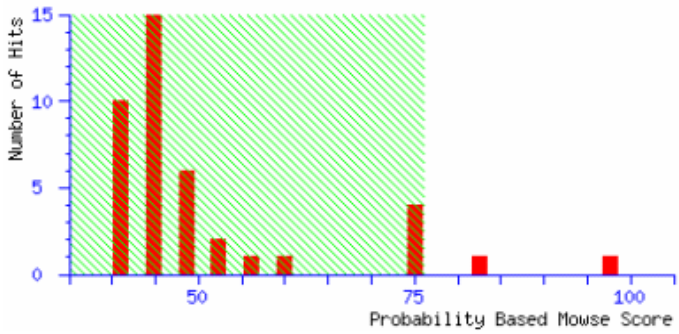
* Oxidation (M)

Fig 18. Peptide Mass Fingerprint of RPV2

Type of search : **Peptide Mass Fingerprint**
Search title : **RPV3**
Database : **NCBInr 20070316 (4739122 sequences; 1635531281 residues)**
Taxonomy : **Bacteria (Eubacteria) (2283968 sequences)**
Top Score : **98 for gi|89241990, ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit [Nostoc sp. PCC 7906]**

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 76 are significant (p<0.05).



1. gi|89241990 **Mass:** 44301 **Score:** **98** **Expect:** 0.0004 **Queries matched:** 20
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Nostoc sp. PCC 7906]
2. gi|89241980 **Mass:** 44516 **Score:** **84** **Expect:** 0.0097 **Queries matched:** 17
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Fischerella muscicola PCC 7414]
3. gi|89242115 **Mass:** 44517 **Score:** 84 **Expect:** 0.0097 **Queries matched:** 17
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Fischerella thermalis PCC 7521]

Match to: **gi|89241990** Score: **98** Expect: 0.0004 **ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit** [Nostoc sp. PCC 7906]
Fixed modifications: Carbamidomethyl (**C**); Variable modifications: Oxidation (**M**)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **97**; Number of mass values matched: **20**; Sequence Coverage: **45%**
Nominal mass (Mr): **44301**; Calculated pI value: **6.66**

Matched peptides shown in **Bold Red**

```
     1 TVWTDLLTDL DRYKGRCYDI EPVAGEDNQY IAYVAYPLDL FEEGSVTNML
    51 TSIVGNVFGF KALRALRLED LRIPVAYLKT FQGPPHGIQV ERDKLNKYGR
   101 PLLGCTIKPK LGLSAKNYGR AVYECLRGGL DFTKDDENIN SAPFQRWRDR
   151 FLFVSEAIAK AQAETGEIKG HYLNVTAPTC EQMLQRAEFA KELKQPIIMH
   201 DYLTAGFTAN TTLARWCRDN GILLHIHRAM HAVIDRQKNH GIHFRVLAKT
   251 LRMSGGDHIH TGTVVGKLEG ERGITMGFVD LLRENYVEQD KSRGIYFTQD
   301 WASMPGVMAV ASGGIHVWHM PALVEIFGDD SVLQFGGGTL GHPWGNAPGA
   351 TANRVALEAC IQARNEGRNL AREGNDVIRE AAKWSPELAV ACEL
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
1 - 14	1739.1020	1738.0947	1737.8938	0.2010	1	-.TVWTDLLTDLDRYK.G
65 - 72	985.6450	984.6377	984.5716	0.0661	1	R.ALRLEDLR.I
80 - 92	1465.8990	1464.8917	1464.7474	0.1443	0	K.TFQGPPHGIQVER.D
80 - 94	1709.0390	1708.0317	1707.8693	0.1624	1	K.TFQGPPHGIQVERDK.L
98 - 110	1502.9910	1501.9837	1501.8439	0.1398	0	K.YGRPLLGCTIKPK.L
121 - 127	910.5240	909.5167	909.4378	0.0789	0	R.AVYECLR.G
128 - 146	2124.1970	2123.1897	2122.9920	0.1978	1	R.GGLDFTKDDENINSAPFQR.W
135 - 148	1748.0550	1747.0477	1746.8074	0.2404	1	K.DDENINSAPFQRWR.D
149 - 160	1395.8840	1394.8767	1394.7558	0.1209	1	R.DRFLFVSEAIAK.A
170 - 186	2034.2420	2033.2347	2032.9459	0.2888	0	K.GHYLNVTAPTCEQMLQR.A*
192 - 215	2704.6850	2703.6777	2703.4054	0.2723	1	K.ELKQPIIMHDYLTAGFTANTTLAR.W
192 - 215	2720.6830	2719.6757	2719.4003	0.2754	1	K.ELKQPIIMHDYLTAGFTANTTLAR.W*
219 - 228	1187.7660	1186.7587	1186.6571	0.1017	0	R.DNGILLHIHR.A
237 - 245	1136.7210	1135.7137	1135.5999	0.1138	1	R.QKNHGIHFR.V
239 - 245	880.5290	879.5217	879.4464	0.0754	0	K.NHGIHFR.V
273 - 283	1221.7750	1220.7677	1220.6587	0.1090	0	R.GITMGFVDLLR.E
273 - 283	1237.7520	1236.7447	1236.6537	0.0911	0	R.GITMGFVDLLR.E*
273 - 291	2243.3020	2242.2947	2242.0940	0.2007	1	R.GITMGFVDLLRENYVEQDK.S*
284 - 291	1024.6620	1023.6547	1023.4509	0.2038	0	R.ENYVEQDK.S
355 - 364	1130.7020	1129.6947	1129.5914	0.1034	0	R.VALEACIQAR.N

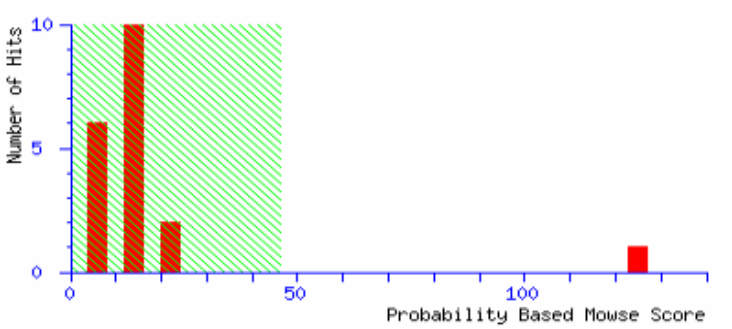
*Oxidation (M)

Fig 19. Peptide Mass Fingerprint of RPV3

Type of search : **MS/MS Ion Search**
Search title : **RPV3-MS/MS**
Database : **NCBInr 20070504 (4900652 sequences; 1692193060 residues)**
Taxonomy : **Bacteria (Eubacteria) (2353538 sequences)**
Protein hits : gi|142082 ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit
gi|121608123 Enoyl-CoA hydratase/isomerase [Verminephrobacter eiseniae EF01-2]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.Individual ions scores > 46 indicate identity or extensive homology (p<0.05).Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Match to: **gi|142082** Score: **125; ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit** NCBI BLAST search of gi|142082 against nr; Fixed modifications: Carbamidomethyl (**C**); Variable modifications: Oxidation (**M**); Cleavage by Trypsin: cuts C-term side of KR unless next residue is P; Sequence Coverage: **6%**; Nominal mass (**Mr**): **53573**; Calculated **pI value: 6.38**

Matched peptides shown in **Bold Red**

```
     1 MSYAQTKTQT KSGYKAGVQD YRLTYYTPDY TPKDTDILAA FRVTPQPGVP
    51 FEEAAAAVAA ESSTGTWTTV WTDLLTDLDR YKGRCYDIEP GPGEDNQSIA
   101 YIAYPLDLFE EGSITNVLTY IVGNVFVFKA LRALRLEDIR FPVAYIKTFQ
   151 GPPHGIQVER DKLNKYGRPL LGCTIKPKLG LSAKNYGRAV YECLRGGLDF
   201 TKDDENINSA PFQRWRDRFL FVADAITKAQ AETGEIKGHY LNVTAPTCEE
   251 MLKRAEYAKE LKQPIIMHDY LTAGFTANNT LARWCRDNGL LLHIHRAMHA
   301 VIDRQKNHGI HFRVLAKALR LSGGDHIHTG TVVGKLEGER GITMGFVDLL
   351 RENYVEQDKS RGIYFTQDWA SLPGVMAVAS GGIHVWHMPA LVEIFGDDFV
   401 LQFGGGTLGH PWGNARGATA NRVALEACVQ ARNEGRNLAR EGNDVIREAA
   451 KWSPELAVAC ELWKEIKFEF EAMDTV
```

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
1	1465.6270	1464.6197	1464.7474	-0.1277	0	62	0.0018	1	K.TFQGPPHGIQVER.D
2	2124.4960	2123.4887	2122.9920	0.4968	1	63	0.00099	1	R.GGLDFTKDDENINSAPFQR.W

Proteins matching the same set of peptides:
gi|223640 Mass: **53484** Score: **125** Queries matched: **2**
carboxylase,RBP
gi|23126258 Mass: **53449** Score: **125** Queries matched: **2**
COG1850: Ribulose 1,5-bisphosphate carboxylase, large subunit [Nostoc punctiforme PCC 73102]
gi|75910111 Mass: **53399** Score: **125** Queries matched: **2**
methionine sulfoxide reductase A [Anabaena variabilis ATCC 29413]
gi|89241966 Mass: **44500** Score: **125** Queries matched: **2**
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit[Anabaena sp.'WH Schoolst.isolate']
gi|89241970 Mass: **44370** Score: **125** Queries matched: **2**
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit[Chlorogloeopsisfritschii PCC 6912]
gi|89241974 Mass: **44322** Score: **125** Queries matched: **2**
ribulose-1,5-bisphosphate carboxylase/oxygenaselargesubunit[CylindrospermumlicheniformeUTEX2014]
gi|89242000 Mass: **44304** Score: **125** Queries matched: **2**
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Tolypothrix sp. CCMP1185]

Fig 20. Peptide fragmentation data of RPV3

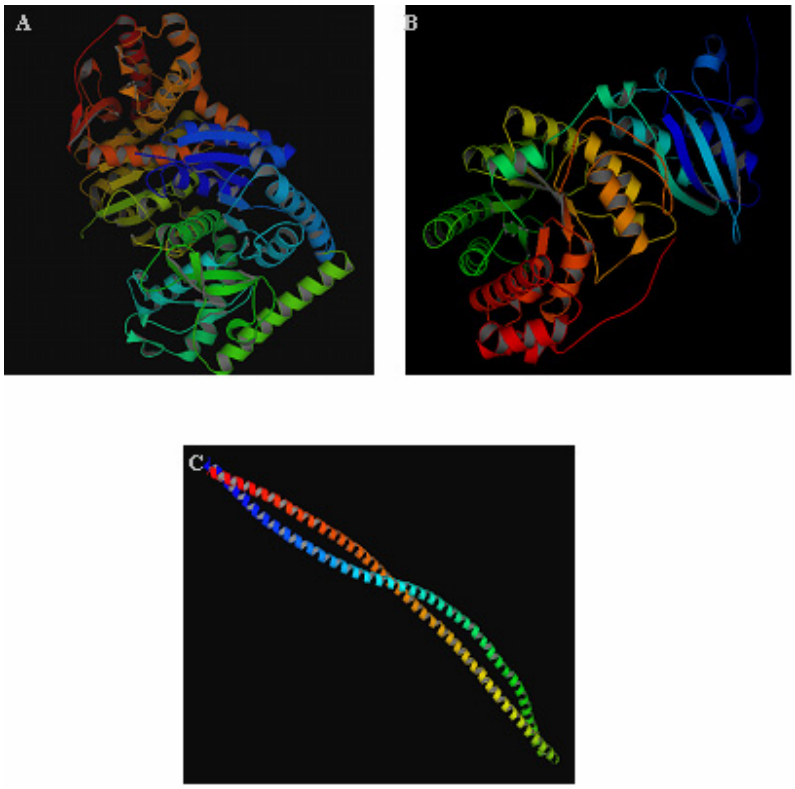


Fig 21. Protein structures. (A) Malate Dehydrogenase (MDH); (B). Ribulose-1,5-bisphosphate carboxylase/oxygenase - Large subunit (RubisCO) and (C) Methyl accepting chemotaxis protein (MCP) of *Nostoc punctiforme* ATCC 29133. These structures were obtained by homology modeling using MODELLER.

Chapter III

Development of Recombinant N. calcicola for Metal Bioremediation

Chapter III

DEVELOPMENT OF RECOMBINANT *N. CALCICOLA* FOR METAL BIOREMEDIATION

3.1 Introduction

The genetically engineered microorganisms (GEM) have shown potential for applications in soil, groundwater and activated sludge environments (Gadd, 2000). However, the vast majority of studies pertaining to genetically engineered microbes are supported by laboratory based experimental data and relatively few examples of GEM applications in environmental ecosystems exist (Gadd, 2000; Barkay and Schaefer, 2001). A wide variety of strategies have been demonstrated on exploitation of GEM to remediate contaminated environments including metal containing waste water. Among the different methods for such strain improvement techniques, research on the use of metal binding proteins, in particular the "metallothionein" has taken its right in the area of metal bioremediation.

The metallothionein (MT) is a low molecular weight, cysteine-rich, metal binding protein which plays an important role in heavy metal detoxification in mice (Kagi and Schaffer, 1988; Vallee, 1991). Since the characterization of mouse metallothionein and its role in metal sequestration (Kagi and Schaffer, 1988), cell surface modification or intracellular expression of metallothionein has been studied in bacterial systems like *E. coli* (Chen and Wilson, 1997), *Staphylococcus* sp., (Kotrba et al. 1999), *Deinococcus radiodurans*, (Brim et al. 2000) *Ralstonia eutropha* CH34 (Valls et al. 2000), *Pseudomonas* sp., (de Lorenzo, 2000) and cyanobacteria (Erbe et al. 1996). Prokaryotic metallothionein *smtA* has been cloned and expressed in *E. coli* (Sode et al. 1998; Valls et al. 1998). The recent publications have proposed novel variations on the use of metallothioneins in bioremediation (Pazirandeh et al. 1998). Expression of MT with a nickel transporter (*nixA* of *Helicobacter pylori*) in *E. coli* has enhanced rate of intracellular Ni^{2+} bioaccumulation (Krishnaswamy and Wilson, 2000). Similar results were obtained with mercury transporter (Pazirandeh et al. 1998).

Cyanobacteria are photosynthetic microorganisms that can fix atmospheric nitrogen and grow in a wide variety of environmental niches including extremes of temperature, salinity, aridity and pH (Paerl et al. 2000). Cyanobacterial responses to metals, nutrients, UV-radiation, heat shock and metabolic stress have been thoroughly reviewed (Tandeau de Marsac and Houmard, 1993). Genetic tools for manipulation of some cyanobacterial strains have been developed (Koksharova and Wolk, 2002) and offer an economical method for treating large volumes of low level waste water which will otherwise be too costly by using conventional technologies. The use of such organisms would avoid addition of organic nutrients to the inoculated environment; thus making it most economical and less maintenance requiring for the effluent treatment (Kargi and Özmihci, 2002). Typically, microbial strains chosen for metal bioremediation should have flexible metabolism, metal resistance and adaptation to changing environmental conditions. Cyanobacteria suit the above criteria (Chojnacka, 2003), in addition to being unique among prokaryotes in that they possess physiological properties (Meeks and Elhai, 2002) otherwise found only in eukaryotic plants.

Nostoc calcicola is a filamentous cyanobacterium which has been studied for its response to metals (Verma et al. 1993, Verma and Singh, 1995; Raveender et al. 2002), herbicides and stress tolerance (Agrawal and Singh, 2002). Hence, *N. calcicola* was chosen to genetically engineer and express metallothionein protein for applications in heavy metal bioremediation.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids and culture media

The bacterial strains and plasmids used are listed in Table 12. *E. coli* DH5α was used as a general host for most recombinant DNA protocols. *E. coli* HB101 was used for transformation of *N. calcicola* with the recombinant plasmids. Axenic cultures of *E. coli* DH5α and *E. coli* HB101 were procured from Bangalore GENEI, Bangalore. These were grown in Luria Bertani (LB) broth (Himedia, Mumbai) or LB supplemented with 1.5% agar under standard culture conditions.

Table 12. Bacterial strains and plasmids used.

S. No.	Bacterial strains/Plasmids	Properties
1.	*E. coli* DH5α	Host for recombinant plasmids
2.	*E. coli* DH10B	Host for recombinant plasmids; F^- conjugal strain
3.	*E. coli* HB101	Host; F^- conjugal strain; $mcrB^-$
4.	E. coli BL21(DE3)pLysS	Expression host
5.	RP4[a]	Inc P plasmid; Am^r, Km^r and Tet^r
6.	pRL623[a]	Helper plasmid, carries genes for DNA methylases
7.	pRL439[a]	*psbA* promoter (*A. hybridus*)
8.	pRL488[a]	pDU1 replicon; Km^r
9.	pBxMT[b]	pBS SK+; carries mMT gene; Am^r
10.	pUC19	Cloning vector; Am^r
11.	pRSET(B)[c]	Source for flanking BamHI/EcoRI sites; Am^r
12.	pRSKV12	Recombinant plasmid; pDU1; psbA-mMT; Km^r
13.	pET29a-*smtA*[d]	*Synechochocus sp*. PCC 7942, smtA protein

Kind gift of [a]Prof. C.P.Wolk; [b]Prof. R. Palmitter; [c]Dr. J. Scaria, USA and [d]Prof. N. Robinson, UK.

3.2.2 Enzymes and chemicals

DNA manipulations were carried out using standard molecular biology protocols as described in Molecular Biology Laboratory manual by Sambrook and Russel (2001). All the enzymes used for DNA manipulations and cloning were purchased from New England Biolabs or MBI Fermentas (USA). The metal salts used were of analytical grade and these were purchased from either Sigma, USA; BDH England and/or Merck Germany. Metal stock solutions were prepared in MilliQ water and digested using 1 – 2% nitric acid. Atomic spectral standards for all the metals were purchased from National Physical Laboratory, New Delhi, and were used for preparing working standard for atomic absorption spectroscopy and metal estimations during bioaccumulation studies. All glassware and plastic wares were cleaned before use with cedepol, followed by overnight soaking in 50% nitric acid.

3.2.3 Metal ion analysis

Metal ion concentrations were determined in a Perkin Elmer (A Analyst 300) atomic absorption spectrophotometer using air-acetylene or nitrous oxide-acetylene flame. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. The instrument was first calibrated with the standard metal solutions in the range covering the concentrations likely to be found in the samples. The samples were analyzed in triplicates to maintain reproducibility.

3.2.4 Construction of recombinant *N. calcicola*

3.2.4.1 Isolation of plasmid DNA

Plasmid DNA from *E. coli* was isolated with a Qiagen plasmid isolation kit as follows: 10 ml of Luria Bertani broth (LB broth) containing respective antibiotics was inoculated with a single colony of an overnight grown *E. coli* culture from LB agar plates. The flasks were incubated overnight at 37 °C with 200 rpm in an orbital shaker incubator. 2 ml of this overnight culture was transferred to 2 ml micro centrifuge tubes and spun at 10000 rpm for one minute. The supernatant was discarded and the pellet was resuspended in 300 µl of resuspension buffer Pl. 300 µl of Lysis buffer P2 was then added and the contents were mixed gently by inversion and incubated at RT for 5 min. 300 µl of

prechilled buffer P3 was added, followed by incubation on ice for 8 min. The contents were then spun at 10000 rpm for 10 min at 4 °C and the supernatant was transferred to the Qiagen column equilibrated with buffer QBT. After washing the column with buffer QC, the DNA was eluted using 1 ml of buffer QF in a fresh 2 ml micro centrifuge tube. DNA was precipitated by the addition of 0.75 volume of isopropanol and centrifuged. The DNA pellet was washed with 1 ml of 70% ethanol and spun again as before. The supernatant was discarded and tubes were air dried for 30 min. The DNA was resuspended in 30 µl of 10 mM Tris-Cl, pH 8.0 and stored at -20 °C until use.

3.2.4.2 Agarose gel electrophoresis of DNA

Electrophoresis of DNA was carried out on 1 % agarose gels prepared in 1x Tris Acetate EDTA Buffer (TAE buffer) and electrophoresed with 1x TAE buffer in the gel tank at 90 Volts (V) for 1 h. The resolved gels were visualized and documented with a Syngene Genesnap Gel Documentation system.

3.2.4.3 Restriction enzyme digestion and purification of DNA

DNA to be purified after restriction enzyme digestions at 37 ^{o}C in a circulating water bath was loaded onto a 1% agarose gel in TAE and electrophoresed at 90 Volts (V) for 1 h. The desired DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a micro centrifuge tube and 3 volumes of buffer QG was added to 1 volume of gel (100mg=100µl). Gel slice with the buffer was incubated at 52 °C until the gel was completely dissolved. After the dissolution of gel, 1 volume of isopropanol was added and mixed. To bind DNA, the sample was applied to Qia quick column placed in a 2 ml collection tube and was centrifuged at 13000 rpm for 1 min in a micro centrifuge (Eppendorf 5415D). The flow-through was discarded and the column was placed back in the same collection tube. 0.5 ml of buffer QG was added to the column and centrifuged as in the previous step. The column was further washed twice with 0.75 ml of buffer PE. To elute DNA, the column was placed on top of a 2 ml micro centrifuge tube, and 30 µl of 10 mM Tris-Cl (pH 8.0) was added and centrifuged at 13000 rpm for 1 min. The purified DNA was stored at -20 °C until use.

3.2.4.4 Dephosphorylation of vector DNA

To reduce the probability of self-ligation of the linearised vector, after digestion with restriction enzymes, 1 unit of Calf Intestinal Alkaline Phosphatase (CIAP) was added to

the restriction endonulease digestion mixture and was incubated for another 30 min at 37 °C. The dephosphorylated DNA was then purified using a Qia quick column as described in section 3.2.4.3.

3.2.4.5 Ligation

Ligation of the insert DNA and dephosphorylated vector DNA was carried out with T4 DNA ligase. A 10 µl reaction mixture was set up as per the composition given in Table 13. For obtaining the maximum number of recombinants, a vector: insert molar ratio of 1:2 was maintained in the reaction mixture. The above mixture was prechilled in a 0.5 ml micro centrifuge tube and it was incubated at 16 °C for 12 h in a circulating water bath. Total reaction mixture was used to transform competent cells of *E.coli* DH5α.

Table 13. Composition of ligation mixture used to clone insert DNA into plasmid vectors

Component	**Stock concentration**	**Volume added (µl)**
Plasmid DNA	20 ng μl^{-1}	2.0
Insert DNA	40 ng μl^{-1}	1.6
T4 DNA ligase	1.0 U μl^{-1}	1.0
T4 DNA ligase buffer	10 x	1.0
Sterile MilliQ water	-	4.4
	Total	10.0

3.2.5 Transformation

3.2.5.1 Preparation of competent cells

For the preparation of competent cells of *E. coli* DH5α, HB101 and DH10B, each strain was streaked separately on LB agar plates and incubated at 37 °C for 12 h. A single colony was transferred to 5 ml LB broth in a 20 ml conical flask and was incubated at 37 °C with 200 rpm in an orbital incubator shaker for 14 h. 1 ml of this starter culture was used to inoculate 50 ml of LB broth in a 500 ml conical flask and was incubated at 37 °C with 200 rpm shaking till OD_{600} reached 0.5. The flask was then chilled on ice for 10 min and culture was transferred to a 50 ml Oakridge tube. This was centrifuged in a refrigerated centrifuge at 8000 rpm for 10 min at 4 °C. The supernatant was discarded

and the pellet was resuspended in 12 ml of prechilled 0.1 M $CaCl_2$ solution. The cell suspension was incubated on ice for 30 min and centrifuged at 8000 rpm for 8 min at 4 °C. The supernatant was discarded and pellet was resuspended in 1 ml of 0.1 M $CaCl_2$ and prechilled glycerol was added to a final concentration of 15 %. The cell suspension was frozen in a mini cooler and stored at -70 °C until use.

3.2.5.2 Transformation

10 µl of the ligation mixture or 1 µg of Qia column purified plasmid DNA was added to a 0.5 ml micro centrifuge tube and was allowed to chill on ice for 10 min. 100 µl of competent cells were added to this tube and incubated on ice for 30 min. The mixture was subjected to heat shock at 42 °C for 60 – 90 sec in a water bath followed by incubation on ice for 2 min. 500 µl of LB broth was added to the cell suspension and the tube was incubated at 37 °C for 1 h. The tube was then centrifuged at 8000 rpm for 5 min and 500 µl of supernatant was removed and cell pellet was resuspended in remaining 100 µl of supernatant and the cell suspension was then plated on LB agar plates containing antibiotics. The plates were incubated at 37 °C for 14 h and screened for transformants.

3.2.5.3 Screening of the transformants for recombinants

After incubation, the resulting colonies were replica plated in duplicates on LB agar plates containing antibiotics. The bacterial colonies were picked at random, subcultured in 10 ml LB broth and incubated at 37 ^{o}C for 12 h in an orbital shaker with 200 rpm. 2 ml of the grown culture was used to isolate plasmids and the plasmid DNA was electrophoresed as described in section 3.2.4.1 – 2. The recombinants were selected based on the gel mobility shift and DNA without the insert DNA served as control. Recombinant plasmids showing gel mobility shift were selected and the presence of insert was confirmed by digestion with respective restriction enzymes. The resultant recombinant plasmid was named as pRSKV12. The overall strategy of the recombinant plasmid construction is portrayed in Fig. 21 and 22. psbA-mMT DNA fragment cloned into pUC19 was sequenced by ABI Prism DNA sequencer using M13 universal primers.

3.2.6 Transformation of *N. calcicola*

3.2.6.1 Growth conditions for recipient cells

The triparental mating was used to transform *N. calcicola* (Wolk et al. 1984). The filamentous cyanobacterium *N. calcicola* was grown as described in section 2.2.1. The cells were harvested by centrifugation at 6000 rpm at RT for 10 min, washed with fresh sterile AA medium and fragmented by cavitation to 2 – 3 cells per filament by sonication (Microson XL2000 Ultrasonic Cell disruptor). These cells were washed, resuspended in AA medium and incubated for 24 h at culture room conditions for recovery. These were harvested and used for triparental mating.

3.2.6.2 Preparation of conjugal and cargo strains

The overnight grown *E. coli* conjugal strain (*E. coli* HB101 containing the plasmid RP4) and cargo strain (*E. coli* HB101 transformed with cargo plasmid i.e. pRSKV12 and pRL623) were harvested and subcultured at 1:40 dilution in fresh LB supplemented with respective antibiotics. These were harvested when the OD_{600} reached approximately 0.6 – 0.8 (2.5 – 3.0 h). For each plate mating, 10 ml of conjugal and cargo strains were harvested by centrifuging at 6000 rpm for 8 min. These cells were washed twice with sterile LB without antibiotics to remove residual antibiotics from the original culture medium. The cell suspensions of these two were mixed together, centrifuged and the cell pellet was resuspended in 200 µl of LB and allowed the mixture at RT until use.

3.2.6.3 Conjugal transfer of recombinant DNA

A 50 ml culture of cavitated *N. calcicola* cells were harvested for multiple plate matings. The cell pellet was resuspended in minimal volume (10 µl should contain ca. 10 µg *chl a*) and it was serially diluted in sterile AA medium. For each plate matings, 10 µl of the diluted *N. calcicola* cells were mixed with 200 µl of *E. coli* (conjugal and cargo strain). This was plated onto a sterile Millipore HATF membrane (Immobilon-NC, 0.45µm, surfactant free filters) laid onto AA agar (1.0%) plates without antibiotics and supplemented with 5.0% sterile LB. The plates were incubated in a low light area (covered with muslin cloth) for 2 days. Filters were then transferred carefully onto fresh AA agar plates supplemented with respective antibiotics and allowed to grow under normal culture conditions for 10 – 15 days.

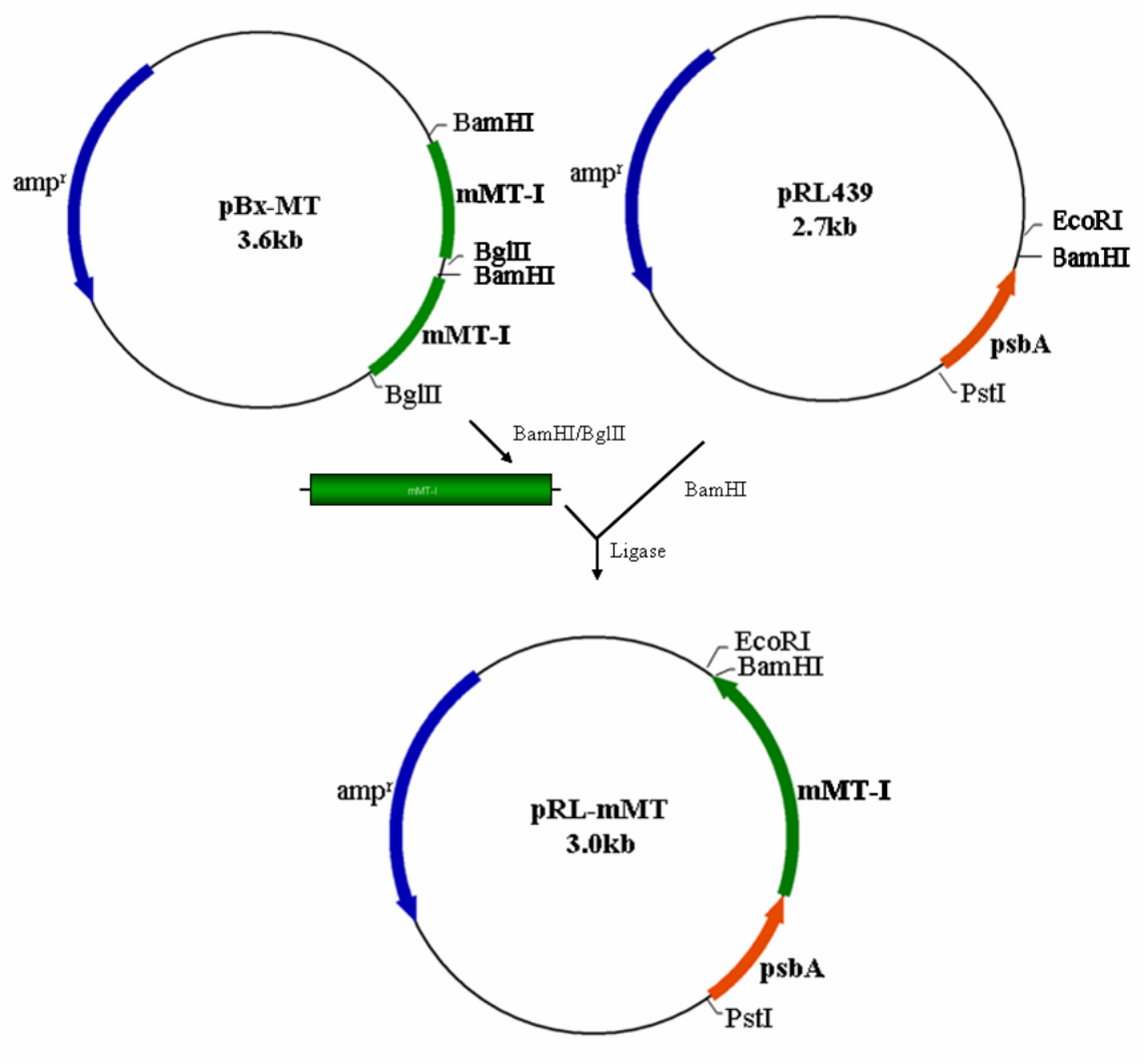


Fig 22. Cloning strategy for construction of the plasmid pRL-mMT. Mouse metallothionein gene from pBx-MT was digested with BamHI/BglII and was cloned into the BamHI site on plasmid pRL439. The resulting pRL-mMT which contains the mMT gene in frame with the promoter *psbA* was transferred into *E. coli* DH5α to express the metallothionein protein.

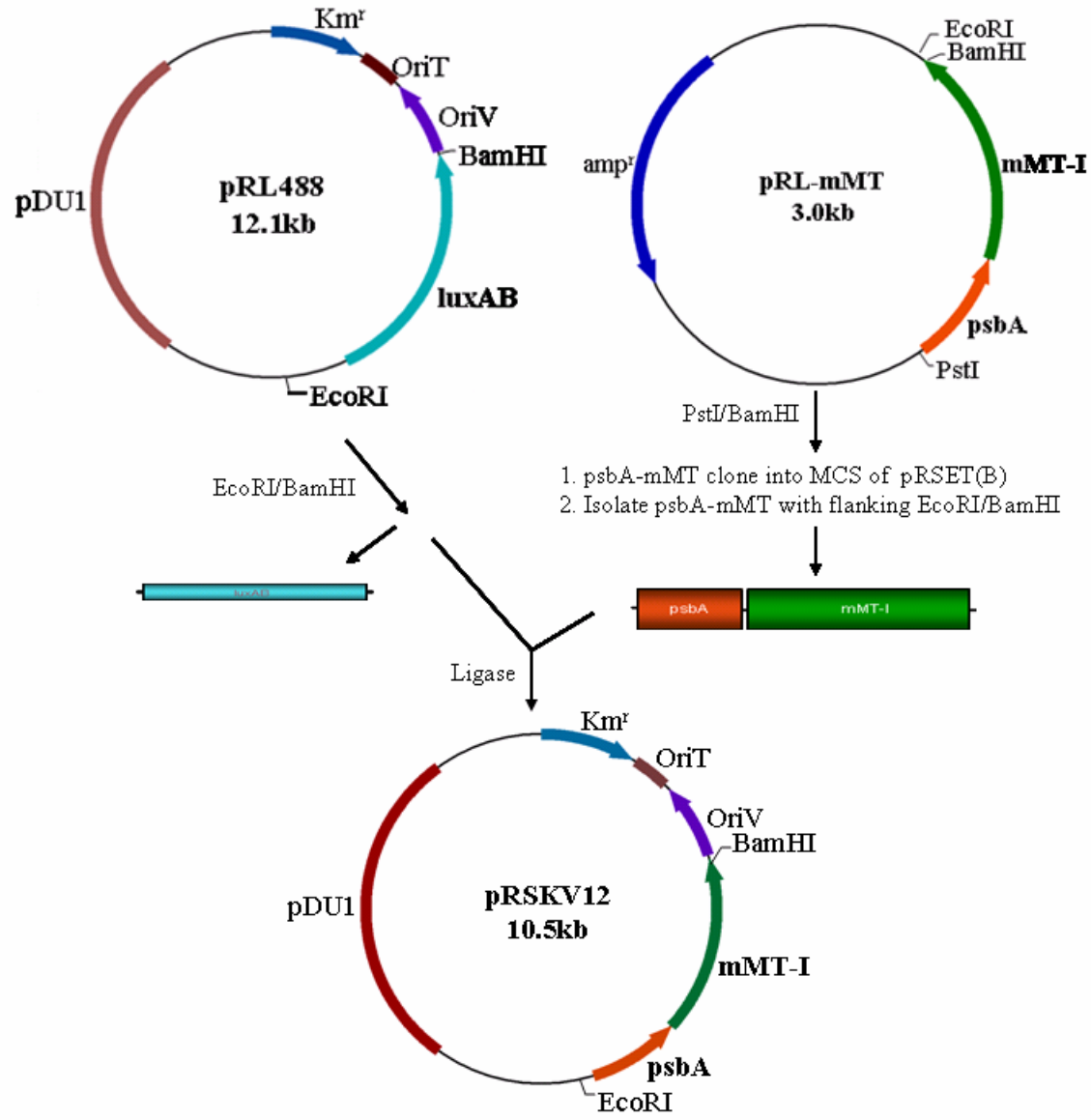


Fig 23. Cloning strategy for the construction of the plasmid pRSKV12. The plasmid pRL-mMT was digested with PstI/BamHI and the psbA-mMT DNA fragment was cloned into pRSET(B) digested with similar enzymes. psbA-mMT with flanking EcoRI/BamHI from pRSET(B) was cloned into the pRL488. The resulting pRSKV12 which contains the mMT gene in frame with promoter *psbA* was transferred into *E. coli* DH5α and HB101. *E. coli* HB101-pRSKV12 was co-transformed with pRL623 for conjugal transfer of recombinant plasmid into *N. calcicola* to express metallothionein protein.

3.2.6.4 Purification of exconjugants

Exconjugant colonies from AA agar plates were subcultured in AA medium containing Kanamycin (Km) 30 µg ml^{-1} and frequently transferred to fresh AA medium with antibiotics to rid of any *E. coli* associated with *N. calcicola*. Such cultures were streaked on nutrient agar plates and incubated in dark conditions for testing the presence of contaminating *E. coli*. The axenic recombinant *N. calcicola* was used for further experiments.

3.2.6.5 Screening for recombinants

2 ml of the axenic culture of recombinant *N. calcicola* was used to isolate plasmid DNA (2.2.6.3) and electrophoresed as described in section 3.2.4.2. The presence of insert, psbA-mMT was confirmed by digestion with EcoRI/BamHI restriction enzymes.

3.2.6.6 Expression of metallothionein in *N. calcicola*

The recombinant *N. calcicola* grown on culture medium supplemented with metals and antibiotics were harvested and stored at -70 ^{o}C. Protein samples for electrophoretic separation were prepared as outlined in section 2.2.8.1 and total protein was determined by BCA method (2.2.3.2). *Synechococcus sp.* PCC 7942 metallothionein was induced from *E. coli* BL21(DE3)pLysS pET29a-SmtA using Isopropyl-ß-D-thiogalacto pyranoside (IPTG) at 0.5 mM for 3 h and plasmid free host cell lysate was used as control. A 50 µg of total protein was separated on a 15% SDS-PAGE (2.2.10.2), stained with CBB and protein bands were visualized. The protein gel image was documented using Syngene gel documentation system.

3.2.7 Characterization of recombinant *N. calcicola*

3.2.7.1 Growth

3.2.7.1.1 Spectrophotometric method

The growth of recombinant strain was monitored spectrophotometrically at OD_{750}. *N. calcicola* was inoculated in fresh AA medium containing Km 30 µg ml^{-1} and incubated in the culture room in an orbital shaker at 100 rpm. 1 ml culture was withdrawn for every 24 h and its absorbance measured at OD_{750} for 15 days. Sterilized plain medium was used to dilute cultures when OD_{750} reached 1.0.

3.2.7.1.2 Determination of proteins

The growth of recombinant *N. calcicola* was monitored in terms of total protein. *N. calcicola* was inoculated in fresh AA medium containing Km 30 µg ml^{-1} and incubated in the culture room in an orbital shaker at 100 rpm. 1 ml culture was withdrawn for every 24 h and used for protein determination (2.2.2.1) and continued for 15 days after inoculation.

3.2.7.1.3 Determination of chlorophyll *a*

Growth of recombinant *N. calcicola* was monitored in terms of total chlorophyll *a* content. *N. calcicola* was inoculated in a fresh AA medium containing Km 30 µg ml^{-1} and incubated in the culture room in an orbital shaker at 100 rpm. 1 ml culture was withdrawn for every 24 h and used for chl *a* determination (2.2.2.2).

3.2.8 Evaluation of transgenic *N. calcicola*

3.2.8.1 Metal resistance

Recombinant *N. calcicola* expressing mouse metallothionein protein was tested for metal resistance properties. *N. calcicola* was inoculated at an initial OD_{750} of 0.6 in AA medium supplemented with metals at various concentrations. It was allowed to grow under culture room conditions. Metal toxicity and resistance properties of recombinant cells were determined along with wild type and *N. calcicola* without insert DNA as control. Cell viability was tested with 1% TTC solution and the growth was monitored regularly by spectrophotometric method and in terms of concentrations of protein and chl *a* content (3.2.7.1.1 – 3).

3.2.8.2 Metal uptake

Exponentially growing recombinant cells were inoculated in AA medium supplemented with metals at sub lethal concentrations and allowed to grow under culture room conditions. Metal uptake by the growing cells of recombinant cells were determined as described in section 2.2.7.2

3.2.8.3 Extracellular accumulation

The concentration of cell wall bound metals were determined as in section 2.2.7.5.

3.2.8.4 Intracellular bioaccumulation

Intracellular sequestration of metals by the recombinant *N. calcicola* expressing mouse metallothionein was determined as in section 2.2.7.6.

3.2.9 Plasmid stability

3.2.9.1 Plasmid DNA isolation

2 ml culture of exponential phase *N. calcicola* after 10 generations without antibiotics and cells grown in presence of Km 30µg ml^{-1} were harvested for plasmid DNA isolation as in section 2.2.6.3. The plasmid DNA was electrophoresed on a 1% agarose gel and results were documented.

3.2.9.2 Restriction enzyme digestion of pRSKV12

The purified plasmid DNA was used for restriction enzyme digestion with EcoRI/BamHI to verify presence of the insert DNA *psbA*-mMT. DNA was electrophoresed on a 1% agarose gel and the results were documented.

3.3 Results and Discussion

3.3.1 Construction of recombinant *N. calcicola*

The wild type *N. calcicola*, an "extremophile" is originally a rice field isolate (Singh et al. 1989). Though it possesses putative uncharacterized endogenous plasmid(s) (2.2.6.3), the genetic tools for metal resistance gene transfer have not been developed for *N. calcicola*. An attempt was made to exploit the well defined triparental conjugation procedure for transfer of a replicating or suicide vector to the host for filamentous cyanobacterial strains. A broad host range replicating shuttle expression plasmid pRL488 (Wolk et al. 1993) containing the *E. coli* pBR322 origin of replication *oriV* and a cyanobacterial origin of replication pDU1 (*Nostoc* sp. PCC 7524) was used. It was transferred to *N. calcicola* by triparental mating as outlined by Wolk and Zhou (2003). Out of four trials, *N. calcicola* could be transformed with a frequency *ca.* 1.4 x 10^3 colony forming unit (cfu). This method was used to obtain transformation in subsequent studies. The exconjugants were able to grow in AA medium supplemented with Km at 30 µg ml^{-1} whereas the wild type cells were sensitive at 2 µg ml^{-1}. The plasmid pRL488

from transformants was isolated, digested with restriction enzymes and it was confirmed that the transformants were "true exconjugants".

The overall strategy of cloning and expression of mMT-I in *N. calcicola* (as portrayed in Figure 22 and 23 of materials and methods) encompassed three stages: i) cloning of MT-I cDNA into *psbA*, ii) preparation of *psbA*-mMT-I DNA cassette with flanking BamHI/EcoRI, and iii) cloning of the insert *psbA*-mMT-I (BamHI/EcoRI) into pRL488.

(i) The MT-I cDNA (300 bp) was obtained from the plasmid pBxMT; it contains two copies of MT-I cloned into BamHI/BglII site of the multiple cloning site (MCS) of pBS-SK+. pRL439 containing the *psbA* promoter was digested with BamHI, MT-I ligated at BamHI site and transformed into *E. coli* DH5α. The transformants were selected based on Amp^r. The presence of insert was checked by restriction digestion of the plasmid. The recombinant plasmid was designated as pRL-mMT (Fig. 24).

(ii) The *psbA*-mMT (450 bp) was released from pRL-mMT with BamHI/PstI digestion; the DNA fragment was cloned into MCS of pRSET(B) to obtain a flanking BamHI/EcoRI (Fig. 25).

(iii) pRL488 is a 12.1 kb plasmid DNA possessing MCS and a promoter less *luxAB* gene (2.1 kb) which is used to analyze the strength of bacterial promoters. The *luxAB* can be released from pRL488 without disturbing the cyanobacterial origin of replication pDU1, *OriT*, *OriV* and *npt* gene. pRL488 was digested with BamHI/EcoRI and the resulting 10 kb fragment devoid of the 2.1 kb *luxAB* gene was used. *psbA*-mMT-I (450 bp flanking BamHI/EcoRI) was ligated into 10 kb fragment of pRL488, resulting *ca*. 10.5 kb plasmid was transformed into *E. coli* DH5α. The transformants were selected based on Km^r, the presence of insert was checked by restriction digestion of the plasmid. The recombinant plasmid was designated as pRSKV12 (Fig. 26).

To verify the orientation and coding frame of the construct, the 450 bp *psbA*-mMT (BamHI/EcoRI DNA fragment) was cloned into pUC19. Using M13 universal primers,

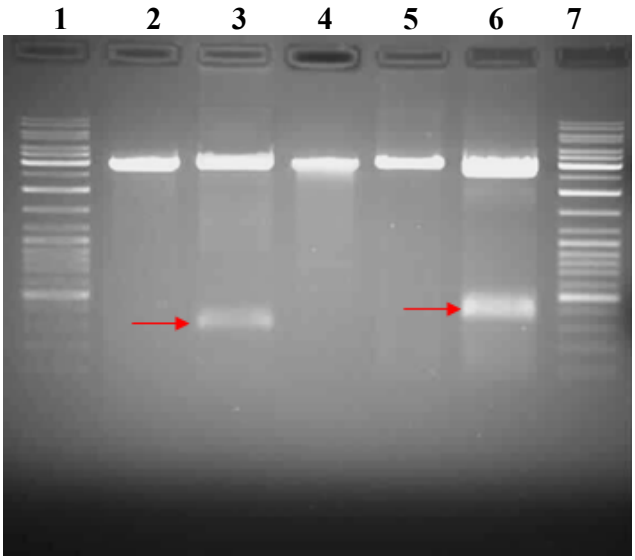


Fig 24. Cloning of mouse metallothionein gene into pRL439. Lane 1, DNA marker*; lane 2, pBxMT (3.3kb); lane 3, pBxMT double digest with BamHI/BglII, arrow shows a 300bp mMT fragment; lane 4, pRL439 (2.7kb); lane 5, pRL439-mMT (3.0kb); lane 6, pRL439-MT double digest with BamHI/PstI, arrow shows a 450bp *psbA*-mMT fragment and lane 7, DNA marker (*MBI Fermentas, SM0331, 1 kb ladder). The DNA samples were separated on a 1.0% agarose gel.

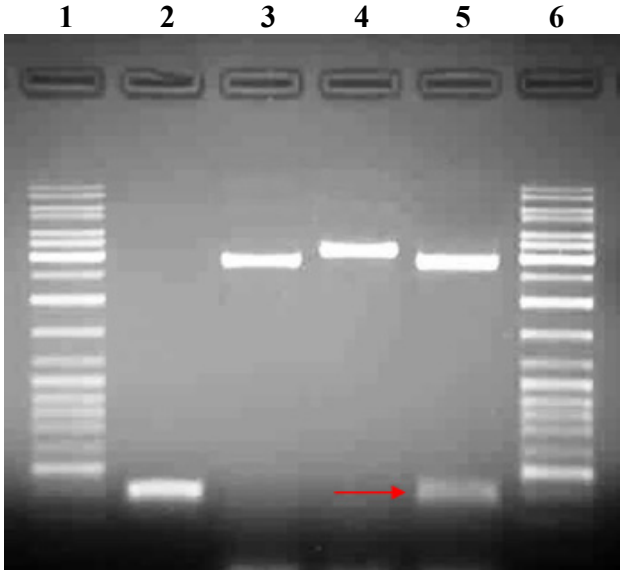


Fig 25. Cloning of *psbA*-mMT into pRSET(B). Lane 1, DNA marker*; lane 2, DNA fragment *psbA*-mMT (450bp); lane 3, pRSET(B) digested with BamHI/pstI (2.9kb); lane 4, pRJ-mMT (3.2kb); lane 5, pRJ-mMT digested with BamHI/EcoRI, arrow shows a 450bp *psbA*-mMT fragment and lane 6, DNA marker (*MBI Fermentas, SM0331, 1 kb ladder). The DNA samples were separated on a 1.0% agarose gel.

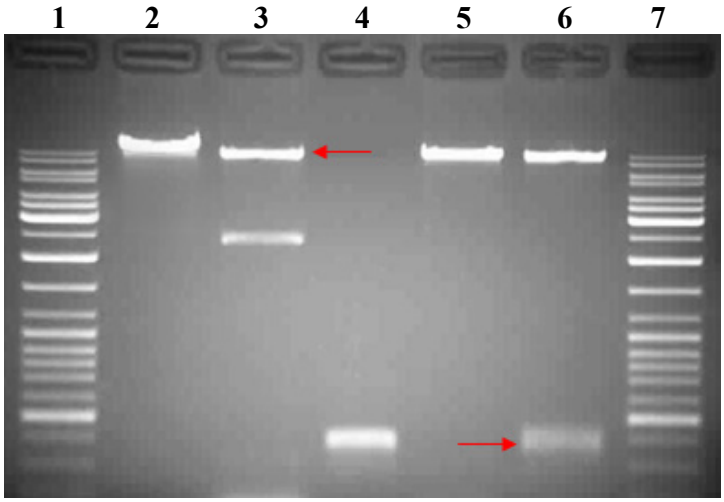


Fig 26. Cloning of *psbA*-mMT into pRL488. Lane 1, DNA marker*; lane 2, pRL488 (12.1kb); lane 3, pRL488 double digest with BamHI/EcoRI, arrow shows a 10kb DNA fragment; lane 4, DNA fragment *psbA*-mMT (450bp); lane 5, pRSKV12 (10.5kb); lane 6, pRSKV12 double digest with BamHI/EcoRI, arrow shows a 450bp psbA-mMT fragment and lane 7, DNA marker (*MBI Fermentas, SM0331, 1 kb ladder). The DNA samples were separated on a 1.0% agarose gel.

the complete sequence of the expression cassette was obtained by automated Sanger's dideoxy DNA sequencing. It was found that *psbA*-mMT was in the correct orientation in the order: promoter with SD sequences (RBS), the start codon, a full length metallothionein polypeptide and a stop codon (Appendix: VI).

3.3.2 Transformation of *N. calcicola*

N. calcicola is a filamentous cyanobacterium i.e. individual cells are tightly connected together to form a chain like structure. Each filament is composed of nearly 20 – 30 cells inclusive of 2 – 3 heterocysts; thus, transforming such an organism requires individual cells for obtaining true transformants rather than pseudo exconjugants. To break the filaments into single cells or possibly 2 – 3 cells per filament, the exponential phase cultures were harvested by centrifugation and sonicated aseptically; the progress of breakage of filaments otherwise called "cavitation" was monitored by observing under light microscopy. The procedure was continued at the lowest possible settings to achieve minimal cellular damage in the mean time to allow cells to separate apart. The contents were centrifuged at 1500 rpm in a laboratory centrifuge to remove unbroken filaments and clumps. The supernatant containing predominantly the single cells was centrifuged at 8000 rpm, washed to remove cell debris and allowed to recover in fresh culture medium for 24 h under culture room conditions. Such cells were harvested and used for triparental mating using the conjugal plasmid RP4.

E. coli HB101 was transformed with the helper plasmid pRL623 [which provided 3 different methylases that methylate DNA on C and G (Elhai et al. 1997)] and co-transformed with the metallothionein expression plasmid pRSKV12. Methylation of pRSKV12 was essential to protect it from host mediated DNA restriction enzymes. It was found that unmethylated pRSKV12 could not be transformed into *N. calcicola* indicating the presence of uncharacterized DNA restriction enzymes. *Nostoc* sp. PCC 7120 which contains three restriction endonucleases (Elhai et al. 1997) and *Nostoc* sp. ATCC 27896 requires *in vivo* methylation of foreign DNA whereas the *Nostoc punctiforme* PCC 79133 does not require such modifications (Cohen et al. 1994). The selection of exconjugants on antibiotics is a critical step; as the cells were plated on Millipore HATF membrane it was

easier to be lifted from a non-antibiotic LB supplemented AA agar plates to selective medium. A minimum of 4 – 5 days of incubation under low light was required in the absence of antibiotics for a stable expression of *npt* gene from pRSKV12. Stress conditions like limited light exposure, excess *E. coli* and nutrient limiting environment may contribute to reduction in the survival of exconjugants transferred from a non-selective medium to Km AA agar plates. 5 days duration in a non-selective medium allows sufficient growth (as seen by the green patches), maximum number of conjugal events and also increases the frequency of successful exconjugants to appear under selective pressure. The recombinant *N. calcicola* appeared as "pin-head" colonies on the membrane (Fig. 27) and subsequently transferred to AA medium.

To eliminate *E. coli* from the recombinant *N. calcicola*, the cultures were repeatedly plated on AA agar plates. An axenic culture of the genetically engineered *N. calcicola* (Fig. 28) was grown in culture medium supplemented with Km at 30 µg ml^{-1}. 2 ml culture was used to isolate plasmid (Fig. 29) and the presence of pRSKV12 in the recombinant cells was confirmed by restriction digestion. The wild type cells did not show any plasmid band on gel electrophoresis; it should be recalled that the endogenous plasmid(s) of *N. calcicola* (section 2.2.6.3) are low copy number and maintained in the absence of selection pressure. *Nostoc* sp. PCC 7120 has 6 endogenous mega plasmids; these are under stringent copy number control and close to 1 each per cell (Kaneko et al. 2001). So that, a 2 ml culture of *N. calcicola* used may not be sufficient to observe its native plasmids.

3.3.3 Expression of metallothionein in *N. calcicola*

Recombinant *N. calcicola* grown on AA medium supplemented with Zn^{2+} or Km was harvested, lysed and the total protein was electrophoresed on a 15% denaturing polyacrylamide gel. The CBB staining of the gel showed a prominent protein band at 19 kD and a diffused band at 6.4 kD. The mouse metallothionein is a 6.4 kD protein; since it is low molecular weight protein, a distinct band may not be possible to obtain from the total cell lysate. In addition, the *N. calcicola* contains excess chlorophyll, polysaccarides, lipids, cellulose like heteropolysaccharides and other low molecular weight substances

Fig 27. Recombinant *N. calcicola* colonies. *N. calcicola* transformed with the plasmid pRSKV12 was selectively grown on Immobilon-NC (HATF) membrane placed on AA agar plates containing 30 µg ml^{-1} kanamycin.

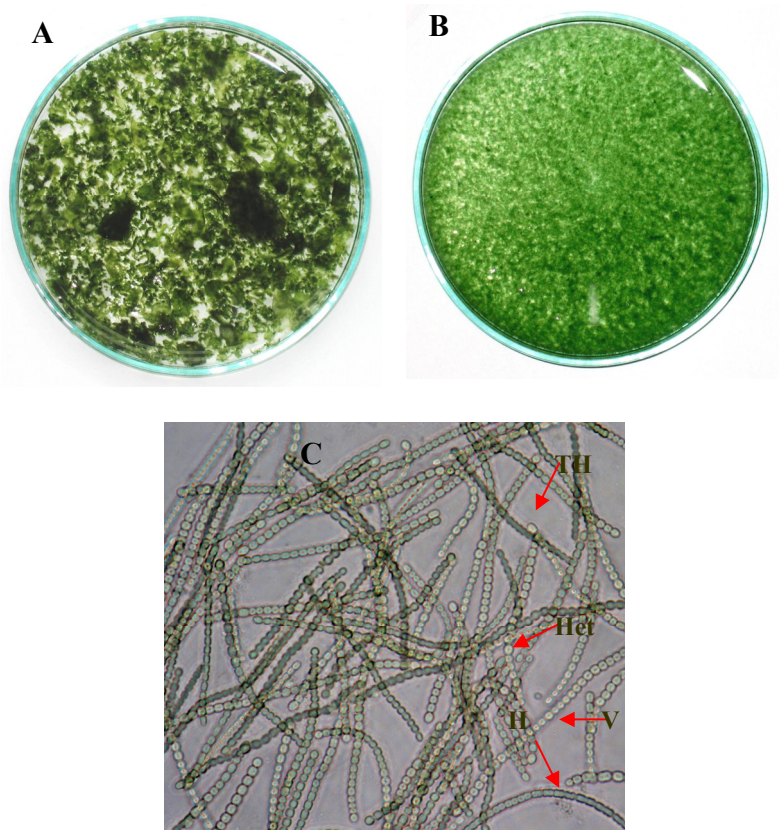


Fig 28. Wild type and genetically engineered *N. calcicola*. Wild type (A) and recombinant cells (B) grown in AA medium. Light microscopy of the *N. calcicola* (C), arrows show hormogonia (H), hererocysts (Het), terminal heterocysts (TH) and vegetative filaments (V) (400x).

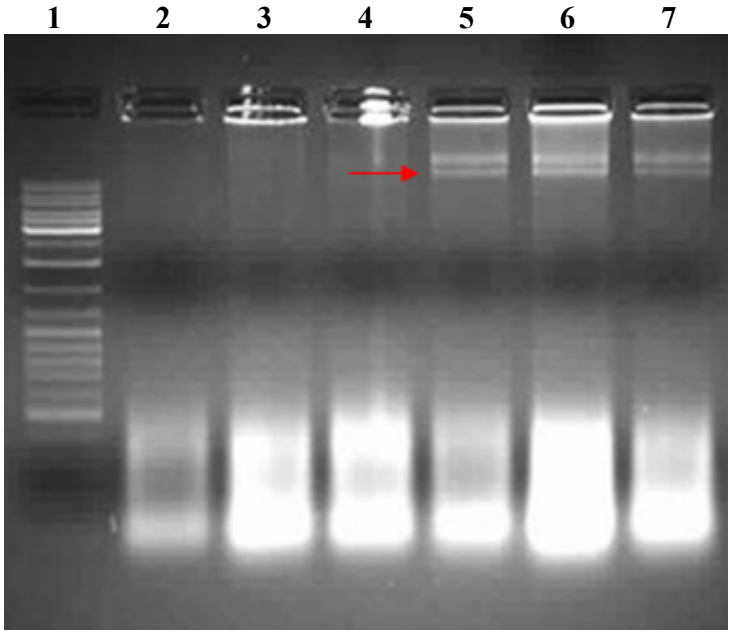


Fig 29. Plasmid DNA pRSKV12 isolated from recombinant *N. calcicola.* Lane 1, DNA marker*; lane 2 – 4, wild type *N. calcicola*; lane 5 – 7, pRSKV12 from recombinants. (*MBI Fermentas, SM0331, 1 kb ladder). DNA was electrophoresed on 1.0% agarose gel.

that migrate faster and concentrate at the top of the gel and mask metallothionein. Although, there was a discernible 6.4 kD metallothionein in this region, an intense protein band with an apparent molecular mass 19 kD protein was observed in the recombinant *N. calcicola* (Fig. 30).

MT is rich in sulfhydryl residues of cysteines, it easily forms inter- and/or intramolecular linkages following the aggregation and configuration changes of the molecules. The electrophoretic patterns of MTs are obtained as broad, obscure bands in the denaturing polyacrylamide gel (Kimura, 1991). MT-I dimer showed an apparent molecular weight of about 19 kD (Vallee, 1991) whereas the cyanobacterial metal binding peptides showed apparent molecular weights, dimer 19.5 kD, and trimer 26.5 kD (Guo et al. 1997). Therefore, the 19 kD and 6.4 kD protein bands observed in the recombinant *N. calcicola* was the dimer and monomer of metallothionein expressed under the control of strong promoter *psbA* from the plasmid pRSKV12.

3.3.4 Evaluation of transgenic *N. calcicola*

3.3.4.1 Growth

The total protein and Chl *a* content of the recombinant *N. calcicola* were used to determine the growth. Although, there was no change in the generation time, 24 h as of wild type *N. calcicola* and the growth profile; a significant culture characteristics was observed. Unlike the wild type cells that used to aggregate and float on the surface of the medium, the recombinant cells grown as homogeneous. The microscopic observation of the recombinant cells showed healthy filaments with all the cellular differentiation patterns. There was no abnormal morphology, heterocyst development and the characteristic cell numbers of each filament. Such a change in culture characteristics could be due to the exogenous plasmid DNA in *N. calcicola*.

3.3.4.2 Metal resistance

The expression of MT-I have increased the heavy metal resistance in *N. calcicola*. The minimum inhibitory concentrations (MIC) were in the order Cd>Co>Cu>Ni>Sr>Zn (Table 14). For applications of genetically engineered *N. calcicola* metal bioremediation,

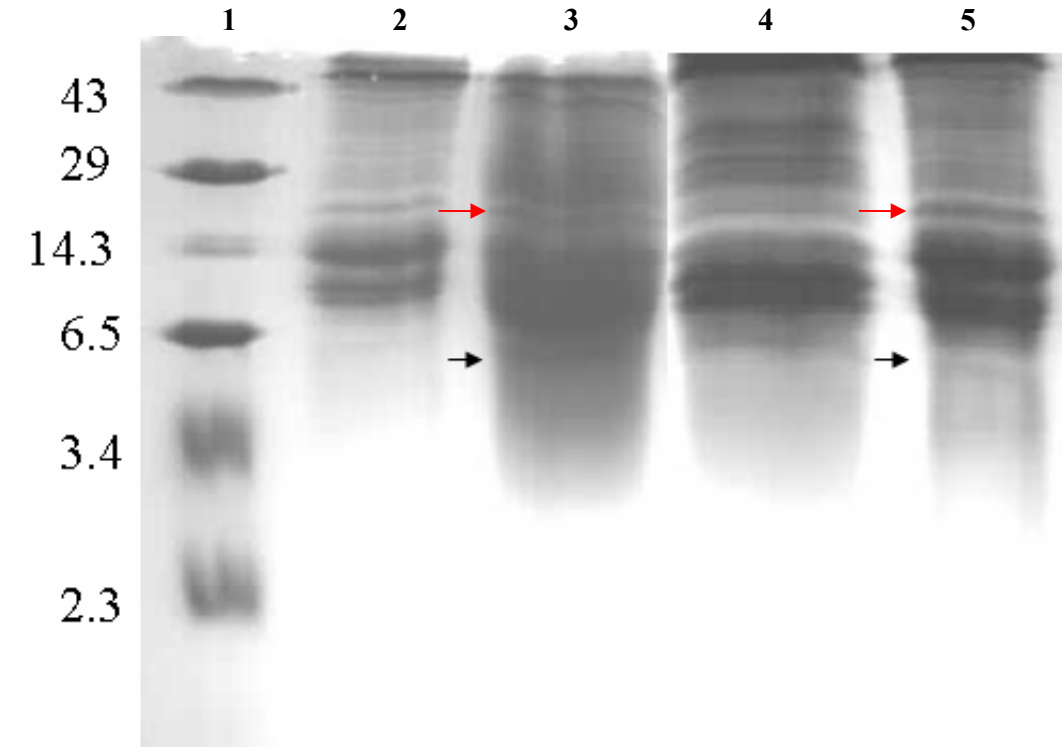


Fig 30. SDS-PAGE of the cell lysate of recombinant *N. calcicola*. Lane 1, protein molecular weight marker; lane 2, wild type cells; lanes 3, recombinant *N. calcicola* grown on AA medium supplemented with Zn^{2+} at 500 µM expressing mouse metallothionein, *ca.* 6.4 kD; lane 4, wild type and lane 5 recombinant cells grown on AA medium supplemented with Km at 30 µg ml^{-1}. The arrow (red) showing prominent protein band at 19 kD is a dimer of MT-I. 50 µg total proteins was electrophoresed on a 12% SDS-PAGE and stained with CBB.

the multiple metal resistance is considered to be an essential factor. In this context, the recombinant cells expressing MT-I will be an added advantage. MT-I confers resistance to an array of heavy metals including Zn, Cd, Cu, Ag, Hg, Co and Mn in microorganisms (Hou et al. 1988) whereas MT-II in *E. coli* JM105 conferred resistance to Cd, Zn and Cu (Odawara et al. 1995). Similar reports on the enhanced resistance to Cd were observed upon expression of metallothionein in *Nostoc* sp. PCC 7120. The elevated MIC of the recombinant cells was due to the internal sequestration of metals by the metallothionein (Ren et al. 1998; Shao et al. 2002). SmtA expression in a marine unicellular cyanobacaterium *Synechococcus* sp., conferred Cd resistance at 2 µM (Blindauer et al. 2002). The metal sequestration by cyanobacterial class II and III metallothioneins have been reported as the key mechanism of metal resistance in many cyanobacterial strains (Robinson, 1989). Recent report suggests that most prokaryotic metal resistance mechanisms are controlled by the SmtB/ArsR family of metalloregulatory transcriptional repressors (Busenlehner et al. 2003).

3.3.4.3 Metal bioaccumulation

The recombinant *N. calcicola* have acquired elevated metal resistance. To compare the metal accumulation capacities, the cells were grown on AA medium supplemented with sub-lethal concentrations of metals (as of wild type cells). The culture medium was a simple mineral medium and added metals were readily available for binding and/or transport across the membranes for intracellular bioaccumulation. The metal removal was determined based on the amount of metals deposited at the cell wall (EDTA washable fraction) and intracellular metal sequestration. It was found that the recombinant *N. calcicola* could accumulate higher amount of metals than that of wild type cells. Among the metals Zn, Cd and Co were preferred for internal sequestration by metallothionein expressed in recombinant *N. calcicola* (Table 15). Among the recombinant cyanobacterial strains (Ren et al. 1998; Blindauer et al. 2002), the *N. calcicola* showed highest efficiency in terms of intracellular bioaccumulation and in the order Zn 242.2 µM, Sr 16.5 µM, Co 5.4 µM Cd 2.78 µM, Cu 0.8 µM and Ni 0.3 µM. A comparison of metal accumulation by previously reported uptake studies on *Nostoc* PCC 7120 by Ren et al. (1998) and the recombinant *N. calcicola* developed by us clearly establishes

Table 14. The minimum inhibitory concentrations (MIC) of metals for recombinant *N. calcicola*. Cells were grown on AA medium supplemented with different concentrations of respective metals and incubated in the culture room conditions for 15 days. Growth was determined by total protein content.

S. No	Metals	MIC (µM)	
		***N. calcicola* wild type**	**Recombinant *N. calcicola***
1	Cobalt(II)	35	52.5
2	Nickel(II)	10	12.5
3	Copper(II)	10	15
4	Strontium(II)	70	100
5	Zinc(II)	750	1000
6	Cadmium(II)	20	67.5

Table 15. Metal bioaccumulation properties of recombinant *N. calcicola* expressing mouse metallothionein. Cells were grown in AA medium supplemented with sub-lethal concentrations of respective metals and incubated in the culture room conditions for 15 days. Total metal bioaccumulation capacities of the cells were determined by harvesting 10 ml of cells, washed with EDTA and oven dried. The cells were acid digested for metal quantification by AAS.

S. No	Metals	Initial concentration (µM)	Intracellular bioaccumulation (µM metal per mg protein)	
			***N. calcicola* wild type**	**Recombinant *N. calcicola***
1	Cobalt(II)	25	1.29	5.405
2	Nickel(II)	7.5	0.108	0.307
3	Copper(II)	7.5	0.409	0.81
4	Strontium(II)	50	14.325	16.465
5	Zinc(II)	500	166.19	242.235
6	Cadmium(II)	15	1.19	2.835

supremacy of the recombinant strain. The metal sequestration and metal resistance due to MT-I gene transfer have been reported by Sode et al. (1998) in *Nostoc* sp. PCC 7120, *Synechococcus* sp. NKBG 15041cc.

3.3.5 Plasmid stability

The *N. calcicola* was transformed with the plasmid pRSKV12 containing origin of replication pDU1, 1.75 kb DNA fragment derived from a naturally occurring cryptic plasmid of *Nostoc* sp. PCC 7524. The stability of pRSKV12 in *N. calcicola* and its use without selection pressure were not characterized, as this was the first report being transformed with such an exogenous plasmid. The recombinant *N. calcicola* was grown several generations in the absence of Km; every sub-culturing the plasmid DNA was isolated using the standard protocol described (2.2.6.3). At the same time, such cultures were inoculated into nutrient medium supplemented with Km at 30 μg ml^{-1}. Surprisingly, it could retain the recombinant plasmid and was able to grow on AA medium containing Km. Such a stable maintenance of the plasmid may be attributed to recognition of origin of replication pDU1 by the host *N. calcicola*.

It is well known that pDU1 based vectors have been used for *Nostoc* sp. (Wolk, 1984) and the copy number of pDU1 was maintained between 170 – 340 per cell in *Nostoc* sp., PCC 7120 (Lee et al. 2003). Schaefer et al. (1993) reported that *Ori* of a plasmid from *Fremyella diplosiphon* UTEX 481 was highly conserved with pDU1. The DNA sequence analysis revealed that *F. diplosiphon* plasmid replication origin is structurally very similar to and shares significant identity with the 1.75 kb replication origin of the plasmid pDU1 isolated from the morphologically distinct cyanobacterium *Nostoc* sp. PCC 7524. Many cyanobacterial strains, both unicellular and filamentous, possess one or several (up to eight) cryptic plasmids (Lau and Dolittle; 1979; Lau et al. 1980; Lambert et al. 1984). Several geographically distinct but genetically related cyanobacterial strains possess identical plasmids with respect to plasmid size, restriction endonuclease digestion patterns and DNA homology (Felkner and Barnum, 1988). More interestingly, similar analyses show that some genetically unrelated strains appear to harbor identical or nearly identical plasmids (Van den Hondel et al. 1979). These studies, in conjunction with those

that demonstrate the widespread distribution of the strains suggest a possible interspecific or intergeneric transmission of some cyanobacterial plasmids in nature.

Although the cyanobacterial plasmids do not possess known function or their removal by plasmid curing has not made any significant morphological or phenotypic changes over time under laboratory conditions (Wolk et al. 1984); it is astonishing to note for their stable inheritance throughout its complex life. Another interesting feature of cyanobacterial plasmids is the stringent control of copy number. The average copy number of pDU1 was found to be 170 – 340 per cell in *Nostoc* sp. PCC 7120 (Lee et al. 2003) and several other strains have varying numbers (Kaneko et al. 2001). Cyanobacteria, unlike *Escherichia coli*, are thought to carry several genome equivalents of DNA in each cell. An estimate of 24 genome equivalents per cell in *Calothrix* sp. strain PCC 7601 (Tandeau de Marsac, 1994), *Nostoc* sp. 7120, *Synechococcus elongates* and *Anabaena variabilis* has approximately 10 genome equivalents per cell (Lee et al. 2003; Kaneko et al. 1996; Craig et al. 1969). The genes that regulate these processes in cyanobacteria have not been characterized.

Lee et al. (2003) reported that a deletion of the gene *all1076* which codes for the protein PlmA, a transcriptional regulator, reduced the numbers of copies per chromosome of several endogenous plasmids in *Nostoc* sp. PCC 7120. Moreover, the deletion of PlmA also led to reduction of exogenous pDU1 based plasmid pAM1691 to less than 25% of its wild-type level and the plasmid was rapidly lost. The PlmA is a 36 kD protein, which shows high similarity to members of the GntR family of transcriptional regulators and play a role in regulating plasmid maintenance. Phylogenetic analysis revealed a new domain topology within the GntR family. PlmA homologs, all coming from cyanobacterial species, form a new subfamily that is distinct from the previously identified subfamilies.

BLAST search of PlmA in Cyanobase (http://bacteria.kazusa.or.jp/cyanobase/) and NCBI databases revealed that all sequenced (in addition to partially sequenced) genome of unicellular and filamentous strains of cyanobacteria possess a highly conserved homolog

of PlmA. Phylogenetic analysis shows distinct distribution of PlmA among unicellular and filamentous cyanobacteria, in specific *Nostoc* sp. (Fig. 31). *Nostoc calcicola* comes under the later category and may possess such a protein homolog that may have influence on stable inheritance of pDU1 based plasmid pRSKV12. A general problem of using the recombinant strains of heterotrophic bacteria in bioremediation technology is the instability of cloned genes when borne on plasmids. To overcome this major drawback, the use of so-called mini-transposons for the stable integration of genes into the chromosome of recipient strains has been proposed (de Lorenzo et al. 1998). The usefulness of mini-transposon method has recently been evidenced by the construction of highly stable recombinant strains carrying genetic expression cassettes on their chromosomes (Panke et al. 1999) resulting in quasi-natural strains bearing exclusively the DNA segment encoding the phenotype of choice (Panke et al. 1998). The disadvantage of such chromosomal integration is the limited copy number(s) unlike the plasmids. Transposon mutagenesis for *Nostoc* sp. is well established (Meeks et al. 1994). Like other bacterial strains, the chromosomal integration of industrially important genes is possible; when expression of genes from a stable pDU1 based vectors are available such attempts are not required for overexpression of heterologous proteins in *Nostoc* sp.

Thus, advantages of using pDU1 based high level expression of metallothioneins not only include increased capacity to remove metals from the solution, but also the greater stability of cloned genes in plasmid and the potential applications in natural environment.

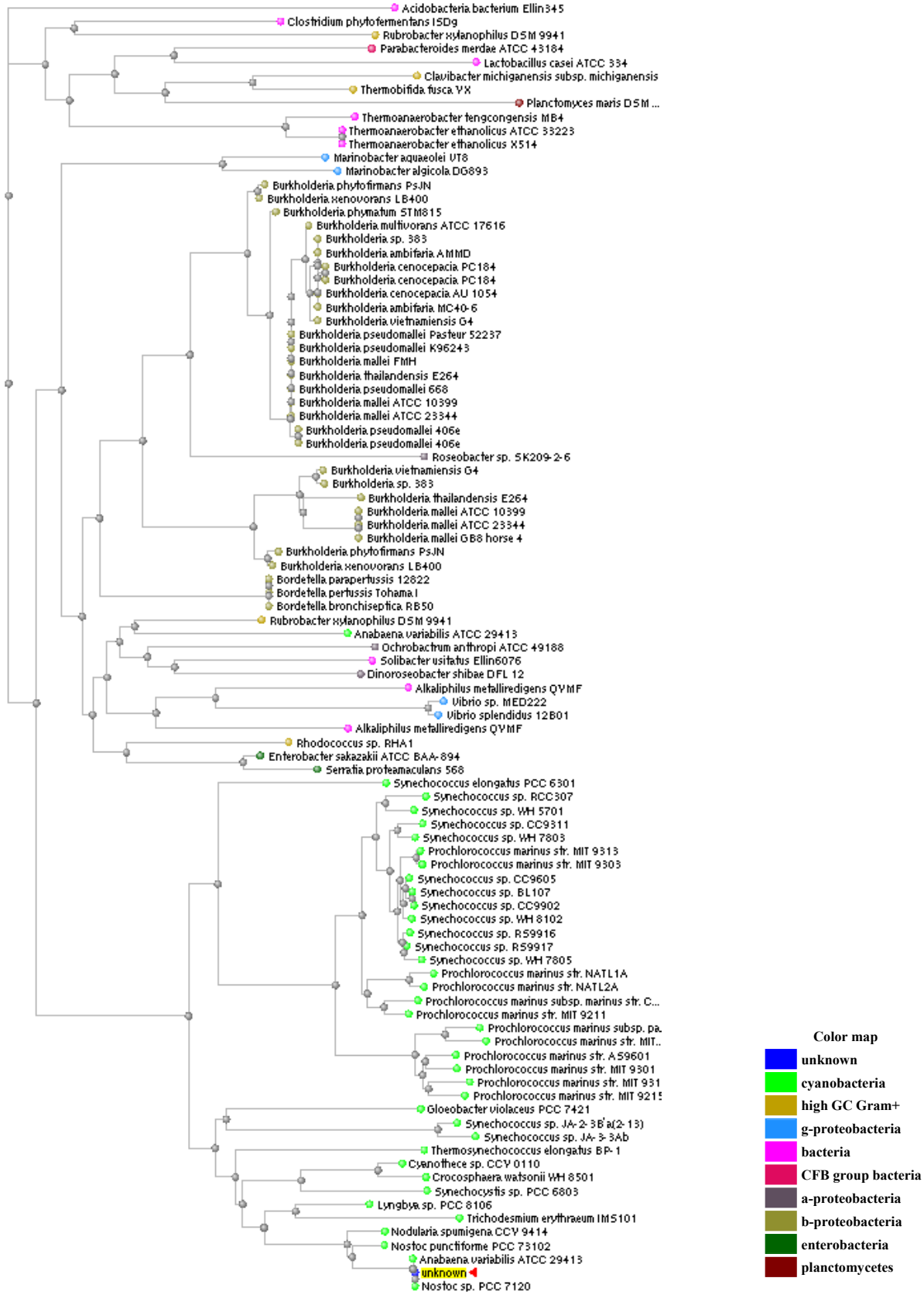


Fig 31. Phylogenetic tree view of PlmA; a new member of the GntR family transcriptional regulators.

Chapter IV

Industrial Applications of Recombinant N. calcicola

Chapter IV

INDUSTRIAL APPLICATIONS OF RECOMBINANT *N. CALCICOLA*

4.1 Introduction

In the previous chapter III, we have clearly established that the recombinant *N. calcicola* expressing metallothionein has distinct advantages over wild type cells in terms of metal resistance and intracellular metal bioaccumulation. Although, such genetically engineered microorganisms (GEM) have been used extensively for bioremediation technologies, the free cells are less suitable for metal removal and recovery processes due to low density, small particle size and problem of its separation from the liquids (Hu and Reeves, 1997). The immobilization of cells *per se* in a defined support has advantages such as high biomass loading, minimal clogging under continuous-flow conditions and efficient regeneration (Hu and Reeves, 1997; Macaskie, 1991).

The most commonly used matrices for immobilization of microbial cells for bioremediation applications have been achieved by natural or synthetic polymers including alginate, cellulose, polyacrylamide, polysulfone, polyvinyl alcohol (Gadd and White, 1993; Gadd, 1998) and even silica gels (Al-Saraj et al. 1999). Of the above, calcium alginate (CAG) beads are widely used due to their versatility and easy preparation. However, the CAG beads are inhomogeneous as they possess cavities and fractures, and the gel strength decreases from capsule to its core, which may result in fast leaching of immobilized cells from the gel (Ortega et al. 1998; Mohapatra and Hsu, 2000). Moreover, CAG are sensitive to chelating compounds, especially phosphates, EDTA and sodium ions (Smidsrod and Skjak, 1990). To overcome these problems, a number of techniques like mixing gelatin (Fadnavis et al. 2003) or hyaluronic acid (Bae et al. 2006), freeze-drying (Tal et al. 1997), coating CAG with calcium phosphate (Leveque et al. 2002) and silica (Coradin et al. 2003) were adopted to improve mechanical stability and to prevent cell leakage.

Silica gel encapsulation is another popular method of immobilization as it can be made non-toxic and environment-friendly (Coradin and Livage, 2007). Recent reviews on silica gel for immobilization technology describe its various applications (Livage and Coradin, 2006; Bottcher et al. 2004). Silica gel has excellent mechanical strength, chemical inertness, resistant to microbial attack and negligible swelling properties in aqueous solutions when compared to organic polymers. Efficient bioaccumulation of heavy metals by entrapped yeasts (Al-Saraj et al. 1999) and *Bacillus sphaericus* cells, spores and S-layers (Raff et al. 2003; Soltmann et al. 2003) in silica gels was already reported. Thus, the objective of this work was aimed at preparation, characterization and applications of novel silica-coated calcium alginate and silica gel immobilized *Nostoc calcicola* for metal removal and recovery from metal containing effluents.

4.2 Materials and Methods

4.2.1 *N. calcicola* and growth conditions

Wild type *N. calcicola* was grown in Allen and Arnon's (AA) medium as described in section 2.2.1 and 3.2.6.1. The culture medium was supplemented with kanamycin (Km) 30 μg ml^{-1} for growing recombinants cells.

4.2.2 Immobilization of *N. calcicola*

4.2.2.1 Preparation of *N. calcicola* for cell immobilization

The exponentially grown *N. calcicola* cells were harvested by centrifugation at 6000 rpm at RT for 10 min and washed twice with Millipore water to remove culture medium components. For metal bioaccumulation experiments, the living cells (wet biomass) were used. The frozen cell pellet of *N. calcicola* was lyophilized (MAXI dry lyo, Heto, Germany) for metal biosorption studies.

4.2.2.2 Calcium alginate (CAG) immobilization

1.5 g of sodium alginate (Fluka, USA) was dissolved in 100 ml of sterile distilled water and then mixed with 5 g of *N. calcicola* cell pellet for bioaccumulation experiments or 1 g lyophilized cells for metal biosorption studies. The mixture was added dropwise into

0.2 M $CaCl_2$ solution using a peristaltic pump (Gallenkamp). CAG immobilized beads (average diameter 4 mm) were allowed to mature at RT for 2 h.

4.2.2.3 Alginate coated CAG immobilization

CAG beads were washed extensively with 50 mM Tris-Cl (pH 7.2) followed by soaking in 0.05% solution of polyallylamine hydrochloride (Aldrich, USA) for 30 min. These beads were washed with 50 mM Tris-Cl and placed in a beaker containing 0.03% sodium alginate solution for 6 min. Alginate coated CAG beads were washed with 50 mM Tris-Cl (pH 7.2) and suspended in AA medium and incubated under culture room conditions.

4.2.2.4 Silica coated CAG (Si-CAG) immobilization

Silica-coated microcapsules were prepared as described by Coradin et al. (2003) with minor modifications. CAG beads were washed extensively with 50 mM Tris-Cl (pH 7.2) followed by soaking in 0.05% solution of polyallylamine hydrochloride (Aldrich, USA) for 30 min. These beads were washed with 50 mM Tris-Cl and placed in a beaker containing 30 mM sodium silicate solution for 2 h at RT. Silica coated CAG beads were washed with 50 mM Tris-Cl (pH 7.2) and suspended in AA medium and incubated under culture room conditions.

4.2.2.5 Silica gel immobilization

Silica gel immobilization of *N. calcicola* was carried out by mixing 10 ml of 0.4 M sodium silicate (Sigma, USA) with 10 ml of 8.5 M colloidal silica (LUDOX HS-40, Aldrich) as described by Nassif et al. (2002). The solutions were mixed thoroughly by using a magnetic stirrer at 300 rpm and pH of the solution was adjusted to 7.0±0.05 with 4 M HCl. A 10% (w/v) suspension of late log-phase cyanobacterial cells was washed with sterile Millipore water, and added immediately to the solution. The mixture was stirred well and silica gel formed within 2 – 3 min at RT. Living cells entrapped in silica gel were supplemented with fresh sterile AA medium and incubated under culture room conditions. For metal biosorption experiments, lyophilized cells immobilized on silica gel were incubated for 2 h at RT followed by overnight drying at 60 ^{o}C. The dried silica gel was powdered to 40 – 60 mesh size for use in metal accumulation experiments. For testing mechanical strength and corrosion resistant properties of the silica gel, different eluants for metal desorption processes were used.

4.2.3 Characterization of immobilized *N. calcicola*

4.2.3.1 Cell viability assays

The viability of *N. calcicola* after immobilization on different matrices were tested by reduction of 1% 2,3,5-triphenyl tetrazolium chloride (TTC) solution.

4.2.3.2 Light microscopy

The nature of *N. calcicola* immobilized on CAG, Si-CAG and silica gel matrices was observed under light microscope (Olympus CKX41). Image was captured using Olympus camera and recorded.

4.2.3.3 Scanning electron microscopy

Silica coated calcium alginate and silica gel immobilized *N. calcicola* cells were washed several times with 0.1 M phosphate buffer (pH 7.4) and fixed by submerging in 2.5% glutaraldehyde in phosphate buffer for 6 h at 4 ^{o}C. Samples were washed repeatedly with the same buffer, dehydrated in a graded ethanol (50 – 100% v/v) and acetone to critical point drying. These were then placed on a carbon coated aluminium stub and gold coated at 0.06 – 0.08 mbar for 45 sec and viewed under a scanning electron microscope (PHILIPS, LEO 435 VP).

4.2.3.4 Transmission electron microscopy

Silica gel immobilized *N. calcicola* cells were washed several times with 0.1 M phosphate buffer (pH 7.4). Primary fixation of the sample was done by submerging in 2.5% glutaraldehyde in phosphate buffer for 6 h at 4 ^{o}C followed by post fixation with 1.0% OsO_4 in 0.1 M phosphate buffer for 2 h at 4 ^{o}C. The samples were washed repeatedly with the same buffer, dehydrated in a graded ethanol (50 – 100% v/v) and acetone to critical point drying. It was embedded on araldite and thin sections of 60 – 70 nm were cut with an ultramicrotome (Reichart Ultracut E, Germany) with diamond knives, which were then mounted on copper grids, contrasted with uranyl acetate and lead citrate and viewed under transmission electron microscope at 80kV (PHILIPS Morgagni 268).

4.2.3.5 Mechanical stability of immobilization matrix

Silica coated CAG capsules were tested for their mechanical stability and cell leakage. Mechanical strength of the hollow beads was tested by counting number of beads fractured upon agitation. For this, silica and alginate coated beads were treated with 50

mM sodium citrate solution for 10 min followed by a wash with 50 mM Tris-Cl. The beads were added separately in conical flasks containing 50 ml of fresh AA medium, placed in an orbital shaker at 50 rpm at RT and the number of fractured capsules was counted at selected time intervals. Amount of cell leakage from microcapsules was determined by inoculating 100 beads in 50 ml of AA medium in triplicates in the orbital shaker as above. A sample volume of 1 ml was withdrawn at different time intervals, centrifuged briefly at 6000 rpm (Eppendorf, 5415D) and the cell pellet was resuspended in 200 µl AA medium. This was plated on 1.5% AA agar plates; after 15 days of incubation under culture room conditions, the number of colonies formed was counted.

4.2.3.6 Physical properties

The porosity of plain silica gel and silica gel immobilized *N. calcicola* was obtained by nitrogen sorption experiments performed at 77 K with a Micrometrics 2010 sorptometer. Prior to analysis, samples were first degassed at 60 °C under 3 µm Hg pressure. Specific surface areas (S_{BET}) were determined by the Brunauer-Emmett-Teller (BET) method in the 0.05 - 0.3 relative pressure range (Brunauer et al. 1938). Porous volume (V_p) and average pore size (D_p) were calculated by Barrett-Joyner-Halenda (BJH) model on the desorption branch (Barrett et al. 1951). The compression test on silica gel was done using the universal testing machine, UNITEK-94100.

4.2.4 Metal stock solutions and metal ion analysis

Metal stocks and determination were preformed as described in sections 2.2.4 - 2.2.5.3.

4.2.5 Industrial applications of recombinant *N. calcicola*

4.2.5.1 Metal biosorption

The lyophilized free cells, CAG, Si-CAG and silica gel immobilized *N. calcicola* were used to evaluate their efficiency for Cu^{2+}, Cd^{2+}, Cr^{3+} and Ni^{2+} biosorption. pH of the working solutions was adjusted by adding 0.1 M HNO_3 or NaOH. Biomass concentrations of *ca*. 1 mg ml^{-1} with an initial metal concentration of 100 mg L^{-1} in conical flasks were placed in an orbital shaker at 150 rpm at 25±2 ^{o}C for 2 h. Samples were withdrawn after equilibration, centrifuged to remove particulate matter and diluted for metal ion analysis by AAS.

4.2.5.2 Metal desorption

25 ml of 100 mg L^{-1} copper solution and biomass concentration equivalent to 25 mg was used for multiple biosorption-desorption cycles. Experiments were carried out at pH 5.0, 150 rpm and 25±2 ^{o}C. The bound metal was desorbed from matrices by treating with 25 ml of 10 mM HCl. Each biosorption or desorption cycle was given 2 h of solid-liquid contact for reaching the equilibrium. After each cycle, the beads were collected, rinsed with Millipore water and reused. Metal concentrations at initial, final and after elution in the solutions were determined by AAS.

4.2.5.3 Metal bioaccumulation

The living cells immobilized on Si-CAG and silica gel was used to evaluate the efficiency of Cu^{2+}, Cd^{2+}, Cr^{3+} and Ni^{2+} bioaccumulation from the at optimum pH. 50 ml culture medium containing respective metal at 100 mg l^{-1} and metal less control were inoculated with an equivalent of ca. 500 mg wet biomass (equivalent to 25 mg dry wt) in conical flasks which were placed in an orbital shaker at 150 rpm for 2 h in the culture room conditions. Samples were withdrawn after equilibration and centrifuged to remove particulate matter for metal ion analysis by AAS.

4.2.6 Continuous removal and recovery of metals from simulated copper mine effluents using up-flow packed-bed columnar bioreactor

4.2.6.1 Preparation of simulated metal containing effluents

Metal containing industrial effluent was collected from Khetri copper mine complex, Rajasthan, India. The industrial waste water was filtered to remove any particulate substances and stored at RT. It was used for the determination of pH, total dissolved substances and concentrations of metals using AAS. Simulated metal laden solution was prepared with respective metals at concentrations which were found in the real mine effluents. This was subsequently used for testing the efficiency of metal removal using immobilized recombinant *N. calcicola* packed in a columnar bioreactor.

4.2.6.2 Bioreactor packed with recombinant *N. calcicola* on Si-CAG

The exponentially grown culture of *N. calcicola* immobilized on Si-CAG was used for the continuous removal and recovery of metals from simulated copper mine effluent. The dimensions of the glass column were 32 cm in length with an internal diameter of 3 cm.

The column and accessories were washed with 10% nitric acid followed by rinsing with deionized water. The column was packed with 3260 Si-CAG beads (*ca*. 5 g wet cells). Millipore water was pumped through the column prior to run in order to wash the packing material and to minimize the air pocket formation. Simulated metal containing influent solution was prepared using Millipore water and concentrations of Cu^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} at 4.04, 0.06, 0.15 and 0.05 mg L^{-1} respectively, the pH was adjusted to 5.8 ±0.05. The rate of flow of the solution through the column was controlled with a peristallic pump at 6 ml min^{-1}. Samples were collected every 2 min for first half an hour followed by 30 min until the column was saturated with metals.

4.2.6.3 Metal desorption

After saturation of biomass in the column with metals which were accumulated from simulated industrial effluent, the column was regenerated in order to recover the bound metals in a minimal volume. A 10 mM calcium nitrate or 10 mM hydrochloric acid solution was passed through the column at 6 ml per min. It is a mild eluant which rips metals from the cells effectively with minimal damage to cell integrity. The metal concentrations in the solutions were measured by AAS.

4.2.7 Growth of recombinant *N. calcicola* on culture medium containing mine effluents and metal removal

To determine the ability of recombinant *N. calcicola* to grow and accumulate metals from real time effluents, the metal laden waste water was added to culture medium at 10 – 80 times dilution and at neutral pH. The cells were inoculated without kanamycin and incubated under standard culture room conditions. Growth was measured spectrophotometrically at OD_{750} and metal concentrations were determined using AAS.

4.3 Results and Discussion

4.3.1 Immobilization of *N. calcicola* on CAG and silica gel

Alginate, the most commonly used matrix for the immobilization of cells, defines an extracellular environment that is beneficial to the cell viability and protection for prolonged biological activity (Coradin et al. 2003). The sensitivity of CAG towards phosphates, citrates, chelating compounds and monovalent cations such as sodium and potassium is the bottleneck for its industrial applications. Coating of CAG with polycations and silica can be used to minimize these limitations (Coradin et al. 2003). Thus, silica coated CAG was used for the immobilization of recombinant *N. calcicola* cells (Fig. 32A – B). The silica membrane over CAG bead as observed under microscope (Fig. 32C) can be represented as shown in Figure 32D.

The silica gel used for the immobilization of the cells for applications in metal removal is shown in the Figure 33A – D. The cell free silica gel was a transparent material and the cells immobilized in the matrix were used for metal accumulation experiments as granules (Fig. 33D) to increase the surface area and to facilitate the mass transfer. Silicates are known to be non-toxic and environment-friendly chemicals. The conventional silica gel immobilization methods are very tedious and reaction conditions required for chemical synthesis limit their applicability for whole cells encapsulation (Coiffier et al. 2001). To overcome these problems, silica gel in the present study was made by mixing sodium silicate and colloidal silica as described in the material and methods. Silica gelation under neutral conditions with sodium silicate and colloidal silica was found to be very fast (*ca*. 2 min) and much easier when compared to other conventional methods (Hu and Reeves, 1997). Moreover, the cell entrapment occurred in solvent-free media, and in suitable osmolarity conditions. In this method, no sulfate or alcohol is formed as a by-product and the amount of Na^+ ions is also very low that minimizes the osmotic shock to the immobilized cells (Nassif et al. 2002). This leads to the maintenance of cell integrity and cell viability as shown by 1% TTC reduction test and the cells were culturable in nutrient medium (data not shown). Thus, interactions between the immobilized species with silica matrices do not appear to be detrimental to their biological activity.

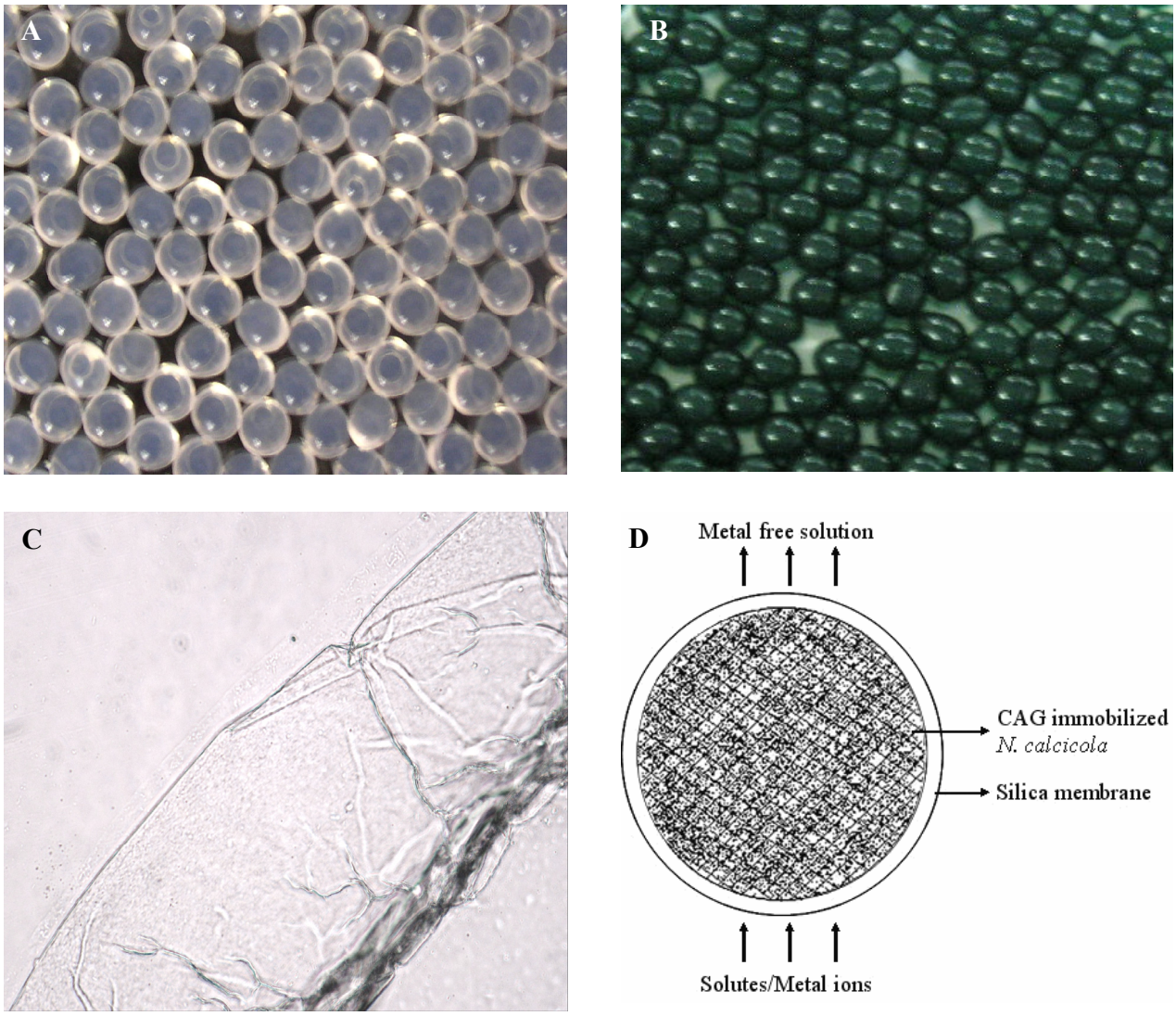


Fig 32. Silica coated CAG immobilized *N. calcicola*. (A) cell-free beads; (B) *N. calcicola* immobilized on CAG (C) light microscopy of the silica membrane over the CAG (400x) and (D) schematic diagram representing silica-coated CAG beads.

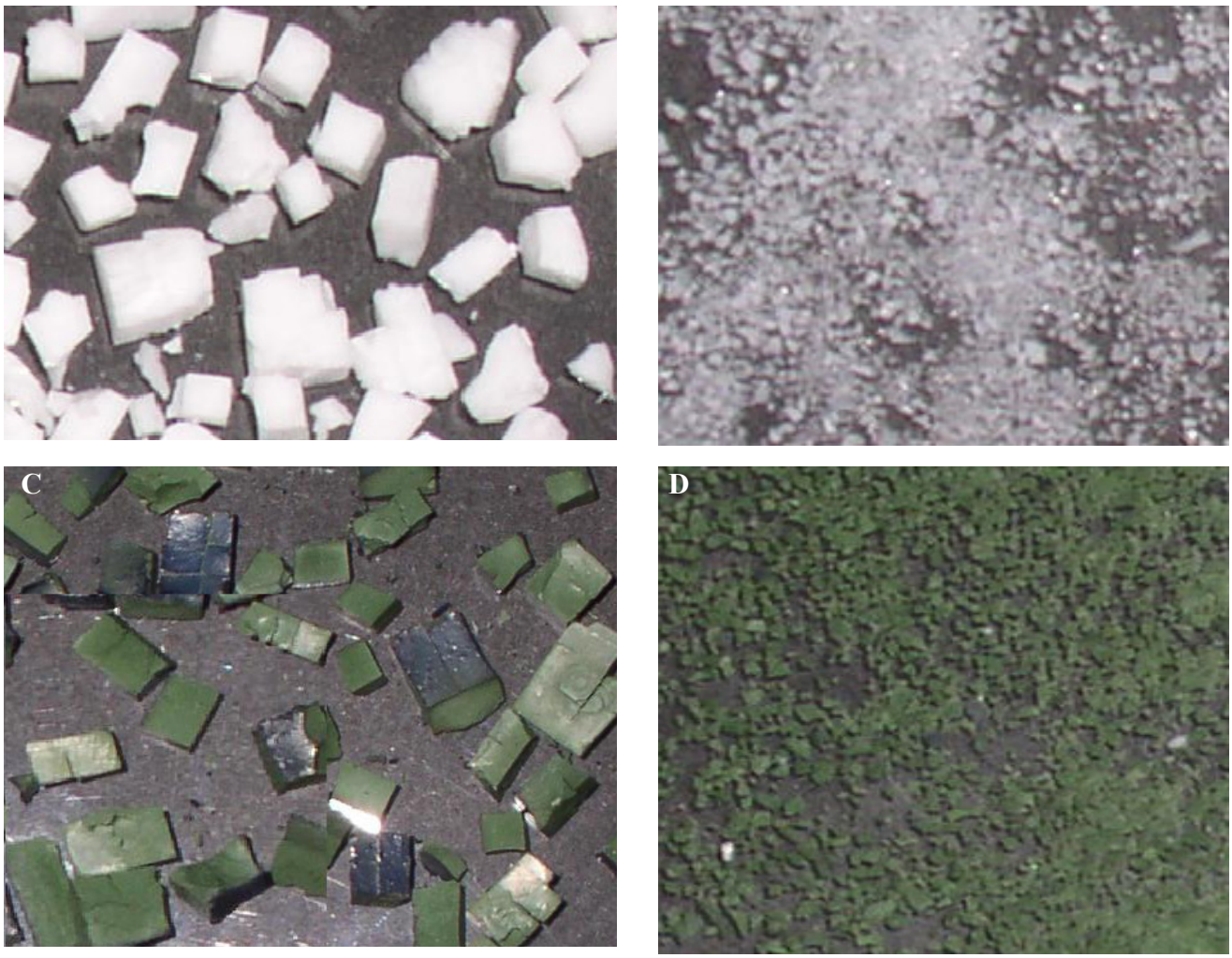


Fig 33. Silica gel immobilized *N. calcicola*. (A) cell free silica gel (B) cell-free silica gel granules (C) *N. calcicola* immobilized on silica gel and (D) *N. calcicola* immobilized on silica gel granules.

4.3.2 Characterization of immobilized *N. calcicola*

4.3.2.1 Ultrastructural studies

The examination of cell-free CAG without silica coating by SEM revealed the presence of large pores and channels on the bead surface (Fig. 34A). *N. calcicola* containing CAG bead without silica coating showed that the bead surface was covered by large cell clusters (Fig. 34B – C). Such conditions may lead to the loss of cells as well as leakage of cellular material from the beads. In the present study, polyallylamine (PAA) was used as a polycation in place of poly-L-lysine (PLL) (Coradin et al. 2001) for coating alginate beads in order to decrease the cost of the process. However, in agreement with previous data on poly-amines mediated precipitation of silica (Coradin et al. 2001), PAA appears as effective as PLL to induce silicate condensation. SEM examination of silica-coated CAG beads showed that the cells are glued by a homogeneous and dense mineral layering (Fig. 34D – E). A cross section of the outer silica layer showed that *ca*. 1 µm thick silica was deposited over a 10 µm organic layer (Fig. 34F). SEM investigations of silica gel indicated that they appear homogeneous and exhibit a granular surface with a discernable array of macropores (Fig. 35A – C). TEM observations suggested the additional presence of mesopores, *ca*. 100 Å (Fig. 35D). Such a porous network on the immobilization matrix can facilitate tight entrapment of immobilized species while allowing solutes like metal ions to pass through easily.

4.3.2.2 Mechanical stability

The stability of silica-coated beads was further examined by studying the cell loss from CAG beads with or without silica coating. Stability of silica coating over CAG was tested by mechanical agitation using sodium citrate treated hollow beads. It was found that the silica membrane was stable against shearing forces generated due to agitation created at 50 rpm at RT and prevented cell leakage from the capsules (Fig. 36). The larger extent of cell loss by the uncoated beads could be attributed to the loose binding of biomass onto CAG (Fig. 34B – C). Hence, the smaller number of colonies formed from the silica coated beads during stability tests were due to the highly stable silica membrane while being porous enough to freely allow metal cations nutrients through the membrane to immobilized cells. In addition,

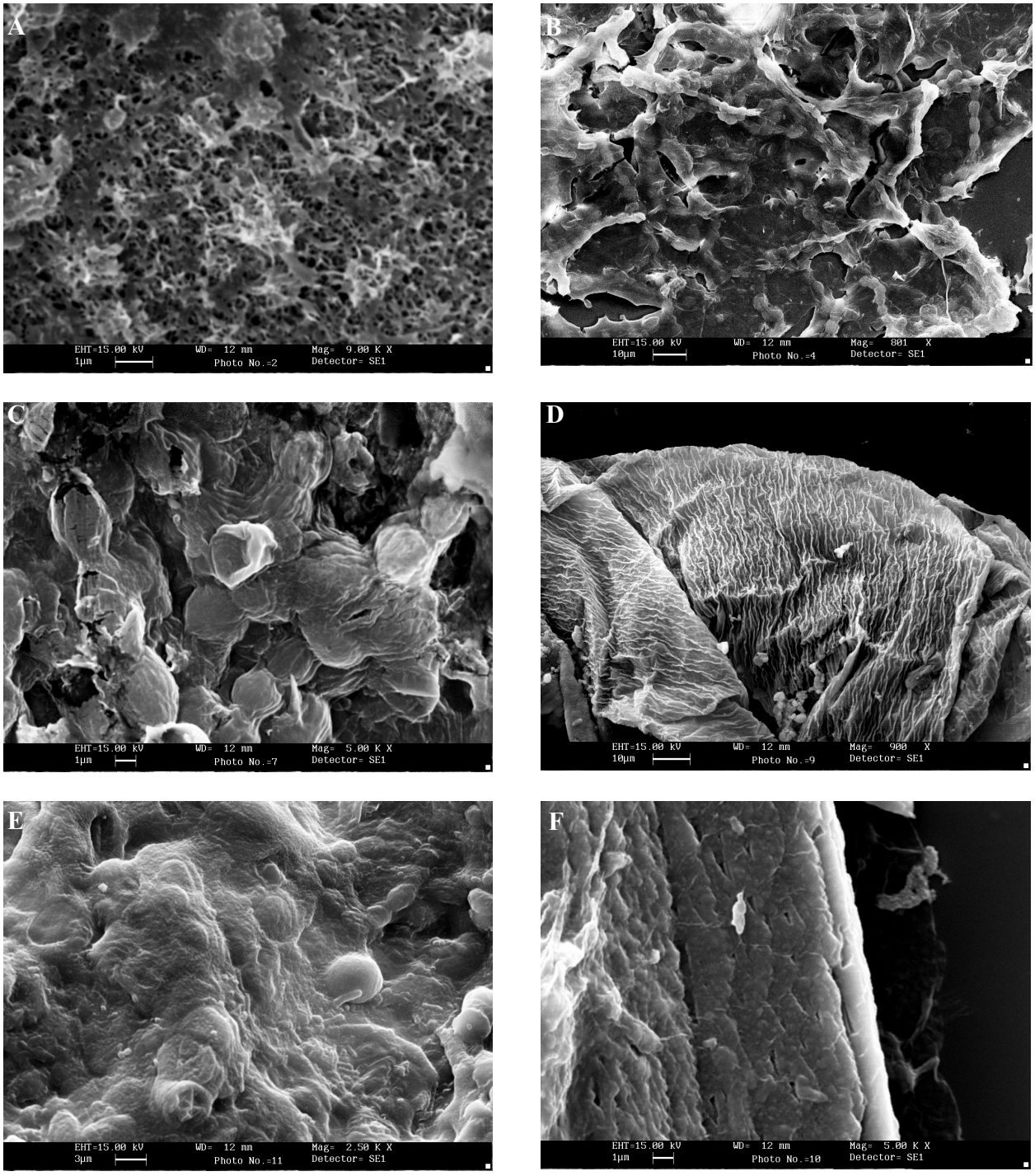


Fig 34. Characterization of CAG beads through scanning electron microscopy (SEM). (A) cell-free calcium alginate beads (9000x); (B, C) CAG immobilized *N. calcicola* (800, 5000x); (D, E) CAG immobilized *N. calcicola*, coated with silica (900, 2500x); and (F) cross-section of silica coated CAG (5000x).

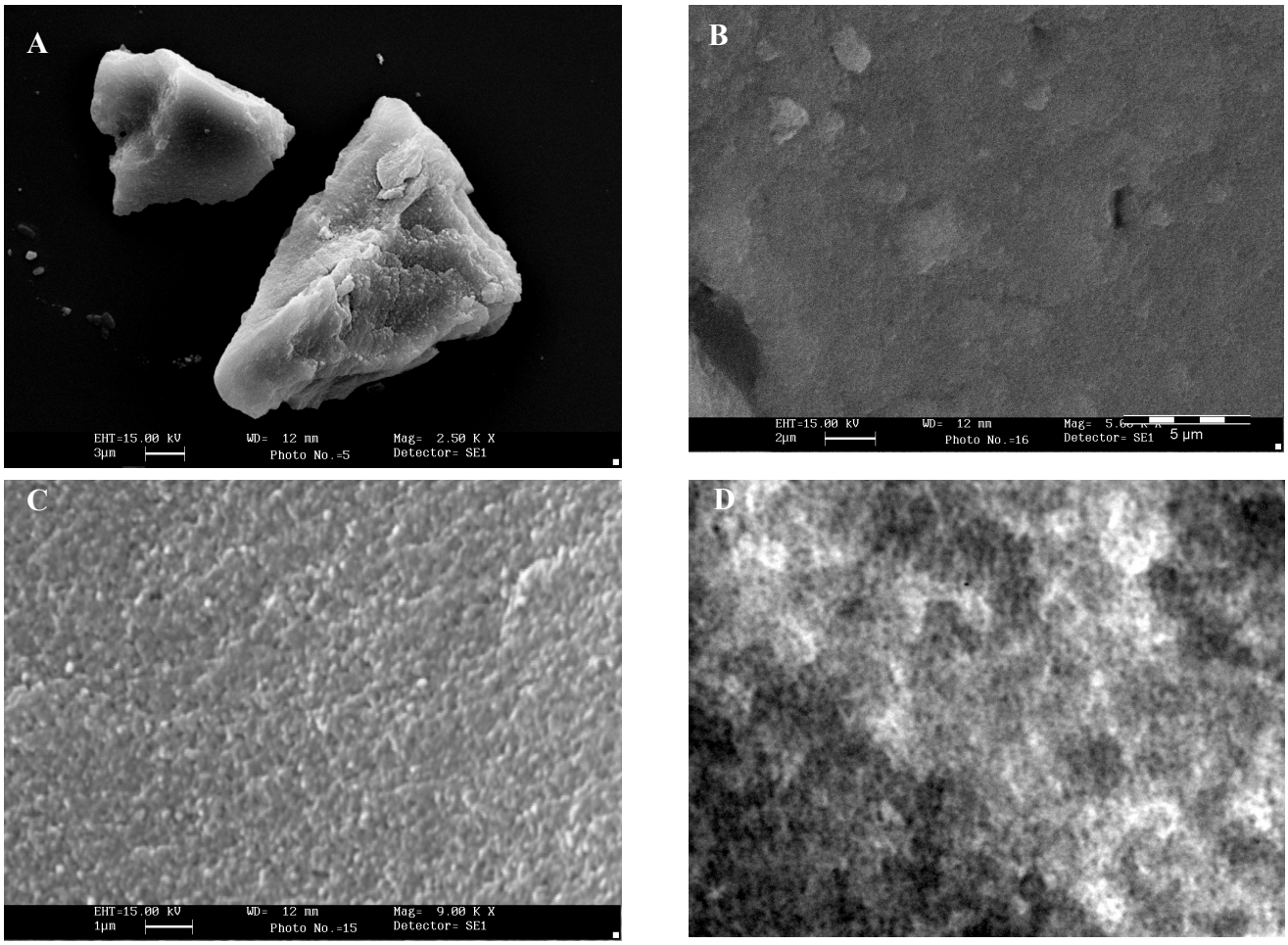


Fig 35. Characterization of silica gel. (A) SEM of the silica gel granules (2500x); (B) surface morphology of the silica gel (5600x); (C) macropores of the silica gel (9000x); (D) mesopores (*ca*. 100Å) of the silica gel as seen by TEM (18000x).

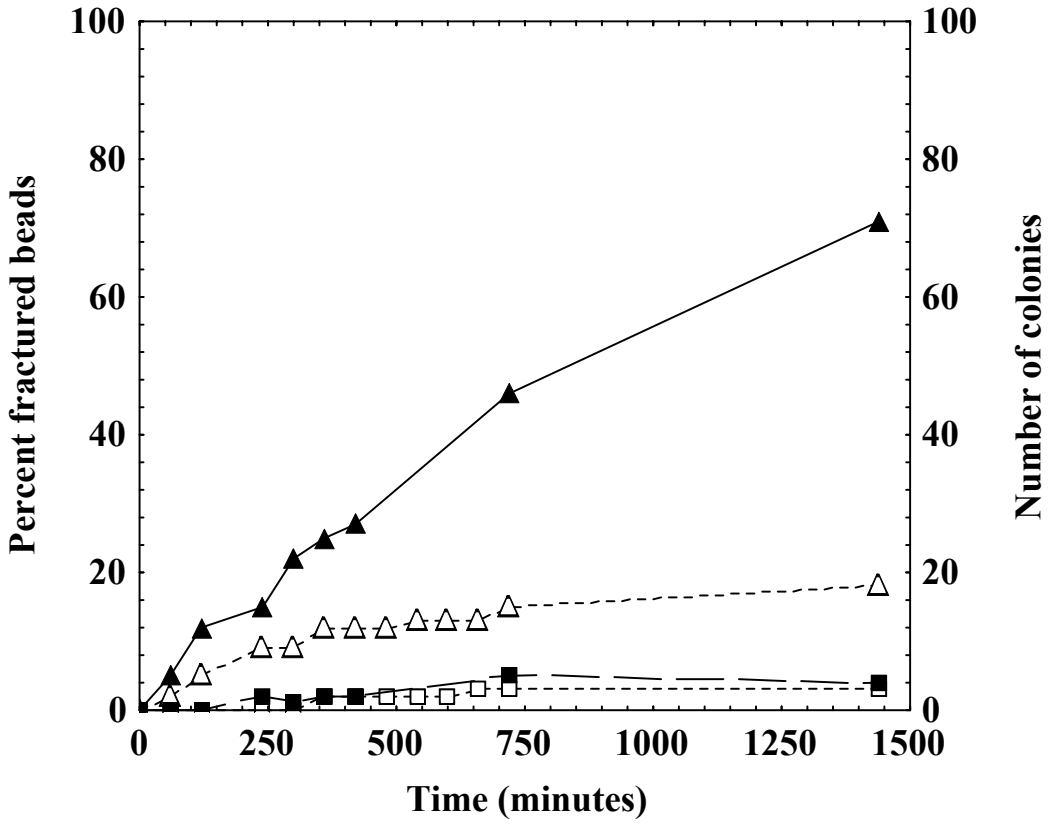


Fig 36. Mechanical stability of CAG beads. The percentage fractured CAG beads (white symbols) vs. number of colonies formed (dark symbols) for silica-coated alginate beads (□, ■) and alginate-coated beads, (Δ, ▲). Mechanical strength of the hollow beads was tested by counting number of beads fractured upon agitation. Silica and alginate coated beads treated with 50 mM sodium citrate solution were added separately in conical flasks containing AA medium and placed in an orbital shaker at 50 rpm at RT; the number of fractured capsules was counted. Amount of cell leakage was determined by plating the solution on 1.5% AA agar plates, after 15 days of incubation under culture room conditions; the number of colonies formed was counted

the process of silica coating did not affect the cell viability of immobilized *N. calcicola* as tested by 1% TTC (data not shown) and the cells were further culturable when plated on AA agar plates. The silica gel was found to be stable upon virgorous agitation on a rotary shaker for 24 h and there was no significant loss of cells from the matrix.

4.3.2.3 Physical properties

Though, the CAG have been coated with a tough 1 µm thick silica membrane, it did not have tensile strength and physical properties like the silica gel. Howerer, the silica coating prevented cell loss and leaching of low molecular weight protein phycocyanin and phycoerythrin (*ca.* 15 kD) from the CAG (data not shown) while allowing the metal ions to cross the membrane for accumulation by the entrapped cells.

The porosity, surface area and pore diameter of the silica gel was confirmed by nitrogen sorption measurements (Table 16). The cell free silica gel had specific surface area S_{BET} of 200 $m^2\ g^{-1}$, a porous volume V_p of 0.65 $cm^3\ g^{-1}$ and an average pore size D_p of 120 Å. It is interesting to note that the presence of cells does decrease both specific surface area (S_{BET} = 160 $m^2\ g^{-1}$) and porous volume (V_p = 0.40 $cm^3\ g^{-1}$) but that the average pore size remains in the mesoporous range ($D_p \approx$ 100 Å). The compression test on two different batches of silica gel showed a breaking load of 0.526 kN and 0.015 kN (Fig. 37A – B) whereas hardness remains same at 11 kg cm^{-1}.

4.3.2.4 Chemical properties

The chemical stability of the silica membrane was also studied in the presence of commonly used metal desorption reagents. The results concluded that silica-coated CAG beads were found to be stable (Table 17) whereas uncoated beads were found to be sensitive to calcium chelating compounds within 4 h. In the present study, it was observed that, even after treating with such agents, silica-coated beads maintained their integrity, whereas control beads without silica membrane were either dissolved completely or partially damaged upon such exposure. The chemical stability of the

Table 16. N_2-sorption porosity measurements of silica gel in the absence and presence of cells: BET specific surfaces (S_{BET}), porous volume (Vp) and average pore size (Dp) as calculated using BJH models.

S. No	Sample	S_{BET} ($m^2 g^{-1}$)	V_p ($cm^3 g^{-1}$)	D_p (Å)
1	Silica gel	200	0.65	120
2	Silica immobilized *N. calcicola*	160	0.40	100
3	Control*	5	0.05	250

* Silica gel synthesized by means of acid hydrolysis

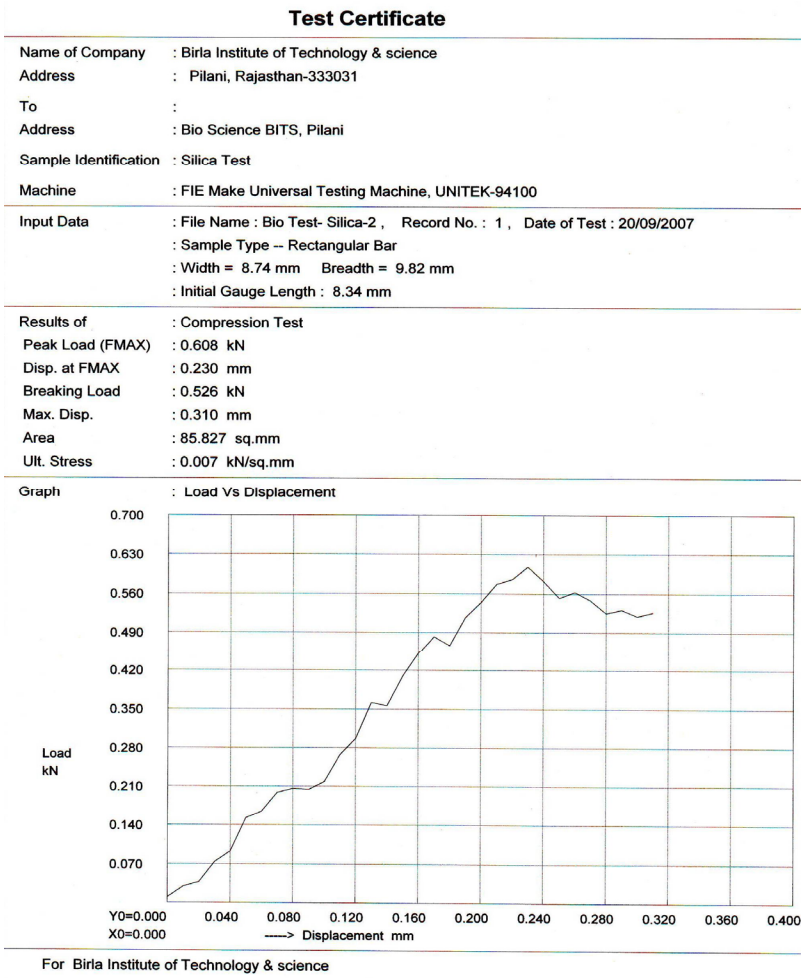


Fig 37A. The compression test results of silica gel. Silica gel dried at 80 ^{o}C and the hydrated gels were used for compression test on a Universal Testing Machine, UNITEK 94100.

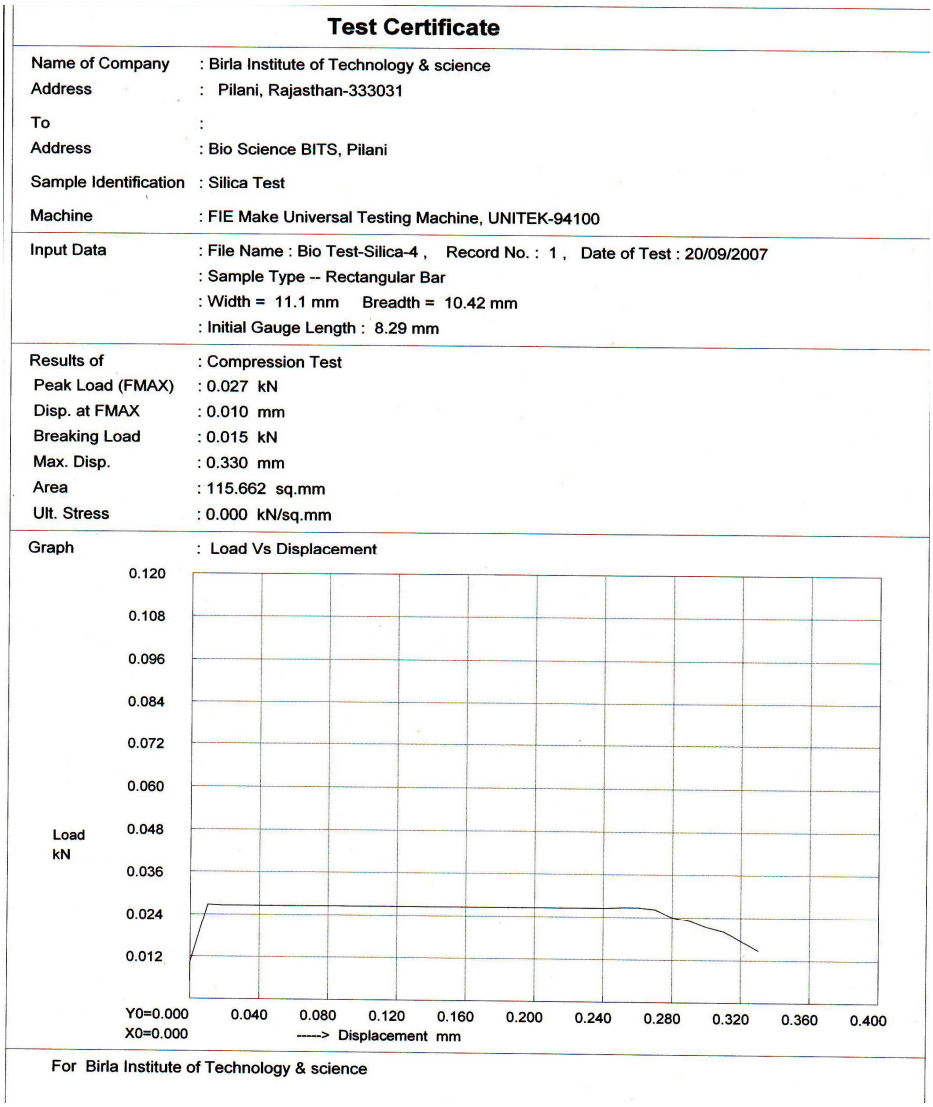


Fig 37B. The compression test results of silica gel. Silica gel dried at 80 ^{o}C and the hydrated gels were used for compression test on a Universal Testing Machine, UNITEK 94100.

Table 17. Stability of the calcium alginate, silica coated calcium alginate and silica gel in different eluants tested for desorption of heavy metals. The immobilization matrices were treated with 25 ml of respective eluants and incubated at 25±2 °C, 50 rpm for 4 h.

S. No	**Eluants**	**CAG immobilized *N. calcicola***	**Si-CAG immobilized *N. calcicola***	**Silica gel immobilized *N. calcicola***
1	H_2SO_4 (0.1 M)	Stable	Stable	Stable
2	HNO_3 (0.1 M)	Stable	Stable	Stable
3	HCl (0.1 M)	Stable	Stable	Stable
4	$Ca(NO_3)_2$ (0.1 M)	Stable	Stable	Stable
5	KH_2PO_4 (0.1 M)	Unstable	Stable	Stable
6	K_2HPO_4 (0.1 M)	Unstable	Stable	Stable
7	EDTA (10 mM)	Unstable	Stable	Stable
8	$CaCl_2.2H_2O$ (0.1 M)	Stable	Stable	Stable
9	$CaCO_3$ (0.05 M)	Stable	Stable	Stable
10	$NaHCO_3$ (0.1 M)	Stable	Stable	Stable
11	Na_2CO_3 (0.1 M)	Unstable	Stable	Stable
12	Na-Citrate (0.05 M)	Unstable	Stable	Stable
13	NaCl (0.1 M)	Stable	Stable	Stable
14	NaOH (0.1 M)	Stable	Stable	Unstable

silica gel was also studied in the presence of desorption reagents. As shown in Table 17, the silica gel was found to be affected only by the action of strong alkali (0.1 N NaOH) while being inert towards rest of the tested eluants even after 24 h.

4.3.3 Metal sorption and desorption by wild type and recombinant *N. calcicola*

4.3.3.1 Metal biosorption

The metal accumulation properties of *N. calcicola,* as free cells, within coated and non-coated CAG, and silica gels are shown in Figure 38. For all evaluated cations, an enhanced accumulation was observed for cyanobacterial cells immobilized in silica-coated beads compared to uncoated. In contrast, silica gel immobilized cells showed *ca*. 5% less metal accumulation when compared to free cells except for Ni^{2+}. Indeed, the metal adsorption involves ion binding to both the entrapped cells and the immobilization matrix. CAG immobilized microbial biomass has been used to recover metals from the aqueous solutions (Garnham et al. 1992; Chang and Huang, 1998) and the efficiency of this approach is partly due to the fact that alginate itself enhances metal accumulation due to the presence of carboxyl groups and also known to effectively bind a range of heavy metals like Cu, Cd, Co, Pb and Zn (Jodra and Mijangos, 2003; Jan et al. 1995; Brower et al. 1997). The slight increase in metal uptake by silica-coated beads could be attributed to the silanol groups that are exposed on the material surface. Such an adsorption should also occur for silica gels, as earlier reported (Al-Saraj et al. 1999). It was observed that a faster metal removal (biosorption kinetics) of metal ions from the solution by silica gel immobilized *N. calcicola*. However the observed slight decrease in metal accumulation when compared to free cells suggest that other factors like effect of tight packing of cells, decreased accessibility and secreted polymers within the encapsulation network are to be taken into account.

4.3.3.2 Metal desorption

Studies involving regeneration of silica-coated beads with continuous copper sorption and desorption have demonstrated clear superiority over plain beads (Fig. 39). The uncoated beads showed a significant decrease in accumulation efficiency over 10 cycles,

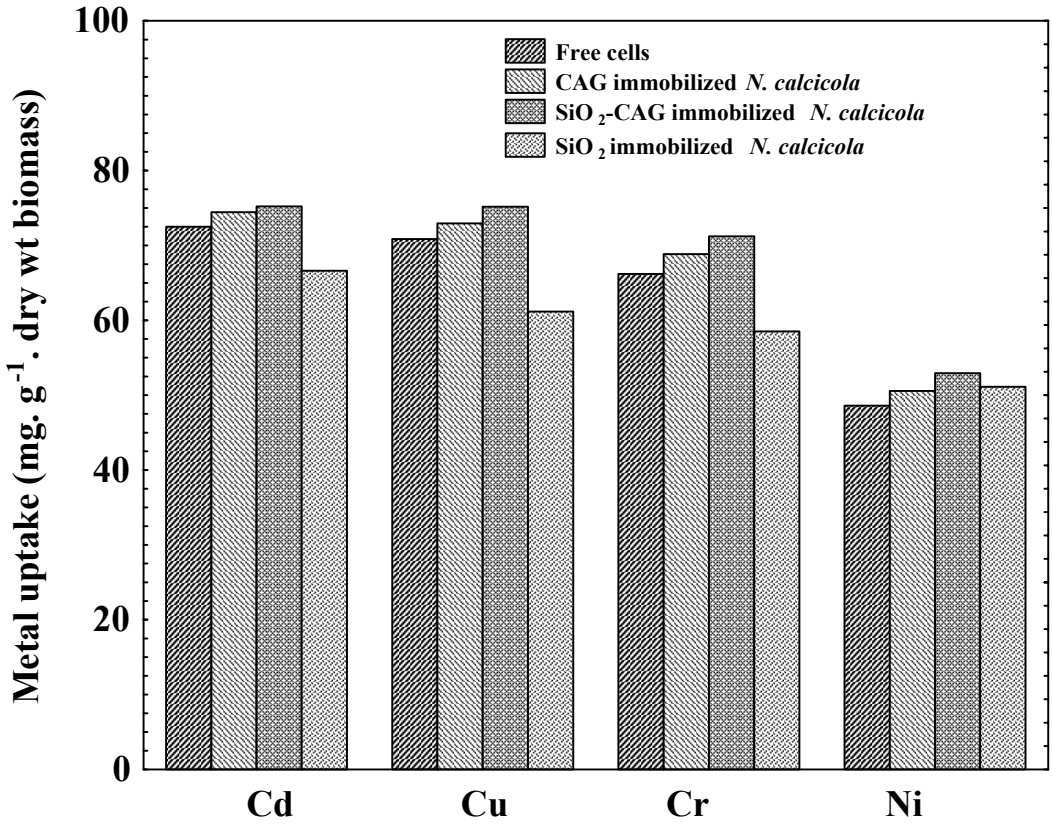


Fig 38. The metal accumulation properties of the free cells, calcium alginate, silica coated calcium alginate and silica gel immobilized *N. calcicola.* The beads were placed in contact with 100 mg L^{-1} of the respective metal for 2 h. Optimum pH, Cd, 7.0; Cu, 5.0; Cr^{3+}, 5.0 and Ni, 7.0; at 150 rpm, and temperature 25±2 ^{o}C. The samples were withdrawn after equilibration and metal concentrations were estimated using AAS.

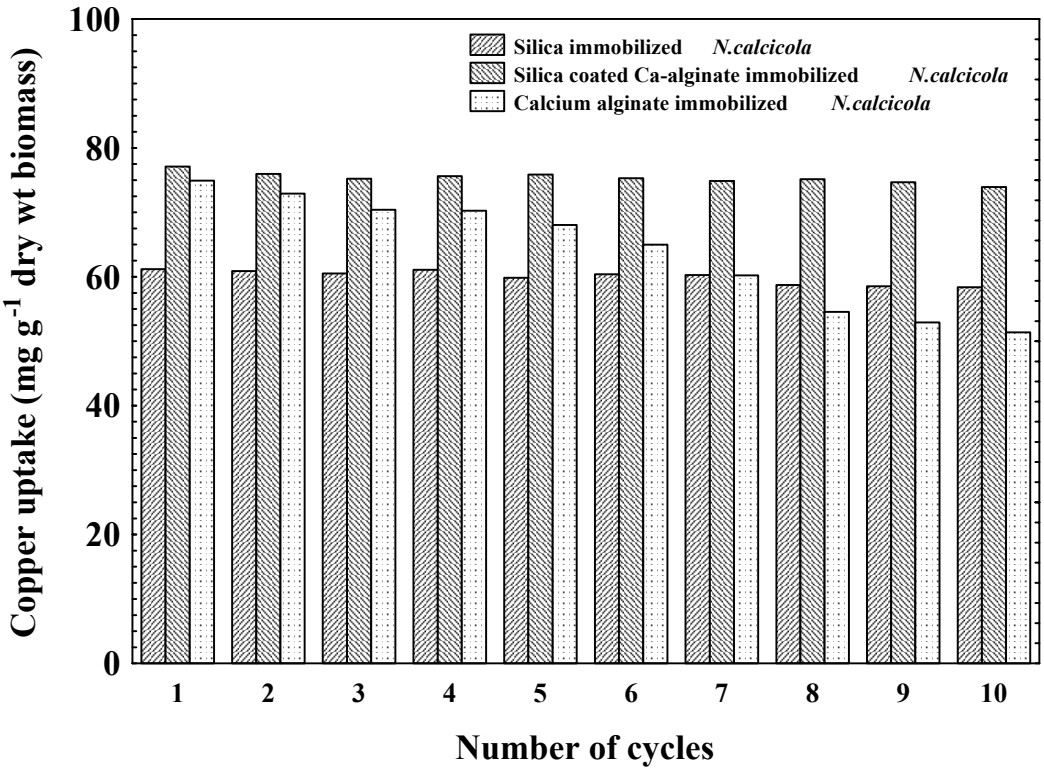


Fig 39. Multiple sorption/desorption of copper using *N. calcicola* immobilized on silica gel, silica coated CAG and CAG matrices. The beads were placed in contact with 100 mg l^{-1} of Cr^{2+} for 2 h at 150 rpm and temperature 25±2 ^{o}C. The bound metal was desorbed using 10 mM HCl and matrices were washed with distilled water before each cycle.

but the silica-coated CAG or the silica gel did not show such loss of sorption properties. Therefore, even if initial accumulation capabilities of silica gel-immobilized *N. calcicola* are lower than within plain beads, their relative efficiency is reversed after 7 cycles. The lower regeneration capacity of CAG beads without silica membrane could be due to slow leaching of cells and biomolecules such as proteins and cell-wall polysaccharides which are responsible for metal binding from the gel matrix after treating with desorbing agents. In contrast, the presence of silica either on the bead surface or as a bulk host should limit this leaching process.

4.3.3.3 Metal bioaccumulation

Metal bioaccumulation by the exponentially grown recombinant cells immobilized on silica-coated CAG and silica gel at optimal pH was shown in the Table 18. The recombinant cells showed high efficiency towards Cd, Cu and Ni whereas Zn was found to be lowest in terms of intracellular bioaccumulation. These results show numerous benefits of using silica-based materials for immobilization. The enhanced mechanical and chemical stability in CAG and silica gel could be attributed to the intrinsic properties of inorganic materials. Microorganisms and algal cells release metabolic and structural components including organic acids and other exudates such as polypeptides and siderophores to the culture medium (Claessens and Cappellen, 2007; Xue and Sigg, 1990). These affect chemical speciation of metal ions in solutions thereby metal precipitation and accumulation at the cell wall. Such molecules can be retained in the silica coated CAG whereas in the uncoated beads these leach out of the matrix. Moreover, these hybrid capsules could accumulate more metals than un-mineralized beads and this may be due to the surface-exposed silanol groups. Such a beneficial effect was not observed for silica gel-entrapped cells, probably due to steric hindrance of the surrounding mineral network.

4.3.3.4 Continuous removal and recovery of metals from simulated copper mine effluents in a packed-bed columnar bioreactor

Metal containing industrial effluent was collected from Khetri copper mine complex, Jhunjhunu, Rajasthan. pH of the effluents was 4.81±0.1 and total dissolved substances was found to be 3.562 g l^{-1}. The major metallic species found in the waste water was copper followed by nickel, cadmium and zinc (Table 19). They are far above the

maximum permissible limits allowed by regulatory authorities for disposal (http://envfor.nic.in/ and www.epa.gov).

In order to treat the waste water using a bioreactor packed with immobilized *N. calcicola*, a simulated metal laden solution was prepared with respective metals at concentrations which were found in the real mine effluents. This was subsequently used for testing the efficiency of metal removal in an up-flow columnar bioreactor packed with 3260 Si-CAG beads containing 5 g wet cells. The column parameters were previously optimized for flow rate, biomass concentrations and pH (Vannela and Verma, 2003). Initial experiments on simulated effluents containing copper at 141.5 mg l^{-1} (as observed in the real time effluents) showed faster column saturation and was found to be inefficient (data not shown). To maximize the copper removal using the bioreactor, different dilutions were employed. The bioreactor performance was found to be excellent in the removal of all the metals as observed with a simulated effluent: water ratio at 1: 35 i.e. final metal concentrations of metal at this dilution was Cu 4.042, Ni 0.147, Cd 0.0621 and Zn 0.052 mg l^{-1} (Fig 40). At this dilution, the silica-coated CAG immobilized *N. calcicola* packed bioreactor was saturated after passing *ca.* 32 L of feed at the flow rate of 6 ml min^{-1} (Fig 41). To recover the bound metals from the column, 10 mM HCl was used and it completely eluted the metals bound in the column with 1.25 L at 6 ml min^{-1} (Fig 42A – B). Similar results were reported with *P. aeruginosa* immobilized on polyurethane beads for uranium removal and recovery (Hu and Reeves, 1997).

4.3.4 Growth of recombinant *N. calcicola* on effluent containing AA medium

The current methods of biosorption or bioaccumulation in bioreactor based technologies are sensitive to pH, ionic strength and the presence of organic or inorganic substances (Ashley and Roach, 1990). Moreover, for treating large quantities of metal contaminated waste water (Fig. 43), the above methods may not suffice for effective metal bioremediation. Under these conditions, application of actively growing cells might be a better option due to their ability of self-replenishment and continuous metabolic

Table 18. Metal bioaccumulation by the immobilized recombinant *N. calcicola.* The immobilized cells were placed in contact with 100 mg L^{-1} of the respective metal for 2 h, at optimum pH Cd, 7.0; Cu, 5.0; Cr^{3+}, 5.0, Ni, 7.0 and Zn 5.5; at 150 rpm, temperature 25±2 ^{o}C. The samples were withdrawn after equilibration and metal concentrations were estimated using AAS.

S. No.	Metals (Initial concentration 100mg L^{-1})	Si-CAG immobilized *N. calcicola* (q_{max} mg g^{-1})	Silica gel immobilized *N. calcicola* (q_{max} mg g^{-1})
1.	Cadmium(II)	86.0	82.1
3.	Chromium(III)	66.0	58.8
2.	Copper(II)	80.3	75.3
4.	Nickel(II)	61.7	60.1
5.	Zinc(II)	52.3	42.5

Table 19. Metal composition of the copper mine effluents.

S. No	Metal	Concentration (mg L^{-1})	Maximum Permissible Limits (mg L^{-1})
1.	Copper	141.5	0.3
2.	Nickel	5.15	0.05
3.	Cadmium	2.18	0.005
4.	Zinc	1.83	0.5

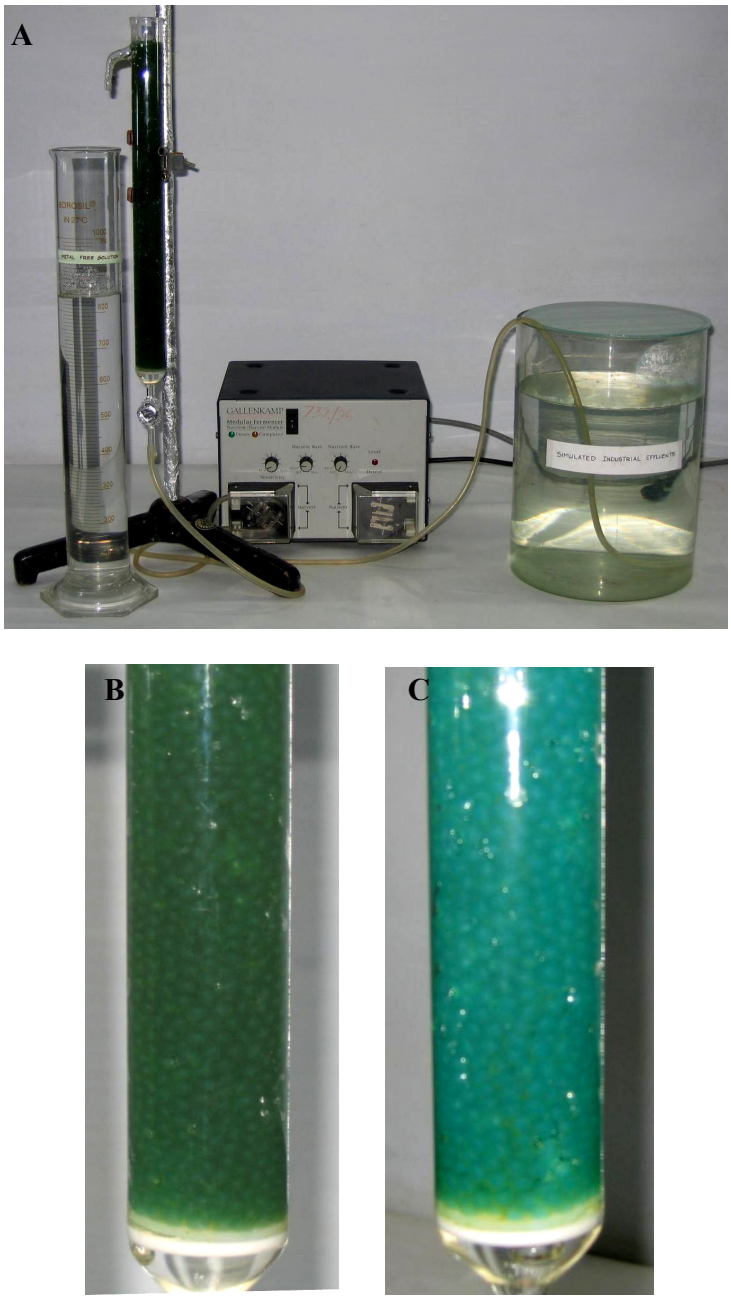


Fig 40. Up-flow packed bed columnar bioreactor. (A) complete assembly of the bioreactor packed with recombinant *N. calcicola* immobilized on Si-CAG; (B) before metal loading; and (C) copper accumulation by the cells in bioreactor from simulated metal containing effluents.

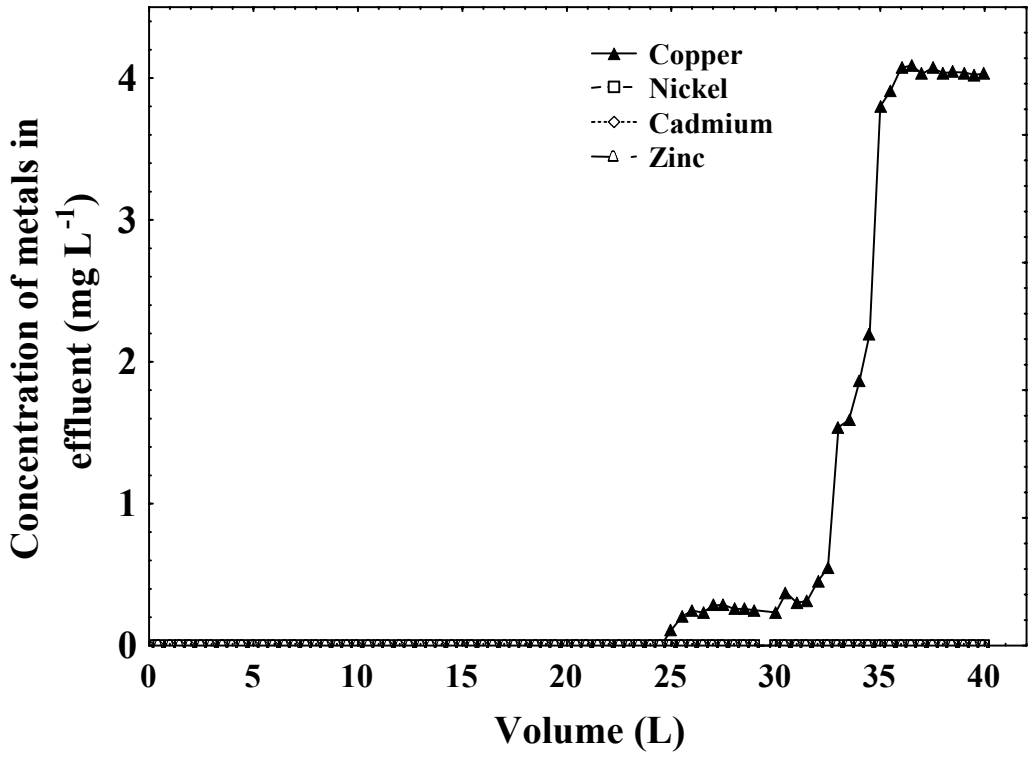


Fig 41. Continuous removal of metals from the simulated metal containing effluent in an up-flow columnar bioreactor packed with recombinant *N. calcicola* immobilized on the Si-CAG. Flow rate 6 ml min^{-1}.

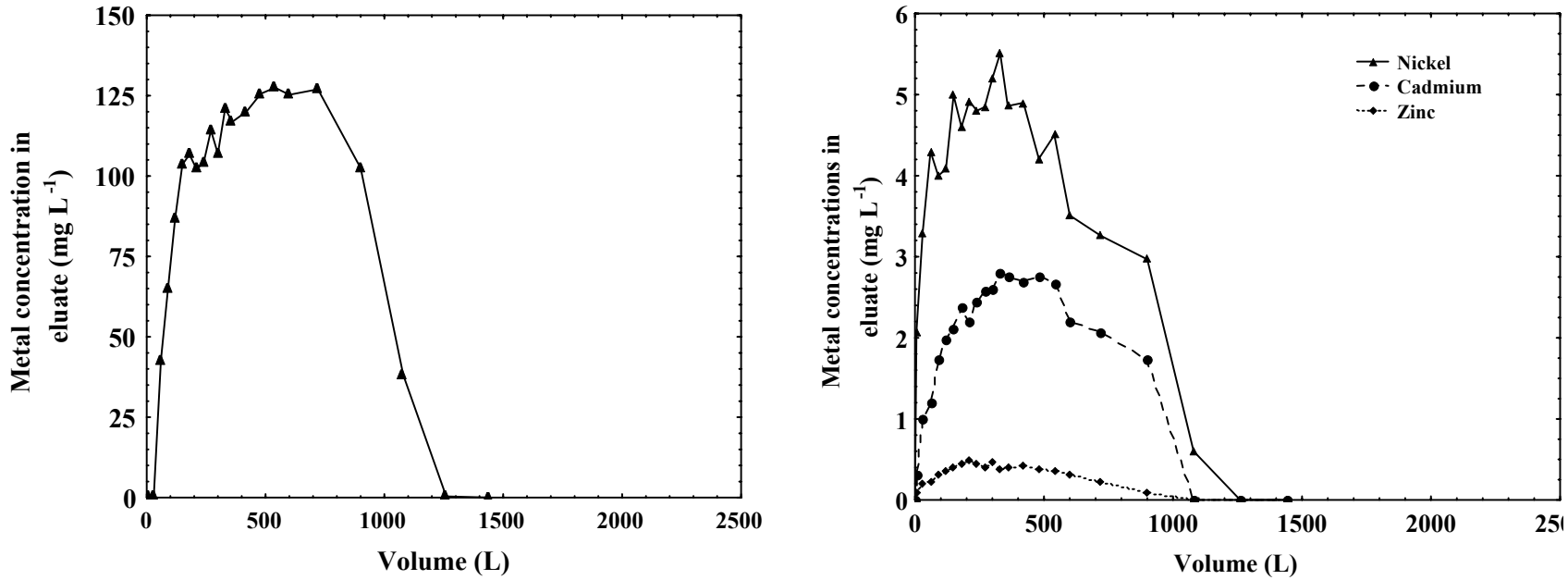


Fig 42A and B. Elution of metals from the bioreactor packed with recombinant *N. calcicola* on Si-CAG using 10 mM HCl; flow rate 6 ml per min.

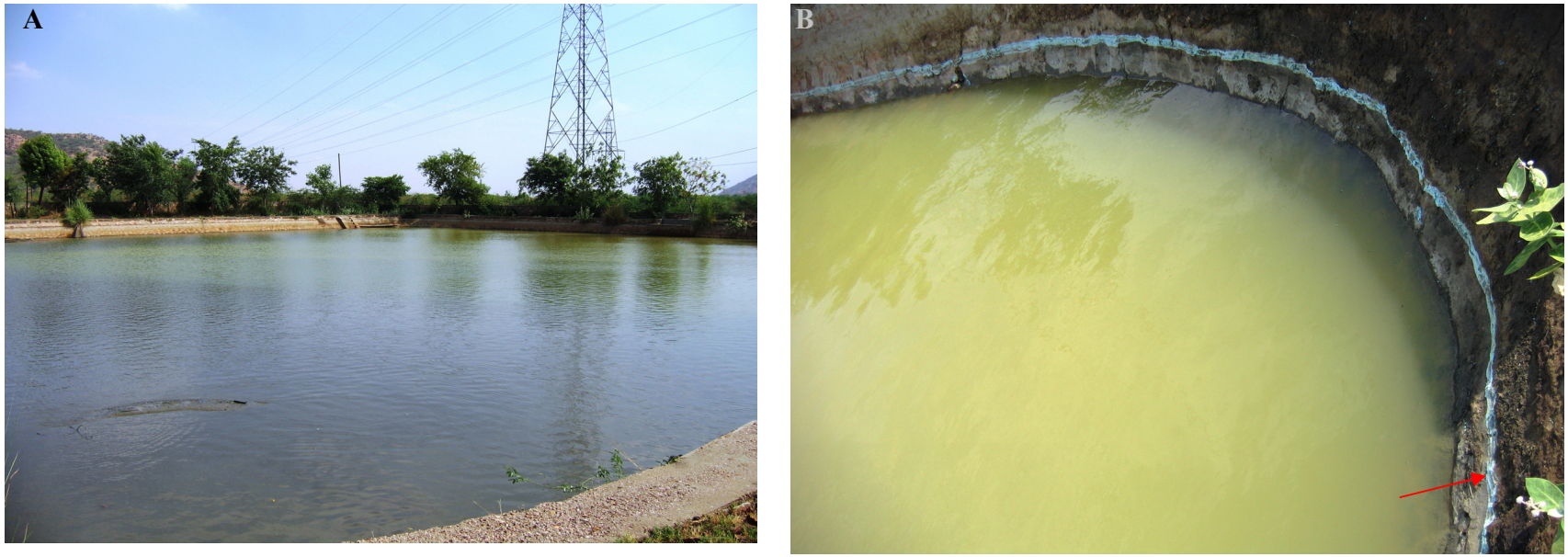


Fig 43. The copper mine effluents pond. Large open pond to store the wastewater (**A**) and excess copper deposited on the wall (**B**).

uptake of metals that lead to a single-stage process for removal of most of the pollutants present in industrial effluents (Malik 2004). Also, the growing cells have unlimited capacities to cleave organo-metallic complexes, degrade organic compounds as well as take up other inorganic ions (Malik, 2004).

N. calcicola could adapt to metal stress conditions (chapter-II) and genetically engineered cells show resistant to most metals (chapter-III). To examine for its ability to grow in such real time metal containing waste water, the cells were inoculated into the copper mine effluents (without any modifications/dilution) and to the culture medium containing effluents at different concentrations (1.25 – 20%). Although, the *N. calcicola* was sensitive to the real time effluents, it grew in culture medium containing effluents. Interestingly, the growth of the cells was rapid at lowest concentrations (1.25%) followed by a decrease upon increase in waste water i.e., the metal concentrations and completely inhibited in growth medium containing effluents at 20% due to toxic effects of copper (Fig. 44 and 45). The activation of growth at low concentrations of effluents could partly be due to the growth promoting properties of additional copper and such enhanced growth of cyanobacteria in metal polluted environment is a common phenomenon (Baptista and Vasconcelos, 2006). The growth inhibitory properties of the effluents on recombinant cells were not only due to the nutrient limitations but also low pH, high concentrations of metals (Table 19) and other interfering agents (total dissolved substances: 3.562 g l^{-1}).

Adjusting the wastewater composition for bioremediation is a common practice and it is called "biostimulation" where the ambient conditions like pH, nutrient levels and addition of carbon source are employed in order to promote microbial growth (Malik, 2004). The advantages of such methods are that the cells can be harvested and metals can be recovered. This process is irreversible as the metals are confined to intracellular proteins including metallothionein and ensure less risk of metal releasing back to the environment.

Recently, nitrogen-fixing cyanobacterium expressing mosquitocidal toxins of *Bacillus thuringiensis* have been developed for use in field conditions (Boussiba et al. 2000). Thus, application of growing cultures of recombinant *N. calcicola* in bioremediation of

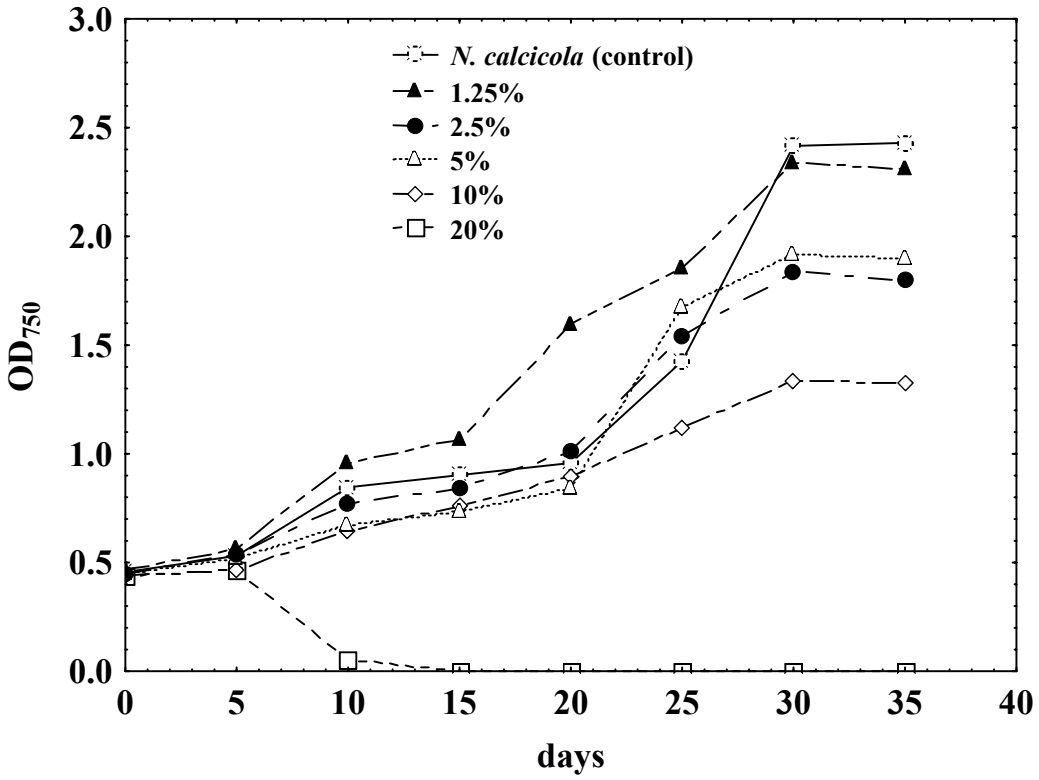


Fig 44. Growth of recombinant *N. calcicola* on AA medium supplemented with copper mine effluents. The cells were inoculated in AA medium devoid of antibiotics and the effluents were added to the growth medium at different concentrations. Cells were incubated under culture room conditions; growth was monitored by measuring the absorbance at OD_{750}.

Fig 45. The recombinant *N. calcicola* grown in AA medium supplemented with copper mine effluents. Metal containing effluents were added to the AA medium at various concentrations (10 – 80 times), cells were inoculated without antibiotics and incubated under culture room conditions. Growth was measured spectrophotometrically at OD_{750}.

heavy metals from industrial effluents could avoid the need for a separate biomass production, in addition, the nitrogen-fixing cyanobacteria are well known that they release beneficial substances (Baptista and Vasconcelos, 2006) like exopolysaccharides, plant growth promoting substances, anti-microbials and fixed nitrogen into the culture. Hence, the treated water can be safely disposed into the environment or used for applications like plantations.

Chapter V

Summary

Chapter V

SUMMARY

The "metallome" refers to the entirety of individual metal species in a cell. Metallome includes distribution of metal, the biomolecules involved in incorporation or complexation and concentrations of the metal inside the cell. The "comparative metallomics" i.e., monitoring the changes of the metallome as a function of time and exposure assist in cellular metal localization. The present study involves the response of *N. calcicola* to metals with special emphasis on metal uptake, compartmentalization, and the stress proteins which play a major role in intracellular distribution of metals. The major results finding are as follows:

1. Zn tolerant strain of *N. calcicola* was developed which grew at 750 μM, the concentration found to be lethal to wild type cells. Such cells showed a two fold increase in the minimum inhibitory concentrations (MIC) for Zn, Mn, Cd, Co and Cu but MIC remained unchanged for Ni, Sr and Cr. Zn equilibrated tolerant cells when suspended in 10 mM phosphate buffer could efflux more than 93% of the metal accumulated in to the medium when compared to the wild type cells. The addition of metabolic inhibitors has reduced the efflux of Zn from the tolerant cells. Similar trends were observed for Cd and Co. Thus, the tolerant cells acquired energy- dependent efflux as a mechanism to maintain the intracellular metal concentrations.

2. Metal treated cells overexpressed superoxide dismutase (SOD) and the malate dehydrogenase (MDH). These enzymes may protect the cells from free radicals and oxidative stress. The high level expression of SOD (1.7 U mg^{-1} protein) and MDH (4.0 mU mg^{-1} protein) upon exposure to Cr(VI) could be attributed to metal specific induction.

3. The ultrastructural studies on *N. calcicola* exposed to metals revealed the localization of metals on the cell wall and cytoplasm thus leading to metal induced cellular damage.

4. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) of the total proteins of *N. calcicola* have led to identification of more than 200 protein spots and most of the proteins could be separated with a pH gradient 4 – 7. The proteome analysis of metal stressed *N. calcicola* showed an induction of Malate dehydrogenase (MDH), Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and Methyl accepting chemotaxis protein (MCP).

5. *N. calcicola* was genetically engineered to express metallothionein from a stable expression plasmid based on pDU1 replicon. The recombinant strain showed resistance to Co, Ni, Cu, Sr, Zn, Cd and the MIC were raised to 52.5, 12.5, 15, 100, 1000 and 67.5 μM as compared to 35, 10, 10, 70, 750 and 20 μM for wild type cells. These are higher than those for the wild type cells.

6. Silica coated calcium alginate (Si-CAG) and silica gel (SiO_2) were developed for whole-cell immobilization. These matrices were characterized for their pore size, chemical and physical stability as well as mechanical strength. Both these matrices were found to be mesoporous in nature with a pore size of *ca.* 100 Å. Si-CAG and SiO_2 were stable in commonly used chemical eluants and extremes of pH. Silica immobilized *N. calcicola* could be used repeatedly for 10 cycles of metal sorption-desorption without any loss of metal accumulation under batch conditions.

7. *N. calcicola* immobilized on silica matrices packed in an up-flow columnar bioreactor completely removed Cu from a simulated copper containing effluent (copper concentrations 4 mg L^{-1}). 5 g of wet biomass could remove *ca.* 140 mg of copper present in 32 liters of solution at pH 4.8. The cell bound copper was eluted in *ca.* 1.2 L of 10 mM HCl.

8. The recombinant strain grows in the medium supplemented with real time effluent containing 14.01 mg L^{-1} of copper. The intracellular bioaccumulation of Cd, Zn and Co were 2.835, 242.235, 5.405 μM metal mg^{-1} protein.

Future scope of the work

The studies on cyanobacterial proteome with respect to metal stress regulation remain in its infancy. Metal induced proteins in *N. calcicola* like Malate Dehydrogenase (MDH), Methyl-Accepting Chemotaxis Protein (MCP) and Ribulose-1,5-bisphosphate carboxylase/ oxygenase (RubisCO) warrants further work to establish its role in metal stress regulation.

Application of the recombinant strain for metal bioremediation technology and the immobilization matrices open the door for future research on developing high affinity biosorbents for metal removal and recovery from real time industrial effluents. The design of biosorbent and biosorption technology largely depends on composition of waste water; thus, a correlation between the effluent composition and genetic trait of biomass need to be established for any effective field applications.

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Appendix I

List of Publications

Manuscripts published/submitted

1. Vaneela R, **Ramachandran S**, Verma SK (2005) Optimization of process parameters for continuous removal and recovery of Cu^{2+} by immobilized *Spirulina platensis*. *ICFAI Intl. Journal of Science and Technology*, 1: 90 – 100

2. **Ramachandran S**, Jain PK, Coradin T, Verma SK (2007) Immobilization of *Nostoc calcicola* in silica gels and silica-coated alginate beads for applications in heavy metal biosorption. *Biotechnology and Bioengineering*. (submitted)

3. **Ramachandran S,** Jain PK, Verma SK (2007) Chromium(VI) induced cellular damage, protein expression and mutagenesis in the multicellular cyanobacterium *Nostoc calcicola*. *J Bacteriol.* (submitted)

Manuscripts in preparation

1) **Ramachandran S,** Jain PK, Verma SK (2007) Zinc(II) and Mn(II) inducible proteins in the cyanobacterium *Nostoc calcicola*.

2) **Ramachandran S**, Jain PK, Coradin T, Verma SK (2007) Genetically engineered *Nostoc calcicola* immobilized on silica coated calcium alginate and silica gel for applications in heavy metal bioaccumulation.

3) **Ramachandran S,** Sarma MVRK, Verma SK (2007) Biosorption of Cr(VI) and Cr(III) by *N. calcicola* immobilized on silica gel.

4) **Ramachandran S** and Verma SK (2007) Rapid non-enzymatic reduction of Chromium(VI) by *Nostoc calcicola* immobilized on mesoporous silica gel.

5) Jain PK, **Ramachandran S,** Verma SK (2007) Isolation and characterization of multiple metal and antibiotic resistant bacterial strains from a copper mine complex.

6) **Ramachandran S,** Jain PK, Verma SK (2007) Transposon mutagenesis as a tool for the identification of metal regulated genes in cyanobacteria.

Publications in Proceedings/ Symposia/Conferences

1. **Ramachandran S**, Pankaj Kumar Jain, Anubha Rai and S.K. Verma (2007) Mechanisms of chromium(VI) induced cytotoxicity, oxidative stress and mutagenesis in *Nostoc calcicola*. 27th Annual Conference of Society of Toxicology (STOX), Oct 6 – 7, Karnataka Veterinary, Animal and Fisheries Sciences University, Bangalore. p. 64

2. Pankaj Kumar Jain, Vikas Kumar Rai, Anubha Rai, **Ramachandran S** and S.K. Verma (2007).Multiple antibiotic resistant bacterial strains from a copper mining industry: An environmental health hazard. 27th Annual Conference of Society of Toxicology (STOX), Oct 6 – 7, KVAFSU, Bangalore. p. 90

3. **Ramachandran S,** Jain PK, Prasanna A, Verma SK (2007) Chromium(VI) and Zinc(II) regulated protein expression in the cyanobacterium *Nostoc calcicola*. ASM 107th General Meeting, May 21 – 25, Toronto, Canada.

4. **Ramachandran S,** Jain PK, Verma SK (2007) Genetically engineered *Nostoc calcicola* immobilized on silica coated calcium alginate and silica gel for applications in heavy metal bioaccumulation. ASM 107th General Meeting, May 21 – 25, Toronto, Canada.

5. **Ramachandran S**, Jain PK, Verma SK (2006) Chromium(VI) induced damage to the cytoskeleton and mutagenesis in *Nostoc calcicola*, International Conference on Toxicology, Toxicogenomics and Occupational Health (ICTTOH), Oct 9 – 11, Jiwaji University, Gwalior. p. 30

6. Jain PK, **Ramachandran S** and Verma SK (2006) Cytotoxic and Genotoxic Properties of Chromium(VI) in the Cyanobacterium *Nostoc calcicola,* International Conference on Applied Phycology" Algae in Biotechnology and Environment", Feb 14 – 15, University of Delhi. p. 258

7. **Ramachandran S,** Jain PK and Verma SK (2006) Synthesis and characterization of silica gel and silica coated calcium alginate matrices for biotechnological applications, International Conference on Applied Phycology"Algae in Biotechnology and Environment", Feb 14 – 15, University of Delhi. pp. 97 – 98

8. Verma SK, Jain PK and **Ramachandran S** (2006) Biosorption Technology for Removal of Co^{2+}, Cu^{2+}, Zn^{2+} and Sr^{2+}, Proceedings of National Conference on Environmental Conservation, Sep 1 – 3, BITS-Pilani. pp. 543 – 550

9. **Ramachandran S**, Jain PK, Sarma MVRK and Verma SK (2005) Characterization of Silica Coated Calcium Alginate and Silica Gel for Applications in Heavy Metal Bioremediation, Proceedings of National Conference on "Sustainable Development of Environment for Future Generation", KCT, Coimbatore. pp. 131 – 140

10. **Ramachandran S,** Jain PK and Verma SK (2006) Metal biosorption by silica gel immobilized *Nostoc calcicola*. All India Seminar on Advances in Botanical, Biotechnological and Microbiological Research in India (AISBBMR), University of Bikaner, Bikaner, Rajasthan, India. p. 43

11. **Ramachandran S,** Mukund T, Bharagavi M, Nirmala H, Scaria J, Verma SK (2005) A reporter gene based whole cell bacterial biosensor for the detection of tetracycline. International Symposium on Recent advances in drug design & drug delivery systems. Feb 26 – 27, BITS-Pilani. pp. 96 – 97

12. **Ramachandran S,** Sarma MVRK, Kokila K and Verma SK (2005) Isolation of His-tagged Proteins using a novel immobilized metal affinity chromatography (IMAC). International Symposium on Recent advances in drug design & drug delivery systems. Feb 26 – 27, BITS-Pilani. pp. 94 – 95

13. **Ramachandran S**, Sarma MVRK, Coradin T and Verma SK (2004) Use of biocompatible silica gel immobilized cyanobacterial cells for biotechnological applications. National Symposium on Recent Trends in Algal Biology and Biotechnology, Feb 4 – 5, Punjabi University. p. 83

14. **Ramachandran S,** Singh H, Verma SK (2003) Role of extracellular proteins in bioremediation of heavy metals/radionuclides. International Symposium on Emerging Trends in Genomics and Proteomics, Education and Research. Jan 12 – 13, Birla Institute of Technology and Science, Pilani. p. 37

Appendix II

Biography of Prof. S.K Verma

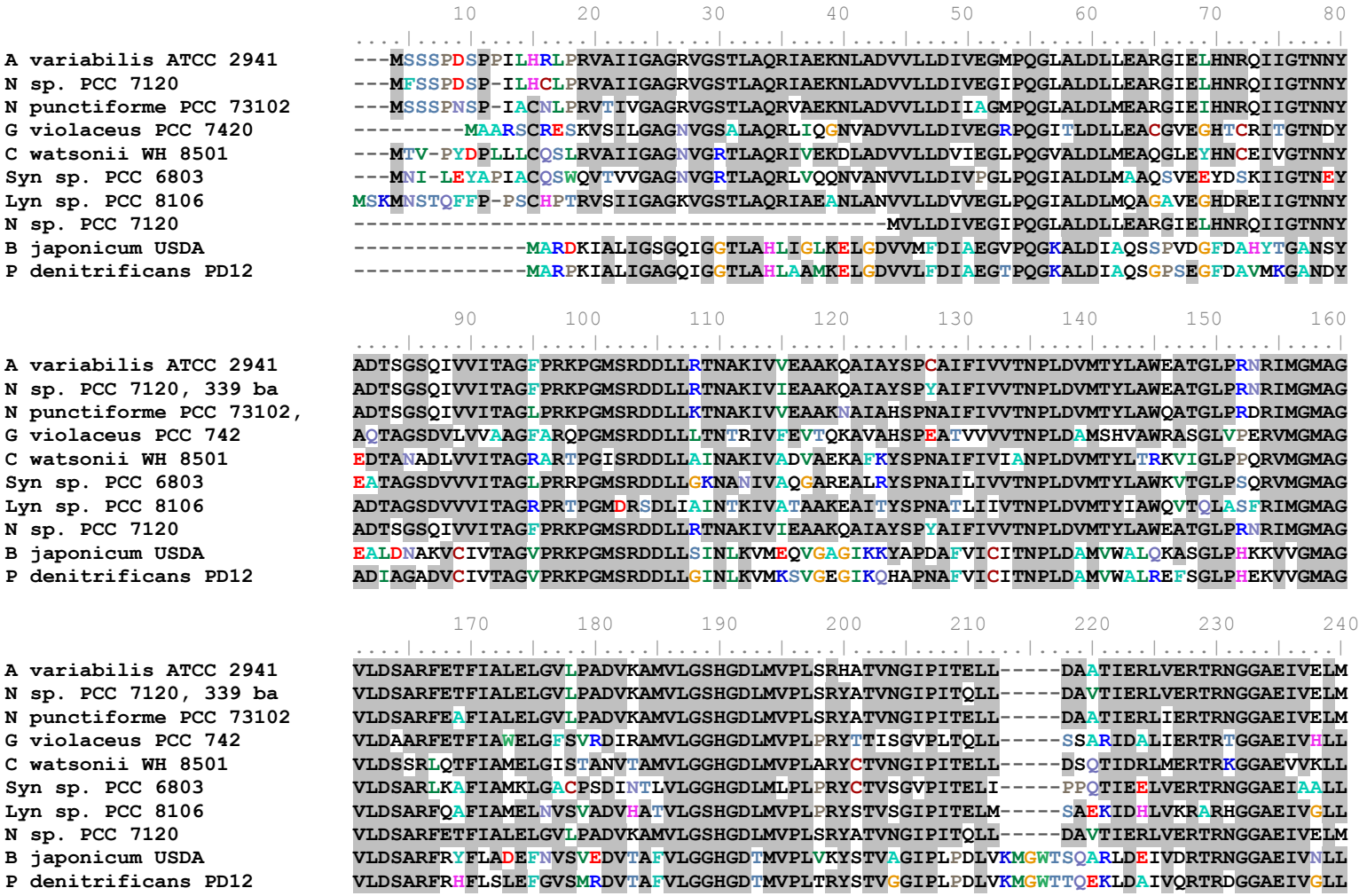
Prof. Sanjay Kumar Verma has obtained master's degree in Genetics from Banaras Hindu University (BHU), Varanasi with specialization in cyanobacteria and applied phycology before completing Ph.D in the area of Environmental Biotechnology from the same university. He worked as post-doctoral research fellow at University of Hyderabad in the area of microbial and molecular genetics. Prof. Verma has handled several research projects related to bioremediation and biodegradation of toxic industrial waste, development of recombinant bacterial biosensors for environmental applications, funded by Bhabha Atomic Research Centre (BARC), University Grants Commission (UGC) and Department of Science & Technology (DST). Currently he is working as a Faculty, Biological sciences group and Chief Warden, Birla Institute of Technology & Science, Pilani. His major research interest lies in biochemistry of cyanobacteria, environmental biotechnology and molecular biology.

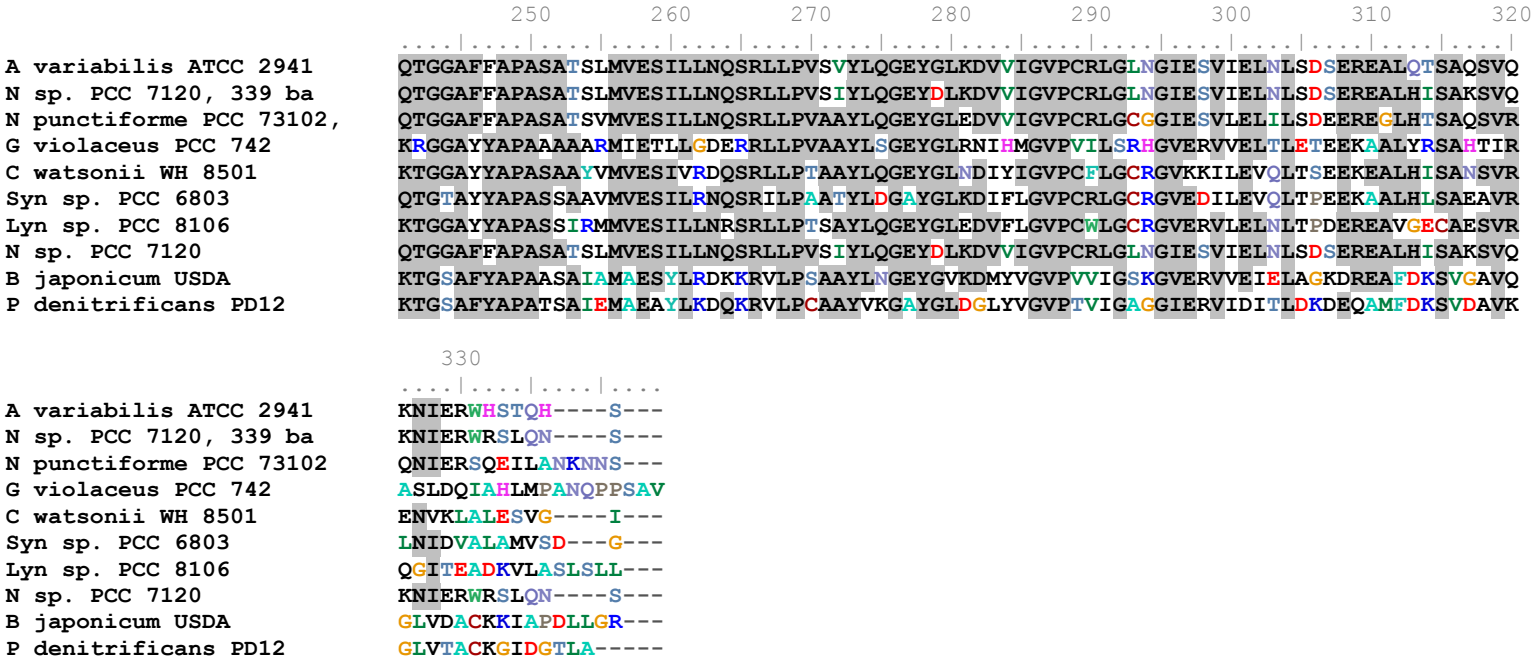
Biography of Mr. S. Ramachandran

Mr. Ramachandran completed M.Sc. Biochemistry from MIET Arts College, Bharathidasan University and M.E. Biotechnology from Birla Institute of Technology and Science, Pilani. He has continued for Ph.D programme of this institute from 2002-2007. He was a recipient of Council Scientific and Industrial Research (CSIR) senior research fellowship. Ramachandran has an active interest in environmental biotechnology and proteomics. He has also been involved in teaching various courses of Biological sciences group, BITS-Pilani. He has published research articles in renowned International journals and presented papers in various National and International conferences.

Appendix-III

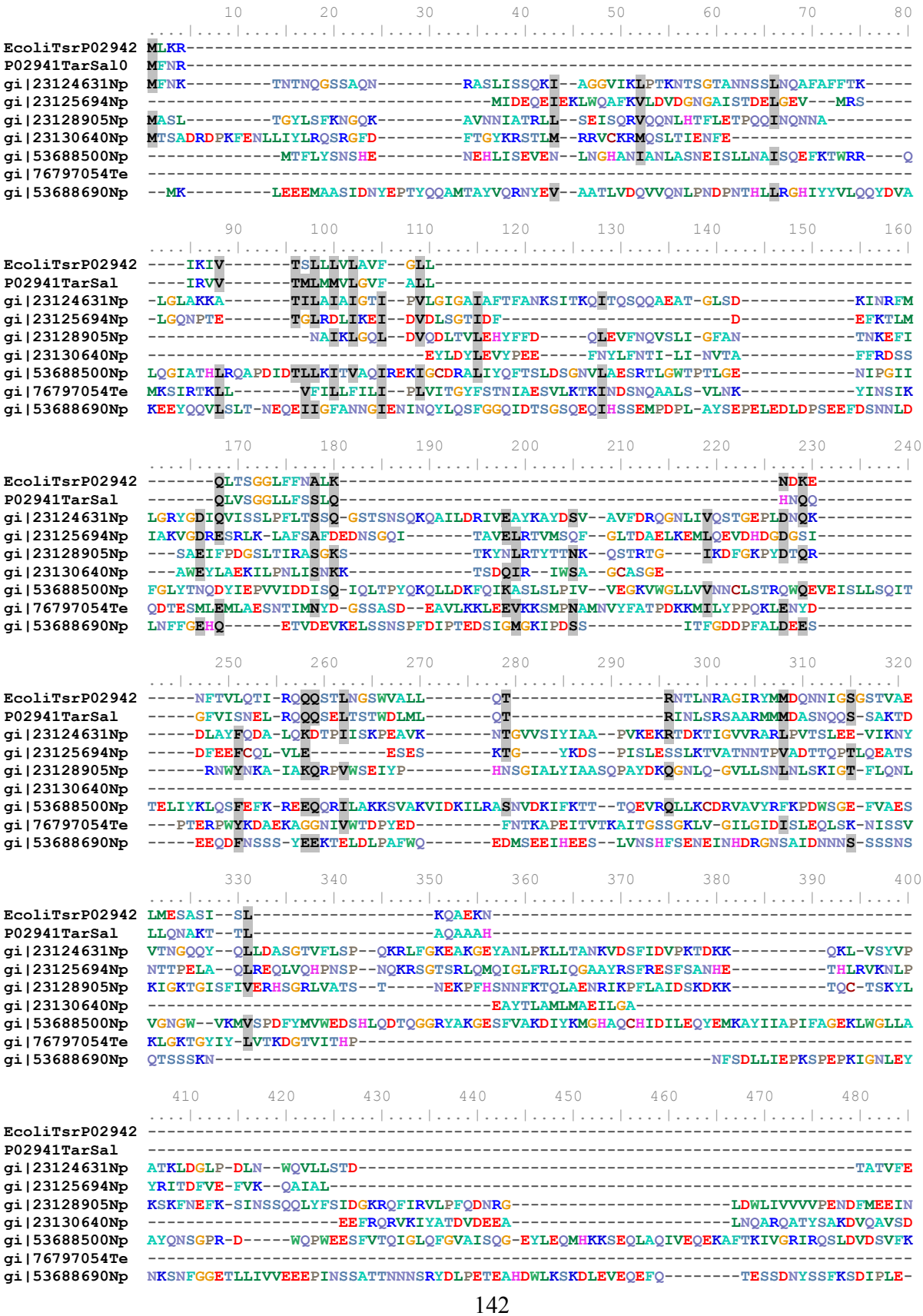
Multiple Sequence Alignment of the protein malate dehydrogenase with cyanobacterial proteins

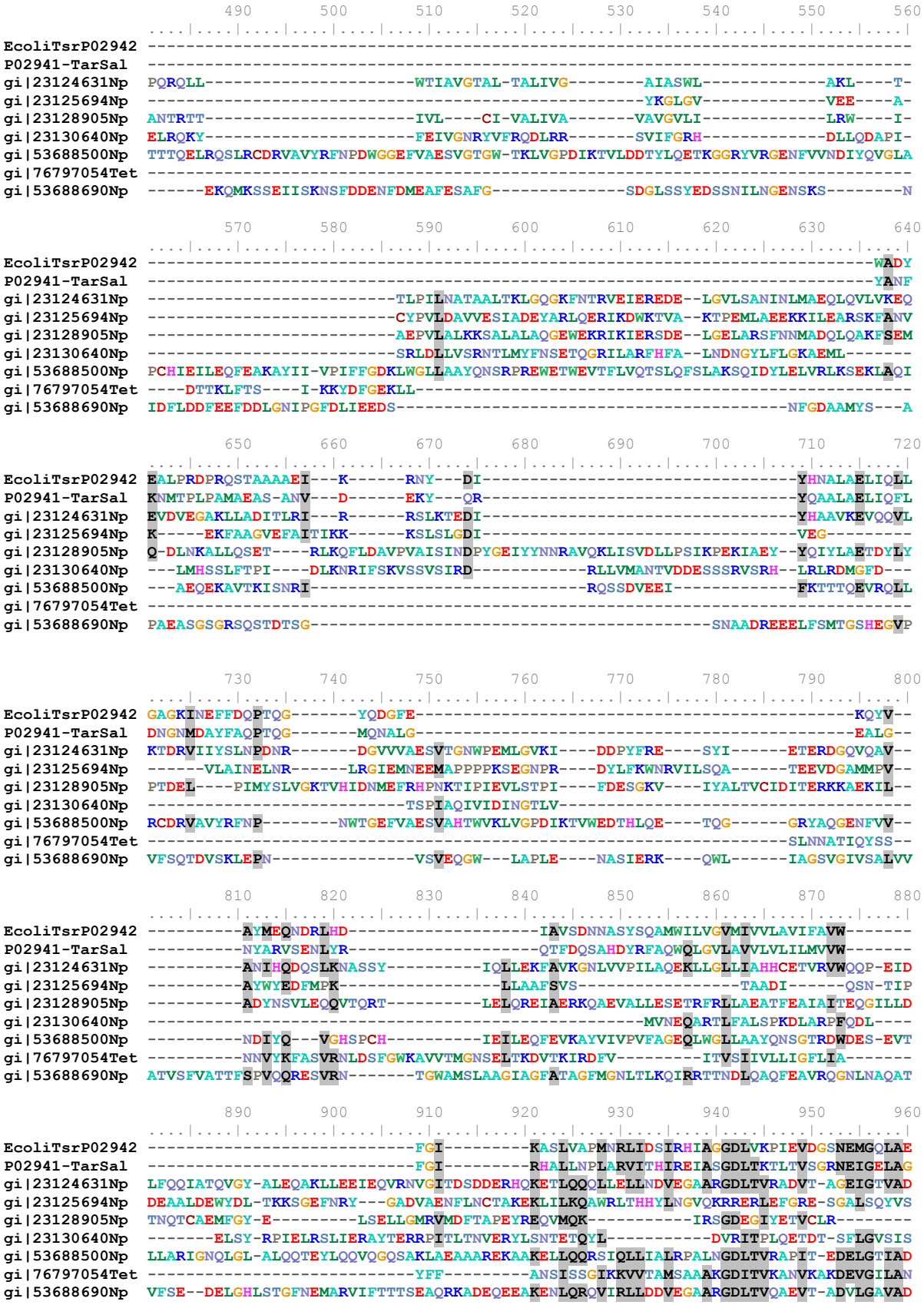


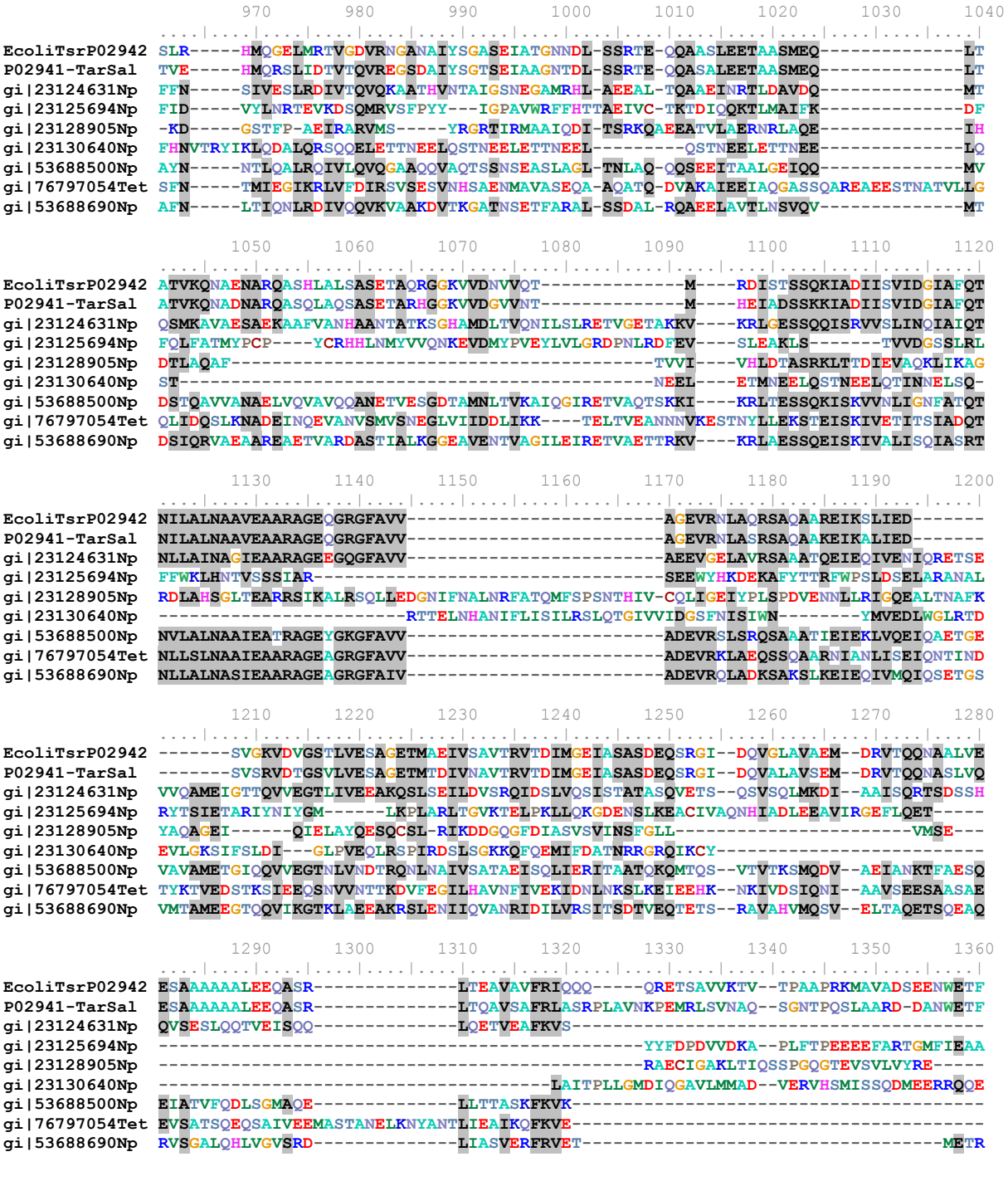

250 260 270 280 290 300 310 320
A variabilis ATCC 2941 QTGGAFFAPASATSLMVESILLNQSRLLPVSVYLQGEYGLKDVVIGVPCRLGLNGIESVIELNLSDSEREALQTSAQSVQ
N sp. PCC 7120, 339 ba QTGGAFFAPASATSLMVESILLNQSRLLPVSIYLQGEYDLKDVVIGVPCRLGLNGIESVIELNLSDSEREALHISAKSVQ
N punctiforme PCC 73102, QTGGAFFAPASATSVMVESILLNQSRLLPVAAYLQGEYGLEDVVIGVPCRLGCGGIESVLELILSDEEREGLHTSAQSVR
G violaceus PCC 742 KRGGAYYAPAAAAARMIETLLGDERRLLPVAAYLSGEYGLRNIHMGVPVILSRHGVERVVELTLETEEKAALYRSAHTIR
C watsonii WH 8501 KTGGAYYAPASAAYVMVESIVRDQSRLLPTAAYLQGEYGLNDIYIGVPCFLGCRGVKKILEVQLTSEEKEALHISANSVR
Syn sp. PCC 6803 QTGTAYYAPASSAAVMVESILRNQSRILPAATYLDGAYGLKDIFLGVPCRLGCRGVEDILEVQLTPEEKAALHLSAEAVR
Lyn sp. PCC 8106 KTGGAYYAPASSIRMMVESILLNRSRLLPTSAYLQGEYGLEDVFLGVPCWLGCRGVERVLELNLTPDEREAVGECAESVR
N sp. PCC 7120 QTGGAFFAPASATSLMVESILLNQSRLLPVSIYLQGEYDLKDVVIGVPCRLGLNGIESVIELNLSDSEREALHISAKSVQ
B japonicum USDA KTGSAFYAPAASAIAMAESYLRDKKRVLPSAAYLNGEYGVKDMYVGVPVVIGSKGVERVVEIELAGKDREAFDKSVGAVQ
P denitrificans PD12 KTGSAFYAPATSAIEMAEAYLKDQKRVLPCAAYVKGAYGLDGLYVGVPTVIGAGGIERVIDITLDKDEQAMFDKSVDAVK
330
A variabilis ATCC 2941 KNIERWHSTQH----S---
N sp. PCC 7120, 339 ba KNIERWRSLQN----S---
N punctiforme PCC 73102 QNIERSQEILANKNNS---
G violaceus PCC 742 ASLDQIAHLMPANQPPSAV
C watsonii WH 8501 ENVKLALESVG----I---
Syn sp. PCC 6803 LNIDVALAMVSD---G---
Lyn sp. PCC 8106 QGITEADKVLASLSLL---
N sp. PCC 7120 KNIERWRSLQN----S---
B japonicum USDA GLVDACKKIAPDLLGR---
P denitrificans PD12 GLVTACKGIDGTLA-----

Appendix-IV

Multiple Sequence Alignment of the protein Histidine kinase with cyanobacterial proteins



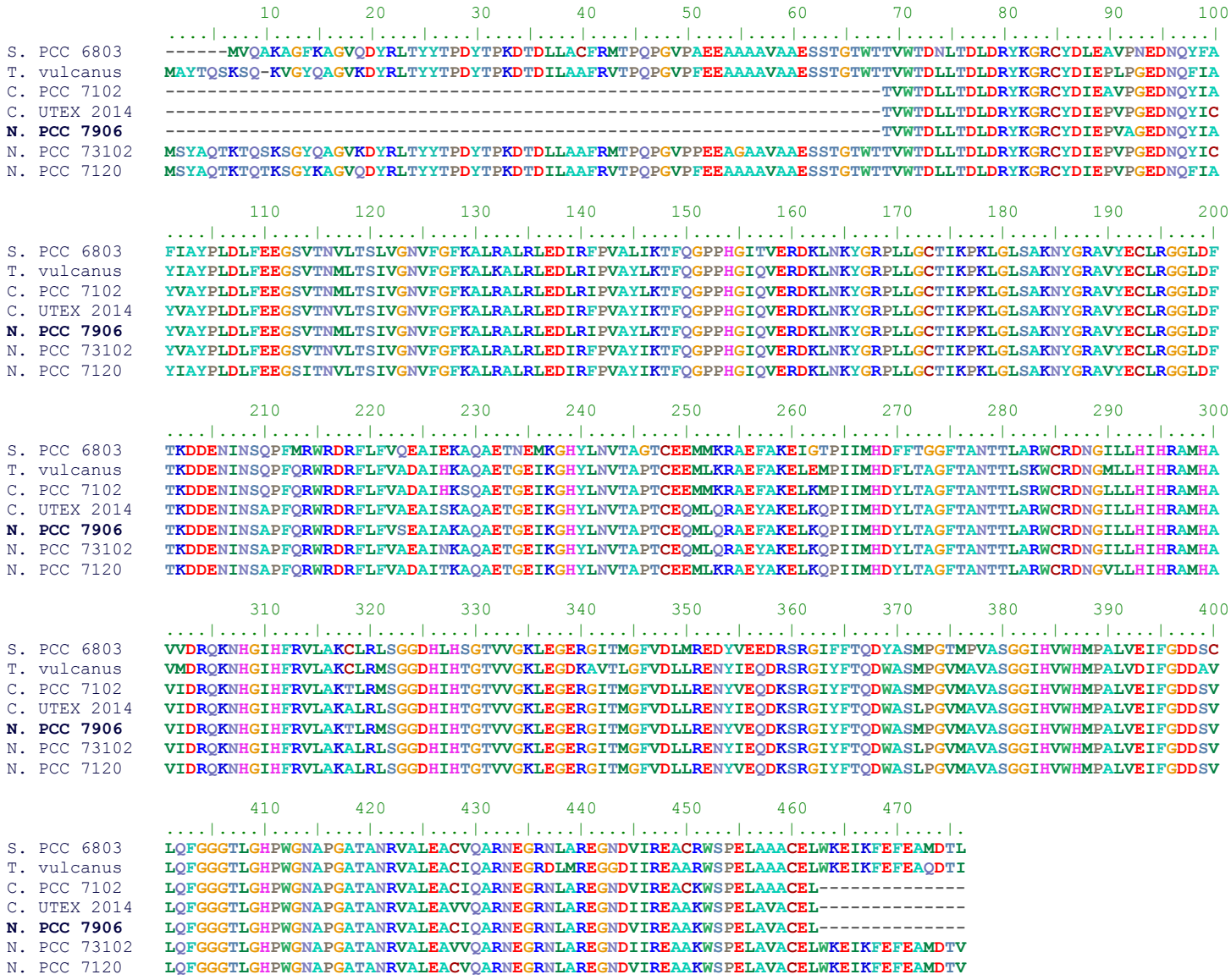

490 500 510 520 530 540 550 560
EcoliTsrP02942
P02941-TarSal
gi|23124631Np
gi|23125694Np
gi|23128905Np
gi|23130640Np
gi|53688500Np
gi|76797054Tet
gi|53688690Np
570 580 590 600 610 620 630 640
650 660 670 680 690 700 710 720
730 740 750 760 770 780 790 800
810 820 830 840 850 860 870 880
890 900 910 920 930 940 950 960



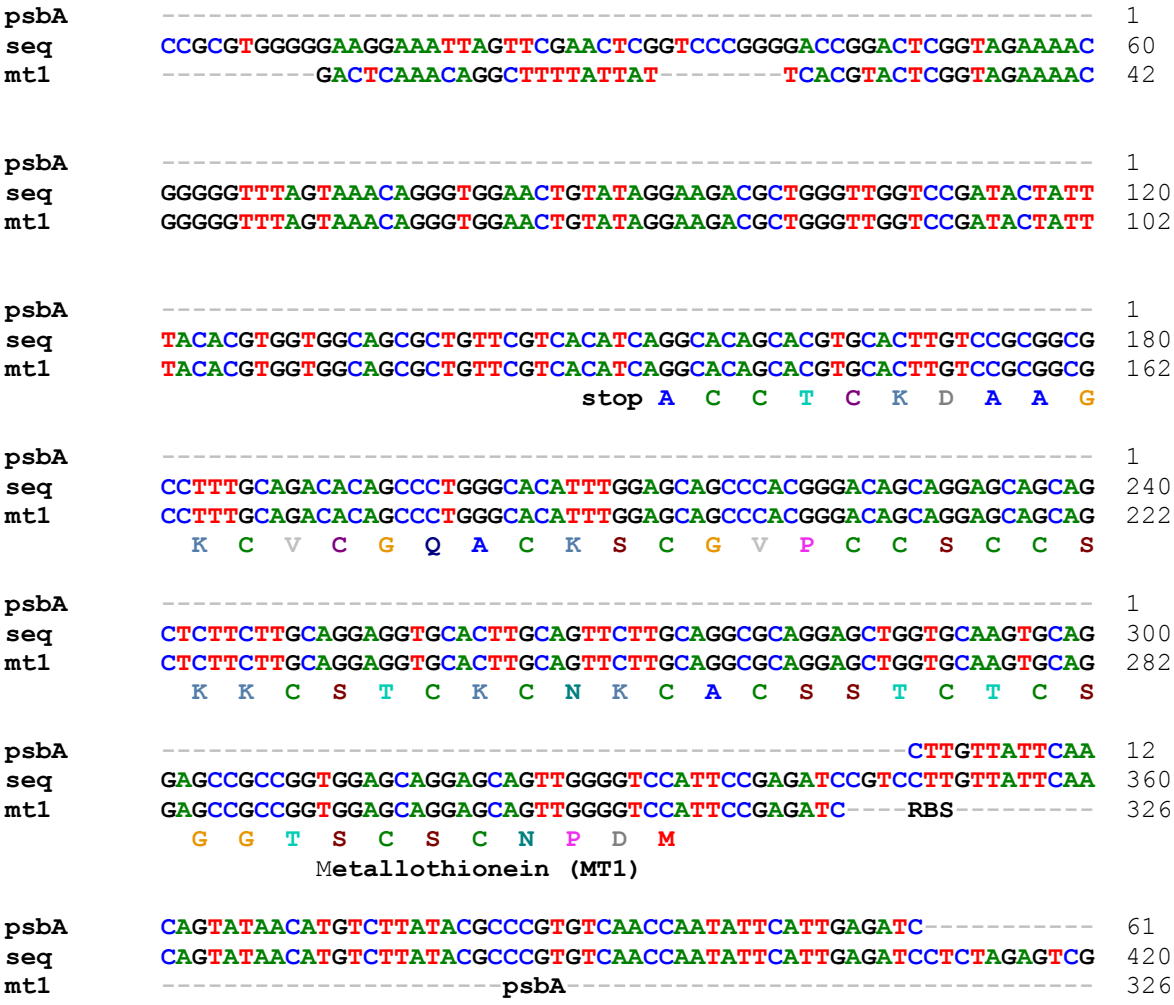
MCP-II of (1) *E. coli*-TsrP02942;(2) P02941-TarSal, *S. typhimurium*; (3 - 9) gi|23124631Np, gi|23125694Np, gi|23128905Np, gi|23130640Np, gi|53688500Np, gi|76797054Tet, gi|53688690Np, *Nostoc punctiforme* PCC 73102

Appendix-V

Multiple Sequence Alignment of the protein RubisCO with cyanobacterial proteins



Appendix: VI



Nucleotide sequence of psbA-mMT in pRSKV12. Seq - Nucleotide sequence of the psbA-mMT* construct present in pRSKV12. **psbA -** psbA promoter from *Amaranthus hybridus* (NCBI Acc. No. S67731). **MT1 -** Mouse metallothionein sequence (MT1)(NCBI Acc. No.NM_013602). *The psbA-mMT fragment flaking with PstI/BamHI from pRL-MT was cloned into pUC19; DNA sequencing was done using M13 universal primer on ABI Prism Automated DNA sequencer.