Chapter 3

Metal Resistance and Tolerance
The utility of strain improvement for metal resistance arises because of the possible suppression of the rate limiting steps within the metabolic pathways mediating, the uptake of metals in cyanobacteria or due to production of specific proteins or enzymes that have evolved having no function other than to protect the organism. Strain improvement for metal resistance can be generally classified as any scientific technique that allow the isolation of cultures exhibiting a desired phenotype. Most commonly, the ability of the strain to exhibit increased product formation is the desired phenotype. However the spectrum of improvements can include other traits such as elimination of toxic metabolites or ability to degrade waste materials.

Classical mutagenesis and screening can offer significant advantage over genetic engineering approaches by yielding gains with minimal start up time and sustaining such gains despite lack of detailed knowledge concerning the physiology of the microorganism. This empirical approach has a long history of success.

In metal uptake phenomena, development of metal resistance in cyanobacteria arises due to alteration in rate limiting steps of various metabolic pathways. In response to metal stress, cyanobacteria after prolonged period of incubation extending up to three months develop resistance and depends on the concentration and type of metal ion taken for treatment. Of the whole microbial cells in culture, initially a few cells develop resistance which can be visualised by the greening of a few cells of the microbial biomass in *Aulosira fertilissima* which is
usually dark brown in color. Subsequent to greening of few cells and due to the disintegration of the wild type cells, the resistant cells could colonise the culture media in due course of time.

The observation of metal resistance and in cyanobacteria and the underlying mechanism can be explained in dictating terms of molecular biology. The major question in the study of heavy metal resistance is whether specific detoxifying mechanism/proteins/enzymes/pathways have evolved which have no other function other than to protect the organism under stressed conditions. Cyanobacteria require a balance between uptake of sufficient metal ions to maintain growth and the ability to protect sensitive cellular activity from excessive concentration of essential and nonessential metals. Evolution has fashioned many proteins either to have rigid binding sites which accept some ions while rejecting others or to have flexible binding sites in which the stereochemistry of the ion determines the final shape of the proteins. Evidence is now emerging that metal homeostasis and metal tolerance are programmed and metal binding protein and metal regulated gene expression play in this process. Some heavy metals are essential micronutrients acting as prosthetic groups in a wide range of enzymes, but like all of the other metals, at elevated concentration reduce microbial growth or in the extreme totally inhibit growth. The effect of various heavy metals vary on growth and evolution of nitrite because of the chemical characteristics of each metal.

Metal tolerance in cyanobacteria in the present study indicates more than one mechanism mentioned above defence. In essence there
are two main strategies. Evidence maintains a low intracellular concentration, either by preventing metal ions from entering cyanobacteria or by reduced uptake or active efflux. In addition sequestration allows high intracellular concentration to be tolerated, by binding of metal to ligands, thus separating the ion from sensitive cellular metabolism. Metal binding ligands like, metallothionein, phytochelatin, siderophores etc have been the focus of attention of most research.

Metallothionein are cysteine rich metal binding gene coded protein first isolated from renal equine source (Thiele 1992). The counter parts of Metallothionein in plant kingdom is phytochelatin or structural name poly gamma-glutamyl cysteiny1 glycine and are not gene encoded, their synthesis is from glutathione by a specific enzyme by a specific enzyme glutamyl cysteine dipeptidyl transferase commonly known as phytochelatin synthase. (Loeffller et al 1989, Zenk 1996).

\[
\text{Glutathione} + \text{Glutathione} \rightarrow \text{Phytochelatin} + \text{glycine}
\]

Or

\[
\text{Phytochelatin}
\]

The mechanism proposed for phytochelatin mediated detoxification is the transport of phytochelatin bound with metal to vacuoles where they are sequestered. But cyanobacteria being prokaryotes without vacuoles phytochelatin mediated heavy metal detoxification does not work at the molecular level.
Upon exposure of cells to heavy metals phytochelatin are synthesised and glutathione level drops immediately (Scheller et al 1987). Recent studies have shown that thioredoxin and thioredoxin like genes can confer resistance against heavy metals in microorganisms (Garbin et al 1996). Thioredoxins are small protein found in many organisms from bacteria to humans. They are characterised by highly reactive disulphide of conserved Cys-X-X-Cys active site. (Holmgren 1985, Jacquot et al 1997). Upon illumination of photosynthetic electron transfer chain generates reduced ferredoxin which transfer its electrons to many acceptors including thioredoxin via ferredoxin-thioredoxin reductase. Reduced thioredoxin in turn are able to reduce several key enzymes of carbon metabolism (Jacquot et al 1997). Several studies have suggested that thioredoxin could be involved in the response of oxidative stress but also in cell division cycle (Regad 1996).

Cyanobacteria subjected to heavy metals are prone to oxidative stress as metals can participate in metal catalysed Fenton type reaction with superoxide or peroxide molecules to generate highly toxic hydroxyl radicals. A similar mechanism was reported by Stadtman (1993) for metal catalysed reaction.

Present studies have revealed that cyanobacteria could negate heavy metal toxicity on prolonged incubation with metals irrespective of the metal used within a concentration range of 20-200 ppm. The different concentration of metals that could be tolerated by cyanobacteria is revealed in dictating terms of growth by chlorophyll estimation.

Table 3.1 shows that the concentration within which a few resistant cells could be found initially after a treatment period of
30 days. These resistant cells subsequently showed as much growth rate as control cells. However no development of further resistance was noticed on exposing the cells again to still higher concentrations of heavy metals than that can be tolerated. This gives a clear indication that their exist some inbuilt programmed mechanism to limit the tolerance level of stressors in both cyanobacteria studied. Masigocladium being thermophilic has natural defence mechanism against high temperature up to 75°C. But the resistance against the stressor temperature could not mount resistance against metal toxicity. Further we observed that resistance against one metal was not enough to develop resistance against another metal.

**Table 3.1: Maximum concentration limit for development of resistance**

<table>
<thead>
<tr>
<th></th>
<th>Copper</th>
<th>Cadmium</th>
<th>Nickel</th>
<th>Chromium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fertilissima</em></td>
<td>50ppm</td>
<td>50ppm</td>
<td>20ppm</td>
<td>200ppm</td>
</tr>
<tr>
<td><em>M. laminosus</em></td>
<td>20ppm</td>
<td>50ppm</td>
<td>20ppm</td>
<td>200ppm</td>
</tr>
</tbody>
</table>

Co-tolerance of metals were noticed where tolerance to one or more metal in the environment, albeit at a lower concentration suggested are given in table 3.2. From the review of literature and from the results of our experimentation on growth of cyanobacteria with different heavy metals, it is noted that metal tolerance mechanism operates in a programmed manner. The possible operation of metallothioneine and phytochelatin mode of defence is totally absent in cyanobacteria as their exist no vacuole for further sequestration of heavy metals. The possible explanation for the observation of tolerance are thioredoxin defence are discussed in the beginning of the chapter, induction of stress proteins, synthesis of osmoprotective compounds like glycine, betaine, mannitol etc.
Table 3.2: Co-tolerance of metals

<table>
<thead>
<tr>
<th></th>
<th>Cu and Cd</th>
<th>Cu and Ni</th>
<th>Cu and Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M.laminosus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A.fertilissima</em></td>
<td>Cd and Ni</td>
<td>Cd and Cu</td>
<td>Cr and Ni</td>
</tr>
</tbody>
</table>

Although the molecular basis of the mechanism involved in cyanobacterial metal tolerance depend heavily on cyanobacterial osmoprotective compounds and stress proteins. Some further probable operation of stress evading mechanism have emerged from the present study. Among these probable mechanisms are (1) efflux of heavy metal ions and prevention of intracellular metal ion accumulation. (2) positively charged chemical entities on the surface of bacteria which repel metal ions and (3) ion channel blockage is induced by metal ions resulting in defective uptake.

Resistance to metal can be coded by genes which, like antibiotic resistance can be found on transposable elements and can also seen be on conjugative plasmids. The metal resistance genes are of the type found in plasmids and prevent the uptake of metals due to impermiability. From the point of view of cyanobacteria, this is the safest method of preventing damage. The inability of *Mastigocladus* to uptake heavy metals as revealed by uptake kinetic studies discussed in the following chapter clearly points to the operation of genetic mechanism through synthesis of compounds in developing resistance, even before the exposure of bacteria to heavy metals. The development of tolerance to heavy metals by *Mastigocladus* on prolonged treatment further authenticates this fact.