Research Paper

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Exploration of A Metallothionein in Cyanobacteria and Bacteria for Chromium Remediation From Industrial Wastes

Microbiology

KEYWORDS : Microorganisms, Chromium, heavy metal binding, bioremediation, metallothionein

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ABSTRACT

Environment pollution by heavy metals which are released into the environment through various anthropogenic activities is one of the world's major environmental problem. The presence of heavy metals like Chromium (Cr) in cyanobacteria isolated from water sample of Wetland Ecopark, Bantala and bacteria isolated from soil of coal mine areas was confirmed by Atomic Absorption Spectroscopy (AAS) and Chromate reductase assay. After incubation with cyanobacteria the chromium content of water was decreased as observed from the results of AAS. Chromate reductase assay showed the increased conversion of Cr (VI) to Cr (III), the less toxic form by the isolated soil bacteria. In response to chromium stress the Nitric Oxide Synthase (NOS) activity increased than control at 10mg/l--25mg/l and decreased beyond 50mg/l in case of cyanobacteria. NOS activity was also affected in case of soil bacteria for Cr (VI). Upon metal stress thiol containing proteins are activated and hence the expression of metallothionein has been observed in case of soil bacteria and cyanobacteria with transition metal Cr. SDS PAGE followed by Western Blot analysis confirms the presence of metallothionein in bacteria with Cr treatment by a single band near 14 KDa protein marker. Morphological changes due to treatment were studied by Scanning Electron Microscopy (SEM) in cyanobacteria. FTIR study reveals the interaction of metals with cell surface components both in cyanobacteria and bacteria. Here is a comparative studies to achieve more useful and active immobilized metallothionein to remove Cr from waste water.

Introduction:

Environment pollution by heavy metals which are released into the environment through various anthropogenic activities is one of the world's major environmental problem. Among these heavy metals chromium causes carcinogenic, genotoxic and immunotoxic effects on humans and animals [1]. Conventional methods used for removal of hexavalent chromium is chemical procedures which are expensive and lack specificity. Biological approaches using microorganisms provides a potential for a highly selective method of removal of toxic metals coupled with considerable operational flexibility. Cyanobacteria is the largest and most diverse group of photosynthetic prokaryotes. Studies have confirmed that cyanobacterium both adsorbs and take up metal ions [2]. An important resistance mechanism may be production of metallothioneins (MTs). Certain soil bacteria possess metallothionein to bind toxic heavy metals [3]. Utilising cyanobacteria and bacteria provides an alternative means to clean up environment without creating harmful wastes.

MTs are low molecular mass proteins (14 KDa) found in animal kingdom with unique abundance of Cysteine residues.The main function of MTs in organism is metal ion transport, maintenance of the oxidative-reducing conditions and expression regulation. Thiols including MT and glutathione are known as effective free radical scavengers creating optimal oxidative-reducing conditions to protect cell compartments and biologically important compounds including cell-cycle-enzymes or DNA. MT regulates free radical level also indirectly by binding of metal ions which are potential radical producers, e.g. Cu. As confirmed by several studies, MT expression in cells is induced also by superoxide and hydroxyl radicals generated by gamma-radiation.

Historically, MTs were ordered in three classes.Class I comprising all proteinaceous MT with locations of Cysteine closely related to those in mammals. Some molluscs and crustacean MT belong to this class.Class II including proteinaceous MT lacks this close similarity to mammalian MTs.Class III consisting of non- proteinaceous MTs in to which some authors included plant heavy metal-binding peptides called phytochelatins. Bacterial MTs belong to class II.

Uptake and adsorption of metals takes place through gelatinous sheath of Cyanobacteria. It can efflux of heavy metals from cytoplasm. Complexation of heavy metal ions inside the cell occurs by various substances, for example organic acids, amino acids, ferritins, phytochelatins, heat shock protein and metallothioneins. Biochemical stress defence responses such as the induction of oxidative enzymes takes place in cyanobacteria.

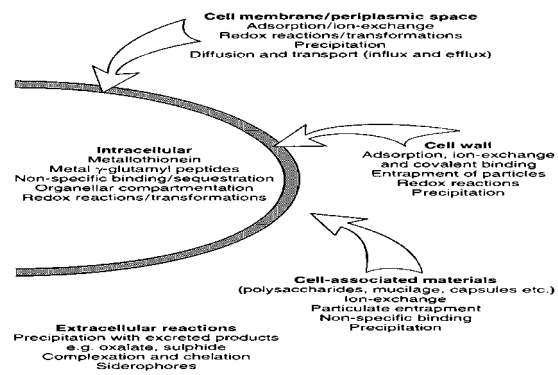

Fig. 1 Processes contributing to microbial uptake and detoxification of toxic metals

The main objective was to study the survival strategies of the microorganisms under experimentation by various methods under heavy metal stress.

Methods and Materials:

Collection of samples: Samples were collected from the soil in Raniganj coal mining area and water from Wetland Ecopark, Bantala.

Isolation of the bacteria and cyanobacteria:

Bacterial cultures were isolated from a pristine soil sample from Raniganj coal mining area, West Bengal, India ,using the standard dilution plate technique. Active charcoal was used as the sole carbon source in minimal media for isolation.

Cyanobacterial consortium isolated from water sample of Wetland Ecopark, Bantala was grown in BG-11 medium for 14 to 21 days in the laboratory under optimal conditions.

Microscopic study:

Bright field microscopy:Gram staining [4]of the isolated bacteria was done and observed under bright field microscope. Cyanobacterial consortium stained with lactophenol and cotton blue was viewed under bright field microscope.

***Phase contrast microscopy*:** It was performed to determine the motile nature of the bacteria.

***Scanning Electron Microscopy*:** Morphological details of cyanobacteria was studied by SEM. For this purpose, cells were suspended in 3% Glutaraldehyde in 0.1M cacodylate buffer(pH-7.2). The SEM was performed [5] with these samples.

Bioremediation of Cr (VI) by Chromate Reductase assay and Atomic Absorption Spectroscopy (AAS):

Chromium reductase activity [6] of bacteria was ascertained to find its metal bioremediation property. For AAS, Chromium solution was prepared as 50ml stock solution (1gm /50ml) by adding 1gm of $K_2Cr_2O_7$.Algal cell was added in 25ml BG11 medium and Chromium concentration was maintained as 10mg/l, 25mg/l, 50mg/l, 75mg/l, 100mg/l, 200mg/l, 300mg/l.The final volume of heavy metal was maintained at 50ml by adding only medium. The pH of the medium was maintained at pH-5.5,7.5 and 9.5 respectively and AAS was done [7] at 356.9nm.

Estimation of total thiol content (Ellman's method):

For total thiol estimation [8] in both bacteria and cyanobacteria cell extract was prepared from both the samples and optical density (O.D) was taken at 420 nm immediately within 5 seconds.

Estimation of Nitric Oxide Synthase (NOS):

NOS activity [9] was determined and readings were taken at 430 nm.

Detection of adsorption of metal on bacterial cell surface by FTIR:

Cell pellets were collected for the bacterial and cyanobacterial cultures and were lyophilyzedin Hahntech (Korea) Freeze dryer. FTIR [10] was performedwith a thin uniform film of the lyophilised bacterial culture in the instrument having model number L1600300 spectrum Two FTIR Sl. No. 94372(Perkin Elmer, U.S.).

Extraction of MT from bacteria, SDS PAGE and Western blotting

For extraction of MT [11] from all the bacterial and cyanobacterial cultures, 500 µL of equivalent mixtures of dithiolthreitol (DTT), 10 mM/L, phenyl methane sulfonyl fluoride (PMSF), 5 µM/L, and glycine buffer, 5 M/L, at pH=8.5 were added to 0.5 g of wet weight of above samples (harvested at the end of log phases and centrifuged at 10000rpm for 10 min at 4 °C).Then were lysed in sonicator for 5 min(30 seconds of sonication with a gap of 30 seconds) and cooled on ice. The samples were centrifuged at 10000 rpm for 10 minutes. The sonicated sample was then further used for SDS PAGE [12]. 12% SDS PAGE was run using protein marker in both the cases.Western Blot of metallothionein[13] from soil bacteriaand cyanobacteria was done with Anti Metallothionein UC1MT (ab 12228) as primary antibody and anti-mouse AP tagged secondaryantibody.

Plasmid isolation from control bacterial culture showing increase in growth and their gel electrophoresis

To check the possible reason for the increase in growth of microorganisms the presence of plasmid was checked which might be helping the cells to resist the metal stressed environment [14]. Gel electrophoresis of the isolated plasmid was performed.

Results and Discussion:

Isolation of the bacteria and cyanobacteria:

Purified colonies were observed after first subculture of bacteria isolated from coal mine areas (Fig. 2).Cyanobacterial growth was observed in BG 11 medium after 14 to 21 days (Fig. 3).



Fig. 2 Growth of bacteria in minimal media using charcoal as sole carbon source

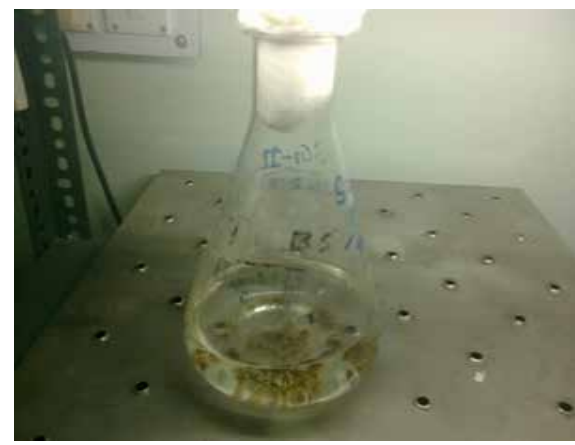

Fig. 3 Growth of Cyanobacteria in BG-11 medium

Microscopic study:

Gram negative coccus was observed under Bright field microscope (Fig. 4a and Fig. 4b). Non motility was confirmed under Phase Contrast Microscope (Fig. 4c). Fig. 5a and Fig. 5b were the Cyanobacterial consortium observed under the bright field microscope

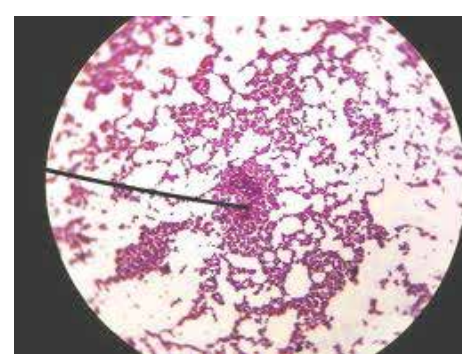

Fig. 4a Control bacteria

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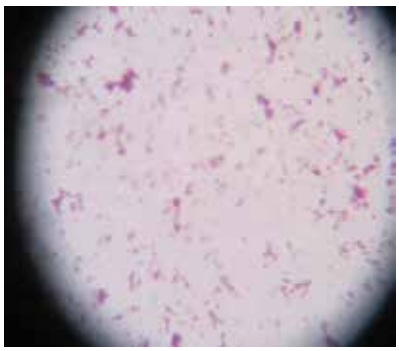

Fig. 4b Cr treated bacteria
Fig. 4a and Fig. 4b under bright field microscope

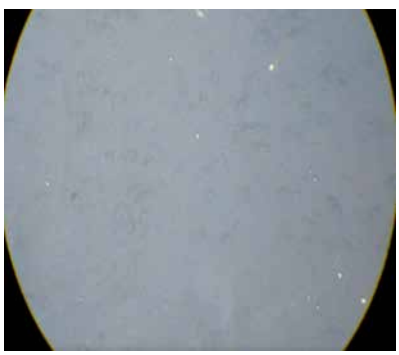

Fig. 4c Cr treated bacteria under phase contrast microscope

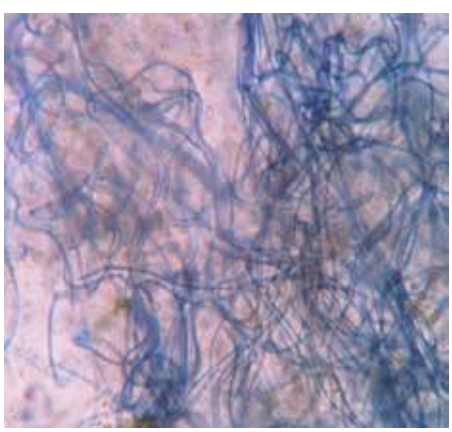

Fig. 5a *Lyngbya sp*. **under 40X**

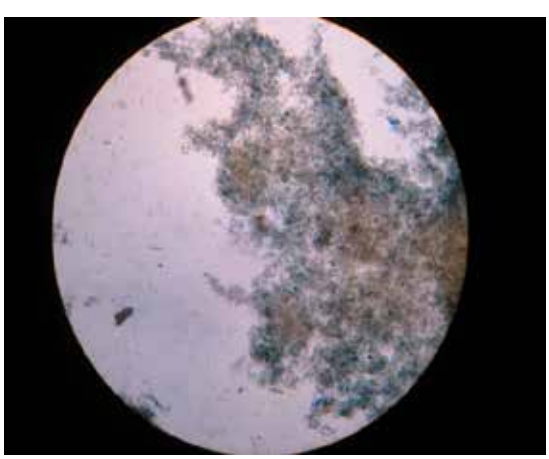

Fig. 5b *Microcystis sp*. **under 40X**

Bioremediation of Cr (VI) by Chromate Reductase assay and AAS:

Isolation of chromium reducing bacteria which are Cr (VI) resistant can be used for environmental clean-up and bioremediation of heavy metals contaminated industrial wastes by evaluating their Cr (VI) reducing ability. Chromium VI is harmful for the living system whereas chromium III is not, instead it acts as an essential micronutrient in humans. Chromate reductase reduces toxic Cr (VI) to non-toxic Cr (III).The chromium reductase assay showed 10 fold reduction in the Cr (VI) in the medium containing the isolated bacteria (Table 1).Graphical representation of the Cr uptake by isolated cyanobacteria at pH 5.5, 7.5 and 9.5 by AAS are shown in Fig. 6, Fig. 7 and Fig. 8 respectively. The Cr content of water decreased after incubation with cyanobacteria even up to pH 9.5.

Table 1 Fold reduction of the amount of Cr (VI) by the isolated bacteria

	Chromium remaining by resting cells			
	O.D.	µM	%	Fold reduction
Control	0.00	25	100	0
Experiment	0.05	2.5	10	10

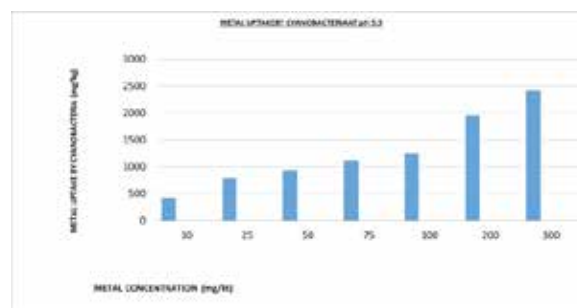

Fig. 6 Metal uptake by cyanobacteria at pH 5.5

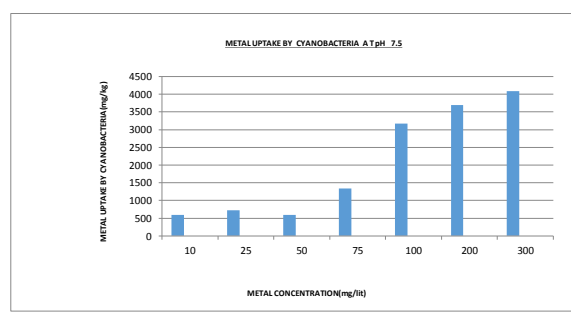

Fig. 7 Metal uptake by cyanobacteria at pH 7.5

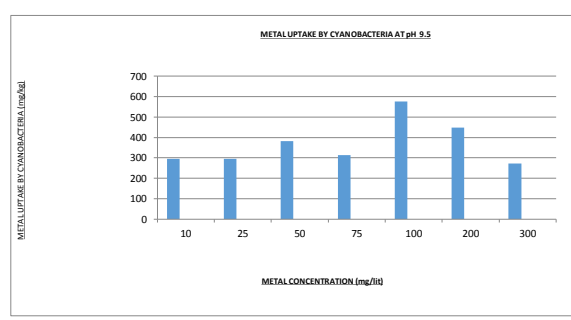

Fig. 8 Metal uptake by cyanobacteria at pH 9.5

Estimation of total thiol content (Ellman's method):

The thiol content in bacteria at 1g/l and 1.75g/l of Cr treatment

were found to be increased compared to control (Fig. 9). The thiol content in cyanobacteria were found to increase at 25 and 50 mg/l Cr treatment (Fig. 10). This indicated that metal stress increases thiol content in bacteria and cyanobacteria.

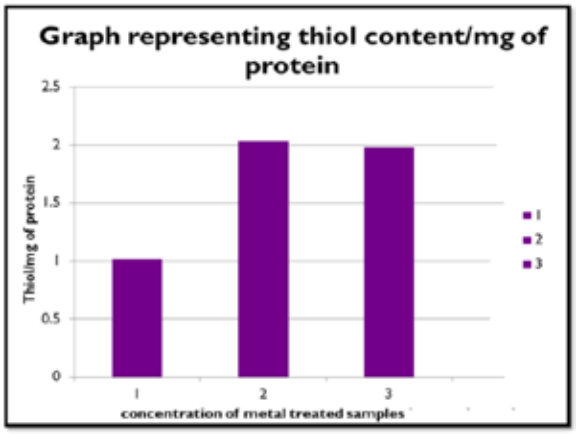

Fig. 9 Thiol content at 1g/l and 1.75g/l Cr treatment in isolated bacteria

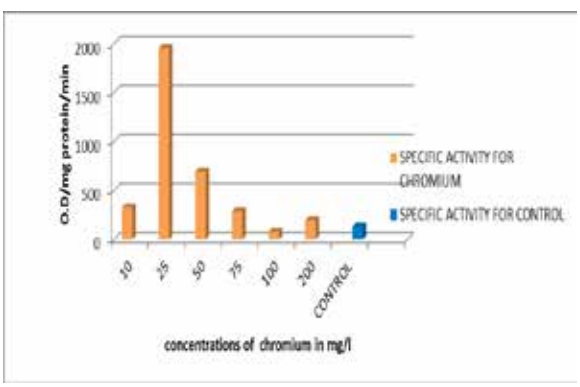

Fig. 10 Thiol content at various concentrations of Cr in cyanobacteria

Estimation of Nitric Oxide Synthase (NOS):

Bacterial NOS activity increased at 1 g/l and 1.75 g/l Cr (VI) compared to control (Fig. 11). Under metal stress the NOS activity increased than control at 10mg/l--25mg/l and decreased beyond 50mg/l in case of cyanobacteria (Fig. 12).

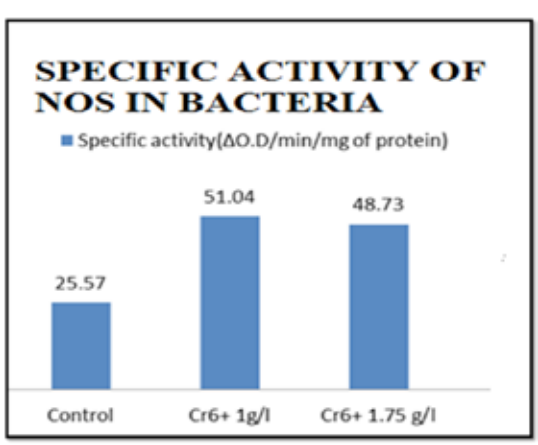

Fig. 11 NOS activity at various concentrations of Cr in bacteria

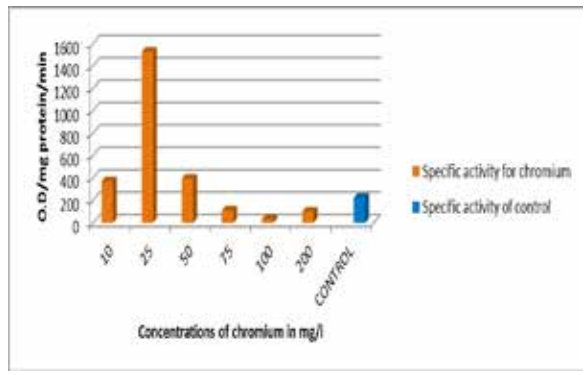

Fig. 12 NOS activity at various concentrations of Cr in cyanobacteria

Detection of adsorption of metal on bacterial cell surface by FTIR:

FTIR in both bacteria and cyanobacteria have shown shift of peaks in Cr (VI) treatment which indicated that Cr interacts with the cell surface components (Fig. 13, 14, 15 and 16).The trough at 1658.5 cm^{-1} in the control (metal untreated bacteria, Fig. 13) for (C=0 group) shifted towards 1633.75 cm^{-1} after binding Cr6+ when bacteria was treated with 1g/L Cr6+ (Fig. 14) (The IR spectrum of dried whole cell in cyanobacteria showed major peak at 1538 cm^{-1} in the control corresponding to carbonyl group (Fig. 15). Existence of carbonyl group is high in the membrane of the organism which is supposed to interact with the metal.At the conc. of 10mg/l of chromium (Fig. 16) treated sample the peak shifted towards 1562.91 cm^{-1} from 1538 cm^{-1}.

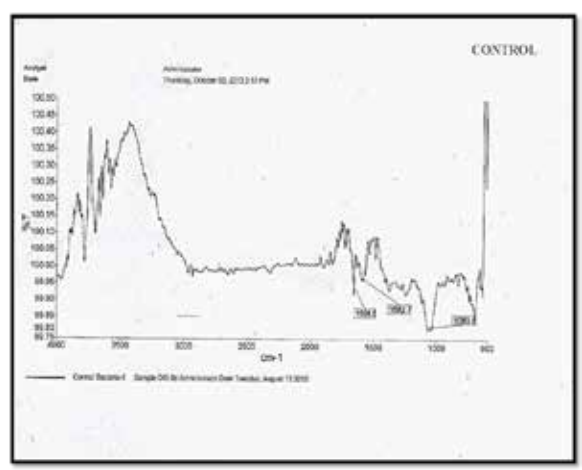

Fig. 13 FTIR of Control bacteria

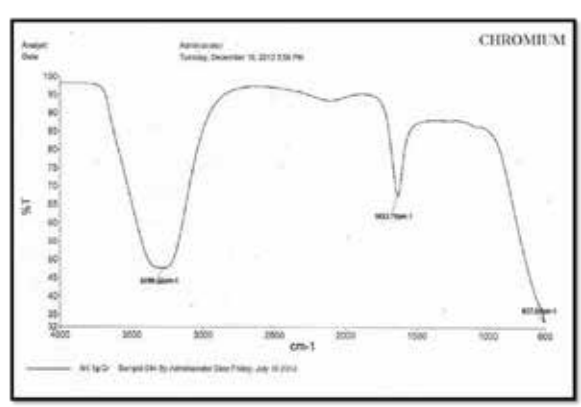

Fig. 14 FTIR of Cr (VI) treated bacteria

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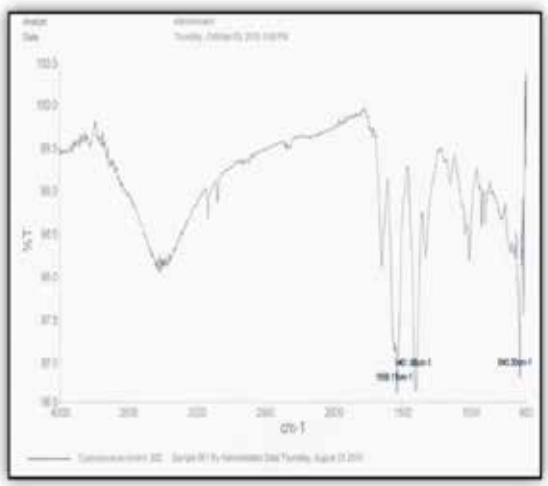


Fig. 15 FTIR of Control cyanobacteria

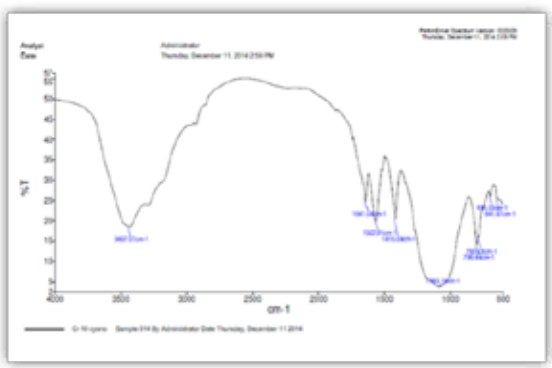
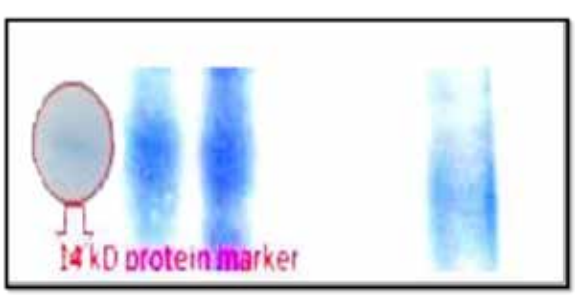


Fig. 16 FTIR of CR (VI) treated cyanobacteria

Analysis of MT from bacteria and cyanobacteria by SDS PAGE and Western blotting:
Prominent bands could be seen in the gel in case of bacteria, with respect to protein marker at 14 KD in Cr treated samples in SDS PAGE compared to control (Fig. 17).Since the protein reacted with anti metallothionein antibody, so bands were observed in Western Blot (Fig. 18), hence it was concluded that the sample contained metallothionein.

Since cyanobacteria can withstand high concentration of Cr (200 mg/l), whereas no considerable amount of MT was observed in SDS PAGE as well as Western Blot near 14 KDa band.. Although some high molecular weight bands were prominent in Western blotting indicating polymerization of MT in cyanobacteria under metal stressed condition. Therefore another type of survival strategy must exist in cyanobacteria for the protection against high concentration of Cr.



Lane 1 2 3 6
Fig. 17SDS PAGE of MT in bacteria (From left to right, Lane 1- Control, Lane 2 -1g/l Cr, Lane 3-1.75g/l, Lane 6- Control bacteria)

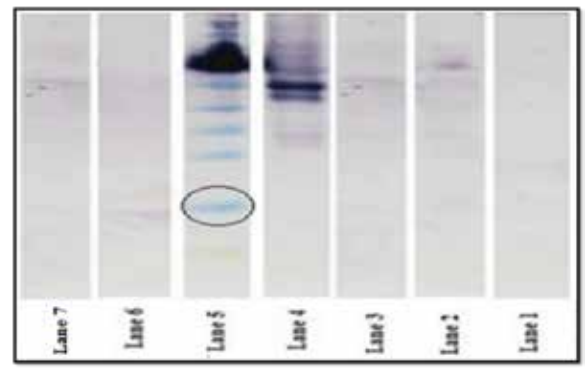
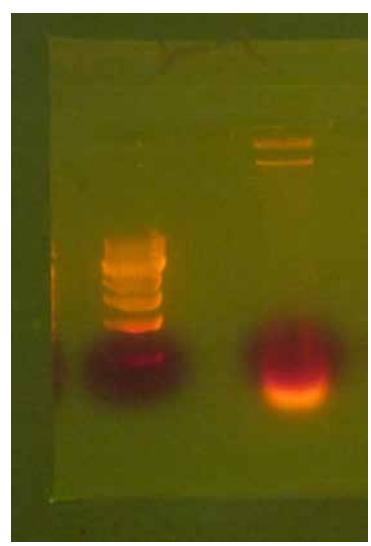


Fig. 18Western Blot of MT (From right to left of the blot, Lane 1-sample buffer, lane 2-Control Cyanobacteria, Lane 3-Control bacteria,lane 5- Protein marker, Lane 6- Cr treated bacteria)

Plasmid isolation from control bacterial culture showing increase in growth and their gel electrophoresis:
It was investigated whether the mechanism for survival of bacteria depends on the thiol content or NOS activity or help plasmid generated product. Bacteria is known for plasmid production under stress condition. Since bacteria was collected from metal stressed condition bacteria is already equipped with plasmid. Plasmid was isolated from control bacterial culture and bands were observed as shown in Fig. 19. Presence of high molecular weight plasmid indicated a plasmid mediated resistance of bacterial culture which might have helped in survival in metal stressed cultures.



Lanes 1 2

Fig. 19 Plasmid isolated from control bacteria (Lane 1-Marker DNA, Lane 2- Plasmid from Control culture)
It is evident that NOS activity increases under heavy metal stress, as a result NO production increases which in turn transactivates MT gene and so MT protein synthesis increases. MT now can bind with metals in metal stressed condition. Bacteria is showing the above relationship between MT production and NOS activity. Although cyanobacteria cannot show MT of 14KDa in presence of Cr but large amount of remediation is possible as is evident from AAS and FTIR studies. Therefore polymerization of MT may support the survival of cyanobacterial culture in high Cr concentration.

Conclusion:

It can be concluded that the MT produced from bacteria can be immobilised to remove Cr from waste water in small scale. But large scale removal of Cr from industrial waste can be done by cyanobacteria.

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