ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

# SIGNAL RESPONSE BETWEEN NITRIC OXIDE SYNTHASE AND METALLOTHIONEIN IN CHROMIUM AND LEAD TREATED BACTERIA ISOLATED FROM COAL MINE AREA

**Rini Roy**

*Department of Microbiology, Bidhannagar College, Kolkata, West Bengal, India*

**Corresponding author: for_rini_roy@yahoo.co.in*

## ABSTRACT

In the study an attempt was made to reveal signal cross talk between metallothionein (MT) and Nitric Oxide Synthase (NOS) in the bacteria isolated from soil of Raniganj Coal mine area after treatment of bacteria with Chromium (Cr) and Lead (Pb). Cr(VI) reducing ability of the bacteria and hence its metal remediation was studied by the Chromate reductase activity of the bacteria. Lead bioremediation was assayed by the Atomic absorption spectroscopy (AAS) after treatment of bacteria with Pb(II). MT was extracted from bacteria isolated from coal mine area. Induction of protein like MT having molecular weight 14 kD occurs in isolated bacteria upon treatment with heavy metals like Cr(VI) and Pb(II) as evidenced from SDS PAGE. The thiol content in metal treated bacteria increased in comparison with the control (metal untreated bacteria). Metals induce NOS activity when it is compared to the control. NOS binds transition metals and demonstrates increment in expression of NOS activity in presence of metals like Cr(VI) and Pb(II). In this study, the effects of these heavy metals on the activity of NOS and the effects of NOS on the thiol content of proteins have been discussed. The mechanism of action of xenobiotics like Pb and trace metals like Cr are to some extent different in terms of thiol content and NOS activity. A hypothesis regarding the relationship between MT and NOS has been proposed here. Bacteria where MT synthesis is regulated by NOS can be used in sites contaminated with higher concentrations of heavy metals.

**Keywords:** Metallothionein, Lead, Chromium, bacteria, Nitric oxide synthase.

## 1. INTRODUCTION

Cr(VI) is harmful for the living system whereas Cr(III) is not, instead it acts as an essential micronutrient in humans. 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 to Cr(III) through chromate reductase assay. In the present study, the effect of Chromium on bacteria isolated from the coal mine area is observed and its chromate reductase activity and thus its metal remediation capacity are ascertained [1].

Chromium toxicity is one of the major causes of environmental pollution emanating from tannery effluents. The Cr(III) species predominantly existing as hydroxides, oxides, or sulphates, are less water soluble, mobile (100 times less toxic), and (1,000 times less) mutagenic. Chemical reduction and precipitation, adsorption on activated carbon, ion exchange, and reverse osmosis, in a basic medium are the principal techniques for recovering or removing Cr(VI), from wastewater. However, these methods have certain drawbacks, namely, high cost, low efficiency, and generation of toxic sludge or other wastes that require disposal and imply operational complexity [2,3]. Bioremediation of Cr(VI) by bacteria is an eco-friendly approach.

Lead is a ubiquitous toxic metal which have mutagenic, carcinogenic, genotoxic, anthropogenic, and phytotoxic effects [4]. Lead is a xenobiotic heavy metal present as a pollutant in the environment which must be remediated. The use of fossil fuels including past use of leaded gasoline, some types of industrial facilities, and past use of lead-based paint in homes are the sources of lead exposure. Pb(II) can be also bioremediated by bacteria.

The defining feature of a nitric oxide synthase (NOS) is a heme and pterin-binding oxygenase domain, and enzymes that possess this domain are found in animals and bacteria. Recent progress in defining the functions of bacterially derived nitric oxide (NO), notably in protection from various stresses and as a potential transcriptional regulator is described [5].

The nitric oxide synthases are family of enzymes cataly-

zing the production of NO from L arginine. NO is an important cellular signaling molecule. Nitric oxide (NO) produced by bacterial NOS has recently been shown to protect the Gram-positive pathogens *Bacillus anthracis* and *Staphylococcus aureus* from antibiotics and oxidative stress [6].

Nitric Oxide (NO) in mammals is involved in several physiological and pathological processes, mainly in cell to cell signalling and cell-host response. Bacteria as a unicellular organism, does not have a similar need for cell-to-cell signalling or cell-host response, and as a result does not synthesize NO through the same enzymatic process found in mammals [7].

Functions of bacterial NOS involve nitration of different metabolites, protection against oxidative stress and to act transcription regulator.

Metallothionein is a family of cysteine-rich, low molecular weight (MW ranging from 3.5 kDa to 14 kDa) proteins which have the capacity to bind both physiological (such as Zinc, Copper, Selenium) and xenobiotic (such as Cadmium, Lead, Mercury, Silver and Arsenic) heavy metals through the thiol group of its cysteine residues, which represents nearly the 30% of its amino acidic residues. Although, the biological functions of MTs have not been fully elucidated, they are thought to play an important role in detoxification of toxic elements such as $Cd^{2+}$, $Pb^{2+}$ and $Hg^{2+}$. MTs also display antioxidant function and are involved in $Zn^{2+}$ homeostasis [8].

Thiol rich proteins protect the bacteria from oxidative and metal stress. Some of the proteins that are transactivated by Nitric oxide synthases has significant biological function.

Metallothionein (MT) is a critical target for nitric oxide (NO) which increases in labile Zn. The activity of the metal responsive transcription factor MTF-1 is affected by NO donors. In response to physiologically relevant increases in intracellular metal MTF-1 translocates from the cytosol to the nucleus and transactivates MT gene expression. It has been suggested that MTF-1 act as an intracellular metal sensor.

In recently identified two organisms that contain a predicted NOS, H-NOX, and downstream histidine kinase, which are constituents in a two-component signalling system, suggest that these organisms are capable of both NO synthesis and sensing [9].

The main objective of this study was to extract metallothionein from the bacterial culture isolated from the coal mining areas, since the microorganisms present in the soil of these areas are naturally exposed to a number of various heavy metals as drainage and further to study the expression of metallothionein by SDS-PAGE technique using two heavy metals like Cr(VI) and Pb(II) in the growth medium. To study bioremediation capacity of the bacteria its Chromate reductase activity was assayed. Lead bioremediation was studied by Atomic Absorption Spectroscopy. Thiol content and NOS activity in the isolated bacteria after Cr(VI) and Pb(II) treatment was observed. FTIR study was carried out to obtain information of the possible cell metal ion interaction. Signal cross talk between MT and NOS was established.

## 2. MATERIAL AND METHODS

### 2.1. Sample and strains used

Metals used in the study were Chromium and lead by using salts potassium chromate and lead nitrate respectively. Bacterial cultures were isolated from a pristine soil sampled 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. The lyophilised culture was the source.

### 2.2. Isolation of bacteria from coal mine area

Soil sample was brought from the mine areas at Raniganj in aim of isolating soil bacteria [10] acquired the ability to grow in presence of metal, this is important to understand the effects of metal on the biological activity of the enzyme Nitric oxide synthase.

Bacterial cultures were isolated from a pristine soil sampled from coal mining area, West Bengal, India. A minimal media plate with active charcoal as carbon source (Composition: Active charcoal 10 g/L, Sodium chloride 0.1 g/L, Magnesium sulphate 0.4 g/L, Dipotassium phosphate 0.5 g/L, Ammonium nitrate 1 g/L and Agar 15 g/L) was used for isolation. This process was done using serial dilution technique and spreading on minimal media agar plates supplemented with active charcoal as the sole carbon source. Colonies with different morphological appearance were selected from these culture plates and purified by further streaking on the same media.

To augment the growth of the isolated bacteria Nutrient Broth (NB) was chosen. 50 mL each of Nutrient Broth was prepared with different concentrations of potassium chromate & lead nitrate. The flasks having concentration of both the salts were 1g/L and 1.75 g/L, & a control

set (NB without any metal salt) were incubated at 37°C for 24 hours. Using Hahntech (Korea) Freeze dryer lyophilization of bacterial culture was done. The lyophilized culture was used for all the study performed with the isolated strain.

### 2.3. Characterization of microorganisms

The organisms were characterized based on detailed colony morphology, cellular arrangements, gram nature, cell morphology.

### 2.4. Preparation of cell extract of the metal treated and control bacterial culture:

Each culture tube containing different concentration of metals Cr(VI) and Pb(II) and the control (without metal) was centrifuged at 10000 rpm for 10 mins. Supernatant containing media was discarded and the pellet was weighed. Equal volume (in mL) of Dithiothreitol (DDT), Phenylmethylsulfonyl fluoride (PMSF) and Glycine was added to the pellet. Each mixture was vortexed, sonicated and centrifuged using above condition. Supernatant was used as cell extract [11] for further study.

### 2.5. Chromate reductase assay and lead assay

The cell extract of metal treated, and control bacterial culture were subjected to chromate reductase assay [12]. Dissolved trivalent Cr(III) can be colorimetrically determined by reaction with diphenylcarbazide in acidic medium forming red violet colour.

The amount of lead present in a sample was detected by Atomic Absorption Spectroscopy (AAS) [13].

### 2.6. Detection of adsorption of metal on bacterial cell surface by FTIR

A thin uniform film of the lyophilised bacterial culture was drawn on a cover slip and FTIR [14] was performed. The IR spectra of dried whole cell were recorded with instrument having model number L1600300 spectrum Two FTIR Sl. No. 94372 (Perkin Elmer, U.S.). The sample was scanned between 600-4000 wave number in $cm^{-1}$ at transmittance mode taking air as reference.

### 2.7. Estimation of total thiol content

For total thiol, total cell extract prepared was used to measure total thiol content of protein by Ellman's method [15]. Protein was measured by Folin-Lowry method [16].

### 2.8. Biochemical assay for Nitric Oxide Synthase

Nitric Oxide Synthase was assayed from the cell extract (enzyme source) following standard protocol [17].

### 2.9. Confirmation of MT by SDS PAGE

SDSPAGE (12%) was run using 14.3-97.4 kDa protein markers [18].

## 3. RESULTS AND DISCUSSIONS

### 3.1. Isolation of bacteria from coal mine area

As shown in the fig. 1, desired colony was taken from the plates and streaked onto another minimal media plate to get pure colony of bacteria.

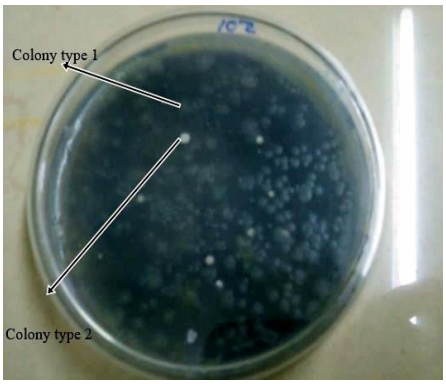


**Fig. 1: Minimal media plates depicting two types of bacterial colonies isolated from the soil sample**

### 3.2. Characterization of microorganisms

The isolated bacteria was stained pink by taking up the counterstain safranin, hence the bacteria was found to be Gram negative in nature in both the Cr(VI) and Pb(II) treated samples in fig. 2a and fig. 2b respectively. The cells were round, coccus in shape.

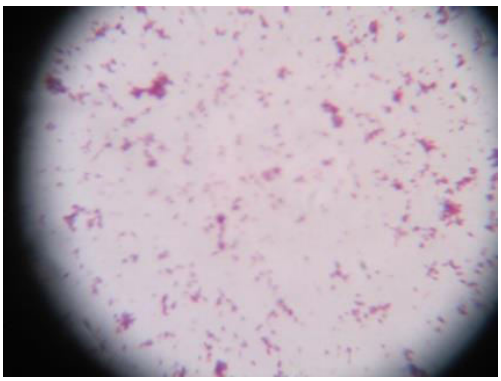

**Fig. 2a: Gram staining of Chromium treated bacteria under X 400 magnification**

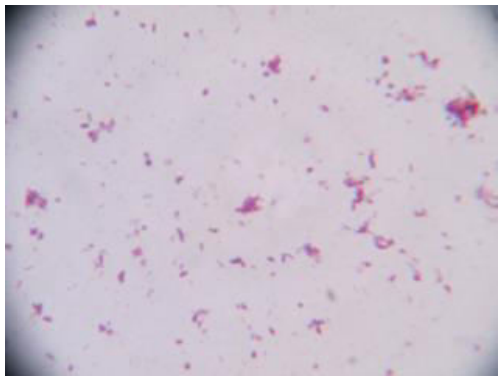

**Fig. 2b: Gram staining of Lead treated bacteria under X 400 magnification**

### 3.3. Preparation of cell extract of the metal treated and control bacterial culture

The cell extract was further used for bioremediation assays for Cr(VI) and Pb(II), FTIR, thiol estimation, NOS assay and SDS-PAGE analysis.

### 3.4. Chromate reductase assay

Compared to the control sample, the specific activity of chromate reductase was higher in both concentrations of chromium treated samples suggesting the fact that chromium increases the enzyme activity. Ten fold reduction in chromium concentration was observed in the experimental set compared to control [18].

### 3.5. Lead assay by AAS

Lead (47.3%) was remediated by immobilized MT extracted from isolated bacteria after one month as detected by AAS [13].

### 3.6. Detection of adsorption of metal on bacterial cell surface by FTIR

The shifting of peak in case of Pb(II) (Control 1658.5 $cm^{-1}$ to Pb(II) treated sample 1605 $cm^{-1}$) evident from fig. 3 and fig. 4 is more compared to Cr(VI) [18] so it can be concluded that the binding of Pb(II) to bacterial membrane is more effective compared to Cr(VI).

### 3.7. Estimation of total thiol content

Comparing both the metal treated cultures viz; Chromium and Lead treated, it could be seen from fig. 5 that the thiol content in Chromium treated bacterial culture is to some extent higher than that of the Lead treated bacterial culture.

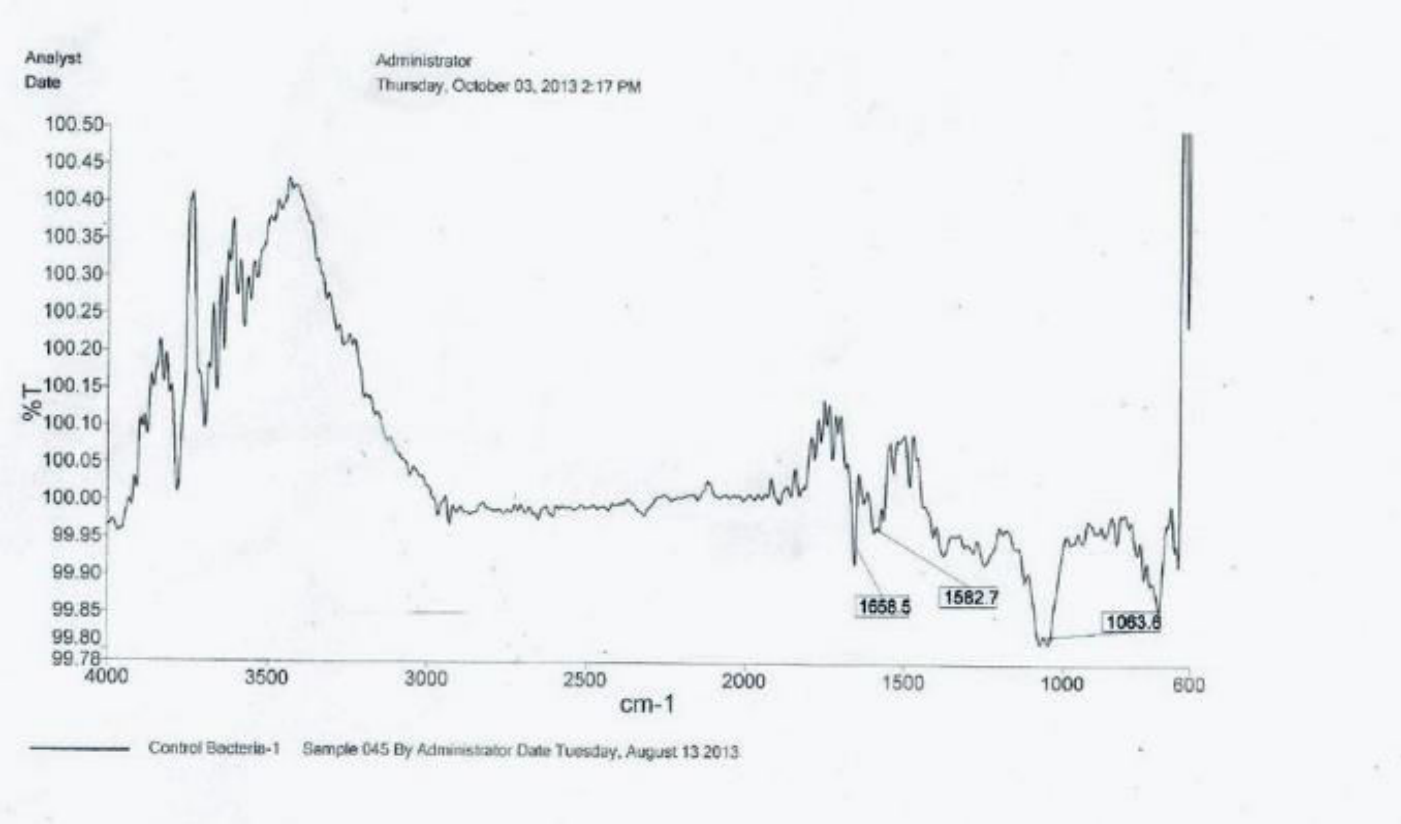


**Fig. 3: FTIR of the Control bacterial sample**

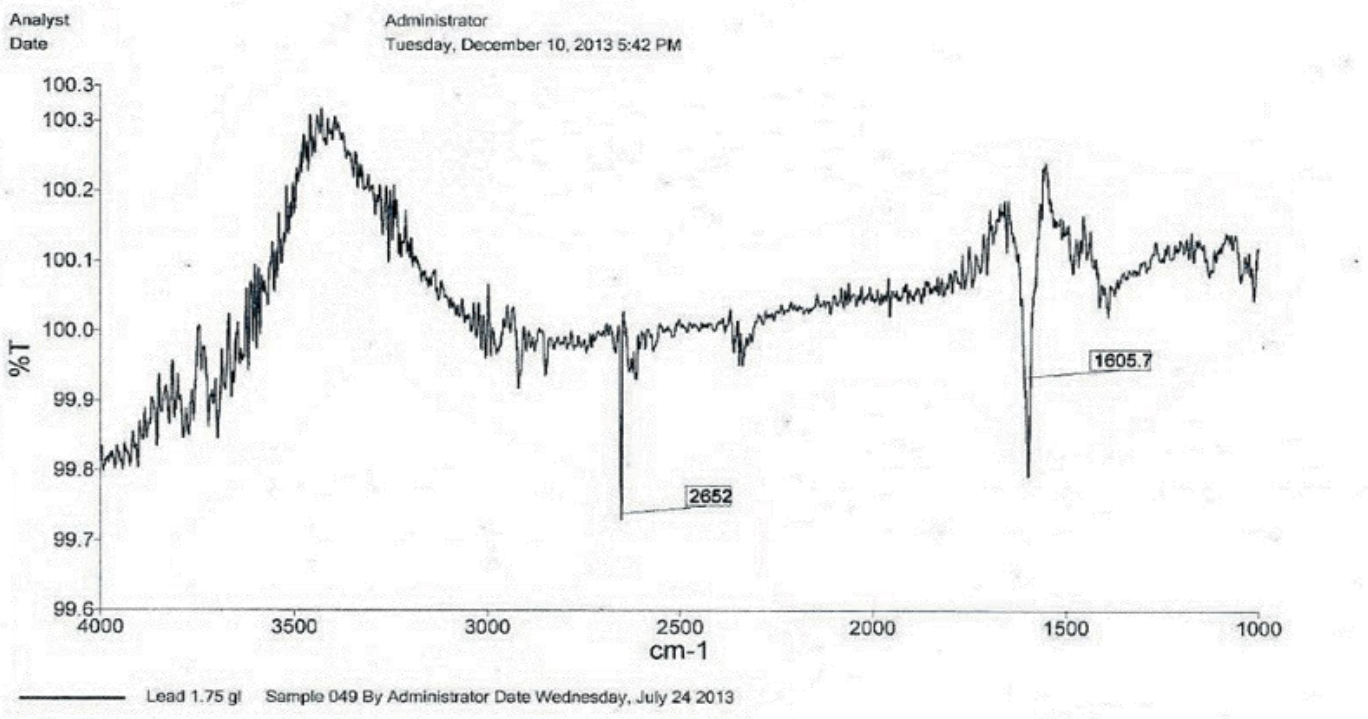


**Fig. 4: FTIR of Lead 1.75 g/L treated bacterial culture**

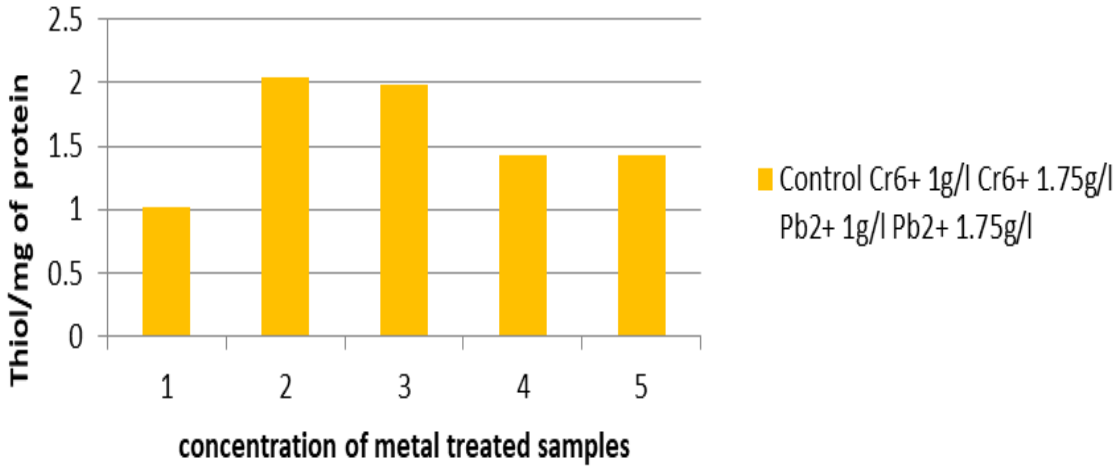


**Fig. 5:Thiol content of the Cr(VI) and Pb(II) treated bacterial samples**

### 3.8. Biochemical assay for Nitric Oxide synthase

Chromium, which is a transition metal, more efficiently induces the NOS activity when compared with Lead as shown in fig. 6. NO affects the expression of thiol rich protein.

Nitric oxide, the product of Nitric Oxide Synthase transactivates the expression of thiol rich proteins. Specific activity of NOS was found to be more in Chromium treated bacterial sample than in Lead treated bacterial sample. Thiol content was also found to be more in Chromium treated bacterial sample than in lead treated sample. When the NOS specific activity increases in chromium treated bacterial sample, thiol content also increases and as the specific activity of NOS decreases in lead treated sample, thiol content also decreases as depicted in fig. 7. These results establish the fact that NOS affects the expression of thiol rich proteins.

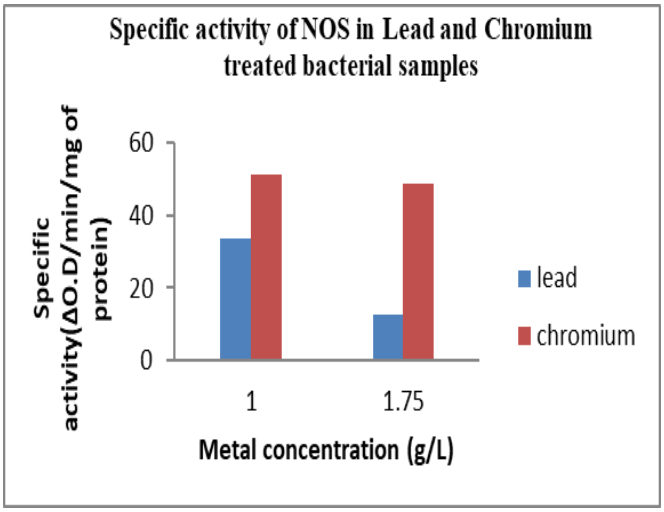


**Fig. 6: Comparison of the specific activities of NOS in the Lead and Chromium treated bacterial culture**

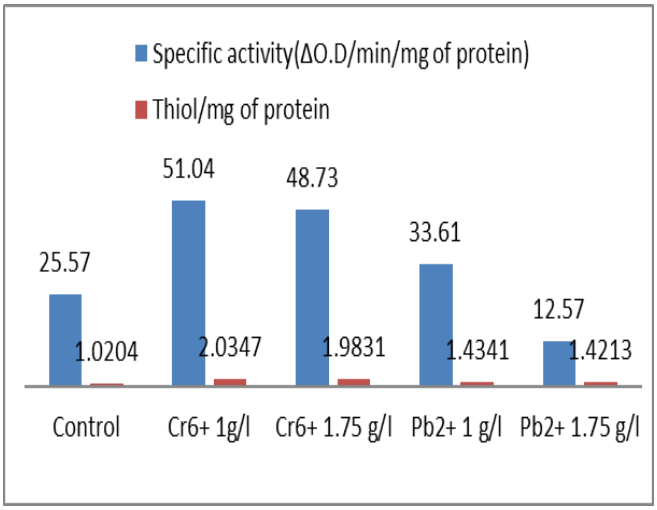


**Fig. 7: Specific activity of the NOS enzyme and the respective thiol content in the 5 samples**

### 3.9. Confirmation of MT by SDS PAGE

Prominent bands could be seen on the gel which confirmed the presence of 14 kDa molecular weight protein in the Cr(VI) and Pb(II) treated bacterial samples [18]. It can be concluded that induction of protein like MT having molecular weight 14 kDa occurs in isolated bacteria upon treatment with heavy metals like Cr(VI) and Pb(II).

## 4. CONCLUSION

The cross talk between MT and NO can be shown in the following flow chart.

Nitric Oxide Synthase (NOS) activity ↑

↓

Nitric oxide (NO) synthesis ↑

↓

NO transactivates Metallothionein (MT) gene

↓

MT protein synthesis ↑

↓

MT now can bind with metals in metal stressed condition.

↓

Bacteria where MT synthesis is regulated by NOS can be used in sites contaminated with higher concentrations of heavy metals.

Gram negative cocci isolated from mines area can balance the high metal ion induced free radical generation, thiol containing protein synthesis and viability of cells. From the band patterns observed in SDS PAGE with respect to the protein marker, it could be concluded that metallothionein activity is induced in lead treated and the chromium treated cultures [18]. Thiol content was increased due to heavy metal challenge in all the cases for Pb(II) and Cr(VI) concentrations. Bioremediation capacity of the isolated bacteria was revealed from AAS for Pb(II) and chromate reductase assay for Cr(VI).

NOS activity was increased except at high concentration of Pb(II). Finally, it has been found that the mechanism of action of xenobiotics like Pb and trace metals like Cr are to some extent different. One of the useful aspects could be the use of the isolated bacteria to clean up Pb(II) contaminated sites and for Cr(VI) contaminated site because of its chromate reductase activity. The understanding of the NOS and metallothionein relationship further elucidates the fact that MT synthesis is induced by the trans activator NO upon treatment of the isolated bacteria with heavy metals like Cr(VI) and Pb(II).

## 5. ACKNOWLEDGEMENT

Author express sincere gratitude to Dr. Aditi Nag Chaudhuri, Associate Professor and Head of the Microbiology Department, Lady Brabourne College for her help, guidance and inspiration to carry out the study in the Department of Microbiology, Lady Brabourne College, Kolkata.

### *Conflict of interest*

There is no conflict of interest to influence the results and interpretation of the manuscript.

## 6. REFERENCES


1. Cheunga KH, Gua JD. *International Biodeterioration & Biodegradation*, 2007; **59**:8-15.
2. Polti MA, Amoroso MJ, Abate CM. *The Journal of General and Applied Microbiology*, 2010; **56(1)**:11-18.
3. Desai C, Jain K, Datta M. *Process Biochemistry*, 2008; **43**:713-721.
4. Alvarez-Ayuso E, Otones V, Murciego A, Sánchez García A. *Science of The Total Environment*, 2012; **439C**:35-43.
5. Rafferty S. *The Open Nitric Oxide Journal*, 2011; **3(Suppl 1-M4):**25-32.
6. Gusarov I, Starodubtseva M, Wang Z-Q, McQuade L, Lippard SJ, Stuehr DJ et al. *J Biol Chem*, 2008; **283**:13140-13147.
7. Pant K, Bilwes AM, Adak S, Stuehr DJ, Crane BR. *Biochemistry*, 2002; **41**:11071-11079.
8. Murthy S, Bali G, Sarangi SK. *African Journal of Biotechnology*, 2011; **10(71)**:15966-15972
9. Rao M, Smith BC, Marletta MA. *mBio*, 2015; **6:**e00206-15.
10. Raja CE, Selvam GS, Omine K, International joint symposium on Geo disaster prevention and Geo environmental in Asia, 2009, JS Fukuoka.
11. Enshaei M, Khanafari A, Sepahy Akhavan A. *Iran J Environ Health Sci Eng*, 2010; **7(4)**:287-298.
12. Jhunjhunwala P, Roy R, Roy S, Chaudhuri AN. *International Journal of Applied Research*, 2016; **2(11):** 249-256.
13. Roy R, Baid G and Chaudhuri AN. *Education, Research and Analysis*, 2018; **5(1.1):**73-76.
14. Chen Y, Xie MY, Li SP, Nie C, Wang YX. *Food Chemistry*, 2008; **107**:231241.
15. Ellman GL, Courtney KD, Andres V, Featherstone RM. *Biochem Pharmacol*, 1961; **7**:88-96.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. *J Biol Chem*, 1951; **193**:265-272.
17. Wang W, Inoue N, Nakayama T, Ishii M, Kato T. *Analytical Biochemistry*, 1995; **227(2)**:274-280.
18. Roy R, Gupta M, Chaudhuri AN. *International Journal of Scientific Research*, 2015; **4(06)**:11-16.