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Research Article

EFFECT OF CADMIUM AND ZINC HEAVY METALS ON THE SOIL BACTERIA ISOLATED FROM COAL MINE REGION

Rini Roy

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

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

ABSTRACT

Study of growth pattern of isolated soil bacteria from Raniganj coal mine area shows an unusual increase in growth rate of the isolated bacteria when treated with heavy metal stress in the culture media. The metals used were Cadmium (Cd) and Zinc (Zn). Utilization of metal ions by the bacterial cells was studied by detection of leftover metal in the culture medium after optimal growth, by Dithizone method and surface adsorption of metal ions on bacterial cell by FTIR technique. The amount of total thiol and non-protein thiol of the bacterial culture was assayed for the presence of thiol containing protein like Metallothionein. Metallothionein production was also studied using SDS-PAGE and Western blot technique to find if the cells were stressed in the presence of increasing concentration of Zn^{2+} and Cd^{2+}. Metallothioneins (MTs) are proteins rich in cysteine residues having low molecular weight. They perform different functions like scavenging of free radicals, involvement in maintaining metal balance, regulation of metabolic activities and protective role against damage caused by heavy metals. Metallothioneins can be correlated with heavy metal contamination of an environment and thus may be considered as bio-marker for environmental pollution.

Keywords: Cadmium, Zinc, Dithizone, Bacteria, Metallothionein.

1. INTRODUCTION

Persistence of heavy metals having toxicity in biogeochemical cycle largely depends on microbes. Microbes also help to remove contamination of toxic heavy metals. Metals possessing atomic density greater than 4000 Kg/m^3 are known as heavy metals [1]. At high concentration, Zinc, Nickel, Copper, Cobalt and Manganese have toxic effect on human health and different organisms [2]. On the other hand, Cadmium, Mercury, Lead etc. do not have any biological role and are harmful to the organisms even at minute concentration [3]. The existence of heavy metals occurs both in bioavailable and non-bioavailable forms. Mobility of heavy metals depends on the metallic element precipitating as positively charged ions as well as the one, which constitute negatively charged part of salt. Detrimental effects are observed on the environmental microbes when the concentration of heavy metals exceeds threshold levels. Otherwise, microorganisms might develop higher resistance against toxic heavy metals when they are exposed to the increased concentrations of these metals [4-6]. Additionally, various means have been developed by the microorganisms dwelling in metal polluted soils to withstand metal stress. Such metal resistant microorganisms can show strong bioremediation capacity. To survive in the metal stressed conditions, bacteria have devised various pathways to resist the intake of heavy metal ions. The pathways adopted for withstanding the heavy metals are accumulation and complexation of the metal ions inside the cell, reduction of the heavy metal ions to a less toxic state [6,7,8] and metal ions efflux outside the cell. Reports already exists on the different metal-resistant bacteria. Isolation of bacteria was done from contaminated sediments, soils, and waters.

Margoshes and Valee discovered Metallothioneins in 1957 as newly invented proteins isolated from the tissue [9] of a horse renal cortex. These proteins possess high degree of homology in whole animal kingdom. Similar proteins are expressed by bacteria, fungi and even plants express similar proteins. MTs are low molecular weight (from 5 to 14 kDa) proteins possessing cysteine residues (higher than 30 % of its amino acidic residues) along with 7-12 metal atoms per molecule [10, 11]. Aromatic amino acids are absent in Metallothioneins.

MTs bind several trace elements like Cadmium (Cd), Zinc (Zn), Mercury (Hg), Silver (Ag) and Platinum (Pt)

due to the presence of thiol groups and gives protection to cells and tissues against heavy metal toxicity [12]. The main function of MTs in organism is maintenance of the oxidative-reducing conditions, regulation of expression and metal ion transport. Thiols like MT and glutathione can scavenge free radicals effectively to create excellent oxidative-reducing conditions to protect cell compartments and cell-cycle-enzymes or DNA. Metallothioneins in bacteria can bind, sequester, and buffer excess intracellular zinc. They are thought to play an important role in detoxification of toxic elements such as Cd^{2+} and Hg^{2+}. MTs also display antioxidant function and are involved in Zn^{2+} homeostasis although, the biological functions of MTs have not been fully elucidated. Zinc coordination in bacterial Metallothioneins [13] is achieved by the imidazole groups of histidine residues, in addition to the of cysteine thiol residues. Zinc ions are silent to most physicochemical probes, e.g., NMR and Mossbauer spectroscopies.

The mass spectrometry has capability to provide information on zinc complexes having vivid applications, potentiality in biochemistry [14]. The study of metal ion resistances elucidates environmental processes and helps to understand basic living processes, bacterial efflux mechanisms for inorganic metal cations and anions, constitutes the resistance systems in bacteria isolated from metal-polluted environments. In Gram-positive bacteria, resistance to Cd^{2+} and Zn^{2+} depends on a P-type efflux ATPase which is related to the copper transport P-type ATPases of bacteria and humans. In Gram-negative bacteria, it is dependent on the action of proton-cation antiporters, members of a newly recognized protein family that performs diverse functions such as metal resistance, cell division, and nodulation of legumes [15]. FTIR spectroscopic techniques were applied to determine the overall structural and compositional changes in bacterial cells after heavy metal stress [16].

In the present study, the effects of metal stress on soil bacteria isolated from soil of Raniganj coal mining area and the growth characteristics of the bacteria at increasing concentration of metal were observed and also their metal tolerance capability and mechanism used by them to protect themselves from high metal concentration in their growth medium were explored. Cd is taken into consideration as the xenobiotic metal and Zn as the physiological metal. Metallothionein were extracted from the bacterial cells and the isolated metallothionein were identified by SDS PAGE and Western Blotting.

2. MATERIAL AND METHODS

2.1. Chemicals

Dithizone was purchased from Sisco Research laboratory. All other chemicals and media components used were obtained from Himedia and were of analytical grade.

2.2. Growth medium

Minimal media (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, Agar 15 g/L, Final pH adjusted to 7.4) and Nutrient broth medium (Composition: Peptic digest of animal tissue 5 g/L, Sodium chloride 5 g/L, Beef extract 1.5 g/L, Yeast extract 1.5 g/L, Final pH adjusted to 7.4) was used for initial isolation of bacterial strain and for all subsequent experiments.

2.3. Sample

Metals used in the study were Zinc(II) and Cadmium(II) by using salts Zinc sulphate and Cadmium nitrate respectively.

2.4. Isolation of the bacteria

Isolation of bacterial cultures from a pristine soil was done from Raniganj coal mining area, West Bengal, India using the standard dilution plate technique. 10-fold dilutions of fresh soil (1 g) were made in phosphate buffered saline (pH 7.5) and 0.1 mL from each of these dilutions were spread on minimal media agar plates supplemented with active charcoal as the sole carbon source. Plates were incubated at 37°C for 2-3 days. Colonies with different morphological appearance were selected from these culture plates and purified by further sub culturing in the same media.

2.5. Use of growth promoting medium for the isolated bacteria

Four mL of the culture was inoculated in 100 mL of Nutrient Broth (NB) medium. After 24 hrs incubation period, huge growth of bacteria was observed. 50 mL of the cultures was centrifuged and pelleted down for lyophilization. The lyophilized culture was used for all the study performed with the isolated strain.

A 250 ml of NB was inoculated with a pinch of lyophilized culture of bacteria isolated from coal mining area of Raniganj, West Bengal and incubated at 37°C for 24 hours. Dense growth was observed after 24 hours. 50 mL each of NB was prepared with different concentrations of Zinc sulphate and Cadmium nitrate.

The concentrations were 2.5 µg/mL, 10 µg/mL, 25 µg/mL and a Control (NB without any metal salt) for each of the salt. The flasks containing the different concentrations of heavy metals with isolated bacteria were incubated at 37°C for 24 hours. Growth was observed after 24 hours.

2.6. Gram Character of isolated bacteria

Gram staining [17] of the revived culture was done to find out their gram character.

2.7. Motility of isolated bacteria

Nutrient agar (NA) plates were made by creating a partition in between. Slide was placed vertically along the diameter of the plate where the slide fits the cord of the plate. Nutrient agar was poured into the plates on either side of the partition. The slide was removed slowly and carefully after the agar had solidified. One part of the plate was streaked with the culture [18]. The plates were incubated for 24 hours at 37°C for growth.

2.8. Growth Curve

A 50 mL each of nutrient broth was prepared in seven conical flasks. One flask was kept as Control (without any metal salt) and the other six were provided with metal salts; Zinc sulphate and Cadmium nitrate each with concentration 2.5µg/mL, 10 µg/mL, and 25 µg/mL. Time was taken as 0 min when no inoculum was added. After addition of the inoculum (bacterial culture) in each of the flask it was incubated at 37°C for 180 mins for the cells to enter the log phase. Thereafter optical density was taken at 600 nm wavelength in spectrophotometer after every 30 mins till the cells enter the stationary phase [19]. Thus, growth curve was obtained.

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

Each culture tube containing different concentrations of metals Zinc and Cadmium and the Control (without any metal) was centrifuged at 10000 rpm for 10 mins. Supernatant containing media was discarded and the pellet was weighed. Equal volume of Dithiothreitol (DDT), Phenylmethylsulfonyl fluoride (PMSF) and Glycine addition was done to the pellet. Vortexing to each mixture was carried out and transferred to a small beaker and sonicated. Sonication was done for 30 seconds with 30 seconds gap repeated 4 times. Each sonicated mixture was transferred to fresh eppendorfs and centrifuged at 10000 rpm for 10 mins [20]. Supernatant was transferred into new eppendorfs and used as cell extract.

2.10. Protein estimation by Folin-Lowry method

Bacterial cell extract from the above procedure was used for protein estimation treated with different concentrations of metals Zn^{2+} and Cd^{2+}. Protein was estimated by Folin-Lowry method [21] at 660 nm.

2.11. Estimation of total thiol content

For total thiol, bacterial cell extract prepared was used to measure total thiol content of protein. Thiol was estimated by Elman's method [22,23] using Dithionitrobenzoic acid (DTNB), Sodium bicarbonate, 0.1 M Phosphate buffer (pH - 7.4). The solution gave a light-yellow colour and optical density (O.D.) was taken at 420 nm immediately within 5 seconds.

2.12. Estimation of Non-protein thiol content

To the bacterial cell extract, 6% TCA was added in the ratio 3:1 (extract: TCA). The cells were then centrifuged at 10000 rpm for 10 mins. pH of the supernatant was adjusted to 7 using the phosphate buffer (0.1 M, pH-7.4). Then the non-protein thiol was estimated by Elman's method using DTNB [22,23].

2.13. Estimation of protein thiol content

Protein thiol content was estimated by subtracting the non-protein thiol content from the total thiol content.

2.14. Plasmid isolation from Cadmium and Zinc containing cultures

From the growth curve experiment, an increase in growth was observed in the cultures containing Cd^{2+} at concentration 2.5µg/mL and Zn^{2+} at concentration 25 µg/mL from the control sample culture which was devoid of any heavy metal stress. Thus, to check the possible reason for the increase in growth, presence of plasmid was checked which might be helping the cells to resist the metal stressed environment. For this, plasmid isolation was carried out [24].

2.15. Detection of adsorption of metal on bacterial cell surface by FTIR

Lyophilisation of the bacterial culture treated with Cd^{2+} (2.5µg/mL), Zn^{2+} (25µg/mL) and Control (without metal salt) was then performed using Hahntech (Korea) Freeze dryer. A thin uniform film of the lyophilised bacterial culture was drawn on a cover slip and FTIR

[25] 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.

2.16. Measurement of the uptake of Zinc by bacteria by measuring the leftover metal by Dithizone method

Measurement of leftover Zinc was done using Dithizone method [26] with Zinc stock (1 mg/mL) of Zinc Sulphate, Sodium-potassium-tartarate (25 mg/mL), Sodium Hydroxide (1N), Hydroxylamine hydrochloride (20 mg/mL), Acetate buffer (pH 5.5), SDS (0.6 M), Dithizone (10 µg/mL). Measurement of the optical density was done at 540 nm.

2.17. Determination of leftover Cadmium by measuring the leftover metal by Dithizone method

Dithizone method was used to measure left over Cadmium [27,28]. The reagents Cadmium nitrate (1 mg/mL), Sodium-potassium-tartrate (25 mg/mL), Sodium Hydroxide, Hydroxylamine hydrochloride (20 mg/mL) and Dithizone (10 µg/mL in chloroform) were added in measured amount in the test tubes. The chloroform layer finally was transferred in the cuvette and absorption was measured at 450 nm.

2.18. Gel Electrophoresis of the proteins extracted from the Control and metal treated culture

The presence of the protein metallothionein was examined by running the sample in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus. SDS-PAGE was carried for the isolation of metallothionein. 12% SDS PAGE was run using 14.3-97.4 kDa protein markers [29]. The gels were stained overnight in Coomassie Brilliant Blue R-250, fixed in 0.5% acetic acid and destained in destaining solution prior to scanning for documentation.

2.19. Western Blotting to elucidate the presence of metallothionein

Western Blotting was performed for the detection and confirmation of metallothionein from the un-lyophilised sample (bacterial cultures containing 25 µg/mL of Zn^{2+} and 2.5 µg/mL of Cd^{2+}) with Anti Metallothionein UC1MT (ab 12228) using the standard protocol [30].

3. RESULTS AND DISCUSSION

3.1. Isolation of the bacteria

Purified bacterial colonies was isolated on minimal media supplemented with active charcoal as carbon source [31] as in Fig. 1.

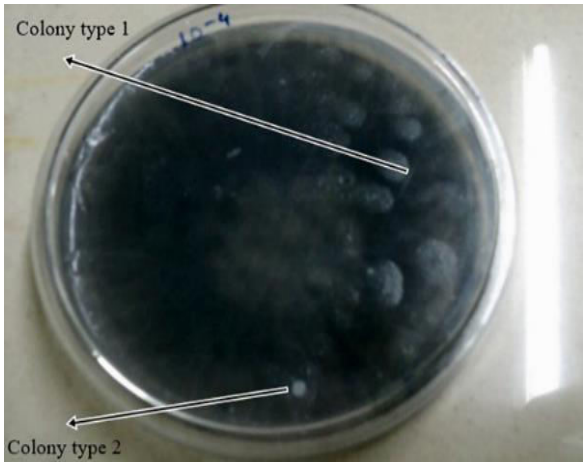


Fig. 1: Two types of bacterial colonies were isolated in minimal media plate

3.2. Use of growth promoting medium for the isolated bacteria

Dense growth of isolated bacteria was obtained when the minimal medium was changed to Nutrient Broth medium depicted in Fig. 2.

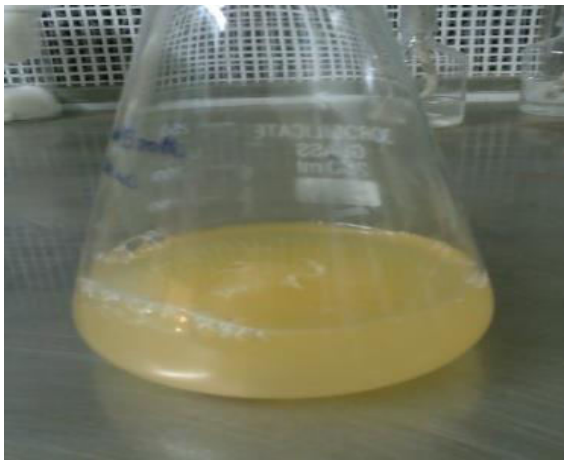


Fig. 2: Dense growth of isolated bacteria in Nutrient Broth medium from coal mine area after 24 hours

3.3. Gram Character of isolated bacteria

From the Gram staining as shown in Fig. 3 it was found that the isolated culture was of gram-negative character, short coccus in shape.

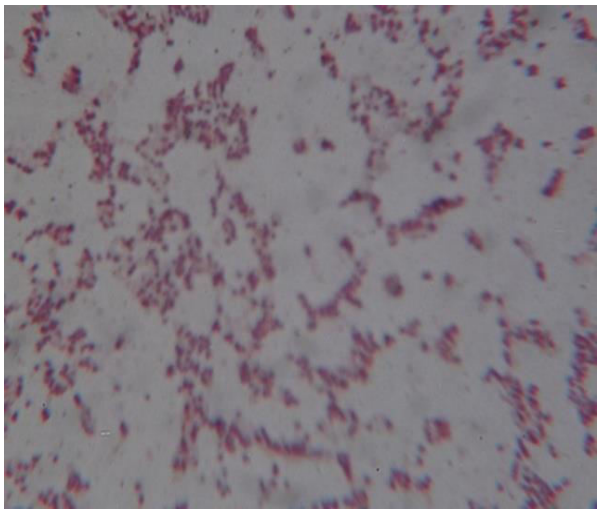


Fig. 3: Gram negative coccus under X 400 magnification

3.4. Motility of isolated bacteria

The part of the nutrient agar (NA) plate where bacteria was streaked showed growth only. There was no growth on the other side of the agar in Fig. 4 indicating the isolated bacteria to be non-motile.

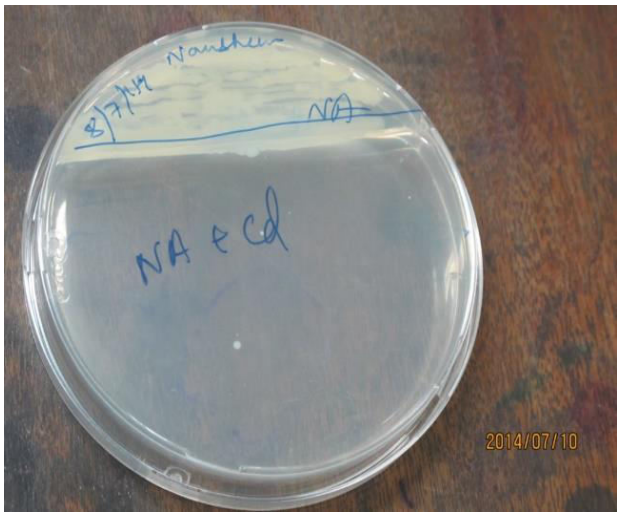


Fig. 4: Petri plate showing the cells to be non-motile

3.5. Growth Curve

Growth curve results as shown in Fig. 5 and Fig. 6, showed that there was an increase in growth of the isolated bacteria in the medium containing Cd^{2+} 2.5 µg/mL and Zn^{2+} 25 µg/mL when compared to the Control which did not contain any heavy metal in the medium. Thus, possible reason for the increase in growth might be due to a resistance system used by the bacteria to overcome the heavy metal stress. Possible resistance mechanism might be due to a plasmid mediated efflux system or surface adsorption on the bacterial cell surface. It was further tried to establish the probable reason experimentally.

At 2.5µg/mL and 25µg/mL, Cd^{2+} and Zn^{2+} acted as trace element promoting growth whereas higher concentrations become toxic.

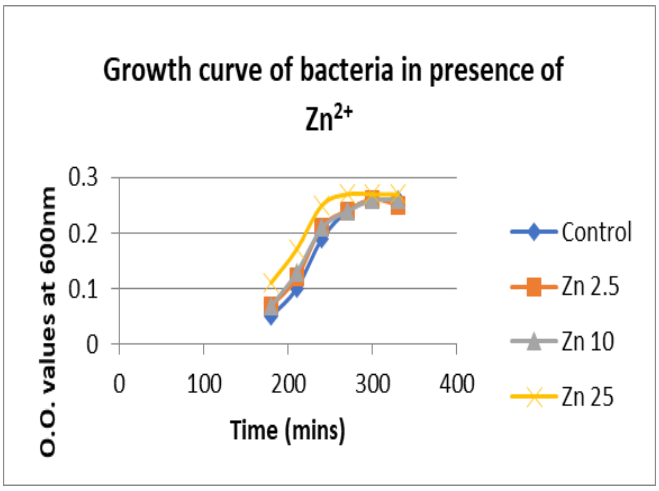


Fig. 5: Growth curve of isolated bacteria using Zn^{2+} in the medium

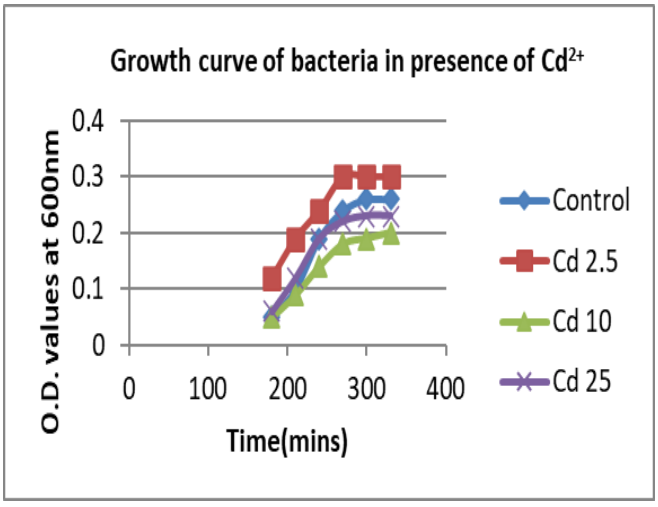


Fig. 6: Growth curve of isolated bacteria using Cd^{2+} in the medium

3.6. Estimation of total thiol content

It was observed from Fig. 7 that there was an increase in the total thiol content of the culture containing metals Cd^{2+} and Zn^{2+} from the control sample culture. This indicated that there might be an increase in protein rich in thiol group such as metallothionein and glutathione.

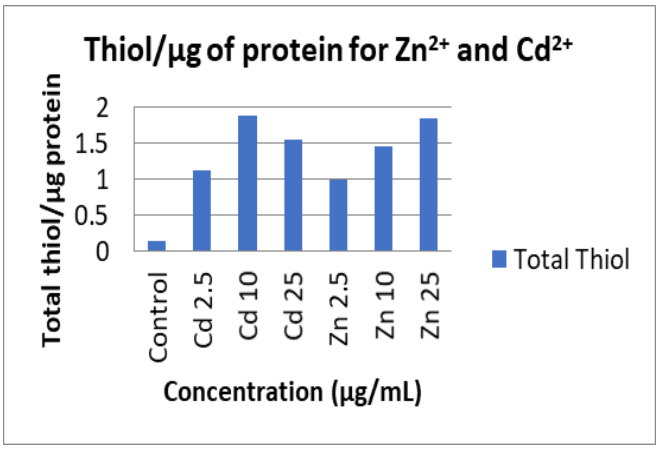


Fig. 7: Total thiol protein of bacteria upon Zn^{2+} and Cd^{2+} treatment

3.7. Estimation of Non-protein thiol content

It was observed from Fig. 8 that there was an increase in the non-protein thiol content of the culture containing metals Cadmium and Zinc from that of the control sample culture. This indicated that there might be an increase in non-protein thiol.

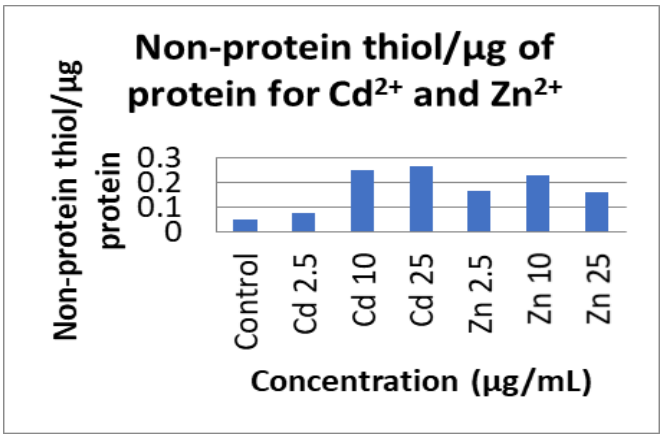


Fig. 8: Total non-protein thiol of bacteria upon Zn^{2+} and Cd^{2+} treatment

3.8. Estimation of protein thiol content

Fig. 9 clearly shows that the protein thiol content of the bacterial culture was increased indicating the presence of protein rich in thiol group like metallothionein.

3.9. Plasmid isolation from Cadmium and Zinc containing cultures

Presence of high molecular weight plasmid in Fig. 10 in lanes 2, 3 and 4 indicated a plasmid mediated resistance of bacterial culture which might have helped in the increase in growth in metal stressed cultures [31].

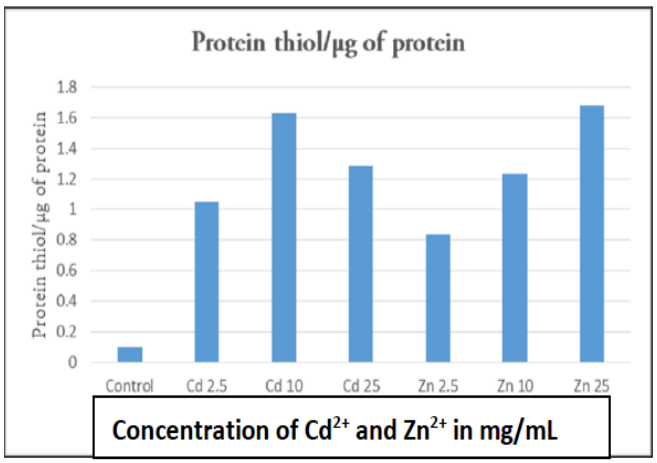


Fig. 9: Protein thiol content of the cultures containing Cd^{2+} and Zn^{2+} were significantly greater than control sample culture

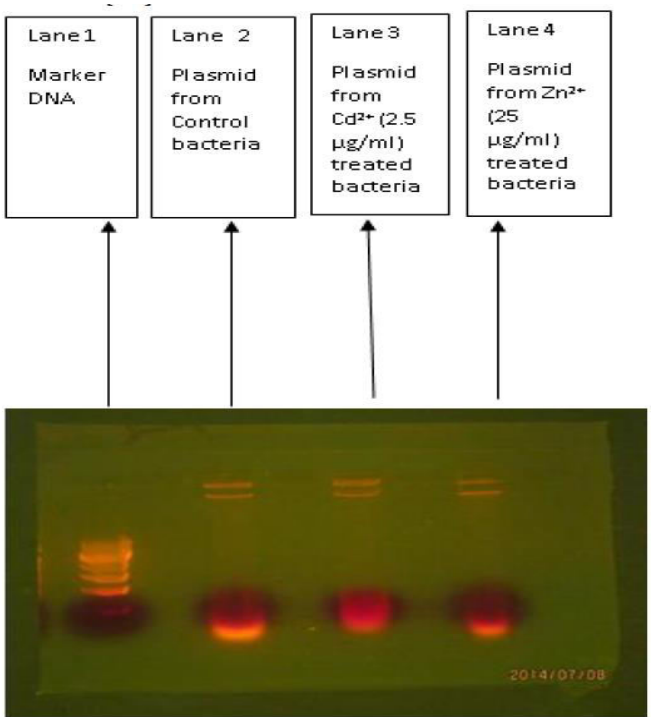


Fig. 10: Plasmids isolated in the control bacteria and Zn^{2+} and Cd^{2+} treated bacterial cultures

3.10. Detection of adsorption of metal on bacterial cell surface by FTIR

FTIR Study was performed to obtain information about the possible cell metal ion interaction. From Fig. 11, Fig. 12, and Fig. 13 it can be predicted that when Zn^{2+} is remediated (Fig. 13) the change in adsorbed material has been observed at 1613 cm^{-1}, 1523 cm^{-1}, 1414 cm^{-1} compared to control bacteria (Fig. 11) and carboxyl group is the interacting group.

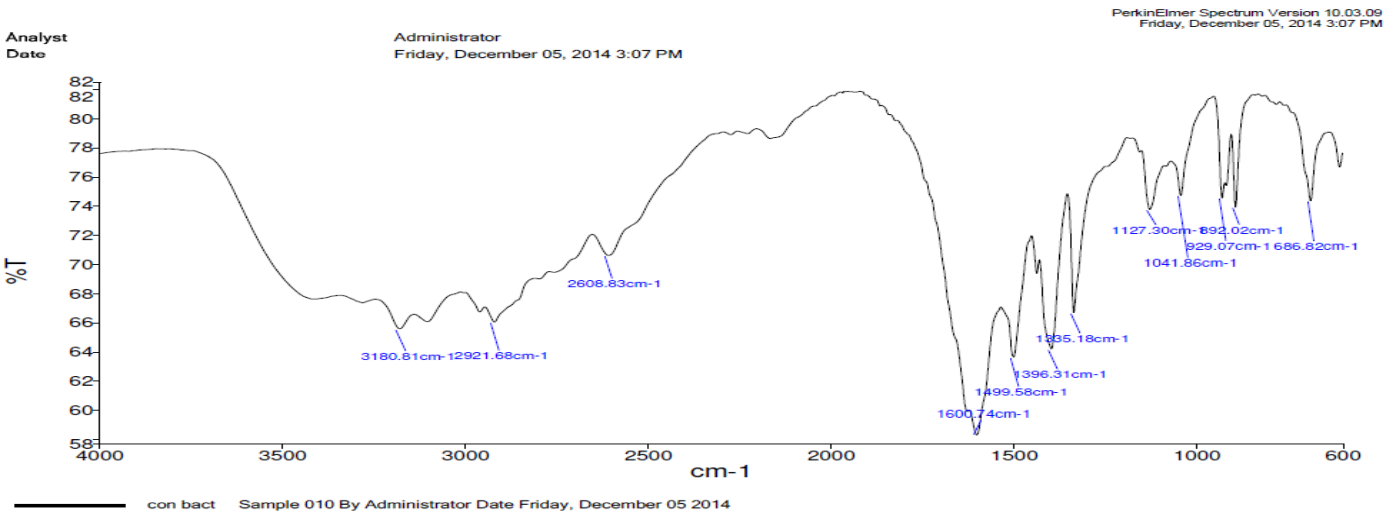


Fig. 11: FTIR of Control bacteria (without any metal treatment)

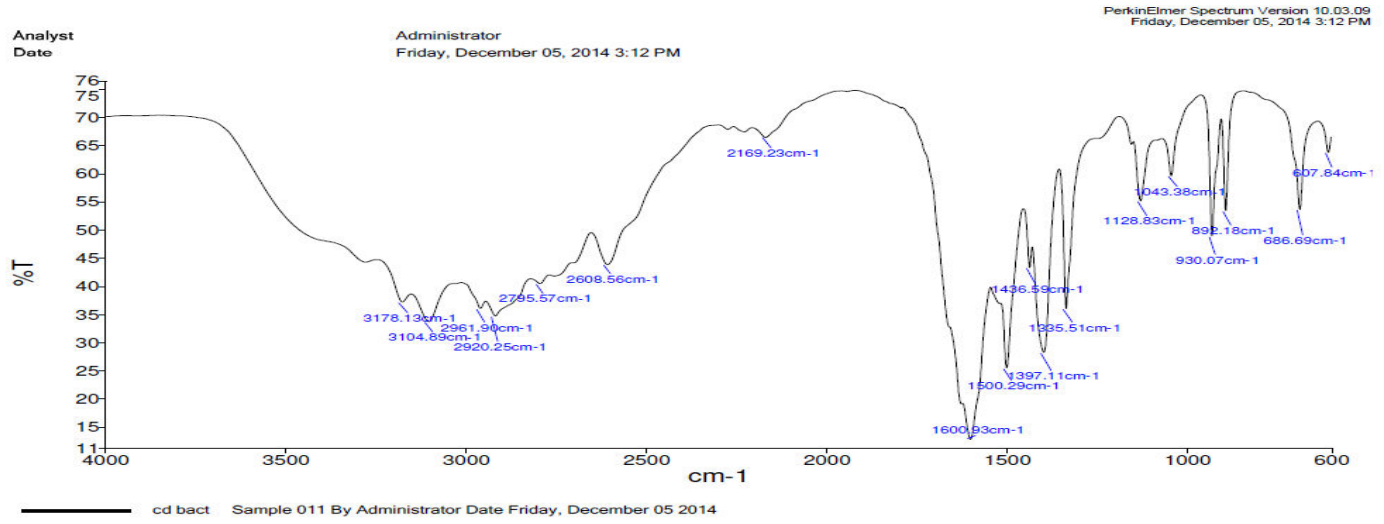


Fig. 12: FTIR of the bacteria treated with Cd^{2+}

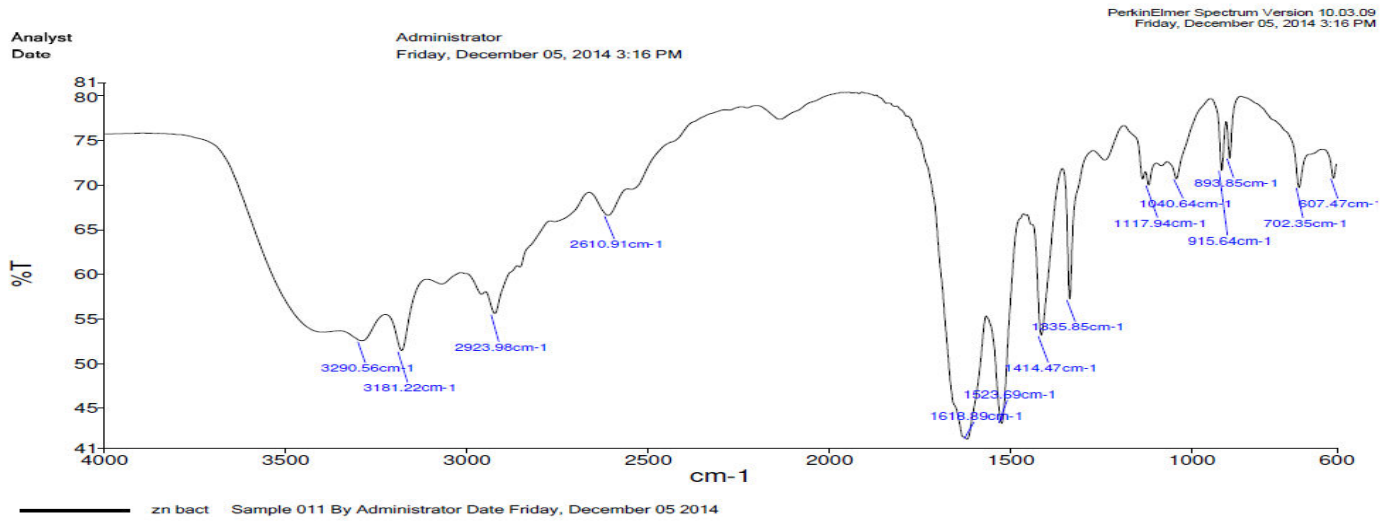


Fig. 13: FTIR of the bacteria treated with Zn^{2+}

3.11. Measurement of the uptake of Zinc by bacteria by Dithizone method (Fig. 14)

From Standard curve of Zn^{2+} using Dithizone method
1 O.D = 13.33 µg/mL
Initial concentration of Zn^{2+} in the medium=25 µg/mL
Concentration of leftover of Zn^{2+} in the medium = 3.68 µg/mL (OD=0.276)
Therefore, total metal uptake=(25-3.68)µg/mL= 21.32 µg/mL
It was observed that most of the Zn^{2+} (85.28%) was remediated by the bacterial cell.

3.12. Measurement of leftover Cadmium by Dithizone method (Fig. 15)

From standard curve of Cd^{2+} using Dithizone method
1 O.D = 5.17 µg/ml
Initial concentration of Cd^{2+} in the medium=2.5 µg/mL
Concentration of leftover Cd^{2+} in the medium = 1.4 µg/mL (OD = 0.25)
Therefore, total metal uptake = (2.5-1.4) µg/mL = 1.1µg/mL
It was observed that the Cd^{2+} (44%) was remediated by the bacterial cell.
Metallothionein remediation by isolated bacteria is more efficient in case of Zn^{2+} than in case of Cd^{2+}

3.13. Gel Electrophoresis of the proteins extracted from the Control and metal treated culture to find the presence of Metallothionein

Bands were observed in all the three lanes (Fig. 16) containing control, Zn^{2+} and Cd^{2+} treated bacterial cultures near 14 kDa marker, which indicates metallothionein proteins have been expressed.

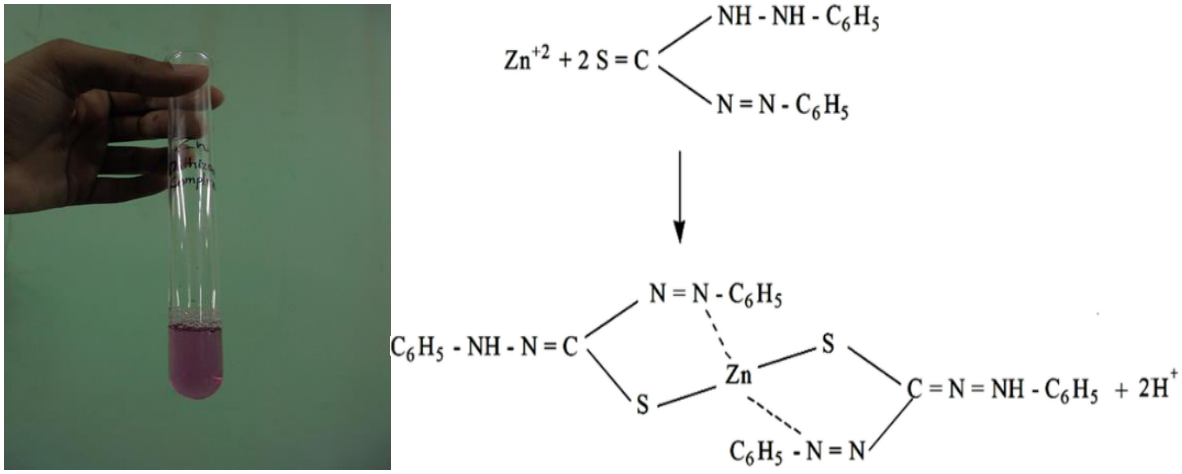


Fig. 14: Pink coloured Zn^{2+}-Dithizone complex after Dithizone reaction for Zn^{2+}

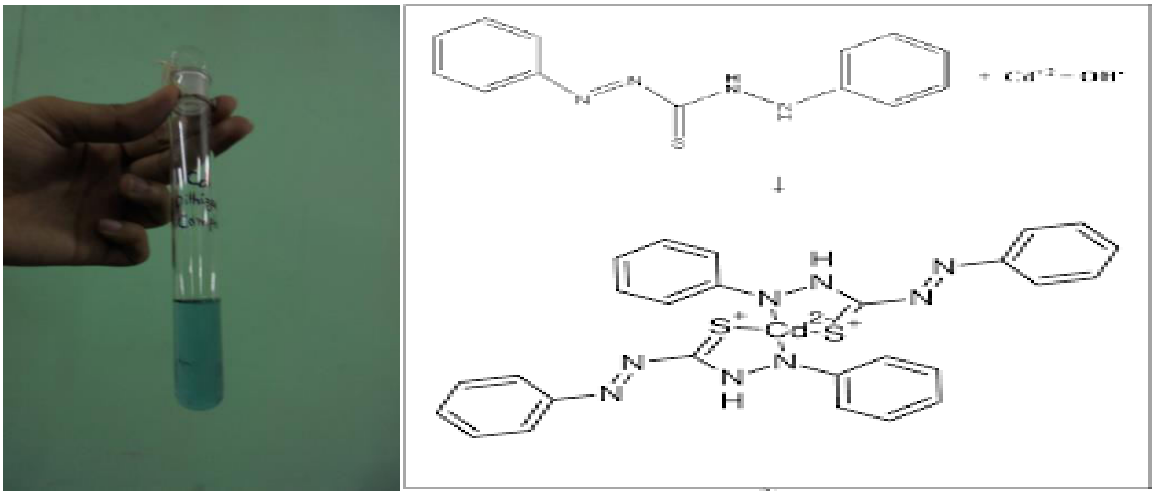
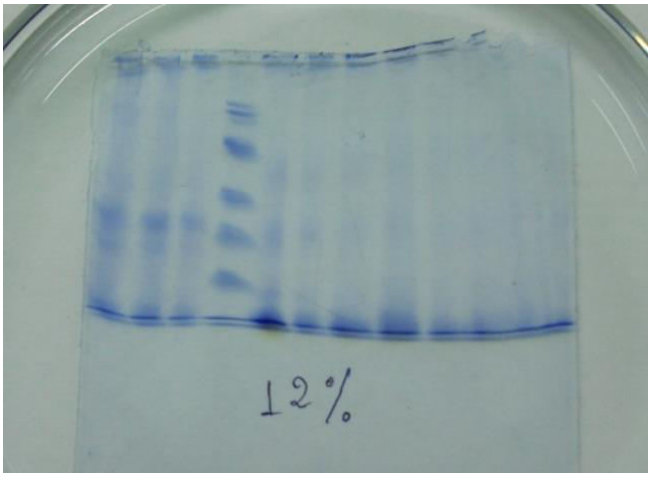


Fig. 15: Green coloured Cd^{2+}-Dithizone complex after Dithizone reaction with Cd^{2+}



Lanes 1 2 3 4

Fig. 16: SDS PAGE: Lane 1-Control bacteria, Lane 2- Cd^{2+} treated bacteria, Lane 3-Zn^{2+} treated bacteria, Lane 4- Protein molecular weight marker)

3.14. Western Blotting to elucidate the presence of metallothionein

The nitrocellulose membrane was treated with NBT-BCIP buffer solution, until a purple colour was observed in the lanes.

Since the protein reacted with anti-metallothionein antibody, so bands were observed, obtained at 14 kDa so it can be concluded that metallothionein was present in the bacterial cell extract which has the bioremediation capacity.

4. CONCLUSION

Cultured bacterial cells isolated from coal mine areas were gram negative coccus and non-motile. Cultured bacterial cells treated with Zn^{2+} metal at concentration 25 µg/mL and Cd^{2+} metal at concentration 2.5 µg/mL showed increase in growth from Control (metal untreated culture). Metal treated bacterial cultures showed increase in total thiol content of the cell from Control. Metal treated cultures showed an increase in non-protein thiol from Control. The difference of the total thiol content and the non-protein thiol gave the protein thiol content of the cultures containing Cd^{2+} and Zn^{2+} which showed significant increase compared to control bacterial culture. This result indicated the presence of metallothionein, protein rich in thiols in isolated bacteria involved in heavy metal binding. High molecular weight plasmid was isolated from bacteria indicating plasmid mediated resistance of bacteria under stress of heavy metals in the growth medium. FTIR study revealed that for Zn^{2+} remediation by bacteria, carboxyl groups on the bacterial surface are responsible mainly for adsorption of the metal. Most of the Zn^{2+} was taken up by the Zn^{2+} treated bacterial cell compared to Cd^{2+} treated bacterial cell. Most of the metal uptake by the Zn^{2+} treated bacterial cell was found to be adsorbed on the bacterial cell surface. Bands observed at the 14 kDa position in SDS-PAGE denoted the presence of metallothionein. Metallothionein Western Blot may be used as marker to clear the confusion of the presence of high concentration of Zn^{2+} and Cd^{2+} on a pool. Metallothionein thus can be used as bio-environmental or bioremediation markers for heavy metal contaminated sites. Further research can be done to establish the efficiency of metallothionein as marker in heavy metal bioremediation. The bacterial culture, which was labelled as SAMPLE showed similarity with *Acinetobacter calcoaceticus*, strain 174B (C1M2) (Accession Number: KF254603.1) based on nucleotide homology and Phylogenetic analysis [32].

5. ACKNOWLEDGEMENT

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Conflict of interest

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

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