Remediation of Mixed Heavy Metals using Acido-tolerant Bacterial co-cultures

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Abstract

Background: Contaminated soils are common environmental problems throughout the world. Bioremediation is a process which helps to transfer the environment using microbes and alter the contaminants and alter the contaminants from a less hazardous to its original state within permissible limits. Objective: This present study focuses on the remediation of mixed heavy metals by acido–tolerant bacterial co–cultures. Methods: The removal of Heavy metals were analysed with UV spectroscopy and Atomic adsorption spectroscopy. Morphological identification by Scanning Electron Microscopy. The acido-tolerant cocultures were biochemically characterized and molecularly identified by 16 s r DNA Sequencing. Result: The isolated bacterial co-cultures could remove mixed metals (Cr and Zn) at 50 mg/L concentration was observed to be 81% and 80.5% for Chromium and Zinc respectively. The Acido- tolerant bacterial co-cultures consisted of two strains, which were identified through biochemical tests and 16s rRNA sequencing as Paracoccus sereniphilus and Paracoccus haeundaensis. The isolated bacterial co-cultures could remove Mixed metals (Cr and Zn) at the concentrations of 50 mg/L to 500 mg/L concentrations. The concentration that showed maximum removal was observed at 100 mg/L up to 76% for both Chromium and Zinc respectively. The isolated Acido-tolerant bacterial co-cultures could remove mixed metals (Cr and Zn) at pH 4.5 at 35°C. The growth of the bacterial co-culture and the removal of mixed metals (Cr and Zn) on supplementing with different carbon showed maximum removal with Lactose up to 82% and 78.56% for Chromium and Zinc respectively. Among nitrogen sources used in the present study, potassium nitrate could enhance the growth of the bacterial co-cultures showing the removal of Mixed metals (Cr and Zn) 82% for Chromium and 79.9% for Zinc. Conclusion: Thus such isolated acido-tolerant bacterial co-culture appears to have great potential for bioremediation of mixed metals from the contaminated sites.

Keywords: *Mixed Heavy Metals, Bioremediation, Acidotolerant, Co-cultures, Anthropogenic, Chromium, Zinc*

I. INTRODUCTION

Heavy metal contamination in the environment is of huge concern globally due to their threat to human life and environment. This is mainly due to natural and anthropogenic sources. Rapid industrialization led to polluting the environment and causing severe degradation in pedosphere, hydrosphere and atmosphere (Paranthaman and Karthikeyan, 2015). Heavy metal pollution occurs directly by effluent outfalls from industries, refineries and waste treatment plants and indirectly by the contaminants that enter the water supply from soil/ground water systems and from the atmosphere via rain water (Vieira and Volesky, 2000).

The heavy metals mostly found in contaminated sites include cadmium, chromium, cobalt, copper, manganese, mercury, nickel, silver, zinc, arsenic and lead (Gawali *et al*, 2014). The heavy metals can also cause adverse effect on humans. Some of the metals are mostly at low concentrations but when the concentration level exceeds the desired amount or increases it becomes toxic (Garbisu and Alkorta, 2003). Chromium (VI) is a heavy metal ion release into the environment mainly due to chrome tanning processes, electroplating, paint and pigmenting manufacturing industries. Compared to trivalent chromium, hexavalent chromium is highly toxic, mutagenic and carcinogenic (Sangeetha *et al*., 2012). Zinc (Zn) is an essential micronutrient and that has been shown to be essential for growth, at excessive levels it is potentially harmful (Chui Wei *et al.*, 2011*)*.

The need for the industrial waste treatment has increased due to environmental problems. Conventional process has been considered for the treatment, but the industries has to invest large sums of money and also it will be a barrier in eco-friendly way, hence the need for biological process for waste treatment. The other disadvantages in physio-chemical treatment or conventional method includes high operational costs due to the chemicals used, high-energy consumption and handling costs for toxic sludge disposal (Gunatilake, 2015).

Bioremediation comprises the use of plants or microorganisms, non-viable or viable, natural or genetically engineered to treat environments polluted with organic molecules that are problematic to break down (Xenobiotics) and to mitigate toxic heavy metals, by altering them into elements with little or no toxicity (Bahig and Abdullah, 2009). The microorganisms may be indigenous to the contaminated area or they may be isolated from

elsewhere and brought to the contaminated site (Kumar *et al*., 2011).

Bioremediation is of two types, in situ bioremediation involves the treatment of contaminants where they are located where the microorganisms come into direct contact with the dissolved and sorbed contaminants and use them as substrates for transformation. Ex situ bioremediation involves utilization of specially constructed treatment facility and is more expensive than in situ bioremediation (Satinder *et al*., 2006).

The microbes mostly employed are the ones isolated from almost any environmental conditions and can adapt and grow at subzero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen and anaerobic conditions, with the presence of hazardous compounds or on any waste stream (Gosa Girma, 2015). They have also progressed diverse approaches to overcome the toxic effects of metals and metalloids, utilizing accumulation, resistance or, more interestingly, by reducing their bio-availability or toxicity through bio-methylation and transformation (Abhijit *et al*., 2016).

Mesophilic bacteria are those organisms which grow at prevailing room temperature, i.e. 28-37ºC. The most popular mesophilic strain which is widely used is *Acidothiobacillus ferroxidans*. It derives energy for its growth by oxidizing Fe (II) to Fe (III) and sulfur, sulphide and different oxyanion of sulphur to sulphate (Valdes *et al*, 2008). Most of the strain in mesophilic bacteria showed a temperature range of 28-37ºC as its optimal growth condition (Debraj and Young, 2010).

Microorganisms which can grow in extreme climatic conditions are called extremophilic bacteria. Extremophiles mostly includes Thermophiles, Alkaliphiles, Barophiles, Acidophiles, Psychrophiles and Halophiles. Conventional microorganisms existing in natural environment will not have the ability to grow in acidic pH and degrade or remove such complex heavy metals. When the mesophilic bacterium fails in the removal of these heavy metals there is a need for exploring organism from extreme environment. Acidophilic strains are extremophiles which can grow in an environment with acidic pH.

Hence this present study aims on the removal of mixed heavy metals by acido–tolerant bacterial co-cultures which were isolated from contaminated site. A batch study was conducted with different concentrations of the mixed heavy metals with the co-cultured bacterial strains. Their growth was optimized by enhancing parameters like pH, temperature, NaCl, carbon and nitrogen sources. Finally, the acido-tolerant bacterial co – cultures were biochemically characterized and identified using molecular techniques. Such isolated co-cultures would be of great importance in the remediation of ex-situ environment.

II. MATERIALS AND METHODOLOGY

A. Sample Collection

Soil samples were collected from Pallavaram tannery effluent treatment company. Soil samples were collected and transferred to sterile zip lock covers and immediately transported to the laboratory.

B. Acclimatization and Isolation of Individual Cultures

About 1 gram of the soil sample was weighed and aseptically inoculated into 100 ml of 9K medium (Ammonium sulphate 3g/L; K_2 HPO_4 0.5 g/L; $MgSO_4.7H_2O$ 0.5 g/L; $KCl_2$0.1 g/L; $Ca(NO_3)_2$ 0.01 g/L; $FeSO_4.7H_2O$ 45 g/L) containing mixed metals i.e 50 ppm of Potassium-di-chromate and Zinc nitrate and in a sterilized 250 ml conical flask in a shaker at room temperature. The medium was maintained at pH 4.5 to isolate acido-tolerant co-cultures.

Pure cultures were isolated by platting it on the nutrient medium and autoclaved at 15 psi at 121ºC for 15 minutes. Pour plate was done on sterilized petri plates and incubated for 24 hours under room temperature. Further screening is done by platting it on nutrient agar medium supplemented with 50 ppm of Potassium-di-chromate and Zinc nitrate.

C. Total Protein Content of Bacterial Consortium

For analysis of total cell protein, samples were centrifuged at 10,000 rpm for 10 mins and washed with fresh mineral medium, then centrifuged and washed few times to remove the substrate. The pellet from each sample was then disrupted by sonification at 30% amplitude for a total of 3 minutes on an ice-water bath. Sample (0.5 ml) was added to 0.5 mL Coomassie Blue protein dye and the absorbance were measured at 595nm. The total protein concentration was determined by calibration with bovine serum albumin standard according to Bradford (1976).

D. Measurement of Removal of Mixed Metals (Cr and Zn) by Bacterial Co-Cultures

Removal of Chromium: Removal of chromium by bacterial consortium was observed till 72 hour. Estimation of chromium removal by Diphenylcarbazide method: The Reagent used for the estimation of chromium was 1,5-Diphenylcarbazide – 500 mg of 1,5-Diphenyl Carbazide was mixed in 100 ml of acetone and the pH was adjusted to 2±0.5 by adding 10% H_2 SO_4. To obtain a standard graph 10 ppm to 100 ppm of potassium-di-chromate was taken in a 100 ml volumetric flask and one flask with no chromium was served as blank. 10 ml of 5% H_2 SO_4 was added to the flasks and diluted to 40 ml. 4 ml of Diphenyl carbazide was added to this and diluted to mark with 5% H_2 SO_4. Absorbance was measured after 5 mins at 540 nm. 5 ml of culture (0 to 72 h)

was taken in a sterilized 1.5 ml vials and it was centrifuged at 10,000 rpm for 5 minutes. 1 ml of supernatant was taken in a sterilized test tube and 9 ml of distilled water was added to it. To this 1 ml of 1,5-diphenyl carbazide was added and shaken immediately. The absorbance was measured at 540 nm (Calomiris et al., 1984).

E. Optimization of Growth Condition for Removal of Mixed Metals by Bacterial Co–Cultures

Removal of Zinc: Removal of zinc by bacterial consortium was observed till 72 hour. Estimation of zinc removal by 3-hydroxybenzylamino benzoic acid method: the Reagent used for the estimation of zinc is 3-hydroxybenzylamino benzoic acid-1g of 3-hydroxybenzaldehyde was dissolved in 25 ml of double distilled water and mixed in a flask with 4 – aminobenzoic acid and refluxed for 3 hour. A pale yellow crystal product formed which is filtered and dried at room temperature which is re-crystalized using ethanol. To obtain a standard graph, 1 ml of 100 ppm to 500 ppm of zinc nitrate solution was taken in a volumentric flask and one flask with no chromium. 3 ml of buffer was added and 2 ml of 3–hydroxybenzylaminobenzoic acid was added. The aqueous phase was brought up to 10 ml by double distilled water. The absorbance was measured at 460 nm. 5 mL of culture (0 to72 h) was taken in a sterilized 1.5 vials and it was centrifuged at 10,000 rpm for 5minutes. 1 mL of supernatant was taken in a sterilized test tube and 3 mL of buffer and 2 mL of 3-hydroxybenzylaminobenzoic acid. The aqueous phase was brought up to 10 mL by double distilled water. The absorbance was measured at 460 nm (Kiran, 2012).

F. Effect of pH and Temperature On the Removal of Mixed Metals (Cr And Zn)

In order to study the effect of pH and temperature**,** the sterilized 100 ml conical flask was taken with sterilized 9K medium supplemented with 50 ppm of Potassium-di-chromate and 50 ppm of Zinc Nitrate. The media was maintained from pH 3 to pH 5. A volume of 1mL of overnight culture was inoculated in the flasks and incubated in a shaker at 37°C. The effect of temperature was studied by inoculating overnight culture and incubating in a shaker at 25ºC, 35ºC, 45ºC and 55ºC with medium maintained at pH 5. Protein estimation was done at 595 nm from 0^{th} day till 5^{th} day. Determination of mixed metals: Chromium was measured by Diphenyl carbazide method (Calomiris *et al*, 1984); Zinc was estimated by 3-hydroxybenzylaminobenzoic acid (Kiran, 2012).

G. Effect of Carbon and Nitrogen Sources on the Removal of Mixed Metals (Cr and Zn)

The effect of carbon sources was studied using various compounds, such as, Sucrose, Lactose and Mannitol, at a concentration of 1% and they were added individually as a supplement to the 9K medium for the removal of mixed metals (Cr and Zn). A volume of 1mL of overnight culture was inoculated in the flasks and incubated in a shaker at 37°C. Nitrogen sources, such as Sodium nitrite, Potassium nitrate and yeast extract were added to 9K medium at a concentration of 1% and 1 mL of overnight culture was incubated at 37°C. Protein estimation was done at 595 nm from 0^{th} day till 5^{th} day. Determination of mixed metals Chromium was measured by Diphenyl carbazide method (Calomiris *et al*, 1984) and Zinc was estimated by 3-hydroxybenzylaminobenzoic acid (Kiran, 2012).

H. Characterization and Identification of Bacterial Co-Cultures by SEM Analysis

The sample preparation for Scanning Electron Microscopy (SEM) was carried out according to the method of Prior and Perkins (1974). The isolated bacterial and yeast strains were grown individually on MSM for 24 hours. The bacterial strains in the Mineral Salts Medium were centrifuged at 8000 rpm for 10 minutes and the pellets were immediately re – suspended in 2% Glutaraldehyde with 0.05 M phosphate buffer and 4% sucrose (pH – 7.3). Fixation was obtained overnight at 4°C. After 24 hours the pellets were centrifuged at 8000 rpm for 10 minutes, washed 4 times with distilled water and placed on aluminium foil. The samples were then dehydrated with series of gradient ethanol (10%, 20%, 30% till 90%) air dried and finally the dried flakes were analysed under the Scanning Electron Microscope (FEI Quanta 200 F).

I. 16S rRNA Partial Gene Sequencing

Pure genomic DNA was isolated from single and pure (without contamination with other microorganisms) by Spin Column Method. Using consensus/ universal oligos, the ~1.5 kb 16S rDNA fragment from isolated rDNA, was amplified using *Taq* DNA polymerase enzyme. The PCR product was run on Agarose gel and the specific band was excised using sterile sharp knife/ cutter to purify through Spin Column Method using Gel DNA extraction Kit. The PCR conditions used were an initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one minute, and a final extension at 72°C for 10 minutes and the purified extension products were separated in the ABI 3730xl DNA Analyzer by capillary electrophoresis. Sequence data analysis was done using BLAST. The output file of the sequence alignment was used to compute phylogenetic trees for aligned sequences of 16s r-DNA sequencing results of the bacterial. Neighbour-joining method was used for tree building with MEGA 6.0 software. To access the reliability of the phylogenetic tree, MEGA provides bootstrap test which used the bootstrap

resampling strategy. The user has to input the number of replicates. In this experiment, 500 replicates were used.

III. RESULTS

A. *Screening and Isolation of Bacterial Consortium*

Soil samples were collected from heavy metal contaminated site, which were enriched with mixed metals to isolate Chromium and Zinc utilizing acido-tolerant bacterial co-cultures. During isolation period, several (seventeen) bacterial strains were isolated from the medium which could grow on heavy metals (50 mg/L). After successive transfer during enrichment period, only two acido-tolerant bacterial strains co-existed and were named (RMKVG1, RMKVG2), which could survive and remove Chromium and Zinc as a sole source of carbon and energy. Figure 1 shows the acido-tolerant bacterial strains utilizing Chromium and Zinc on nutrient agar in 10^{-6}dilutions.

The growth pattern of the co-cultures was monitored at every 24 hour's interval. The Figure 2 depicts that there was an increase in the total protein from 0^{th} day (4.5 mg/mL) and by the end of 4^{th}day, there was decrease in total protein (3.2 mg/ml) indicating that the bacterial co – cultures could not survive after 5^{th} day. The Chromium at 50 mg/l of concentration showed maximum removal i.e up to 47.56% by the end of 24 h and the final degradation that is on 5^{th} day showed 81% removal respectively. The removal of Zinc for the concentration 50 mg/L was 33.6% by the end of 24 h and for the 5^{th} day the maximum removal was up to 80.5%. Hence for all the experiments the total protein and the removal of Chromium and Zinc was checked up to 5^{th} day.

B. *Removal of Various Concentrations of Mixed Metals (Cr and Zn) by Acido-Tolerant Bacterial Co-Cultures*

The ability of bacterial co–culture to use Chromium and Zinc as sole source of carbon and energy was studied at 100 mg/L to 500 mg/L (100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L concentrations) of both Chromium and Zinc. Chromium at 100 mg/L of concentration showed maximum removal i.e up to 42 % by the end of 24 h and the final degradation being 76.23 % by the end of 5^{th} day. This was followed by 200 mg/L of Chromium concentration showing degradation up to 62 %, for 300 mg/L the removal of Chromium was up to 53.6 %, the Chromium removal was up to 42 % at 400 mg/L and the maximum Chromium removal for 500 mg/L was observed to be 29 % on 5^{th} day. Zinc at 100 mg/L of concentration showed maximum removal is up to 42.3 % by the end of 24 h and the final degradation being 76 % by the end of 5^{th} day. This was followed by 200 mg/L of Zinc concentration showing degradation up to 72.3%, 300 mg/L of zinc concentration was up to 52%, for 400 mg/L the degradation was upto 40.67 % and for 500 mg/L the maximum Zinc removal was observed to be 45 % on 5^{th} day.

The maximum protein content observed in 100 mg/L during 24 h was 4.8 mg/ml to 48 hour was 5.6 mg/ml of incubation and on the end of the 4^{th} day the protein content was 3.4 mg/ml. Hence from the Figure 3 it was observed that as the concentration of Chromium and Zinc were increased there is decrease in the protein content of the bacterial co-cultures and also decrease in the removal of the heavy metals.

Optimization of growth conditions on the removal of Mixed metals (Cr and Zn) by the Acido-tolerant bacterial co-cultures

C. *Effect of Ph On the Removal of Mixed Metals*

The study was carried out to determine the effect of pH (pH 3 to pH 5) on the removal of Chromium and Zinc at 50 mg/L concentration. The Figure 4 predicts the protein content of bacterial co–cultures at different pH from pH 3 to 5, where maximum removal of Chromium was achieved at pH 4.5 showing up to 90 % and for Zinc removal maximum concentration was observed to be 81.3% at pH 4.5 at the end of 5^{th} day. The total protein was found to be 6.3 mg/ml and 7.5 mg/ml by the end of 2^{nd} day at pH 4.5. The bacterial co–cultures were able to remove Chromium and Zinc at optimum pH 4.5.

D. *Effect of Temperature on the Removal of Mixed Metals*

To determine the effect of temperature on the growth of bacterial co–cultures a study was conducted at different temperatures (25°C, 35°C, 45°C and 55°C) at optimum concentration of 50 mg/L and pH 4.5. The Figure 9 predicts at 35°C there was maximum removal of Chromium up to 89 % by the end of 5^{th} day and for Zinc maximum removal was seen at the same temperature up to 83.67 %. The total protein was found to be 6.4 mg/ml by the end of 2^{nd} day. There was not much difference seen in the removal of Zinc and Chromium at temperature 25°C. But it was observed that there was a decrease in the bacterial co–cultures at 55°C which proved that the co-culture could grow optimally at 35°C, higher temperature inhibited the growth of the acido-tolerant co-cultures.

E. *Effect of Carbon Sources in the Removal of Mixed Metals*

To examine the influence of carbon sources on the removal of Chromium and Zinc at optimum concentration of 50 mg/L, carbon sources like Sucrose, Lactose and Mannitol were supplemented in the medium along with Chromium and Zinc. The Figures 6 shows that, almost all the carbon sources were able to enhance the removal Chromium. While

in the presence of lactose as carbon sources the Chromium removal was up to 82% and for Zinc the maximum removal was up to 78.56 %. This was followed by Mannitol were Chromium removal was up to 70.5 % and for Zinc maximum removal was up to 80.45%. By using Sucrose as the carbon source, Chromium removal was up to 69.4 % and for Zinc showed removal was up to 69.45%. Maximum total protein was observed in Lactose (5.6 mg/ml) supplemented media followed by Mannitol (4.6 mg/ml) and sucrose (4.3 mg/ml). Lactose as the carbon source showed maximum removal up to 82% and Zinc removal up to 78.5 %.

F. Effect of nitrogen sources on the removal of Mixed metals

To examine the influence of nitrogen sources on the removal of Mixed metals (Cr and Zn) at 50 mg/L concentration, various nitrogen sources like Yeast Extract, Sodium Nitrite and Potassium Nitrate were supplemented in medium along with Mixed metals. Figure 7 shows that the removal of Chromium was maximum seen in Potassium Nitrate showing maximum removal up to 82%, followed by Sodium Nitrite up to 72.1 % and Yeast Extract up to 70.1 % by the end of 5th day. For Zinc, maximum removal was observed in Potassium Nitrate up to 79.9 %, followed by Sodium Nitrite up to 70.14 % and Yeast Extract up to 69.4 %. Removal of Mixed metals (Cr and Zn) showed maximum removal with Potassium Nitrate as the nitrogen source. Maximum total protein by the co-cultures proves that Potassium Nitrate as the nitrogen source showed maximum growth (4.9 mg/ml)

G. SEM Analysis of the Acido – Tolerant Bacterial Strains

SEM analysis was carried out to determine the morphological structures of bacterial strains. The ultra structure of bacterial strains was observed under 60,000 X and 80,000 X magnifications. Figure 13 shows that the structure of the bacterial strain RMKVG1 and RMKVG2 was observed under 80,000 X and 60,000 X magnifications respectively. It was seen that both the strain showed coccus shaped morphology. The genomic DNA was isolated from each bacterial isolates. PCR amplification was performed and the unknown bacterial strains were identified through 16s rDNA sequencing. The bacterial isolates were identified from the sequence using BLAST tool. Figure 9 represents the Phylogenetic tree computed based on 16s r-DNA sequencing results of the two strains. RMKVG1 and RMKVG2 were identified as *Paracoccus serriniphilus* and *Paracoccus haeundaensis* respectively.

IV. DISCUSSION

Heavy metal contamination refers to the excessive deposition of toxic heavy metals in the soil caused by human activities. Most soils of rural and urban environments may accumulate one or more of the heavy metals above defined background values high enough to cause risks to human health, plants, animals, ecosystems, or other media. Hence a need for treating these contaminated soil (Chai Su *et al*, 2014). Bioremediation is an emerging technology, which utilize the potentiality of microorganism to degrade the chemical compounds which are toxic in nature. Bacterial consortia and bacterial strains were used for the removal of heavy metals from the contaminated soil (Rajendran, 2003).

Ebtesam *et al* (2013) isolated *Enterobacter sp.*, *Streptrophomonas sp.*, *Comamonas sp* and *Ochrobactrum sp* were able to resist mixed metals such as 275 mg Cu/L, 320 mg Cd/L, 140 g Co/L and 29 mg Cr/L respectively. In the present study maximum degradation was seen only in 100 mg/L of mixed metals i.e Cr up to 76.23% and Zn up to 76% respectively. As the concentration of mixed metals (Cr and Zn) increased up to 500 mg/L there was decrease in the growth of the bacterial co-cultures and in the removal of the mixed metals. Merina *et al* (2016) isolated *Enterobacter sp*. and *Klebsiella sp*., were able to remove lead maximum up to 78 % and 85 % at pH 4 and temperature 31°C by 48 hours. In the present study the bacterial co-cultures isolated were able to remove mixed metals (Cr and Zn) at pH 4.5 and temperature 35°C were 89% for Chromium and 83.67% for Zinc by the end of 5th day, which show the acido-tolerant nature of the isolated bacterial co-cultures.

Ashwini *et al* (2016) reported on the effects of carbon source (1% Fructose, Lactose, Glucose and Mannitol) on the bacterial consortium by the removal of Chromium. The bacterial consortium that has grown maximum and removal of Chromium was seen in Fructose up to 97.85%. This was followed by Mannitol, Lactose and Glucose having 97.7%, 96.77% and 96.45% respectively. In the present study, among the carbon sources used (1% of Lactose, Sucrose and Mannitol) maximum growth and removal of mixed metals (Cr and Zn) by the bacterial co-cultures were seen in Lactose up to 82% of Chromium and 78.56% of Zinc removal. This was followed by Mannitol and Sucrose 70.5% and 69.4% for Chromium and 80.45% and 69.45% for zinc removal respectively.

Among nitrogen sources (1% of Yeast extract, Sodium nitrite, Potassium nitrate and Ammonium nitrate) used in the study by Ashwini *et al* (2016) for the optimization, Yeast extract showed maximum removal of Chromium up to 96.77% by the end of 5th day. In the present study Potassium nitrate showed maximum removal of Mixed metals (Cr and Zn) up to 82% for Chromium and 79.9% for Zinc. Qiuzhuo Zhang *et al* (2014) isolated *Pseudomonas*

putida, Cupriavidus necator, Exiguobacterium sp., Bacillus aquimaris, B. cereus, and Alcaligenes sp. from Yangtze River in china to study the level of tolerance to the heavy metals. In the present study, bacterial co-cultures were isolated from heavy metal contaminated soil to test its tolerance against mixed metals and through 16s rDNA sequencing results, they were identified as *Paracoccus serinipHilus* (RMKVG1) and *Paracoccus haeundaensis* (RMKVG2).

V. CONCLUSION

Heavy metals are the major source of contamination of soil through anthropogenic sources. Mostly heavy metals in the contaminated sites are mixed metals and the commonly found metals in the soil are Chromium and Zinc. These metals are highly toxic to the environment and poses severe threat to the biological systems. Hence there is a need for the removal of mixed metals (Cr and Zn) from the contaminated soil in an eco-friendly manner. The use of micro-organisms as co-cultures in the removal of mixed metals is safe, cost effective and eco-friendly methods used for removing such hazardous compounds. From this study, it can be concluded that the enriched bacterial co-cultures could be used for the removal of Mixed metals (Cr and Zn) at optimum concentration of 50 mg/L at pH 4.5 and temperature 35°C. Supplementing the media with Lactose as carbon source and Potassium Nitrate as nitrogen sources could be greatly enhanced the growth and removal of mixed metals (Cr and Zn) by bacterial co-cultures. On the basis of these results, bacterial co-cultures can be effectively applied in the biological systems for the effective removal of Mixed metals (Cr and Zn) from heavy metal contaminated site.

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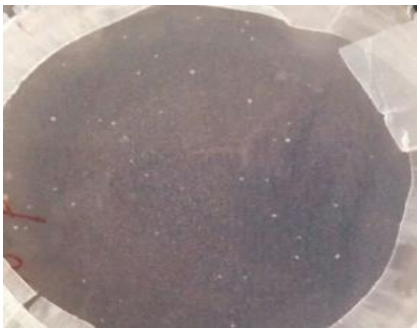

Figure 1 Enriched Mixed Metals, Utilizing Acido-Tolerant Bacterial Co-Cultures on MSM

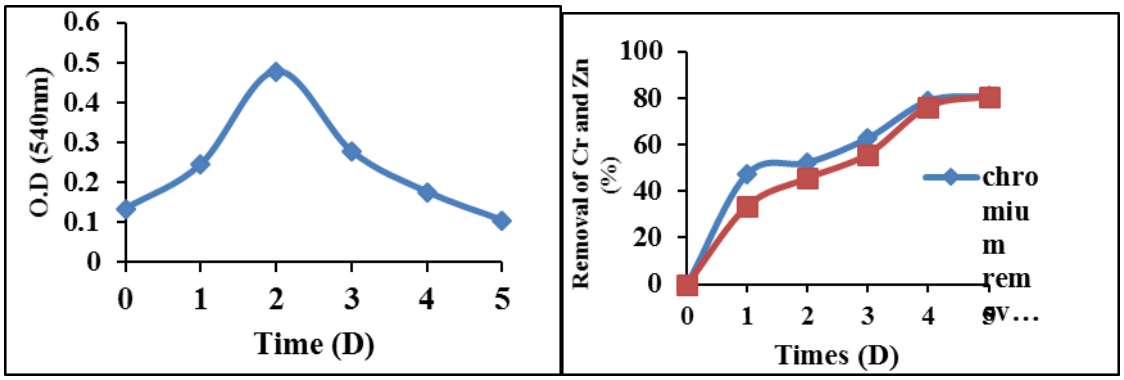

Figure 2 Growth Pattern and Removal of Mixed Metals (Cr And Zn) by Acido-Tolerant Bacterial Co-Cultures During Enrichment Period

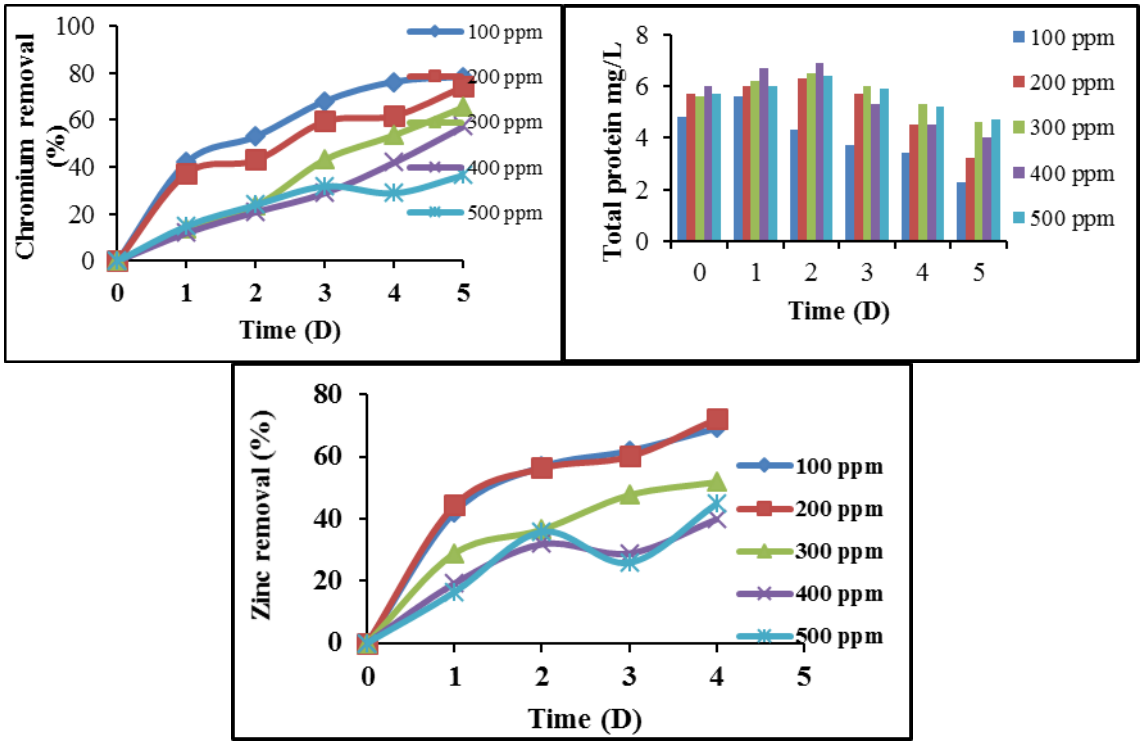

Figure 3 Removal of Different Concentrations of Mixed Metals (Cr And Zn) and Total Protein Content by the Acido-Tolerant Bacterial Co-Cultures

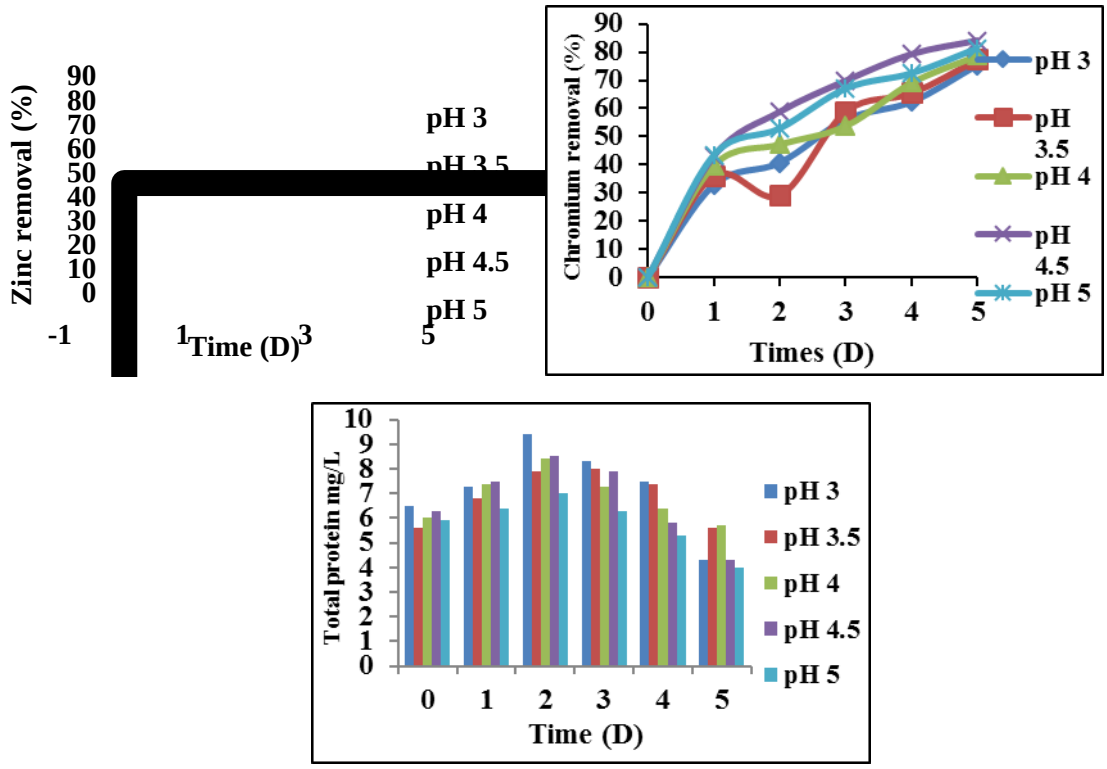

Figure 4 Effect Of Ph on the Removal of Mixed Metals (Cr And Zn) and Total Protein by Acido-Tolerant Bacterial Co-Cultures

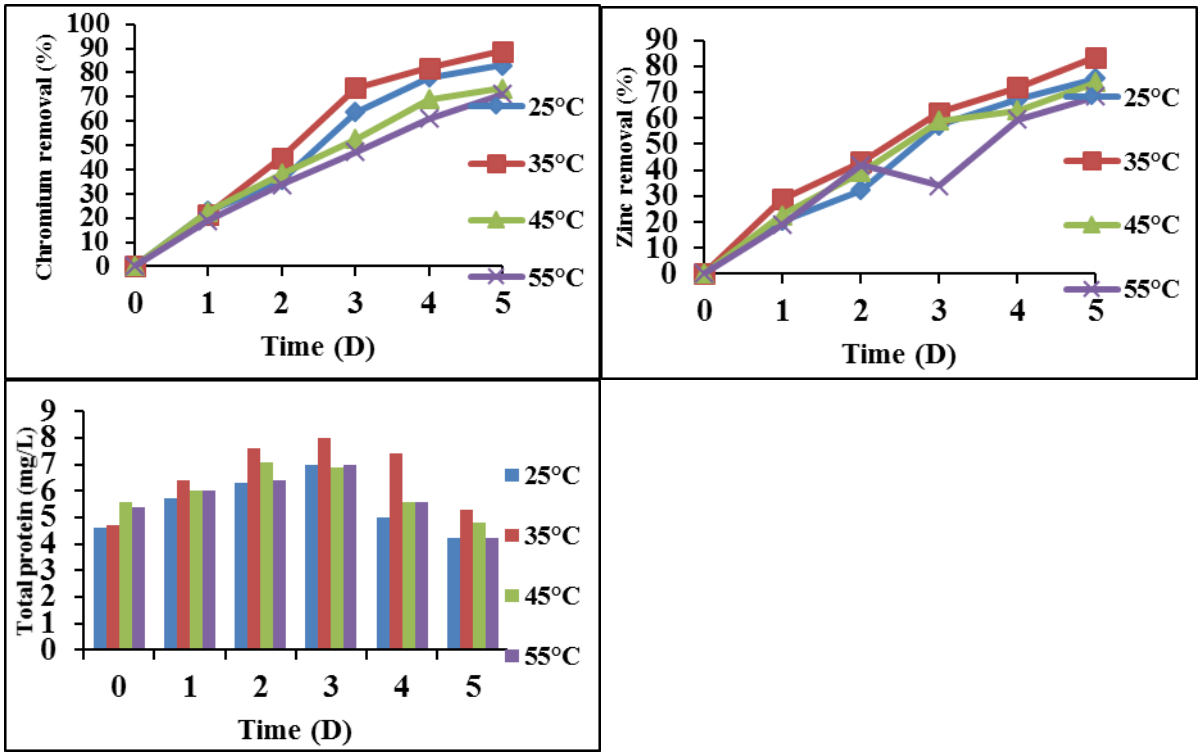

Figure 5 Effect of Different Temperatures on the Removal of Mixed Metals (Cr And Zn) and Total Protein by the Acido-Tolerant Bacterial Co–Cultures

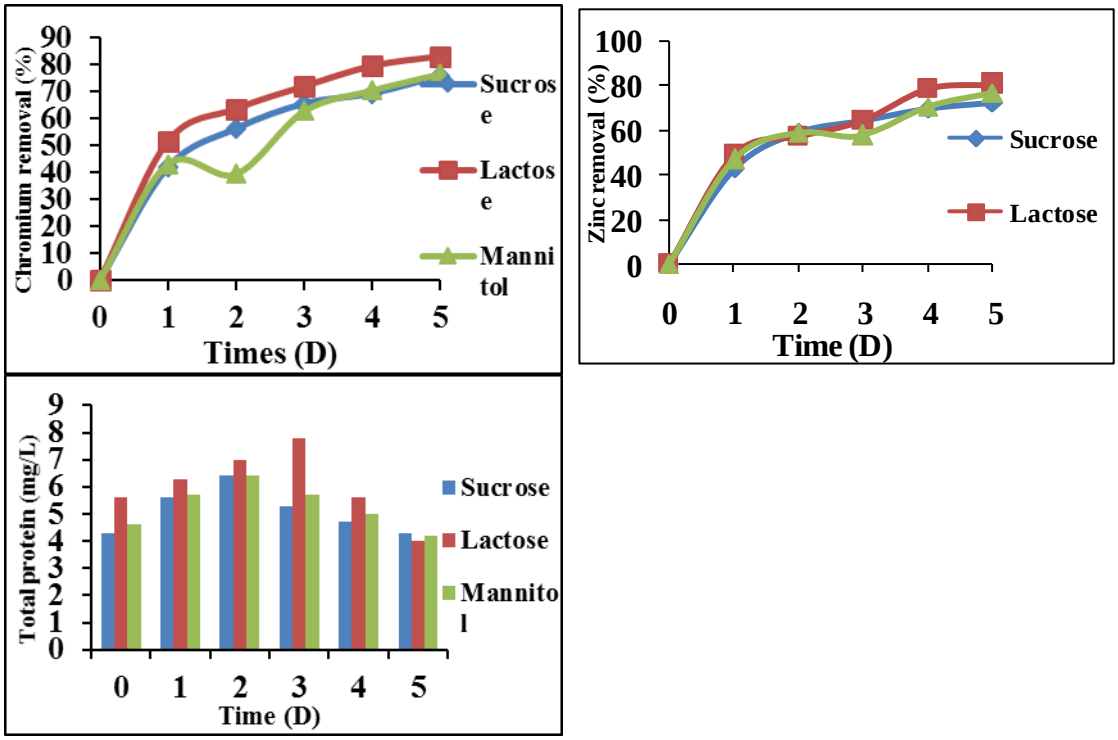

Figure 6 Effect of Carbon Sources on Removal of Mixed Metals (Cr And Zn) and Total Protein by the Acido-Tolerant Bacterial Co–Cultures

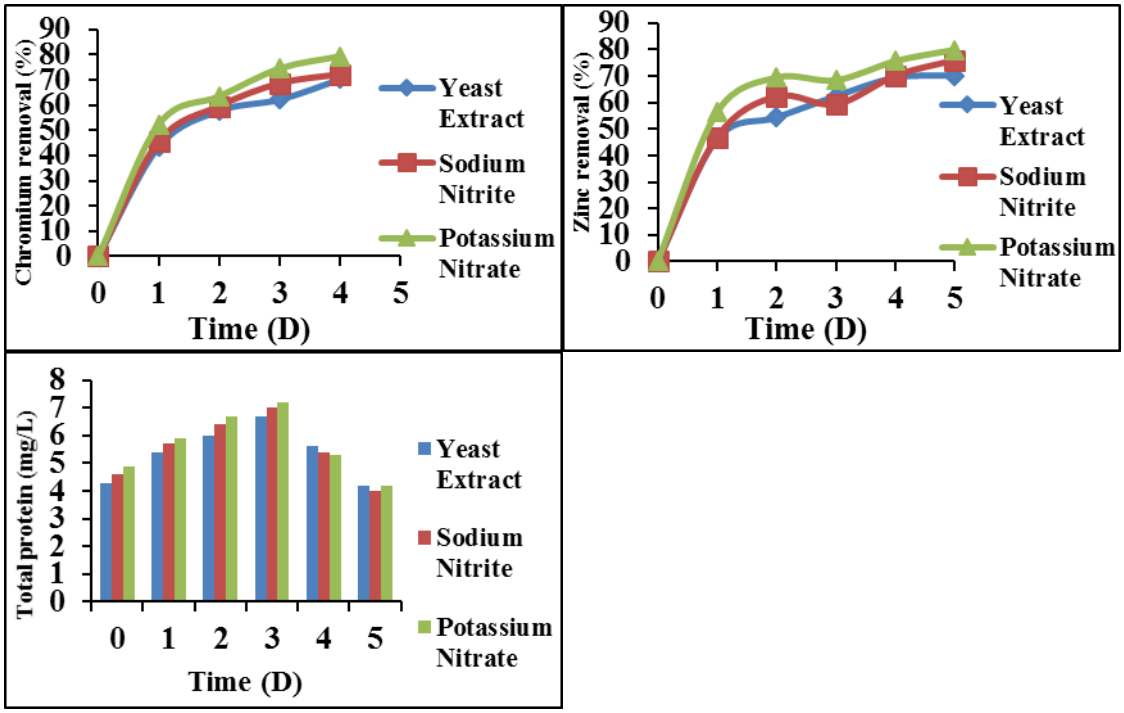

Figure 7 Effect Of Nitrogen Sources on the Removal of Mixed Metals (Cr And Zn) and Total Protein by the Acido-Tolerant Bacterial Co–Cultures

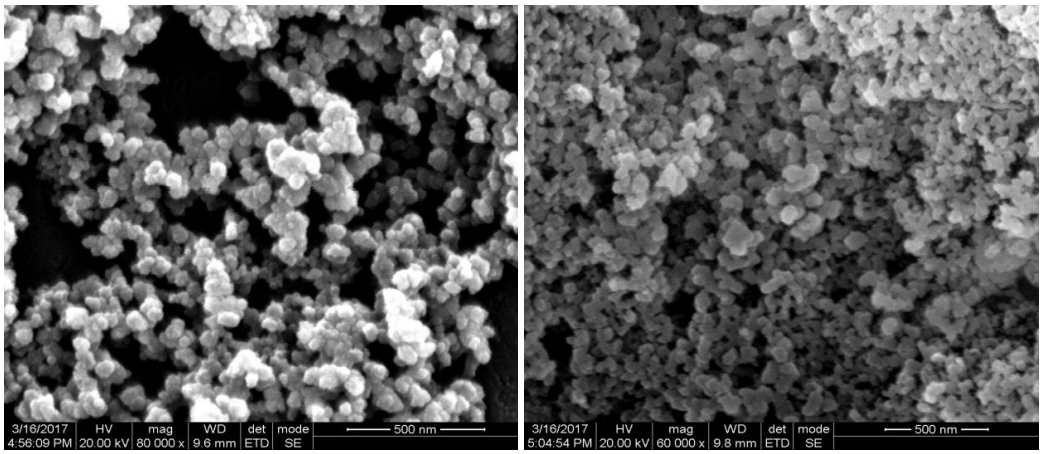

Figure 8 Shows the Morphology of the Bacterial Strains RMKVG1 (Above) and RMKVG2 (Below) Under 80,000 X and 60,000 X Magnifications Respectively

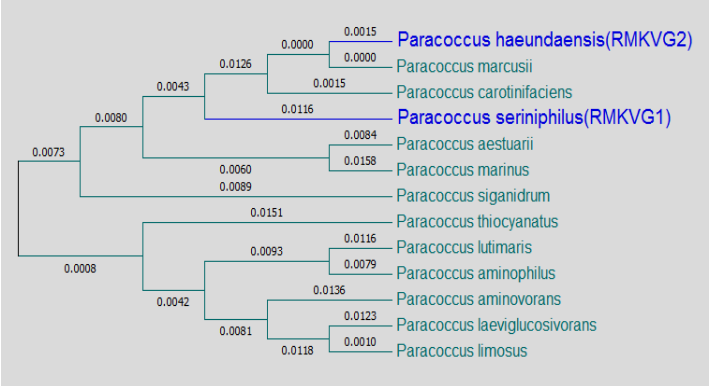

Figure 9 Phylogenetic Tree of the Isolated Bacterial Strains Identified Through 16s R-DNA Sequencing Showing Evolutionary Relationship