Isolation of Bacteria from Waste water Resistant to Heavy Metals

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Abstract: The basic objective of the study was to utilize our available resources and time in a proper way to study and isolate those strains of bacteria that are found to be resistant to heavy metals. Total amount of Co was determined by High Pressure Liquid Chromatography (HPLC Perkin Elmer model). Samples were run through reverse phase C18 column by using 66% acetonitrile in water (mobile phase) at a flow rate of 0.800 mL. Cobalt was detected by their absorbance in the UV range at wave length(?) of 211 nm. Volume of the samples injected was 20 µL. Growth of bacterial strain was determined by taking OD at 600nm of bacterial cultures grown for 24 hours in different concentration (0, 300, 400 and 500µgmL−1) of Co. Red Bean seeds (stressed with different concentration of Co) were inoculated with our bacterial isolates. Control seeds were without inoculation and without any stress. It was noticed that the isolated reduced seed germination as compared to control in the absence of Co.

Key words: Heavy Metals • Bacterial Strains • Red Bean Seeds • Premier Sugar Mills • Waste Water

INTRODUCTION

Heavy metals are trace metals with a density at least five times that of water [1-3] and defined as the 53 metals with a density above 4-5 g/cm³. Kennish [4] classified heavy metals as elements having atomic weights between 63.546 and 200.590. Out of the 90 naturally occurring elements, 21 are nonmetals, 16 are light metals and the remaining 53 are heavy metals [5]. Although heavy metals comprise the major part of the elements, the understanding of the metabolism of heavy metals in biological systems and their biotechnological use are in their infancy [6].

Heavy metals in surface water systems can be from natural or anthropogenic sources, with anthropogenic inputs of metals exceed natural inputs. Surface runoff from mining operations usually has a low pH and contains high levels of metals such as iron, manganese, zinc, copper, nickel and cobalt. The combustion of fossil fuels pollutes the atmosphere with metal particulates that eventually settle to the land surface, urbanstorm water runoff often contains metals from roadways and atmospheric fallout [9]. Determination of copper concentrations in Greenland ice dated from seven millennia ago to the present, show values exceeding natural levels, beginning about 2500 years ago [9].

Availability of heavy metals in the cell must be carefully controlled due to their potential to form radicals and their tendency to bind to biological macromolecules [10]. Microorganisms use a number of mechanisms to maintain the correct equilibrium, including the uptake, chelation and extrusion of metals.

While some of the heavy metals are purely toxic with no known cellular role [11], other metals are essential for life at low concentration but become toxic at high concentration [12], high concentration of all the heavy metals inhibits the activity of sensitive enzymes. Out of 17 most important heavy metals Fe, Mo and Mn are classified to have low toxicity, Zn, Ni, Cu, V, Co, W and Cr are categorized to have average toxicity, while As, Ag, Sb,
Cd, Hg, Pb and U are grouped in highly toxic heavy metals. Wide range of essential cell components is potential targets for metal induced damage such as DNA for replication as a result of which cell death can occur [13].

Although microbial tolerance to heavy metals has been studied over the past 30 years, the last 15 years have been outstanding with respect to discoveries at molecular level. As a consequence, the amount of work and information is substantial.

Bacteria, being one of the most primitive life forms on earth, naturally developed tolerance to a wide range of toxic heavy metals including As, Cd, Co, Cr, Cu, Hg, Ni, Sb, Te and Zn [14, 15] in its genome. Copper, nickel, cobalt and iron are essential to bacteria because of their use as catalytic and structural elements in enzymes and other molecules. In many cases, the first response to toxic metal contamination is a large reduction in microbial activity [16]. This is confirmed by the fact that habitats that have had high levels of metal contamination for years still have microbial populations and activities that are smaller than the microbial populations and activities in uncontaminated habitats. Moreover, resistance mechanisms do not offer protection at extremely high levels of free metal ions and a lethal toxic effect is observed [17]. Copper is required for the function of several important enzymes in Escherichia coli including the cytochrome oxidase [18], copper-zinc superoxide dismutase and amine oxidase[19]. Essential metals for microorganisms as trace nutrients such as Zn, Co and Ni must be transported into cells against concentration gradient. Highly specific Ni uptake systems were found in R. eutropha [20]. Some bacteria have evolved mechanisms to detoxify heavy metals and some even use them for respiration. Microbial interactions with metals may have several implications for the environment. Microbes may play a large role in the biogeochemical cycling of toxic heavy metals also in cleaning up or remediating metal-contaminated environments.

Resistance to cobalt in Gram-negative bacteria always seems to be the byproduct of another heavy metal resistance, either to nickel [21] or to zinc. Co^{2+} is detoxified by RND-driven systems and exceptionally by a HoxN-type transporter in Gram-negative bacteria and by CDF-transporters in eukaryotes and Gram-positive bacteria [22].

The basic objective of this study was to isolate those strains of bacteria that are found to be resistant to heavy metals.

**MATERIALS AND METHODS**

**Preparation of Medium:** Media used to isolate, screen and maintain bacterial cultures was L-agar and L-broth (Tables 1 and 2). All glassware and media were autoclaved at 121°C at 15 lb/Inch for 15 mints. Stalk solution of heavy metal (Co) were prepared in distilled water and autoclaved.

**Isolation of Resistant Bacteria from Waste Water:** For isolation of Co resistant bacteria, waste water was collected from the main effluent drain of the Premier Sugar Mills located in Mardan. Samples were brought in sterile containers into Plant Physiology lab of the Department of Botany, Abdul Wali Khan University Mardan. To isolate bacterial strains, the samples were diluted by the procedure of serial dilution in sterile distilled water. Dilutions obtained (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were spread on the surface of LB agar (containing 300 μg mL^{-1} of Co) in Petri plates with help of spreader. Colonies obtained were picked and purified by many round of streaking.

**Determination of Cobalt:** Total amount of Co was determined by High Pressure Liquid Chromatography (HPLC Perkin Elmer model). Samples were run through reverse phase C18 column by using 66% acetonitrile in water (mobile phase) at a flow rate of 0.800 mL. Cobalt was detected by their absorbance in the UV range at wave length(λ) of 211 nm. Volume of the samples injected was 20 μL.

**Standard Curve:** To prepare standard curve of Co, LB broth was supplemented with 100 μg mL^{-1} to 500 μg mL^{-1} of this metal. 2 mL of samples were treated with 5 mL concentrated HNO_{3} for 24 hours. Samples were dried on water bath and reconstituted to 25 mL with dH_{2}O. Samples were run on C18 column at chromatographic conditions mentioned above. Concentration (Y) was plotted against peak area and equation of the straight line was derived by regression analysis of the data obtained.

**Recovery of Cobalt:** Percent recovery of the selected metal and matrix effect was determined simultaneously by comparing peak areas of metal processed as mentioned in the above section to the peak areas obtained for metal dissolved in dH_{2}O directly by the following equation:

\[ % \text{recovery} = \frac{\text{conc. of metal in LB media}}{\text{conc. of metal in LB media}} \times 100 \]
Table 1: Recipe of L- Agar Media

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Components</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tryptone</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Yeast Extract</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>NaCl</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>Agar</td>
<td>1.5/100ml</td>
</tr>
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</table>

Table 2: Recipe of L -Broth Media

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Components</th>
<th>gm/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tryptone</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Yeast Extract</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>NaCl</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 3: Recipe for nutrient solution

<table>
<thead>
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<th>S.N</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>KN03</td>
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<tr>
<td>2</td>
<td>Ca(No3)</td>
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</tr>
<tr>
<td>3</td>
<td>KH2PO4</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>MgSO47*H2O</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
<td>Fecl3</td>
<td>0.1</td>
</tr>
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</table>

Seed Germination: Red Bean seeds (Hybrid petal seeds) procured from Mardan was used for plant growth experiment. Seeds were washed before used. Seeds were surface sterilized with 0.1%HgCl and incubated with bacterial suspension in 10 mM MgCl, set at OD of 0.2 for two hours. Petri plates were washed, dried and filter paper was placed in each plate. The plates were autoclaved and labeled for each strain and for different concentration of cobalt (0, 500, 1000, 1500 µg mL⁻¹). Five seeds of bean (previously sterilized and inoculated) were placed in each Petri plate [23]. All plates were kept in dark at room temperature and seed germination was checked daily. Seeds dipped in dH₂O without bacteria or metal were taken as positive control. Seeds treated with metal solution without any bacterial were taken as negative control. Each treatment was replicated thrice and experiment was repeated twice.

RESULTS

Five CO resistant bacteria strains that could resist very high concentration of the metal were isolated from waste water (Table 4).

Minimal Inhibitory Concentration (MIC) of the Studied Metal: The lowest concentration that prevented bacterial growth was considered the MIC. For this purpose, bacterial strains were grown at different concentration (300, 400, 600, 1000 and 1500 µg mL⁻¹). It was noted that growth was gradually decreased with increasing metal concentration. Bacterial growth was determined in terms of OD at 600nm (Table 4). Decrease in OD below 0.1 was taken as considerable growth inhibition by the selected metal. Our isolates could resist up to 1500 µg mL⁻¹ of Co. Growth of the strains was inhibited by Co at this concentration. ODs of bacterial strains Co1, Co2, Co3, Co4 and Co5 were 0.0058, 0.0043, 0.0059, 0.0111 and 0.0075 respectively at 1500 µg mL⁻¹ of Cr₂O₃ in the media.

Metal Uptake by the Strains: Media was prepared with different concentration of Co(300, 400, 500 µg mL⁻¹). All strains were inoculated and harvested from overnight cultures. Growth of the strains was determined by taking optical density (OD) at 600nm. After finding optical density, the cultures were centrifuged at 12000 rpm for two minutes to harvest bacterial cells. Pellet (bacterial cells) was oven dried at 70°C for 48 hours and bacterial culture supernatant was kept in water bath for drying. 0.5 gm dry pellet was treated with 1 mL H₂SO₄ and 4 mLHNO₃ overnight. The next day samples were heated for one hour and filtered through Whatman No. 1 filter paper to remove debris. The filtrate was diluted with distilled water up to 25 mL. 10 mL concentrated HNO₃ was added to 50 mL of bacterial culture supernatant in a glass beaker. Volume of the mixture was reduced to 40 mL by heating on a water bath followed by filtration with Whatman No. 1 filter paper. Glass distilled water was added to the filtrate to make its final volume up to 50 mL. Bacterial culture supernatant form the same strain grown on metal few medium was used to prepare blank solution by the same procedure. Determination of the metal was carried out as described earlier.

Minimal Inhibitory Concentration (MIC) of Cobalt: To check resistant level of isolated strains toward Co, Broth was prepared with different concentration of Co(300 - 1500 µg mL⁻¹). After overnight incubation, OD of the cultures was taken at 600 nm.
Table 4: Growth of Co bacterial strains with different concentrations of Co

<table>
<thead>
<tr>
<th>S.N</th>
<th>Strain</th>
<th>300µg/mL</th>
<th>400µg/mL</th>
<th>600µg/mL</th>
<th>1000µg/mL</th>
<th>1500µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Co1</td>
<td>0.0923</td>
<td>0.1543</td>
<td>0.0813</td>
<td>0.1252</td>
<td>0.0058</td>
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<tr>
<td>2</td>
<td>Co2</td>
<td>0.1948</td>
<td>0.1634</td>
<td>0.2451</td>
<td>0.0088</td>
<td>0.0043</td>
</tr>
<tr>
<td>3</td>
<td>Co3</td>
<td>0.2243</td>
<td>0.2026</td>
<td>0.0161</td>
<td>0.0063</td>
<td>0.0059</td>
</tr>
<tr>
<td>4</td>
<td>Co4</td>
<td>0.2286</td>
<td>0.2324</td>
<td>0.0193</td>
<td>0.0816</td>
<td>0.0111</td>
</tr>
<tr>
<td>5</td>
<td>Co5</td>
<td>0.1356</td>
<td>0.1919</td>
<td>0.0455</td>
<td>0.0911</td>
<td>0.0075</td>
</tr>
</tbody>
</table>

Fig. 1: Concentration of Heavy metal in pellet and supernatant for bacterial strains in 300 concentration

Fig. 2: Concentration of Heavy metal in pellet and supernatant for bacterial strains in 400 concentration

of Co in their Biomass and left 72.66% in the culture media. At this 300µg/mL concentration the Co3 absorbed 13.33% of Co metal in their Biomass and left 86.66% in the culture media. 87.33% of this metal was removed from the culture media by Co4 leaving 72.66% behind at 300µg/mL concentration. The accumulated 16.66% of the metal at their Biomass and left 83.33% in the culture media at 300µg/mL.

Increasing metal concentration to 400µg/mL, Co1 isolate absorbed 12.5% of Co metal in their Biomass and left 87.5% in the culture media. The strain Co2 accumulated &2% of Co in from culture media containing 400µg/mL. Co3, Co4 and Co5 could absorb 92, 90 and 98% of the heavy metal from culture media supplemented with 400µg/mL concentration of the pollutant respectively (Fig. 2).

Increasing metal (500µg/mL) in the culture media reduced metal extraction ability of the strain to 10%. The Co1 accumulate 15.6% of Co in their Biomass and left 84.4% in the culture media at 500µg/mL. The strain Co2
Fig. 3: Concentration of Heavy metal in pellet and supernatant for bacterial strains in 500 concentration

Fig. 4: Optical density of Co bacterial strains with different concentrations (300, 400 and 500) and control

absorbed 92% of Co from the culture media containing 500µg mL⁻¹. Co3 accumulate 2% of the metal in its Biomass when grown in the presence of 500µg mL⁻¹. Reduction in its metal binding potential was recorded at higher concentration of Co. It could absorb 91 and 86% of the heavy metal from culture media at 500µg mL⁻¹ of the pollutant respectively (Fig. 3).

**Bacterial Growth Determination:** Growth of bacterial strain was determined by taking OD at 600nm of bacterial cultures grown for 24 hours in different concentration (0, 300, 400 and 500µg mL⁻¹) of Co. Values of optical density of bacterial growth with different concentrations (300, 400 and 500µg mL⁻¹) were shown on graph. Growth of Co1 at 0 concentration of Co was 0.4 (OD₆₀₀) after 24 hours of incubation. A decrease in comparison to control was noticed which was 1% in the growth of the strain when concentration of Co was increased to 400 and 500µg mL⁻¹. The strain Co2 growth in media having 300µg mL⁻¹ of Co was slightly lesser than the control and increase in the metal concentration could not cause further increase in the growth of this strain. For instance, growth of Co3 was greater in the presence of 300µg mL⁻¹ of Co than its growth in media containing 400µg mL⁻¹. 54% more growth than control was observed when culture media of Co3 was supplied with 300µg mL⁻¹ of this metal. Additional of more amount of this metal gradually reduced the growth. The strain Co4 showed a reduced growth in media containing 300µg mL⁻¹ of Co. However maximum decrease in its growth occurred when provided with 400µg mL⁻¹. Growth in media having 500µg mL⁻¹ of Co was slightly lesser than the previous concentration.
Growth of Co5 gained 47% more biomass in culture media containing 300µg/mL of Co than media having no additional Co. Trend of gradual reduction was observed in the growth of Co5 by further increasing the amount of this metal in the media (Fig. 4).

**Plant Growth Experiment**

**Seed Germination:** Red Bean seeds (stressed with different concentration of Co) were inoculated with our bacterial isolates. Control seeds were without inoculation and without any stress. Unstressed seeds inoculated with our isolates were also tested for judgment. Another comparison was made with seeds treated with metal but not inoculated with our strains. Control seeds (without bacteria) showed maximum germination in the absence of heavy metal contamination. Co harmfully affects the germination of seeds inducing up to 75% reduction in seed germination (Table 5). It was noticed that the isolated reduced seed germination as compared to control in the absence of Co. Although, reduction in seed germination was observable.

**Table 5: Germination %age of bacterial strain for Co**

<table>
<thead>
<tr>
<th>S.N</th>
<th>Strain</th>
<th>Germination %age</th>
<th>Absorbance</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 0</td>
<td>500</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Co1</td>
<td>100</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Co2</td>
<td>70%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>3</td>
<td>Co3</td>
<td>60%</td>
<td>30%</td>
<td>20%</td>
</tr>
<tr>
<td>4</td>
<td>Co4</td>
<td>80%</td>
<td>30%</td>
<td>30%</td>
</tr>
<tr>
<td>5</td>
<td>Co5</td>
<td>92%</td>
<td>40%</td>
<td>30%</td>
</tr>
</tbody>
</table>

**Discussion**

Heavy metals have established increasing consciousness in the last decades. The quantitative method as we used it in this paper has provided highly fruitful information and versatile results. In present study, we isolated 5 metal resistant bacterial strains from the polluted water of Premier sugar Mills Mardan for the evaluation of their bioremediation potential of toxic heavy metals. These bacteria could resist a properly evaluated of their bioremediation potential of toxic heavy metal polluted water of Premier sugar Mills Mardan for the study of their metal resistance. These strains were observed. Control seeds (without bacteria) showed maximum germination in the absence of heavy metal contamination. But reductions were observed in it with Co resistant bacteria strain. Under 500µg mL⁻¹ minimum bean seeds failed to germinate, suggesting a strong inhibitory effect of Co exposure. A 50% reduction was observed when compared with control. Bacteria are not exclusive in having to protect themselves against the toxic effect of metal. Thus a variety of tolerance and resistance mechanisms have developed, including avoidance or elimination, which minimizes the cellular accumulation of metals and tolerance, which
allows bacteria to survive while accumulating high concentration of metal. Metal (Co) was determined in the bacterial biomass as well as in the culture supernatant after growing our strains in metal supplemented media for 24 hours. To determine Co, the sample were run on reverse phase C18 column of HPLC and eluted with 66% acetonitrile. Five of our isolates were able to accumulate high amount of Co, suggesting their tolerance toward this metal. Bacterial isolate Co-1 accumulated 80% of Co in its biomass and left 20% in the culture media at 300 µg mL⁻¹. The amount of metal accumulation by the cell was directly proportional to the amount of initial metal supply in medium. It was found that amount of metal accumulation by the cell increased with increase in concentration of metal. Howlett and Avery [28] and Askwith and Kaplan [29].

**CONCLUSION**

It may be concluded that our isolated bacterial strains could tolerate a high amount of Co, showing their history of exposure to the said metal. Furthermore, their inhibitory effect on seed germination and plant growth indicated that the strains may not be suitable for treating agriculture lands contaminated with Co.

**REFERENCES**