BIOREMEDIATION OF HEAVY METAL POLLUTED ENVIRONMENT USING RESISTANT BACTERIA

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ABSTRACT
Heavy metals are the most hazardous pollutants present in industrial and domestic waste water. Microbe related technologies may provide an alternative method of metal removal. The goal of present study was to isolate heavy metal tolerant microorganisms and studied their heavy metal resistance potential. Heavy metal tolerant microorganism was isolated from sewage water collected from different localities (domestic, industrial and agricultural site) of Jabalpur, India. In present study, out of twenty five, only 4 bacterial isolates shows heavy metal resistance potential. These selected isolates were identified as Bacillus carotarum, Bacillus cereus, Bacillus lentus and Bacillus licheniformis according to Bergey’s manual of systemic bacteriology and PIB computer kit by using Bacillus identification matrix. Identity of the isolates was further confirmed at genetic level by DNA homology. Heavy metal tolerance test showed maximum microbial tolerance to Pb and minimum tolerance to Zn and Cr. Our results strongly recommended that the isolated Bacillus sp. has the properties to resist a wide range of heavy metals and antibiotics. These heavy metals may be harmful to human being as well as to the animals. The present investigation has widened the scope for research and development of metal tolerance and antibiotic resistance from bacterial origin. This heavy metal resistant organism could be a potential agent for bioremediation of heavy metals polluted environment.

Key Words : Heavy metal tolerance, DNA homology, Heavy metal resistance potential, Micro-organisms, Bioremediation

INTRODUCTION
The term Heavy metal is collectively applied to a group of metals (metal- like elements) with density greater than 5g/cm³ and atomic number above 20. These kinds of metals are directly related with environmental pollution and biological toxicity problems. Heavy metals are widespread pollutants of great concern as they are non-degradable and thus persistent. These metals are used in various industries from which effluents are consequently discharged into the environment. Some heavy metals are essential trace elements, most can be, at high concentrations, toxic to all forms of life including microbes, humans and animals. Lead (Pb) a major pollutant that is found in soil, water and air is a hazardous waste and is highly toxic to human, animals, plants and microbes.1 Hexavalent chromium (Cr (VI)) and trivalent chromium (Cr (III)) are the most prevalent species of chromium in the natural environment.2 Major sources of chromium pollution include effluents from leather tanning, chromium electroplating, wood preservation, alloy preparation and nuclear wastes due to its use as a corrosion inhibitor in nuclear power plants.3,4 Removal of excess of heavy metal ions from wastewater is essential due to their extreme toxicity towards aquatic life and humans.5-7 Bioremediation processes are very attractive in comparison with physico-chemical methods such as electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption for heavy metal removal techniques because they can have lower cost and higher efficiency at low metal concentrations. There are a number of bio- materials that can be used to remove metal from waste water such molds, yeasts, bacteria, and seaweeds.8,9 The ability of microbial stains to grow in the presence of heavy metals would
be helpful in the waste water treatment where microorganisms are directly involved in the decomposition of organic matter in biological processes for waste water treatment because often the inhibitory effect of heavy metals is a common phenomenon that occurs in the biological treatment of waste water and sewage. The application of heavy metal tolerant microorganisms is a promising approach for increasing heavy metal bioavailability in heavy metal amended waste water.

AIMS AND OBJECTIVES
To isolate heavy metal tolerant microorganisms and studied their heavy metal resistance potential. This heavy metal resistant organism could be a potential agent for bioremediation of heavy metals polluted environment.

MATERIAL AND METHODS
Sampling and isolation
The sewage samples were collected under sterilized conditions from different localities (domestic, industrial and agricultural site) of Jabalpur, India. Samples were aseptically processed for isolation of bacterial sp. having heavy metal resistance potential by serial dilution and pour plating method.

Assessment of their heavy metal resistance potential
Bacterial isolates were screened for their heavy metal resistant potential against heavy metals (such as Pb, Zn and Cr) by the plate diffusion method.

Heavy metal solution preparation
For preparation of heavy metal concentrations (lead, Chromium and zinc) distilled water was used along with the following:
- Lead nitrate - Pb(NO\(_3\))\(_2\) 1% (1 g in 100 ml of the distilled water)
- Zinc sulphate - ZnSO\(_4\) 1% (1 g in 100 ml of the distilled water)
- Potassium dichromate - K\(_2\)Cr\(_2\)O\(_7\) 1% (1g in 100 ml of the distilled water)

Screening by plate diffusion method
Heavy metal resistance potential of bacterial isolates was determined by the plate diffusion method against heavy metals (such as Pb, Zn, and Cr). According to this method, NAM (Nutrient Agar media) plate was swabbed with overnight culture of each bacterial isolate and in each plate wells were prepared with a sterile cork borer. 100 µl of appropriate heavy metal solution were poured in each well and plates were incubated at 37°C for 24 h. After incubation, the zone of inhibition was then measured. A zone size less than 1mm scored as heavy metal resistance strains.

Identification of selected bacterial isolates
Bacterial isolates, exhibited the heavy metal resistance potential were identified according to Bergey’s manual of systemic bacteriology and PIB computer kit. Their identities were further confirmed at generic level by DNA homology using standard bacterial cultures.

Maximum Tolerable Concentration (MTC) of bacterial isolate
Maximum Tolerable Concentration (MTC) of heavy metals was designated as the highest concentration of heavy metals that can be tolerated by the resistant bacteria isolate. MTC for the resistance bacterial isolate was evaluated by plate diffusion method. NAM plate was swabbed with overnight culture of each resistance bacterial isolate and in each plate wells (5mm) were prepared with a sterile cork borer. 100 µl of appropriate heavy metal solution were poured in each well and plates were incubated at 37°C for 24 h. Until the bacterial isolates failed to give zone of inhibition on the NAM plate. The concentration of heavy metal was gradually increased.

Determination of growth profile of bacterial isolate
Growth studies of resistance bacterial isolates was studied in 250 ml flask containing 100ml nutrient broth supplemented with 1% of lead nitrate, 0.01% of zinc sulphate and 0.01% of potassium dichromate. Flask were inoculated with 5ml of overnight culture and agitated on an orbital shaker (180 rpm) at 30°C. After every 60 minutes interval optical density was taken at 620 nm.
RESULTS AND DISCUSSION
Isolation and screening of heavy metal resistant bacterial isolates
25 bacterial isolates were isolated from nine samples of sewage that were collected from different localities (domestic, industrial and agricultural sites) of Jabalpur, Madhya Pradesh, India. After screening, out of these 25 isolates only 4 bacterial isolates exhibited varying degree of heavy metal resistance potential against selected three heavy metals (Fig. 1 (a) and Fig. 1 (b)). Hence, these isolates were selected for further study. The difference in the toxicity toward the bacterial isolates could be explained by the conditions of bacterial isolation and the nature and physiological characteristics of each bacterial isolate.15

Identification of the selected isolates
These selected isolates were tentatively identified as Bacillus carotarum (IS-15), B. cereus (IS-8), B. lentus (IS-6), B. licheniformis (IS-2) (Table 1).

DNA homology
Identity of isolated strain Bacillus cereus was further confirmed at generic level by DNA homology (Fig. 2).

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**Fig. 1(a)**: Screening of bacterial isolates against Pb, Positive result: No zone of inhibition

**Fig. 1(b)**: Screening of bacterial isolates against Pb, Negative result: Zone of inhibition
Table 1: Biochemical and physiological characteristics of the bacterial isolates

<table>
<thead>
<tr>
<th>Tests</th>
<th>IS-8</th>
<th>IS-6</th>
<th>IS-2</th>
<th>IS-15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polymyxin resistance</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Streptomycin resistance</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose acid</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose acid</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Raffinose acid</td>
<td>-</td>
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<tr>
<td>Salicin acid</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Lactose acid</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Xylose acid</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Growth at 50°C</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Growth in 10% NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Oxidase reaction</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges-proskauer test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ID score (By PIB)</td>
<td>0.97023</td>
<td>0.98161</td>
<td>0.99894</td>
<td>0.95282</td>
</tr>
<tr>
<td>Taxa</td>
<td>Bacillus cereus</td>
<td>Bacillus lentus</td>
<td>Bacillus licheniformis</td>
<td>Bacillus carotarum</td>
</tr>
</tbody>
</table>

Note: (+) Positive; (-) Negative

Fig. 2: DNA homology

Maximum Tolerable Concentration (MTC) of heavy metals

Heavy metal tolerance test showed maximum microbial tolerance to Pb and minimum tolerance to Zn and Cr (Table 2 and Fig. 3). Since heavy metals are all similar in their toxic mechanism, multiple tolerances are common phenomena among heavy metal resistant bacteria. In this piece of work they observed that the *Bacillus* sp. has multiple
heavy metal tolerance property and resistance to Cd\(^{2+}\), Cr\(^{6+}\), Ni\(^{2+}\) and Co\(^{2+}\). Chromium appeared as more toxic than cadmium. The combined resistance to heavy metals was also reported.\(^\text{16}\)

**Determination of growth profile of the selected isolates**

*Bacillus carotarum*, *Bacillus cereus*, *Bacillus lentus*, and *Bacillus licheniformis* showed the high peak values with Pb and Zn as compared to Cr (Fig. 4 to Fig. 7). Growth rate of the sewage isolates in the presence of heavy metal (Cd, Ni, As and Pb) were consistently slower than that of the control similar results have been reported earlier.\(^\text{17,18}\)

### Table 2: Maximum Tolerable Concentration (MTC) of selected bacterial isolates

<table>
<thead>
<tr>
<th>S/N</th>
<th>Sewage bacteria</th>
<th>Heavy metal</th>
<th>Lead</th>
<th>Chromium</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Bacillus carotarum</em></td>
<td>1%</td>
<td>0.01%</td>
<td>0.01%</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td><em>Bacillus cereus</em></td>
<td>1%</td>
<td>0.01%</td>
<td>0.01%</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td><em>Bacillus lentus</em></td>
<td>1%</td>
<td>0.01%</td>
<td>0.01%</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td><em>Bacillus licheniformis</em></td>
<td>1%</td>
<td>0.01%</td>
<td>0.01%</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3:** Maximum Tolerable Concentration (MTC) of selected bacterial isolates

**Fig. 4:** Growth profile of isolates: *Bacillus lentus*
**Fig. 5:** Growth profile of isolates: *Bacillus cereus*

**Fig. 6:** Growth profile of isolates: *Bacillus licheniformis*

**Fig. 7:** Growth profile of isolates: *Bacillus carotarum*
CONCLUSION

In the present investigation, it was concluded that out of 25 bacterial isolates only four bacterial isolates viz. Bacillus cereus, Bacillus carotarum, Bacillus lentus and Bacillus licheniformis, showed a wide range of resistance to different heavy metals such as lead, zinc and chromium. Moreover these isolates can be genetically engineered to reach better results in removal of lead, zinc and chromium. However, before exploiting the strain as an efficient biotechnological tool for lead, zinc and chromium detoxification further investigation needs to be carried out in laboratory scale.

REFERENCES