SCREENING AND CHARACTERIZATION OF HEAVY METAL RESISTANT BACTERIA FOR ITS PROSPECTS IN BIOREMEDIATION OF CONTAMINATED SOIL

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ABSTRACT

Developing bioremediation processes for soils and effluents polluted by heavy metals is one of the serious concern. For the same, extensive screening for the bacteria having resistance to heavy metals such as lead, nickel, cobalt, chromium, copper, manganese and zinc was attempted. During this SGPB1, PGNI2, PGCO3, KGCR4, PGCU5, PGMN6 and SGZN7 isolates were obtained having maximum threshold level of tolerance to lead (0.26 mM), nickel (2.20 mM), cobalt (0.51 mM), chromium (0.01 mM), copper (0.09 mM), manganese (8.0 mM) and zinc (1.22 mM) respectively. Maximum growth of these isolates at threshold concentration of respective metal was found at pH 7.5 and temperature 30°C except for the isolate PGCU 5 which showed maximum growth in presence of copper at pH 6 and 30°C. Besides metal tolerance further antibiotic resistance pattern of these isolates were also studied. The results revealed that antibiotic resistance and metal resistance were highly coupled. Isolates PGNI2, PGCO3, KGCR4 and PGCU5 were found to have multiple antibiotic resistance. Based on the morphological, cultural and biochemical characterization, it was found that the isolates SGPB1, KGCR4 and SGZN7 were belong to genus Pseudomonas, the isolates PGNI2 and PGMN6 were belongs to Escherichia genus and isolates PGCO3 and PGCU5 were of Bacillus species.

Key Words: Bioremediation, Heavy metals resistance, Antibiotics, Pseudomonas, Escherichia, Bacillus

INTRODUCTION

Heavy metals are those elements with a molecular weight greater than 53, a density greater than 6 g cm⁻³ and an atomic number greater than 20. They occur naturally in rocks and soils but concentrations are frequently elevated as a result of pollution. They are also called trace elements, which are toxic to living organisms at excessive concentrations but some including Zn, Cu, Mn and so on, at low but critical concentrations are micronutrients used in the redox processes, regulation of the osmotic pressure and also enzyme components which are essential for the normal healthy growth and reproduction by living organisms. At high concentrations, these micronutrients damage DNA and membrane as well as loss of functions of enzyme. However, heavy metals like Ni, Co and Pb cause oxidative stress, lipid peroxidation, carcinogenesis, mutagenesis and neurotoxicity on humans, animals and plants at low concentrations. Elevated concentration of heavy metals are introduced into the environment through metalliferous mining, metal smelting, activities of metallurgical industries, waste disposals, corrosion of metals in use and agriculture and petroleum exploration among others. The discharge of effluents containing heavy metals mounts pressures on the ecosystem and consequently causing health hazards to plants, animals, aquatic life and humans. Upon surface contamination, the toxic metals are transported to groundwater and bioaccumulated.
Introduction of certain concentrations of heavy metals into the environment kills the majority of the micro flora selecting a few cells that would have evolved resistance mechanisms to the metals.

The introduction of heavy metals in various forms in the environment can results in considerable modifications of the microbial communities and their activities.\(^5\) Heavy metals may exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions or modifying the active confirmations of biological molecules.\(^6\) Numerous studies have examined the heavy metal sensitivity or resistance of bacteria isolated from different habitats and its mechanisms to adapt the toxic metal during exposure.\(^7,8\) Some microorganisms have been reported to evolved mechanisms to detoxify heavy metals and some even use them for respiration thereby becoming resistant to such metals.\(^9\) The resistance mechanisms used by microorganisms to tolerate heavy metal stress include permeability barriers, intra and extracellular sequestration, efflux pumps, enzymatic detoxification and reduction. Living organisms absorb sub lethal concentrations of heavy metals upon pollution by diffusion of ions or complexes, mediated transport, endocytosis of particulate metal and pinocytosis of organometallic aggregates. In some cases, resistance to metal ions has been reported to be plasmid mediated and observed to be encoded by genes in close proximity to antibiotic resistance genes.\(^10\)

In polluted environment, the response of microbial communities to heavy metals depends upon the concentrations and availability of metals as well biological factors such as the type of metal, nature of medium and microbes.\(^11,12\) Furthermore, the frequency of appearance of resistant bacteria to specific heavy metals may be correlated with increasing loads of metals in the environment. As a result heavy metal resistant bacteria may be used as biological monitors or bioindicators of environmental contamination.\(^13\) Bioindicators have been shown to be a sensitive and reliable tool in detecting the sub lethal toxicity of these polluting compounds.\(^14\)

In recent years, it has become obvious that chromosomal and plasmid borne determinants for heavy metal resistance can transfer freely within an ecological system such as soil. For this reason bacteria in environment contaminated with numerous heavy metals may evolve or acquire a number of heavy metal resistance determinants resulting in multiple resistant. These metal resistant microbes develop the mechanisms which help in detoxification or cleaning-up of the metal from that environment. This possible environmental application to remove dangerous heavy metal from the contaminated soil generated present interest to screen out the metal resistant microbes from the contaminated site and its characterization to explore it further for its suitability for bioremediation of heavy metal contaminated soil.

**MATERIAL AND METHODS**

**Collection of soil samples**

Twenty five different soil samples of industrial waste disposal site were collected from different localities such as Sachin GIDC (SG), Pandesara GIDC (PG), Kadodara GIDC (KG), Akshat paper mill samples (S1) and Garage samples from Surat (S2). Soil samples from soil surface (0-5 cm) and at a depth (approximately 20 cm) were taken in sterilized polyethylene bags using sterilized spatula and stored at 4°C until examination.

**Media and chemical used**

For isolation and purification, strains were routinely grown in Luria Bertani (LB) (component g/l:10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, pH 7.5) medium at 30°C. The stock solutions (100 mg/ml) of following metal salts namely CuCl\(_2\), MnCl\(_2\), CoCl\(_2\), NiCl\(_2\) and K2Cr2O7, ZnSO\(_4\), (CH\(_3\)COO)\(_2\)Pb were prepared in distilled water, sterilized by filtration through membrane filters and stored in sterile flasks in the dark at 4°C for no longer than 1 day.\(^15\)

**Isolation of heavy metal resistant bacteria**

Each 10 g soil sample was added to 90 ml sterilized water and mixed on the magnetic blender for 30 min to separate bacteria from the soil completely. After being deposited for 20 min, 1 ml suspension was added to LB broth and incubated at 30°C for 24 h on 120 rpm. For the
selective isolation of heavy metals resistant bacteria, heavy metals incorporated media were used. The LB agar incorporated with each of heavy metal was prepared. The concentration of each heavy metal was maintained at 300 μg/ml of the medium. The enriched sample directly streaked on LB media and incubated at 30°C for 24 h. After the incubation period the plates were observed for any kind of growth on the media. The isolated and distinct colonies were sub cultured and obtain in the form of pure culture.

**Determination of threshold level of metal resistance**

For the determination of MTC, heavy metal resistant bacterial isolates were grown on LB agar plates with gradually increasing the concentration of the respective heavy metal. The initial minimum concentration used was 300 μg/ml. Each Subsequent transfer to LB agar plates with increasing the metal concentration by 50 μg/ml until the isolate failed to grow. This concentration above which bacterial isolates failed to grow was considered as threshold level of metal resistance.

**Optimization of growth parameters**

**pH**

The optimal growth condition with reference to pH was also determined. The isolates were grown in LB medium with different pH values (6.0, 6.5, 7.0, 7.5, 8.0) and incubation was carried out at temperature 30°C for 24 h. The optical density of the log phase growing cultures (8-10 h) was noted at 600 nm to determine the growth.

**Temperature**

The optimal growth condition with reference to temperature was determined. The isolates were grown in LB medium with different temperature values (25°C, 28°C, 30°C, 32°C, 35°C) and incubation was carried out for 24 h. The optical density of the log phase growing cultures (8-10 h) was noted at 600 nm to determine the growth.

**Determination of antibiotic resistance pattern**

Antibiotic resistance of the heavy metal resistant isolates was assayed by disc diffusion method. Antibiotic-impregnated discs (6 mm diameter, Hi Media, Mumbai, India) were placed on nutrient agar plates poured with bacterial isolates and incubated at 30°C for 24 hrs. The diameter of the inhibition zones around the disc was measured. The organisms were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone given in standard antibiotic disc chart.

**Characterization of bacterial isolates**

As per the Bergey's manual of determinative bacteriology, the selected isolates were studied for the morphological, cultural and biochemical test.

**RESULTS AND DISCUSSION**

**Isolation of heavy metal resistant bacteria**

The extensive sampling was carried out to determine the presence and numbers of metal resistant bacterial population. From the soil sample total 30 potent isolates were obtained. 5 bacterial isolates i.e. SGPB1, PGPB1, KGPB1, S1PB1 and S2PB1 were found to show lead resistance 10, 6.5, 0.12, 2.5, 5 (mg/ml) respectively. Isolates which showed nickel resistance were SGNI2, PGNI2, KGNI2, S1NI2 and S2NI2 and the value of resistance were 30, 55, 0.5, 4, 12 (mg/ml) respectively. Cobalt resistance was found in SGCO3, PGCO3, S1CO3 and S2CO3 isolates respectively 8, 10, 1.5, 1.8 (mg/ml). Chromium resistance was observed in isolate KGCR4 and S1CR4 which was 1.1, 0.9 (mg/ml) respectively. Cobalt resistance was found in SGCU5, PGCU5, KGCU5, S1CU5 and S2CU5 isolates were found to have copper resistance 1.2, 1.5, 1.2, 0.6, 0.9 (mg/ml) respectively. Manganese resistance was reported in SGMN6, PGMN6, KGMN6, S1MN6 and S2MN6 isolates 50, 200, 10, 2.5, 2.5 (mg/ml) respectively. Zinc resistance was found in isolate SGZN7, PGZN7, KGZN7 and S1ZN7 shows the value of resistance 35, 18, 4, 1.8 (mg/ml) respectively. The results are shown in Fig. 1.

**Selection of metal resistant bacteria**

From these 30 isolates obtained from the diverge location, screening was carried out for most potential metal resistant bacterial isolate. The study was carried on the basis of maximum threshold limit for the respective metals by the isolates. The results are shown in Fig. 1.
Each single bacterial isolate selected were showed maximum tolerance to each respected metal (Fig. 2). SGPB1 isolate was found to show maximum lead tolerance (10 mg/ml), while PGNI2 was found to have maximum nickel tolerance (55 mg/ml) in comparison to all other isolates. Cobalt resistance was reported maximum in PGCO3 (10 mg/ml) which was quite high in comparison to rest of isolates. Chromium tolerance (10 mg/ml) was observed maximum by KGCR4. Copper resistance reported maximum in PGCU5 (1.5 mg/ml), for manganese PGMN6 isolate was selected showing maximum manganese tolerance (200 mg/ml) which was approximately 4 fold higher than the SGMN6, for zinc maximum tolerance was reported in SGZN7 (35 mg/ml). By analyzing the data, it can be concluded that sample SG and PG were highly contaminated sites with metal ions and most prominent source of maximum metal tolerant isolates.

Fig. 1: Threshold level of metal tolerance of bacterial isolates obtained from the different sampling sites

Fig. 2: Growth of bacterial isolate in LB broth supplemented with metals (from left Mn, Cr, Zn, Ni, Pb, Co, Cu)
Optimization of growth parameters

pH

The pH was optimized to for maximum growth in presence of respective metal at a threshold concentration of the selected bacterial isolates. Growth response of bacterial isolates at different pH was analyzed by spectrophotometrically at 600 nm. Fig. 3 indicates that optimum pH for SGPB1, PGNI2, PGCO3, KGCR4, PGMN6, SGZN7 was 7.5 while optimum pH for isolate PGCU5 was 6.5.

![Graph A: pH vs. O.D. at 600 nm for different bacterial isolates]

**Fig. 3 :** pH and temperature optima of the selected metal resistant isolates

Temperature

Selected bacterial isolates found to show different characteristic growth on nutrient broth at different temperature. So temperature was optimized for maximum growth of the bacterial isolates in the presence of respective metal at threshold concentration. Fig. 3 indicates that 30°C temperature was optimum for all the isolates.

Resistance to antibiotics

In order to determine the resistance to antibiotics, each bacterial isolate was tested against 27 different antibiotics by the disk diffusion method. After incubating for 36–48 h, the plates with antibiotics disk are observed for zone of inhibition and resistance pattern of each isolate was shown in Table 1.

![Graph B: Temp. vs. O.D. at 600 nm for different bacterial isolates]

**Table 1 :** Antibiotic resistance pattern of selected metal resistant isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Resistance pattern*</th>
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<tbody>
<tr>
<td>SGPB1</td>
<td>Nil</td>
</tr>
<tr>
<td>PGNI2</td>
<td>Cl&lt;sup&gt;15&lt;/sup&gt; Ce&lt;sup&gt;10&lt;/sup&gt; Ct&lt;sup&gt;5&lt;/sup&gt;B&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>PGCO3</td>
<td>A&lt;sup&gt;10&lt;/sup&gt;Cx&lt;sup&gt;5&lt;/sup&gt;Cd&lt;sup&gt;10&lt;/sup&gt;Ag&lt;sup&gt;10&lt;/sup&gt;P&lt;sup&gt;10&lt;/sup&gt;Ct&lt;sup&gt;5&lt;/sup&gt;E&lt;sup&gt;10&lt;/sup&gt;Cp&lt;sup&gt;10&lt;/sup&gt;V&lt;sup&gt;50&lt;/sup&gt;S&lt;sup&gt;10&lt;/sup&gt;R Cm&lt;sup&gt;10&lt;/sup&gt;B&lt;sup&gt;10&lt;/sup&gt;Pb&lt;sup&gt;50&lt;/sup&gt;G&lt;sup&gt;10&lt;/sup&gt;N&lt;sup&gt;30&lt;/sup&gt;</td>
</tr>
<tr>
<td>KGCR4</td>
<td>A&lt;sup&gt;10&lt;/sup&gt;Cx&lt;sup&gt;5&lt;/sup&gt;Cd&lt;sup&gt;10&lt;/sup&gt;P&lt;sup&gt;10&lt;/sup&gt;Ct&lt;sup&gt;30&lt;/sup&gt;Cc&lt;sup&gt;30&lt;/sup&gt;Cp&lt;sup&gt;10&lt;/sup&gt;V&lt;sup&gt;50&lt;/sup&gt;Cm&lt;sup&gt;10&lt;/sup&gt;B&lt;sup&gt;10&lt;/sup&gt;Pb&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>PGCU5</td>
<td>A&lt;sup&gt;10&lt;/sup&gt;Cd&lt;sup&gt;10&lt;/sup&gt;P&lt;sup&gt;10&lt;/sup&gt;Cc&lt;sup&gt;30&lt;/sup&gt;Cp&lt;sup&gt;10&lt;/sup&gt;Cm&lt;sup&gt;10&lt;/sup&gt;B&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>PGMN6</td>
<td>Nil</td>
</tr>
<tr>
<td>SGZN7</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Cl = Clarithromycin, Ce = Cefpodoxime, Cfx= Cefixime, B= Bactracin, A= Ampicillin, Cx= Cefuroxime, Cd= Cephaloxil, Ag= Augmentin, P= Penicillin, Ct= Cephotaxime, E= Erythromycin, Cp= Cefpodoxime, V= Vancomycin, S= Streptomycin, R= Rifampicin, Cm= Clindamycin, Pb= Polymyxin B, G= Gentamycin, N= Neomycin

From Table 1, it could be concluded that isolates PGNI2, PGCO3, KGCR4 and PGCU5 were found to have multiple antibiotic resistance while isolates SGPB1, PGMN6 and SGZN7 were resistance to only few of the antibiotics. The isolates SGPB1, PGMN6 and SGZN7 were sensitive to antibiotics tested. Exposure of bacterial isolates to the heavy metals for a long time may change its structure and functions to adapt antibiotic resistance. The resistance was due to acquisition of resistance factors or by gene mutation. The
antibiotic resistance was coupled with metal resistance, the genes for resistance were found on the plasmid. The multiple plasmids responsible for antibiotic resistance could be transformed to the wild type strain for the transformation.

**Characterization of bacterial isolates**

**Morphological and cultural characterization**

Selected strains were microscopically studied. PGCO3 and PGCU5 isolates were gram positive and producing large, flat, opaque colonies typical to *bacillus* on nutrient agar media. The isolate SGPB1, KGCR4 and SGZN7 were gram negative showing large, irregular, undulated, low convex colonies with greenish blue pigment on nutrient agar media. The PGIN2 and PGMN6 were gram negative isolates with small, colorless, low convex and transparent colony on nutrient agar media.

**Biochemical characterization**

Selected strains were studied for their biochemical tests which were needed for characterization of the strains. The results are shown in **Table 2**. All 7 isolates were tentatively identified following Bergey’s Manual of Determinative Bacteriology.¹⁶

**Table 2**: Biochemical characteristics of the selected metal resistant bacterial isolates

<table>
<thead>
<tr>
<th>Biochemical characteristics</th>
<th>Selected metal resistant isolates</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SGPB1</td>
</tr>
<tr>
<td>Sugar utilization</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
</tr>
<tr>
<td>Manose</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>Methyl Red test</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Urea hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Hugh and Leifson’s</td>
<td>+</td>
</tr>
<tr>
<td>Triple Sugar Iron agar slant</td>
<td>Slant/</td>
</tr>
<tr>
<td></td>
<td>H₂S</td>
</tr>
<tr>
<td></td>
<td>Gas</td>
</tr>
</tbody>
</table>

+ = positive result, - = negative result, R/R = No fermentation and aerobic, Y/Y = Sucrose and/or lactose fermentation and facultative anaerobes, R/Y = Only glucose fermentation

The isolates PGCO3 and PGCU5 were positive for gelatin liquefaction, VP test and catalase test and thus tentatively identified as *Bacillus* spp. The isolate SGPB1, KGCR4 and SGZN7 were positive for gelatin liquefaction, urease production, Hugh and Leifson’s, nitrate reduction, catalase test and citrate test. Thus, based on morphological and biochemical
characteristics SGBP1, KGCR4 and SGZN7 were tentatively identified as *Pseudomonas* spp. The PGNI2 and PGMN6 isolates were positive for nitrate reduction, indole production, catalase test, MR test and TSI agar slant. Based on morphological and biochemical characteristics, PGNI2 and PGMN6 isolate were tentatively identified as *Escherichia* spp.

**CONCLUSION**

Out of these 30 isolates from 25 soil contaminated sample, SGBP1, PGNI2, PGCO3, KGCR4, PGCU5, PGMN6 and SGZN7 were found to show metal tolerance to lead (0.26 mM), nickel (2.20 mM), cobalt (0.51 mM), chromium (0.01 mM), copper (0.09 mM), manganese (8.0 mM) and zinc (1.22 mM) The SGBP1, KGCR4 and SGZN7 were identified as *Pseudomonas* spp., while PGNI2 and PGMN6 isolates were of *Escherichia* spp. and PGCO3 and PGCU5 were belong to *Bacillus* spp. These studies revealed the potential of heavy metal resistant isolates. The metabolic active system of metal resistance by these isolates is novel and represents a point of interest for possible environmental applications to remove dangerous heavy metal from the contaminated soil.

**REFERENCES**

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