Isolation of heavy metal resistant bacterial strains from the battery manufactured polluted environment

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Abstract

Removal of heavy metals from contaminated sites using microorganisms is a cheaper alternative to chemical technologies. There is therefore a need to isolate, identify and characterize the microorganisms that exist and interact in contaminated environment and to isolate the genetic determinants of resistance, frequently located on plasmids and transposons.

Investigations were carried out to isolate microbial strains from the battery manufactured polluted soil and to test their metal tolerance to cadmium, mercury, lead, zinc and nickel (Cd, Hg, Pb, Zn, Ni). In the primary screening were isolated 24 aerobic bacterial strains that were tested on higher concentrations of Cd, Pb, Zn and Ni (up to 10 mM), the widespread hazardous chemicals used by battery industry. It has been noticed that the intracellular accumulation of Pb changes the color of colonies grown on media with lead. The selected 8 strains were morphologically and physiochemically characterized and the plasmid profile determined. Data indicated that the isolated bacteria have a great potential in heavy metals bioremediation.

Keywords: battery manufactured polluted soil, heavy metal, resistant bacteria, plasmid profile

Introduction

Environmental biotechnology strategies must address and solve in a long-term perspective the formidable environmental problems now facing the world, such as soil contamination with pesticides, metals or hydrocarbons, disposal of animal manures, treatment of waste waters or recovery of reusable products and energy from wastes (8). Thus, bioremediation is becoming increasingly used mostly in case of removal of heavy metal from contaminated environment, as a cheaper alternative to chemical technologies (8). On the other hand microorganisms capable of biodegrading or detoxify heavy metals usually are already present in contaminated soils and groundwater. There is therefore a need to isolate, identify and characterize the microorganisms that exist and interact in contaminated environment and to isolate the genetic determinants of resistance, frequently located on plasmids and transposons (3, 5, 8, 10).

The present study described the isolation, screening and genetic characterization of the heavy metal resistant bacterial strains isolated from the battery manufactured polluted soil.

Materials and Methods

Sample collection and isolation of heavy metal resistant bacteria

The soil samples were collected from three different locations of the battery manufactured contaminated environment, at the depth of 0 - 15 cm below surface. All samples (named P1/2/3) were kept in clean sterile bags, labeled accordingly and stored at 4°C. Isolation of cultures from the soil samples was carried out using enrichment isolation procedure. In the first step, in the case of each experimental set (P1A/B/C, P2A/B/C,
P3A/B/C), 1 g of soil sample was incubated on LB medium, at the temperature of 28°C for 24 hours and the microbial density determined spectrophotometrically (600 nm). To isolate metal resistant bacteria, 1 ml of microbial suspension was spread on 1 mM, 3 mM and 5 mM PYE-metal (Cd, Ni, Pb, Zn, and Hg) media. The growth of the bacterial colonies was measured after 24 - 48 hours of incubation at 28°C. Morphologically dissimilar colonies were randomly selected and maintained at 4°C for bacterial characterization (1, 4, 9). PYE agar media were prepared by dissolving 1 g of peptone, 0.5 g of yeast extract, 1 g of NaCl and 1.5 g of agar in 100 ml distilled water, pH 7.2 and the medium was autoclaved at 121°C for 15 minutes. Sterile stock solutions of metal (CdCl₂, NiCl₂, PbSO₄, and ZnSO₄) were added to PYE media after sterilization to obtain the desired concentration of metal. However, 1 mM PYE-Hg medium was prepared by dissolving HgCl₂ into agar media before autoclaving.

**Screening of the bacterial strains with metal tolerance**

In order to test the bacterial tolerance to metals, isolated colonies were picked up and streaked on PYE agar medium with successively higher concentrations (5 mM, 8 mM and 10 mM) of metal (Cd, Ni, Pb, and Zn). The strains capable of growing under these conditions were selected for further experiments.

**Plasmid profile screening**

Plasmid DNA was isolated from selected strains using PureYield Plasmid Midiprep System (Promega, USA) and visualized on 1 % agarose gel, in the presence of BenchTop 1kb DNA ladder (Promega, USA).

**Reproducibility of the data**

The coefficient of variation (CV) was calculated from the ratio of standard deviation (SDV) and arithmetic mean in three independent experiments. The CV (%) < 10% shows there is not significant biological variation within the probe in experiments where the isolation of metal resistant bacteria was performed.

**Results and Discussion**

**Isolation of heavy metal resistant bacteria**

Investigations were focused on the isolation and characterization of the aerobic bacterial strains with metal tolerance to identify potential candidates for heavy metals bioremediation.

Soil samples were collected from the battery manufactured polluted environment, where lead, zinc, nickel and cadmium are the widespread hazardous chemicals used by battery industry. Similarly, Fagade et al. (1999) described the isolation of two Pseudomonas strains that effectively accumulated lead from a battery manufactured effluent (5).

The enrichment isolation technique was mostly used to isolate metal resistant bacteria (1, 4). The microbial density of the experimental sets (P 1/2/3 A/B/C) kept at 28°C in enrichment media for 24 hours were determined by spectrophotometer and the biological variation calculated. The heterotrophic bacterial counts range from 10⁷ - 10⁹ CFU/ml. The coefficient of variation (CV) per sample was less than 10 % (4.296, 7.253, and 7.166), showing that there is no significant variation within the experimental sets, regardless of the date when the analysis was done. However, a value of 26.23% for CV total proved that samples isolated from three different locations had different microbial densities (Table 1).
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Table 1. Microbial density in samples isolated from heavy metal polluted soil

<table>
<thead>
<tr>
<th>Experimental set</th>
<th>Date</th>
<th>OD 600 nm</th>
<th>OD 600 nm</th>
<th>OD 600 nm</th>
<th>Average</th>
<th>Average/ sample</th>
<th>SDV/ sample</th>
<th>CV/ sample (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1a</td>
<td>0.812</td>
<td>0.660</td>
<td>0.703</td>
<td>0.7250</td>
<td></td>
<td>0.7146</td>
<td>0.0311</td>
<td>4.2960</td>
</tr>
<tr>
<td>P1b</td>
<td>0.830</td>
<td>0.184</td>
<td>1.025</td>
<td>0.6796</td>
<td></td>
<td>0.8047</td>
<td>0.0607</td>
<td>7.2530</td>
</tr>
<tr>
<td>P1c</td>
<td>0.785</td>
<td>0.656</td>
<td>0.777</td>
<td>0.7393</td>
<td></td>
<td>0.8117</td>
<td>0.0576</td>
<td>7.1660</td>
</tr>
<tr>
<td>P2a</td>
<td>0.978</td>
<td>0.535</td>
<td>1</td>
<td>0.8376</td>
<td></td>
<td>0.8047</td>
<td>0.0607</td>
<td>7.2530</td>
</tr>
<tr>
<td>P2b</td>
<td>0.911</td>
<td>0.542</td>
<td>1.073</td>
<td>0.8420</td>
<td></td>
<td>0.8117</td>
<td>0.0576</td>
<td>7.1660</td>
</tr>
<tr>
<td>P2c</td>
<td>0.875</td>
<td>0.518</td>
<td>0.811</td>
<td>0.7346</td>
<td></td>
<td>0.8047</td>
<td>0.0607</td>
<td>7.2530</td>
</tr>
<tr>
<td>P3a</td>
<td>0.723</td>
<td>0.695</td>
<td>0.996</td>
<td>0.8046</td>
<td></td>
<td>0.8047</td>
<td>0.0607</td>
<td>7.2530</td>
</tr>
<tr>
<td>P3b</td>
<td>0.660</td>
<td>0.596</td>
<td>1.018</td>
<td>0.7580</td>
<td></td>
<td>0.8117</td>
<td>0.0576</td>
<td>7.1660</td>
</tr>
<tr>
<td>P3c</td>
<td>0.699</td>
<td>0.900</td>
<td>1.019</td>
<td>0.8726</td>
<td></td>
<td>0.8117</td>
<td>0.0576</td>
<td>7.1660</td>
</tr>
<tr>
<td>Control (PYE)</td>
<td>0.041</td>
<td>0.082</td>
<td>0.039</td>
<td></td>
<td></td>
<td>0.7770</td>
<td>0.2038</td>
<td>26.2314</td>
</tr>
</tbody>
</table>

A total of 24 different bacterial strains were isolated from the contaminated soil samples on PYE-metal (Cd/Ni/Pb/Zn and Hg) media with concentration of 1 mM, 3 mM and 5 mM (Table 2). Mercury had the highest toxicity on the bacteria as no colonies developed on 3 mM Hg media. Also, the cadmium-media were very selective and microorganisms did not grow in one third of the samples. Altogether, five bacterial strains were isolated on each medium containing mercury, lead and zinc, six strains on PYE-Cd and only three strains on nickel medium.

Isolated colonies were picked up from plates according to their different form and purified by subculturing onto fresh nutrient PYE-metal agar plates using the streak-plate technique. Colonies grown on plates were studied with respect to size, color, opacity, form, elevation, and margin (data not shown). It has been noticed that medium with 3 mM Pb changed the color of the colony that become brownish, probably because of the intracellular lead accumulation. Two isolated Pseudomonas strains that adsorb and accumulate lead and membrane-associated accumulation of Ni/Cd in Pseudomonas aeruginosa or Cd-induced siderophore production have been reported (2, 5, 7).

Table 2. The aerobic bacterial strains isolated on PYE-metal media (Cd, Zn, Ni, Pb, Hg)

<table>
<thead>
<tr>
<th>Sample</th>
<th>3mM Cd</th>
<th>1mM Hg</th>
<th>3mM Ni</th>
<th>3mM Pb</th>
<th>3mM Zn</th>
<th>5mM Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1A</td>
<td>A9</td>
<td>A12</td>
<td>A19</td>
<td>A23</td>
<td>A1</td>
<td>A25</td>
</tr>
<tr>
<td>P1B</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>A22</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P1C</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>A24</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P2A</td>
<td>A4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P2B</td>
<td>-</td>
<td>A13</td>
<td>+</td>
<td>A21</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P2C</td>
<td>A7</td>
<td>-</td>
<td>A18</td>
<td>+</td>
<td>A2</td>
<td>A26</td>
</tr>
<tr>
<td>P3A</td>
<td>A10</td>
<td>A14</td>
<td>A17</td>
<td>A20</td>
<td>A3</td>
<td></td>
</tr>
<tr>
<td>P3B</td>
<td>A11</td>
<td>A15</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>P3C</td>
<td>A8</td>
<td>A16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

+microbial colonies; - without microbial colonies

Screening of the bacterial strains with heavy metal tolerance

The heavy metal tolerance of the bacterial isolates was tested in undefined nutrient agar medium (PYE) with increasing concentration of metal (5 mM, 8 mM and 10 mM). Figure 1 shows that 5 mM PYE-Cd medium is very selective and only 38.10% of the tested
strains tolerate this concentration, while nickel or lead in the same concentration do not significantly inhibit the bacterial isolates. The order of toxicity on 5 mM solidified medium was found to be Cd>Zn>Pb>Ni.

Figure 1. The preliminary screening of bacterial strains on 5 mM PYE-metal (Cd/Zn/Ni/Pb) media

A number of 15 bacterial strains developed in the presence of 5 mM heavy metals were selected for the second screening on 8 mM and 10 mM PYE-metal (Cd/Zn/Ni/Pb) media (Figure 2). At the concentration of 10 mM cadmium only 20% of the bacterial isolates developed, while the maximum concentrations of lead, zinc or nickel tested were tolerated by 60 – 70% of bacterial strains. Strains A12 and A23 can grow on all media PYE-metal (Cd/Zn/Ni/Pb) 10 mM.

Figure 2. The number of resistant bacterial strains on 5 mM, 8 mM and 10 mM PYE-metal (Cd/Zn/Ni/Pb) media

**Plasmid profile screening**

Bioremediation of heavy metals may be used in conjunction with genetic engineering, potentially reducing both the cost and environmental impact and are considered a potential approach to biological remediation. The presence of plasmids conferring resistance to heavy metals in microorganisms isolated from contaminated sites has been demonstrated. Thus, the bacterial strain *Pseudomonas putida* PhCN contains two plasmids, the 120 kb plasmid that encode for breakdown of phenol (pPhCN1) and the 100 kb pPhCN2 plasmid that code for cadmium and copper resistance (3). Similar, *Jobling et al.* (1988) isolated aquatic gram-negative bacteria with mercury resistance coded by conjugative plasmids with sizes of 75 kb to >250kb. The plasmids were classified by restriction mapping into three distinct groups: pMER11, pMER327 and pMER610, that seems to be obtained by genetic rearrangements (6). Moreover, *Zolgharnein et al.* (2007) proved that the frequency of the occurrence of plasmids in heavy metal resistant bacteria was more than that in the common bacteria. The study showed that about 66% of bacterial strains isolated from sediment and water samples from Persian Gulf and enclosed industrial area carried large (38-62 kb) and/or small sized (>2kb) plasmids, some of them were involved in removal of cadmium or lead from solution (10).

Therefore, we determined the plasmid profile of the eight selected bacterial strains (A12, A13, A14, A15, A16, A17, A19 and A23) (Figure 3). The results showed in Figure 3 confirm the presence of plasmids over 10kb in strains A14, A15, A19 and A23. The bacterial strain A12 contains two plasmids, with molecular weights of ~4.5kb and 6kb, respectively.
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Conclusion

In our work, we isolated 24 aerobic bacterial strains from the battery manufactured contaminated soil samples. The microbial strains were tested on higher concentration of cadmium, lead, zinc and nickel (up to 10 mM) and several bacterial strains with potential in heavy metal bioremediation were selected. A number of 8 bacterial strains were able to grow on 8 mM heavy metal media, while only two strains (A12 and A23) developed on media with 10 mM Cd, Pb, Zn, and Ni. The plasmid profile screening shows the occurrence of plasmids in strains A12, A14, A15, A19 and A23.

Acknowledgements

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References