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Heavy metal resistance of bacteria and its impact on the production of antioxidant enzymes

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Microorganisms exposed to heavy metal stress are vulnerable to the toxic conditions caused by reactive oxygen species. The aim of this study was to investigate the tolerance of stressed bacteria and detect the ability of some resistant bacterial strains to produce antioxidant enzymes under heavy metal stress. The minimum inhibitory concentration (MIC) of heavy metals and antibiotic sensitivity were investigated for some bacterial strains isolated from a heavy metal-polluted area. Moreover, the activities of antioxidant enzymes produced in the resistant bacterial strains were measured. The most toxic heavy metals found using the MIC test were Cd\(^{2+}\) followed by Co\(^{2+}\). The MIC of all heavy metals except Pb\(^{2+}\) and Zn\(^{2+}\) against the most metal-resistant bacteria species ranged from 300 to 500 mg/L. In addition, 100 mg/L Zn\(^{2+}\) or Pb\(^{2+}\) increased and induced the activity of the antioxidant enzymes catalase, peroxidase and ascorbate peroxidase. The strain *Pseudomonas putida* KNU5 greatly induced ascorbate peroxidase (1,030%) under the stress of 100 mg/L Zn\(^{2+}\), while the activity of ascorbate peroxidase decreased to 115% under 100 mg/L Pb\(^{2+}\). Thus, heavy metals cause oxidative stress in *P. putida* and antioxidant enzymes appear to play a pivotal role in combating oxidative stress, providing protection to *P. putida*. Furthermore, the results of this study show that antioxidant enzymes can be used to biologically monitor heavy metal pollution.

Key words: Heavy metals, resistant bacteria, antioxidant enzymes.

INTRODUCTION

Heavy metals are toxic because they interfere with the normal biochemical reactions of the human body. The release of heavy metals into the environment causes an environmental pollution problem because they are nonbiodegradable and hence accumulate in living organisms. Soils are usually regarded as the ultimate fate for heavy metals released into the environment; however, until recently, relatively little has been known regarding the way heavy metals are bound to soils and the ease with which they may be released (Banat et al., 2005).

Some metals (e.g., Cd, Co, Cr, Cu, Fe, Ni, Pb and Zn) are essential metals, which serve as microelements; they are used for redox reactions to stabilize molecules through electrostatic interactions, as components of various enzymes, and as regulators of osmotic pressure (Bruins et al., 2000). However, the presence of high concentrations of heavy metals in the environment can be detrimental to a variety of living species. The most important features that distinguish heavy metals from other toxic pollutants are their nonbiodegradability and propensity in living materials (Orhan and Buyukgungor, 1993). Metal processing, finishing and plating are the main sources of metal wastes; however, there are also many other processes from which heavy metals originate.

Heavy metals have been reported to inhibit bacterial growth, as indicated by an extension of growth rate and

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generation time (Mahapatra and Banerjee, 1996). They also have been shown to decrease the dry weight of fungi and the total protein concentration of bacterial cells (Fahmy et al., 2001; Mohamed, 2005). Metal uptake by microorganisms is a complicated process that depends on the chemistry of the metal ions, surface features of the microorganisms, cell physiology and physicochemical influences from the environment, e.g., pH, temperature and metal concentration (Mapolo et al., 2005; Goyal et al., 2003; Klimmek et al., 2001).

Heavy metals and other pollutants increase the production of reactive oxygen species such as $O_2^-$, $OH^-$, and $H_2O_2$ (Kwon and Anderson, 2001). Antioxidant enzymes are able to prevent oxidative stress by scavenging reactive oxygen species or reducing oxidized glutathione to its reduced form (Lenartova et al., 1998). For example, superoxide radicals ($O_2^-$) generated are converted to $H_2O_2$ by the action of superoxide dismutase. In addition, cellular accumulation of $H_2O_2$ is prevented by catalase, peroxidase or the ascorbate glutathione cycle in which ascorbate peroxidase reduces it to $H_2O$ (Puneli et al., 2003).

The objectives of this study were to isolate resistant bacterial strains from heavy metal-contaminated sites in South Korea and investigate the effect of some heavy metals on the antioxidant enzymatic activity of these resistant strains.

MATERIALS AND METHODS

Soil physical and chemical properties

Four soil samples were collected from different mining areas in Chuncheon City, Gangwon-do, South Korea, and directly transferred to the laboratory via clean zipper bags placed in an ice box for physicochemical property analyses. A fifth soil sample was collected from an agricultural area. The soil pH and electrical conductivity of 1:5 soil to water mixtures were determined using a pH meter (Orion 3 Star, Thermo, USA). The exchangeable cations were analyzed by inductively coupled plasma (ICP) spectrometry after 1 M NH$_4$OAc extraction (Sumner and Miller, 1996). Soil samples were air-dried and shaken through a 2-mm sieve. The total concentration of heavy metals was estimated by digestion in 10 mL of 60% HNO$_3$ and microwave oven-drying at 200 ± 5°C for 20 min (Mars-X, HP-500 plus, CEM Corp.), according to EPA Method 3051 (USEPA, 1994). The concentrations of Co, Cr, Cu, Fe, Ni, Pb and Zn were determined by inductively coupled plasma/atomic emission spectroscopy (ICP-AES; Perkin Elmer Optima, USA).

Microbial counting in soil

Fresh soil samples were stored at 4°C for microbiological analyses. Heterotrophic bacterial and fungal populations were estimated by the plate count method. Soil suspensions and dilutions were prepared in sterilized and distilled water. Media of nutrient agar (NA) and modified potato dextrose agar (MPDA) were prepared in the laboratory and used to determine the total soil bacterial and fungal populations, respectively (Alef and Nannipieri, 1995). Each solid medium was inoculated with 0.1 mL of diluted soil and incubated at 27°C. The numbers of colonies on each medium were counted and reported as colony-forming units (CFU)/g of soil.

Bacteria and growth conditions

Bacterial strains were isolated from the different soil samples. Pseudomonas Agar selective medium was used to obtain the Pseudomonas species. The medium was prepared by adding pancreatic digest of casein (10 g), proteose peptone (10 g), K$_2$HPO$_4$ (1.5 g), MgSO$_4$·7H$_2$O (1.5 g) and agar (15 g) to 1,000 mL of distilled water. The different bacterial isolates were stored and maintained on nutrient agar at 31°C.

Identification of isolates

Several methods such as 16S rRNA gene sequence detection in the National Center for Biotechnology Information (NCBI) database and biochemical tests were used for identification of microorganisms. Biochemical reaction assays, e.g., β-galactosidase, H$_2$S, urease and indole production, were conducted in order to characterize and identify the bacterial strains. These assays were carried out using commercially available bioMérieux API 20 E strips. Furthermore, all the bacterial strains were identified using the 16S rRNA molecular technique for quick and accurate identification. The genomic DNA was isolated from bacteria according to the manufacturer’s instructions using a genomic DNA Prep Kit (SolGent, Daejeon, Korea). The isolated DNA was then used as a template for polymerase chain reaction (PCR) to amplify the 16S rRNA gene. A universal bacterial primer set of 27F (5′-AGA GTT TGA TCC TGG CTC AG 3′) and 1492R (5′-GGTAC TTG GTT ACC ACT T3′) was used to amplify the nearly complete 16S rRNA gene (Weisburg et al., 1991). The partial 16S rRNA gene sequence was compared with the full sequence available in the GenBank database using a BLAST search (NCBI) to identify the isolated bacteria.

Determination of the minimum inhibitory concentrations (MICs)

In order to isolate the most resistant strains, the MIC experiment was conducted on plates of Tris-minimal medium and was confirmed on broth medium, according to the method described by Yilmaz (2003). The appropriate heavy metal concentrations were adjusted in 5 mL of Tris-minimal solution, and the media were autoclaved (20 min at 121°C and 1.5 bars) and inoculated after cooling. The minimum concentration of metal that completely prevented growth was determined to be the MIC. The bacterial strains were classified into resistant and sensitive strains according to their MIC values.

Antioxidant enzymatic activity assay

One milliliter of the tested bacteria, with an optical density of 0.9 at 600 nm, was grown in 250 mL of Tris-minimal solution at 37°C. Heavy metal was added after 2 h of incubation. After 24 h, approximately 0.05 g of cells were harvested, washed with saline solution, and ground in a mortar and small pestle with liquid nitrogen and 50 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, 0.1% (W/V) polyvinyl pyrrolidone and 0.1% (W/V) ascorbic acid. The extract was centrifuged at 10,000 rpm for 10 min with cooling. The obtained supernatant was used to measure antioxidant enzymatic activity.

Catalase activity was determined according to the method of Abassi et al. (1998). In brief, the decrease in absorbance at 240 nm was monitored after the addition of 0.1 mL of enzyme extract to 1.9 mL of buffer (12.5 mM H$_2$O$_2$ in 50 mM sodium phosphate buffer, pH 7.0). Enzyme solution containing 50 mM sodium phosphate buffer (pH 7.0) was used as a control ($ε = 39.4$ mM⁻¹ cm⁻¹). Peroxidase and ascorbate peroxidase activities were determined according to the...
Table 1. The physicochemical properties and the microbial density of the studied soils

<table>
<thead>
<tr>
<th>S/N</th>
<th>Site</th>
<th>pH (1:5)</th>
<th>EC‡</th>
<th>NH₄-N</th>
<th>NO₃-N</th>
<th>Exchangeable cations</th>
<th>Microbial density CFU/ g soil x 10⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baeknyeon mine</td>
<td>4.7</td>
<td>1.30</td>
<td>11.38</td>
<td>6.21</td>
<td>Ca²⁺  122.6</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Jucheon mine</td>
<td>8.0</td>
<td>0.20</td>
<td>15.75</td>
<td>1.14</td>
<td>Mg²⁺  12.1</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Hanheung mine</td>
<td>8.1</td>
<td>0.23</td>
<td>21.88</td>
<td>5.86</td>
<td>K⁺</td>
<td>143</td>
</tr>
<tr>
<td>4</td>
<td>Bongwha mine</td>
<td>6.1</td>
<td>0.10</td>
<td>35.88</td>
<td>1.66</td>
<td>Na⁺</td>
<td>4.8</td>
</tr>
<tr>
<td>5</td>
<td>Agricultural soil</td>
<td>6.7</td>
<td>0.14</td>
<td>14.88</td>
<td>7.10</td>
<td>Bacteria</td>
<td>36.951</td>
</tr>
</tbody>
</table>

† Organic matter; ‡ electrical conductivity; § total carbon; ¶ total nitrogen.

Table 2. Heavy metals content of the contaminated sites and the control soil (mg kg⁻¹).

<table>
<thead>
<tr>
<th>S/N</th>
<th>Site</th>
<th>Cd</th>
<th>Pb</th>
<th>Cr</th>
<th>Ni</th>
<th>Cu</th>
<th>Zn</th>
<th>Co</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Baeknyeon mine</td>
<td>3.8</td>
<td>348</td>
<td>8.5</td>
<td>5.0</td>
<td>122.6</td>
<td>143</td>
<td>1.8</td>
<td>13.316</td>
</tr>
<tr>
<td>2</td>
<td>Jucheon mine</td>
<td>67.3</td>
<td>3.521</td>
<td>13.5</td>
<td>12.1</td>
<td>265.4</td>
<td>4.383</td>
<td>4.8</td>
<td>36.951</td>
</tr>
<tr>
<td>3</td>
<td>Hanheung mine</td>
<td>2.3</td>
<td>90</td>
<td>14.2</td>
<td>8.3</td>
<td>76.5</td>
<td>99</td>
<td>3.5</td>
<td>12.214</td>
</tr>
<tr>
<td>4</td>
<td>Bongwha mine</td>
<td>3.9</td>
<td>1.231</td>
<td>10.2</td>
<td>5.1</td>
<td>105.1</td>
<td>539</td>
<td>4.7</td>
<td>17.715</td>
</tr>
<tr>
<td>5</td>
<td>Control (Agr. soil)</td>
<td>1.7</td>
<td>43</td>
<td>8.8</td>
<td>4.4</td>
<td>14</td>
<td>105</td>
<td>2.5</td>
<td>11.926</td>
</tr>
</tbody>
</table>

methods of Cakmak and Marschner (1992) and Dalton et al. (1987), respectively. All enzymatic activities were calculated according to the law derived by Whiteley and Lee (2006): Activity = ΔA V εtv

where ΔA is the change in absorbance, V is the total volume of the assay mixture, v is the volume of the sample, ε is the extinction coefficient in mL µmol⁻¹ cm⁻¹ and t is the time in min.

Antibiotic sensitivity of bacteria

Seven of the most common antibiotics were applied via the disc diffusion method using susceptibility test discs (BD BBL™ Sensi-Disc™). The antibiotics used and their disc doses were as follows: neomycin (30 µg), tetracycline (5 µg), ciprofloxacin (5 µg), cefazidime (30 µg), vancomycin (30 µg), amikacin (30 µg) and gentamicin (10 µg). These antibiotics have different modes of action against bacteria. The cultures were incubated at 31°C for 24 to 48 h according to the species.

Statistical analysis

Antioxidant enzymatic activity measurements for each treatment were repeated three times. The analysis of variance was carried out by using Statistical Package for the Social Sciences (SPSS) 10.0 and the data were reported as means ± standard error (SE) (n=3). Different letters indicate significance according to Duncan’s test (P<0.05).

RESULTS AND DISCUSSION

The physicochemical properties of soil samples

The pH, electrical conductivity, and NH₄-N, NO₃-N, Mg²⁺ and K⁺ concentrations of the four contaminated soils and the uncontaminated agricultural soil (control soil) were measured and are listed in Table 1. According to the chemical analyses of the collected soils, the exchangeable cations did not vary greatly between the heavy metal-contaminated and the uncontaminated control soils.

Heavy metal concentrations

It was observed that Pb²⁺ and Zn²⁺ were the most abundant metals in the polluted soils, while Ni²⁺ and Co²⁺ showed lower levels (Table 2). It is well known that long-term exposure of water and soil to heavy metals can considerably modify their microbial populations, reducing their activities and their numbers (Doelman et al., 1994). In addition, heavy metal-resistant microorganisms play an important role in the bioremediation of heavy metal-contaminated soils (Ray and Ray, 2009; Abou-Shanab et al., 2007). In the present study, various microorganisms were isolated from soil samples in the studied sites where heavy metals and other pollutants have been emitted in industrial effluents for several years.

Microbial density

As shown in Table 1, the greatest microbial number was counted in the control sample; while the bacterial counts in the collected polluted samples as compared to the control sample were remarkably different. For example, in area No.1 (Baeknyeon mining area), the number of bacteria was 2 × 10⁶ CFU/g of soil; whereas in the control (unpolluted soil), the number of bacteria was 2.1 ×
10^7 CFU/g. Similarly, the amount of fungi was less in the heavy metal-polluted soil, e.g. 4.5 \times 10^5 CFU/g in the mining area of Baeknyeon, as compared to 7.0 \times 10^5 CFU/g in the uncontaminated control soil. These values were expected, since it has been reported that heavy metals prolong bacterial growth rate, exponential phase of growth, lag period and generation time, leading to decreased bacterial counts (Mahapatra and Banerjee, 1996).

### Identification of isolates

According to the 16S rRNA analysis, isolates *Pseudomonas KNU5* and *Pseudomonas KNU8* had 99% homology with *Pseudomonas putida*, and isolate *Bacillus subtilis* Kh had 99% homology with *B. subtilis*; therefore, only these three isolates were selected for the antioxidant enzyme investigation (The GeneBank accession numbers are KC207085, AB675368 and CP002183, respectively). Moreover, the genetic identifications of these isolates were in agreement with their biochemical reaction results.

### Determination of the MIC

The MIC determination of heavy metals to ward bacteria was first carried out on solid media, followed by broth media. The MIC values determined were greater on solid media than in liquid media, possibly due to chelation between agar and the heavy metals. To investigate the interactions between microbes and toxic metals, a better understanding of the behavior of heavy metals in the culture medium being used and how the medium reflects the natural environmental conditions must be determined (Jonas et al., 1984; Knotek-Smith et al., 2003). Rathnayake et al. (2013) formulated a novel minimal medium in order to provide a greater free heavy metal ion concentration in solution, resulting in the maximum toxicity to bacteria. We selected the most metal-resistant bacteria species to carry out the MIC determination in liquid media (Table 3). Then, we selected the most resistant and the most sensitive strain to pursue the following tests. With our metal-resistant bacteria species, the MIC ranged from 300 to 500 mg/L for all of the heavy metals, except Pb^{2+} and Zn^{2+}, in which the MIC ranged from 600 to 700 mg/L for each. However, with the metal-sensitive bacterial species, the MIC ranged from 100 to 300 mg/L in all of the heavy metal solutions, and from 100 to 500 mg/L in both Pb^{2+} and Zn^{2+} solutions.

The high tolerance of the metal-resistant species to Pb^{2+} and Zn^{2+} might be due to their adaptation to the surrounding environment or to mutations in the bacterial plasmid. In particular, Pb^{2+} and Zn^{2+} showed the greatest concentration of all of the heavy metals analyzed in the soil samples collected from the polluted sites. Our results are in agreement with those by Hassan et al. (2008), who stated that the difference in toxicity toward bacterial isolates could be explained by the conditions of bacterial isolation and the nature and physiological characteristics of each bacterial isolate. Moreover, zinc is an essential component of select prokaryotic and eukaryotic proteins. A zinc-containing bacteriochlorophyll has been discovered in photosynthetic bacteria of the genus *Acidiphilium* (Hiraishi and Shimada, 2001). Furthermore, zinc is essential for DNA synthesis (MacDonald, 2000).

### Antibiotic resistance of bacteria

After incubation for 24 to 48 h, the bacterial cultures were examined for antibiotic resistance. Amikacin was the most potent antibiotic, and it was effective against all the metal-resistant bacterial strains except *P. putida* KNU5. *P. putida* KNU5 was resistant to all antibiotics used.
Figure 1. The effect of different antibiotics and their dose per disk on the growth of two bacterial strains (A) *P. putida* KNU5; and B) *P. putida* KNU8 for the tested antibiotics (neomycin 30 µg (N), octafloxin 30 µg (OFX), ciprofloxacin 5 µg (CIP), cetafazidime 30 µg (CAZ), vancomycin 30 µg (VA), amikacin 30 µg (AN) and gentamicin 10 µg (GM). The antibiotic name abbreviation is shown on its disk, as well as, the dose per disk.

Table 4. Response of the heavy metals resistant strains to the antibiotic actions.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Ofloxacin (5 µg)</th>
<th>Ciprofloxacin (5 µg)</th>
<th>Cefazidime (30 µg)</th>
<th>Neomycin (30 µg)</th>
<th>Vancomycin (30 µg)</th>
<th>Amikacin (30 µg)</th>
<th>Gentamicin (10 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resistant strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> KNU5</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td><em>P. putida</em> KNU8</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>P. putida</em> KNU9</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><em>P. putida</em> KNU12</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> Kh</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><strong>Sensitive strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em> KNU1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><em>P. putida</em> KNU14</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Bacillus methylotrophicus</em> Kh</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Arthrobacter nigatensis</em></td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

 except gentamicin, while *B. subtilis* Kh was resistant to all the antibiotics used except gentamycin and amikacin (Figure 1). Thus, the two antibiotics gentamycin and amikacin seemed to be the most effective against our metal-resistant bacterial strains (Table 4). Many investigators have reported that metal-tolerant environmental isolates are resistant to a wide array of antibiotics. Shafiani and Malik (2003) studied seven different antibiotics toward bacteria isolated from wastewater-irrigated soils and found that most isolates showed resistance to five different antibiotics. Similarly, Yilmaz (2003) found that *Bacillus* EB1 was resistant to six antibiotics and susceptible to seven antibiotics. Furthermore, Ahmed et al. (2001) found that all strains of *Bradyrhizobium* sp. were resistant to one or more antibiotics. In our study, the sensitive bacterial strains only showed some resistance to vancomycin, and they were almost sensitive to the antibiotics used (Table 4). There is a correlation between heavy metal resistance and antibiotic resistance. Sabry et al. (1997) have found that there is a correlation between metal tolerance and antibiotic resistance for isolates from sea water. Similarly, nickel-resistant bacteria isolated from serpentine soil in Anadaman, India, have shown resistance to multiple antibiotics (Pal et al., 2004). Bezverbnaya et al. (2005) have shown that 220 Enterobacteriaceae and
The production of antioxidant enzymes by metal-resistant bacterial strains under Zn\(^{2+}\) and Pb\(^{2+}\) stress

Heavy metals and other pollutants increase the production of reactive oxygen species (Kappus, 1987). Our results showed that Zn\(^{2+}\) and Pb\(^{2+}\) are pollutant metals that induce the production of reactive oxygen species and cause an imbalance in the cellular oxidative status of bacteria. The antioxidant enzymatic activities of the isolates obtained ranged from high activity (1,030%) to low activity (15.8%) as described in Table 5, which were determined by the percentage or μmol min\(^{-1}\) g\(^{-1}\) protein for each gram of dry weight of cells minus the control. In general, the results demonstrated that 100 mg/L Zn\(^{2+}\) or Pb\(^{2+}\) increased and induced the activity of the antioxidant enzymes catalase, peroxidase and ascorbate peroxidase (Table 5). The greatest percentage of catalase was observed for P. putida KNU8 with 100 mg/L Zn\(^{2+}\) (311%). Both other strains, P. putida KNU5 (142%) and B. subtilis Kh, showed the same catalase activity (141%) under Zn\(^{2+}\) stress (Figure 2). Under Pb\(^{2+}\) stress, catalase was induced but at a reduced percentage as compared to under Zn\(^{2+}\) stress; for P. putida KNU8, a greater percentage of catalase activity (229%) was observed, as compared to the other two strains.

Antioxidants are compounds that are able to overcome biologically toxic reactive oxygen species without undergoing conversion to harmful free radicals (Lurie,
The induction of peroxidase is less than catalase and ascorbate peroxidase enzymes under Zn\(^{2+}\) or Pb\(^{2+}\) stress. Only a high percentage of peroxidase was observed for *B. subtilis* Kh with 100 mg/L Pb\(^{2+}\) (260%) and 100 mg/L Zn\(^{2+}\) (160%). *P. putida* KNU8 and *P. putida* KNU5 showed less activity for peroxidase under both heavy metal conditions (Figures 3 to 4). Peroxidases are produced under a variety of stressful conditions such as water stress (Zhang and Kirkham, 1994), chilling (Prasad et al., 1995), salinity (Mittal and Dubey 1991), gamma-radiation (Wada et al., 1998) and under toxic levels of Al, Cu, Cd or Zn (Shah et al., 2001; Chaoui et al., 1997).

The strain *P. putida* KNU5 showed high induction for ascorbate peroxidase (1,030%) under the stress of 100 mg/L Zn\(^{2+}\), while the activity of ascorbate peroxidase was decreased to 115% under 100 mg/L Pb\(^{2+}\) (Figure 4).
addition, Pb^{2+} induced ascorbate peroxidase for *P. putida* KNU8. The bacterial strain *B. subtilis* Kh showed less enzymatic activity as compared to the other strains. Chen et al. (2000) have found that CuSO_{4} elevates the production of ascorbate peroxidase and peroxidase without affecting the release of catalase. In addition, our results are in agreement with the data of Lee and Shin (2003) and Lee (1997). They have reported that the activity of catalases slightly increases, followed by that of ascorbate peroxidase and peroxidase after Cd treatment.

Thus, the present study suggests that heavy metals cause oxidative stress in *P. putida* and that antioxidant enzymes appear to play a pivotal role in combating oxidative stress, providing protection to *P. putida*. In addition, antioxidant enzymes can be used to biologically monitor heavy metal pollution.

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