CHAPTER-IV
4.1. Soil texture analysis

Soil samples were collected from contaminated crop field of Cachar district of Assam, nearby paper industry, garages and petrol pumps and other polluted sites. After performing the texture analysis of soil samples, most of them were clay-to-clay loam in lowlands (clay>50%). The soil composition revealed the occurrence of highest percentage of clay followed by sand and silt in the crop field soils. Bulk density of waste water irrigated soil nearby Cachar Paper Mill, Panchgram was found to be between 1.1-1.3 g/ml. Sample collected from Village Karatigram of Cachar District showed 0.7g/ml indicating high organic content. The colour of the soil was brown to dark black. In the Munsell soil colour chart, its value was 4 and chroma 2 (Fig. 10), indicates the fact that the soil is well humified and developed under humid grassland. On an average, the water holding capacity of all cultivated and irrigated soil was found to be between 28% - 34%.

Interestingly, the uncultivable crop field in Silchar (nearby garage area in Kathal point, Silchar town) town has clay textured soil with high bulk density but because of little porosity (14%) it is very difficult for roots to penetrate.

Village Najathpur accounts for highest productivity and good yield as reported according to the field survey during soil sample collection. The soil was much fertile, but due to lack of irrigation facility farmers have to depend on rainfall. The N, P, K content was medium, medium and low. The pH range of the soil was found to be 4.8 – 5.2, indicates strongly acidic nature of the soil. Acidification can be the result of the excessive use of fertilizer, or it can also occur naturally.

Fig. 10: Munsell colour chart, indicating value 4 and chroma 2.
4.2. Isolation and enumeration of bacteria

Total viable counts ranges from $4.5 \times 10^4$ (CFU/g) in site-1 sample to $20 \times 10^4$ (CFU/g) in site-4 sample as shown in Table 4. At 1000 µg/ml concentration of CdCl$_2$, the frequencies of resistance to cadmium varied from 48% in site-6 to 79.2% in site-7. The CFU for lead resistant bacteria at concentration 100µg/ml depicts frequencies of resistance to lead ranges from 48.9% (site-4) to 75.3% (site-8). It has been observed that highest frequency for lead resistance was observed at site-8 with percentage of lead resistance bacteria as 90% (at 50µg/ml) and 75.3% (at 100µg/ml).

Table 4: Enumeration of Bacteria with percentage of Cadmium and lead Resistant Bacteria

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Bacterial Count (CFU/g)</th>
<th>Cadmium Resistant Bacteria</th>
<th>Lead Resistant Bacteria</th>
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<tr>
<td></td>
<td>(CFU/g)</td>
<td>% of Resistant Bacteria</td>
<td>(CFU/g)</td>
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<td>Site-1</td>
<td>20.0 X 10$^4$</td>
<td>17.6 X 10$^4$</td>
<td>88.0</td>
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<tr>
<td>Site-2</td>
<td>11.3 X 10$^4$</td>
<td>9.2 X 10$^4$</td>
<td>81.4</td>
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<tr>
<td>Site-3</td>
<td>14.0 X 10$^4$</td>
<td>11.0 X 10$^4$</td>
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<td>Site-4</td>
<td>4.5 X 10$^4$</td>
<td>3.7 X 10$^4$</td>
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<td>Site-5</td>
<td>17.0 X 10$^4$</td>
<td>14.0 X 10$^4$</td>
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<td>Site-6</td>
<td>12.5 X 10$^4$</td>
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<td>Site-7</td>
<td>4.8 X 10$^4$</td>
<td>4.1 X 10$^4$</td>
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<td>Site-8</td>
<td>15.0 X 10$^4$</td>
<td>12.8 X 10$^4$</td>
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4.3. Morphological and cultural characteristics of isolated starins

Sixty-two bacteria were isolated from soil samples, identified by their colony characterization, gram staining and biochemical testing (Holt et al., 1994; Sneath, 1986), and were pre-assumed as *Pseudomonas* sp., *Klebsiella* sp. and *Bacillus* sp. The observed colony morphological characteristics pertaining to color, shape and surface are collectively displayed at Table 5. The highest prevalence was observed by *Pseudomonas* sp. (45.2%) followed by *Bacillus* sp. (30.6%) and *Klebsiella* sp. (24.2%).

Table 5: Gram Staining and Morphological Characteristics of bacterial Isolates.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Media used</th>
<th>Gram staining</th>
<th>Colony characterization</th>
<th>Size</th>
<th>Colour</th>
<th>Margin</th>
<th>Surface</th>
<th>Code given</th>
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4.4. Biochemical characterization of isolated strains

After performing the biochemical tests, it was deciphered that all Ps isolates (Ps-1 to Ps-28) and all KI isolates (KI-1 to KI-15) were gram-ve rod while all Ba isolates (Ba-1 to Ba-19) were gram +ve rod. Positive results for nitrate reduction test were shown by all the strains except Ps-9, Ps-18 and Ba-10. Almost all the Ps and Ba isolates were found to be indole negative, MR negative (Table 6). With a major exceptions in KI isolates (KI-1, KI-2, KI-6, KI-8 to KI-15), all the isolates were urease negative. Oxidase tests for Ps isolates were positive indicating them to be aerobic strains, while others were negative. All Ba and KI isolates were variable in VP test, while all Ps isolates gave negative results. Hydrolysis of starch was seen in all Ba isolates and Ps-19 (Fig. 14).

Table 6: Biochemical characteristics of the isolated strains

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<th>Indole</th>
<th>MR</th>
<th>VP</th>
<th>Citrate</th>
<th>Nitrate Reduction</th>
<th>Starch Hydrolysis</th>
<th>Limous Milk Test</th>
<th>Urease Test</th>
<th>Catalase Test</th>
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**Fig. 11:** Gram staining of some of the bacterial isolates

After performing different biochemical tests and gram staining (Fig. 11), the isolates were confirmed as *Pseudomonas* sp., *Klebsiella* sp. and *Bacillus* sp. and very few remains unidentified.
Fig. 12: IMViC test of bacterial isolates.
(a) Control, (b) Ps-1, (c) Kl-8 and (d) Ba-6

Fig. 13: Nitrate Reduction test of the bacterial isolates

Fig. 14: Starch hydrolysis
4.5. 16S rDNA sequencing of bacterial isolates

Five bacterial isolates were identified on the basis of their 16S rDNA sequencing followed by nucleotide homology and phylogenetic analysis. The isolates Ps-1, Ps-4, Ps-11 and Ps-17 were identified as *Pseudomonas aeruginosa* whereas Ba-6 as *Bacillus cereus*.

The sequence were first analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for finding the closest homologous sequences and aligned. All the relevant sequences were saved in a fasta format (*.fas, *.fasta or *.txt) and aligned using CLUSTAL X2. The output of CLUSTAL (i.e., *.aln file) become the input for MEGA. In MEGA, a distance matrix was made based on nucleotide sequence homology (using Kimura-2 Parameter) followed by construction of phylogenetic tree using Neighbour Joining (NJ) method.

Four sequences were submitted to NCBI-GenBank and accession number has been obtained for the isolated strains. The detailed information regarding these strains along with their GenBank accession number are given below:

<table>
<thead>
<tr>
<th>Strain Code</th>
<th>Identified as</th>
<th>GenBank Acc. No.</th>
<th>Alignmnet View</th>
<th>Nucleotide similarity and distance matrix</th>
<th>Phylogenetic tree (NJ method)</th>
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<td>Table 7</td>
<td>Table 8</td>
<td>Fig. 15</td>
</tr>
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<td>KF031123</td>
<td>Table 9</td>
<td>Table 10</td>
<td>Fig. 16</td>
</tr>
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<td><em>Pseudomonas aeruginosa</em> SN4</td>
<td>KF447770</td>
<td>Table 11</td>
<td>Table 12</td>
<td>Fig. 17</td>
</tr>
<tr>
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<td>KF447771</td>
<td>Table 13</td>
<td>Table 14</td>
<td>Fig. 18</td>
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<td>Ba-6</td>
<td><em>Bacillus cereus</em></td>
<td>To be submitted</td>
<td>Table 15</td>
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>Pseudomonas aeruginosa strain SN1 16S ribosomal RNA gene sequence

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Table 7: Alignment view of SN1 using combination of NCBI GenBank and RDP database

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Table 8: Nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample ‘SN 1’ and ten other closest homologous microbes

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</table>

Fig. 15: Phylogenetic tree of SN1 constructed using neighbor Joining Method

Based on nucleotides homology and phylogenetic analysis the microbe (SN 1) was detected to be *Pseudomonas* sp (Fig. 15). Nearest homolog was found to be *Pseudomonas aeruginosa* (GeneBank Accession Number: JQ866912.1).

After submitting the sequence to NCBI-GenBank, the accession number obtained was KF031122.
### Pseudomonas aeruginosa strain SN3 16S ribosomal RNA gene sequence

```
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### Table 9: Alignment view of SN3 using combination of NCBI GenBank and RDP database

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Table 10: Nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample ‘SN 3’ and ten other closest homologous microbes

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<td>0.025</td>
<td>0.000</td>
<td>0.025</td>
</tr>
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<td>0.079</td>
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<td>0.011</td>
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<td>0.025</td>
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</tr>
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<td>0.041</td>
<td>0.052</td>
<td>0.034</td>
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<td>0.053</td>
<td>0.032</td>
<td>0.047</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Fig. 16: Phylogenetic tree of SN3 constructed using neighbor Joining Method

Based on nucleotides homology and phylogenetic analysis the microbe (SN 3) was detected to be *Pseudomonas aeruginosa* (Fig. 16). Nearest homolog was found to be *Pseudomonas* sp. (GeneBank Accession Number: JX416374.1).

After submitting the sequence to NCBI-GenBank, the accession number obtained was KF031123.
>Pseudomonas aeruginosa strain SN4 16S ribosomal RNA gene sequence

```
GTCGAGCGGATGAAGGAGGCTTGTCCCTGGAATTCGACGCCGGACGGTTAGTAAAGCTGAGAATGATCAGAGTATTCCACGACTCTGGAACAGGAGGCTTCTATTGCTCGAGGATACGATGAGCTGCAGCTTCGAGGCAGCGGAGGCTTCTATTGCTCGAGGATACGATGAGCTGCAGCTTCGAGGCAGCGGAGGCTTCTATTGCTCGAGGATACGATGAGCTGCAGCT
```

Table 11: Alignment view of SN4 using combination of NCBI GenBank and RDP database

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<tr>
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</tr>
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</tr>
<tr>
<td></td>
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<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td></td>
<td>KC417305.1</td>
<td>0.99</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td></td>
<td>KC197062.1</td>
<td>0.99</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td></td>
<td>KC121042.1</td>
<td>0.99</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td></td>
<td>JF901362.1</td>
<td>0.99</td>
<td>Endophytic bacterium</td>
</tr>
<tr>
<td></td>
<td>JO894531.1</td>
<td>0.99</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td></td>
<td>JN572122.1</td>
<td>0.99</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td></td>
<td>HQ841508.1</td>
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<td>Pseudomonas sp.</td>
</tr>
<tr>
<td></td>
<td>JF281099.1</td>
<td>0.99</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td></td>
<td>FM997334.1</td>
<td>0.99</td>
<td>Uncultured bacterium</td>
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</table>
Table 12: Nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample ‘SN 4’ and ten other closest homologous microbes

<table>
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<td>0.016</td>
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<td>0.099</td>
<td>0.029</td>
<td>0.053</td>
<td>0.076</td>
</tr>
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<td>KC522362</td>
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<td>0.018</td>
<td>0.017</td>
<td>0.028</td>
<td>0.034</td>
<td>0.068</td>
<td>0.008</td>
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<td>0.044</td>
</tr>
<tr>
<td>3</td>
<td>KC417305</td>
<td>0.039</td>
<td>0.018</td>
<td>0.000</td>
<td>0.023</td>
<td>0.023</td>
<td>0.033</td>
<td>0.062</td>
<td>0.010</td>
<td>0.025</td>
<td>0.038</td>
</tr>
<tr>
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<td>0.017</td>
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<td>0.000</td>
<td>0.037</td>
<td>0.047</td>
<td>0.084</td>
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<td>0.060</td>
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<td>0.023</td>
<td>0.037</td>
<td>0.000</td>
<td>0.029</td>
<td>0.061</td>
<td>0.028</td>
<td>0.018</td>
<td>0.037</td>
</tr>
<tr>
<td>6</td>
<td>JF901362</td>
<td>0.062</td>
<td>0.034</td>
<td>0.033</td>
<td>0.047</td>
<td>0.029</td>
<td>0.080</td>
<td>0.051</td>
<td>0.039</td>
<td>0.021</td>
<td>0.030</td>
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<tr>
<td>7</td>
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<td>0.062</td>
<td>0.081</td>
<td>0.051</td>
<td>0.061</td>
<td>0.000</td>
<td>0.071</td>
<td>0.054</td>
<td>0.024</td>
</tr>
<tr>
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<td>0.008</td>
<td>0.010</td>
<td>0.013</td>
<td>0.028</td>
<td>0.039</td>
<td>0.071</td>
<td>0.000</td>
<td>0.031</td>
<td>0.048</td>
</tr>
<tr>
<td>9</td>
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<td>0.030</td>
<td>0.025</td>
<td>0.039</td>
<td>0.018</td>
<td>0.021</td>
<td>0.054</td>
<td>0.031</td>
<td>0.000</td>
<td>0.030</td>
</tr>
<tr>
<td>10</td>
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<td>0.044</td>
<td>0.038</td>
<td>0.060</td>
<td>0.037</td>
<td>0.030</td>
<td>0.024</td>
<td>0.048</td>
<td>0.010</td>
<td>0.000</td>
</tr>
<tr>
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<td>0.056</td>
<td>0.046</td>
<td>0.066</td>
<td>0.057</td>
<td>0.056</td>
<td>0.032</td>
<td>0.056</td>
<td>0.048</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Fig. 17: Phylogenetic tree of SN4 constructed using neighbor Joining Method

Based on nucleotides homology and phylogenetic analysis the microbe (SN 4) was detected to be *Pseudomonas aeruginosa* (Fig. 17). Nearest homolog was found to be *Pseudomonas aeruginosa* (GeneBank Accession Number: JN572122.1).

After submitting the sequence to NCBI-GenBank, the accession number obtained was KF447770.
>Pseudomonas aeruginosa strain SN5 16S ribosomal RNA gene sequence

AAATCGAGCGGATGGGAGCTTGATCTCTGAGAATCGGCCGCGGAGTTAGTCTAGG
AAATCTGCTGATGAGGGGAGTAAAATGTCGCGAAACCGGCGTCATACGCTGACCGG
GAAAGTGAGGGGCGATTCGCAACGGCTATGAGAATGGCGCTAGTCTGAGTCTAGG
GGTTAAGAGCCTACCAAGCGAGCGCTACGTCGTAATGCCTGAGGAGTATAGCTA
GAGACAGCCGTCCGACTCCCTACGGGAGAGACAGTGATGGGAAATATTGGCAGAT
GATCCAGCCCGTCGCTGAGTGAAGAAGGCTCGAGCTGGTATTTGAGTGATGTTTG
AGTACAGATGTAAGGCTGGAATTTTCTGCTGAGGCTCGGTAATAGCTAGAAAG
ACACCGATGCGGAGAGGCAGACCTGTGACTGAGACTGAGGAGGTGCAGGTTAGT
CAATTAGTAGATACCTTGGTGAATCCACGCAGGCTACGATAGCCGCTTGGGAGATT
ACCTTACCTGGGTTCACGACTGAGAATTCCTGCTGAGATGTTTGACGCTGGGAA
CACAGGCTGCTATGGCGCTGTGCTGAGATGTTTGACTTCCGGTAACGACCG
CAACCTTGTCTTTAGGACACCTTCCGGGTGGGACCTTAAAGGACTGCTGGTGAC
GAGAAGTGGAAGTTGATCAGCAGTACGTCAATGATCAGCTGGCGCTACCCAGG
TGTCGCTACAAAGGGGTGCGAACTCCGAGAGTGGTAGCTAATCCCTAAAAACCGAT
GATCCAGCTCGTCAACCTGCTGAGGTGGAATCTGCTGACGATATCAGAGAAGTGC
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CTAATGCTAACCAGCAA

Table 13: Alignment view of SN5 using combination of NCBI GenBank and RDP database

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<th>Alignment results</th>
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<td>Pseudomonas sp.</td>
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<td>JQ866912.1</td>
<td>1</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>1</td>
<td>KC417305.1</td>
<td>1</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
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<td>JX416374.1</td>
<td>1</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>1</td>
<td>JX035794.1</td>
<td>1</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>1</td>
<td>JQ891453.1</td>
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<td>Pseudomonas sp.</td>
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<td>1</td>
<td>JN995664.1</td>
<td>1</td>
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<td>JN572422.1</td>
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<td>Pseudomonas aeruginosa</td>
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<td>1</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>1</td>
<td>HQ202541.1</td>
<td>1</td>
<td>Pseudomonas aeruginosa</td>
</tr>
</tbody>
</table>
Table 14: Nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample ‘SN 5’ and ten other closest homologous microbes

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<td>0.057</td>
<td>0.042</td>
<td>0.025</td>
<td>0.124</td>
<td>0.103</td>
<td>0.079</td>
<td>0.032</td>
<td>0.079</td>
<td>0.012</td>
</tr>
<tr>
<td>2 JX094352</td>
<td>0.061</td>
<td>0.000</td>
<td>0.017</td>
<td>0.028</td>
<td>0.041</td>
<td>0.076</td>
<td>0.055</td>
<td>0.032</td>
<td>0.036</td>
<td>0.030</td>
<td>0.051</td>
</tr>
<tr>
<td>3 JQ866912</td>
<td>0.057</td>
<td>0.017</td>
<td>0.000</td>
<td>0.026</td>
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<td>0.078</td>
<td>0.057</td>
<td>0.034</td>
<td>0.031</td>
<td>0.032</td>
<td>0.047</td>
</tr>
<tr>
<td>4 KX417305</td>
<td>0.042</td>
<td>0.028</td>
<td>0.026</td>
<td>0.000</td>
<td>0.018</td>
<td>0.084</td>
<td>0.063</td>
<td>0.040</td>
<td>0.010</td>
<td>0.038</td>
<td>0.030</td>
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<td>0.041</td>
<td>0.037</td>
<td>0.018</td>
<td>0.000</td>
<td>0.101</td>
<td>0.080</td>
<td>0.057</td>
<td>0.008</td>
<td>0.055</td>
<td>0.012</td>
</tr>
<tr>
<td>6 JX035794</td>
<td>0.124</td>
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<td>0.076</td>
<td>0.084</td>
<td>0.101</td>
<td>0.000</td>
<td>0.022</td>
<td>0.049</td>
<td>0.093</td>
<td>0.047</td>
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<td>0.057</td>
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<td>0.022</td>
<td>0.000</td>
<td>0.034</td>
<td>0.072</td>
<td>0.025</td>
<td>0.091</td>
</tr>
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<td>0.034</td>
<td>0.040</td>
<td>0.057</td>
<td>0.049</td>
<td>0.034</td>
<td>0.000</td>
<td>0.050</td>
<td>0.019</td>
<td>0.069</td>
</tr>
<tr>
<td>9 JN882122</td>
<td>0.032</td>
<td>0.036</td>
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<td>0.093</td>
<td>0.072</td>
<td>0.050</td>
<td>0.000</td>
<td>0.048</td>
<td>0.020</td>
</tr>
<tr>
<td>10 JF281099</td>
<td>0.079</td>
<td>0.030</td>
<td>0.032</td>
<td>0.038</td>
<td>0.055</td>
<td>0.047</td>
<td>0.025</td>
<td>0.019</td>
<td>0.048</td>
<td>0.000</td>
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<td>0.112</td>
<td>0.091</td>
<td>0.069</td>
<td>0.020</td>
<td>0.067</td>
<td>0.060</td>
</tr>
</tbody>
</table>

Fig. 18: Phylogenetic tree of SN5 constructed using neighbor Joining Method

Based on nucleotides homology and phylogenetic analysis the microbe (SN 5) was detected to be *Pseudomonas aeruginosa* (Fig. 18). Nearest homolog was found to be *Pseudomonas* sp. (GeneBank Accession Number: HQ202541.1).

After submitting the sequence to NCBI-GenBank, the accession number obtained was KF447771.
> *Bacillus cereus* (SN2) 16S ribosomal RNA gene sequence

GCAAGTCAGCGAATGGATTAAGAGCTTGCTTTGCGATGGAAGTACGCGACGGGGATGCGTAACACGTGGAATGGGGATTAAAGACGCTTTCCGCTCCTGTCATGACATGGGATGACGGCAGGGGTGATC

CCACACTGGAGGACACCGGACCCGACACCTTACCCGGAGGAGCAGTAGGGAATCTCCCGCAATGGACGAAAGTCTGACGGAGCGACGCCGCTGAGTGATGAGTAGGAGGCTTTCCGGGTCTGTAACACTCGTTG

TGGAGGAAGAAACAGCTCATGTTAGTAAAGGCTGGCACCTTTGACGTTACCCAGAAGCCCACCGGTCAACCCGGGTAGGATGACGGTAAATGCATAGAGATATGGAGGAAACAGCTGGCAAGGCGACTTTCTGGTCTGTAACCTGACATGAGGCGGCA

AAGCCTGGGAGCAACAGGATTAGATATCCCTGTTAGCTACCCGAGGCAAGACGATGAGTCTAAGTGGAGATTCCTCCCGTGGAGAATCAGGC

CGAAGGCTGCAAACTCAAAGAATGACCGGGGGGGCAGAAGCGGTGGAGCATGTGCTTATAATCGAAGCAACCGGAGACCTACCCGGAGCATCCTGGAGATGACGGTCCTCC

TTCCGGGAGCAGATGACAGGTTGCTGGCATGTGGTGTGTCGTCAGCTCGTGCTGAGTGATTGGGGTTAACGCGACAAGCAGCAGCTCCTTT

Table 15: Alignment view of SN2 using combination of NCBI GenBank and RDP database

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</tr>
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<td><em>Bacillus thuringiensis</em></td>
<td></td>
</tr>
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<td>KF150341.1</td>
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<td><em>Bacillus anthracis</em></td>
<td></td>
</tr>
<tr>
<td>KC906252.1</td>
<td>0.99</td>
<td><em>Bacillus sp.</em></td>
<td></td>
</tr>
<tr>
<td>KC527059.1</td>
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<td><em>Bacillus cereus</em></td>
<td></td>
</tr>
<tr>
<td>KC960017.1</td>
<td>0.99</td>
<td><em>Bacillus thuringiensis</em></td>
<td></td>
</tr>
<tr>
<td>KC466270.1</td>
<td>0.99</td>
<td><em>Bacillus sp.</em></td>
<td></td>
</tr>
<tr>
<td>KC849454.1</td>
<td>0.99</td>
<td><em>Bacillus cereus</em></td>
<td></td>
</tr>
<tr>
<td>KC484964.1</td>
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<td><em>Bacterium CalalmoE420</em></td>
<td></td>
</tr>
<tr>
<td>KC519422.1</td>
<td>0.99</td>
<td><em>Bacillus anthracis</em></td>
<td></td>
</tr>
<tr>
<td>AB592487.1</td>
<td>0.99</td>
<td><em>Bacillus cereus</em></td>
<td></td>
</tr>
</tbody>
</table>
Table 16: Nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample ‘SN 2’ and ten other closest homologous microbes

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<th>7</th>
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<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
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<td>0.446</td>
<td>0.387</td>
<td>0.464</td>
<td>0.437</td>
<td>0.425</td>
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</tr>
<tr>
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<td>0.042</td>
<td>0.090</td>
<td>0.089</td>
<td>0.070</td>
<td>0.141</td>
<td>0.082</td>
<td>0.095</td>
<td>0.101</td>
</tr>
<tr>
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<td>0.042</td>
<td>0.000</td>
<td>0.090</td>
<td>0.087</td>
<td>0.077</td>
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<td>0.090</td>
<td>0.000</td>
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<td>0.069</td>
<td>0.057</td>
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<td>0.049</td>
</tr>
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<td>0.087</td>
<td>0.029</td>
<td>0.000</td>
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<td>0.046</td>
<td>0.048</td>
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</tr>
<tr>
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<td>0.041</td>
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<td>0.089</td>
<td>0.077</td>
<td>0.057</td>
<td>0.030</td>
</tr>
<tr>
<td>7</td>
<td>KC468227.</td>
<td>0.387</td>
<td>0.141</td>
<td>0.140</td>
<td>0.084</td>
<td>0.046</td>
<td>0.041</td>
<td>0.079</td>
<td>0.046</td>
<td>0.048</td>
<td>0.057</td>
</tr>
<tr>
<td>8</td>
<td>KC849454.</td>
<td>0.464</td>
<td>0.082</td>
<td>0.078</td>
<td>0.057</td>
<td>0.046</td>
<td>0.077</td>
<td>0.114</td>
<td>0.060</td>
<td>0.087</td>
<td>0.093</td>
</tr>
<tr>
<td>9</td>
<td>KC489644.</td>
<td>0.437</td>
<td>0.085</td>
<td>0.079</td>
<td>0.043</td>
<td>0.048</td>
<td>0.057</td>
<td>0.067</td>
<td>0.087</td>
<td>0.000</td>
<td>0.049</td>
</tr>
<tr>
<td>10</td>
<td>KC519422.</td>
<td>0.425</td>
<td>0.101</td>
<td>0.095</td>
<td>0.049</td>
<td>0.057</td>
<td>0.038</td>
<td>0.051</td>
<td>0.093</td>
<td>0.049</td>
<td>0.060</td>
</tr>
<tr>
<td>11</td>
<td>AB592487.</td>
<td>0.303</td>
<td>0.232</td>
<td>0.233</td>
<td>0.165</td>
<td>0.173</td>
<td>0.179</td>
<td>0.164</td>
<td>0.204</td>
<td>0.167</td>
<td>0.152</td>
</tr>
</tbody>
</table>

Fig. 19: Phylogenetic tree of SN2 constructed using neighbor Joining Method

Based on nucleotides homology and phylogenetic analysis the microbe (SN 2) was detected to be *Bacillus cereus* (Fig. 19). Nearest homolog was found to be *Bacillus* sp. (GeneBank Accession Number: KC466270.1).

*Not submitted to NCBI-GenBank*
4.6. Minimum Inhibitory Concentration (MIC) of bacterial strains

All the bacterial isolates exhibited high resistance to heavy metals with minimum inhibitory concentration (MIC) against heavy metals ranging from 20μg/ml to 1800μg/ml (Table 17). *Pseudomonas aeruginosa* SN3 exhibited high resistance with MIC for heavy metals as 1800μg/ml against cadmium, 170μg/ml against lead, 60μg/ml against copper and 1800μg/ml against zinc.

There is a great variation in the tolerance capacity of isolated bacterial strains against cadmium chloride. Around 54.8% of the tested isolates were resistant above 1000μg/ml CdCl₂ concentration (Table 17), 30.6% were found to be between 500-1000 μg/ml and 14.5% were tolerant upto 500 μg/ml CdCl₂ concentrations. Of the 62 strains tested for their response to cadmium chloride, most of the strains showed multi-metal tolerance.

Lead showed the MIC values between 80-180μg/ml which was added in the form of lead acetate. Most of the strains were tolerable at 100μg/ml lead acetate concentration. Surprisingly, it has been observed that the isolates that can easily tolerate 100μg/ml concentration find some extreme stress in surviving at 130-140μg/ml lead acetate concentration. In the presence to zinc stress, around 90% of the bacterial isolates were found to be tolerant at 1000μg/ml zinc concentration. Fig. 23 shows the growth of *Pseudomonas* sp. against zinc at 1500 μg/ml. In copper stress condition, very rarely they survive 50μg/ml copper sulphate concentration. It has been shown in Fig. 25 that one strains *Bacillus cereus* grows in 50μg/ml copper sulphate stress.

*Klebsellia* sp. showed 1700μg/ml against cadmium, 160μg/ml against lead, 50μg/ml against copper and 1500μg/ml against zinc whereas *Bacillus* sp. showed the values similar to *Klebsellia* sp. except lead 150μg/ml.

*Pseudomonas aeruginosa* exhibits multiple tolerance to heavy metals. Heavy Metal Tolerance Test indicated highest tolerance to cadmium by *P. aeruginosa* SN3 (1800μg/ml), lead by *P. aeruginosa* SN3 and *P. aeruginosa* SN5 (170μg/ml).
Table 17: Resistance of bacterial isolates to heavy metals (Cd, Pb, Cu and Zn).

<table>
<thead>
<tr>
<th>Bacterial Isolates</th>
<th>Strain No.</th>
<th>Minimum Inhibitory Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cadmium</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>Ps-1</td>
<td>1700 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-2</td>
<td>1100 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-3</td>
<td>800 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-4</td>
<td>1800 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-5</td>
<td>1000 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-6</td>
<td>1200 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-7</td>
<td>700 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-8</td>
<td>400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-9</td>
<td>1100 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-10</td>
<td>1000 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-11</td>
<td>1400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-12</td>
<td>600 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-13</td>
<td>200 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-17</td>
<td>1400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-15</td>
<td>300 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-16</td>
<td>500 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-17</td>
<td>1400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-18</td>
<td>800 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-19</td>
<td>800 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-20</td>
<td>600 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-21</td>
<td>1200 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-22</td>
<td>600 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-23</td>
<td>400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-24</td>
<td>400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-25</td>
<td>1400 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-26</td>
<td>1200 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-27</td>
<td>200 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Ps-28</td>
<td>1000 µg/ml</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Ba-1</td>
<td>1600 μg/ml</td>
<td>150 μg/ml</td>
</tr>
<tr>
<td>Ba-2</td>
<td>700 μg/ml</td>
<td>130 μg/ml</td>
</tr>
<tr>
<td>Ba-3</td>
<td>800 μg/ml</td>
<td>130 μg/ml</td>
</tr>
<tr>
<td>Ba-4</td>
<td>1500 μg/ml</td>
<td>150 μg/ml</td>
</tr>
<tr>
<td>Ba-5</td>
<td>1600 μg/ml</td>
<td>130 μg/ml</td>
</tr>
<tr>
<td>Ba-6</td>
<td>1700 μg/ml</td>
<td>150 μg/ml</td>
</tr>
<tr>
<td>Ba-7</td>
<td>1700 μg/ml</td>
<td>120 μg/ml</td>
</tr>
<tr>
<td>Ba-8</td>
<td>1300 μg/ml</td>
<td>120 μg/ml</td>
</tr>
<tr>
<td>Ba-9</td>
<td>1400 μg/ml</td>
<td>150 μg/ml</td>
</tr>
<tr>
<td>Ba-10</td>
<td>1500 μg/ml</td>
<td>130 μg/ml</td>
</tr>
<tr>
<td>Ba-11</td>
<td>1500 μg/ml</td>
<td>110 μg/ml</td>
</tr>
<tr>
<td>Ba-12</td>
<td>1500 μg/ml</td>
<td>140 μg/ml</td>
</tr>
<tr>
<td>Ba-13</td>
<td>800 μg/ml</td>
<td>110 μg/ml</td>
</tr>
<tr>
<td>Ba-14</td>
<td>1400 μg/ml</td>
<td>150 μg/ml</td>
</tr>
<tr>
<td>Ba-15</td>
<td>1100 μg/ml</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Ba-16</td>
<td>900 μg/ml</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Ba-17</td>
<td>1000 μg/ml</td>
<td>120 μg/ml</td>
</tr>
<tr>
<td>Ba-18</td>
<td>1400 μg/ml</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Ba-19</td>
<td>1200 μg/ml</td>
<td>110 μg/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Klebsellia sp.</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kl-1</td>
<td>1600 μg/ml</td>
<td>140 μg/ml</td>
<td>50 μg/ml</td>
<td>1500 μg/ml</td>
</tr>
<tr>
<td>Kl-2</td>
<td>1500 μg/ml</td>
<td>160 μg/ml</td>
<td>35 μg/ml</td>
<td>1400 μg/ml</td>
</tr>
<tr>
<td>Kl-3</td>
<td>1100 μg/ml</td>
<td>150 μg/ml</td>
<td>45 μg/ml</td>
<td>1500 μg/ml</td>
</tr>
<tr>
<td>Kl-4</td>
<td>600 μg/ml</td>
<td>90 μg/ml</td>
<td>40 μg/ml</td>
<td>1300 μg/ml</td>
</tr>
<tr>
<td>Kl-5</td>
<td>1300 μg/ml</td>
<td>100 μg/ml</td>
<td>50 μg/ml</td>
<td>1400 μg/ml</td>
</tr>
<tr>
<td>Kl-6</td>
<td>1300 μg/ml</td>
<td>100 μg/ml</td>
<td>40 μg/ml</td>
<td>1500 μg/ml</td>
</tr>
<tr>
<td>Kl-7</td>
<td>1000 μg/ml</td>
<td>110 μg/ml</td>
<td>30 μg/ml</td>
<td>1100 μg/ml</td>
</tr>
<tr>
<td>Kl-8</td>
<td>700 μg/ml</td>
<td>90 μg/ml</td>
<td>30 μg/ml</td>
<td>1100 μg/ml</td>
</tr>
<tr>
<td>Kl-9</td>
<td>300 μg/ml</td>
<td>100 μg/ml</td>
<td>40 μg/ml</td>
<td>1200 μg/ml</td>
</tr>
<tr>
<td>Kl-10</td>
<td>1100 μg/ml</td>
<td>110 μg/ml</td>
<td>50 μg/ml</td>
<td>1200 μg/ml</td>
</tr>
<tr>
<td>Kl-11</td>
<td>1100 μg/ml</td>
<td>130 μg/ml</td>
<td>35 μg/ml</td>
<td>1400 μg/ml</td>
</tr>
<tr>
<td>Kl-12</td>
<td>1700 μg/ml</td>
<td>150 μg/ml</td>
<td>30 μg/ml</td>
<td>1000 μg/ml</td>
</tr>
<tr>
<td>Kl-13</td>
<td>1300 μg/ml</td>
<td>130 μg/ml</td>
<td>50 μg/ml</td>
<td>1000 μg/ml</td>
</tr>
<tr>
<td>Kl-14</td>
<td>1000 μg/ml</td>
<td>130 μg/ml</td>
<td>40 μg/ml</td>
<td>800 μg/ml</td>
</tr>
<tr>
<td>Kl-15</td>
<td>500 μg/ml</td>
<td>110 μg/ml</td>
<td>40 μg/ml</td>
<td>1200 μg/ml</td>
</tr>
</tbody>
</table>
Fig. 20: MIC of *Pseudomonas* sp. against Cd (at 1100 μg/ml).

Fig. 21: MIC of *Pseudomonas* sp. against Cu (at 50 μg/ml).

Fig. 22: MIC of *Pseudomonas* sp. against Pb (at 130 μg/ml).

Fig. 23: MIC of *Pseudomonas* sp. against Zn (at 1500 μg/ml).

Fig. 24: MIC of *Bacillus* sp. against Cd (at 1500 μg/ml).

Fig. 25: MIC of *Bacillus* sp. against Cu (at 50 μg/ml).
Fig. 26: MIC of *Bacillus* sp. against Pb (at 130 µg/ml).

Fig. 27: MIC of *Bacillus* sp. against Zn (at 400 µg/ml).

Fig. 28: MIC of *Klebsiella* sp. against Cd (at 130 µg/ml).

Fig. 29: MIC of *Klebsiella* sp. against Cu (at 50 µg/ml).

Fig. 30: MIC of *Klebsiella* sp. against Pb (at 130 µg/ml).

Fig. 31: MIC of *Klebsiella* sp. against Zn (at 180 µg/ml).
4.7. Determination of Thermal Death Time (TDT)

The isolates having higher minimum inhibitory concentration against cadmium and lead were selected for further investigation. Thermal Death Time (TDT) was determined by exposing the bacterial isolates at 60°C for 10, 20, 30, 40, 50 and 60 (Fig. 32 and Fig. 34) minutes followed by incubation at 37°C for 72 hours. After incubation, it has been observed that four bacterial strains were able to grow at that temperature regime and thus the experiment was further proceeded with exposing temperature of 100°C for 15, 30, 45, 60, 75, 90, 120 and 150 minutes followed by incubation at 37°C for 72 hours (Fig. 33 and Fig. 35).

At 100°C exposing temperature for 1 hour, growth pattern was observed in Ps-11 (P. aeruginosa SN4) and Ps-17 (P. aeruginosa SN5) whereas; isolates Ps-1 (P. aeruginosa SN1), Ps-4 (P. aeruginosa SN3) and Ba-6 (Bacillus cereus) showed the TDT at earlier incubation period at 100°C. The highest TDT was noticed at 90 minutes at 100°C by P. aeruginosa SN4 (Table 18) followed by P. aeruginosa SN5 (Fig. 35) which showed TDT as 75 minutes at 100°C.
Table 18: Effect of temperature on survival of bacterial growth (Determination of Thermal Death Time, TDT)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Bacterial Isolates</th>
<th>Temperature regime</th>
<th>Thermal Death Time (TDT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>100°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 min</td>
<td>10 mins</td>
</tr>
<tr>
<td>1.</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>SN1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 mins at 100°C</td>
</tr>
<tr>
<td>2.</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>SN3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 mins at 60°C</td>
</tr>
<tr>
<td>3.</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>SN4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90 mins at 100°C</td>
</tr>
<tr>
<td>4.</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>SN5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>75 mins at 100°C</td>
</tr>
<tr>
<td>5.</td>
<td><em>Bacillus cereus</em></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 mins at 100°C</td>
</tr>
</tbody>
</table>

+ = positive result (growth)
- = negative result (no growth)
4.8. Effect of Salinity on Bacterial Growth

The salt concentration in an environment is the major contributor to the osmotic effect of ions on growth. Bacteria require ions that are provided by salts and typically moderate salt concentrations. High salt or high sugar in the environment leads to loss of water from cells and, ultimately, to the death. Some bacteria require an astonishingly high level of salt to begin growth, whereas other bacteria would be immediately killed in high levels of salt. The results were shown in Table 19. All the isolates showed 0.5% NaCl tolerance, whereas two strains *Pseudomonas aeruginosa* strain SN3 and strain SN5 showed a moderate tolerance at 10% NaCl concentration.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Control</th>
<th>0.5% NaCl</th>
<th>1% NaCl</th>
<th>5% NaCl</th>
<th>10% NaCl</th>
<th>15% NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em> SN1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> SN3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> SN4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> SN5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = positive result (growth)

- = negative result (no growth)

Fig. 36: Bacterial isolate: Ps-1, Ps-4 and Ps-11 at 10% NaCl

Fig. 37: All 5 isolates at 15% NaCl
4.9. Antibiogram pattern of heavy metal resistant isolates

All the predominant isolates having high MIC values for a set of metals exhibited high resistance pattern towards a group of antibiotics. It was observed that most of the metal tolerant strains (Ps-1, Ps-4, Ps-11, Ps-17 and Ba-6) were resistant to amoxycillin, ampicillin, cefalexin, cefixime, kanamycin, methicillin and tetracycline (Table 20). The present study showed similar results with that of Calomiris et al. (1984) who found a correlation between the resistance to high level of Cu(II), Pb(II), Zn(II) and antibiotic in the bacterial species found in drinking water. This fact was also established by other researchers that multiple metal resistance bacterial isolates exhibits high resistance towards a group of antibiotics (Vajiheh et al., 2003). Complete results showing the antibiotic resistance and susceptibility pattern is shown in Table 20.

Table 20: Antibiogram pattern of some selected bacterial isolates.

<table>
<thead>
<tr>
<th>Antibiotic disc</th>
<th>Ps-1</th>
<th>Ps-4</th>
<th>Ps-11</th>
<th>Ps-17</th>
<th>Ba-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>16 (I)</td>
<td>13 (I)</td>
<td>14 (I)</td>
<td>13 (I)</td>
<td>14 (I)</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>NI</td>
<td>NI</td>
<td>7 (R)</td>
<td>NI</td>
<td>5 (R)</td>
</tr>
<tr>
<td>Cefalexin</td>
<td>7 (R)</td>
<td>4 (R)</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Cefixime</td>
<td>NI</td>
<td>6 (R)</td>
<td>NI</td>
<td>5 (R)</td>
<td>NI</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>12 (I)</td>
<td>7 (R)</td>
<td>25 (S)</td>
<td>13 (I)</td>
<td>25 (S)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>5 (I)</td>
<td>2 (R)</td>
<td>NI</td>
<td>8 (R)</td>
<td>NI</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>21 (S)</td>
<td>17 (I)</td>
<td>19 (S)</td>
<td>23 (S)</td>
<td>21 (S)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Methicillin</td>
<td>NI</td>
<td>NI</td>
<td>4 (R)</td>
<td>7 (R)</td>
<td>NI</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>23 (S)</td>
<td>21 (S)</td>
<td>25 (S)</td>
<td>27 (S)</td>
<td>25 (S)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>NI</td>
<td>4 (R)</td>
<td>5 (R)</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>

NI= no inhibition zone; Diameter of disc=6 mm.

Letters in parenthesis indicate sensitivity; R = Resistance; I = Intermediate; S = Susceptible.
Fig. 38: Control of Ba-6 on MHA plate

Fig. 39: Antibiotic sensitivity and resistance pattern of Ba-6

Fig. 40: Control of Kl-8 on MHA plate

Fig. 41: Antibiotic sensitivity and resistance pattern of Kl-8
Fig. 42: Control of Ps-1 on MHA plate
Fig. 43: Antibiotic sensitivity and resistance pattern of Ps-1

Fig. 44: Control of Ps-4 on MHA plate
Fig. 45: Antibiotic sensitivity and resistance pattern of Ps-4
After isolation and characterization of heavy metal resistant bacteria, the final objective was to evaluate its efficacy by pot experimental studies.

- First, pot experiment were performed to investigate the effect of different soil types, toxic effect of cadmium and lead on seedling growth of rice (*Oryza sativa*) plant.
- Finally, the efficacy of all identified heavy metal resistant strains were tested for remediation purpose, i.e., role of these isolated strains on growth of *Oryza sativa* seedlings inoculated in cadmium and lead incorporated soil at different concentrations.

### 4.10. Effect of heavy metal contaminated soil on growth of rice seedlings

#### 4.10.1. Effect of different soil type

A set of pot experiment were performed to determine the deleterious effect of different soil types (i.e., industrial, garage, manure and pesticide soil) on seedling germination and growth (Fig. 46).

Shoot and root length of the seedlings after 3 days of inoculation are shown in Fig. 47 and were measured upto 17 days from the date of inoculation. It was observed that the pesticide soil and manure soil have a steady growth (24.5±0.3 cm and 23.4±0.4 cm) as compared to industrial and garage soil (Fig. 48).
However, on day 17, all the pots showed a significant difference among each other (P<0.01). Garage soil showed a marked mean difference with manure soil (6.64 cm), pesticide soil (7.74 cm) and industrial soil (4.36 cm), suggests the maximum reduction in seedling growth of *Oryza sativa* after 17 days of inoculation (Table 21).

The lowest growth observed in garage soil and industrial soil infers the toxic effect of effluents, heavy metals and other chemicals on the seedling germination and growth of *Oryza sativa*. The resulting rank order of decreased growth for soil type was:

Pesticide Soil > Manure Soil > Industrial Soil > Garage Soil.

Multiple comparison study showed that there was a significant mean difference in seedling growth of *Oryza sativa* in garage soil compared to other soil types after 10 days of inoculation (P<0.05) (Table 22).
Table 21: Multiple Comparison and Mean Difference between the seedling growth of *Oryza sativa* grown in different types of soil after 10 days and 17 days of seedling inoculation

<table>
<thead>
<tr>
<th></th>
<th>Manure</th>
<th>Pesticide</th>
<th>Industrial</th>
<th>Garage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manure</td>
<td>0</td>
<td>1.10**</td>
<td>2.28**</td>
<td>6.64**</td>
</tr>
<tr>
<td>Pesticide</td>
<td>0.24</td>
<td>0</td>
<td>3.38**</td>
<td>7.74**</td>
</tr>
<tr>
<td>Industrial</td>
<td>0.48</td>
<td>0.72</td>
<td>0</td>
<td>4.36**</td>
</tr>
<tr>
<td>Garage</td>
<td>2.14*</td>
<td>2.38*</td>
<td>1.66*</td>
<td>0</td>
</tr>
</tbody>
</table>

Mean difference at **P < 0.01; *P < 0.05 (ANOVA)
4.10.2. Effect of Cadmium on seedling germination and shoot growth of *Oryza sativa*

Effect of cadmium on seedling germination and shoot growth of *Oryza sativa* was studied by pot experimental studies. After 5 days of seedling inoculation, no significant difference among control and test pots were observed in seedling germination. However, on day 10, a marked mean difference was observed on seedling growth above 20mg/kg cadmium concentration in the soil.

After 3 weeks of inoculation, maximum shoot growth was attained by pot with Cd at 5mg/kg dosage and control having shoot length of 31.66 ± 0.42 cm and 32.02 ± 0.30 cm respectively (Fig. 49). The result thus indicates that low concentration (>5mg/kg) of Cd may have micro-nutrient-like effects on rice plants and the plants appeared to be healthy (P<0.01). The dosage of cadmium at concentration 20, 50 and 100 mg/kg showed a decreased shoot length by 14.6%, 25.7% and 35.4% respectively when compared them with control. Delayed germination was also observed at Cd concentration above 50mg/kg of soil.

![Fig. 49: Effect of Cadmium on seedling growth of *Oryza sativa*](image)

*Values are mean ± standard deviation of five replicates*
4.10.3. Effect of Lead on seedling germination and shoot growth of *Oryza sativa*

A concentration dependent decrease on shoot length of *Oryza sativa* seedlings was observed in all lead incorporated pots. There was a significant mean difference on day 10 onwards among all Pb dosage pots at all concentrations and also when compared with the control set (at P<0.01).

After 3 weeks of inoculation, dosage of lead at concentration 20 and 50 mg/kg showed 29% and 34% decrease in shoot length when compared with control pot, whereas the maximum reduction (55%) was observed in pot having 100mg/kg Pb dosage (Fig. 50). The maximum growth was attained by control having shoot length of 31.74 ± 0.39 cm. The dosage of lead at all experimental concentrations showed a decreased shoot length when compared them with control pots and found to be significant at P<0.01.

![Fig. 50: Effect of Lead on seedling growth of *Oryza sativa*](image)

*Values are mean ± standard deviation of five replicates*
4.11. Evaluation of bacterial isolates by pot trials

The impact of bacterial inoculation on shoot growth of *Oryza sativa* seedlings has been carried out by pot experimental studies. Isolated Cd and Pb tolerant bacteria (*P. aeruginosa* SN1, SN3, SN4, SN5 and *B. cereus*) were selected to study their effect on seed germination and shoot elongation in cadmium and lead incorporated soil (at concentrations 20mg/kg and 50mg/kg). The pots without bacterial inoculum, but subjected to Cd and Pb incorporation were taken as uninoculated control.

4.11.1. Effect of *Pseudomonas aeruginosa* and *Bacillus cereus* on seedling germination and shoot growth of *Oryza sativa* seedlings inoculated in cadmium incorporated soil

Inoculation of plants with *P. aeruginosa* SN1 significantly increases Cd tolerance of *Oryza sativa* at all concentrations of Cd (20mg/kg and 50mg/kg) in soil. After 20 days of plant growth, multiple comparison result reveals that *P. aeruginosa* SN1 attains 16% increased shoot growth as compared to uninoculated control pots. The increased shoot growth by strain SN1 having shoot length of 30.40 ± 0.30 cm at 50mg/kg Cd in soil were further compared with shoot length of pots without Cd and bacterial inoculation (Table 22). The result thus obtained demonstrates *P. aeruginosa* SN1 as a potent isolate for bioremediation purpose. *P. aeruginosa* SN4 and SN5 showed increased shoot length (11% and 8% respectively) and significant mean difference (3.0 cm and 2.1 cm respectively) when compared with uninoculated control at 50mg/kg Cd in soil (P<0.01). An adverse effect was observed in (delayed germination and decreased shoot growth) after inoculation of *Bacillus cereus* at both 20mg/kg and 50mg/kg Cd in soil (Table 22).
Table 22: Shoot length of *Oryza sativa* seedlings inoculated with cadmium resistant bacteria in cadmium incorporated soil and compared them with control set

<table>
<thead>
<tr>
<th>Control Group</th>
<th>Bacterial Strain</th>
<th>Shoot length (in cm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20mg/kg</td>
<td>50mg/kg</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> SN1 + Cd</td>
<td>30.40 ± 0.30*</td>
<td>29.86 ± 1.33*</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> SN3 + Cd</td>
<td>26.40 ± 0.86ns</td>
<td>22.24 ± 1.63ns</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> SN4 + Cd</td>
<td>28.58 ± 0.98ns</td>
<td>28.18 ± 0.49*</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> SN5 + Cd</td>
<td>27.42 ± 2.30ns</td>
<td>27.28 ± 0.53*</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus cereus</em> + Cd</td>
<td>24.48 ± 0.19*</td>
<td>22.82 ± 0.50*</td>
</tr>
<tr>
<td>Uninoculated control (with Cd only)</td>
<td></td>
<td>27.62 ± 0.35</td>
<td>25.18 ± 0.26</td>
</tr>
<tr>
<td>Control without Cd and bacteria</td>
<td></td>
<td>31.66 ± 0.42</td>
<td>31.66 ± 0.42</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of five replicates; ns= non significant; *= significant at P<0.01; compared with uninoculated control.
At 20mg/kg cadmium in soil: It has been observed that *Oryza sativa* inoculated with *P. aeruginosa* SN1 under cadmium stress at 20mg/kg showed a significant increase in shoot length (30.40 ± 0.30 cm) of *Oryza sativa* seedlings when compared with in uninoculated control sets (27.62 ± 0.35). The pots without cadmium amendment and bacterial inoculation showed the maximum growth upto 31.66 ± 0.42 cm after 20 days of seedling inoculation (Fig. 51). There was a decrease growth of *Oryza sativa* after inoculation of *P. aeruginosa* SN3, *P. aeruginosa* SN5 and *Bacillus cereus* having shoot length 26.40 ± 0.86 cm, 27.42 ± 2.30 cm and 24.48 ± 0.19 cm respectively (Fig. 51). However, statistical analysis showed the results attained after inoculation of *Bacillus cereus* as significant, which actually represents the mean difference with uninoculated control sets (cadmium amendment at 20 mg/kg and without bacterial inoculation) at P<0.01.

At 50mg/kg cadmium in soil: *P. aeruginosa* SN1, SN4 and SN5 showed significant mean difference at P<0.01 when compared with uninoculated control pots (cadmium amendment at 50 mg/kg and without bacterial inoculation). The maximum shoot length was attained by *P. aeruginosa* SN1 (29.86 ± 1.33 cm) followed by *P. aeruginosa* SN4 (28.18 ± 0.49 cm). Although *P. aeruginosa* SN3 attains slightly increased shoot length (Fig. 52), but fails prove significant result by statistical analysis. *Bacillus cereus* showed delayed and decreased seedling growth (22.82 ± 0.50 cm after 20 days) illustrates that there may be no interaction/negative interaction between the rhizosphere of *Oryza sativa* plant and the strains of *Bacillus cereus*.

Overall pot experimental studies demonstrated that inoculation of *P. aeruginosa* SN1 at both stress condition (20mg/kg and 50 mg/kg of cadmium in soil) could be beneficial to attain normal seedling growth of *Oryza sativa*. 

Fig. 51: Shoot length of *Oryza sativa* seedlings (in cm) inoculated with heavy metal resistant bacteria in cadmium incorporated soil (20mg/kg Cd in soil) and compared them with control set.
Fig. 52: Shoot length of *Oryza sativa* seedlings (in cm) inoculated with heavy metal resistant bacteria in cadmium incorporated soil (50mg/kg Cd in soil) and compared them with control set.
4.11.2. Effect of *Pseudomonas aeruginosa* and *Bacillus cereus* on seedling germination and shoot growth of *Oryza sativa* seedlings inoculated in lead incorporated soil

The study on plants inoculated with *P. aeruginosa* SN4 and SN5 significantly increases Pb tolerance of *Oryza sativa* seedlings at Pb concentrations of 20mg/kg and 50mg/kg in soil. Statistical analysis and multiple comparison studies highlight the fact that *P. aeruginosa* SN3 and SN4 attains 14% and 16% increased seedling growth as compared to uninoculated control pots at 20mg/kg Pb in soil (Table 23). Significant result were observed at concentration 50mg/kg of lead in soil by strain SN4 and SN5 with increased shoot growth of 11.5% and 10% respectively.

However, *P. aeruginosa* SN1 showed contrasting results at 20mg/kg of Pb in soil having mean difference of 2.46 cm with uninoculated control pots. Interestingly with the increasing concentration of Pb in soil (50mg/kg), no significant mean difference was observed (Table 23). Another striking feature observed in the study was that when *Bacillus cereus* was inoculated at both 20mg/kg and 50 mg/kg Pb concentration in soil adverse effect was observed in seedling growth. It also delayed the process of seed germination of *Oryza sativa*.

The overall result revealed the fact that *P. aeruginosa* SN4 and SN5 could be considered as potential candidate for revegetation purpose in lead contaminated soil.
Table 23: Shoot length of *Oryza sativa* seedlings inoculated with lead resistant bacteria in lead incorporated soil and compared them with control set.

<table>
<thead>
<tr>
<th>Control Group</th>
<th>Bacterial Strain</th>
<th>Shoot length (in cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> SN1 + Pb</td>
<td>27.02 ± 0.19*</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> SN3 + Pb</td>
<td>28.64 ± 0.32*</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> SN4 + Pb</td>
<td>29.28 ± 0.30 *</td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em> SN5 + Pb</td>
<td>27.52 ± 0.28 *</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus cereus</em> + Pb</td>
<td>18.20 ± 0.31 *</td>
</tr>
<tr>
<td></td>
<td>Uninoculated control (with Pb only)</td>
<td>24.56 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>Control without Pb and bacteria</td>
<td>31.66 ± 0.42</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation of five replicates; ns= non significant; *= significant at P<0.01; compared to uninoculated control.
At **20mg/kg lead in soil**: Inoculation of *Oryza sativa* seedlings at 20mg/kg of lead soil attains shoot length of 24.56 ± 0.32 cm, which is quite less as compared to the seedling growth of *Oryza sativa* without Pb and bacterial inoculation (Fig. 53). In the further experiment, inoculation of all four strains of *P. aeruginosa* (strain SN1, SN3, SN4 and SN5) showed a remarkable increase in its length. However, *Bacillus cereus* showed similar results as of cadmium stress attaining delayed and reduced shoot length of 18.20 ± 0.31 cm when compared with uninoculated control sets (lead amendment at 20 mg/kg and without bacterial inoculation) at P<0.01. Maximum seedling growth was observed by *P. aeruginosa* SN4 having shoot length of 29.28 ± 0.30 cm, indicate a potent strain for bioremediation purpose. *P. aeruginosa* SN1, SN3 and SN5 also showed significant result at P<0.01 having shoot length of 27.02 ± 0.19 cm, 28.64 ± 0.32 cm and 27.52 ± 0.28 cm respectively.

At **50mg/kg lead in soil**: All the isolates were tested at 50mg/kg lead in soil and compared them with the control sets. Maximum shoot length was observed by *P. aeruginosa* SN4 (26.70 ± 0.25 cm), followed by *P. aeruginosa* SN4 (26.40 ± 0.22). The result thus obtained by these two strains were further found to be significant when compared with uninoculated control (at P<0.01). After 20 days of seedling inoculation, two strains *P. aeruginosa* SN1 and *P. aeruginosa* SN3, which showed significant result at 20mg/kg of lead in soil, does not show any significant response in 50mg/kg of lead in soil (Fig. 54). *Bacillus cereus* never showed positive results in all the previous pot experimental results. Result thus obtained after inoculation *Bacillus cereus* were delayed and reduced shoot length (16.64 ± 0.42 cm) at concentration 50 mg/kg of lead in soil (Fig. 54). However, based on their higher mean difference with the uninoculated control pots, statistical analysis showed the result to be significant.

The two groups of pot experimental studies demonstrated that inoculation of *P. aeruginosa* SN4 at both stress condition (20mg/kg and 50 mg/kg of lead in soil) could be beneficial to attain the normal growth as compared to the control pots. However, *P. aeruginosa* SN5 also showed contrasting results at 50mg/kg lead in soil.
Fig. 53: Shoot length of *Oryza sativa* seedlings (in cm) inoculated with heavy metal resistant bacteria in lead incorporated soil (20mg/kg Pb in soil) and compared them with control set.
Fig. 54: Shoot length of *Oryza sativa* seedlings (in cm) inoculated with heavy metal resistant bacteria in lead incorporated soil (50mg/kg Pb in soil) and compared them with control set.
### Table 24: Characteristics of isolated bacterial strains.

<table>
<thead>
<tr>
<th>Initial Code</th>
<th>Bacterial isolate</th>
<th>MIC for heavy metals (in µg/ml)</th>
<th>Pot experimental result at P&lt;0.01</th>
<th>16s rDNA sequence analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cd</td>
<td>Pb</td>
<td>Cu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ps-1</td>
<td>*Pseudomonas aeruginosa SN1</td>
<td>1700</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>Ps-4</td>
<td>*Pseudomonas aeruginosa SN3</td>
<td>1800</td>
<td>170</td>
<td>60</td>
</tr>
<tr>
<td>Ps-11</td>
<td>*Pseudomonas aeruginosa SN4</td>
<td>1400</td>
<td>160</td>
<td>60</td>
</tr>
<tr>
<td>Ps-17</td>
<td>*Pseudomonas aeruginosa SN5</td>
<td>1400</td>
<td>170</td>
<td>60</td>
</tr>
<tr>
<td>Ba-6</td>
<td>*Bacillus cereus</td>
<td>1700</td>
<td>150</td>
<td>50</td>
</tr>
</tbody>
</table>

ns - non significant
# - adverse effect
* - significant at P<0.01 (ANOVA)