3.1. Selection of study site

Present study was carried out in Cachar district of Assam, India. Primary data were directly collected from village people and farmers. Agricultural field survey and interaction with associated people gives an overview of the entire scenario of this region. The district lies between 92° 24’ E and 93° 15’ E longitude and 24° 22’ N and 25° 8’ N latitude. The total geographical area of the district is 3,786 Sq. Km. The district covers 1047 villages having 3 agricultural sub-divisions. As per 2011 census, the total population of the district is about 1736319 with population density of 382 persons per sq.km. Among the 15 agro-climatic regions of the country, categorized on the basis of homogeneity in agro-characteristics, Cachar falls in the Barak Valley zone. The agro-climatic conditions of the district are conducive for various agricultural activities. The types of land available in the district are classified as: medium land-69048 hectares, high land-11642 hectares, low land-19512 hectares, very low land-10792 hectares and bheel area- 4735 hectares (Source: District soil testing Department, Cachar, Assam). The use of chemical fertilizer is much common and its application has been increased by 22% during the study period i.e., 2011-12 when compared with previous year.

Geographical information of the study site, climatic condition (temperature and rainfall data), nutrient status (N, P, K content and pH) of the soil were collected from soil testing Department, Cachar District, Assam. Apart of this, total rice productivity (demand and supply), factors effecting rice productivity and other relevant data were collected from the Office of Director of Agriculture, Silchar, Assam and Krishi Vikas Kendra (KVK), Masimpur, Assam.

3.1.1. Agro climatic condition and land area

The economy of Cachar district is basically agrarian in nature with about 80% of the population dependent on agriculture. Paddy is the major crop. Other important crops include oil seeds, pulses, cash crop like jute, vegetables etc. The agro climatic conditions of the district are conducive for various agricultural activities. Agriculture in the district is characterized by over dependence on rainfall, predominance of seasonal crops and traditional methods of cultivation. As per survey report of Director
of Agriculture, Cachar, Assam (2011-12), the total cropped area of Cachar district is 152826 hectares and the net crop sown area was 124000 hectares having 123.2% cropping intensity. Silchar subdivision alone accounts for 49% of net sown area. The district covers 33300 hectares flood prone area and 11500 hectares drought prone area. Total of 48661 numbers of farm families and majority of who are landless and marginal farmers. Only about 2.5% of the net cropped areas are covered by irrigation facilities. According to NABARD projections in the (Potential Linked Credit Plan) PLP-2007, it is estimated that the present rate of cropping intensity at 123.5% can be raised up to 150% by extending assured irrigation facilities. Further the use of certified seed covers only 1.15% of the total cropped area and the fertilizer consumption was only 30.58kg/ha in the district.

3.1.2. Land distribution and cropping system

Having a total geographical area of 377610 hectares, the total forest coverage of this district was 141768 hectares (37.5%). An estimated 33% of total geographical area was suitable for cultivation (Fig. 6). Mono-cropping are usually preferred in this region which covers an estimated area of 98659 hectares followed by double cropping (22456 hectares). Multiple cropping systems are less preferred and all cropping systems are season dependent (Source: Krishi Vigyan Kendra, Masimpur, Cachar, Assam). Total production, supply and requirements of some major food commodities for the year 2012-2013 are shown in Table 1.

![Fig.6: Land coverage of Cachar District of Assam.](image-url)
Table 1: Surplus & deficit status of major food commodities in Cachar district for the year 2012-13

<table>
<thead>
<tr>
<th>Name of the food commodity</th>
<th>Total production (in tonnes)</th>
<th>Requirement per year (in tonnes)</th>
<th>Deficit (in tonnes)</th>
<th>Surplus (in tonnes)</th>
<th>Requirement/person</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td>255928</td>
<td>230180</td>
<td></td>
<td>25748</td>
<td>@454gm/day/adult</td>
</tr>
<tr>
<td>Pulses</td>
<td>1899</td>
<td>23725</td>
<td>21826</td>
<td></td>
<td>@50gm/day/adult</td>
</tr>
<tr>
<td>Edible oil</td>
<td>1279</td>
<td>21353</td>
<td>20074</td>
<td></td>
<td>@45gm/day/adult</td>
</tr>
<tr>
<td>Vegetable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kharif</td>
<td>15632</td>
<td></td>
<td>37588</td>
<td>62050</td>
<td>@100gm/day/adult</td>
</tr>
<tr>
<td>Rabi</td>
<td>84006</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Source: Director of Agriculture, Cachar, Assam*

3.1.3. Rice productivity in Cachar District

There is an increase in total rice productivity during autumn and winter season as compared to 2007-2008. Winter paddy accounts for highest productivity throughout the year, whereas summer paddy reported to be the least. There has been drastic decrease in summer productivity in the year 2009-10 where the yield was only 724 kg/ha. The detailed area, production and productivity during the year 2007 to 2012 are shown in Table 2.
Table 2: Total Area (in hectares), Production and Productivity of rice in Cachar district.

<table>
<thead>
<tr>
<th>Year</th>
<th>Name of the crop</th>
<th>Area (ha)</th>
<th>Production (kg)</th>
<th>Productivity (kg per ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007-08</td>
<td>Autumn paddy</td>
<td>10130</td>
<td>15701</td>
<td>1550</td>
</tr>
<tr>
<td></td>
<td>Winter paddy</td>
<td>79842</td>
<td>157287</td>
<td>1970</td>
</tr>
<tr>
<td></td>
<td>Summer paddy</td>
<td>9680</td>
<td>18941</td>
<td>1957</td>
</tr>
<tr>
<td>2008-09</td>
<td>Autumn paddy</td>
<td>8350</td>
<td>13808</td>
<td>1654</td>
</tr>
<tr>
<td></td>
<td>Winter paddy</td>
<td>93610</td>
<td>199711</td>
<td>2133</td>
</tr>
<tr>
<td></td>
<td>Summer paddy</td>
<td>9560</td>
<td>21126</td>
<td>2210</td>
</tr>
<tr>
<td>2009-10</td>
<td>Autumn paddy</td>
<td>8410</td>
<td>16457</td>
<td>1957</td>
</tr>
<tr>
<td></td>
<td>Winter paddy</td>
<td>94500</td>
<td>204084</td>
<td>2160</td>
</tr>
<tr>
<td></td>
<td>Summer paddy</td>
<td>8902</td>
<td>6448</td>
<td>724</td>
</tr>
<tr>
<td>2010-11</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2011-12</td>
<td>Autumn paddy</td>
<td>7000</td>
<td>14560</td>
<td>2008</td>
</tr>
<tr>
<td></td>
<td>Winter paddy</td>
<td>91912</td>
<td>183824</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>Summer paddy</td>
<td>7497</td>
<td>13645</td>
<td>1082</td>
</tr>
</tbody>
</table>

*Source: Director of Agriculture, Cachar, Assam*
3.1.4. Fertility status of soil

Soil fertility testing for agricultural purpose aims to test the soil and find out the nutrient content particularly nitrogen, phosphorous and potassium. Soil texture and pH are also important parameters in soil fertility status. These factors are very important for fertilizer selection and crop selection. In the present study, data were collected from 19 AEO circles and compiled to get the fertility status of 3 districts (Silchar, Sonai and Lakhipur). Sub-division wise pH range and N, P, K status are represented in Table 3.

Table 3: pH range and N, P, K status of agricultural field of Cachar district.

<table>
<thead>
<tr>
<th>Sub-division</th>
<th>pH Range</th>
<th>N</th>
<th>P</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silchar</td>
<td>4.5-5.8</td>
<td>Medium to high</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Sonai</td>
<td>4.5-5.8</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Lakhipur</td>
<td>4.6-5.7</td>
<td>Medium</td>
<td>Medium</td>
<td>Low</td>
</tr>
</tbody>
</table>

The above table describes the fertility status of soil in Cachar district of Assam, India. NPK indicates Nitrogen, Phosphorous and Potassium, whereas the code H, M and L indicates high, Low and medium respectively. On an average, Cachar district is having a pH range of 4.5-5.8. Nitrogen and phosphorous content in soil were found me be in range (i.e, medium) while the potassium content is low. It has also been observed few places of Silchar sub-division, the nitrogen content in very high. Due to lack of awareness among village people, wrong fertilizer selection is effecting the yield of rice in some areas of cachaer district of Assam.

An outline of soil map of Cachar district was collected from Soil Testing Department of this district and further it was edited using Adobe Photoshop CS5 and represented in Fig.7.
Fig. 7: Soil map of Cachar district

Source: Soil Testing Department, Cachar District, Assam (2012-13)
3.1.5. Climatic condition and rainfall

The seasonal pattern of rainfall and its variations from year to year govern the flood pulse and determine the extent and duration of inundation of the low lying rice fields by the overflowing river waters. During summer, average maximum and minimum temperature of this district is 35°C and 26°C respectively. Cachar district receives an average annual rainfall of more than 2800 mm.

Maximum rainfall occurs between May to August thus lowering the soil pH to its minimum. The relative humidity are also higher in the summer months between May to August (65-90%). Rainfall data for the year 2010, 2011 and 2012 has been collected from the office of Director of Agriculture, Cachar as shown in Fig. 8.

![Graph](image)

Fig. 8: Monthwise average rainfall (mm) of Cachar district during last three years (2010, 2011 and 2012).

In the year 2012, maximum rainfall was observed during the month of June (904.4 mm), which was recorded as maximum during the study period. The average annual rainfall recorded during the year 2012 was 3903 mm.
3.2. Collection and analysis of soil sample

To study the soil profile of the study site, samples were randomly collected from eight different locations during the year 2011-2012. Sampling was done from contaminated crop field nearby paper industry, garages and petrol pumps and other polluted sites. Eight sites were selected for sample collection and are marked as Site-1, Site-2, ……… and Site-8. The details of all the sites are as follows:

**Site-1:** Uncultivable land nearby Panchgram Paper Mill waste disposal sites in between Cachar District and Hailakandi District of Assam

**Site-2:** Agricultural field nearby River Barak, where industrial discharge, household waste are used for irrigation purpose in Cachar District, Assam

**Site-3:** Agricultural land area nearby garage and welding shop in Kathal point, Silchar, Assam

**Site-4:** Cultivable land nearby petrol pump in Meherpur, Silchar, Assam

**Site-5:** Crop field having low productivity and extensive fertilizer use in Katigorah, Cachar District, Assam

**Site-6:** Agricultural land surrounded by brick industry nearby NIT Silchar, Assam

**Site-7:** Crop field nearby Narsingpur area, Cachar District, Assam

**Site-8:** Soil collected from Silcore Tea Estate, Cachar District, Assam

The on-site soil colour was studied by Munsel Soil Colour chart. Soil sample finally collected in sterilized polythene bags, properly labeled and immediately bought to the laboratory for isolation of bacteria and their (soil) physico-chemical tests.

The collected soil samples were air-dried and crushed with pestle and porcelain mortar. The crushed soils were sieved through a 2mm sieve and about 250g of the sieved samples were stored in plastic containers with proper tagging. The physical characteristics (pH, texture and composition, bulk density, particle density, moisture content, water holding capacity etc.) of the soil samples were analyzed following the standard methods of soil analysis (Jackson, 1973). These soil test were carried out by DELUX water and soil testing kit.
3.3. Isolation and enumeration of bacteria

Soil plating was done within 72 hours of sample collection. Isolation and quantitative enumeration of bacteria from soil samples was carried out by the dilution plate count method (Parkinson et al., 1971; Johnson and Curl, 1972). 10 g soil from each sample was aseptically weighed and transferred to a conical flask with 90 ml sterilized distilled water, and were shaken for 30 min at about 150 rpm. Immediately after shaking, a series of dilution of the suspension was made for each sample by pipetting 1 ml aliquot into 9 ml sterilized distilled water. The final dilution selected for isolation of diazotrophic bacteria was $10^{-4}$. 1 ml of this dilution was inoculated aseptically onto petridish containing 18-20 ml Nutrient Agar (NA) media and kept in an incubator at $37^\circ$C for 48 hrs. After incubation period, the colonies growing on the plates were counted using a colony counter and expressed as cfu (colony forming units) per gram of soil.

To check the diversity of cadmium resistant bacteria, separate nutrient agar plates were prepared, incorporated with 500 and 1000 µg/ml of cadmium chloride (CdCl$_2$). The percentage of cadmium resistant bacteria was calculated as:

\[
\text{Percentage (\%)} \text{ Cadmium Resistant Bacteria} = \frac{\text{Cadmium resistant bacteria (cfu/g)}}{\text{Total heterotrophic bacteria (cfu/g)}} \times 100
\]

Similarly, the diversity of lead resistant bacteria was also determined at concentration 50 and 100 µg/ml of lead acetate.

3.4. Selective isolation of cadmium and lead resistant bacteria

For selective isolation of bacteria, samples were streaked on Pseudomonas Isolation Agar (PIA), Klebsiella Isolation Agar (KIA) and Starch agar incorporated with cadmium chloride and lead acetate at concentration 100 and 50µg/ml respectively. The plates were then incubated at $37^\circ$C for 24 hrs. Pure cultures were obtained by repeated sub-culturing (streak plate method) on their respective media.
3.5. Morphological and biochemical characterization of isolated strains

Pure isolates of cadmium and lead resistant bacteria were identified up to species level based on the criteria of Bergey’s Manual of Systematic Bacteriology (Krieg and Holt, 1984) and Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994).

The following morphological, physiological and biochemical tests were performed:

3.5.1. Gram’s staining

This test was done by Gram Stains-Kit (Himedia K001) and microscopic observation were done for determination of two distinguishable characters Gram Positive (+ve) and Gram negative (–ve) strains of Bacteria. In Gram’s staining technique, bacterial samples were smeared on clean slides, air dried and heat fixed by gentle heating. The slides were flooded with crystal violet solution for one minute, washed with water and flooded with Gram’s Decolourizer until no violet colour was visible from drain off solution. Slides were washed with water and counter stained with 0.5% safranin for about one minute and washed with water. The slides were air dried and examined under a microscope (Olympus, Model: CX21FS1) using 100X objective lens. Cells were then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

3.5.2. Morphological and biochemical characterization

Morphological characteristics of all isolates viz., Colony morphology (colour, shape, margin, elevation and surface) and cell morphology (size, shape and arrangement) were studied.

The biochemical characterization of the isolates was performed as outlined by Cappuccino and Sherman (2005) and Harley (2005). The composition of some of the chemicals and reagents that were used in the biochemical tests are given in appendix-I and Appendix-II.
i) Indole test:

Indole test was performed to determine the ability of the organism to convert tryptophan into indole. Some bacteria can produce indole from amino acid tryptophan using the enzyme tryptophanase.

\[
\text{Tryptophan} \xrightarrow{\text{Tryptophanase}} \text{H}_2\text{O} \rightarrow \text{Indole} + \text{Pyruvic acid} + \text{NH}_3
\]

Pure bacterial cultures were inoculated in sterile peptone broth, which contains amino acid tryptophan and incubated overnight at 37°C. Following incubation, about 5 drops of Kovac's reagent was added to the culture broth. The appearance of a red or red-violet color in the surface of the broth signifies the positive result. A negative result appears yellow.

ii) Methyl red test:

On the MR-VP broth (consisting of glucose, peptone, and a phosphate buffer) medium contained in the sterilized test tubes the test bacterial cultures were inoculated and then incubated for 48 hrs at 30°C. After, the period of incubation, around ten drops of methyl red indicator was added to each tube followed by gentle shaking. The formation of red colour in the test tubes after indicated that the bacteria fermented dextrose.

iii) Voges-Proskauer test:

On the MR-VP broth medium contained in the sterilized test tubes the test bacterial cultures were inoculated and then incubated for 48 hrs at 30°C. After incubation, ten drops of Barrit’s reagent-A was added and shaken gently this was followed by adding a few drops of reagent-B. The formation of pink/red colour in the broth was considered as positive test. If, after the reagents have been added, a copper color is present, the result is negative.
iv) Citrate test:

The isolates were inoculated in Simmon’s citrate agar slants and then incubated for 48 hours at 37°C. The tubes were then observed. The change of colour due to the change of pH indicates positive results. No colour change in the tube was considered as negative.

v) Catalase test:

Bacterial isolates were taken in a sterilized clean glass slide and few drops (1 ml) of 3% hydrogen peroxide (H₂O₂) were added. Appearance of gas bubbles confirms positive test.

vi) Nitrite reduction test:

The nitrate broth with the inoculated isolates was incubated at 30°C for 48 hours. After incubation, on addition of 3 drops of nitrite test reagent, 1 drop of sulphuric acid and 1 drop of culture containing test organisms were dispensed on clean porcelain plates. Appearance of blue colour was regarded positive test.

vii) Starch hydrolysis:

This test is used to differentiate bacteria based on their ability to hydrolyze starch with the enzyme α-amylase or oligo-1,6-glucosidase by breaking the glycosidic linkages between the sugar subunits. The microbial test cultures were streaked on the starch agar plates and incubated at 30°C for 24-48 hrs. After, incubation, iodine solution was flooded on the petriplates. The formation of clear zone around the colony was taken as positive test.
viii) Litmus milk test:

Litmus Milk tube was aseptically inoculated using a wire loop with the desired bacterium. These tubes were then incubated for one week at 35-37°C. The tubes were observed every after 2-3 days to check the color of the media (or the pH) and the results were recorded.

ix) Urease test:

This test was used to differentiate organisms based on their ability to hydrolyze urea with the enzyme urease. The bacterial cultures were inoculated to the test tubes containing urea broth and incubated for 24 to 48 hours at 30°C. The appearance of pink colour was considered as positive test.

x) Gelatin liquefaction:

To the nutrient gelatin deep tubes, the test cultures of bacteria were inoculated in the tubes and then incubated for 24 hours at 30°C. The tubes were then put inside the refrigerator at 4°C for half an hour. After refrigeration, the tubes were taken out and observed. The tubes within cultures that remained in the liquid state were said to be undergone gelatin liquefaction and the test bacterial isolates that got solidified on refrigeration were considered as negative.

xii) Oxidase Test:

The microbial test cultures were streaked on the nutrient agar plates/tryp ticase soy agar plates and incubated at 37°C for 24-48 hrs. After incubation, two to three drops of p-aminodimethylaniline oxalate was added on the petriplates. The change of colour from pink to maroon and finally to purple was taken as positive test. No colour implies negative test.
3.6. Identification of bacteria by 16S rDNA sequencing

Pure culture of bacterial isolates was provided to Bangalore GeNei™ for identification of isolates up to species level through 16S rDNA sequencing. In brief, Genomic DNA was isolated using GeneiUltrapure™ Bacterial DNA Purification kit (KT162) using 395BG forward and 396BG reverse primer; the ~1.5 kb 16S rDNA fragment was amplified by Taq DNA Polymerase. The PCR products were loaded on 1.0% agarose gel along with 500bp DNA ladder. The PCR product was sequenced using the same primers. In case of SN1 and SN 5, 13BG internal primer was also used.

The PCR mix composition:

Genomic DNA : ~20ng

dNTP mix (2.5mM each) : 1.0μl

Forward Primer : 100ng

Reverse Primer : 100ng

Taq Buffer A (10X) : 1X

Taq Polymerase enzyme : 3U

Glass distilled water : to make up the volume 50μl

The sequence of the forward, reverse primer and internal primers are:

Forward Primer 395BG : CTGAGCCAGATCAAACCTCT

Reverse Primer 396BG : AAGTCGTAACAAGGTACCCTTA

Internal Primer 13BG : CAGCAGCCCGCGTAAATAC
**Phylogenetic Analysis:** After sequencing, the ~1400 bp sequence were first analysed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ribosomal Database Project (http://rdp.cme.msu.edu/) for finding the closest homologous sequence. The first ten homologous sequences were selected based on their maximum identity score. The sequences were than aligned and a distance matrix was constructed. Finally, a phylogenetic tree was made using Neighbour Joining method.

### 3.7. Determination of minimum inhibitory concentration (MIC)

All the isolates were checked for metal tolerance. MIC was determined against respective heavy metals Cd (CdCl$_2$), Cu (CuSO$_4$·5H$_2$O), Pb [(CH$_3$COO)$_2$Pb·3H$_2$O] and Zn (Zinc Metal Powder)) by gradually increasing the concentration of the heavy metals on Nutrient Agar (NA) plates until the strains failed to give colonies on the plate. The initial concentration used was 50µg/ml and thereby the concentration was gradually increased by 10-15 µg/ml each time on NA plates. The growth of cultures on last concentration was transferred to the higher concentration by streaking on the plate. MIC was recorded when the isolates failed to grow on plates.

The bacterial isolates having higher minimum inhibitory concentrations towards a group of heavy metals (Cd, Pb, Cu and Zn) were selected for further test. These isolates were than passed through some routine test such as selected for determination of thermal death time (TDT), effect of salinity, antibiotic sensitivity and resistance pattern.
3.8. Determination of Thermal Death Time (TDT)

In order to determine the thermal endurance of five bacterial isolates, first they were grown on nutrient broth (each isolate were inoculated in 7 different tubes) and incubated for 24 hrs at 37°C. Thermal Death Time (TDT) was determined on nutrient agar plates by streaking the isolates after exposing them at a 60°C at a particular time interval (Dubey and Maheshwari, 2011).

![Fig.9: Determination of thermal death time of the bacterial isolates](image)

**Procedure:** To test the TDT of a single bacterial isolates, two nutrient agar plates were prepared and each plate was divided into four sectors. The sectors were labeled as O, 10, 20, 30, 40, 50 and 60 (Fig. 9); where O refers to the 0 time or control. The test isolates was first inoculated on O sector of both the plates. All the remaining 6 tubes were kept in a water bath, maintaining the temperature at 60 °C and the incubation time was recorded. After an exposure of 10 mins, 1 tube was taken out, cooled under tap water and streak inoculation of the suspension on sector 10 of the plate was made. The process was repeated after every 10 mins interval and streaked on respective a sector (20, 30, 40, 50 and 60) that corresponds the time of incubation at 60 °C.

Similarly, the TDT was checked at 100 °C at O, 15, 30, 45, 60, 75, 90, 120 and 150 mins. time interval.
3.9. Effect of Salinity on Bacterial Growth

Isolates were tested for osmotic stress. The growth of any bacteria can be affected by the amount of water entering or leaving the cell. If the medium has low amount of solute, it is hypotonic and has high osmotic pressure on the cell. On the other hand, if the solute is high, medium is hypertonic and growth is considerably inhibited. In such cases, cytoplasm dehydrates and shrinks away from the cell wall.

**Procedure:** Nutrient Agar (NA) medium was prepared in five different conical flasks supplemented with NaCl at concentrations 0.5%, 5%, 10% and 15% and one kept as a control. The NA media were poured in petriplates, bacterial strains were streaked each plate and incubated at 37°C for 24 hrs (Dubey and Maheshwari, 2011). The influence of NaCl concentrations on degree of inhibition of bacterial growth were recorded.

3.10. Antibiotic sensitivity and resistance pattern of the bacterial isolates

The isolates were tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method (Bauer *et al.* 1966) and the antibiotics discs were procured from ‘HIMEDIA’. The antibiotic that were used in the present study are Amikacin (30 mcg), Amoxycillin (10 mcg), Ampicillin (25 mcg), Cefalexin (30 mcg), Cefixime (5 mcg), Ceftriaxone (30 mcg), Chloramphenicol (10 mcg), Gentamicin (50 mcg), Kanamycin (5 mcg), Methicillin (30 mcg), Ofloxacin (5 mcg) and Tetracycline (30 mcg).

**Procedure:** The selected isolates were freshly inoculated on saline water or peptone water, their turbidity was checked by comparing with McFarland solution and inoculated on Mueller-Hinton Agar. The selected antibiotics were placed on the plate and incubated at 37°C for 24 hours. The diameter of the inhibition zones was measured to the nearest mm and the isolates were classified as resistant (R), intermediate (I) and susceptible (S) following the standard antibiotic disk sensitivity testing method.
After performing all the above experiments, the final objective was to evaluate the isolated strains by pot experimental studies. The isolates of *Pseudomonas* sp. and *Bacillus* sp. were selected and their effect was studied taking seed germination and growth as a statistical parameter.

### 3.11. Pot experimental studies

Seedling germination and its growth pattern can be easily measured in laboratory condition. Thus, it has been taken under consideration as a general parameter to study the influence of heavy metals (Fodor 2002; Hagemeyer, 2004) on seedling growth.

#### 3.11.1. Preparation of bacterial inoculum

The bacteria showing the highest MIC were taken under consideration for the preparation of bacterial inoculum. The isolates Ps-1, Ps-4, Ps-11, Ps-17 and Ba-6 were inoculated in nutrient broth and kept in shaker incubator at 120 rpm at 28 ± 2°C for 48 hours. After incubation period, 5 ml of broth was added to 45 ml distilled water for the formulation of bio-fertilizer and to carry out the pot experiment.

#### 3.11.2. Collection and pre-sowing treatment of *Oryza sativa* seedlings

Seeds of *Oryza sativa* were collected from Krishi Vikas Kendra, Masimpur, Assam. The seeds sizes and weights were homogenous. Clean seeds were dipped in water; floating seeds were discarded, while seeds settled on bottom of container were selected. Seeds were surface-sterilized with 95% alcohol for 30 seconds, followed by 0.1% (w/v) HgCl₂ for 1–2 min and then washed with sterile distilled water for 5–6 times (Singh *et al.*, 2008). The seeds were then put in a sterile petridish containing Hogland Solution and remain dipped overnight.
3.11.3. Pot trial studies

The earthen pots (24 cm X 12 cm X 12 cm) were filled with sterilized sandy loam soil. Seeds were sown on all the pots to study the following:

- **Effect of cadmium and lead on shoot growth of *Oryza sativa***: The experiment for studying the effect of cadmium in shoot growth of *Oryza sativa* was conducted in 6 earthen pots (taking 5 replicates). Similar experiment was performed to determine the deleterious effect of lead on shoot growth of *Oryza sativa*. Rice seeds were sown on all pots and daily dosage of cadmium and lead was given at concentrations 0, 5, 10, 20, 50, 100mg/kg.

- **Role of cadmium and lead tolerant bacteria on shoot growth of *Oryza sativa sown in cadmium and lead incorporated soil***. Pot experiments were performed to determine the bioremediation potential of cadmium and lead tolerant bacterial isolates. Pot trial was performed in four different experimental groups to determine the shoot length of *Oryza sativa* after three weeks of exposure to:
  
  i. Cadmium (20mg/kg soil) and bacterial inoculums
  
  ii. Cadmium (50mg/kg soil) and bacterial inoculums
  
  iii. Lead (20mg/kg soil) and bacterial inoculums
  
  iv. Lead (50mg/kg soil) and bacterial inoculums

3.12. Statistical Analysis

After performing the pot experiment, SPSS 16.0 was used to perform statistical analysis of the gathered data. Descriptive statistics calculates the means of all replicates with standard error and deviations. Multiple comparison tests were performed to evaluate the effectiveness of each bacterial isolates. When analysis of variance (ANOVA) showed significant effects, Tukey’s-b test (assuming equal variances) and Ggmes-Howell test (assuming unequal variances) was done to make comparison between groups at P<0.05 and P<0.01.