Chapter 3.

Materials

And Methods
3. MATERIALS AND METHODS

3.1. Isolation of nickel tolerant bacteria

Ni resistant bacterial isolate was obtained from the industrial metal waste contaminated soil (pH 6.2, Ni content 25 ppm) nearby Kolkata, India. The soil sample was diluted (10-folds) with sterilized phosphate buffer (pH 7.2) and inoculated on nutrient (Beef Extract, 0.3%; Peptone, 0.5%; NaCl, 0.5% in distilled water; agar 2%, pH 7.2) agar plates having different concentrations of Nickel (0.2 – 20 mM) as NiCl₂, 2H₂O. The colonies that could tolerate 2 mM of Ni were selected randomly; of them which showed maximum level of Ni resistance was finally selected for further experiment in this study and the isolate was designated as KUNi1. The minimum inhibitory concentration (MIC) of Ni was determined using both nutrient broth and liquid minimal medium (3gm K₂HPO₄, 6gm Na₂HPO₄, 5gm NaCl, 2gm NH₄Cl, 0.1gm MgSO₄, 8gm glucose in 1 l distilled water, pH 7.2). The isolate was then purified to have a pure culture by cycles of single colony isolation and liquid culture transfers on minimal medium supplemented with 2 mM Ni and maintained by periodic subculturering on Ni supplemented NA slant.

3.2. Morphological and Biochemical tests

The following biochemical tests were conducted for the identification and characterization of the isolate.

3.2.1. Gram staining. Bacterial smear was heat fixed and covered with crystal violet solution (Crystal violet 5 gm/l) for 1 min. The stain was washed properly and then the slide was covered with iodine solution (Iodine 10 gm/l, Potassium Iodide 20 gm/l) for 1 min. The iodine solution was washed off with absolute alcohol. It was then washed with water and counter stained with aqueous solution of Safranine(5 gm/l), and was then again washed with distilled water. The slide was examined under microscope.

3.2.2. Catalase test. The isolate was grown on nutrient agar plate at 37 °C for 2 days to get distinct colony. Few drops of H₂O₂ were poured on the top of the colony. The effervescence of gas bubbles immediately after addition of H₂O₂ from the colony surface
confirmed catalase test positive. As a negative control H₂O₂ was dropped on the other part of the nutrient agar plate devoid of any bacterial colony.

3.2.3. Methyl Red (MR) and Voges Proskauer (VP) test. The bacterial strain was grown in 10 ml of glucose peptone broth (peptone 5 gm; K₂HPO₄ 5 gm; glucose 10 gm per l; pH 7.4) in test tubes for 2 days at 37 °C. Few drops of methyl red reagent (5 mg dissolved in 30 ml of 95% ethanol and diluted to 50 ml with distilled water) were added to one of the test tube containing culture for MR test. Methyl red positive reaction was indicated by bright red coloration and negative reaction was indicated by yellow or orange color of the culture medium. α-naphthol (Barritt’s reagent A) and potassium hydroxide (Barritt’s reagent B) were added to other set and remained for about 15 min for color development. VP positive reaction was indicated by the development of a red color and negative reaction was indicated by in yellowish to copper color of the culture medium.

3.2.4. Oxidase test. The test was performed by putting bacterial culture on a strip of filter paper impregnated with 1% (w/v) aqueous solution of N-N-dimethyl-p-phenylenediamine. A pink color development within 30 sec indicated the oxidase positive test.

3.2.5. Citrate utilization. The Simon’s citrate medium (NaCl, 5 gm; MgSO₄.7 H₂O, 0.2 gm; NH₄H₂PO₄, 1 gm; K₄HPO₄, 1 gm; Na-citrate, 5 gm; bromophenol blue, 0.02 gm; yeast extract, 50 mg; agar powder 20 gm; distilled water 1 l; pH 6.8) was used for this test. Growth and bright blue coloration of the medium are indicative of positive test and negative, if there is no growth.

3.2.6. Gelatin hydrolysis. For gelatin hydrolysis study, tubes containing sterilized medium (gelatin, 12 gm; beef extract, 3 gm; peptone, 5 gm; distilled water, 1 l; pH 6.8) was inoculated with freshly grown culture and incubated for 5 days at 28 °C. After the growth of the organism, the culture tubes were kept at 4 °C along with uninoculated control. Negative solidification of the tube indicated liquefaction of gelatin.

3.2.7. Urease test. Urease activity of the bacterial strain was carried out in a medium containing (gm/l): Part A – peptone, 1.0; glucose, 1.0; NaCl, 5.0; KH₂PO₄ 2.0; phenol red, 0.12; agar powder, 20.0; pH 8.0; and Part B – urea, 40.0. After sterilization, part A and part B were mixed together (1:1) aseptically and the resultant mixture was poured on
sterilized petriplates. Young culture was streaked on plate and incubated at 37 °C for 6 days. Development of deep red color surrounding the bacterial growth was indicative of positive urease test.

3.2.8. **Nitrate reduction test.** The test organism was inoculated in 5 ml of medium (KNO$_3$, 1.0 gm; peptone, 5.0 gm; pH 7.5, water, 1 l) and kept at 37 °C for 3 days. Formation of nitrite in the culture medium was tested by adding 1 ml of reagent (0.8 gm of sulfanilic acid dissolved in 100 ml of 5 N acetic acid and 0.5 gm of α–napthalmine dissolved in 100 ml of 5 N acetic acid, mixed immediately before use). Red color development denotes a nitrate reduction test positive.

3.2.9. **Amylase test.** Amylase activity of the bacterial strain was carried out in starch agar medium (starch soluble, 20gm/l; peptone, 5gm/l; beef extract, 3gm/l; agar, 15gm/l; pH 7.0, water, 1 l). Starch agar plates were streaked with the young bacterial culture and kept at 37 °C for 24 h. After incubation the plates were flooded with a dilute iodine solution for 1 min. A clear zone around the streaked area of growth indicates positive test for amylase activity.

3.2.10. **Indole production test.** Tryptone water containing (gm/l) Tryptone, 10; NaCl, 5.0 was inoculated using an inoculating needle. After incubating the bacteria for at least 48 h, 0.2 ml of Kovac’s reagent (p-dimethylaminobenzaldehyde, 50 g; isoamyl alcohol 750 ml; conc. HCl, 250 ml) was added to the media to detect indole production by the bacteria. The development of a red/pink layer on top of the media is a positive result (i.e. the bacteria can breakdown tryptophan to form indole).

3.2.11. **Arginine hydrolysis test.** Arginine hydrolysis broth (Tryptone 5 gm/l; Yeast extract 5 gm/l; K$_2$HPO$_4$ 2 gm/l; L-Arginine monohydrochloride 3 gm/l; Glucose 0.5 gm/l) was inoculated with the bacterial isolate at 37 °C for 48 h. Then 5 drops of the Nessler's Reagent (KI 50 gm, HgCl$_2$ saturated 35 ml, distilled water 25 ml, 50% KOH solution 400 ml) was added to the culture tube. Development of orange color upon addition of Nessler's Reagent indicates positive reaction and no color change indicates negative reaction.

3.2.12. **Oxidation-Fermentation test (O/F test).** 5 ml of sterilized Hugh and Leifson’s agar medium was taken in a culture tube followed by addition of 1 ml of 10% sterilized glucose solution. Bacterial suspension was stabbed in two tubes containing the
medium. Small amount of liquid paraffin was added on the top of one of the two tubes. Both the tubes were incubated at 37 °C for 24 h. Yellow coloration of the tube without paraffin indicated positive test that is oxidative utilization of glucose. Acid production in both the tubes resulting yellow coloration indicates fermentation reactions.

3.2.13. *Sulfide production and Motility test.* The bacterial sample was inoculated into SIM (Sulfide Indole Motility medium – peptone 30 mg/ml, beef extract 3 mg/ml, ferrous ammonium sulfate 0.2 mg/ml, sodium thiosulfate 0.025 mg/ml) agar media by stabbing with a needle in such a way that a straight line was formed and the needle was withdrawn very carefully to avoid destruction of the stabbed line. After 36 h of incubation, the migration of the bacteria away from the original line of inoculation was observed. Positive test indicated migration of the bacteria away from original line of inoculation. Appearance of the black color along the line of stab inoculation indicated sulfide production by the strain. A loopful of bacterial culture was inoculated to Kligler’s agar (HiMedia, India) plate and development of black color around the bacterial growth indicated positive test for sulfide production.

3.2.14. *Manitol utilization test.* Mannitol complete medium (K₂HPO₄, 0.5 gm; MgSO₄. 7H₂O, 0.4 gm; NaCl, 0.1 gm; glutamine, 1 gm; NH₄NO₃, 1 gm; mannitol, 10 gm; water 1 l; agar, 1.5%) was inoculated with the young bacterial culture and kept at 37 °C for 24 h. Visual observation of growth in the medium indicated positive result.

3.2.15. *Carbohydrate metabolism.* Using sterile inoculating technique, overnight culture was inoculated into phenol red containing glucose, sucrose, maltose, mannitol, and lactose broth medium supplemented with 5% each of carbon source. One uninoculated each of carbon source tube was used as a control. After incubating the bacteria for at least 24 h at 37 °C, yellow coloration of the tube indicated positive test *i.e.* carbohydrate metabolism of that supplemented carbon source medium. No change of phenol red indicator of the medium represented as carbohydrate metabolism negative.

3.2.16. *Test for cellulase production.* Cellulase production test was performed according to the method of Hankin and Anagnostakis (1977) with slight modifications. A loopful of young culture of the bacterial isolate was inoculated to petriplates containing Czapek-mineral salt agar medium (NaNO₃, 2 gm/l; K₂HPO₄, 1 gm/l; MgSO₄.
7H2O, 0.5 gm/l; KCl, 0.5 gm/l; peptone, 2 gm/l; agar, 20 gm/l; pH 6.5) supplemented with carboxymethyl cellulose (5 gm/l). The plates were incubated at 37 ºC for 4 days. After incubation the plates were flooded with 1% solution of hexadecyltrimethyl ammonium bromide. No zone formation around the growth is indicative of negative result.

3.2.17. Test for phosphate solubilization. Pikovaskya agar (Himedia, India) plates were spot inoculated with a loopful of young culture of the bacterial isolate. Present of clear zone around the bacterial colony after 5 days at 37 ºC indicated positive response to phosphate solubilization (Pikovaskya, 1948).

3.2.18. Detection of siderophore production. Fresh bacterial isolate was inoculated on chrome azurol sulfone (CAS) blue plates, following the protocol of Schwyn and Neilands (1987). For preparing 1 l CAS agar, 60.5 mg CAS dissolved in 50 ml water and mixed with 10 ml iron solution (1 mM FeCl₃ in 10 mM HCl). This solution was slowly mixed in 72.9 mg hexadecyltrimethyl ammonium bromide dissolved in 40 ml water. The resultant dark blue liquid was sterilized in boiling water bath. 100 ml of the CAS solution was mixed with 900 ml of sterilized mannitol complete medium (K₂HPO₄, 0.5gm/l; MgSO₄. 7H₂O, 0.4gm/l; NaCl, 0.1gm/l; glutamine, 1.0gm/l; NH₄NO₃, 1.0gm/l; mannitol, 10.0gm/l; agar 15 gm/l) supplemented with 30.24 gm piperazine-N,N’-bis-(2-ethane sulphonic acid) and adjusting pH to 6.8 with 1 N NaOH. The mixed solution under hot condition was poured on the plates to prepare CAS agar plates. The siderophore produced by the organism scavenges the Fe³⁺ from the CAS–iron complex causes a color change from blue to yellowish-orange around the colony, which indicates siderophore production.

3.2.19. Test for HCN production. Production of HCN was determined following the method of Lorck (1948). Bacterial culture (48 h) was streaked on King’s B medium (HiMedia, India) supplemented with glycine (4.4 gm/l) with the addition of a sterilized filter paper soaked in 0.5% (w/v) picric acid in 1% Na₂CO₃ placed in the upper lid of the petriplate. After incubation at 37 ºC for 24 h, changes in color was examined. Uninoculated plates were used as control.

3.2.20. Quantitative determination of indole acetic acid (IAA) production. IAA was quantified following the method of Gordon and Weber (1951). Bacterial strain was
grown overnight at 37 °C on nutrient broth with or without tryptophan (500 μg/ml) and then the bacterial cells were removed from the culture medium by centrifugation. One ml of the supernatant was mixed thoroughly with 4 ml of Salkowski’s reagent (150 ml H₂SO₄, 250 ml H₂O, 7.5 ml 0.5 M FeCl₃. 6H₂O), and the absorbance was measured at 520 nm with spectrophotometer.

3.3. Molecular biology techniques

3.3.1. Isolation of plasmid DNA. Plasmid DNA was isolated from the bacterial strain following the standard protocol of alkaline lysis method (Birnboim and Dolly, 1979). A single fresh colony was inoculated into 2 ml of NB containing 2 mM Ni in the form of NiCl₂ in plugged tubes and incubated overnight at 37°C with vigorous shaking in a rotary shaker. For rapid preparations, 1.5 ml of the cells grown to a stationary phase, were taken in a microcentrifuge tube placed on ice and harvested by centrifugation at 12,000 rpm for 30 sec at 4°C. The spent media were removed by careful decantation to leave the pellet as dry as possible which was suspended in 100 μl of ice-cold solution I (Glucose 50 mM; Tris-HCl 25 mM; EDTA 10 mM; pH 8.0) by vigorous vortexing until complete dispersion. Next, a brief incubation in ice for 5 min was followed by the addition of 200 μl of solution II (1% Sodium dodecyl sulphate in 0.2 N NaOH), mixing and storing on ice for a minute. Immediately 150 μl of ice-cold solution III (5 M Potassium acetate, 60 ml; Glacial acetic acid, 11.5 ml; water, 28.5 ml; pH 5) was added mixed thoroughly by gentle vortexing for 10 sec in inverted position. Following a further incubation of 10 min on ice, it was centrifuged at 12,000 rpm for 10 min at 4 °C and the clear aqueous phase was transferred to a fresh tube. After sequential extractions of the aqueous phase with equal volumes of (a) buffer saturated phenol–chloroform (1:1) mixture and (b) chloroform, the plasmid DNA was precipitated by adding of two volumes of ice-cold absolute ethanol and allowing to stand 2 min at room temperature and collected by centrifugation at 12,000 rpm for 10 min at 4 °C. The pelleted nucleic acid material was briefly washed with 500 μl of 70% (v/v) ethanol, air dried, reconstituted in minimum volume (50 μl) of TE buffer (Tris, 25 mM; EDTA, 0.25 M; pH 8) and stored at – 20 °C for further use.
3.3.2. Isolation of genomic DNA. Cells from culture suspension of KUNi1 (16 h grown, 100 ml) were harvested by centrifugation at 4000 rpm for 10 min at 4 ℃. The cell pellet was resuspended gently in 9.5 ml TE buffer (Tris, 10mM; Na₂EDTA, 1mM; pH. 8.0). Then 1.5 ml of 10% SDS solution and 50 µl of a 20mg/ml proteinase K solution were added. After vigorous mixing the suspension was incubated for 1 h at 37 ℃. 1.8 ml of a 5M NaCl solution was added to it followed by mixing. 1.5 ml of 10% CTAB (hexadecyl trimethyl ammonium bromide) in 0.7 M NaCl was added; the sample was mixed for 10 min and incubated for 20 min at 65 ℃. An equal volume of TE saturated phenol/chloroform (1:1) solution (pH 8.0) was added and the sample was mixed gently for 30 min. Until the solution become homogeneous. Thereafter, the sample was centrifuged at 10,000 rpm for 30 min at 4 ℃ to separate the phases. The aqueous supernatant was transferred carefully to a fresh tube and to it an equal volume of chloroform/isoamyl alcohol (24:1) followed by gentle mixing. The mixture was then centrifuged at 10,000 rpm for 30 min at 4 ℃. The upper phase was transferred into a new tube and mixed with 0.6 volume of isopropanol until a white DNA pellet precipitated and condensed into a tight mass. The precipitate was transferred into a fresh tube with 1 ml of 70% ethanol using a bent capillary tube and washed three times with 70% ethanol. Finally, the DNA was reconstituted in minimum volume (50 µl) of TE buffer.

3.3.3. Amplification of 16S rDNA sequence. Polymerase chain reaction using oligonucleotide primers specific for the target 16S rDNA sequence was carried out for amplification of target DNA, the source being the isolated chromosomal DNA preparation. The procedures for the preparation of the latter have already been described earlier. A 100 µl reaction mixture in 200 µl capacity tubes was used for carrying out PCR experiments in a thermal cycler (Eppendorf Master Cycler, Germany). The PCR amplification reaction was performed using high fidelity PCR Master Kit (Roche Applied Science, Germany) taking 50 ng of template DNA and 400 ng of each of the primer pair.

The 1.45 kb 16s rDNA fragment was amplified using 16s rRNA bacterial specific forward 27(5ʹ-AGAGTTTGATCCTGGCTCAG-3ʹ) and reverse 1492(5ʹ-TACGGTTACC TTGTTACGACTT-3ʹ) primer sets (Giovannoni, 1991). The mixture
was subjected to an amplification of 35 cycles with each cycle comprising a template denaturation step at 94 °C for 1 min, a primer annealing step between 50 °C for 1 min and a primer extension step at 72°C (for Taq) for 2 min. A pre-denaturation period of heating the reaction mixture for 5 min at 94°C, for complete denaturation of the template, was allowed before initiation of the first cycle. Similarly, a post extension period of 10 min at 72 °C was allowed at the end of the last cycle for any incomplete amplicons (Sambrook et al., 1989). The base sequencing of the PCR product was done at Jenia Biotechnologies Pvt. Ltd., Hyderabad, India. The sequence data were aligned and analyzed to identify the bacterium further.

**PCR Amplification conditions:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>DNA</td>
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</tr>
<tr>
<td>16s Forward Primer</td>
<td>400 ng</td>
</tr>
<tr>
<td>16s Reverse Primer</td>
<td>400 ng</td>
</tr>
<tr>
<td>dNTPs (2.5mM each)</td>
<td>4 µl</td>
</tr>
<tr>
<td>10X Taq DNA Polymerase Assay Buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>Taq DNA Polymerase Enzyme (3U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Water</td>
<td>X µl</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

**Profile:**

- Initial denaturation: 94 °C for 5 min
- Denaturation: 94 °C for 1 min
- Annealing: 50 °C for 1 min
- Extension: 72 °C for 2 min
- Final extension: 72 °C for 10 min
- MgCl₂: 1.5 mM final concentration
- Number of Cycles: 35

3.3.4. **Electrophoresis, staining and visualization of DNA.** Prior to subjecting the DNA samples to agarose gel electrophoresis, a block of agarose gel was prepared by pouring molten agarose [1 gm of agarose powder in 100 ml of Tris-acetate EDTA (Tris base, 24.2gm; glacial acetic acid, 57.1 ml; 0.5 M EDTA (pH 8.0), 100 ml, volume made
up to 1 l by H₂O) buffer (pH 8.0) and heated on a boiling water bath to get desired 1% concentration of agarose cooled down to 50 °C on gel casting trays of appropriate sizes (horizontal type, Bangalore Genei, India). DNA was mixed with 6X DNA tracking dye [25% bromophenol blue; 40% sucrose in H₂O (w/v)] and applied on the agarose gel for carrying out electrophoresis on a horizontal tank containing 1X TAE, by applying a constant voltage (initially 0.5 volt/cm for 15 min and subsequently 2.5 volt/cm), until the tracking dye reached a distance of about 1 cm away from the gel end (anode side). For comparison of molecular sizes, standard 1kb marker DNA for chromosomal DNA and for plasmids were used as reference. Following electrophoresis, staining was done by immersing the gels in an aqueous ethidium bromide solution (0.5 µg/ml) and incubating for 30 min at room temperature. For visualization, gels were rinsed with distilled water and viewed on an UV transilluminator, photographed and documented using a gel documentation system (1000 Gel Doc, BioRad Laboratories).

3.3.5. Sequencing and analysis of the PCR product. The sequencing of the PCR product was done at Bangalore Genei, India using the bacterial specific primers F27(5′-AGAGTTTGATCCTGGCTCAG-3′) and R1492(5′-TACGGTTACCTTGTTACGACTT-3′) for 16s rDNA. Sequence homologies were analyzed using BLAST version 2.2.12 of National Center for Biotechnology Information (Altschul et al., 1990), and the Ribosomal Database Project (Maidack et al., 1997). Multiple sequence analyses were done using ClustalW version 1.83 (Thompson et al., 1994), and a consensus neighbour-joining tree (Saitou and Nei, 1987) was constructed following the majority rule out of 100 phylogenetic trees derived using the program NEIGHBOR of the PHYLIP package (Felsenstein, 1993). The genetic distances were computed by the Kimura’s 2-parameter method. The resulting tree was plotted using TREECON (Van de Peer and De Wachter, 1994).

3.4. Antibiotic sensitivity test

To determine sensitivity to antibiotics, the bacterial strain was tested for its resistance to 10 different antibiotics. The sensitivity to antibiotics was determined by disc diffusion method (Bauer et al., 1966). The bacterial strain was grown on Mueller-Hinton broth (MH, Himedia, India) at 37 °C for 24 h. Young culture was plated on MH
agar plates to have uniform bacterial lawn. The antibiotic discs were placed on freshly prepared lawn of the isolate on MH agar plates and incubated at 37 °C for 48 h. The diameter of the inhibition zones were measured and the bacterium was classified as sensitive (S), resistant (R) and intermediate (I) depending upon the size of inhibition zone following the standard disc sensitivity method (HiMedia, India). The antibiotics used were - Tetracycline (30 µg), Polymyxin-B (30 units), Gentamycin (10 µg), Amikacin (30 µg), Colistin (10 µg), Ciprifloxacin (5 µg), Kanamycin (150 µg), Ampicillin (20 µg), Rifampicin (20 µg), Chloramphenicol (20 µg) and Clavulanic acid (7.5 µg).

3.5. Test for heavy metal tolerance of the strain

Minimum inhibitory concentration (MIC) for each test metal viz. Cd, Co, As, Ni, Zn and Cu were determined both in nutrient broth and chemically defined minimal liquid medium. Both of the medium (pH 7.0) were supplemented with different concentrations of heavy metals. Respective metals were added as analytical grade salts of Cd (CdCl₂, H₂O), Co (CoCl₂, 6H₂O), As (As₂O₃), Ni (NiCl₂, 6H₂O), Zn (ZnCl₂), and Cu (CuSO₄.5H₂O) from their membrane sterilized stocks before. Young bacterial cells were inoculated by sterile inoculating loop containing different concentration of metal salts. Growth was recorded after 3 days of incubation at 37ºC. Development of turbidity in the growth medium was observed using a spectrophotometer and accordingly the MIC was determined. The lowest concentration of metal that completely prevented the growth was considered as the MIC.

3.6. Growth Response of the isolate under Nickel Stress

To assess the effect of different Ni (II) concentrations (0.5, 1, 2.5, 5 and 7.5 mM) on cell growth under aerobic conditions, 0.25 ml of 16 h grown cell suspension was inoculated finally to have ~6 log CFU/ml in nutrient and minimal media supplemented with 2 mM Ni²⁺ as NiCl₂ and incubated on a rotary shaker at 37°C, and compared with respective control set without Ni. Growth responses were measured by determining optical density (A₆₀₀) after every 4 h of incubation, viable cell count was also done for
each treatment regime at 4 h intervals by dilution platecounting on nutrient agar. Changes of pH values of the culture medium during incubation were also monitored.

3.7. **Effect of pH, temperature, salt and other heavy metals on growth of the bacterial strain**

3.7.1. **Effect of pH on growth.** To study the effect of pH on the growth of the isolate, three conical flasks containing 25 ml of minimal media was taken and the pH was adjusted to 5, 7 and 9 respectively by adding dilute 1 M HCl or 1N NaOH. Flasks were then autoclaved and test amount of Ni was added from the stock solution (500 mM, NiCl$_2$) to the flasks to have 1 mM of Ni concentration in the medium. In each of the conical flasks, 0.1 ml of overnight grown culture of KUNi1 (~6 log CFU/ml) were inoculated and incubated in a shaking incubator at 37 ºC. The growth responses were measured as a change in optical density at 600 nm at every 4 h of intervals using a spectrophotometer. The pH of the media was observed after every 4 h of intervals.

3.7.2. **Effect of temperature on growth of the bacterial strain.** To determine the effect of different temperatures on the growth of the bacterial strain, four different temperature values (22ºC, 30ºC, 37ºC and 42ºC) were selected for this study. Eight conical flasks containing 25 ml minimal medium were prepared. The pH of the medium in the conical flasks was adjusted to 7.0 with dilute HCl or 1N NaOH. Flasks were then autoclaved and 0.25 ml of overnight grown culture was inoculated and incubated in a shaking incubator at different temperatures i.e. at 22ºC, 30ºC, 37ºC and 42ºC with or without the presence of 2 mM Ni for each temperature regime. The growth responses were measured as a change in optical density at 600 nm at every 4 h of interval using a spectrophotometer.

3.7.3. **Effect of NaCl on growth of the bacterial strain.** Equal amount of young broth culture of the bacterial strain was inoculated to conical flasks containing 25 ml of minimal media supplemented with varying concentrations of NaCl salt (0 - 200mM) separately. In each flask 2 mM Ni was added as NiCl$_2$. Flasks were kept in a shaker incubator at 37ºC. The growth responses were measured as a change in optical density at 600 nm at every 4 h of interval using a spectrophotometer.

3.7.4. **Effect of different heavy metal combinations on growth.** Different filter sterilized metal concentrations were prepared by dissolving NiCl$_2$, CuSO$_4$, ZnCl$_2$,
CoCl$_2$ and CdCl$_2$ salts in deionized water to have metal concentration of 20 mM. Binary metal solutions were prepared by the use of the five metal concentrations at equimolar ratios in minimal media. All glassware was washed with 0.1 M HCl before and after each experiment to avoid binding of the metal to it. For studying growth response of the isolate in presence of different metal combinations, 0.25 ml 16 h grown cell suspension from minimal media (~6 log number of CFU/ml) was tested with different combination of metal solutions (2 mM each) by incubating on a shaking incubator at 37°C. Optical density at 600 nm was recorded for each culture tube after every 4 h of incubation for 24 h.

3.8. Study of Nickel Removal by KUNi1 under Different Culture Conditions

3.8.1. Effect of different concentrations of Ni in Ni removal. 0.25 ml of 16 h grown cell suspension from minimal medium (~6 log number of CFU/ml) was inoculated to the same medium having different concentrations of Ni viz. 0.5, 1, 2.5, 5 and 7.5 mM and incubated on a shaking incubator at 37°C. Cells were harvested after 24 h of incubation by centrifugation (10000 rpm for 10 min at 4°C). The collected supernatants were digested with concentrated acid mixture (nitric acid: perchloric acid, 4:1) and the metal content was quantified by atomic absorption spectrophotometer (AAS) (GBC-Avanta, Australia). Cell-free sets were maintained for each treatment regime to determine the artifacts that might rise due to the metalsorption on the glass surface of the container. All glasswares were also washed with 0.1 N HCl before and after each experiment to avoid binding of the metal to it. The % of Ni$^{2+}$ removal by the biomass was computed using the following equation:

$$R(\%) = \frac{(C_o - C_e) \times 100}{C_o}; C_o \text{ and } C_e \text{ are the initial and equilibrium concentration of Ni}^{2+} \text{ ion (mg/l) in the solution.}$$

3.8.2. Effect of pH on Ni removal. For studying the effect of pH on the Ni removal, cells were similarly treated in minimal medium having 2 mM Ni but at different pH (5, 7 and 9). The cells were harvested after 24 h of growth and the Ni accumulation was measured as described earlier.

3.8.3. Effect of temperature on Ni removal. To assess the effect of temperature on Ni removal, cells were similarly treated in minimal medium having 2 mM Ni but at
different temperature (22°C, 30°C, 37°C and 42°C). The cells were harvested after 24 h of growth and the Ni accumulation was measured as described earlier.

3.8.4. Effect of biomass on Ni removal. For studying the effect of initial inoculum load on Ni removal, cells (~4, 5 and 6 log CFU/ml) were reinoculated to liquid minimal medium having 2 mM Ni and Ni removal was measured at different time intervals as described earlier.

3.8.5. Effect of different carbon source on Ni removal. This experiment was conducted in search of suitable carbon source to support Ni removal by the bacterial isolate. The isolate was exposed to each of the carbon sources (glucose, sucrose, maltose, mannitol and lactose). Minimal media having different carbon sources (0.8% w/v of the medium, pH 7.2) were supplemented with 2 mM Ni²⁺, inoculated with same volume of young cell suspensions (~6 log CFU/ml) and incubated on a rotary shaker at 37 °C. The Ni removal was measured after 72 h by atomic absorption spectroscopy as mentioned earlier.

3.8.6. Effect of other metals in Ni removal. Different metal concentrations and binary metal solutions in minimal medium were prepared as described above. The KUNi1 inoculated cultures were grown at 37°C for 24 h in a rotary shaker and then harvested by centrifugation and medium supernatants were analyzed using an atomic absorption spectroscope following standard protocol as described earlier. Cell-free sets were maintained as control.

The effect of other metal ions on nickel removal was evaluated following the equation: 
\[ Q = \frac{v (C_i - C_f)}{m} \]
where \( Q \) is the metal uptake (mg/g), \( v \) is the liquid sample volume (ml), \( C_i \) is the initial concentration of the metal in the solution (mg/ml), \( C_f \) is the final concentration of the metal in the solution (mg/ml) and \( m \) is the amount of the cell mass added (mg).

Langmuir sorption model was chosen for evaluation of experimental data of multimetal absorption study following the equation: 
\[ Q = \frac{Q_{max} b C_f}{1+b C_f} \]
and for Linearized Langmuir model 
\[ \frac{1}{Q} = \frac{1}{Q_{max}} \left( \frac{1}{b C_f} + 1 \right) \]
where \( Q_{max} \) is the maximum metal uptake under the given conditions, ‘b’ is a constant related to the affinity between the biosorbent and sorbate. The Coefficient of Determination (\( R^2 \)) was also calculated to
determine the nature of the effect (synergistic or antagonistic or neutral) of other metals on nickel removal ability of the isolate.

3.9. Nickel removal study by live and dead cell mass

In order to ascertain whether live or dead cell mass are involved in nickel removal over time, 0.25 ml of 12 h grown cell suspension from minimal medium (~6 log number of CFU/ml) was inoculated to same medium having 2 mM of Ni and incubated on a shaking incubator at 37°C. Cells were harvested at 24, 48, 72 and 96 h of incubation by centrifugation (10000 rpm for 10 min at 4°C). Because of less cell mass production by the strain at the maximum tolerance level of Ni (10 mM), in this experiment 2 mM Ni was used to have substantial biomass yield. To assess any possibility of the capacity of dead cell mass in removing Ni$^{2+}$, the same protocol was followed for each time regime and each culture was autoclaved to obtain dead cell mass. Assay of nickel accumulation and removal by both live and dead cell mass of each set by atomic absorption spectroscopy was performed by maintaining standard protocol as mentioned above. Cell-free sets were maintained for each time regime to determine the artifacts that might rise due to the metal sorption on the glass surface of the culture container.

3.10. Transmission electron microscopy

Transmission Electron Microscopy (TEM) of the cells was done in order to trace the possible localization of accumulated metal inside the cell. Nickel-free (control) and Ni$^{2+}$ loaded (1 mM) bacterial cells were concentrated by centrifugation at a density of $10^{-8}$-$10^{-9}$ cells/ml, cell pellet washed with Na-phosphate buffer (0.1 M, pH 7.4) thrice followed by resuspension in 1 ml of fixative solution (2.5% glutaraldehyde) in the same buffer and kept for 6 h at 4 ºC. Post fixation, the fixative solution was removed by centrifugation and the cell pellet was washed with sodium phosphate buffer five times, finally resuspended in 1 ml of sodium phosphate buffer and was analyzed by TEM microscopic imaging of whole cell as well as thin sections of cells were performed in a transmission electron microscope (JEOL 100 CX, Japan) at 100 kV AC Voltage.
3.11. Scanning Electron Microscopy and Energy Dispersive X-ray analysis

Intracellular metal accumulation can lead to several changes in surface features of cells. To detect such changes the metal treated (1mM) as well as untreated (control) cells were analyzed by Scanning Electron Microscopy. SEM images have a characteristic three dimensional appearance and are useful for judging the surface structure of the sample. The sample was fixed in the same way as done for TEM. The samples were then coated with a nanometer thick layer of gold after mounting them on studs following formation of smear on glass cover slips and then analyzed by SEM. Scanning Electron Microscopy was performed at 30kV AC Voltage on a SEM. EDXS analysis of the sample was done with EX-64165 JMU, JEOL (Japan).

3.12. X-ray powder diffraction study

The X-ray diffraction pattern of the Ni treated (2 mM) or untreated lyophilized cells after 48 h of growth of the test strain were recorded in a Seifert 3000P Powder Diffractometer using monochromatic Mo Kα1 radiation. Diffraction spectra were recorded in a range between 10° to 90° (2θ) with a step length of 0.02° (2θ). To identify the Nickel salt, the experimental X-ray diffraction pattern was compared with powder diffraction standards.

3.13. Cell fractionation

For localizing the site of intracellular Ni accumulation in the bacterial strain, Ni (2 mM) exposed cells after 48 h of growth were fractionated into cellular and cell-free parts following McLean and Beveridge (2001) with slight modifications by Sau et al. (2008). Cells were harvested by washing thrice in 0.2 M phosphate buffer (pH 7.2) by centrifugation (4000×g at 4°C), resuspended in the same buffer to have a 10 ml suspension and kept in an ice bath. Cells were disrupted with an ultrasonicator. Power was applied five times in 1 min pulses with 100% amplitude. After sonication the suspension was centrifuged at 12000×g for 10 min at 4°C to obtain a soluble fraction (S_{12}). Five ml of S_{12} fraction was centrifuged at 150,000×g for 90 min at 4°C to obtain S_{150} fraction. The pellet fraction was washed twice with 0.2 M phosphate buffer (pH 7.2) and then resuspended in 5 ml of the same buffer to obtain membrane fraction.
(P_{150}). Amount of Ni retained in the sub-cellular fractions was quantified as per standard atomic absorption spectrophotometric protocol operating at 232 nm, 0.2 mm slit width and air-acetylene flame following acid digestion. Protein from each fraction was precipitated with 10% (w/v) cold trichloroacetic acid, washed and dissolved in 10 mM Tris-HCl (pH 8).

3.14. Study of the effect of Mg\(^{2+}\) on growth and nickel removal capacity

Nickel free mid-log phase KUNi1 cell suspension of ~ 6 log CFU/ml was added to medium containing MgSO\(_4\) of concentration ranging from 2 mM to 10 mM. In each conical 2 mM fixed NiCl\(_2\) was added. One control sample was prepared where no MgSO\(_4\) was added, only 2 mM NiCl\(_2\) was present. Then at 4 h interval, the OD values of these samples were taken for growth study. In this experiment the cell culture was maintained at 37 °C in shaking condition. After 24 h the cells were harvested and the pellets were washed with 1X PBS buffer twice. Then the cell pellets were digested with acid mixture for 3 h at 80 °C for atomic absorption spectroscopic study. Similarly, nickel free mid-log phase KUNi1 cell suspension of ~ 6 log CFU/ml was added to medium containing Ni as NiCl\(_2\) of concentration ranging from 0.1 mM to 2.5 mM. In each conical 2mM fixed MgSO\(_4\) was added. One control sample was prepared where no NiCl\(_2\) was added, only 2mM MgSO\(_4\) was present. Samples were incubated at 37°C. After 24 h, the cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C and the pellets were washed with 1X phosphate buffer saline (0.1 M, pH 7.2) twice. Then the cell pellets were digested with concentrated acid mixture (nitric acid: perchloric acid, 4:1) for 3 h at 80°C for atomic absorption spectrophotometric study. All glasswares were also washed with 0.1N HCl before and after each experiment to avoid binding of the metal to it.

3. 15. Study of the activity of Ni dependent hydrogenase enzyme


The isolate was grown in on nutrient broth (pH 7) for 24 h in air tight vacuum tubes with different concentrations of Ni\(^{2+}\) (0.5 mM, 1 mM, 1.5 mM and 2 mM) and without Ni\(^{2+}\) (control) and a syringe was incorporated into each tube for gas collection during incubation period. After 24 h gas is taken from the tube and injected into 1mM KCl
solution under sealed condition for mixing of H₂ (if produced) into KCl which will be converted into HCl and was titrated by 0.1N NaOH for measuring the hydrogenase enzyme activity.

**3.15.2. Effect of Mg²⁺ on hydrogenase activity.** The isolate was grown in nutrient broth for 24 h in air tight vacuum tubes and hydrogenase activity was measured after 24 h as mentioned earlier; but this time either with different concentrations of Mg²⁺ (0-15 mM) and 2 mM Ni²⁺ (fixed) or with different concentrations of Ni²⁺ (0.1-2.5 mM) and 2 mM Mg²⁺ (fixed).

**3.16. Effect of respiratory inhibitors and stimulators on nickel uptake**

Ni-free mid-log-phase cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C. The collected cell pellet was washed with 1X phosphate buffered saline (pH 7.2) twice and resuspended in 1X PBS. 10mM each of Sodium azide and Sodium cyanide (inhibitors) and 20 mM each of Succinate and Gluconate (stimulators) were added to separate 50-ml conical flask containing 5 ml of cell suspension (4 x 10⁸ cells per ml) and each flask was incubated at 37°C for 30 min. Then 1 mM Ni as NiCl₂ was added into the cell suspension and flasks were further incubated at 37°C for 30 min and compared with respective control set without inhibitors and stimulators. Nickel content was determined by using atomic absorption spectrophotometer as described earlier.

**3.17. Estimation of thiol**

Total thiol was estimated in the bacteria following the method of Ellman (1959) with minor modification. 100 µl of young bacterial suspension culture was inoculated to 250 ml nutrient broth (HiMedia, India) supplemented with different concentration of NiCl₂ (0, 1, 2 and 3 mM). The experimental sets were kept separately at 37°C for 48 h. After that the cultures were centrifuged at 6000 rpm for 15 min at 4°C. The cells were washed with 0.001 M phosphate buffer saline [phosphate buffer saline (10X) NaCl, 80 gm; KCl, 2 gm; Na₂HPO₄, 7H₂O, 26.8 gm; KH₂PO₄ 2.4 gm; H₂O 800 ml; pH 7.2] containing 0.025 M EDTA and then with 0.01M phosphate buffer saline. The cell were broken by ultrasonic treatment at 20 Khz for 30 sec on ice and centrifuged at 12000 rpm for 30 min at 4°C. The supernatant solution was used for thiol estimation and protein
estimation. The supernatant was diluted with sterilized water. Six ml of the diluted sample was taken in a large glass tube and to it 4.0 ml of 0.1 M sodium phosphate buffer pH 8.0 (Stock solution A and B were prepared. Solution A consists of 3.561 gm of Na$_2$HPO$_4$, 2H$_2$O dissolved in 100 ml of water. Solution B consists of 3.121 gm NaH$_2$PO$_4$, 2H$_2$O dissolved in 100 ml of water. Then 94.7 ml of solution A was mixed with 5.3 ml of solution B along with 100 ml of water to make a volume of 200 ml) was added. Then 10 ml of water was added to the tube to get a total volume of 20 ml. 3.0 ml of solution was taken from the 20 ml mixture and to it 0.02 ml of DTNB [19.8 mg of 5, 5'-Dithiobis (2-Nitrobenzoic acid) was dissolved in 5 ml of 0.1 M phosphate buffer) was added. The reaction mixture was incubation for 1 h. The resulting solution was vortexed and the absorbance was recorded at 412 nm against a suitable blank. Thiol content was estimated using the extinction coefficient of $p$-nitrothiophenol ($\varepsilon = 13,600$/M/cm at 412 nm) produced after reaction between thiol and DTNB.

3.18. Protein estimation

Protein was estimated following the method of Bradford (1976). Bradford reagent (0.01%) was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95% alcohol and to it 100 ml of 85% (w/v) phosphoric acid was added and the solution was diluted to 1 L with deionized water. Standard protein solution was prepared by dissolving 25 mg of bovine serum albumin (BSA) with standard buffer and the volume was made up 25 ml (1 mg/ml). Test sample or BSA (0.1 ml) was taken in test tubes; 5 ml of the Bradford reagent was added to each tube and mixed thoroughly by vortexing. The buffer (0.1 ml) was taken as blank and treated in same way. After 2 min, the absorbance of the mixture was measured at 595 nm. The amount of protein in the unknown sample was calculated using bovine serum albumin (BSA) as standard.

3.19. Study of ACC degradation ability of the isolate

In this experiment nickel free mid-log phase cell suspension of ~ 6 logs CFU/ml was added to two sample sets. One set contained 0.1 mM ACC in 25 ml media and the other set contained 0.1 mM ACC along with 2 mM NiCl$_2$ in 25 ml media. Then these two sets were incubated at 37 °C for 24 h. After that, cells were harvested by centrifugation at
6,000 rpm for 10 min at 4°C and the cell free supernatant was subjected for ninhydrin assay according to the standard protocol (Li et al., 2011). Ninhydrin reagent was prepared by dissolving 500 mg of ninhydrin and 15 mg of ascorbic acid in 60 ml of ethylene glycol, stored at -20 °C and mixed with 60 ml of 1 mol/l citrate buffer (pH 6.0) prior to use. 0.5 M ACC stock solution was stored at 4 °C and mixed in medium prior to use. In this method 1 ml of supernatant was added to 2 ml ninhydrin reagent in glass test tubes and the glass test tubes were capped and shaken and then placed in boiling water bath. After 15 min, the tubes were moved into a water bath at room temperature for 2 min and then shaken for 30 sec. After standing at room temperature for 10 min, the samples were transferred into cuvette and absorbance was measured at 570 nm spectrophotometrically.

3.20. Assay of phosphate solubilization capacity of the isolate

KUNi1 was grown as a pre-inoculum for 24 hin 50 ml SRSM2 liquid medium, a modified SRSM1 medium, where 1.1 gm/l of dipotassium phosphate-trihydrate replaced the tribasic calcium phosphate (Vazquez et al., 2000) in 250 ml Erlenmeyer flasks under rotary agitation of 120 rpm at 30 ± 2 °C. The SRSM1 enrichment medium is specific for the isolation of phosphate-solubilizing microorganisms. The modified medium consisted of: glucose, 10 gm/l; tribasic calcium phosphate, 5 gm/l; ammonium sulfate, 0.5 gm/l; potassium chloride, 0.2 gm/l; magnesium sulfate heptahydrate, 0.3 gm/l; manganese sulfate, 0.004 gm/l; ferrous sulfate, 0.002 gm/l; sodium chloride, 20 gm/l; yeast extract (HiMedia, India), 0.5 gm/l; bromocresol purple, 0.1 gm/l. The tribasic calcium phosphate was autoclaved first. Then, the other sterile ingredients were aseptically mixed after autoclaving. The final pH was adjusted after autoclaving to 7.2 with sterile 1 N sodium hydroxide. The culture was washed 3 times with 2% sodium chloride solution by centrifugation at 7,000 × g for 10 min. The optical density at 540 nm of the bacterial culture was adjusted to 1.0 ± 0.1 in the saline solution by using a spectrophotometer. An aliquot of 500 µl of this bacterial suspension was added to the 250-ml flasks with 50 ml of SRSM1 enrichment liquid medium containing insoluble calcium phosphate (Sundara-Rao and Sinha, 1963). The control consisted of uninoculated flasks. Five ml cell suspension was taken from each flask and centrifuged (7,000 × g, 27 ± 2 °C, 10 min). The pellet was discarded and 4 ml of the supernatant
was added in 4 ml reagent [6 N H₂SO₄ + H₂O + 2.5% ammonium molybdate + 10% ascorbic acid (1:2:1:1)]. Absorbance was measured at 820 nm after 2 h incubation at 37 °C. A standard curve was drawn in the same way by using dipotassium phosphate-trihydrate as a standard and compared the absorbance of the residual phosphate of the supernatant present in the medium.

3.21. Assay of cellulase activity

Cellulase enzyme degrades plant body parts containing abundant amount of cellulose into glucose units. Plants get these simpler carbohydrates again as carbon source. Cellulase activity was determined by growing the cells in CMC (Carboxyl-Methyl Cellulose) broth (CMC-26gm, NH₄H₂PO₄- 1gm, KCl- 0.2gm, MgSO₄- 1gm, yeast extract- 1gm, for 1000 ml) medium with various Ni²⁺ concentrations (0, 0.5, 1, 1.5 and 2 mM) for 48 h at 37°C. After incubation, medium was centrifuged at 6000 rpm for 5 min. Cell free extract was collected for respective concentrations. Three ml DNS reagent (sodium-potassium tartarate- 182 gm, NaOH- 10 gm, di-nitro salicylic acid- 10 gm, phenol- 2 gm, Na₂SO₄- 0.5 gm in 600 ml distilled water and then volume was adjusted to 1000 ml with distilled water). Test tubes were incubated for 30 min and OD was measured at 540 nm. Dextrose was taken as standard.

3.22. Assay of nitrate reductase activity

Nitrate reductase enzyme converts NO₃⁻ to NO₂⁻. It plays an important role in nitrogen assimilation. For measuring Nitrate reductase activity, cells were grown in YTA Broth (trypoton- 1%, yeast extract- 0.5%, KNO₃- 1 gm, Ca₃(PO₄)₂- 0.23 gm, pH-7, for 100 ml distilled water) for 24 h at 30°C in shaker with different Ni²⁺ concentrations (0, 0.5, 1, 1.5 and 2 mM). After incubation, culture broth was centrifuged at 6000 rpm for 10 min to collect supernatant. 250 µl Griess-I (0.5 gm sulphanilic acid in 150 ml 5N acetic acid) and 250 µl Griess-II (0.5 gm naphthalime in 50 ml distilled water) were added and incubated for 20 min at room temperature. Absorbance was measured at 548 nm. Sodium nitrite was used as the standard.

3.23. Survival of the strain in nickel amended soil

Ni contaminated soil sample was collected from industrial effluent fed field. Then 200 ml nickel was added as NiCl₂ solution to the soil sample to have 0.6 mM and 0.4
mM concentrations separately in two different conical flasks. One ml of young cell suspension culture was added to the experimental sets containing 200gm of soil in sterilized beakers with 0.6 mM and 0.4 mM Ni, and to a control set with no additional Ni was added. The three experimental sets were then kept at 37°C in an incubator. At every 24 h of intervals, representative amount was drawn for counting colony forming unit (CFU) by dilution plating using NA medium (HiMedia, India) containing 2.5 mM of Ni.

3.24. Growth response of KUNi1 in Ni contaminated soil with additional nutrient

Analytical grade of NH₄Cl (300 mg/l), glucose (0.5 gm/l) and KH₂PO₄ (200 mg/l) were supplemented to the nickel contaminated soil sample as N, C and P sources respectively. One ml of young cell suspension culture was added to the sterilized beaker containing 200gm of soil with added Ni (0.6 mM) and soil without nickel. The flasks were kept at 37°C on incubator for 72 h. The viable colony was determined at 24 h of intervals for each set by dilution plate technique on a nutrient agar medium with 2.5 mM Ni as described earlier.

3.25. Nickel removal efficiency of KUNi1 from nickel contaminated soil

Two ml of culture suspension obtained from growing single viable colony from each set (described earlier) was withdrawn at 24 h of intervals and centrifuged at 6000 rpm for 15 min. The Ni content in the supernatant was analyzed as explained before. Percentage of Ni removal was calculated by taking difference of the metal content of the cultures at zero time and at the time of sampling as described earlier.

3.26. In-vitro anti-fungal activity assay

The bacterial strain was tested for its antagonistic property against two test phytopathogenic fungal isolates (*Rhizoctonia solani* and *Phytophthora infestans*) following the dual culture method (Jin and Hee, 1989). All fungal isolates were grown separately in petriplates in potato dextrose agar medium (PDA, HiMedia, India) for 72 h at 30°C so that a uniform fungal mass was produced covering the whole petriplate. Then 3 mm discs of agar containing fungal mycelia were cut from respective plates with the help of a cork borer. PDA plates were prepared in two sets, one set supplemented with 100 µM of FeCl₃ solution and the other set contained no FeCl₃. The sets were
inoculated with 3 mm discs of 72 h old culture of each fungal isolate separately. Young bacterial culture was prepared by inoculating the bacterial isolate to 50 ml nutrient broth and kept overnight at 37°C. Bacterial culture was streaked near the mycelial disc keeping a 2 cm space. Interaction with live bacteria in dual culture and inhibition zone for each isolate was measured after 72 h of growth at 30°C. Final observations from all the replicates were converted to percentage inhibition with respect to the positive control set.

3.27. Survival ability of the bacteria in soil

Kanamycinresistant spontaneous mutant of the bacterial isolate was screened by dilution plating of young bacterial suspension on NA plates containing kanamycin(200µg/ml). Bacterial culture was prepared in batch by inoculating 5 ml of kanamycin resistant young bacterial suspension to 500 ml of nutrient broth supplemented with kanamycin(200 µg/ml). Total 10 conical flasks, each containing 500 ml nutrient broth was inoculated. All the cells were pulled together by centrifugation at 6,000 rpm for 15 min. Bacterial pellets were dissolved in 200 ml of sterilized water. Soil sample (500 gm) was collected from field, air dried and sterilized by autoclaving for 1 h for three consecutive days. The soil was then kept in a sterilized beaker under aseptic condition. The bacterial suspension was inoculated to the sterilized soil and the sets were incubated at 30°C in control chamber to maintain moisture content of the inoculated soil. Survival of the bacterial isolate was determined after 0, 20, 40 and 60 days of interval by dilution plating on kanamycin (200 µg/ml) supplemented NA plates.

3.28. Plant growth promotion assay

In-situ growth promotion of plants and rhizosphere colonization study was done with a stable kanamycin resistant mutant (200 µg/ml) of the bacterial strain KUNi1. Surface sterilized chili seeds and pumpkin seeds were imbibed either with sterilized water or with bacterial suspension for one hour in clean sterilized container and then sown in solarized clay pots of same sizes using sterilized soil and kept in the growth chamber to avoid microbial contamination. Five sprouting seeds were allowed to grow in each pot. To evaluate the effects of Ni toxicity, seedlings were irrigated every 3 days
of interval either with sterilized water (control) or with 200 ml of 200 µM Ni as NiCl₂ solution. The choice of such lowered Ni concentration was for avoiding severe effect on test plants due to accumulation of Ni in the soil as a result of consecutive irrigation with Ni salt. The plants were harvested after 30 days and measurement of growth parameters was done prior to dry weight estimation.

3.29. Estimation of iron content

The total iron content in the leaves was measured following the method of Rangarajan and Kelly (1998) with little modification. One gram of fresh leaves was taken in a crucible and dried at 105°C. The dried sample was acid digested in a mixture of conc. HNO₃ and HClO₄ (4:1, v/v). The iron content in the digest was determined by atomic absorption spectrometer (AAS).

3.30. Estimation of chlorophyll in leaf sample

The chlorophyll content was determined from leaf slices weighing one gram by extraction from leaf tissue in 85% acetone following the method of Arnon (1949). Absorbance of the extract was obtained at 663 nm and 645 nm respectively. Total chlorophyll content was calculated from the obtained data by using the following formula – (mg/gm fresh weight) = [(13×95 × A₆₆₅ – 6×88 × A₆₄₉) + (24×96 × A₆₄₉ – 7×32 × A₆₆₅) ×V]/ (1000 × W), with V being the volume of the extract and W being the fresh weight (gm) of leaf tissue.

3.31. Root colonizing ability assay

To study rhizosphere colonization, soil sample adhering to the roots of the above mentioned plants used for growth promotion assay removed from the roots and bacterial population (CFU/gm of soil) was determined by dilution plating on NA plates with kanamycin (200µg/ml) or without kanamycin for the assessment of contaminants, if any. Rhizoplane colonization was studied by macerating washed roots in sterilized water and the resulting suspension was plated as mentioned before.

3.32. Estimation of Ni in plants

Microorganisms were removed prior to the estimation of Ni in the root and shoot samples by vigorous washing with 0.01 M EDTA and sterilized water to avoid
interference of Ni accumulated by plant associated bacteria. The washed root and shoot samples were then dried at 105°C and were digested in a mixture of concentrated HNO₃ and HClO₄ (4:1, v/v) (Chen et al., 2004). The Ni content in the digest was determined by atomic absorption spectrometer (AAS).