

Original Research Article

Characterization of PGP Traits by Heavy Metals Tolerant *Pseudomonas putida* and *Bacillus safensis* Strain Isolated from Rhizospheric Zone of Weed (*Phyllanthus urinaria*) and its efficiency in Cd and Pb Removal

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ABSTRACT

Keywords

Heavy metals tolerant, Antibiotic resistant, *Pseudomonas sp.*, *Bacillus sp.*, PGPR, Heavy metals removal

In the present study two heavy metal resistant bacterial strains were isolated from rhizosphere of *Phyllanthus urinaria*, uprooted from cotton field and industrial waste dumping field of Gandhinagar, Gujarat. Isolate were identified as *Pseudomonas putida* CG29 (N5) and *Bacillus safensis* KM39 (LT) by 16S rDNA gene sequence analysis. Selected isolates were also evaluated for their plant growth promoting traits, HM (Pb and Cd) resistance and bioaccumulation of heavy metals. Removal of Pb was found to be higher than Cd in laboratory condition. Both strains were found to exhibit multiple antibiotic resistant characteristics. Result showed; the increasing bacterial growth in Pb and Cd amended broth culture medium. Both isolates were screened for qualitative parameters viz. inorganic Phosphate solubilization, production of siderophores, Indole acetic acid, HCN, ammonia, nitrate reduction and nitrification. In the tryptophan amended medium *Pseudomonas sp.* significantly ($p < 0.001$) increased IAA production. Both the isolates were also screened in different parameters of environmental condition viz. NaCl, pH, and UV. The inoculation of bacterial strains with plant, having plant growth promoting capability as well as heavy metal resistance can be considered as stimulator of heavy metals phytoremediation technologies.

Introduction

Soil is one of the most important natural resource on which lives of all plants, animals and microorganisms directly or indirectly dependent. In soil, different microorganisms thrive on abundantly present nutrients therein and through various interactions play a pivotal role in cycling of nutrients and pedogenesis (Ahemad and Kibret, 2013), among pollutant, abundant

amounts of heavy metals (Cd, Cu, Pb Cr, which are known to be toxic in nature) contaminated soils through various natural and manmade activities (Liu et al. 2013), heavy metals found longer time in soil due to non- biodegradable in its nature and pose a risk to human health through come into the food chain (Lal et al.2013). Exceeding threshold limit it affects microbial diversity

and soil fertility (Huang et al. 2009). Many Industries including electroplating, chemical manufacturing, paper making, printing and dyeing, hardware and leather that increase heavy metals concentration in soil. Heavy metals accumulation cannot only lead to the disorder of soil function but it also can leach out and pollute the soil as well as water environment.

Heavy metal pollution of soils is becoming one of the most severe environmental hazards and has negative impact on human health and agriculture. In modern agriculture the use of various agrochemicals is a common practice (i.e., herbicides, pesticides, insecticides, fungicides etc). Heavy metals input to arable soils through fertilizers courses increasing concern for their potential risk to environment. Chemical fertilizers like phosphate fertilizers were generally the major source of trace metals among all inorganic fertilizers (Saber and Hasan, 2014; Lu et al. 1992), and much attention had also been paid to the concentration of Cd in phosphate fertilizers. Thus remediation of such metals stressed soil is of paramount significance as they are rendered inappropriate for agriculture application. Pollution of soil with heavy metals is a great known ecological problem, due to their strong negative effects on all living organisms, including humans. Among heavy metal pollutants lead and cadmium need special attention due to their widespread occurrence and potential for their toxicity.

The rhizosphere is the narrow region of soil that is directly influenced by root secretions and richness of the microorganism's interrelationship. Free living rhizobacteria are root-adhering bacteria associated with rhizospheric region and form symbiotic relationships with many plants and determined by numerous mechanisms,

including plant secretion of specific organic compound competition for nutrients and solid attachment sites (Singh et al. 2013). Soil bacteria, which are found in the rhizosphere and have been considered to be only 1-2% bacteria promote plant growth (Kloepper et al. 1999; Antou and Kloepper, 2001). Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria which is found in the rhizosphere, adhere root surfaces and associated with roots, developed the quality of plant growth directly and /or indirectly (Ahmad et al. 2008).

PGPR is mainly of two types: i) modulating/symbiotic (nodule forming or bacteria which present inside the root surface) PGPR & ii) Free living (associated from outer environment of rhizosphere (root surface) PGPR. phosphate solubilization is the property of PGPR by which plant growth was stimulated and also have the capability to produce phytohormones, asymbiotic N₂ fixation, siderophores production, antibiotics synthesis, enzymes and/or fungicidal compounds and providing nutrients to the plant (Egamberdiyeva, 2007; Bashan et al. 1989; Bashan and Levanony, 1990; Mrkovacki et al. 2001; Ahmad et al. 2006; Bharathi et al. 2004; Cattelan et al. 1999). The common traits include production of plant growth regulators (auxin, gibberellins, ethylene etc.), siderophore, HCN, NH₃, antibiotics and multiple drug resistant (MDR) properties, production of lipase enzyme (Glick et al. 1998; Kloepper et al. 1980; Alstrom and Burns, 1989; Lata, 2003; Bauer et al. 1966). Although several studies are available carried out to isolation and characterization of metal potential bacteria very little information is available about isolation and characterization of heavy metals potential free living PGPR from cotton field (pesticide contaminated soil) and industrial dumping sites.

In this study the rhizobacterial community of *Phyllanthus urinaria*; a wild medicinal weed plant from contaminated soil at GIDC and Pethapur was examined using independent- and culture-dependent techniques. The objectives were to:

- (i) isolation characterization of potential rhizobacteria,
- (ii) screen isolates for siderophore production, Indole acetic acid (IAA), Phosphate solubilization and other PGP characters,
- (iii) determine growth rate of isolates at various conc. of Pb and Cd simultaneously evaluation of metals removing capacity (under UV-VIS spectrophotometer and AAS respectively)
- (iv) determine the impacts of different Environmental conditions (pH, Temperature, Salt and UV rays) on bacterial growth
- (v) Identify the potential bacterial strains by 16S rDNA analysis and heavy metals (Cd and Pb) content in collected soil.

Material and Methods

Collection of samples

Rhizospheric soil samples were collected from- i) an intensive agricultural soil (Pethapur Gandhinagar) [24° 23'90"5 Latitude and 72°76'48"5 Longitudes] used for cotton cropping at the time of sampling & ii) GIDC industrial waste dumping site [23°15'18"longitude 72°39'10"latitude]. *Phyllanthus urinaria* was the most abundant wild weed species that usually grow in agricultural field or in waste field uncultivated grasslands and in landfills. Five randomly located plants uprooted carefully and were placed in sample collection bags (HiMedia), kept in ice box during field survey. Collected samples were kept at 4°C in the laboratory until processed.

Physiochemical analysis of soil samples collected for isolation rhizobacteria

Soil sample were analyzed for physiochemical parameters like pH, moisture, conductivity, Total organic carbon organic matter, total nitrogen, available phosphorus, total phosphate, sulphate, exchangeable ammonium, nitrite etc. (APHA, 2012) heavy metals (Cd & Pb) determined by using the AAS (Atomic absorption spectrophotometer).

Isolation of rhizobacteria from Heavy Metals contaminated sites

The bacterial isolation was carried out same day by the pour plate method (McClellan et al. 2009), excess of soil as removed gentle shaking and the soil adhering to roots formed composite sample. Soil sample were serially diluted, 100 µl of 10⁶ times in buffer solution (1 gm soil + 9 ml saline solution i.e., 50% NaCl) spread on LB (Luria Bertini) plates incubated for 24 hrs at 30°C and counted (cfu g⁻¹). Colonies of different morphological appearances were selected and transferred to Nutrient agar media for *Bacillus* sp. and Kings B media for *Pseudomonas* sp. (Ahmad et al. 2008) for purification through the streak plate method (Prescott and Harley, 2002).

Minimum inhibitory concentration (MIC) determination of isolates

MIC of the bacterial isolates was determined by gradual increase of Cd and Pb (10-30 ppm and 10-50 ppm respectively) (Aleem et al. 2003). This concentration was considered as MIC of respective isolates. After incubation at 28-30°C for 5 days, representative bacterial colony were observed according to the morphological characteristics (pigments, colony form, elevation, margin, texture and opacity)

(Simbert and Krieg, 1981). The strains were maintained in glycerol stocks at -80°C.

Determination of HM resistance and quantification of metals removal from media

Resistance of isolates to Cd(II) and Pb(II) was determined by growing them separately in 20 ml LB broth liquid medium to which Cd(II) (from 70 to 100 ppm) or Pb(II) (from 70 to 150 ppm) was added in increasing concentrations. Two flasks of each concentration along with control (without metals) were inoculated with the appropriate cell suspension (48 hrs old culture) grown in Kings B and LB medium without heavy metals in order to obtain a cell density of approximately 10^6 cells mL⁻¹ (OD₆₀₀ nm of about 0.05). Their growth was evaluated by measuring the OD₆₀₀ nm after 2 to 6 days incubation (30 °C).

Aliquots of the growing cultures (2 ml) were removed in order to measure cell growth by measuring OD₆₀₀ nm; Standard curves were prepared for both Heavy Metals (Cd and Pb).

Removal of heavy metals from the culture medium was determined by following the method of Banerjee et al. (2015). In brief, 7 ml of culture broth were taken from both bacterial strains (N4 and LT) treatment sample containing Cd (II) (100 ppm) or Pb(II) (150 ppm) at 24-h interval for 6 days. Supernatant was collected by centrifugation at 5000rpm for 15 min, mixed 2.5 ml of concentrated HNO₃, and heated at 70 °C until it became transparent. Metals concentration in the supernatant was analyzed by atomic absorption spectroscopy (model Spectra AA55). Broth medium without bacterial strain containing the above mentioned metals was taken as control.

Environmental effects on bacterial growth

Effects of pH on rhizobacteria

pH is a limiting factor, which governs bacterial growth. To determine the pH optima nutrient broth medium meant for growth of the isolates were adjusted to different pH ranging from 4.0 to 9.0 adjusted the pH range of nutrient broth with buffer solution (Sigma Aldrich) maintained the pH of medium, autoclaved and cooled at room temperature inoculated with 0.1 ml 24 hrs fresh broth culture and incubated at 30°C under shaking condition at 120 rpm, growth was measured and compare in terms of OD at 600 nm using UV-VIS spectrophotometer (Dynamica CE model no. DB 20). Growth was observed and compared after 24 hrs; Set up was designed in triplicates.

Effects of Salt (NaCl) concentration

The optimum salt concentration on the bacterial growth was studied at different concentration of NaCl ranging from control (without salt), 1%, 2%, 4%, 5% were used in composition of Ingredients gm / l; peptic digest of animal tissue 5gm, Beef extract 1.5gm, and Yeast extract 1.5gm autoclaved at appropriate temp at 15 lbs for 15 min cooled flasks at room temperature after inoculation with 0.1 ml of 24 hrs old culture, incubated at 30°C at 127 rpm shaker the growth was measured by UV-VIS spectrophotometer (Dynamica CE model no. DB 20) up to 8th days. Growth was measured in terms of OD at 600 nm using UV-VIS spectrophotometer.

Effects of UV light

The effects of UV-B rays on selected potential bacterial growth was observed on NA plate exposed under UV-B rays (laminar

air flow) for 10 min. incubate the plates for 30°C for 24 hrs and observation compared the growth against the control (non-exposed plates).

Qualitative assay (Plate assay) of plant-growth promoting traits of rhizobacteria

Potential rhizobacterial isolate were determined for Plant growth promoting traits by standardized methods.

Evaluation of Phosphate solubilization

Potential bacterial isolates were checked for phosphate solubilization (one of the PGPR activity) on prepared Pikovskaya's (1948) medium agar plate. The appropriate medium was autoclaved adding 0.3g/ml bacteriological agar at 121°C on pressure 15 lbs for 15 min. after solidifying the agar plated were spot inoculated with a 24 hrs broth culture in triplicate using sterilized stainless steel loop and incubate the plate at 30°C for 5-7 days. After incubation at appropriate temperature the halo and colony diameters were measured. Clear zone development around the spot (Montero et al. 2013) after incubation was an index of phosphate solubilization. Phosphate solubilization expressed in terms diameter (cm) of Phosphate solubilization efficiency (% PSE) calculated from following equation-

Where, Z= Diameter of zone (mm)

C= Diameter of colony/bit (mm)

Evaluation of IAA production

Potential bacterial isolates were cultured for 48 hrs at Kings B medium and amended with 0.5mg/ml Tryptophan (precursor of IAA) in test tube. After proper incubation, test tubes were mixed with 2 ml of

Salkowski's reagent components with 150 ml concentrated H₂SO₄, 250 ml, distilled H₂O, 7.5 ml, 0.5 M FeCl₃.6H₂O (Patten& Glick 2002) and allowed to incubate at room temperature in dark for 30 min. Pink colour development indicates the presence of IAA and yellow colour indicate presence of tryptophan. Test has been done in triplicates for each.

Effect of tryptophan on IAA production

Concentration of Indole produced by potential isolates was assayed as described by Patten and Glick (1996). As described above Bacterial isolates were propagated in Kings B medium with L-tryptophan (1, 2 and 5 mg/ml) supplemented with heavy metal as 0.5mM CdCl₂, 2.5mM (PbNO₃)₂ then incubated at 28±2°C for 10 days. Bacterial cells were removed by centrifugation at 7,000 rpm for 15 min at 4°C. After centrifugation supernatant was kept for a min at room temperature. 1 ml of the supernatant was mixed with 2 ml of Salkowski's reagent in the ratio of

1:2 and incubated at room temperature for 20 min. Development of a pink colour indicated indole production by isolate. The absorbance of supernatant mixture (supernatant+ Salkowski's reagent) for Indole production was measured at 530 nm by UV-VIS spectrophotometer (Dynamica CE model no. DB 20). The amount of IAA present in the extract was calculated from the standard curve of analytical grade IAA.

Evaluation siderophore production (PLATE ASSAY)

Chrome azurol sulfonate (CAS) assay (universal assay – Schwyn & Neilands, 1987). The Chrome Azurol Sulfonate (Sigma-Aldrich) assay agar was used for the qualitative screening of siderophore production. After prepared CAS medium

plates were spot inoculated with 24 hrs old culture from Kings B medium onto the blue agar and incubated at 30 °C for 5 to 7 days yellow-orange zone formation around the growth were observed and haloes sized were measured for total siderophore excretion by isolates. Plates were prepared in triplicate for each Bacterium.

Evaluation of Ammonium production

For the production of ammonia test, method was used by Lata (2003). Both of strains (N5 and LT) were grown in peptone agar slant tubes. Tubes were incubated at 30 °C for 4 days. After 4 days, 1ml of Neisser's reagent was added to each tube. Presence of faint yellow colour (+) indicated small amount of ammonia and deep yellow (++) indicated good amount of ammonia production (Becker et al. 2002).

Evaluation of Catalase production

Catalase test was performed by taking a drop of 3% hydrogen peroxide was added to 48 hr old bacterial colony on a clean glass slide and mixed using a sterile tooth-pick. The effervescence indicated catalase activity (Kumar et al. 2012).

Evaluation of HCN production

24 hrs old of potential selected bacterial culture was inoculated on Petridis containing Nutrient Agar supplemented with Glycine. Whatman filter paper soaked in 2% w/v sodium carbonate in 0.5 % (w/v) picric acid solution was plate din side the lid of a petriplate. Incubated the plate for 48-96 hrs at 30 °C, development the colour of filter paper from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively (Alstrom and Burns, 1989).

Nitrification (nitrate forming Test)

Prepared ferrous ammonium sulphate (FAS) broth after autoclaving at 15 lbs (121°C) for 15 minutes and cooled at room temperature poured in test tubes. Inoculated a loop full fresh culture for each test tubes of both bacteria incubate it at 30°C for 1 week. Transfer a drop of the cultures from the ammonium sulphate medium to the 3 drops of Trommsdorff's reagent with 1 drop of dilute sulphuric acid in a culture tube. Appearance of blue black color indicates the presence of nitrite (Dubey & Maheswari, 2012; Cappucino and Sherman, 2008).

Evaluation of Nitrate reduction

Suspend components in distilled water dissolved completely by continuous stirring. Dispensed in culture tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. After cooling inoculated with 1 ml of fresh culture for each incubated for 1-2 days. For the test added few drops of both Nitrate reagents (Sulfanilic Acid and Alpha-Naphthylamine) into the tube containing culture to be tested. A distinct red or pink colour indicates nitrate reduction if no pink colour then it shown negative (ISO 1993).

Antibiotic Sensitivity test performed by rhizobacteria

The susceptibility to antimicrobial agents was tested with antibiotic disks by the method of Bauer et al. (1966). The commercial 6 different antibiotic disks used were streptomycin (10 µg), penicillin (10 µg), Kanamycin (30 µg), Gentamycin (10 µg). Autoclaved Mueller-Hinton medium at 15 lbs (121 °C) for 15 min dipped a sterilized swab into the 24 hrs old culture broth and expressed any excess moisture by pressing the swab against the side of the tube. Swabbed the surface of the agar

completely allowed the surface to dry for about 5 minutes before placing antibiotic disks on the agar with the help of sterilized forceps. Then it was incubated at 30°C for 24 hours or until bacterial growth was observed. At the end of incubation, the diameter (in mm) of each zone (including the diameter of the disk) was measured and recorded. The sensitivity and resistance profiles were determined based on the diameter of the inhibitory zone and the evaluation was done according to the standard chart.

Sequence analysis and phylogenetic tree construction

16S rDNA analysis of potential isolates was done by Xceleris Laboratory (Ahmadabad). The gene sequences 16S rDNA was compared to the entire Gene Bank nucleotide and amino acid databases.

Statistical analysis

All the data were analyzed represented as mean values of three replicates; the data sets generated were analyzed using a statistical package GraphPad Prism (5.0version) to determine the significant difference among means of the treatment at 5 percent significance level. The data were analyzed by analysis of variance (ANOVA) and the means were performed by Turkey test at $P < 0.05$.

Results and Discussion

The contamination of the environment with heavy metals has been a nominal problem all over the world which affecting soil microbial biomass, fertility and plant growth, contributing to food chain bioaccumulation with heavy metals (Giller et al.1998; Muller et al.2001). In present study result showed the exogenous

microbial biomass from the metals contaminated site. Exogenous PGPR (ePGPR) or free living PGPR which has high capacity to tolerate Cd and Pb concentration at 50mg/L and 110mg/L respectively and simultaneously showed Plant Growth Promoting Rhizobacterial traits such as siderophore production, hormone synthesis (IAA), and phosphate solubilization.

Physicochemical properties of soil

Soil samples were collected from rhizospheric region of *Phyllanthus niruri* plants grown in two different metals contaminated site of Gandhinagar. Soil collected from Sabarmati River was used as a alluvial soil for pot experimental setup. Prior to PGPR isolation, physical characterization (moisture content) and soil chemical properties (soil pH, Organic matter, EC, Available phosphorus, Total nitrogen etc.) was determined. Physicochemical characteristics and heavy metals (Cd and Pb) contents of collected soil samples are presented in (Table 1).

Isolation of heavy metals (Pb and Cd) potential Rhizobacteria

The heavy metal tolerant property of the isolated strain was confirmed by growing it on heavy metal containing media. After incubation and enumeration of total cultivable rhizospheric bacteria on Nutrient agar media amended with heavy metals, Pb (110 mg/l) and Cd (50 mg/l) concentration. Colonies with different morphologies were selected from the plates to obtain potential plant growth promoting rhizobacteria bacteria. Two of them were selected for presented study from two different sites LT strain (*Bacillus safensis*) from GIDC and N5 strain (*Pseudomonas* sp.) from cotton agricultural field.

Physical and biochemical characterization of isolated bacterial strain

Colonial & cellular Morphology selected isolates

Colonial morphology on agar medium revealed a translucent round form with 2 - 4 mm in size for strain N5 while strain LT appeared yellowish, round and 5-6 mm in size, isolates stained were gram negative (N5) and gram positive (LT), both were motile. Cells of strain N5 were short rods, observed contrasting with the long rods bacillus-like cells of strain LT. Details for cellular morphology and Gram reaction are given as follows (Table 2).

Biochemical Characterization of selected isolates

Two selected efficient Bacterial isolates were characterized on the basis of morphological and biochemical tests all the results of biochemical characterization such as MRVP, Sugar Test, starch hydrolysis, catalase, citrate utilization oxidase production, lipase production etc. were presented as following (Table 3).

Molecular Identification/Phylogenetic analysis of selected bacterial strains

Identification of selected PGPR strains were done by 16S rDNA gene sequences from the cultures. Four major steps for genotypic characterization of selected PGPR isolates in this study were as follows: genomic DNA extraction (Fig. 1), PCR amplification of 16SrDNA, sequencing of PCR amplicon and 16S rDNA sequence analysis.

The phylogenetic tree of identified bacteria was constructed to determine their affiliations (Fig. 2). Analysis of the 16S rDNA gene sequences revealed that one of

the strain belonged to *Pseudomonas putida* strain CG29 (Accession No.- KF782801.1, BP-1267, Similarity-99%) and another of them belonged to *Bacillus safensis* strain KM39 (Accession No.- JF411315.1, BP-1388, similarity-99%).

Heavy metals (Cd and Pb) tolerance by isolates

The growth of two selected isolates (N5 and LT) as consortium showed good growth with heavy metals (Pb and Cd) at different concentration compared to the single colony. Growth rate of the treatments (N5, LT and Consortium) at OD₆₀₀ was in the following order, Consortium > N5 > LT (Pb supplemented media), Consortium > LT > N5 (Cd supplemented media). Minimum inhibitory concentration of the selected bacterial strain against these two heavy metals Pb and Cd is showing in (Fig. 3 & 4), Cd was more toxic than Pb for bacterial growth. Both the isolate (N5 & LT) are significant at p<0.05 in the presence of heavy metals (Pb & Cd). Metal removal from the culture medium at different time intervals is given in (Table 4).

Metal absorption was recorded to be highest in case of Pb inoculated with LT (day 1, 53.02 %, to day 4, 89.19 %), while N5 inoculated treatment recorded (day 1, 46.60 %, to day 4, 77.76%). Cadmium absorption ability of the bacterial strain N5 was (day 3, 30.59%) while LT showed (day 2, 28.33%), Heavy metals present in medium decreased with increasing growth of bacteria from day 1 up to day 6. Absorption were decrease with decreasing bacterial growth.

Effects of different environmental conditions on bacterial growth

Effects of pH on selected isolates

The isolated strains (*Pseudomonas* sp. and

Bacillus sp.) were found to grow within a pH range pH (4 to 9). The optimum growth of *Pseudomonas* sp. was at pH (6.5 to 7) was found and *Bacillus* sp. grows well at pH (7.5 to 8). Both isolates showed growth at pH 4 and pH 9 (acidic and basic condition) (Fig.5).

Effects of Salt (NaCl) concentration on selected isolates

The effect of NaCl concentration on growth of the isolates was observed. The optimum growth for N5 (*Pseudomonas* sp.) and LT (*Bacillus* sp.) was found at 1% NaCl concentration (Fig.6).

Pseudomonas sp. showed optimum growth from 3 to 5 days and *Bacillus* sp. grew well up to 7 days of incubation.

Effect of UV light on bacterial growth

Effects of UV-B light on bacterial growth were also observed. Agar plates streaked by selected bacterial culture were exposed to UV-B light for 15 minutes. Improper growth was observed on agar plate against the control. Isolates (*Pseudomonas* sp. and *Bacillus* sp.) gave 50% growth on agar medium after 24hrs incubation.

Physiological assay of plant growth promoting characters of selected isolates

The Pb and Cd resistant isolates were screened for IAA production and phosphate solubilization ability under heavy metals (Cd and Pb) stress. The result showed that N5 strains were able to produce IAA with and without L-tryptophan while LT was unable to produce plants hormones. In addition to IAA production both isolates demonstrated the potential for phosphate solubilization; both isolates were able to solubilized inorganic phosphate in plate

assay. The phosphate activity was expressed in terms of cm diameter of zone produced around the colony and also expressed as PSE%. By plate assay method maximum production of phosphate solubilizing activity in terms of solubilization zones (cm diameter) was shown by both isolates (*Pseudomonas* sp. and *Bacillus* sp.). Present study showed positive ammonium production for both Isolates (*Pseudomonas* sp. and *Bacillus* sp.).The isolate *Pseudomonas* sp. was positive in HCN production on nutrient agar while *Bacillus* sp. was negative for the same. The production of low molecular weight, iron-chelating siderophores by heavy metals (Cd and Pb) resistant bacterial strains was detected on blue agar. Both the inoculated bacterial strains posed the ability to chelate Fe⁺² (iron) but, with different strength. Percentage (%) help in evaluation of siderophore production by given bacterial isolates. The highest orange halo zone was produced by *Pseudomonas* sp. (N5) bacteria followed by the *Bacillus* sp. (LT) (Table 5).

The bacterial isolates also differed in their HCN and nitrification test, highest nitrification was found by *Bacillus* sp. (LT) and HCN activity was exhibited by the isolate *Pseudomonas* sp. (N5). While in nitrate reduction activity of N5 found to be positive and LT was negative for the same.

Evaluation of the IAA production of selected isolates

The estimation of IAA level in bacterial culture grown under heavy metal (Cd and Pb) stress condition in the presence of L-tryptophan supplemented media showed utilization of L-tryptophan as a precursor for growth and IAA production in *Pseudomonas* sp. while bacteria

Bacillus sp. was negative in IAA production

shows the trend of increase in IAA production when the culture was supplemented with L-tryptophan, in L-tryptophan addition, the IAA production was higher than heavy metal stress condition (Table 6). The maximum IAA production under the influence of heavy metal stress could be observed in N5 either with or without L-tryptophan supplement.

The data in Table 5 indicated the variation for IAA production in Cd and Pb amended medium. The main effect of each treatment upon IAA production was estimated. It is appeared to be of positive effects of tryptophan Graph (Fig. 7) showed the, positive and negative influence of different conc. of tryptophan on IAA production.

Characterization of IAA producers with tryptophan

Cd and Pb tolerant selected isolates (*Pseudomonas* sp. and *Bacillus* sp.) were used in antibiotic sensitivity test. The experiment used 6 commercialized different antibiotic disks namely Tetracycline (10 mg), Penicillin (10 mg), Kanamycin (10 mg), Streptomycin (20mg), Gentamycin (50 mg), and Voriniczazole (10mg). The sensitivity and resistance profile to antibiotics was based on the diameter of the inhibition zone and the evaluation was done on the basis of National Committee for Clinical Laboratory Standard's (NCCLS) chart provided with the antibiotic kits by HiMedia. The antibiotic profiles of the isolates indicated the sensitivity of the strains to different antibiotics (Table 7). Isolates were resistant to Tetracycline. *Pseudomonas* sp. was quite sensitive to all antibiotics where as *Bacillus* sp. was resistance to 50% of the antibiotics tested.

Microorganisms have developed the mechanisms to cope with a variety of toxic

metals for their survival in the environment enriched with such metals. Heavy metals tolerant and have efficiency for metals removing from soil as well as capable for promoting plant growth (PGPR) can be useful to speed up the recolonization of the plant rhizosphere in polluted soils.

Present study was carried out to investigate some of the Physical and chemical characteristics of the soil collected from the Industrial and agricultural soil. Organic matter, pH, heavy metal (Cd & Pb) etc. analysis of soil was determined in which industrial site showed high pH and high heavy metals due to presence of metals salts (Onojake and Osuji 2012; Patnaik et al.2013). In agricultural soil pH was slightly low and was in acidic condition due to presence of organic matter high organic matter (Dawaki et al. 2013).

Study reflects the preliminary work done in isolation of heavy metal (Cd &Pb) resistant PGPR strain LT and N5 from cotton field and industrial waste dumping field. Whereas Pandey et al. (2011) isolated and characterized two Pb and arsenate tolerant *Bacillus* sp. from slag disposal site. Metal resistance mechanisms in microorganisms is well known, they adopted different mechanisms for metal tolerance as different types of transport channel (Nies, 1999), and compartmentalization within the cell (Ahmad, et al. 2008). Isolated Cd and Pb resistant bacteria having MIC of 150 mg/kg Pb, 100 mg/kg Cd and showed multiple plant growth promoting activities, similarly work done by (Islam et al. 2014, Sharma et al. 2012). Growth rates of the isolates in the presence of heavy metals (Cd and Pb) were consistently slower than that of the control (similar observation have been reported earlier, Fulekar et al. 2012; Pal et al. 2004).

Bacterial heavy metal removal property has

great application in environmental point of view. Banerjee et al. 2015 reported in his study that lead accumulation capacity of the bacterial strain *E. cloacae* B1 was very high compared to Cadmium and nickel

Similar result was found in the present study that both the strain observed high accumulation of Pb in comparison to Cd. Strains (N5 and LT) and were identified as *Pseudomonas putida* CG29 and *Bacillus safensis* KM39 (GenBank Accession was KF 782801.1 and JF 411315.1), 16S rDNA similarity values indicate that strains were 99% identical on the basis of typical phenotypic characters, Gene sequence (16S rDNA) analysis and phylogenetic analysis (Sharma and Fulekar, 2009).

Since salt (NaCl) concentration, UV (ultra-violet) rays and pH have roles to control the efficiency of metabolic activity and enzymatic function, which affects the survival of selected microorganisms (Samanta et al. 2012). The property to modify the pH, UV rays and NaCl are important characteristic, these strains are able to grow under acidic and slightly alkaline conditions and modified pH value close to neutrality (Sanchez-Gonzalez et al. 2011). These environmental factors indicate that strains have ability to survive in any harsh condition (Samanta et al. 2012). Plant rhizosphere is known to be preferred ecological niche for various types of soil microorganisms due to rich nutrient availability. It has been assumed that inoculation with *Pseudomonas* and *Bacillus* sp. enhanced the plant growth as a result having PGP activities such as siderophore production, phosphate solubilization, ammonia production and HCN production

(Ahmad et al. 2008; Lata et al. 2003; Majeed et al. 2015) as result has been found in present study. Negative response of IAA production by *Bacillus* sp. is may be due to gene products involved in IAA metabolism in gram-positive bacteria are completely unknown (Idris et al. 2007). Stimulation of IAA synthesis by *Pseudomonas* sp. with tryptophan was described previously for rhizospheric gram-negative bacteria (Ernsten et al. 1987; Koga et al. 1991). Patten and Glick (2002) used a mutant of the gram-negative plant-beneficial bacterium

Pseudomonas putida, deficient in the ipdc gene product (indole-3-pyruvate decarboxylase) to demonstrate that IAA synthesis in bacteria is dependent on tryptophan concentration.

Metal tolerance holds an association with antibiotic resistance (Verma et al. 2001). A similar kind of multiple antibiotic resistance property were observed in both isolates (*Pseudomonas* sp. and *Bacillus* sp.) which clearly indicates that the high degree of antibiotic resistance might be associated with higher levels of tolerance to various heavy metals (Dhakephalkar et al. 1994; Rosen 1996; Hassen et al. 1998). Under conditions of imposed stress, metal and antibiotic resistance in microorganisms possibly helps them to adopt spontaneously than by mutation and natural selection (Bhattacharjee et al. 1988; Silver et al. 1989). Under stress conditions, bacteria in rhizosphere may enhance the plant growth by different mechanisms such as by optimizing the supply of nutrients, stimulating plant growth by the synthesis of phytohormones IAA and solubilization of inorganic phosphorus (Yang et al. 2009).

Table.1 Characteristics of collected soil used for isolation of rhizobacteria

Serial No.	Soil Parameters	Soil Samples	
		Cotton Field	Industrial field
1	Moisture	52.31±0.32	60.12±0.41
2	pH	7.25±1.43	5.99±1.82
3	EC (mho/cm)	1.09±1.59	0.55±1.63
4	Orthophosphate mg/l)	1.13±2.41	0.92±1.71
4	A. phosphorus(mg/l)	6.01±0.83	3.345±2.72
5	Total phosphate(mg/l)	37.25±1.66	14.37±0.51
6	E. ammonium(mg/l)	3.47±2.96	6.29±1.49
7	Nitrate(mg/l)	2.32±2.81	1.20±0.77
8	Nitrite	11.10±0.61	7.53±0.54
9	Total Nitrogen	19.42±0.87	12.84±0.69
10	Organic carbon (%)	0.45±1.66	0.85±2.84
11	Organic matter (%)	2.68±2.53	1.92±1.61
12	Chloride (%)	0.75±0.75	0.53±0.11
13	Sulphate(mg/l)	9.18±0.89	10.76±0.58
14	CFU/g×10 ⁰	2.1±2.62	1.5±2.53
15	Heavy metals (mg kg⁻¹)		
I	Cd	1.82±1.43	0.59±1.72
ii	Pb	0.61±0.98	2.05±2.60

All the values are mean of five replicates ± SD. where ns = not significant at p < 0.05.

Significant at p < 0.05

Significant at p < 0.01

Table.2 Cellular and colonial morphology of selected isolates

Cell Morphology	N5 strain (<i>Pseudomonas putida</i> strain CG29)	LT(<i>Bacillus safensis</i> KM 39)
Gram behaviour	-ve	+ve
Spores	-	-
Cell motility	+ve	+ve
Shape	Short rod	Rod
Size		
Surface	Smooth	Smooth
Reflect light	Brilliant	Brilliant
Aspect	Wet	Wet
Consistence		
Margin	Entire	Undulate
Elevation	Convex	Raised
Pigmentation	Green	Yellow

Table.3 Biochemical characterizations of selected isolates

S.No.	Biochemical tests	Results	
		<i>Pseudomonas sp.</i>	<i>Bacillus sp.</i>
1	Starch hydrolysis test (Amylase production)	-ve	-ve
2	methyl red test	+ve	-ve
3	Vogous posker test	-ve	-ve
4	TIS(triple sugar iron test)	K/NC	A/NC
5	Catalase test	+ve	+ve
6	Citrate utilization test	+ve	-ve
7	Oxidase production test	+ve	+ve
8	Oxidation Fermentation test (Hugh Lefson Medium) (Motility Test)	+ve	+ve
9	Lipase production Test	-ve	+ve
10	Dehydrogenase producing test	+ve	+ve
11	Motility	+ve	+ve
12	Urease test	-ve	+ve

Note:

K/NC -No fermentation; Peptone used aerobically (Red/no color change)

A/NC-ferment sugar but didn't grows in the anaerobic area of the butt (Yellow/No colour Change).

Table.4 Removal efficiency (%) of heavy metals by selected strain (by AAS)

<i>Pseudomonas sp.</i>				<i>Bacillus sp.</i>		
Sample (in Days)	Pb (150 ppm) present in medium	Amount of Pb absorbed	Removal%	Pb (150 ppm) present in medium	Amount of Pb absorbed	Removal%
Control	82.69	0.0	0.0	82.69	0.0	0.0
1	62.29	29.03	46.60	60.88	32.28	53.02
2	58.71	27.92	47.55	59.22	40.01	67.56
3	52.21	31.32	59.98	56.83	43.33	76.25
4	51.86	40.33	77.76	54.25	48.39	89.19
5	48.97	35.68	72.86	48.80	35.05	71.82
6	48.08	30.33	63.08	43.30	29.31	67.69
15						
	Cd 100 ppm Present in medium	Amount of Cd absorbed		Cd (100 ppm) present in medium	Amount of Cd absorbed	
control	72.33	0.0	0.0	72.33	0.0	0.0
1	66.35	25.08	37.79	61.89	23.82	38.48
2	58.07	7.23	12.46	52.30	28.33	54.17
3	50.82	15.55	30.59	49.01	21.21	43.27
4	49.71	11.21	22.55	46.91	13.00	27.71
5	42.02	9.44	22.47	42.26	10.01	23.36
6	40.09	4.92	12.28	38.72	6.88	17.76

Table.5 Evaluation of plant growth promoting traits by selected isolates

PGP TRAITS		ISOLATES	
		<i>Pseudomonas</i> sp.	<i>Bacillus</i> sp.
Phosphate solubilization	Size of colony(cm)	0.51±0.1	0.57±0.12
	Zone of solubilization(cm)	1.5±0.21	0.85±0.12
	PSE %	150%	85%
Siderophore production	Colony diameter	0.41±0.11	0.55±0.21
	Orange halo diameter(cm)	0.43±0.21	0.25±0.14
	Siderophore production (%)	43%	25%
IAA production		++ve	-ve
HCN production		±ve	-ve
Ammonia production		+ve	+ve
Nitrification		±ve	++ve
Nitrate reduction		++ve	-ve

Value are ± SD (n=3)

Table.6 Quantitative analysis of IAA synthesis by isolates

IAA synthesis(mg/L)			
Conc. of L Tryptophan	Control (<i>Pseudomonas</i> sp.)	Cd + <i>Pseudomonas</i> sp.	Pb + <i>Pseudomonas</i> sp.
Tryptophan(1mg/ml)	10.21±0.62	9.88±0.45	9.99±0.32
Tryptophan(2mg/ml)	11.59±0.32	10.01±0.41	11.03±0.54
Tryptophan(5mg/ml)	14.06±0.74	11.27±1.74	13.17±0.78

Value are ± SD (n=3)

Table.7 Antibiotic sensitivity tests by selected isolates

S. No.	Antibiotics	<i>Pseudomonas</i> sp. (Zone size in mm)	<i>Bacillus</i> sp. (Zone size in mm)
1	Tetracycline (10mg)	(NZ)R	(NZ) R
2	Penicillin (10mg)	(NZ) R	R (5.8±1.03mm)
3	Kanamycin (10mg)	S (15±0.39 mm)	(NZ) R
4	Streptomycin (20 mg)	(NZ) R	R (10±0.62 mm)
5	Gentamycin (50 mg)	S (15 ±0.55mm)	S (8 mm)
6	Voriniczole (10mg)	S (12mm±1.38)	(NZ) R

Value are ± SD (n=3), NZ= no zone, S= Sensitive, R= Resistant
National committee on clinical Laboratory standard (1997)

Fig.1 16S rDNA amplicon band (M) DNA marker, A) LT and B) N5

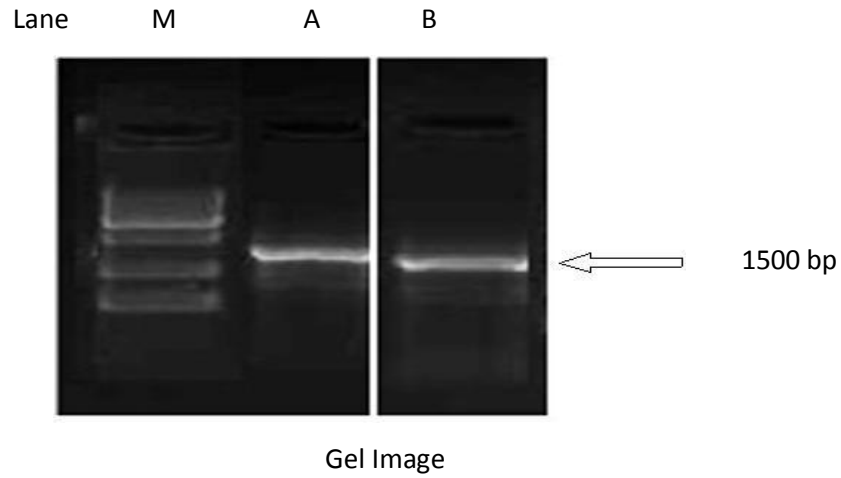


Fig.2 Evolutionary relationship of 11 Taxa of A) N5 (*Pseudomonas putida* strain CG29 and B) LT (*Bacillus safensis* KM39)

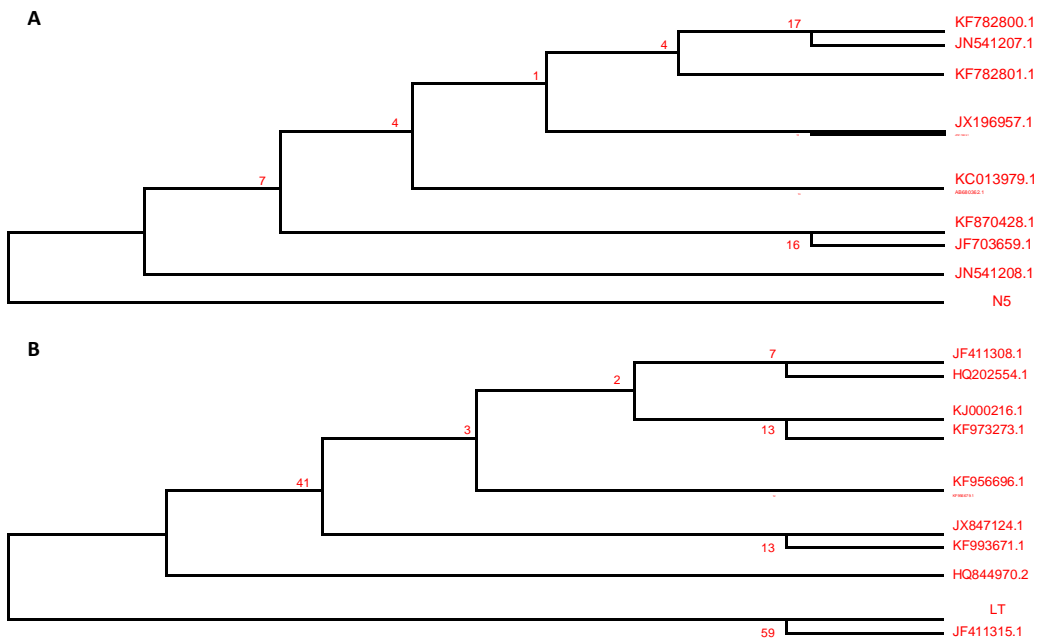


Fig.3 Growth rates of isolates (a) *Pseudomonas* sp. (b) *Bacillus* sp. in presence of Pb

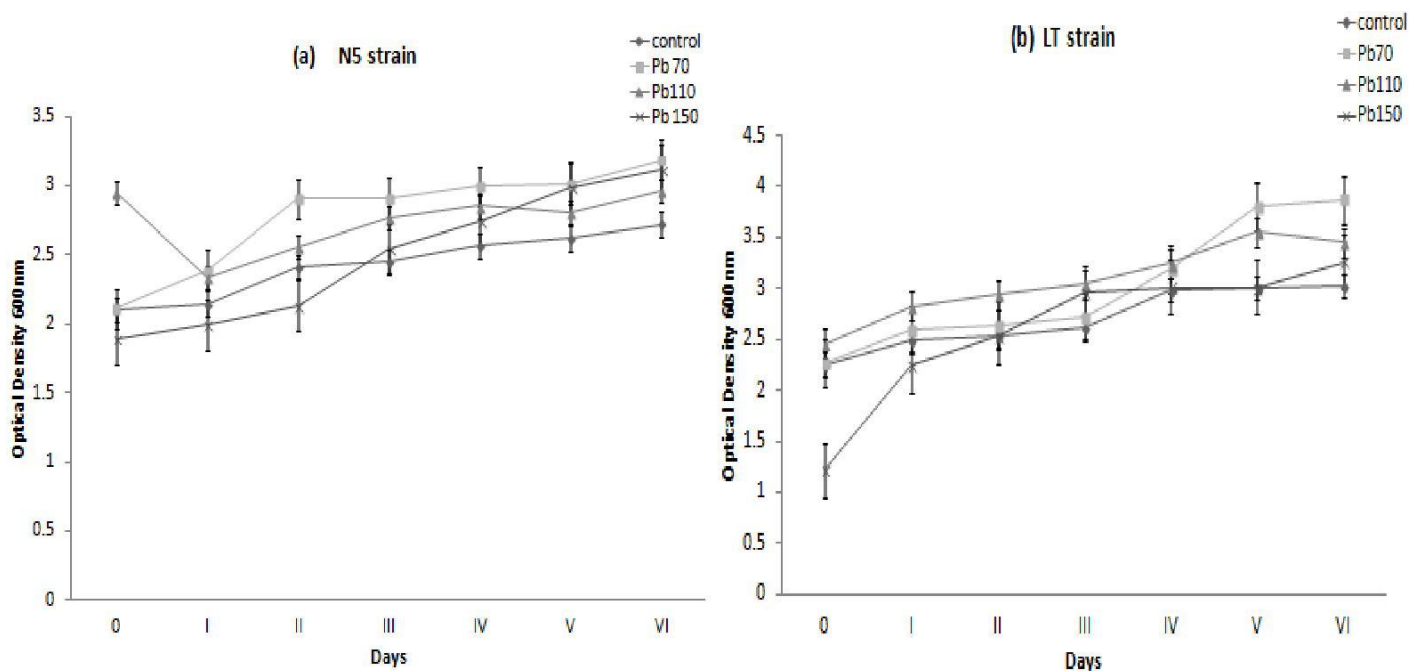


Fig.4 Growth rates of isolates (a) *Pseudomonas* sp. (b) *Bacillus* sp. in presence of Cd

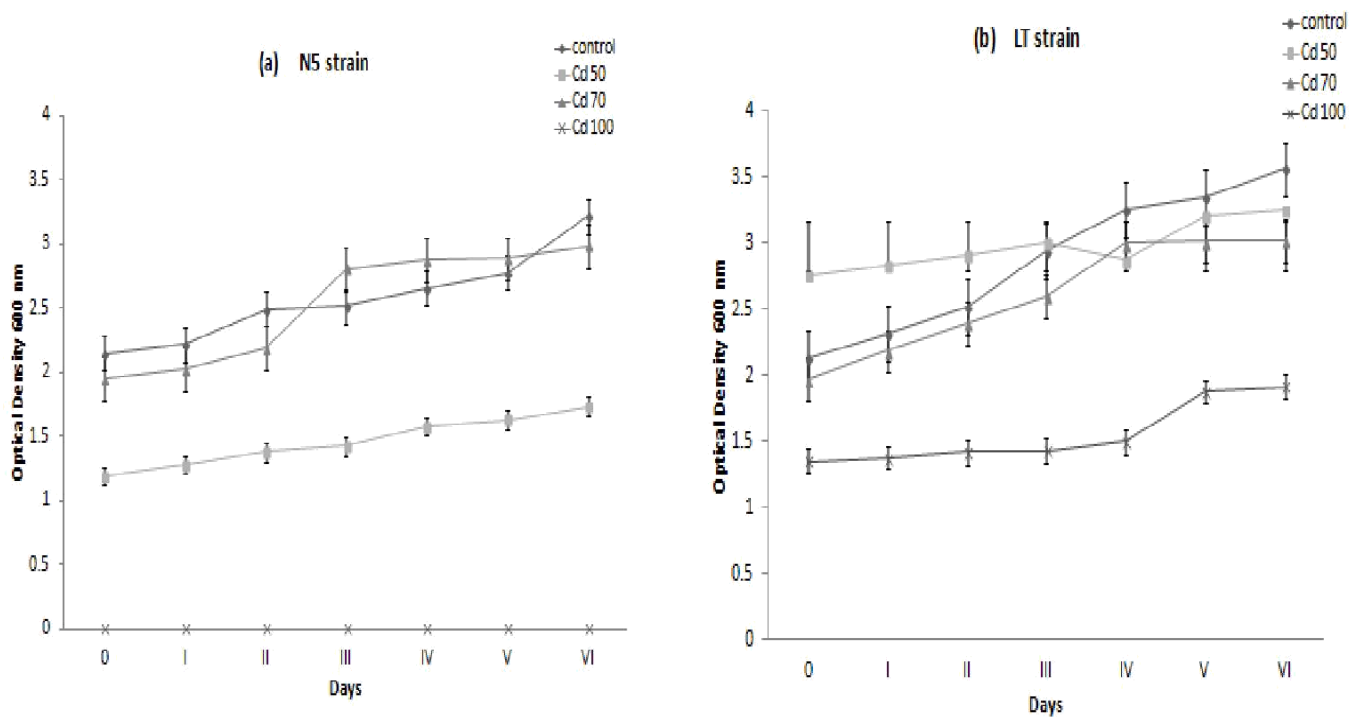


Fig.5 Effects of different pH range on growth of N5 (*Pseudomonas* sp.) and LT (*Bacillus* sp.) Values are means (n=3) ± SD. LT* and N5**, where each point of pH was significant difference with each other

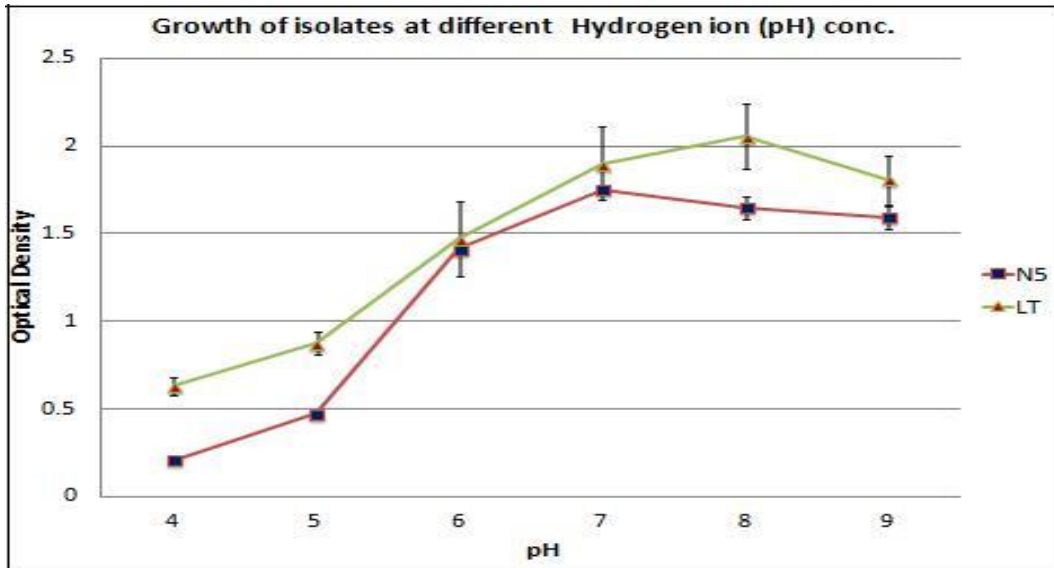
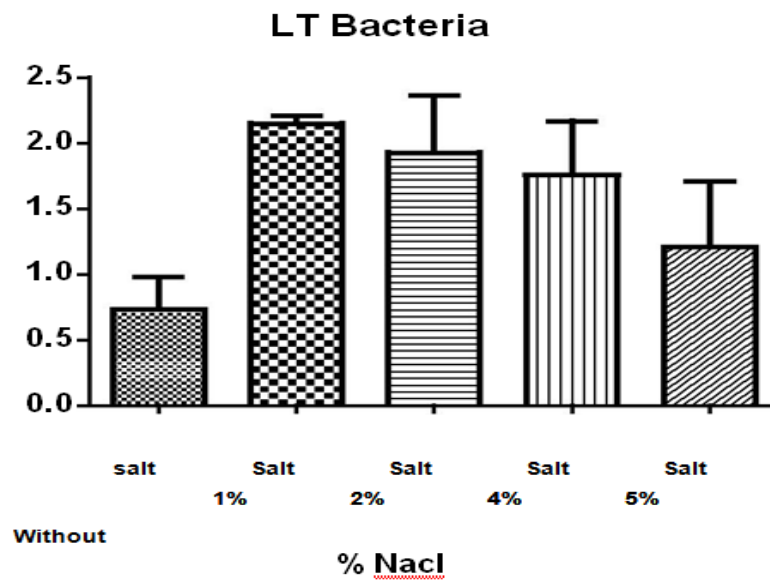
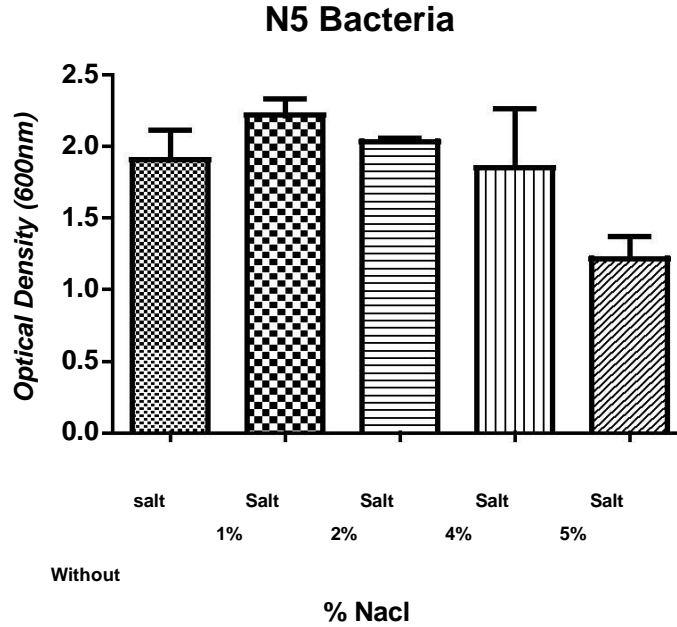


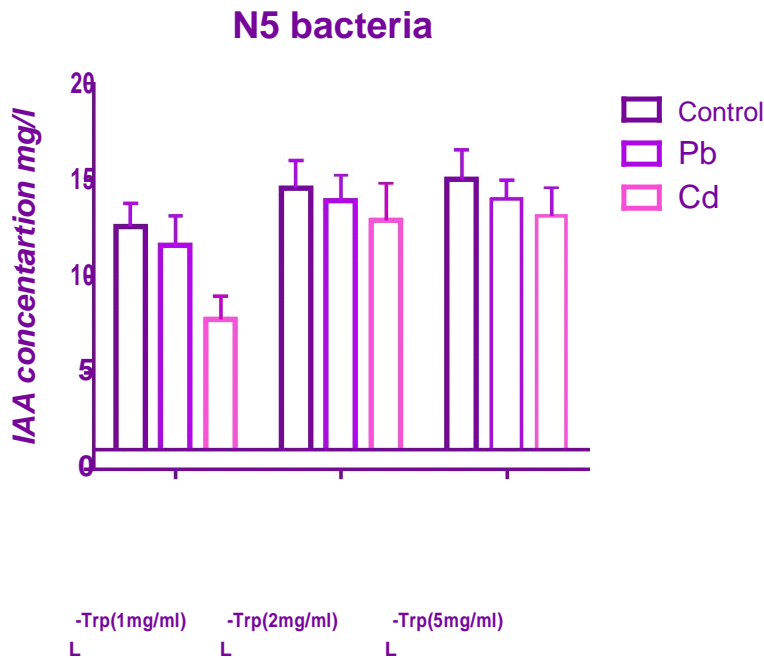
Fig.6 Effects salt concentration on N5 (*Pseudomonas* sp.) and LT (*Bacillus* sp.)





Values are means (n=5) ± SD, LT ***and N5***, where significant differences were found. *** Significant at p < 0.01

Fig.7 Quantitative analysis of IAA synthesis by *Pseudomonas* sp.



Significant different at p < 0.0001 at tukey's multiple comparison tests

Research reports suggested that diverse group of free-living soil bacteria can improve host plant growth and alleviate

toxic effects of heavy metals on the plants (Belimov et al. 2004; Wani et al. 2008). Selection of microorganisms both metal

tolerant and efficient in producing PGP compounds can be useful to speed up the recolonization of the plant rhizosphere in polluted soils. The potent heavy-metal tolerant bacterial species obtained in this study belongs to the genus

Pseudomonas sp., *Bacillus* sp. can potentially be used in the field of phytoremediation due to their PGPR activity like production of phytohormones and nitrogen sources, mineral solubilization simultaneously. Performing environmental parameters for bacterial growth is also showing that bacteria can easily survive in different environmental conditions.

Acknowledgement

Author expresses their sincere gratitude to Prof. BR Glick (Department of Biology, University of Waterloo, Canada) for their valuable support, help and valuable suggestion. Ms. Ratan Singh is grateful to RGNFD fellowship (RGNF-2012-13D-GEN-UTT-56466) provided by UGC.

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