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Plant Growth Promoting Traits Exhibited by Metal Tolerant Bacterial Isolates of Industrial Effluent

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A B S T R A C T

A wide range of microorganisms present in industrial effluent, exposed to different heavy metals, develop tolerance to such metal. Adaptation to the adverse environmental condition make them resistant to different antibiotics and various characters exhibited by them need to be explored. Twenty numbers of bacteria isolated from the effluent of a steel plant showed tolerance to heavy metals like Ni, Cr, Cd, Pb, and Hg and resistance to different antibiotics. Through morphological and biochemical characterization, the isolates were identified as species of *Staphylococcus*, *Psuedomonas*, *Burkholderia*, *Sphingomonas*, *Bacillus*, *Paenibacillus*, *Lysinibacillus* and *Enterococcus*. All the isolates were tried for plant growth promoting traits. Of the twenty isolates R-57 and R-58 showed positive for IAA production, siderophore production, PO_4 solubilization, HCN production and ammonia production.

Introduction

Population explosion coupled with rapid industrialization, urbanization and application of synthetic chemicals in agriculture have created a new order of by product causing environmental pollution by generating innumerable quantity of solid and liquid wastes. Such wastes are often loaded with heavy metal that has emerged as a major factor for reducing plant growth and agricultural productivity worldwide (Ma *et al.*, 2016).

Bacteria have the adaptive capacity to develop resistance/tolerance towards metal and antibiotic due to constant exposure in the environments (Sarma *et al.*, 2010). In these environments the activities like biosorption, bioprecipitation, extracellular sequestration, transport mechanisms, and/or chelation are developed by the microorganism and such resistance mechanisms are the basis for the use of microbes in bioremediation (Haferburg *et al.*, 2007).

Plant growth promoting rhizobacteria (PGPR) are group of microorganism which facilitate plant growth under stress condition including toxic metal different direct and indirect traits and also helps bioremediation of toxic metals (Nadeem *et al.*, 2014). Plant Growth Promoting Bacteria (PGPB) not only enhances plant growth but also increase productivity.

They can produce phytohormones (e.g. indole-3-acetic acid) that promote plant growth, facilitate nutrient availability (e.g. by solubilizing phosphorus and producing ammonia) can alter metal flow dynamics via siderophore release etc. PGPB can also produce 1-aminocyclopropane-1carboxylate (ACC) deaminase, which alleviates plant stress through the reduction of ethylene levels (Glick, 2012). A bacterial isolate with metal tolerant efficiency and plant growth promoting (PGP) properties can provide multiple advantages in crop productivity (Tripathy *et al.,* 2007).

Keeping in view of this an attempt has been taken in the present study to assess the ability of bacteria isolated from effluent of a steel plant on metal tolerance and plant growth promoting traits.

Materials and Methods

Collection of Sample

Effluent sample was collected in a sterile bottle from industrial effluent from a steel plant aseptically and transferred to the laboratory and stored under refrigerated condition for further work.

Enrichment, isolation and screening of bacterial isolates

Effluent enrichment method was performed by placing 10 ml effluent sample in a 250 ml Erlenmeyer flask containing 100 ml sterilized luria bertani media and incubated at 37^0C. After 48 hours of incubation, the turbid broth was spreaded on sterilized Luria bertani agar plate and incubated for 24 hrs.

The isolates were screened on LB agar plate amended with 50 ppm of heavy metals such as Ni, Cd, Cr, Pb and Hg separately and incubated for 48 hours. The growth of heavy metal resistant bacteria colony having different morphology were observed and Purity of the isolates were confirmed by quadrant streak plate method and preserved using 15 % glycerol stocks.

Biochemical characterization and sugar utilization test

Morphological Colony characteristics of the isolates were noted and grams staining were performed. Biochemical tests such as indole, methyl red, voges proskauer, citrate utilization test, oxidase and catalase test, H_2S production, motility test, and Urease production were carried out following standard method. Sugar utilisation was tested using Hicarbo Kit-A, Hicarbo Kit-B and Hicarbo Kit-C (Himedia).

Assessment of metal tolerance

Evaluation of all the bacterial isolate was determined by gradual increase of Cr, Ni, Cd, Pb and Hg metal concentration in LB media until the bacterial isolates failed to grow on plates over 7 days of incubation. The MIC of respective isolates was determined.

Determination of antibiotic sensitivity test

Antibiotic sensitivity pattern of the isolates were tested by Kirby-Bauer disc diffusion method. After incubation the resistance and sensitivity were determined according to the inhibition zone around the colony.

PGPB traits

The metal tolerant bacterial isolate were analyzed for different plant growth promoting traits.

Indole acetic acid producing ability

Bacteria were incubated for 24 h in LB broth and then they were centrifuged. An aliquot of

1 ml of supernatant was taken and transferred to a test tube that contained 2 ml of Salkowski reagent. The mixture was kept in room temperature for 30 min (Gordon and Weber, 1951) to observe change on colouration.

Solubilization of inorganic phosphate

For phosphate solubilization assay, fresh cultures of bacterial strains were inoculated into National Botanical Research Institute Phosphate Medium (NBRIP) (Nautiyal, 1999). Plates were incubated for 7 days and the formed with clear zone around them were considered as positive for the phosphate solubilization.

Ammonia production

Bacterial strains were checked for ammonia production according to Cappuccino and Sherman (1992). Fresh cultures were inoculated in to 10 ml peptone water. The production of ammonia was detected by adding 500µl of Nessler's reagent to each tube.

Siderophore production

Detection of Siderophore production was carried out by inoculating the bacterial isolates on Chrome Azurol S (CAS) plate assays (Schwyn and Neilands, 1987).

HCN production

The HCN production by the bacterial isolates was determined by adapting the method of Ahmad *et al.*, (2008). Fresh cultures were inoculated in LB medium supplemented with glycin (4.4g/lit) for 24 h at 30^0C.On the top of each plate, a sterilized filter paper (Whatman no.1) soaked in 2% sodium carbonate prepared in 0.5% picric acid solution was placed. Plates were incubated at 30^0C to study HCN production.

Results and Discussion

Enrichment, isolation and screening of Bacterial isolates

A total of sixty bacterial isolates (R-1 to R-60) were isolated from effluent sample. Amongst them twenty bacterial isolates were found to be tolerant to Ni, Cr, Cd, Pb, and Hg. Out of these twenty isolates, six bacterial isolates were gram negative and fourteen were gram positive. All the 20 isolates exhibited tolerance against all the five heavy metals up to a minimum concentration of 50 ppm (Table 1).

Biochemical characterization and sugar utilization test

On biochemical variability and sugar utilization, it was observed that among 20 isolates only R-14 was positive for indole production and rest isolates were negative. Ten isolates were positive for methyl red and ten showed negative result.

Eight isolates showed positive for Voges Proskauer reaction and twelve were negative for the test. Twelve of the twenty bacterial isolates utilized citrate. Fourteen bacteria were positive for oxidase, seventeen were positive for catalase and nine isolates could produce hydrogen sulphide.

Among twenty, fifteen isolates were found to motile and thirteen isolates had the ability to produce urease enzyme. Total 35 numbers of sugars were taken for test. The results are described in table 2. 3 and 4. According to above biochemical reaction the isolates were identified by ABIS online are in table 5.

Tolerance for heavy metal concentrations (in ppm)

All the 20 bacterial isolates isolated from industrial effluent were found to be tolerant to

Ni, Cr, Cd, Pb and Hg. Isolates R-3, R-4, R-10, R-22, R-26, R-35, R-43, R-44, showed the highest tolerance of 1350 ppm to the metal Ni (Fig. 1). Isolate R-37 was able to tolerate Cr metal up to the concentration of 1650 ppm (Fig. 2).

Most of the bacteria were tolerant to 120 ppm of Cd concentration (Fig. 3). For Pb all the organisms were found to be tolerant at the concentrations of 75 ppm. Most of the isolates were tolerant to 50 ppm concentration and both R-37 and R-56 were tolerant to 100 ppm of Hg (Fig. 4 and 5).

Antibiotic susceptibility test

Table 6 depicts that all the isolates resisted to all most all the commonly used antibiotics taken for this study. All the isolates are susceptible for penicillin B. All the bacteria were resistant to nalidixic acid, azithromycin, streptomycin, ciprofloxacin, kanamycin, and amoxycilin. A few organisms were susceptible for rifampicin, gentamycin, amikacin, cefonicid and tetracycline. Sarma *et al.*, 2010 opined that the resistance capacity may develop due to the metal contaminated environment in isolates.

Table.1 Grams staining and morphological characteristics of the isolates

Isolates	Gram's reaction	Morphological character
R-3	+ve cocci	Creamy, cerrated, dry round ring at the centre, sorounded by layers
R-4	+ ve cocci	Cerrated margin, ring at centre
R-10	+ ve rod	Small, round, Creamy, shiny, centre elevated and dry
R-14	- ve rod	Small, round, greenish brown, elevated shiny
R-22	+ ve cocci	Creamy, flat, dry, surrounded by wet margin.
R-26	+ ve rod	White, flat surrounded by dry margin
R-35	+ ve cocci	Small, creamy, dry, round, flat
R-37	- ve rod	Light green, spreaded colony
R-43	- ve rod	Flat, cerrated margin
R-44	+ ve cocci	Mucoid colony, folded, wrinkled
R-45	+ ve cocci	White, cerrated margin, ring at centre
R-51	+ ve rod	Small, white, round
R-52	+ ve cocci	Small, Yellow, round
R-53	+ ve rod	Mucoid, cerrated margin
R-54	+ ve cocci	Mucoid, elevated colony,
R-55	+ ve rod	Small, round, white, elevated colony
R-56	- ve rod	Small, round, watery, cerrated margin
R-57	- ve rod	Lemon green, oily, spreaded
R-58	- ve rod	Green, and mucoid colony
R-60	+ ve cocci	Round, offwhite

Table.2 Biochemical characterization of the isolates

Isolates	Indole Test	Methyl red	Voges Proskauer	Citrate utilization	Oxidase	Catalase	H_2S production	Motility	Urea hydrolysis
R-3	-	+	+	-	+	-	-	-	+
R-4	-	+	+	+	-	+	+	+	-
R-10	-	+	+	+	-	+	+	+	-
R-14	+	+	-	+	+	+	-	+	-
R-22	-	+	+	+	-	+	+	-	+
R-26	-	+	-	-	-	-	+	+	+
R-35	-	+	-	+	+	+	+	+	+
R-37	-	-	-	+	+	+	-	+	-
R-43	-	+	+	-	+	-	-	+	-
R-44	-	+	+	+	-	+	-	+	+
R-45	-	+	+	+	+	+	+	-	+
R-51	-	-	-	-	+	+	+	+	+
R-52	-	+	-	-	+	+	-	+	+
R-53	-	-	+	-	+	+	-	-	+
R-54	-	-	-	-	+	+	+	+	+
R-55	-	+	+	-	+	+	-	+	+
R-56	-	-	-	-	-	+	-	+	+
R-57	-	-	-	+	+	+	-	+	-
R-58	-	-	-	+	+	+	-	+	-
R-60	-	-	-	+	+	+	+	-	+

*Legend: + (positive) reaction, - (negative) reaction.

Table.3 Sugar fermentation of the isolates

Isolates	lac	Xyl	Mal	Fruc	Dext	Gal	Raf	Tre	Mel	Suc	L-ara	Man	Inu	N-agl	Glyc	Sal	Dulc	Ins
R-3	-	-	-	+	+	-	+	+	-	+	-	-	+	-	+	-	-	-
R-4	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+
R-10	+	-	-	+	+	-	-	-	+	+	-	-	-	-	+	+	-	-
R-14	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
R-22	-	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	-
R-26	-	+	+	+	+	+	-	-	+	-	+	+	+	-	+	-	-	-
R-35	-	-	+	+	+	-	-	-	-	+	-	-	-	+	+	+	-	+
R-37	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+
R-43	-	-	+	+	+	-	+	+	-	+	+	+	-	-	+	-	-	-
R-44	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	+	+	-
R-45	+	+	+	+	+	-	+	-	-	-	+	+	+	+	-	-	+	-
R-51	-	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	+	+
R-52	-	+	-	-	+	+	-	-	-	-	+	-	-	-	+	+	-	+
R-53	+	+	+	+	+	-	+	+	-	-	-	+	-	+	+	-	+	-
R-54	+	+	-	-	-	-	+	+	+	+	-	+	-	+	-	-	+	-
R-55	-	-	-	-	-	-	+	-	+	-	+	-	+	+	-	+	-	+
R-56	+	+	-	-	-	+	-	-	-	+	+	+	-	+	+	+	-	-
R-57	-	+	-	-	+	+	-	-	+	-	+	+	-	-	-	+	+	-
R-58	-	+	-	-	+	+	-	-	-	-	+	+	-	-	+	-	+	-
R-60	+	+	+	+	+	-	+	+	-	-	+	+	-	+	-	-	-	+

Legend: + (positive) reaction, - (negative) reaction.
Lac-Lactose, Xyl-Xylose, Mal-Maltose, Fruc-Fructose, Dext-Dextrose, Gal-Galactose, Raf-Raffinose, Tre-Trehalose, Mel-Mellibiose, Suc-Sucrose, L-ara-L-arabinose, Man-Mannose, Inu-Inulin, N-agl-Sodium- gluconate, Gly-Glycerol, Sal-Salicin, Dul-Dulcitol, Ins- Inositol.

Table.4 Sugar fermentation of the isolates

Isolates	Sorb	Mant	Ado	Arab	Erythr	α-methyl	Rha	Celli	melz	α-mano	Xylt	ONPG	Esc	D-ara	Citr	Maln	Sorb
R-3	-	+	-	-	-	+	-	+	-	-	-	-	+	-	-	-	+
R-4	+	+	-	-	+	-	-	+	-	-	+	-	+	-	+	-	-
R-10	-	+	-	+	+	-	-	+	+	-	+	+	+	+	+	-	-
R-14	+	+	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-
R-22	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-	-
R-26	+	+	+	-	-	-	+	-	-	-	-	-	+	+	-	-	-
R-35	+	-	-	+	-	-	+	-	-	-	+	-	+	-	+	+	-
R-37	+	+	-	-	+	-	+	-	-	-	-	-	+	-	+	-	-
R-43	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-
R-44	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-
R-45	-	+	-	+	-	-	-	+	-	-	+	-	+	-	+	-	-
R-51	+	+	+	-	-	-	+	+	-	-	+	-	+	-	+	-	-
R-52	+	-	+	-	-	+	+	-	-	-	-	-	+	-	+	-	-
R-53	+	+	+	-	+	+	-	+	-	-	+	-	+	-	+	-	-
R-54	+	-	+	+	-	+	-	+	-	-	-	-	+	-	+	-	-
R-55	-	+	-	+	-	+	+	-	-	-	+	-	+	-	-	+	-
R-56	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-
R-57	+	-	-	+	+	-	-	-	-	-	+	-	+	-	+	+	-
R-58	-	-	-	-	-	+	-	-	-	+	+	+	+	-	+	+	-
R-60	-	+	+	-	-	-	+	+	-	+	+	-	+	-	+	+	-

Legend: + (positive) reaction, - (negative) reaction.
Sorb- Sorbitol, Mant- Mannitol, Ado- Adonitol, Arab- Arabitol, Erythr- Erythritol, α-methyl- alpha-Methyl-D-glucoside, Rha-Rhamnose, Celli- Cellobiose, melz- Melezitose, α-mano- alpha-Methyl-D-Mannoside, Xylt-Xylitol, ONPG-ortho-Nitrophenyl-β-galactoside, Esc-Esculin, D-ara- D-Arabinose, Citr- Citrate, Maln- Malonate, Sorb- Sorbose.

Table.5 Biochemical identification of the isolates

Isolates	Identification
R-3	*Staphylococcus sp.*
R-4	*Staphylococcus sp.*
R-10	*Bacillus sp.*
R-14	*Burkholderia sp.*
R-22	*Staphylococcus sp.*
R-26	*Bacillus sp.*
R-35	*Staphylococcus sp.*
R-37	Unknown taxon
R-43	*Sphingomonas sp.*
R-44	*Staphylococcus sp.*
R-45	Unknown taxon
R-51	*Paenibacillus sp.*
R-52	*Staphylococcus sp.*
R-53	*Paenibacillus sp.*
R-54	Unknown taxon
R-55	*Lysinibacillus sp.*
R-56	Unknown taxon
R-57	*Pseudomonas sp.*
R-58	*Pseudomonas sp.*
R-60	*Enterococcus sp.*

Table.6 Antibiotic susceptibility of the metal tolerant bacterial isolates

Isolates	PEN	RIF	NA	AZM	STR	GEN	CIP	KAN	AMC	CID	TET	NV	AMX
R-3	+	-	-	-	-	-	-	-	-	-	-	-	-
R-4	+	-	-	-	-	-	-	-	-	-	+	-	-
R-10	+	-	-	-	-	-	-	-	-	-	-	-	-
R-14	+	-	-	-	-	+	-	-	-	+	+	+	-
R-22	+	-	-	-	-	-	-	-	+	-	+	-	-
R-26	+	-	-	-	-	-	-	-	+	-	-	-	-
R-35	+	-	-	-	-	-	-	-	-	+	-	-	-
R-37	+	+	-	-	-	-	-	-	-	-	+	-	-
R-43	+	-	-	-	-	-	-	-	-	-	-	-	-
R-44	+	-	-	-	-	+	-	-	-	-	-	-	-
R-45	+	-	-	-	-	-	-	-	-	-	+	-	-
R-51	+	-	-	-	-	-	-	-	-	-	-	-	-
R-52	+	-	-	-	-	-	-	-	-	-	-	-	-
R-53	+	-	-	-	-	-	-	-	-	-	-	-	-
R-54	+	-	-	-	-	+	-	-	-	-	-	-	-
R-55	+	-	-	-	-	+	-	-	-	-	-	-	-
R-56	+	-	-	-	-	-	-	-	-	-	-	-	-
R-57	+	-	-	-	-	-	-	-	-	-	-	-	-
R-58	+	-	-	-	+	-	-	-	-	-	+	-	-
R-60	+	-	+	-	+	-	-	-	-	+	-	-	-

Legend: + (Susceptible), - (Resistant).
PEN-penicillin G, RIF-Rifampicin, NA-Nalidixic acid, AZM-Azithromycin, STR-Streptomycin, GEN-Gentamycin, CIP-Ciprofloxacin, KAN-Kanamycin, AMC-Amikacin, CID-Cefonicid, TET-Tetracyclin, NV-Novobiocin, and AMX-Amoxycilin

Table.7 Plant growth promotion traits of the metal tolerant bacterial isolates

Isolates	IAA	Phosphate solubilization	Ammonia production	Siderophore production	Hydrogen Cyanide production
R-3	-Ve	-ve	+Ve	-Ve	-Ve
R-4	-Ve	-ve	+Ve	-Ve	-Ve
R-10	-Ve	-ve	+Ve	-Ve	-Ve
R-14	+Ve	-ve	+Ve	+Ve	-Ve
R-22	-Ve	-ve	-Ve	-Ve	-Ve
R-26	-Ve	-ve	-Ve	-Ve	-Ve
R-35	-Ve	-ve	+Ve	+Ve	-Ve
R-37	+Ve	-ve	+Ve	+Ve	-Ve
R-43	-Ve	-ve	+Ve	-Ve	-Ve
R-44	-Ve	-ve	+Ve	-Ve	-Ve
R-45	-Ve	-ve	+Ve	-Ve	-Ve
R-51	-Ve	-ve	+Ve	-Ve	-Ve
R-52	-Ve	-ve	-Ve	-Ve	-Ve
R-53	-Ve	-ve	-Ve	-Ve	-Ve
R-54	-Ve	-ve	+Ve	-Ve	-Ve
R-55	-Ve	-ve	-Ve	-Ve	-Ve
R-56	+Ve	-ve	+Ve	+Ve	-Ve
R-57	+Ve	+ve	+Ve	+Ve	+Ve
R-58	+Ve	+ve	+Ve	+Ve	+Ve
R-60	-Ve	-ve	+Ve	-Ve	-Ve

Fig.1 Growth of 20 isolates at different concentrations of Ni metal

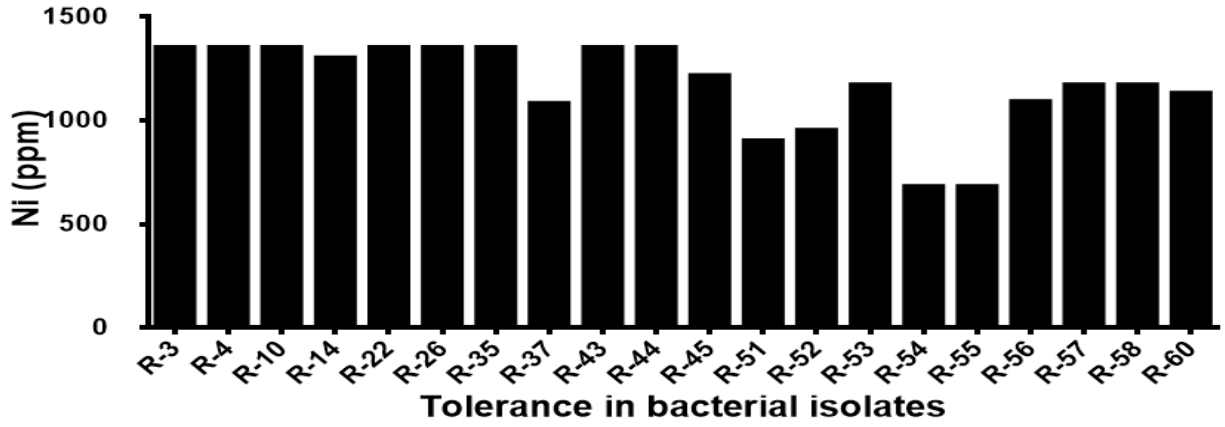


Fig.2 Growth of 20 isolates at different concentrations of Cr metal

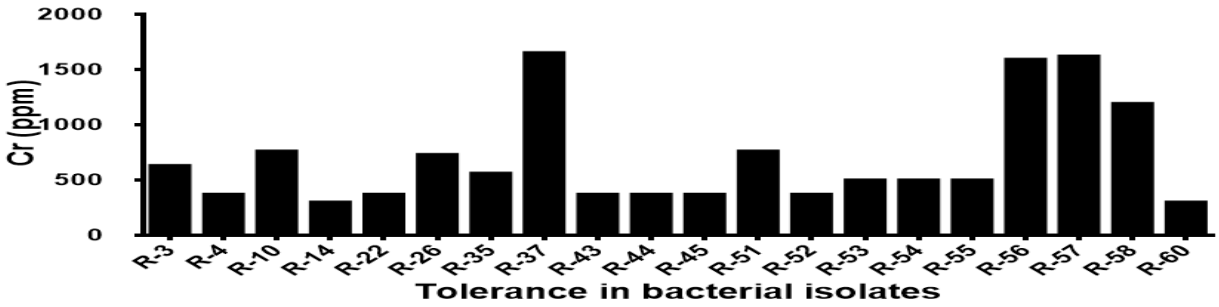


Fig.3 Growth of 20 isolates at different concentrations of Cd metal

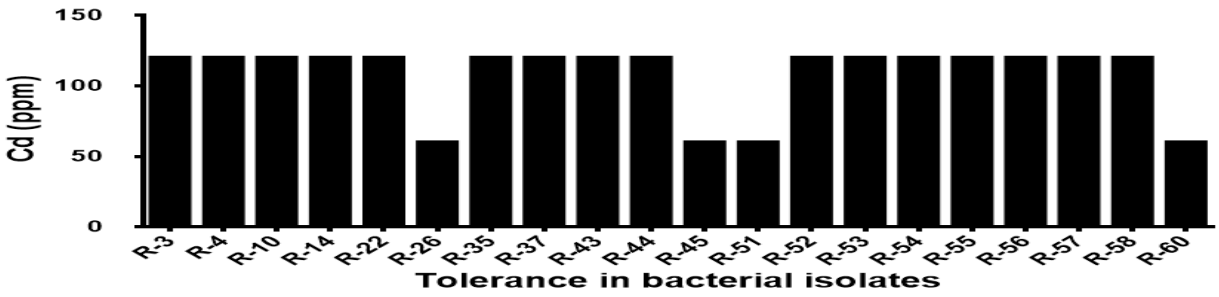
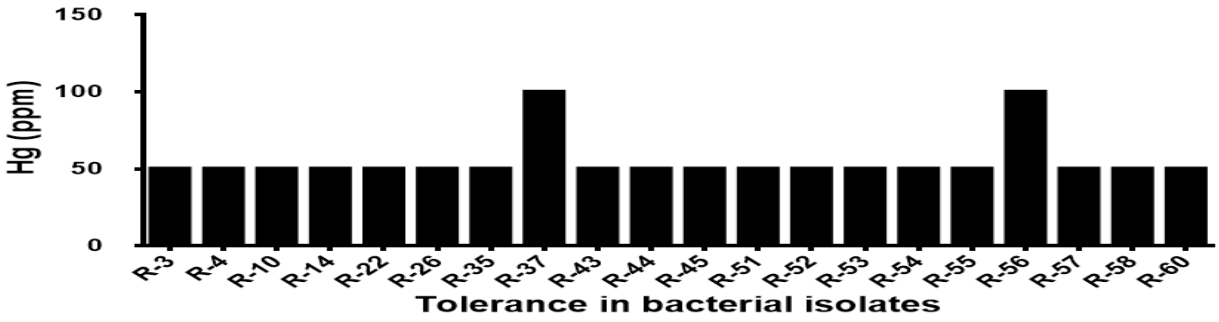


Fig.4 Growth of 20 isolates at different concentrations of Pb metal

Fig.5 Growth of 20 isolates at different concentrations of Hg metal



Plant growth promotion activities

Observation of pink colour in IAA tube indicates positive result. R-14, R-37 and R-56, R-57 and R-58 were found to be positive for IAA production. NBRIP Plates were incubated for 7 days and the formed with clear zone around the colony were considered as positive for the phosphate solubilization (Table 7). R-57 and R-58 showed positive result for PO_4 solubilisation. After adding Nessler's reagent in the ammonia tube, the development of yellow to brown colour was considered as positive result for ammonia production. Isolates R-22, R-26, R-52, R-53 and R-55 showed positive result for ammonia production. The presence of yellow to orange halo around the bacterial growth after incubation for 72h indicated a positive result for siderophore production. R-14, R-35, R-37, R-56, R-57 and R-58 were found to be positive for siderophore. Change in colour of filter paper to orange colour was considered as positive result for HCN production. Both the isolates R-57 and R-58 were found to be positive for HCN production.

In the present investigation it is observed that all 20 bacterial isolates isolated from the industrial effluent were biochemically identified as *Staphylococcus spp*., *Psuedomonas spp*., *Burkholderia sp*., *Sphingomonas sp*., *Bacillus spp*., *Paenibacillus sp*., *Lysinibacillus sp*. and *Enterococcus sp.,* and were found tolerant to 60-120ppm of Cd, 75 ppm Pb and 50-100 ppm Hg metals. These microorganisms have high degree of tolerance capacity against chromium ranging from 300-1650ppm and for nickel 680-1350ppm. Pandit *et al.,* (2013) isolated six number of Cu, Cd, Cr and Ni tolerant bacteria from industrial effluent sample. So in this study it is evident that most of the bacterial isolates were found to be tolerant to the metal salts such as Ni, Cr, Cd, Pb and Hg with high concentrations. Pramanik *et al.,* (2016) reported the chromium tolerant bacteria showing metal tolerant along with plant growth promotion (PGP) traits which might be helpful for plant growth promotion in chromium contaminated soil.

The results obtained in this study revealed that multiple heavy metal tolerant isolates are resistant to several antibiotics such as nalidixic acid, azithromycin, streptomycin, ciprofloxacin, kanamycin, and amoxycilin, rifampicin, gentamycin, amikacin, cefonicid and tetracycline. All the isolates are resistant to several antibiotics taken in this study except penicillin B. Kaur *et al.,* (2015) reported that bacteria isolated from industrial effluents were found to be resistant against ampicillin, ciprofloxacin, erythromycin, kanamycin, tetracycline, vancomycin, methicillin, gentamycin, and chloramphenicol. Due to cross resistance mechanism the bacteria have tolerance more than one antimicrobial agent such as antibiotics and heavy metals (Champan, 2003). These were seemed to be an efficient plant growth promoting bacteria that could produce IAA, siderophore, ammonia, HCN and solubilize phosphate. Reports available also indicate that the potential of soil microorganism to assist plant establishment on contaminated environment through mediating nutrient mineralization and uptake by plants, production of plant growth hormones and siderophores. The beneficial effects caused by the indigenous isolates may be used as a bioinoculant that facilitates the plant growth and thus increases phytoextraction efficiency for the remediation of heavy metal contaminated sites. *Psuedomonas sp*. Showed potentials in metal absorption as well as PGP and biocontrol activity (Sarma *et al.,* 2012).

The potential of the isolates can be considered as a probable candidate for the concerted

approach in the bioremediation as well as phytoremediation technology to convert metal contaminated sites to productive land and increase crop prodictivity.

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