Scientific Journal of Biology

Research Article

Application of Heavy Metal Tolerance Plant Growth Promoting Bacteria for Remediation of Metalliferous Soils and their Growth Efficiency on Maize (*Zea mays* L.) Plant -

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Submitted: 22 July 2021; **Approved:** 28 September 2021; **Published:** 01 October 2021

Cite this article: Chowdhury SK. Application of Heavy Metal Tolerance Plant Growth Promoting Bacteria for Remediation of Metalliferous Soils and their Growth Efficiency on Maize (*Zea mays* L.) Plant. Isolates. Sci J Biol. 2021 Oct 01;4(1): 039-050.
doi: 10.37871/sjb.id23

Abstract

Environmental rhizobacteria play an important role in bioremediation against various heavy metal pollutants in soil and wastewater. Phytoremediation is taken into account as a completely unique environmentally friendly technology that uses plants to get rid of or uptake heavy metals. The utilization of heavy metal tolerance Plant Growth-Promoting Rhizobacteria (PGPB) forms a key technology for increasing biomass preparation and defense of the plants to multiple heavy metals. The present study isolated 58 bacteria strains from the soil and roots of maize plant rhizosphere irrigated with normal and waste water. The bacteria were tested for heavy metals resistance, salt tolerance, and PGP attributes. Pot culture experiments were carried on under greenhouse environment and all data regarding growth attributes, physiological attributes, multiple heavy metal tolerant indexes, and accumulation of heavy metal in maize plant parts were recorded. Among the 58 isolated strains, 8 strains were preliminarily screened as multiple heavy metal resistance, salt tolerance, indole-3-acetic acid, phosphate solubilization, and siderophore production, and lastly, WW-40 strain was selected as the potent PGPR. Application of this strain in greenhouse conditions significantly increased highest 52% of seed germination, 1078 % of vigour index, 68.57% of shoot length, 71% root length, 44.44% of shoot fresh weight, 50% of root fresh weight, 52.38% of shoot biomass, and 66.66% of root biomass excess as compared to heavy metal treatment maize seedling. The photosynthetic pigments of maize seedlings in heavy metal contaminated pots were reduced as compared to the consortium (WW-40 + Heavy metal) pots. The chlorophyll contents increased 68.75% excess in consortium with Zn than Zn inoculated pot. Similarly, the carotenoid contents increased 57.89% excess in Zn consortium pot and xanthophylls contents increased 65.62% excess in Ni consortium pot as compared to other metal treatment pots. The uptake of heavy metal by root and shoot of maize plants increased by inoculation of WW-40 strain as compared to plants in multiple heavy metal contamination without inoculation. Thus, the heavy metal tolerance bacterial isolate distinguish in our research have promise applications for rectifying metal contagion soils which is the potential of PGPR strain for both bioremediation and promotion of growth of crop plants have importance in the control of environmental contamination.

Keywords: Phytoremediation; Heavy metals; PGPR; Metal accumulation; *Zeya mays*

INTRODUCTION

The growing urbanization and industrialization in the present world have resulted in serious environmental situations due to the discharge of pollutants especially heavy metals pollutants released regularly into the soil environment [1]. The contamination of normal water resources and agricultural soil is affected by multiple heavy metals due to the use of different fungicides, land chemical fertilizers, wastewater irrigation, and sewage sludge [2,3]. Thus, the protection of the environment from toxic effects is necessary for heavy metals through bioremediation [4]. The traditional physicochemical approach for metal rectification such as, electrochemical processes, filtration, acid leaching, or ion exchange is costly and may not be very effective. The development of sustainable and environmentally friendly technologies is used to extract and remove toxic heavy metals from water and soil [5]. The less cost-effective and environment-friendly method, bioremediation, clean-up the heavy metal through the use of microorganisms, plants, or other biological systems [6]. Among the bioremediation, phytoremediation is the most common method for soil remediation which utilizes plants to remove toxic metals from soils. The extraction of the standard quantity of pollutants from superficial soil surfaces and water needs to hyper-accumulator plants but it grows slowly in metals stress because of toxicity of heavy metals and attains very low biomass [7,8]. So the maintenance standard healthy state of heavy metal in soils can take years for reconstruction and depends on the concentration of heavy metals and types of soil of the particular area. At present, numerous plant species like an alpine weed (*Thlaspi caerulescens*), helianthus (*Helianthus annuus*), Indian mustard (*Brassica juncea*), and willow (*Salix* spp.), are being employed to soak up and accumulate serious metals from the soil [9]. The capability of phytoremediation can be enhanced by the application of PGPR in metal contamination soils [10] which are termed as rhizoremediation apply plant-microbe for interactions to develop the potentiality of phytoremediation [11]. Root colonization capacity of numerous beneficial plant growth-promoting rhizobacteria promotes the growth of plants by the production of growth regulators like auxin, cytokinin, and gibberellin [12]. Auxins enhance plant growth and metal uptake in metal stress conditions [13], and ethylene-induced seed germination or root growth by lowering the metal stress level. The most common PGPR strains *Bacillus* and *Pseudomonas* have been strongly utilized as metal uptake agents due to their high metal-binding capability [14]. Morphological peculiarities of bacteria give them metal-binding capability because of the charge of the cell wall properties, the ratio of high surface-volume, protein of S-layer, and proteins of metal-binding [15]. PGPR bacteria multiple metal resistances in the rhizosphere of plants considerably enhance metal adsorbing in plants and decrease metal toxicity [16,17]. Plant growth attributes viz. growth hormones, siderophores, solubilizing phosphate by PGPR increase the availability of soluble iron, phosphate, IAA for improving total metal uptake by plants through reduction of metal stress in the rhizospheric soil [18,19]. Metal bioavailability increased by metals solubilizes microbes and specific plants and which are not seen in a single PGPB [20,21].

The aims of this research are to i) screening of multiple heavy metals and salt tolerance rhizobacteria from contaminant maize field; ii) characterization, and pot experiment for determination of different plant growth parameters to increase phytoremediation capability in metal toxicity soils; and iii) evaluation of enhancement of Photosynthetic pigment contents, metal index, and accumulation surrounds the maize root surface.

MATERIALS AND METHODS

Soil sampling and chemical analyses

The soil samples were collected from the rhizosphere of maize cultivated irrigated with industrial and municipal wastewater and irrigated with normal water maize fields in the English Bazar Block of Malda District. The collected sample was placed in an autoclaved container and brought to the laboratory for analysis. The physicochemical parameters of soil samples like pH, conductivity, total organic carbon, organic matter, total nitrogen content, total phosphorous content, total potassium content were analyzed using the standard method [22]. The samples were dried and run through a 2 mm sieve before measuring pH, organic matter content, and the concentrations of available nitrogen (N), potassium (K), and phosphorus (P) [23]. Alkali N-proliferation method applied for soil available N detection whereas and available K and P measure with the ASI method. The K_2CrO_7 $2H_2SO_4$ oxidation method was used to assess the soil organic matter. The air-dried soil samples were run through a 2 mm nylon sieve to extract heavy metals and dissolve by 1:2:2 (V:V:V) HNO_3 : HCl : $HClO_4$. Vanadium (V), titanium (Ti), iron (Fe), zinc (Zn), nickel (Ni), lead (Pb), manganese (Mn), copper (Cu), chromium, arsenic (As), (Cr), and cadmium (Cd) were measured by inductively coupled.

Isolation and screening of multiple heavy metal tolerance rhizobacterial strains

To perform this experiment, 20 soil samples were collected from the maize rhizosphere of wastewater and normal water irrigated maize (*Zea mays* L., variety DHM-1) field, and samples were placed individually in plastic bags and brought to the laboratory. Soil plastic bags were open gently and 10 g of the soil sample were transferred into sterile flasks containing 90 ml of double sterile distilled water and kept on the rotary shaker at 150 rpm for 20 min. Then, serial dilution was made up from 10^{-1} to 10^{-9} using dilutions technique and 10 µl aliquot was inoculated in fresh sterile Nutrient Agar (NA) plate and incubated into 37° C biological incubator for 48 h. The strains were selected on the basis of their morphological features and tested for their resistance against five heavy metals viz. cadmium, lead, zinc, copper, and nickel. The heavy metal resistance assay was performed by spot-inoculating on nutrient agar medium amended with the respective metal salts. Minimum Inhibitory Concentration (MIC) values were calculated by adding $CdCl_2$, $Pb(NO_3)_2$, $CuSO_4$, $ZnCl_2$, and $NiCl_2$ in standard medium at 200, 400, 600, 800 and 1000 mg L^{-1} heavy metal concentrations. After incubation at 28° C for 5 days, the MIC value of the viable Colony-Forming Units (CFU) was observed after 48h of incubation at 30° C [24].

Salt tolerance test of isolates

Assessment of salt tolerance test of all strains, NaCl (1-20%) amended with NA medium and allow to solidification in a biological incubator. The overnight fresh culture of each isolate with a uniform population (5 x 10^8 cells ml^{-1}) was spotted on the fresh sterile NA agar plates and the petridishes were kept in the incubator at 37°C after sealing. Morphological characterization on the basis of colonies diameter and appearance of isolated strains were investigated after 3, 6, and 9 days incubation. Salt tolerance of each isolates to different percentages of salinity was recorded by founding the quality of the bacterial colonies grown in the control plates [25,26].

Plant growth promoting attributes of the isolates

Plant growth promoting parameters such as IAA production, solubilization of inorganic phosphate, and siderophore were assessed using different methods. IAA production was measured by Sheng, et al. [27] methods of each PGPR isolated strain. The potent bacterial strains were cultured in 0.5 mg ml^{-1} L-tryptophan containing SMS medium for 4 days at 37°C at 200 rpm. Then, 2 ml Salkowski's chromogenic reagent was gently mixed with 1 ml cell suspension [28] and place it dark for 30 min at 28°C and colour changes of the cell suspension were recorded. The ability of phosphate solubilization of isolated bacteria strains was determined by inoculating the bacterial culture in NBRIP medium at 28°C for 7 days and measurement the solubilization zone around each bacterial colony [29]. Production of Siderophore was determined using blue agar medium with Chrome Azurol S (CAS) and formation of halo zone around the colony was measures in terms of diameters [30].

In vivo Greenhouse experiment

Inoculum preparation and seed inoculation: The strains were cultured in a 250 ml conical flask individually containing 200 ml LB media and keep it at 37°C in the orbital shaking incubator (100 rpm) for 48 h. The fermenting broth was centrifuged at 6000 rpm for 6 min. The cell pellet was cleaned two times with sterile distilled water, and suspended in 10 ml sterile distilled water approximately 10^6 CFU/seed (O.D = 0.8), vortex, and used for seed treatment. Approximately 10-15 maize seeds were surface sterilized with 5% sodium hypochloride (NaoCl, Merk, India) for 1 min and washed thrice in sterile distilled water. Seeds were air-dried and soaked in bacterial suspension, and the preparation was stirred frequently for 5 min. Bacterized seeds were gently spread on a Petridish and air-dried overnight at room temperature. The total number of bacterial cells per seed was counted via serial dilutions and was set to approximately 10^6 CFU/seed (O.D = 0.8).

Experimental setup: The pots of earthen were filled with 8 kg soil with sandy clay loam texture having pH 7.64, organic matter 0.63%, EC 1.29 dS m1, saturation percentage 38.6%, extractable potassium 125.6 ppm, available phosphorous 7.5 ppm and while lead was not detectable in that soil. Before filling of the pot, this soil was polluted by lead using lead nitrate ($PbNO_3$) salt, cadmium using cadmium chloride ($CdCl_2$) salt, zinc using zinc chloride ($ZnCl_2$) salt, cupper using copper sulphate ($CuSO_4$) salt and nickel using nickel chloride ($NiCl_2$) salt at a concentration of 200, 400, 600, 800 and 1000 mg L^{-1}. Total 5 seeds were swan in each pot and kept at normal room temperature.

Analysis of plant growth attributes: Plant growth attributes such as seed germination and vigour index, shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight was recorded. Shoot and root dried in hot till the parts were lost their internal cell water. The percentage of germination was evaluated with the following formula [31]:

Germination rate (%) = (number of seeds germinated/total number of seeds) × 100
Vigour index = % of germination × total plant length.

Assessment of heavy metal tolerance index

Evaluation of heavy metal tolerance index was measured according to the method of Balint, et al. The treated and control maize seedling samples were taken and dried at 50°C in a hot air oven. The heavy metal tolerance index was calculated using the formula:

% Heavy metal tolerance index = Dry weight of treated plants / Dry weights of control Plants × 100

Determination of photosynthetic pigments

To an analysis of photosynthetic pigments, 100 mg leaf tissues were collected from the heavy metal treated and control plant separately and then cut into small pieces and mixed with 7 ml of DMSO (dimethyl sulphoxide) in test tubes at 65°C for 3 h. After crushing the samples, DMSO was poured onto make up the volume up to 10 ml, and the absorbance of the filtered extract was recorded by UV-vis Spectrophotometer (UV-Vis 1800, Shimadzu, Japan) at 645 and 663 nm marking blank as pure DMSO [32]. Extraction and estimation of Photosynthetic pigments from leaves of maize plants were ground with 80% acetone. Estimation of the chlorophylls and carotenoids has followed the method of Chowdhury, et al. [33]. Photosynthetic pigments viz. total chlorophyll, chlorophyll a, chlorophyll b, and carotenoids were expressed as mg/g of fresh leaf tissue. The absorbance for chlorophylls a & b and carotenoid was recorded at 663, 645, and 470 nm, respectively.

The content of xanthophylls estimation in maize leaves was done according to Lawrence [34] method. Samples were oven-dried, crush and take 50 mg powdered to keep in a 100 ml flask. Then add the 30 ml of a combined extract of hexane (10 ml): acetone (7 ml): absolute alcohol (6 ml): toluene (7 ml)) was done and the flask was shaken for 15-20 min. After the addition of 40% methanolic KOH (2 ml) to the flask, it was then kept in the water bath (58°C) for 20-25 min and the samples were placed under dark conditions for 1 h. For this purpose, 30 ml of hexane and 10% sodium sulfate were mixed to a volume of 100 ml and then vigorously shaking for a minute. The flask was again incubated under dark conditions. After that, the upper portion was transferred into a 50 ml volumetric flask, and the volume was makeup using hexane, and absorbance was recorded at 474 nm.

Determination of multiple heavy metal accumulation

The seedlings of maize were collected and their separated roots and shoots parts were allowed to dry in the oven at 65°C for 48 hrs. The oven-dried samples were crushed to make powder and dissolve according to the Allen, et al. [35] method. For this purpose, 200 mg of dust form sample was taken and digested in aquaregia (H_2SO_4: HNO_3: $HClO_4$, v/v) in ratio 1:3:1 with help of beakers using a hot induction plate. Then, dissolve samples were allowed to be cooled and filtered through 0.22 µm pore containing nylon syringe filters. The dilution of samples was done by using double distilled water and the final volume make up to 50 ml. After that, these digested samples were stored at room temperature and estimated the roots and shoots heavy metal accumulation in the plant through Atomic Absorption Spectrophotometer (Shimadzu 6200).

Statistical analysis

All the statistical analysis was performed using the statistical program SPSS v. 13.0 (SPSS, 2004).

RESULTS

Soil character analysis

The soil analysis result showed that different physicochemical properties of waste water irrigated soil were significantly higher than normal water irrigated soil which was shown in table 1. Similarly, the heavy metal content analysis of waste water irrigated soil showed higher mg L^{-1} in the context of normal water irrigated soil as shown in table 1.

Screening of Heavy metal tolerance bacterial strains

Assessment of heavy metal tolerance of bacteria strains from maize rhizosphere as shown in table 2. A total of 48 bacteria isolated from maize rhizosphere irrigated with industrial and municipal wastewater showed were resistance to multiple heavy metals and 10 strains isolated from maize plants cultivated with normal water were mainly susceptible to heavy metals. The bacterial strains WW-09, WW-16, WW-22, WW-25, WW-40, WW-48, WW-51 and WW-55 were potent against multiple heavy metals.

Table 1: Different physico-chemical properties of normal water and polluted water irrigated maize field soil.

Physicochemical Properties		
Physicochemical Properties	**waste water irrigated Soil**	**Normal water irrigated Soil**
pH	6.6	5.1
Electrical Conductivity (m)	0.2	0.2
Organic Carbon (%)	2.64	0.84
Available N (kg/ha)	200.7	553.93
Available P(kg/ha)	116.16	3.24
Available K(kg/ha)	247.5	4.95
Heavy metals content (mg L^{-1})		
Heavy metals (mg L^{-1})	**Waste water irrigated Soil**	**Normal water irrigated Soil**
Lead (Pb)	14.99	3.36
Zinc (Zn)	11.47	5.08
Cadmium (Cd)	13.60	4.74
Cupper (Cu)	11.61	3.66
Nickel (Ni)	10.38	5.38
Calcium (ca)	10.75	2.0

Salt tolerant test

The result of the salt tolerance test of potent 8 bacterial strains is shown in figure 1. The result in this assessment showed that a significant percentage of rhizospheric isolates were resistant to high salinity percentages (up to 20 %). WW-40 isolated strains showed higher salt tolerance than WW-09, WW-16, WW-22, WW-25, WW-40, WW-48, WW-51 and WW-55. In addition, due to high salts concentration, the rhizosphere isolates were not able to grow in a 21% NaCl medium.

Maximum tolerable concentration assay of the potent PGPR strains

The maximum tolerable concentration of multiple heavy metal tolerance 8 bacterial strains are shown in table 3. According to the results, strains WW-09, WW-16, WW-22, WW-25, WW-40, WW-48, WW-51, and WW-55 showed good heavy metal tolerance potentiality against Pb, Cd, Zn, Cu, and Ni in MIC assay. All selected bacteria were observed to tolerate the tested heavy metals with different capabilities ranging from 1000 to 1600 mg L^{-1} for Pb, 600 to 1000 mg L^{-1} for Cd, 400 to 600 mg L^{-1} for Zn, 200 to 500 mg L^{-1} for Cu, and 300 to 1000 mg L^{-1} for Ni. Among the 8 strains, WW-40 showed the highest multiple heavy metal tolerable capabilities than other isolated bacteria.

Plant growth promoting activity

The results showed that the potent bacterial strains growing in medium amended with tryptophan were able to produce IAA as shown in table 4. Strain WW- 40 produced the highest (7.68 g/ml) whereas WW-16 produced the lowest (1.47 g/ml) IAA among the eight isolates tested. In addition to IAA production, all 8 strains exhibited the potential capability for phosphate solubilization (Table 4). The highest phosphate solubilization was observed by the isolated strain WW- 40 (129.33 g/ml). The lowest quantity of phosphate solubilization was observed by the isolate WW-22 (60.78 g/ml).

Table 2: Heavy Metal tolerant assay of the isolated strains from normal water and polluted water irrigated maize field.

Strains	Pb	Cd	Zn	Cu	NI	Co		Strains	Pb	Cd	Zn	Cu	NI	Co
NW- 30	–	–	–	–	–	–		WW- 1	+	++	+	++	+	+
NW- 31	–	–	–	–	–	–		WW- 2	+	+	+	+	+	+
NW- 32	–	–	–	–	–	–		WW- 3	+	++	+	+	+	+
NW- 33	–	–	–	–	–	–		WW- 4	+	+	+	+	+	+
NW-34	–	–	–	–	–	–		WW- 5	+	++	++	+	+	+
NW- 35	–	–	–	–	–	–		WW- 6	+	++	+	+	+	+
NW- 36	–	–	–	–	–	–		WW- 7	+	+	+	+	+	+
NW-37	–	–	–	–	–	–		WW- 8	+	+	+	+	+	+
NW-38	–	–	–	–	–	–		WW - 9	+++	+++	++	++	++	++
NW-39	–	–	–	–	–	–		WW- 10	+	+	+	+	+	+
WW- 40	+++	+++	++	++	++	++		WW- 11	+	+	+	+	+	+
WW- 41	+	+	+	+	+	+		WW- 12	+	+++	+	+	+	+
WW- 42	+	+	+	+	+	+		WW- 13	+	+	+	+	+	+
WW- 43	+	+	+	+	+	+		WW- 14	+	+	+	+	+	+
WW- 44	+	+	+	+	+	+		WW- 15	+	+	+	+	+	+
WW- 45	+	+	+	+	+	+		WW- 16	++	+++	+	++	+	++
WW- 46	+	+	+	+	+	+		WW- 17	+	+	+	+	+	+
WW- 47	+	+	+	+	+	+		WW- 18	+	+	+	+	+	+
WW- 48	++	+++	+	++	++	++		WW- 19	+	+++	+	+	+	+
WW- 49	+	+	+	+	+	+		WW- 20	+	+	+	+	+	+
WW- 50	+	+	+	+	+	+		WW- 21	+	+	+	+	+	+
WW- 51	++	+++	++	+	++	++		WW- 22	+++	+++	++	+++	++	++
WW-52	+	+	+	+	+	+		WW- 23	+	+	+	+	+	+
WW- 53	+	+	+	+	+	+		WW- 24	+	+	+	+	+	+
WW-54	+	+	+	+	+	+		WW- 25	++	+++	++	+	+	++
WW-55	+++	+++	++	++	++	++		WW- 26	+	+	+	+	+	+
WW- 56	+	+	+	+	+	+		WW- 27	+	+	+	+	+	+
WW-57	+	+	+	+	+	+		WW- 28	+	+	+	+	+	+
WW- 58	+	+	+	+	+	+		WW- 29	+	+++	+	+	+	+

Here +++ indicated highly heavy metal tolerant isolates, ++ indicated moderate heavy metal tolerant isolates and + indicated low heavy metal tolerant isolates. NW: Normal Water irrigated soil; WW: Waste Water irrigated soil.

Table 3: Tolerance to heavy metals MIC (mg L^{-1}).

Strains	Pb	Cd	Zn	Cu	Ni
WW-09	1000	800	500	200	400
WW-16	1200	600	400	300	500
WW-22	1200	1000	600	300	500
WW-25	1400	1000	400	400	500
WW-40	1600	1200	600	500	1000
WW-48	1400	1000	500	400	500
WW-51	1200	800	400	300	400
WW-55	1000	600	400	200	300

Table-4: IAA production and phosphate solubilization by bacterial isolates without metal stress. All the values are mean of three replicates ± standard deviation (SD).

Strains	IAA	PS
WW-09	5.40 ± 0.23	72.98 ± 0.53
WW -16	1.47 ± 0.05	114.48 ± 1.86
WW - 22	5.43 ± 0.11	60.78 ± 0.20
WW -25	1.86 ± 0.04	102.42 ± 0.80
WW - 40	7.68 ± 0.45	129.33 ± 1.01
WW -48	2.52 ± 0.22	81.23 ± 0.92
WW - 51	1.85 ± 0.09	126.42 ± 0.62
WW -55	6.33 ± 0.25	106.58 ± 0.87

Furthermore, the application of WW-40 PGPR strain in the presence of heavy metal for phosphate solubilization and IAA production was also investigated (Figure 2). Results from this study focus that the existence of 0.129 g/ml zinc, 0.190 g/ml copper, and 0.176 g/ml Ni did not decrease the IAA production (Figure 2A). Although, IAA production was decreased by Pb and Cd. Phosphate solubilization potentiality was inhibited by lead, cadmium, zinc, and copper (Figure 2B) and not suppressed by nickel.

The production of siderophores by the 8 multiple-metal tolerance bacterial strains was observed by the absorbance at 400 nm, as described in materials and methods. The production of siderophore was observed that WW 40 and WW 48 showing the maximum and minimum siderophore levels released in the supernatant (Figure 3A). In addition, various absorbances at 400 nm were shown between bacterial cultures grown in the presence versus the absence of iron. Furthermore, the effect of heavy metals on siderophore production by the bacterial isolates was showed in figure 3B. Results showed that except Nickel, all the heavy metals inhibited the production of siderophore in comparison to control.

In vivo Plant growth parameters

The percentage of seed germination under various treatment conditions of Maize seedling is shown in figure 4A which is focused on the potentiality of WW-40 strain to induced maize seed germination in the presence of Pb, Cd, Cu, Zn, and Ni. Results showed that the highest percentage of seed germination occurs in only WW 40 strain in comparison to control. The application of WW-40 strain with Pb, Cd, Cu, Zn, and Ni heavy metals, seed germination increases 2 fold approximately in comparison to Pb, Cd, Cu, Zn, and Ni inoculated pot. Similarly, vigour index of Maize seedling was observed highest in case of WW-40 strain inoculated pot than control as shown in figure 4B. Application of consortium (WW 40 strain + heavy metals) showed that the vigour index increased approximately 5 fold of maize seedling compared with Pb, Cd, Cu, Zn, and Ni inoculated pots.

The effect of strain WW 40 up to 30 days maize seedlings under Pb, Cd, Cu, and Ni stress was assessed in terms of shoot length, root length, shoot fresh weight, root fresh weight, shoot biomass, and root biomass as shown in figure s4A-C. Results on root and shoot growth of maize seedling showed that the application of strain WW-40 increases with multiple heavy metal 2 fold length increased in Pb inoculated pot followed by 3 fold increased in Cd, 2.5 fold increased in Cu, 2.7 fold increased in Zn and 2.8 fold in Ni inoculated pots with comparison to Pb, Cd, Cu, Zn, and Ni inoculated pots as shown figure 5A. Similarly, shoot fresh weight increased excess 15 cm in Pb inoculated pot followed by 10 cm, 15 cm, 20 cm and 21 cm in Cd, Cu, Zn and Ni inoculated pots. The root fresh weight observed highest excess growth 9 cm in Ni and Zn inoculates maize seedling whereas lowest growth 3 m by Cd and Cu inoculated pots as shown in figure 5B. The shoot biomass excess increase by the application of strain WW 40 in Pb pot is 2.2 cm followed by 2 cm, 2.3 cm, 3 cm and

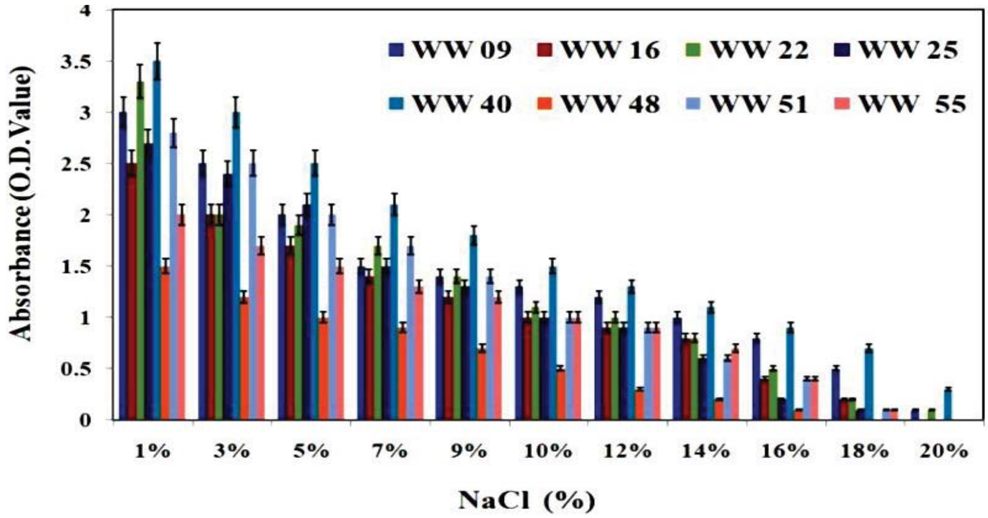

Figure 1: Salt tolerance (%) assay of maize (*Zea mays* L.) rhizospheric isolates irrigated with industrial and municipal waste water.

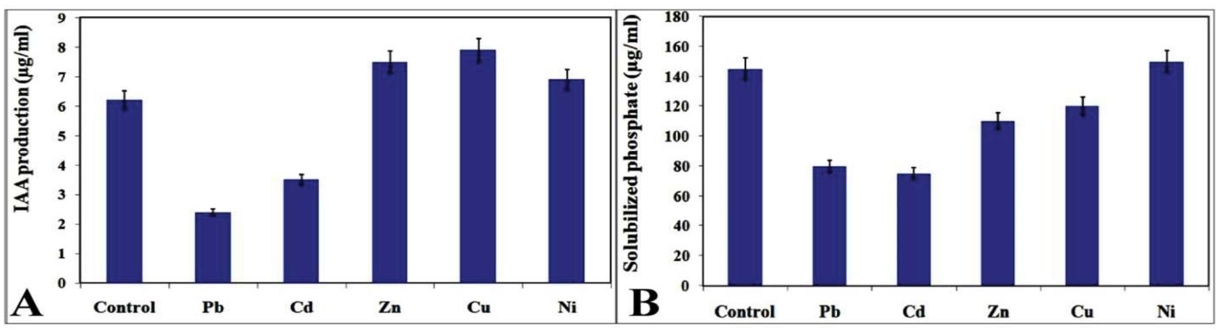

Figure 2: A) IAA production by WW-40 and B) phosphate solubilization by WW-40 in the presence of heavy metals. Control: absence of heavy metals.

2.7 cm in Cd, Cu, Zn and Ni pots whereas root biomass significantly increased 0.9 cm in Pb pot followed by 0.7 cm, 0.5 cm, 0.6 cm and 1.2 cm as shown in figure 5C.

Photosynthetic pigment

The photosynthetic pigments were determined by analyzing total Chlorophyll, Carotenoid, and Xanthophyll contents as shown in figures 6A-C. Total chlorophyll content was observed lower 0.2 mg/g FW in Pb stress followed by 0.25 mg/g FW, 0.3 mg/g FW, 0.36 mg/g FW, and 0.4 mg/g FW in the presence of Cd, Cu, and Ni as compared to control seedlings as shown in Figure-6a. Application of strain WW 40 with Pb inoculated seedling observed that the chlorophyll content is significantly increased 1.5 -fold higher than Pb inoculated maize seedling. Similarly, in case of Cd, Zn, Cu and Ni inoculated pots, chlorophyll content increased 3- fold, 2.5 -fold, 1.5 -fold, and 2 -fold respectively for application of WW 40 strain. Total carotenoid content is observed lower 0.4 mg/g FW in Pb stress followed by 0.35 mg/g FW, 0.35 mg/g FW, 0.4 mg/g FW, and 35 mg/g FW in the presence of Cd, Cu, Zn, and Ni as compared to control seedlings as shown in figure 5B. Application of strain WW 40 in all heavy metal pot with maize seedling, carotenoid content is increased 1.75 -fold in Pb followed by 2.25- fold, 2.5 -fold, 2.25 -fold and 2- fold in Cd, Cu, Zn, and Ni inoculated pots. Total xanthophylls content was observed lower 10 mg/g FW in Pb stress followed by 9 mg/g FW, 12 mg/g FW, 11 mg/g FW, and 13 mg/g FW in the presence of Cd, Cu, Zn, and Ni as compared to control seedlings as shown in figure 5C. The content of xanthophylls is increased 2.2 -fold in Pb- inoculated pot, 3 -fold in Cd, 2- fold in Cu, 2.3 -fold in Zn by the application of PGPR strain WW 40 in all heavy metal inoculated pots.

Heavy metal tolerance index and accumulation.

The tolerance index of maize plant against Pb, Cd, Cu, Zn, and Ni heavy metal was observed 100% in control seedling and 57.77% in Pb stress followed by 64.44 %, 60 %, 55.55 and 48.88 % in the presence of Cd, Cu, Zn, and Ni as compared to control seedlings as shown in figure 7. The tolerance index of WW-40 strain amended with Pb, Cd, Cu, Zn, and Ni stressed seedling is 137.77%, 142.22%, 111.11%, 133.33% and 135.55% respectively.

Heavy metal accumulation in the shoot of maize plant observed the highest reduction in Cd inoculated pot and lowest reduction in Cu inoculated pot by the application of PGPR strain WW-40 as compared to only heavy metal inoculated pot as shown in figure 8A. Similarly, 50% Cd accumulation reduces by the PGPR strain WW 40 which is the highest and lowest reduction observed in the case of Pb as shown in figure 8B.

DISCUSSION

In recent years, PGPR is widely utilized for phytoremediation of metalliferous soils of the agricultural field which help the growth promotion of crops under stress conditions and reduce the heavy metals toxicity of crop plants in different ecosystems [36,37]. Production of crop losses increases the Pb, Cd, Zn, Cu, and Ni content in the soil that affects the growth of the crop plants in the agricultural field [38]. According to Kotoky, et al. [39] reports, it has been well established that no plant can grow well without the presence of microorganisms. The present study focuses on the rhizospheric bacteria colonization on maize plant root surface to help the growth and tolerance of these plants from environmental stresses. There is no toxicity effect of heavy metals on maize plants observed and so plants

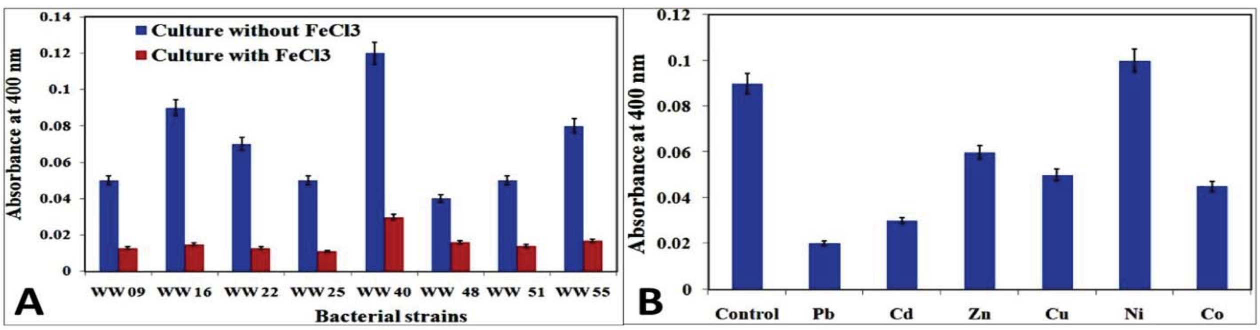

Figure 3: A) Production of siderophore by the bacterial strains fermenting with and without FeCl3, respectively.
B) Production of siderophore by WW-40 amended of heavy metals without FeCl3. Control: absence of heavy metals.

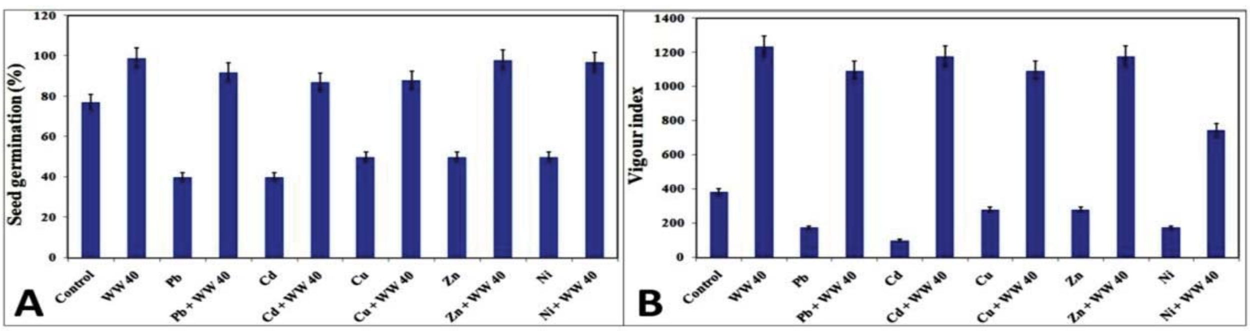

Figure 4: *In vivo* growth attributes of maize seedling. A) Seed germination (%) B) Vigour index.

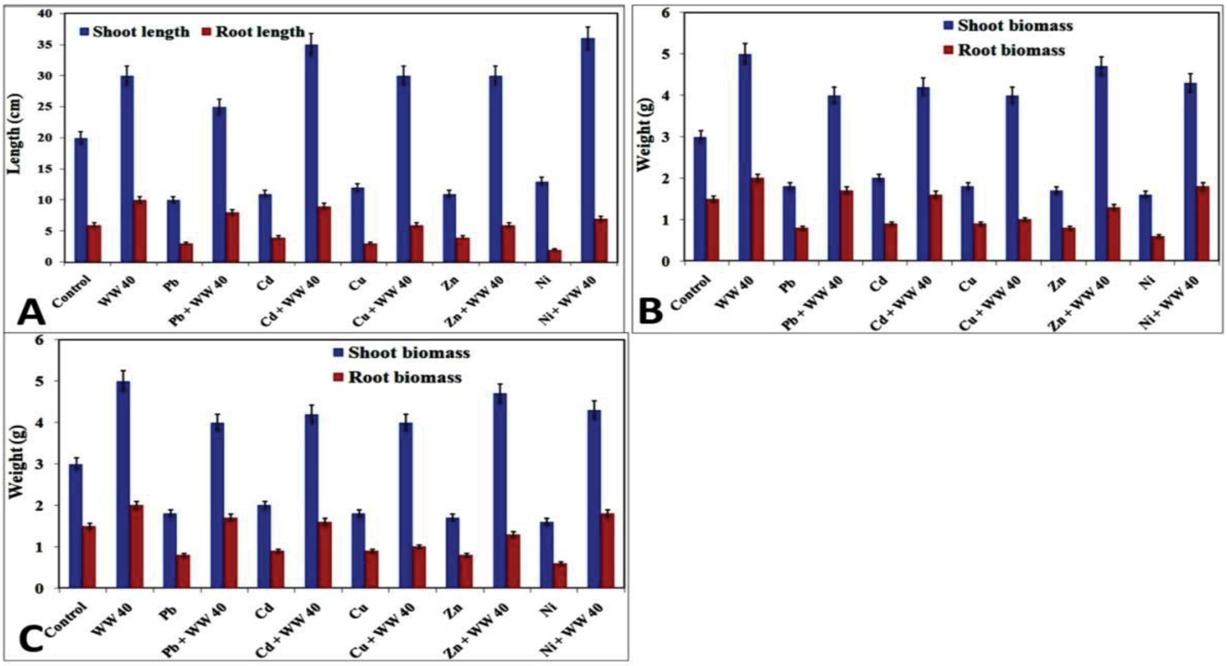

Figure 5: *In vivo* growth attributes of maize seedling. A) Root length and shoot length B) Shoot fresh weight and root fresh weight C) Shoot biomass and root biomass.

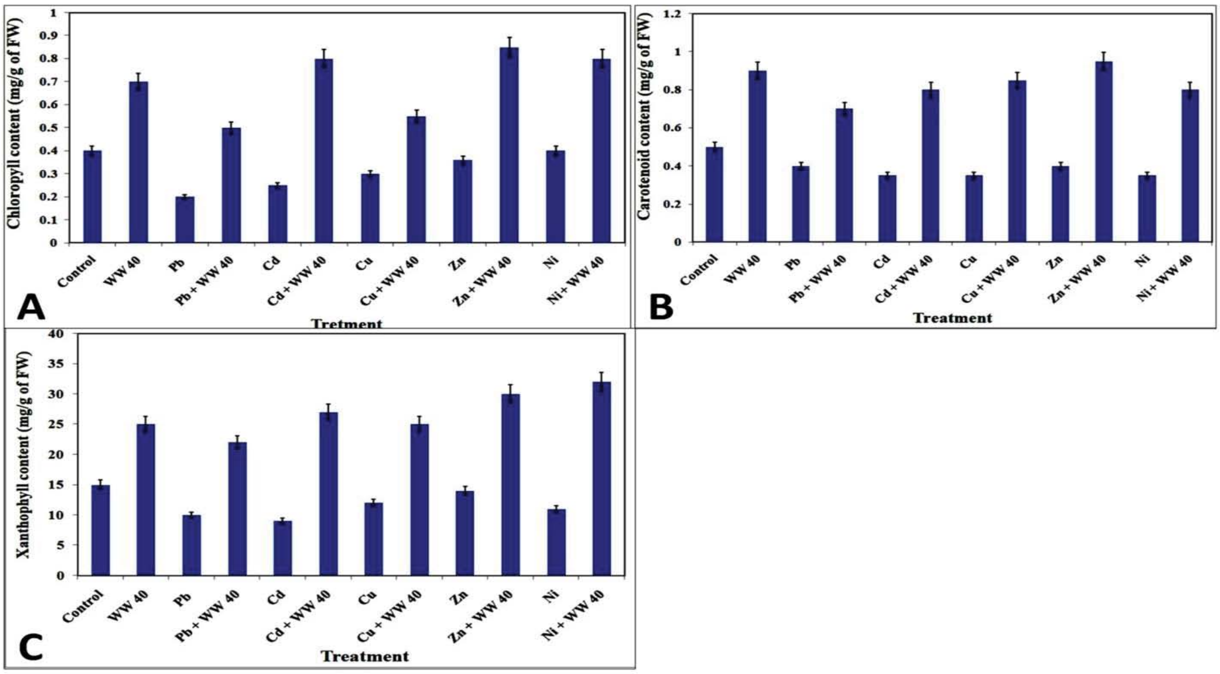

Figure 6: Effect of PGPR strains WW 40 on photosynthetic pigments. A) Chlorophyll content B) Carotenoid content C) Xanthophyll content in 20-days old *Zea maize* seedlings under five heavy metal stresses.

become healthy. The previous report revealed that PGPR adapted to heavy metal toxicity for their survival in places contaminated metalliferous soils [40]. The potency of heavy metal resistance of contaminated environments isolates has higher resistance than normal areas [41]. Hence, it can be expected that wastewater irrigated maize plants rhizospheric bacterial isolates are inhabitants in highly metal–resistant soil, and their population is affected by biological and non–biological agents such as heavy metal stress [42]. The maize field isolated strains of this study also showed remarkable tolerance to salinity. Previous reports showed that few halophilic bacteria resistant to heavy metals [43] and six heavy metal resistant bacteria isolates viz. *Chryseobacterium indoltheticum, Pseudomonas helmanticensis, Cupriavidus oxalaticus, Bacillus almalaya, Bacillus mycoides,* and *Acinetobacter tjernbergiae* were able to tolerate 1-7% salinity [44].

Salt tolerance reports observed that these isolated strains have the potential to be used in areas of high salinity and metalliferous polluted areas (Figure 1).

In this study, 8 rhizospheric bacterial isolates showed different PGP attributes such as IAA production, siderophore and phosphate solubilization (Table 4) (Figures 2&3). Past studies on rhizospheric bacteria from various crop plants that were potentially tolerant to heavy metals also showed these PGP traits [45]. Multiple heavy metals–stressed plants associated with bacterial isolates actively shown production such PGP properties of *Zea mays* cultivated with wastewater in this study. IAA production by the 8 strains is in different quantities. The previous report documented that 80% of rhizospheric bacteria isolated from various plants rhizosphere had the capability to synthesis IAA as secondary metabolites [46]. However, IAA production can help the bacteria to interact with the plant that has no specific role in bacterial cells [47]. Rhizospheric bacteria attached with the epidermal cell of the root surface and increase the number of root hairs initiation by the production of bacterial IAA which loosens the cell wall of the plant and increases the number of roots that help the take nutrients from the surrounding soil [48]. Siderophore production is one of the most essential characters of rhizobacteria which is synthesized under very low iron stress, acts as specific ferric iron-chelating agents and makes them more powerful in the challenge with other microorganisms in the environment and helping plant-bacteria for root colonization [49]. In this study PGPR significantly produce siderophore in the heavy metal condition which is shown in figure 3. The most potent strain WW40 produces the highest siderophore than control under multiple heavy metal conditions. Phosphorus is a necessary mineral nutrient that helps various physiological roles for plant growth and development. However, a large amount of total phosphate stock in soils but plants can be utilized a poor amount of the total phosphorus terms as plant-available phosphorus [50]. The soil stress due to heavy metal can also be a barrier to the absorption of plant-available phosphate and suppressed plant growth. Although, Phosphate availability could also be in the soil enhance the solubilization potentiality of phosphate by many PGPR through increasing the mobile of inorganic P to available phosphate [51]. The amounts of absorption of soil phosphorous by plants enhance and improved plant growth promotion. We found that WW-34, WW-36, WW-37, and WW-58 significantly increased the total P content in shoots and roots of *Zea mays* growing in WW-34, WW-36, WW-37, and WW-58 contaminated soil compared to control plants (Figure 2B). Seed bacterization of *Zea mays* with WW-34, WW-36, WW-37, and WW-58 strains significantly increased the rate of the seed germination and vigour index in comparison to the untreated control (Figures 4A-B). The percentage (92%) of seed germination observed in Pb inoculated pot with WW-40 strain is 52% greater than Pb inoculated pot only (40%). The application of WW-40 strain in Cd, Zn, Cu, and Ni pots significantly increased 47%, 38%, 38, 47%. Similarly, the vigour index of *Zea mays* seedling significantly increase 918%, 1078%, 812%, 896%, and 569% by the application of WW-40 strain in Pb, Cd, Cu, Zn, and Ni inoculated pots. Previous research focuses on the inhibition of seed germination in various cereal crops such as rice, wheat, and barley in response to different heavy metals which is probably due to the morphological and physiological changes in roots that result in reducing heavy metal tolerance [52]. Our results also demonstrated that there was a significant increase in plant growth parameters like shoot and root length, shoot and root fresh weight, and biomass (Figures 5A-C). The shoot length of maize seedling increased 60%, 68.57%, 66.66%, 63.33% and 91.66% and root length increased 62.5%, 55.55%, 50%, 33.33%, and 71.42% excess by the application of the strain WW 40 in Pb, Cd, Cu, Zn, and Ni inoculated pots. The highest shoot fresh weight increased excess 46.51%, root fresh weight 50%, and root biomass (63.93%) was observed in Ni inoculated pots and shoot biomass 63.82% in Zn inoculated pot by application of WW 40 strain. The effect of heavy metals on seeds with various growth abnormalities viz. germination, reduced root, and shoot elongation [53,54] was reported by earlier researchers.

The pigments content of *Zea mays* seedling (Chlorophyll ‘a’, ‘b’, Xanthophyll and Carotenoids content) are reduced by Pb, Cd, Zn, Cu, and Ni contamination pots (Figure 6). The pigments content might be reduced due to the insertion of multiple heavy metals within the phytoporphyrin ring of the pigments chlorophyll and decreases the production of chlorophyll [55,56]. The synthesis of chlorophyll molecule decrease either by reducing the potentiality of chlorophyllase enzyme or lowering the adsorption of Fe and Mg by plants [57,58]. Cd, Zn, Cu and Ni also degraded the chlorophyll molecule [59]. The photosynthetic pigments of crop plants were observed to enhancement by the application of plant growth-promoting bacteria that increases nutrient uptake in plants through phosphate solubilization and exudating essential substances that play a crucial role in the synthesis of photosynthetic pigments necessity for light-harvesting complex and its photo assimilation [60]. The chlorophyll levels increasing due to inoculation of *Klebsiella pneumoniae* in *V. mungo* under Cd stress [61]. The chlorophyll contents of *Zea mays* plants also increased by the application of *Azotobacter chrococcum* with Cu and Pb [62]. Heavy metal tolerance index showed that consortium (Pb + WW-40) applied *Zea mays* seedling tolerate 43.83% higher than Pb inoculated pot. Similarly, the application of in Cd, Cu, Zn, and Ni pot significantly increases the metal tolerance index 29.64%, 56%, 47.82% respectively (Figure 7). The result also signifies that the treatment of maize seedlings with microbial strains WW-40 in each heavy metal pot mitigates Pb, Cd, Cu, Zn, and Ni and reduces the heavy metal tolerance index. The application of *Methylobacterium oryzae* and *Burkholderia* sp. with Cd and Ni inoculated tomato seedlings decrease the Heavy metal tolerance index uptake [63].

In the current study, PGPR microorganisms are reduced heavy metal accumulation in the shoot of maize plant observed at 37.5% in Pb inoculated pot, 40% in Cd, 34.21% in Cu, 38.46% in Zn and 37.8% in Ni pot as compared to Pb, Cd, Cu, Zn, and Ni pot as shown in figure 8A. Similarly, in case of root, heavy metal accumulation lowered 38.4% in Pb inoculated pot, 40% in Cd, 50% in Cu, 43.75% in Zn, and 47.05% in Ni pot by the application of PGPR strain WW 40 as shown in Figure-8b. The mechanism of accumulation of Pb, Cd, Cu, Zn, and Ni reduction could be enhancement by the potential application of rhizobacteria. Previous studies suggested that *Z. mays* when contaminated with *A. chroococcum* bacterium lowered Cu and Pb accumulation in plant parts which is most likely due to the synthesis of various metabolites, protons and exudates that act as metal chelators and immobilize [64]. Moreover, it was suggested that *Bacillus megaterium,* a metal tolerant strain, decreases the Ni translocation [65], and As-resistance Exiguobacterium decrease as translocation in *Vigna radiata* plants by accumulation at the root parts [66]. It has been observed that *Acinetobacter lwofi* promotes growth and reduces uptake in *V. radiata* [67].

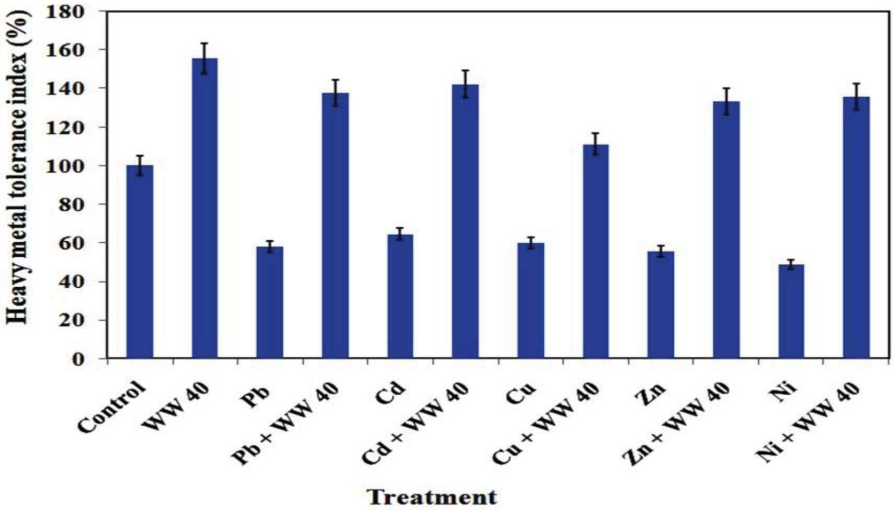

Figure 7: Heavy metal tolerance index in 20-days old *Zea mays* seedlings under five heavy metal stresses.

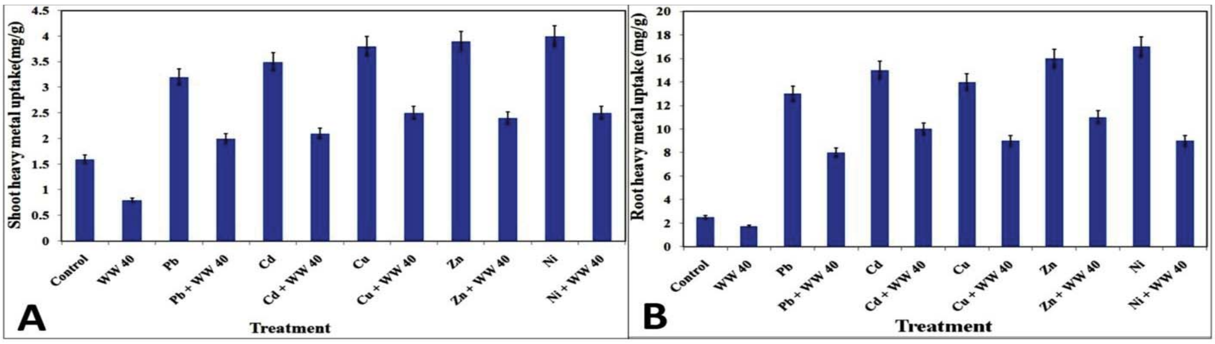

Figure 8: Effect of WW 40 on A) Shoot metal uptake B) Root metal uptake.

CONCLUSIONS

The maize field isolated multi-metals resistant and salt tolerance bacterium appeared to be potent plant growth-promoting that would turn out IAA, siderophores, solubilize the phosphate. In this study, the WW-40 strain enhance the rate of seed germination and vigour index in the metalliferous soil and promoted growth of maize seedling in terms of root length, shoot length, root fresh weight and shoot fresh, root biomass and shoot biomass subjected to Pb, Cd, Cu, Zn, and Ni metallic element stress. Application of the strain in Pb, Cd, Cu, Zn, and Ni pots observed a significant increase in the photosynthetic pigment content of seedlings like chlorophyll, carotenoid, and xanthophylls. The Pb, Cd, Cu, Zn, and Ni metal accumulation capability reduced by the maize seedling found in the presence of WW 40 strain in the rhizosphere. The decreased levels of Pb, Cd, Cu, Zn, and Ni resulted in the alleviation of Pb, Cd, Cu, Zn, and Ni toxicity by decreasing its bioavailability. Therefore, all these traits act as a driving force in enhancing the growth of plants in a metal-polluted environment. The present study, therefore, projects the contribution of micro-organisms in decreasing Pb, Cd, Cu, Zn, and Ni toxicity and accumulation, implicating their roles for achieving the goal of lower Pb, Cd, Cu, Zn, and Ni concentrations in maize plants with better growing conditions. It could be also a good choice for application in microbially assisted phytoremediation approaches for the pollution of multi-metals contaminated soils.

ACKNOWLEDGMENT

Authors are gratefully to Department of Botany, Balurghat College for their help. The financial assistance provided by the University Grants Commission File No: PSW-029/14-15(ERO), New Delhi, Government of India is gratefully acknowledged.

COMPLIANCE WITH ETHICAL STANDARDS

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author's contributions

SKC designed the whole study including sample collection, antibacterial assay, antifungal assay, synergistic effect at Department of Botany, Sreegopal Banerjee College and prepares the manuscript.

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