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Research Article

Cellular Damage, Plant Growth Promoting Activity and Chromium Reducing Ability of Metal Tolerant *Pseudomonas aeruginosa* CPSB1 Recovered from Metal Polluted Chilli (*Capsicum annuum*) Rhizosphere

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Abstract

Heavy metals adversely affect plants, microbes and human health. Considering these, plant growth promoting rhizobacteria was isolated from metal polluted chilli rhizosphere and characterized. Indigenous strain CPSB1 identified as *Pseudomonas aeruginosa* by 16S rRNA gene sequence analysis showed higher tolerance to Ni (800:400 µg/ml), Zn (2000:800 µg/ml), Pb (1800:1000 µg/ml) and Cr (1000:400 µg/ml) on solid: liquid medium, respectively. *Pseudomonas aeruginosa* solubilized 12.3 and 13.3 µg/ml P with 400 µg/ml of Zn and Cr, respectively. Strain CPSB1 secreted IAA, siderophores, cyanide, ammonia and ACC deaminase under metal stress. Plant growth regulators gradually decreased with increasing concentrations of metals but were retained even at higher concentrations. *Pseudomonas aeruginosa* reduced Cr (VI) under aerobic conditions which completely reduced Cr (VI) after 120h at pH 8 and 35°C. Scanning electron microscopy (SEM) and Fourier Transform Infrared (FT-IR) Spectroscopy revealed cellular damage and alteration in cell surface functional groups, respectively. The metals uptake by *P. aeruginosa* CPSB1 was validated by energy dispersive X-ray (EDX) spectroscopy. The results suggest that *P. aeruginosa* endowed with plant growth promoting activities and chromium reducing ability could play a significant role in reducing chromium toxicity and supplying essential nutrients and hormones to plants, even in metal contaminated soils.

Keywords: *Pseudomonas aeruginosa*; Cellular Damage; Plant Growth Regulators; Hexavalent Chromium; Bioremediation

Abbreviations

PGPR: Plant Growth Promoting Rhizobacteria; SEM: Scanning Electron Microscopy; FE-SEM: Field Emission Scanning Electron Microscopy; EDX: Energy Dispersive X-ray; FT-IR: Fourier Transform Infra-Red; IAA: Indole 3 Acetic Acid; ACC: 1-Aminocyclopropane-1-Carboxylate; CAS: Chrome Azurol S

Introduction

Industrial effluents, tanning industries, sewage wastes and many other metal discharging industries are the major sources of environmental pollution that adds considerable amount of toxic metals to the environment including soils. The heavy metals which are biologically non-degradable and persist in the environment, cause a serious threat to plants [1], soil fertility [2], microbial populations and their associated activities [3] and via food chain, human health [4]. These problems warrant urgent attention so that the health of soils and concurrently the production of crops are preserved. In this context, certain microorganisms especially belonging to plant growth promoting rhizobacteria (PGPR) group endowed with the distinctive property of heavy metal tolerance and plant growth promotion have been identified [5]. The soil microbes showing tolerance to one or multi-metals detoxify/reduce the deleterious effects of heavy metals by several mechanisms including exclusion, extracellular and intracellular sequestration of metal ions and transformation of heavy metals to less toxic forms [6,7]. In addition to metal tolerance, certain bacteria also show resistance to various antibacterial drugs (antibiotics) largely due to alterations in the genetic architecture of the organism [8]. This is due to the fact that antibiotics and heavy metal resistance genes are transferred together during conjugation [9]. The possession of dual properties i.e., the ability to tolerate higher level of metals and to display resistance against antibiotics by any PGPR makes such organism an interesting one in crop production practices.

Among the most prominent heavy metal contaminants, chromium, a product of chrome plating, wood processing, metal chelating and many other industries is one of the most hazardous metals [10] and adversely affects both plants [11] and microbes [12]. Among nine oxidation states, a highly stable Cr (VI) is more toxic to microbes [13] and plants [14] as compared to other forms of chromium. Chromium in general is inhibitory due to- (i) high wa-

ter solubility (ii) permeability through cell membrane and (iii) ability to interact with macromolecules like DNA and proteins. However, metal tolerant bacteria can reduce the toxicity of chromium in polluted environments for example, industrial effluents and contaminated soils [15] by transforming Cr (VI) to Cr (III). Chromium reduction is usually carried out by microbial enzymes or may occur through complex formation with metabolites [16]. Apart from metal reduction, such PGPR supply essential macro and micronutrients and hormones to plants while growing in metal stressed soils.

Application of metal reducing and plant growth promoting organisms therefore, becomes extremely significant in soils which are poor in nutrients but rich in contaminants. Considering the deleterious effects of heavy metals on one hand and the metal detoxification ability of PGPR on the other hand, the present study aimed at identifying metal tolerant PGPR possessing plant growth promoting activity and chromium reducing potential. Also, the impact of heavy metals on cellular morphology, cell surface functional groups and metal uptake by test bacterium was analyzed using SEM, FT-IR and EDX respectively.

Materials and Methods

Bacterial isolation, heavy metal tolerance and antibiotic resistance/sensitivity

The soil samples collected from chilli (*Capsicum annum*) rhizosphere irrigated continuously with lock industries effluents were serially diluted and plated on solid agar plates by standard spread plated method and incubated at 28 ± 2°C for two days. The bacterial colonies were picked and re-streaked three times on the same medium to obtain a pure culture. The isolated bacterial strains were maintained on King's A agar until use. The resistance/sensitivity of bacterial strains towards various heavy metals was determined by agar plate dilution method using nutrient agar medium amended with increasing concentrations (0-2400 µg/ml) each of Ni ($NiCl_2.6H_2O$), Zn ($ZnSO_4.H_2O$), Pb [$Pb(CH_3.COO)_2.3H_2O$] and Cr ($K_2Cr_2O_7$). The metal salts of Ni (CAS No. 7791-20-0; Mol. wt. 237.69), Zn (CAS No. 7446-19-7; Mol. wt. 179.45) and Cr (CAS No. 7778-50-9; Mol. wt. 294.18) were obtained from Qualigens, India, whereas the Pb was procured from Sisco Research Laboratories (SRL) Pvt. Ltd., India. A- 10 µl of log phase bacterial suspension containing approximately 10^8 cells/ml was spot inoculated onto the metal amended plates. The plates were incubated at 28 ± 2°C for 48h and the highest concentration of each metal supporting bacterial growth was defined as the maximum tolerance level (MTL). Of the total 17 bacterial strains, strain CPSB1 exhibiting highest tolerance towards the tested heavy metals was further tested for resistance/sensitivity towards 14 antibacterial drugs (mcg/disc): streptomycin (10), ampicillin (10), rifampicin (15), chloramphenicol (30), nalidixic acid (30), ciprofloxacin (10), erythromycin (15), nitrofurantoin (300), norfloxacin (10), doxycyclin (10), penicillin-G (10), tetracycline (30), methicillin (10) and gentamicin (10) by the disc diffusion method [17]. The antibiotic discs were obtained from Hi- Media, Pvt. Ltd., Mumbai, India. For this, a 100 µl of overnight grown bacterial culture was spread evenly onto nutrient agar plates. Later on, antibiotic discs of known potency were mounted aseptically onto the plates and the plates were incubated at 28 ± 2°C for 48h. Following incubation, the zone of inhibition (mm) was measured.

Assessment of cytotoxic damage, cell surface functional groups and metals uptake

The cellular damage to *P. aeruginosa* CPSB1 exposed to 400 µg/ml each of Ni, Zn and Pb and 200 µg/ml of Cr was observed under a Field Emission Scanning Electron Microscope (FE-SEM) embedded with EsB detector (Model ULTRA plus, Carl Zeiss, Germany). For this, the cell pellets were washed with phosphate buffered saline (PBS) and fixed overnight at 4°C in fixation buffer (0.1 M PO_4^{2-} buffer+ 2.5% glutaraldehyde+ 2% paraformaldehyde). The fixed cell pellets were again washed with PBS followed by washing with ethanol gradients (30, 50, 70, 90 and 100%). The pellets were then dissolved in 20 µl sterile double distilled water. A thin smear of the sample was prepared on a glass cover slip and observed under a field emission scanning electron microscope. An untreated control sample was also run in a similar manner for comparison. After cellular damage, the impact of heavy metals on cell surface functional groups of test bacterium was determined by FT-IR spectroscopy. The FT-IR analysis of strain CPSB1 prepared both from metal-treated and untreated biomass was performed with a FT-IR spectrometer (Thermo Nicolet, Nexus 670). For this, dried bacterial biomass (2.5 mg) was blended and ground with KBr (75 mg) in an agate mortar. The translucent discs were prepared by pressing the ground material with the help of 8 tonnes of pressure bench press. The respective discs were immediately analysed with a spectrophotometer in the range of 1000 - 4000 cm^{-1} with a resolution of 5 cm^{-1}. The influence of atmospheric H_2O and carbon dioxide was always subtracted. The EDX was used to determine metal uptake by bacterial cells.

Bacterial identification

Genus level identification of metal tolerant bacterial strain CPSB1 was performed using standard morphological and biochemical tests [18]. The morphological characterization included Gram's staining, shape and size of the cell and the pigment formation while the biochemical tests involved indole reaction, methyl

red, Voges-Proskauer, citrate utilization, nitrate reduction, catalase, oxidase and urease tests, starch and lipid hydrolysis and mannitol utilization. For molecular characterization, 16S rRNA partial gene sequencing was done commercially by a DNA sequencing service (Macrogen, Seoul, South Korea) through 16S-rDNA sequencing using universal primers, 785F (5/-GGATTAGATACCCTGGTA-3/) and 907R (5/-CCGTCAATTCMTTTRAGTTT-3/). The DNA sequence obtained was analyzed and compared with the sequences of other genera already available at the National Centre for Biotechnology Information (NCBI) server using BLASTn tool. The nearest neighbour sequence was identified and a phylogenetic tree was constructed using MEGA 6.0 software employing the bootstrapped neighbour-joining approach.

Assay of Plant Growth Promoting Active Biomolecules under Metal Stress

Indole acetic acid, siderophores and P- solubilization

The assay of all plant growth promoting substances secreted by CPSB1 was performed in the presence and absence of heavy metals at 28 ± 2°C. The concentration of Cr, Ni, Zn, and Pb added to respective medium were (µg ml^{-1}): 0, 25, 50, 100, 200 and 400. The secretion of indole-3-acetic acid (IAA) by CPSB1 was detected quantitatively by the method of [19] later modified by [20]. For this, a- 100 µl of overnight grown culture of CPSB1 containing approx. 108 cells/ml was inoculated in 25 ml of Luria Bertani broth (gl^{-1}; tryptone 10; yeast extract 5; NaCl 10 and pH 7.5) (Hi- Media, Pvt. Ltd., Mumbai, India) amended previously with 200 µg tryptophan ml^{-1} and varying concentration of Ni, Zn, Pb and Cr. The metal treated and bacterium inoculated broth was incubated for 48h on a shaking incubator with 125 rpm. Following incubation, 5 ml culture from each treatment was centrifuged at 10,000g for 15 min. and 2 ml supernatant was taken. The supernatant was then mixed with 2 - 3 drops of orthophosphoric acid and 4 ml of Salkowsky reagent prepared by adding 2% 0.5 M $FeCl_3$ to 35% $HClO_4$. All samples were incubated at 28 ± 2°C in darkness for about 1h. The pink colour of supernatant was quantified at 530 nm using a UV-Vis spectrophotometer (UV-2450, Shimadzu). A calibration curve was prepared using pure IAA as standard.

Bacterial siderophores was estimated qualitatively on Chrome Azurol S (CAS) (Hi-Media, Pvt. Ltd., Mumbai, India) agar plates amended with Ni, Zn, Pb and Cr [21]. The metal treated/untreated CAS agar plates were incubated for five days and the development of yellow to orange halo around the bacterial growth was recorded. P- solubilization by strain CPSB1 was measured both qualitatively and quantitatively [22] using Pikovskaya medium (Hi-Media Pvt. Ltd., India). Furthermore, the solubilization index (SI) and solubilizing efficiency (SE) of *P. aeruginosa* CPSB1 was calculated by the formula of [23]:

Solubilization index (SI) = (colony diameter + zone of halo)/ colony diameter; Solubilizing efficiency (SE) = (zone of halo/colony diameter) × 100.

Synthesis of cyanogenic compounds, ammonia and ACC deaminase

Standard methods were used to detect hydrogen cyanide (HCN) [24] and ammonia [25]. The 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 4.1.99.4) was qualitatively assayed by spot inoculation of 10 µl of overnight grown bacterial culture into DF salts minimal medium (g/l: KH_2PO_4 4; Na_2HPO_4 6; $MgSO_4.7H_2O$ 0.2; glucose 2; gluconic acid 2 and citric acid 2 with trace elements: $FeSO_4.7H_2O$ 1 mg/l; $H_3BO_3$10 mg/l; $MnSO_4.H_2O$ 11.19mg/l; $ZnSO_4.7H_2O$ 124.6 mg/l; CuSO4.5H2O 78.22 mg/l; MoO_3 10 mg/l; pH 7.2) [26]. The plates were enriched with 3 mM ACC (Sigma Aldrich, USA) which served as the sole source of N. Plates containing DF medium but without ACC served as negative control and those supplemented with (NH4)2SO4 (0.2% w/v) served as positive control. The plates were incubated at 28 ± 2°C for 72h and were observed for bacterial growth. *Mesorhizobium* strain LMS-1 containing pRKACC plasmid [27] was included as a positive control. ACC deaminase was also quantitatively assessed [28]. *Pseudomonas aeruginosa* CPSB1 was inoculated in 10 ml DF medium amended with different concentrations of Ni, Zn and Pb and incubated at 28 ± 2°C for 24 - 48h. The culture was spun (9000 r/min.) and the cell pellets were collected. The pellets were washed twice with 0.1 M Tris-HCl (pH 7.5) and re-suspended in 2 ml of modified DF medium supplemented with 2 mM of ACC. Following this, the culture was again incubated at 28 ± 2°C for 36 - 72h. After incubation, the bacterial cells were harvested by centrifugation (3,000g for 5 min.) and washed twice with 0.1 M Tris-HCl (pH 7.5). The cell pellets were re-suspended in 200 µl of 0.1 M Tris-HCl (pH 8.5) and were toluenized with 5% toluene (v/v). The cell suspension was incubated with 5 µl of 0.3M ACC solution in an eppendorph tube at 28 ± 2°C for 30 min. A- 50 µl of cell suspension without ACC served as negative control while 50 µl of 0.1M Tris- HCl (pH 8.5) with 5 µl of 0.3M ACC served as blank. The samples were later acidified with 500 µl of 0.56 N HCl and mixed thoroughly. Following this, the cell debris was removed by centrifugation (12,000g for 5 min.). A 400 µl of 0.56N HCl and 150 µl of DNF solution (0.1g 2, 4- dinitrophenyl hydrazine prepared in 100 ml of 2N HCl) were added to 500 µl aliquot of the supernatant and the mixture was again incubated at 28 ± 2°C for 30 min. After incubation, one ml of 2N NaOH was added to each sample and absorbance was measured at 540 nm. The concentration of α -ketobutyrate produced by the bacteria was determined by plotting a calibration curve of α-ketobutyrate as standard. The ACC deaminase activity was expressed as the amount of α-ketobutyrate produced per milligram

of protein per hour. Each individual experiment was repeated three times so as to minimize any experimental error.

Hexavalent chromium reduction

Bioreduction of Cr (VI) by *P. aeruginosa* CPSB1 was assessed by growing cells (100 µl of exponentially grown culture) in nutrient broth treated with varying concentrations (25, 50, 100, 200 and 400 mg ml^{-1}) of $K_2Cr_2O_7$ at different temperatures (25, 35 and 40°C), pH (5, 6, 7, 8 and 9) and incubation periods (up to 120 h). For Cr (VI) reduction analysis, two ml culture from each tube was centrifuged (6000 rpm for 20 min.) at 10°C. Post centrifugation, a 400 µl of supernatant was taken and the volume was made to 1 ml using double distilled water. The Cr (VI) remaining in the supernatant was quantified by 1, 5-diphenyl carbazide method [29, 30, 31].

Statistical Analysis

The results were statistically analysed using Duncan's multiple range test (DMRT) and the least significant difference (LSD) was calculated using Minitab 17 statistical software at 5% probability level.

Results and Discussion

Heavy metal tolerance, antibiotic resistance and strain identification

A total of 17 bacterial strains were recovered from the rhizospheric soils of chilli cultivated in metal contaminated soils. The bacterial isolates were further screened for their ability to tolerate different metals such as nickel, zinc, lead and chromium. Of these, one isolate (CPSB1) showed maximum tolerance to Ni (800:400 µg/ml), Zn (2000:800 µg/ml), Pb (1800:1000 µg/ml) and Cr (1000:400 µg/ml) when grown on nutrient agar plates: nutrient broth medium. In general, the tolerance to heavy metals was greater on nutrient agar plates compared to those recorded for nutrient broth. The greater heavy metal tolerance by strain CPSB1 while growing on nutrient agar could possibly be due to poor availability of metals due to polymeric nature of solid agar medium as also reported by others [32]. On the contrary, due to greater solubility of such metals in aqueous environments (nutrient broth), metals might have been easily available to bacteria. However, whatever has been the reason, the elevated levels of heavy metals inhibit bacterial population due possibly to the surface binding of metal ions and disruption of membrane functions.

Moreover, the variation in bacterial ability to resistance/tolerate one or more heavy metals could be attributed to multiple mechanisms evolved within bacteria such as metal efflux by ATPases, complexation of various intracellular compounds, binding of metal to bacterial cell envelopes or reducing the toxic metal forms to less toxic forms [33,34]. In a similar study, a Gram-Negative *Rhizobium halophytocola* strain RT7 and a Gram-Positive *Bacillus circulans* isolated from industrial soils have been shown to tolerate higher levels of manganese and other metals [35]. In another study, *Pantoea agglomerans* tolerated Cd up to 3000 µg/ml whereas, *Enterobacter asburiae* was found to tolerate 2000 µg/ml Ni when grown in nutrient broth medium [36]. The metal tolerant strain CPSB1 was further tested to assess its antibiotic sensitivity/resistance potential. Interestingly, strain CPSB1 showed resistance to multiple antibiotics. However, strain CPSB1 was susceptible to five antibiotics namely, streptomycin, rifampicin, ciprofloxacin, norfloxacin and gentamycin and the zone of inhibition varied between 11.6 mm (rifampicin) to 27.3 mm (norfloxacin). In a similar study, *Acinetobacter baumannii* HAF - 13 and metal tolerant isolates of *Bacillus* and *Pseudomonas* sp. showed variable resistance to multiple antibiotics [37,38]. The multi heavy metal tolerance and multiple antibiotics resistance is likely to enhance the ability of strain CPSB1 to survive in the contaminated soil environment which might prove helpful in the maintenance of antibiotic resistance genes by enhancing the selective pressure of the environment [39]. Furthermore, the antibiotic resistance among bacteria could be used as a marker for identifying metal tolerant microbes in metal enriched environment. Considering the high multi-metal tolerance and multiple antibiotic resistance ability of strain CPSB1 it was characterized further. Strain CPSB1 was Gram negative, rod shaped and exhibited green fluorescence when grown on King's A agar medium. Moreover, the strain tested positive for citrate utilization, nitrate reduction, catalase, oxidase and could hydrolyse lipids. On the other hand, it gave a negative reaction towards indole, methyl red, Voges-Proskauer, urease, mannitol utilization and starch hydrolysis (Table 1). Based on morphological and biochemical tests, strain CPSB1 was identified to genus level as *Pseudomonas* sp. which was further identified to species level as *Pseudomonas aeruginosa* (GenBank Accession Number KX821717) by 16S rDNA sequence analysis. A phylogenetic tree (drawn using the neighbour joining programme in MEGA 6.0) between different bacteria and isolate CPSB1 is represented in Figure 1 and sequence data is deposited in NCBI GenBank.

Morphological characteristics	
Colony shape	Round
Colony colour	Yellowish
Colony Morphology	Smooth
Pigmentation Gram reaction Cell shape	Florescent green Gram negative Short rods
Biochemical characteristics	
Indole	-
Methyl red	-
Voges-Proskauer	-
Citrate utilization	+
Nitrate reduction	+

Catalase	+	
Oxidase	+	
Urease	-	
Mannitol utilization	-	
Starch hydrolysis	-	
Lipid hydrolysis	+	
Tolerance to heavy metals (µg ml $^{-1}$)		
	Nutrient agar	**Nutrient broth**
Nickel	800	400
Zinc	2000	800
Lead	1800	1000
Chromium	1000	400

Table1: Heavy metal tolerance and morphological and biochemical properties of *P. aeruginosa* strain CPSB1. '+' and '-' indicate positive and negative reactions respectively.

Figure 1: Phylogenetic Analysis based on 16S rRNA gene comparison of 840 bp showing relationship between *Pseudomonas aeruginosa* CPSB1 (GenBank accession no. KX821717) and other closely related bacteria. The bar indicates distance values calculated in MEGA 6.0 and values at node represents percentage of 1000 Bootstrap Replicates.

In- vitro assay of indole acetic acid, siderophores and P-solubilization

The ability of metal tolerant and antibiotic resistant *P. aeruginosa* CPSB1 to produce one or more plant growth promoting active biomolecules was evaluated in the absence and presence of varying doses of heavy metals. The metal tolerant *P. aeruginosa* strain CPSB1 produced a significant amount of plant growth promoting substances both in the presence and absence of heavy metals which however, declined with increasing concentrations of Ni, Zn, Pb and Cr (Table 2). When grown in the presence of 400 µg/ml Cr, *P. aeruginosa* 1 synthesized 47.4 µg/ml IAA. While comparing the toxic impact of three concentrations of test metals on IAA production, it was found that Pb at 400 µg/ml was most toxic and caused a significant decrease (66.4%) in IAA which was followed by 400 µg/ml of Zn (55.6%) and Ni (52.2%) when compared with control. Despite the toxicity shown by Ni, Zn and Pb, the synthesis of IAA by *P. aeruginosa* CPSB1 continued even at higher concentrations of three metals tested in this study. However, the reduction in IAA production at higher concentrations of the three metals could possibly be due to slower bacterial growth.

Treatment	Metal dose rate (µg/ml)	*IAA (µg/ml)	P-solubilization				Siderophores Zone on CAS agar (mm)	ACC deaminase activity (µM α ketobutyrate mg^{-1} protein $hour^{-1}$)	HCN	NH_3
			P- zone diameter (mm)	P-solubilized in liquid medium (µg/ml)	SI	SE				
P. aeruginosa CPSB1	00	74.7 ± 1.3a	20.7 ± 0.6a	85.0 ± 2.9a	2.9	188.2	20.7 ± 0.6a	71.2 ± 5.2a	+	+
CPSB1 + Ni	25	43.5 ± 0.9e	15.3 ± 0.6c	17.7 ± 0.4b	2.4	139.1	20.0 ± 1.0b	61.8 ± 2.9b	+	+
	50	40.2 ± 0.9f	15.0 ± 1.0c	17.2 ± 3.9b	2.4	136.4	18.7 ± 0.6c	41.8 ± 2.3e	+	+
	100	39.1 ± 0.8f	12.7 ± 0.6f	16.3 ± 0.4b	2.2	115.5	17.3 ± 0.6e	41.2 ± 1.7e	+	+
	200	37.0 ± 1.3h	11.3 ± 0.6g	15.3 ± 0.7d	2.0	102.7	14.0 ± 1.0h	39.7 ± 1.2f	+	+
	400	35.7 ± 0.2h	ND	ND	0	0	13.3 ± 0.6h	39.1 ± 8.2f	+	-
CPSB1 + Zn	25	37.5 ± 0.4g	15.7 ± 0.6c	18.5 ± 0.9b	2.4	142.7	16.7 ± 0.6e	47.2 ± 2.6e	+	+
	50	35.1 ± 0.3i	14.3 ± 0.6d	16.0 ± 0.6c	2.3	130.0	15.7 ± 0.6f	38.8 ± 2.9f	+	+
	100	35.0 ± 0.8j	14.0 ± 1.0e	15.9 ± 0.4c	2.3	127.3	15.0 ± 1.0g	29.0 ± 4.8h	+	+
	200	33.7 ± 0.6k	11.7 ± 0.6g	14.1 ± 0.6e	2.2	117.0	14.7 ± 1.2g	25.3 ± 4.4h	+	+
	400	33.2 ± 0.7k	11.3 ± 0.6g	12.3 ± 0.7h	2.1	113.0	13.0 ± 1.0h	18.7 ± 1.0i	+	+
CPSB1 + Pb	25	36.1 ± 0.9j	12.7 ± 0.6f	18.4 ± 1.5b	2.2	115.5	19.0 ± 1.0b	66.9 ± 1.4a	+	+
	50	32.3 ± 0.9l	12.0 ± 0.0f	14.9 ± 0.7e	2.1	109.1	17.7 ± 0.6d	67.8 ± 1.5a	+	+
	100	30.1 ± 2.1m	11.3 ± 0.6g	14.3 ± 0.1e	2.0	102.7	16.0 ± 1.0f	64.9 ± 0.7a	+	+
	200	27.6 ± 1.2n	ND	ND	0	0	15.7 ± 1.2f	40.7 ± 1.7e	+	-
	400	25.1 ± 2.5o	ND	ND	0	0	14.0 ± 1.0h	39.9 ± 3.5f	+	-
CPSB1 + Cr	25	52.1 ± 0.9b	17.3 ± 0.6b	18.1 ± 0.9b	2.6	157.3	ND	57.5 ± 15.5c	+	+
	50	50.2 ± 1.6b	15.7 ± 0.6c	14.6 ± 0.7e	2.4	142.7	ND	49.1 ± 10.4d	+	+
	100	49.8 ± 0.4c	11.7 ± 0.6g	13.9 ± 0.6f	2.1	106.4	ND	31.8 ± 2.7g	+	+
	200	47.5 ± 0.9d	11.3 ± 0.6g	13.4 ± 1.2g	2.0	102.7	ND	21.7 ± 3.9i	+	+
	400	47.4 ± 1.7d	11.0 ± 0.0h	13.3 ± 0.7g	2.0	100.0	ND	10.3 ± 0.8j	+	-
LSD (P ≤ 0.05)		1.9	0.8	2.2			1.2	8.7		

Table 2: Plant growth promoting traits of *P. aeruginosa* CPSB1 grown in the presence and absence of heavy metal stress.Values are mean of three independent replicates; ± indicates standard deviation. Mean values denoted with different letters are significantly different according to Duncan's multiple range test (DMRT). * IAA production was recorded at 200 µg/ml concentration of tryptophan. SI and SE indicate solubilization index and efficiency, respectively. ND indicates 'not detected'.

The secretion of IAA by *P. aeruginosa* strain CPSB1 could play a significant role in facilitating cell division, root initiation, phototropism and apical dominance of plants when grown in metal enriched soils [7]. A similar decline in IAA production by *Bradyrhizobium japonicum* in the presence of lower concentrations of metals is reported [40].

Siderophores, a low molecular weight iron chelating compound is yet another important biomolecule which sequester iron within soil and indirectly facilitate plant growth. Due to this, the siderophore producing ability of *P. aeruginosa* CPSB1 was further assayed using CAS agar plates supplemented with varying concentrations of metals. The size of orange halo generally decreased with progressive increase in concentrations of Ni, Zn and Pb and Cr. Interestingly,

the zone size was inversely proportional to the concentrations of the three heavy metals used. *P. aeruginosa* CPSB1 produced orange halo around the bacterial growth even in the presence of 200 µg/ml each of Ni (Figure 2a), Zn (Figure 2b) and Pb (Figure 2c) added to CAS agar plates. The zone was however, more obvious in metal free medium (Figure 2d). The highest zone size was observed in the presence of 25 µg/ml of Ni (20 mm) whereas 400 µg/ml Zn produced the lowest halo (13 mm) (Table 2). Interestingly, siderophore production by *P. aeruginosa* CPSB1 was completely inhibited at all concentrations of Cr ranging from 25 - 400 µg/ml and therefore, no halo was formed on Cr treated CAS agar plates. Production of siderophores by *P. aeruginosa* strain CPSB1 indicates that this strain can be used as a biocontrol agent for suppressing the growth of phytopathogens affecting the crops adversely, thereby indirectly promoting the plant growth. In a similar experiment, Cu among different metals exhibited highest toxicity to siderophore production by *P. aeruginosa* ZGKD3 [41].

Figure 2: Siderophore Production by *P. aeruginosa* CPSB1 on CAS Agar Plates treated with 200 µg/ml each of Ni (a), Zn (b), Pb (c) and without metal (d).

Like siderophores, the size of zone of P- solubilization (halo) declined with increasing concentrations of metals and the impact was more pronounced at higher rates of each metal. As an example, the halo size was 12.7 mm at 25 µg Pb/ml while zone of P- solubilization was completely diminished at 400 µg/ml of the same metal (Table 2). When grown in the presence of 400 µg/ml Cr, *P. aeruginosa* CPSB1 solubilized 13.3 µg/ml of P. While comparing the impact of metals at 400 µg/ml, the toxicity followed the order: Pb > Ni > Cr > Zn. Moreover, the amount of P-solubilized in liquid Pikovskaya medium declined progressively with consequent increase in metal concentrations added to growth medium. The P-solubilization was declined by 80.8%, 81.3%, 83.2% and 83.6% when strain CPSB1 was grown in Pikovskaya broth medium treated with 100 µg/ml each of Ni, Zn, Pb and Cr respectively, relative to control (Table 2). However, even the higher concentrations of each metal did not diminish completely the solubilization of insoluble P. The zone of P- solubilization and the amount of P solubilized in liquid medium were significantly correlated (r = 0.69). The constant decrease in P solubilization following metal application, however, suggests some regulatory role of metals in P solubilization by *P. aeruginosa.* The solubilization index and solubilization efficiency of *P. aeruginosa* CPSB1 also declined progressively in a manner similar to those of other active biomolecules. Results by [42] revealed a high P- solubilization efficiency of lead tolerant *P. aeruginosa* HMT 51 as 200, 281.81 and 314.28 after 24, 48 and 72h of growth, respectively.

ACC deaminase, hydrogen cyanide and ammonia

The ACC deaminase secreted by many PGPR is an important trait that indirectly promote plant growth by lowering the excessively higher levels of ethylene generated in plants while growing under stressed environment [43]. In the present study, *P. aeruginosa* CPSB1 exhibited a positive ACC deaminase activity when grown on DF medium amended with/without various concentrations of Cr, Ni, Zn and Pb after four days of incubation. Strain CPSB1 revealed maximum production of ACC deaminase in DF medium devoid of any metal compared to those treated with varying rates of each metal. As the concentration of metal was increased, the ACC deaminase activity decreased gradually. As an example, in the presence of 400 µg/ml Cr, the ACC deaminase activity was reduced by 85.5% when compared with untreated control (71.2 µM α-ketobutyrate mg^{-1} protein h^{-1}). Among metals, zinc at 400 µg/ml however, decreased ACC deaminase activity by 73.7% over control. Lead at 400 µg/ml, on the contrary, had poor effect and reduced ACC deaminase release by 43.9% only when compared with control (Table 2). The order of toxicity of the heavy metals was: Cr > Zn > Ni > Pb. In other study by [44] the ACC deaminase activity and IAA production by *Escherichia* sp. N16, *Enterobacter* sp. K131 and N9 and *Serratia* sp. K120 have been shown to be

negatively affected by Ni, Cd, and Mn. The different doses of heavy metals however, did not affect the production of HCN adversely by *P. aeruginosa* CPSB1. On the other hand, *P. aeruginosa* CPSB1 synthesized ammonia under both metal free and metal stressed conditions but the production of NH3 was inhibited at 200 and 400 µg/ml of Pb. However, *P. aeruginosa* CPSB1 did not secrete ammonia when grown in medium treated with 400 µg /ml of Ni and Cr (Table 2).

Assessment of cellular damage, surface functional groups and uptake of metals

Scanning electron microscopy (SEM) revealed significant distortions in the morphological structures of *P. aeruginosa* CPSB1 grown in the presence of 400 µg/ml each of Ni (Figure 3a), Zn (Figure 3c), Pb (Figure 3e) and 200 µg/ml of Cr (Figure 3g) compared with untreated control. Also, the SEM micrographs of *P. aeruginosa* CPSB1 exhibited reduction in cell size, irregular margin, clumping and aggregation of cells when exposed to heavy metals as compared to uniform, normal rods with smooth margin and undamaged cells while grown in metal free medium (Figure 3i). A similar reduction in the size of Pb treated cells of *Bacillus licheniformis* [45] and an irregular cell morphology and aggregation in Ni treated cells of *Sinorhizobium* sp. [46] under SEM has been reported. The presence of peaks in EDX spectra further validated the uptake of heavy metals like Ni (Figure 3b), Zn (Figure 3d), Pb (Figure 3f) and Cr (Figure 3h) by *P. aeruginosa* CPSB1 (Figure 3i).

Figure 3: Scanning Electron Micrographs and EDX Spectra of *P. aeruginosa* CPSB1 observed at 400 µg/ml of (a,b) Ni (c,d) Zn (e,f) Pb (g,h) 200 µg/ml Cr and (i) Untreated Control.

In a similar study, binding of metals onto the surface of *Saccharomyces cerevisiae* was observed through EDX analysis [47]. Furthermore, the change in functional groups of bacterial surface proteins and lipids was detected by FT-IR technique. The FT-IR analysis of metal loaded dry biomass of *P. aeruginosa* CPSB1 was performed in the range of 1000 - 4000 cm^{-1}. The results revealed significant deviations in the peaks corresponding to various functional groups attached to bacterial cell surface when grown in the presence of 100 µg/ml each of Cr, Zn, Ni and Pb (Figure 4). A broad peak in the range of 3287 - 3404 cm^{-1} could be assigned to bonded H and free OH of alcohol (Table 3). The FTIR spectrum of broad and strong bands was observed in the region of 3404 - 1659 cm^{-1} (Figure 4). Certain changes were observed in the FTIR spectrum of the heavy metal loaded bacterial cell biomass. Narrowing and shifting of peaks was observed along with peak disappearance in the region 1451 - 1464 cm^{-1}. A similar disappearance of peaks was observed in the 1200 cm^{-1} - 1300 cm^{-1} regions and at 1450 cm^{-1} in case of Pb treated *B. japonicum* [40]. The shift in peaks (compared to control) could possibly be due to the change in functional groups present on bacterial cell surface as a result of interaction with various metal ions. In another study, several peaks were detected in the range of 650 - 1750 cm^{-1} in the biomass of *P. aeruginosa* corresponding to alterations in carbohydrates, lipids and proteins. In addition, other peaks representing -OH groups were also present in the synchronous and asynchronous spectra [48]. The SEM micrographs and FT-IR spectra of untreated and metal treated biomass of *P. aeruginosa* CPSB1 thus clearly confirmed that heavy metals had severe inhibitory effects on microbial cells and caused structural damage leading to disturbances in the biochemical composition of the cells.

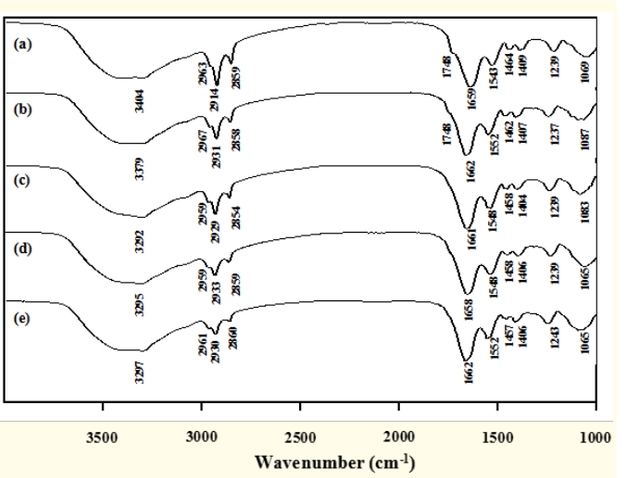

Figure 4: FT-IR Spectra of Biomass of *Pseudomonas aeruginosa* CPSB1 after 48h growth in Nutrient Broth treated in the absence (a) and presence of 100 µg/ml each of Cr (b), Ni (c), Pb (d) and Zn (e).

Assay of chromium reducing ability

Bio reduction of hexavalent chromium by chromium tolerant bacteria is considered important for growing crops in chromium contaminated environment. The Cr reduction is largely an enzyme mediated process which involves chromate reductase, an enzyme found in many chromium resistant bacteria [49]. Chromate reduc-

tase lowers the toxicity of chromium by transforming Cr (VI) to less toxic Cr (III). Considering the toxicity of chromium on one hand and importance of PGPR in reducing the chromium toxicity on the other hand, the metal tolerant *P. aeruginosa* CPSB1 was assayed further for its ability to reduce toxic hexavalent chromium to less toxic form at different- (i) chromium concentration (ii) temperature and (iii) pH. The results revealed 84.9% reduction of Cr (VI) by *P. aeruginosa* CPSB1 after 24h growth at 35°C which significantly increased even further to 99.6% after 48h bacterial growth. Chromium was however, completely reduced after 120 h growth. While comparing the impact of pH on Cr (VI) reduction, maximum reduction by *P. aeruginosa* CPSB1 was observed at 100µg Cr/ml (Figure 5a) at pH 8 (Figure 5b) and 35°C after 120h (Figure 5c). Generally, Cr reduction was poor at acidic pH compared to neutral or slightly alkaline pH. These optimum conditions determined for Cr reduction were in accordance with findings reported by other workers [50,51].

Peaks in untreated control	Peaks in metal loaded biomass of *P. aeruginosa* CPSB1(Wavenumber cm^{-1})				Assignments	Reference
	Cr	Pb	Ni	Zn		
3287-3404	3297-3419	3295-3424	3292-3429	3297-3431	H bonded and free OH of alcohol	[52]
2963	2970	2959	2959	2961	CH_3 asymmetric stretching from lipids, proteins, polysaccharides and nucleic acids	[53]
2914	2930	2933	2929	2930	Stretching vibrations of CH_2 & CH_3 of phospholipids, cholesterol	[54]
2859	2860	2859	2854	2860	Alkyl and CHO group	[55]
1659	1662	1658	1661	1662	Alkenes	[40]
1543	1552	1548	1548	1552	COO^- of the carboxylate group	[55]
1464	1462	1458	1458	1457	CH_2 scissoring mode of the acyl chain of lipids	[56]
1409	1407	1406	1404	1406	COO^- symmetric stretching from amino acid side chains and fatty acids	[53]
1239	1237	1239	1239	1243	Amino groups	[40]
1069	1087	1065	1083	1085	C–O (or) SO and PO stretching vibrations	[57, 58]

Table 3: FT-IR assignments of *Pseudomonas aeruginos*a CPSB1 in the presence and absence of heavy metals.

Figure 5(a): Reduction of Hexavalent Chromium by *P. aeruginosa* CPSB1 in the Presence of (●) 25 µg/ml (°) 50 µg/ml (▼) 100 µg/ml (Δ) 200 µg/ml and (■) 400 µg/ml of Cr (VI).

Figure 5(b): Reduction of Hexavalent Chromium by *P. aeruginosa* CPSB1 at Varying pH after different Time Intervals.

Figure 5(c): Reduction of Hexavalent Chromium by *P. aeruginosa* CPSB1 at Varying Temperatures after (●) 24 h (°) 48 h (▼) 72 h (Δ) 96 h (■) 120 h.

Conclusion

This study demonstrated that *P. aeruginosa* CPSB1 recovered from metal polluted chilli rhizosphere tolerated different heavy metals and also displayed resistance to multiple antibiotics. Also, the ability of *P. aeruginosa* CPSB1 to produce plant growth promoting active biomolecules was retained even in the presence of higher rates of heavy metals. However, cytotoxic damage, alteration in cell surface functional groups and uptake of metals by *P. aeruginosa* CPSB1 were revealed by SEM, FT-IR and EDX, respectively.

Moreover, *P. aeruginosa* CPSB1 very effectively reduced the hexavalent chromium which differed with chromium concentration, temperature and pH. Conclusively, *P. aeruginosa* CPSB1, due to its inherent dual properties of multi metal tolerance and multiple antibiotic resistance and ability to produce active plant growth promoting biomolecules and to reduce the toxicity of hexavalent chromium could be developed as a novel bioinoculant for enhancing the production of crops in soils even contaminated with metals.

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Conflict of Interest

There is no conflict of interests.

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