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Research Article

Heavy metal, salinity and azo dye tolerant, Cr (VI) reducing, plant growth-promoting *Pseudomonas aeruginosa* R32 reverses Cr (VI) biotoxic effects in *Vigna mungo*

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Abstract
Hexavalent chromium [Cr (VI)], derived from various industries, including fly ash from coal-based Thermal Power Plants, can be a source of toxic pollution of land and water bodies. This study aimed to bioremediation of such pollutant dump sites using bacteria capable of both Cr(VI) reduction and plant growth-enhancing substance production. The bacteria were isolated from the rhizospheric fly ash of a Thermal Power Plant, Kanpur. One of the rhizospheric isolate, *Pseudomonas aeruginosa* R32 showed high minimum inhibitory concentration (MIC) for Cr(VI) (1250 µg/ml), heavy metal tolerance ($ZnCl_2$, $CdCl_2$, $Pb(NO_3)_2$) up to 100 µg/ml, Acid Red 249 (AR) tolerance and halotolerance (6% NaCl). The isolate R32 also produces plant growth-promoting (PGP) hormones in the absence or presence of Cr (VI). R32 could completely reduce Cr(VI) at a tested dose of 100 and 500 µg/ml after 24h and 72h, respectively. However, decolorization of AR was observed after 48 hours at an initial concentration of 100 µg/ml and confirmed by Fourier transform infrared spectroscopy analysis. *Vigna mungo* seed inoculation with isolate R32 showed increased rootling growth compared to shoot after 7 d treatment with 0, 100, 500, and 1000 µg/ml of Cr(VI) concentrations, respectively. Root length tolerance index in Cr(VI) treated *V. mungo* seedlings was reduced to 56%, 35%, and 29%, respectively, when treated with 100, 500, and 1000 µg/ml Cr(VI) in comparison to control. Cr(VI) sub-MIC concentrations can affect the plant growth-promoting properties of rhizospheric bacteria. Herein, we report the isolation of rhizospheric bacteria *P. aeruginosa* R32 showing concurrent PGP substance production and Cr(VI) bioreduction capabilities in the presence of PGP inhibitory Cr(VI) concentrations.

Keywords: Cr(VI) bioreduction, Heavy metal toxicity, Plant growth promotion, *Pseudomonas aeruginosa*, *Vigna mungo*

INTRODUCTION

Hexavalent chromium [Cr(VI)] is a water-soluble toxic heavy metal with mutagenic, teratogenic, and carcinogenic properties due to its high oxidizing properties (Oliveira, 2012). One of the major sources of Cr(VI) contamination is fly ash derived from coal combustion in thermal power plants (Gianoncelli *et al.*, 2013; Mohanty and Patra, 2015; Verma *et al.*, 2016). Fly ash generated from coal-based power plants is typically disposed of using either dry techniques such as embankments or reused in the manufacture of cement-based products such as bricks etc., or deposited in ash ponds (Senapati, 2011).

There is a growing concern over the environmental pollution created by fly ash pollutant leaching in surrounding water drainage bodies, soils, and air and its effect on the health of all life forms (Alterary and Marei,

2021). Two findings in a U.S. Environmental Protection Agency (USEPA) report regarding chromium in coal ash are alarming and state that "nearly 100% of chromium leaching from coal ash is hexavalent chromium" and "chromium leachate exceeds EPA threshold for hazardous waste at 5000 ppb" (Lisa Evans, 2011). The use of fly ash as an additive to agricultural soil to improve soil properties, nutrient levels (K, Na, Mg, Zn, Fe) and crop productivity is also being considered where heavy metal concentration (Cr(VI), Cd, Pb etc) remains a problematic issue (Wong and Wong, 1986).

In addition to physico-chemical methods for reducing toxic Cr(VI), several biobased remediation strategies, such as microbes and plant-based bioremediation are promising, eco-friendly, and effective (Kour *et al.*, 2021). Phytoremediation enhanced by microbial bioreduction uses diverse chromate-resistant microorganisms to reduce Cr(VI) to relatively less toxic, less reactive, and insoluble Cr(IV) and Cr(III) speciation forms and form an important strategy for ensuring environmental sustainability (Kumar *et al.*, 2016). Bacterial tolerance and subsequent reduction of Cr(VI) has been reported by several mechanisms, which include intracellular or extracellular chromate reductase enzyme activity, non-enzymatic redox reduction by bacterial surfaces, bioaccumulation in biofilms, extrusion by chromate efflux pumps (Viti *et al.*, 2014; Thatoi and Pradhan, 2018). The role of plants in the phytoremediation of pollutants using methods of phytostabilization, phytovolatilization, phytodegradation, and phytoextraction has been reported (Parveen *et al.*, 2022). Additionally, integrated bacteria-plant-mediated remediation of Cr(VI) is now being explored (Kumar *et al.*, 2016). Resident bacteria of plant rhizospheric regions also produce plant growth-promoting (PGP) substances, such as indole acetic acid, siderophores, and mineral solubilizing enzymes, that help in plant growth, development and enhance immunity to toxic substances, including remediation potential (Khatoon *et al.*, 2020). Concurrent chromate reducing and PGP bacteria are potentially useful in microbe-assisted phytoremediation of contaminated soil and water from effluents of tanneries, fly ash, and electrochemical industries. A major drawback to augmenting polluted sites with bioremediating bacteria is their low in-field survival rates in the face of several environmental variations and stressors (Bodor *et al.*, 2020). The isolation of hardy bioremediating species that can withstand multiple stresses such as halostress, cross heavy metal as well as industrial azo dye compounds can ensure long term survival and bioremediation efficacy of the strains in contaminated agricultural soils (Tiwari and Lata, 2018). The present study aimed to isolate Cr(VI) reducing and PGP substance producing bacteria from fly ash exposed rhizospheric soils around Panki Thermal Power Plant, Kanpur, and their characterization for use in Cr(VI) bioremediation.

MATERIALS AND METHODS

Sampling, microbial isolation and characterization

Samples were obtained from fly ash-contaminated rhizospheric soils around the Panki Thermal Power Plant, Kanpur (Uttar Pradesh) (26.4705° N, 80.2407° E). Samples were serially diluted and plated on tryptone soya peptone agar (TSA) supplemented with 100 µg/ml Potassium dichromate ($K_2Cr_2O_7$) by standard spread plate method. Microbial identification until genus level was performed using biochemical characterization as per Bergey's determinative manual (Holt *et al.*, 1994). Media and analytical chemicals were procured from HiMedia and Merck, India.

Minimum inhibitory concentration (MIC), Chromate reduction assay, Surface FTIR spectroscopy

Isolates were subcultured on Trypticase Soya Agar growth medium supplemented with increasing concentrations of Cr(VI) up to 1250 µg/ml. The bacterial growth was recorded until day 5 post-inoculation to determine the MIC value of Cr(VI). The reduction of Cr (VI) was determined using the 1,5 diphenyl carbazide method (Sagar *et al.*, 2012). Briefly, Tryptone soya peptone broth (TSB) media with varying concentrations of Cr(VI) was inoculated with 24 h log culture (10% v/v) of different microbial isolates. The samples from bacterial cultures were centrifuged at 10,000 rpm for 10 min. The supernatants were acidified using 6N H_2SO_4 followed by the addition of 25% (w/v) of 1, 5 diphenyl carbazide in acetone and the Cr(VI) presence was determined by measuring absorbance at 540 nm wavelength using a UV-Vis spectrophotometer (Thermo Scientific, Spectronics). The functional groups on the particle surface responsible for the Cr(VI) adsorption were identified by Fourier transform infrared spectrometric (FTIR) analysis (Quintelas *et al.*, 2008). Samples for FTIR measurement were prepared by exposing an exponentially grown culture of R32 isolate with 0, 100, and 500 µg/ml Cr(VI) for 24 h. Following exposure, a bacterial pellet was prepared by centrifugation (10,000 rpm for 10 min) followed by washing with ddH_2O. The spectra of control and Cr(VI) treated bacterial pellets were recorded within 4000 to 500 cm^{-1} using FTIR spectrometer (Tensor 27 Bruker, Germany) at the Center of Environmental Science and Engineering, Indian Institute of Technology, Kanpur.

Antibiotic sensitivity

Antibiotic susceptibility tests for each of Cr(VI) resistant isolates were performed by Kirby Bauer disk diffusion method as per CLSI nomenclature (Humphries *et al.*, 2018). Mueller Hinton Agar plates (HiMedia, Mumbai) were spread with cultures and antibiotic disc (HiMedia, Mumbai) applied and incubated at 37 °C. The inhibition zones were measured after 18-24 h. Isolates were

characterized as resistant, moderate and sensitive compared with standard CLSI charts. The antibiotics tested in the assay were: A/S - Ampicillin Sulbactam; Ac- Amoxycillin Clavulanic acid; Ce- Cefotaxime; Cs- Cetoperazon Sulbactum; Cu- Cefuroxime; Pt- Piperacillin/Tazobactum.

Tolerance of Cross heavy metal, NaCl, and Acid Red (AR)

Heavy metal tolerance was determined for each isolate in TSA medium supplemented with 100 µg/ml of different heavy metal ions ($ZnSO_4$, $CdCl_2$, $HgCl_2$, $Pb(NO_3)_2$) and 100 µg/ml of Cr(VI). Plates were incubated at 30 ° C for up to 72 h and bacterial growth was measured as described above. For halotolerant measurement, the isolated bacteria were plated and grown on the TSA medium supplemented with an increasing concentration (1-8%) of NaCl, and the growth was measured. The isolates were cultured on TSA media amended with 100 µg/ml AR and the ability to grow and decolorize dye was observed.

Decolorization and degradation analysis of Acid Red (AR)

AR decolorization and degradation were analyzed by UV-Vis spectrophotometer and FTIR, respectively. TSB media was amended with 100 µg/ml each of AR and Cr (VI) and inoculated with log phase culture (10% v/v) of R32 for 48 h. The bacterial culture was centrifuged at 10,000 rpm for 10 min. AR decolorization efficiency of R32 in the supernatant was analyzed using a UV-Vis spectrophotometer (MULTISKAN EX, Thermo Fisher Scientific, USA) at a fixed wavelength (520 nm). Further, FTIR analysis was carried out in control (uninoculated AR media) *versus* R32 treated and the spectrum was recorded at a range 4000 to 500 cm^{-1}.

Plant growth promontory (PGP) activities

The following PGP tests were performed in the absence and presence of 100 µg/ml of Cr(VI).

Phosphate solubilization

Phosphate solubilization was checked on Pikovskaya's medium by measuring the clearance zone (Pikovskaya, 1948). The quantitative bioassay was carried out in Erlenmeyer flasks (100 ml) containing 10 ml of National Botanical Research Institute's phosphate (NBRIP) broth medium inoculated with the 10% bacteria cell (Nautiyal, 1999). The flasks were incubated for 5 d at 30 °C on a shaker at 120 rpm. Available phosphorus content in the filtered supernatant and control (supernatant obtained without bacteria inoculation) was estimated using the vanado-molybdate colorimetric method by measuring the absorbance at a wavelength of 450 nm.

Indole acetic acid (IAA)

Indole acetic acid production was tested using tryptone broth containing 0.1% tryptophan. The medium was incubated with 10% log culture (1×10^7 cell/ml) for 2 days at 30 °C with shaking. The bacterial culture was centrifuged at 10,000 g for 10 minutes. IAA production was analysed using 1 ml of supernatant mixed with 2 ml of Salkowasky's reagent, and the optical density was measured at 550 nm (Patten and Glick, 1996).

Hydrogen cyanide (HCN)

Qualitative test was performed on Kings' B agar media by spreading isolates and observing a colour change in Whatman filter paper soaked with 0.5% picric acid and 2% Na_2CO_3 in the petri dish lid from yellow to brown as a positive test for HCN production as described previously (Lorck, 1948).

Siderophore

Screening of siderophore production was performed on Chrom Azurol Media (Schwyn and Neilands, 1987). Siderophore production by the isolates was tested quantitatively by a CAS-shuttle assay. Succinate medium inoculated with log phage culture was incubated for 48 h at 30 ºC with constant shaking at 120 rpm. Centrifuged cell-free supernatant was mixed with 0.5 ml of CAS reagent, and the absorbance was measured at 630 nm against a reference. Siderophore unit in aliquot was calculated by using the following formula: [(Ar – As)/Ar] x 100, where Ar is the absorbance at 630 nm of reference (CAS assay solution+ uninoculated media) and As is the absorbance at 630 nm of the sample (CAS assay solution + supernatant) (Payne, 1994).

Ammonia production

Ammonia production was determined using peptone water. The bacterial isolates were inoculated in peptone water at 30 ºC for 2 days. Following incubation, 1 ml of Nessler's reagent was added into each tube and the development of yellow color indicates ammonia production (Wani *et al.*, 2008).

Biofilm formation

Biofilm formation was determined using Congo red agar (CRA) and crystal violet assays with some modification (Melo *et al.*, 2013). Briefly, isolates were streaked on CRA, and the presence of black precipitate was recorded as an indication for the exopolymer production in the biofilm. For the crystal violet assay, 500 µl of log-phase cultures collected in microcentrifuge tubes were incubated at 30 ºC for 24h. Following incubation, the cultures were stained with crystal violet (1% w/v) dye for 20 min. Next, an excess crystal violet dye was washed and dimethyl sulphoxide (DMSO) was added to mobilize crystal violet stained bacteria, and

absorbance was measured using UV-Vis Spectrophotometer at 630 nm wavelength.

Molecular characterization of R32

Isolated genomic DNA of R32 isolate was amplified by PCR using a universal 16S rDNA bacterial primer set (Bangalore Genei, India). The DNA sequence obtained was analyzed at National Center for Biotechnology Information server using BLAST tool and the corresponding sequences were downloaded (www.ncbi.nih.nlm.in). The phylogenetic tree was constructed using the neighbor-joining method in MEGA X at the bootstrap value of n = 500 (Kumar *et al.*, 2018).

Plant bioassay

The experiment was performed to evaluate the toxic effects of Cr(VI) on seed germination and seedling development of *Vigna mungo* in the presence or absence of R32 isolate (Sagar *et al.*, 2012). Healthy seeds were firstly surface sterilized in a solution of 0.1% $HgCl_2$ and then washed with sterile water. Log phase culture (10^8 CFU /ml) was used to inoculate sterile seeds, while in control treatments, seeds were placed in sterile saline water for a duration of 20 min. 2 ml of varying $K_2Cr_2O_7$ concentrations (0, 100, 250, 500, and 1000 µg/ml) was added in autoclaved Petri plates lined with Whatman filter paper. Petri plates were placed at room temperature for germination at 28 ºC for 7 d and moisture content was maintained by adding 2 ml sterile water on alternate days. After 1 week, seedlings were harvested to determine shoot length, root length, and fresh biomass. Tolerance indices (TI) of seedlings were determined with the help of the following formula.

T.I.= Mean root of treated seedling/ mean length of control seedlings x 100 Eq.1

Statistical analysis

Experiments were performed in triplicates. Student's t-test and One-way ANOVA were used wherever applicable. The results were considered significant when $p \leq 0.05$. Statistical analysis was carried out using Graph Pad Prism software (version 6.0, USA).

RESULTS AND DISCUSSION

Microbial screening for Cr(VI) tolerant and plant growth-promoting isolates

The preliminary microbial screening for Cr(VI) tolerant and plant growth-promoting isolates showed that 1 g of soil contained about 56 isolates, of which Gram-positive bacilli (30/56) predominated, followed by Gram -positive cocci (11/56), Gram-negative bacilli (10/56), and Gram-negative cocci (3/56). Fifty-six isolates were screened for their ability to produce plant growth promontory products such as siderophores, indole acetic acid (IAA), hydrogen cyanide (HCN), ammonia, as well as potassium and phosphate solubilization activity using qualitative assays as described in materials and methods. Fig 1 shows that in the absence of Cr(VI), 35.7% were IAA-producing isolates and 39.28% were siderophores producers, while 19.6% and 16% isolates produced IAA and siderophores respectively, in the presence of 100 µg/ml Cr(VI). Only 12.5% isolates solubilized phosphate in the presence of Cr(VI), in contrast to 23.3% in the absence of Cr(VI). Approximately 50% of the isolates showed inhibition in producing IAA, siderophores, and phosphate solubilization enzymes in the presence of Cr(VI). The inhibitory effect of Cr(VI) presence was not observed on ammonia and HCN production.

Based on their ability to produce PGP products in the presence of 100 µg/ml Cr(VI), six isolates (R15, R27, R30, R31, R32, and R34) were selected for further characterization. In the absence and presence of 100 Cr(VI), IAA production has been shown by R15, R30, R32, and R33; siderophore production by R27, R30, R31, R32, R33 and R34; HCN production by R32, R33; ammonia production by R15, R27, R30, R31, R32, R33 and R34 ; and phosphate solubilization by R32 and R33. According to Bergey's manual of determinative bacteriology, microbiological and biochemical characterization was performed for genus-level identification (Holt *et al.*, 1994). Isolates R15 belonged to *Bacillus* species, R27 and R34 to *Proteus* species, R30 was an unidentified Enterobacteriaceae, R33 and R32 to *Pseudomonas* species, and R31 was a Gram-positive *Staphylococcus* species. Microbial characterization of bacteria from fly ash samples capable of heavy metal tolerance and PGP products have been reported previously. In a previous study, *Staphylococcus pasteuri* isolated from contaminated fly ash sites were reported to be resistant to heavy metals and capable of producing indole acetic acid, siderophores, and phosphate solubilizing enzymes (Roy *et al.*, 2017). The fly ash-derived

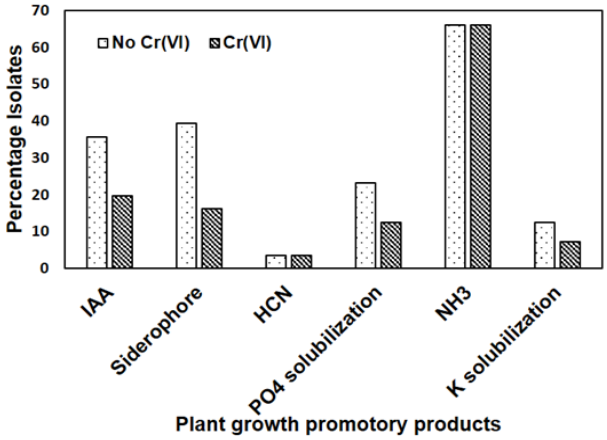

Fig. 1. *Percentage plant growth promoting isolates from fly ash exposed rhizospheric soil samples in the presence and absence of 100 µg/ml Cr(VI)*

Table 1. Minimum inhibitory concertation (MIC) for Cr(VI), antibiotic sensitivity, biofilm formation, cross heavy metal tolerance, halotolerance, acid red tolerance and decolorization

Isolates ↓	$K_2Cr_2O_7$ [Cr(VI)] µg/ml					Antibiotic Sensitivity		$K_2Cr_2O_7$ [Cr(VI)] 100 µg/ml						
								Cross Heavy Metal Tol^{ce} 100 µg/ml				Acid Red 100 µg/ml		Halo-Tol^{ce}
Concentration →	500	750	1000	1250	1500		CRA$^{\#}$ Biofilm	$ZnCl_2$	$CdCl_2$	$Pb(NO_3)_2$	$HgCl_2$	Tol^{ce}	D 48 h	NaCl (%)
R15	++	+	-	-	-	$A/S^SAc^RCe^SCs^SCu^SPt^S$	-	+	+	+	-	+	-	6
R27	++	+	-	-	-	$A/S^SAc^RCe^SCs^SCu^RPt^S$	+	+	+	+	-	+	-	6
R30	++	-	-	-	-	$A/S^SAc^RCe^SCs^SCu^RPt^S$	-	+	+	+	-	+	-	6
R31	++	+	+	+	-	$A/S^SAc^RCe^SCs^SCu^RPt^S$	-	+	+	+	-	+	-	8
R32	+++	++	+	+	-	$A/S^RAc^RCe^SCs^SCu^RPt^S$	+	+	+	+	-	+	+	6
R33	+	+	+	-	-	$A/S^RAc^RCe^SCs^SCu^RPt^S$	-	+	+	+	-	+	-	6
R34	+	+	-	-	-	$A/S^SAc^RCe^SCs^SCu^RPt^S$	-	+	+	+	-	+	-	8

+++: Better growth; ++:Intermediate growth; +: Poor growth; -: no growth; #CRA: Congo Red Assay; Tolce: Tolerance; D: Decolorization; AS: Antibiotic Sensitivity, A/S: Ampicillin Sulbactam; Ac: Amoxyllin clavulanic acid; Ce: Cefotaxime; Cs: Cetoperazon sulbactum; Cu: Cefuroxime; Pt:Piperacillin/Tazobactum; R:Resistant; S: Sensitive

isolates *Bacillus anthracis* from the rhizosphere of *Saccharum spontaneum* was shown PGP features like the production of ammonia, siderophore, hydrocyanide, and indole acetic acid (Mukherjee *et al.*, 2017).

Cr(VI) MIC, Biofilm formation, Cross heavy metal, Acid red, halo tolerance and antibiotic sensitivity

The six isolates were subsequently adapted to grow on higher Cr(VI) concentrations in a successive increase of 250 µg/ml of Cr(VI) until no further growth was observed. Table 1 shows that R31 and R32 had strong growth and Cr(VI) tolerance up to 1250 µg/ml concentrations, while most isolates could tolerate only up to 750 µg/ml Cr(VI). Two isolates R27 and R32 showed the ability to form biofilms based on Congo red agar plates. All the isolates could tolerate cross-heavy metals (100 µg/ml of $ZnCl_2$, $CdCl_2$, $Pb(NO_3)_2$) in the presence of 100 µg/ml Cr(VI) except for $HgCl_2$. All the isolates were cross inoculated to test for any antagonistic action and none were observed (data not shown). The ability of the isolates to tolerate high salt concentrations was also checked, as textile dye and leather effluents are rich in brine and a source of Cr(VI) pollution (Garg *et al.*, 2012). All the isolates could tolerate up to 6% salinity, while R31 and R34 tolerated up to 8% salinity (Table 1). Industrial pollution is also marked by recalcitrant azo dyes that are difficult to degrade and cause phytotoxicity (Ngo and Tischler, 2022). Although all isolates showed growth on TSA amended with 100 µg/ml AR, only R32 was found to show a zone of decolorization in 48 h. The use of indigenous microorganisms that are adapted to extreme environments shows higher tolerability and efficacy and is useful for environmental bioremediation due to their inherent stability to environmental stressors (Tahri Joutey *et al.*, 2015). High tolerance levels to metals, azo dyes, and salts are also acquired by the ability to form biofilms that protect against environmental stressors (Mohapatra *et al.*, 2019). Since many of the rhizospheric isolates belonged to Pseudomonads and the Enterobacteriaceae group, antibiotic sensitivity was tested using the disk diffusion assay, and results are interpreted as per CLSI norms (Table 1). 100% resistance was reported for Amoxycillin clavulanic acid, 75% resistance for Cefuroxime, and 25% for Ampicillin Sulbactam. All the isolates were sensitive for Cefotaxime, Cetoperazon sulbactum, and Piperacillin/Tazobactum. The increasing antibiotic resistance amongst environmental gram-negative bacteria isolated from rhizospheric soils is a cause of concern (Waseem *et al.*, 2017).

Quantification of PGP activity

Experiments were designed to test the effect of Cr(VI) on growth as well as the ability of select isolates to produce indole acetic acid, siderophores, and phosphate solubilization at different time points post-inoculation. Fig 2a shows that 100 µg/ml Cr(VI) concentration significantly affected IAA production in R15 and R30 without affecting growth, while no effect was observed on IAA production of R32 and R33. Fig 2b shows the repressive effect of Cr(VI) on phosphate solubilization in a time-dependent manner for isolates R32 and R33 (phosphate solubilizing isolates). Fig 2c compares siderophore production in the absence and presence of 100 µg/ml Cr(VI) for isolates found to be positive in the qualitative test. The influence of 100 µg/ml Cr(VI) on siderophores production differed amongst the isolates. It remained unaffected in isolates R27, R32, and R33 while showing a significant decrease in R30, R31, and R34 isolates. Fig 2d shows the ability of the isolates to form biofilms in the absence or presence of 100 µg/ml Cr(VI). 71.4% of the isolates showed decreased biofilm formation ability, while isolates R30 and R31 were unaffected. The presence of stress in the form of 100 µg/ml Cr(VI) causes negative regulation of PGP such as IAA, siderophore, and phosphate solubilization even in isolates that tolerate and grow in the presence of 100 µg/ml Cr(VI). Hence, it can be concluded that in a general population, 100 µg/ml Cr(VI) concentration can also decrease PGP abilities of rhizospheric organisms without affecting their growth. Further, we report that even though 100 µg/ml and 250 µg/ml Cr(VI) were completely reduced by bacterial isolates, PGP production was inhibited even when the bacteria showed growth in the absence of Cr(VI) or reduced Cr(VI) in the media. This was surprising as chromate reduction results suggest that Cr(VI) has been completely reduced to less toxic species at 100 µg/ml or even higher concentrations . Hence, Cr(VI) stress, even at sub-lethal concentration or its reduced by-products, may cause the repression of PGP genes, which may reduce the efficacy of such biofertilizer formulations. In the present experiments, the log phase inoculum was always maintained in TSA media containing 100 µg/ml Cr(VI). Subsequently, PGP production was studied using such isolates in media containing no or increasing Cr(VI) concentrations. The results showed that re-inoculation of bacteria in media free of Cr(VI) restored PGP production abilities whereas sub-lethal Cr(VI) concentrations or its reduced by-products showed concentration-dependent repression of PGP production. Similar biotoxic effects of Cr(VI) have been reported on PGP production except for exopolysaccharide production in *Cellulosimicrobium funkei* Strain AR8 (Karthik and Arulselvi, 2017). In indole acetic acid and phosphate solubilization were reduced in the presence of 100 µg/ml and 150 µg/ml Cr(VI) in *Bacillus* sp. (Wani and Khan, 2010). Cr(VI) toxicity may also be causing transcriptional downregulation of PGP producing genes due to oxidative stress (Ackerley *et al.*, 2006) . The PGP production was found to be suppressed when *Pseudomonas aeruginosa* ATCC P15442 (P15) was treated with 100 µg/ml Cr(VI); however, no effect on the growth and bioremediation ability (Kumar *et al.*, 2020). Hence, the release of effluents with permissible Cr(VI) concentrations may cause a reduction in soil fertility by affecting the PGP production of microbial isolates without affecting their growth. Therefore, the present study brings forth the importance of testing the PGP inhibitory concentration of Cr(VI) for bioremediation candidate organisms in addition to MIC of heavy metals only for growth.

Cr(VI) bioreduction ability

The presence of growth on Cr(VI) amended media only indicates the isolates' ability to tolerate high concentrations of heavy metals. Growth of the isolates in the presence of 100 and 500 µg/ml Cr(VI), respectively, as well as the ability of the isolates to reduce Cr(VI) was determined using diphenyl carbazide assay. As indicated in Fig 3a, the isolates R32 showed a similar growth profile in media control and amended with 100 µg/ml Cr (VI); however, reduction in growth at 500 µg/ml Cr(VI) during 72 h incubation. The isolate took longer to grow, likely due to the initial bioreduction of toxic Cr(VI) to concentrations that supported their growth. As seen in Fig 3b, the isolate R32 could completely reduce 100 µg/ml Cr(VI) in 24 h, while at 500 µg/ml Cr(VI) the isolate showed complete reduction at 72 h post-treatment.

Molecular characterization of R32 Isolate

Amongst the screened bacteria, PGP-producing R32 isolate was capable of Cr(VI) bioreduction as well as AR decolorization. Molecular characterization of the rhizobacterial strain R32 was performed by PCR amplification of genomic DNA using a universal 16S rDNA bacterial primer set (Bangalore Genei, India) and its sequencing. The phylogenetic tree was constructed using the neighbor-joining method in MEGA X. R32 was identified as *P. aeruginosa* R32 based on the 16 S rRNA gene sequence analysis and is submitted with NCBI GenBank accession number MK779771 (Fig 4).

FTIR spectroscopy cell surface of R32

FTIR spectroscopy of untreated control, 100 and 500 µg/ml Cr(VI) exposed R32 was carried out to identify functional groups and chemical bonds that play a role in Cr(VI) adsorption on the R32 cell surface (Fig 5). Strong absorption bands were observed at 844, 1643 and 3659 cm^{-1} for both Cr(VI) treated and controlled untreated cells indicating symmetric stretching of cell

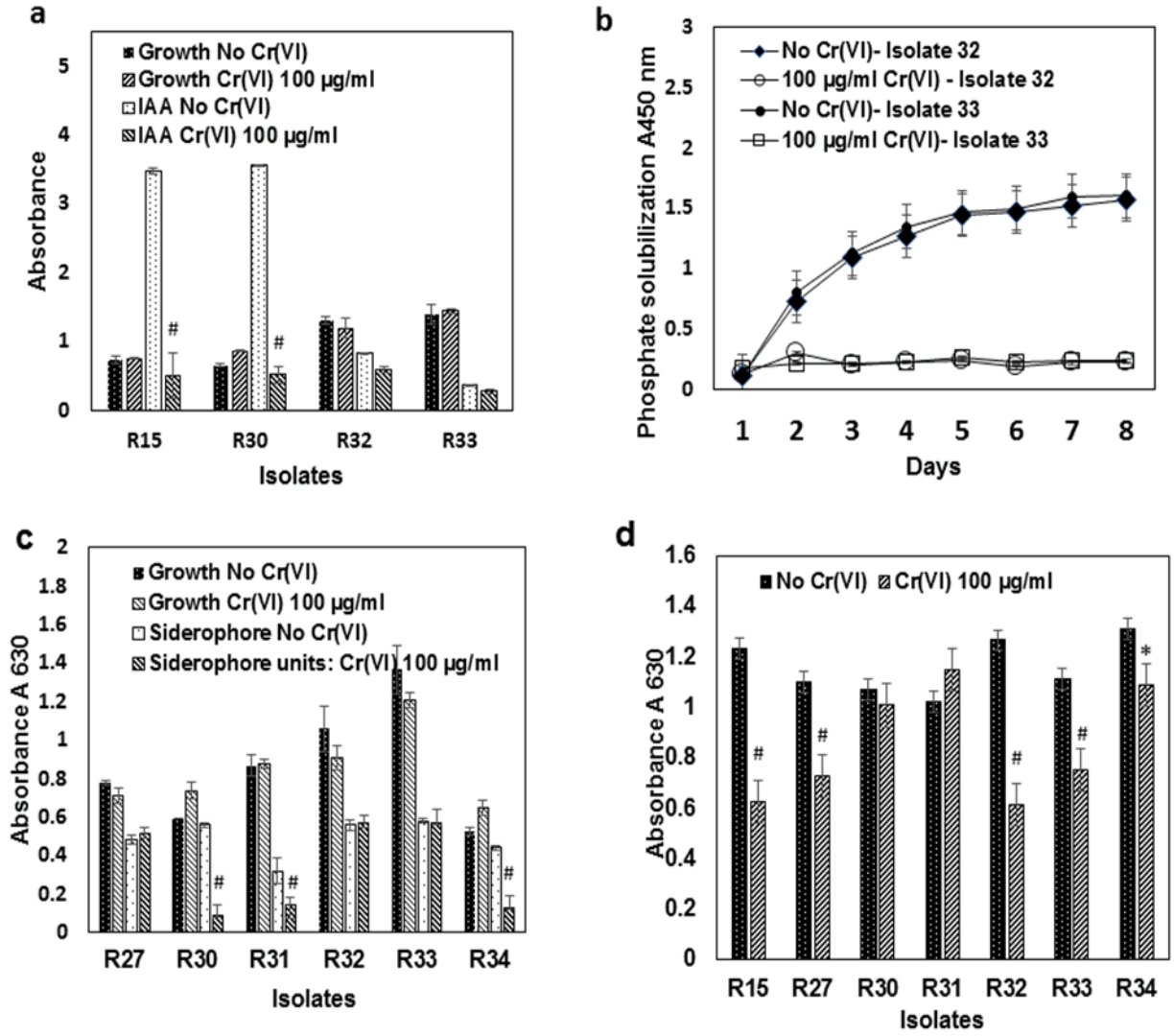

Fig. 2. *Effect of 100 µg/ml Cr(VI) on the production of PGP and growth in rhizospheric isolates. (a) IAA in tryptone broth with 0.1% tryptophan at 2 days post inoculation (b) phosphate solubilization in NBRIP media (c) siderophore production in CAS media at 2 days post inoculation (d) 24h biofilm formation in the absence and presence of Cr(VI) in TSB media. Data are mean of three replicates with standard deviation. * p ≤ 0.05, # p ≤ 0.001*

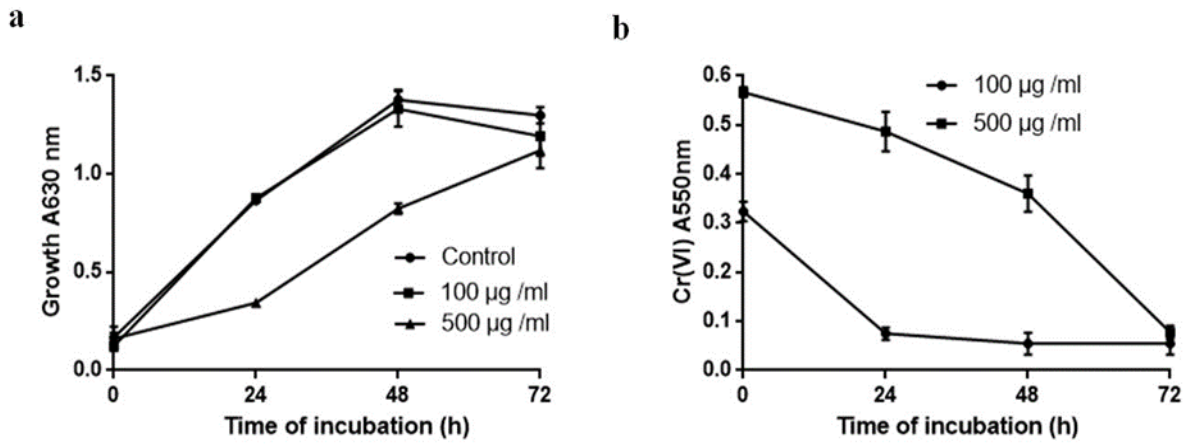

Fig. 3. *(a) Effect of 100 µg/ml and 500 µg/ml Cr(VI) on growth of isolate R32 (b) Chromium reducing ability of R32 in media amended with 100 and 500 µg/ml Cr(VI) at 24, 48 and 72 h post incubation. Data are mean of three replicates with standard deviation*

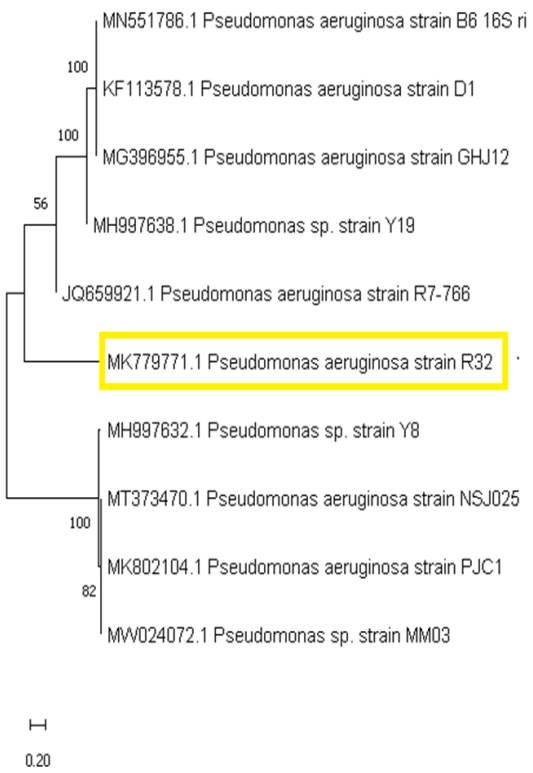

Fig. 4. *Phylogenetic tree showing the relationship of Pseudomonas aeruginosa (MK779771.1) with other closely related bacterial strain. The 16S rRNA gene sequence of closely related species was obtained from NCBI Genbank database. The tree was constructed using neighbor- joining method of Mega X, at the bootstrap value of n = 500.*

surface proteins, lipids, and polysaccharides. In 100 and 500 µg/ml Cr(VI) treated R32, an increase in absorption peaks at 997, 1491, 3568, and 3777 cm^{-1}, indicate Cr(VI) chelating with carboxyl groups, alkene stretches, nitro groups, amino groups, and hydroxyl groups of cell surface polysaccharides and proteins. Similar Cr(VI) adsorption properties have been reported in the case of *Bacillus coagulans* biofilm (Quintelas *et al.*, 2008). The strong absorption peak at 3659 cm^{-1} indicates the binding of chromium metal with functional groups exhibited on the surface of the bacterial cell (Mishra *et al.*, 2021).

AR decolorization and Biodegradation analysis

AR decolorization in TSB containing 100 µg/ml of AR and Cr(VI) was found to be 99% in 48 h of the incubation period (Fig 6a). Furthermore, biodegradation by the isolate was confirmed by scanning the control and decolorizing AR+Cr(VI) supernatants using ATR FTIR spectroscopy. A shift in FTIR spectra within the range of wavenumber 500-4000 cm^{-1} of decolorized AR+Cr (VI) depicted changes in functional groups after decolorization compared to untreated sample (Fig 6b). The FTIR spectrum of control dye AR showed peaks at 1562, 2365, 3443, and 3642 cm-1, representing N=N stretching, N-H+ stretching, N-H stretching and O-H stretching, respectively. In decolorized samples, these peaks were absent and peak shifts were found at 2924, 3082, 3137, 3310, and 3468 cm^{-1} indicating AR degradation. Peaks appeared at 3673 cm^{-1} (-OH groups), 3418 cm^{-1} (secondary amide), and 2924 cm^{-1} (C-H stretching), further confirming the decolorization of AR. Similar observations using FTIR have been shown elsewhere in *Pseudomonas putida* treated with monoazo dye orange II, where peaks around 3500-m^{-1} indicate to the -OH and -NH_2 groups in decolorized samples (Kumar Garg *et al.*, 2012). The presence of peaks at 1118, 1637, and 3434 cm^{-1} indicates the production of primary amines and secondary amide during consortia's catabolic degradation of azo dye (Desai *et al.*, 2009). In a previous study, *Pseudomonas* strain WS-D/183 has been shown to cause concomitant decolorization of reactive red 120 and reduction of Cr(VI) (Hussain *et al.*, 2020). Concomitant decolorization of acid Red 249 and reduction of hexavalent chromium by *Klebsiella pneumoniae* SK1 was cultured in a microaerophilic condition within 48 h of incubation (Kumar *et al.*, 2021).

Efficacy of Plant growth-promoting and Chromium detoxification of Isolate R32: Isolate *P. aeruginosa* R32 was assayed for its ability to affect the seed germination and vegetative growth of *Vigna mungo* in the presence of an increasing concentration of Cr(VI) as described in materials and methods. Cr(VI) toxicity was

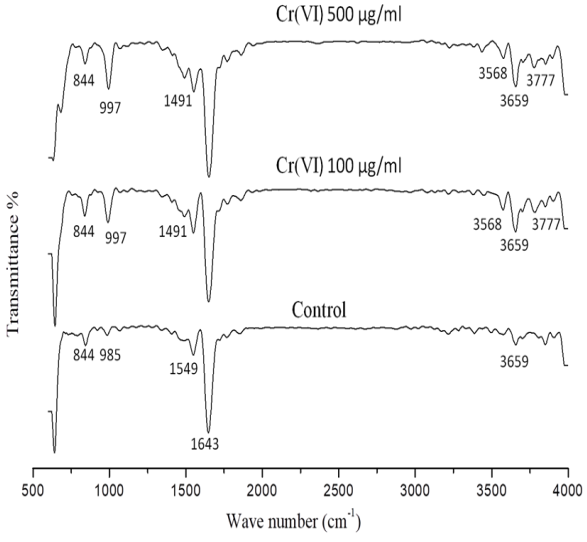

Fig. 5 . *FTIR analysis of control, 100 and 500 µg/ml treated P. aeruginosa R32 cell surface*

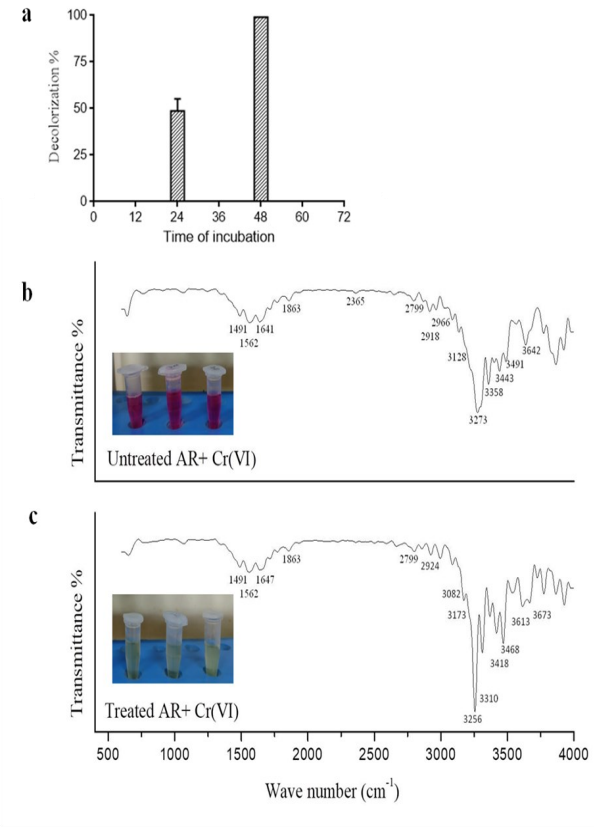

Fig. 6. *(a) AR decolorization in TSB media contain 100 µg/ml AR+Cr(VI). (b) FTIR profile of untreated100 µg/ml AR+Cr(VI) (c) and its decolorized products obtained after 48 hours incubation with R32*

evident wherein shoot length reduction by 22%, 28%, and 43% and root length reduction by 43%, 64%, and 70% was observed in the presence of 100, 500, and 1000 µg/ml Cr(VI) respectively (p≤0.5). According to Fig 7a, 46% increase in shoot length and 127% in root length was observed in 100 µg/ml Cr(VI) treated and R32 inoculated seeds in comparison to 100 µg/ml Cr (VI) treated but non-inoculated control. In the presence of 500 µg/ml Cr(VI), R32 inoculated seeds showed a 38% increase in shoot length and a 133% increase in root length in comparison to the control. R32 inoculated seeds treated with 1000 µg/ml Cr(VI) showed a 50% increase in both shoot length and root length, which was statistically significant compared to non-inoculated 1000 µg/ml Cr(VI) treated seeds. These results indicate that Cr(VI) bioreduction by the isolate, in addition to PGP activities, was more beneficial for root development than shoot production. The tolerance index (per cent ratio of root length of Cr(VI) treated versus untreated seeds) in seedlings of *V. mungo* to Cr(VI) treatment were reduced with the values 56%, 35%, and 29 % when treated with 100, 500 and 1000 µg/ml chromium concentration as compared to control, respectively (Fig 7b). The ability of *P. aeruginosa* R32 to produce indole acetic acid (rooting hormones) in addition to HCN and siderophores in the presence of Cr(VI) showed a reversal of toxic effect with similar root and shoot length as well as fresh biomass as observed in water-treated control *V. mungo* seeds. The results indicate that the primary beneficial effect on seed germination was obtained due to the ability of R32 to reduce Cr(VI) and detoxify the environment. The phytohormones not only control morphogenetic response but also reduce phytotoxicity of heavy metals (Saini *et al.*, 2021). The exogenous augmentation of 10 µM indole acetic acid decreased Cr(VI) phytotoxicity in *Pisum sativum*, increasing growth and nitrogen metabolism (Gangwar and Singh, 2011). The production of IAA, siderophore, and solubilization of phosphate and potassium by *Bacillus subtilis* increases the growth of root-shoot and biomass of *Cicer arietinum* in artificially added chromium in a

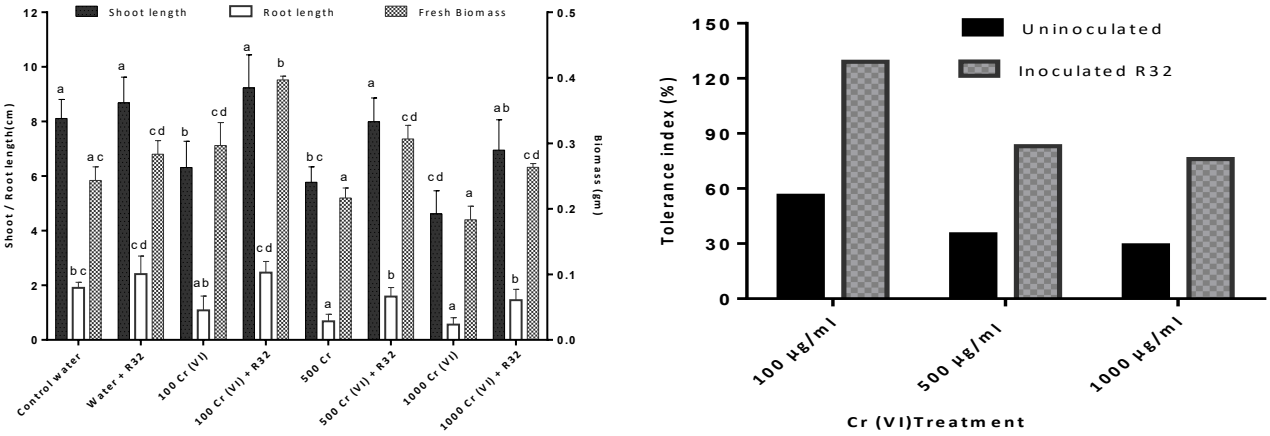

Fig. 7. *(a) Influence of P. aeruginosa (R32) inoculation on shoot length, root length and fresh biomass of Vigna mungo in the absence (control) and in the presence of different concentration (100, 500 and 1000 µg/ml) of Cr(VI). For the same parameters, values with different letters are significantly different (P < 0.05). The multiple comparisons among treatments were made using ANOVA with Tukey's multiple comparison test (b) Percentage of tolerance index in V. mungo using different concentration (100, 500 and 1000 µg/ml) of Cr(VI) in the absence and presence of R32*

pod (Shreya *et al.*, 2020). Bioinoculation consortia of *Bacillus endophyticus*, *Paenibacillus maceranns* and *Bacillus pumilis* into the rhizosphere of *Typha latifolia* was found to enhance phytoextraction of Fe, Cd, Pb, Cr, Ni, Cu and Zn from fly ash (Tiwari *et al.*, 2013). Similarly, PGP-producing *Staphylococcus arlettae* strain MT4 has been shown to positively influence the growth of *Helianthus annuus* L. in the presence of 100 mg/L chromate (Qadir *et al.*, 2020). *Pseudomonas aeruginosa* strain OSG41 was reported to reduce up to 1800 µg/ml Cr(VI) and showed decreased production of indole acetic acid, tricalcium phosphate, and exopolysaccharide with increasing Cr(VI) concentration even as it improved the growth of chickpea plant (Oves *et al.*, 2013). PGP bacteria can indirectly enhance seedling's growth and development by detoxifying Cr(VI) effects and directly by producing PGP substances. These findings suggest that *P. aeruginosa* R32 can help to reduce Cr(VI) phytotoxicity, ameliorate plant growth with PGP products, and be useful for microbe-enhanced phytoremediation processes.

Conclusion

Heavy metal Cr(VI) bioremediation using hardy and indigenous rhizospheric plant growth-promoting and heavy metal-reducing bacterial isolates is currently considered viable and effective for sustainable bioremediation strategies. The present study successfully isolated a bacterial strain *P. aeruginosa* R32 from the fly ash exposed rhizospheric region of a Thermal Power Plant. The strain showed potentially high PGP activities and can enhance the growth of *V. mungo* even in the presence of Cr(VI) at 100 µg/ml. It could completely reverse the toxic effects of Cr(VI) at 100 µg/ml by reducing it to a less or non-toxic species. In addition, the strain showed high tolerance to heavy metals and salinity and decolorizes azo dye Acid red 249. Overall, the isolated *P. aeruginosa* R32 can be a potentially useful candidate for bioremediation of soil and water contaminated with Cr(VI).

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Conflict of interest

The authors declare that they have no conflict of interest.

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