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Characterization of Cd^{2+} Resistant *Pseudomonas aeruginosa* AF2 and Its *In Vitro* Plant Growth Promoting Effects on *Amaranthus viridis* L.

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Abstract: Cadmium (Cd^{2+}) contamination in agricultural soils poses a critical threat to global food security and plant productivity. This study examined Cd^{2+} contamination in soils irrigated with municipal wastewater near an open disposal site in Raiganj, Uttar Dinajpur, West Bengal, and isolated eleven Cd^{2+}-resistant bacterial strains from the rhizosphere of *Amaranthus viridis* L. Among these, strain AF2 exhibited superior multi-metal resistance and was selected for detailed characterization. 16S rRNA gene sequencing identified AF2 as *Pseudomonas aeruginosa*, with 100% sequence identity to strains BQ26 and NCTC 13628. Growth kinetics in Cd^{2+}-amended LB medium revealed concentration-dependent growth delays, while biofilm formation was stimulated at low Cd^{2+} levels (up to 1 mM) but inhibited at higher concentrations. SEM-EDAX analysis confirmed Cd^{2+} bioadsorption via multiple binding sites on the cell wall. AF2 also exhibited diverse plant growth-promoting (PGP) traits, including IAA production, ACC deaminase activity, nitrogen fixation, siderophore production, phosphate solubilization, and Zn solubilization, as well as ammonia and HCN production. *In vitro* application of AF2 significantly enhanced germination (1.1 to 8-fold), root growth (1.5 to 3.3-fold), fresh weight (1.3 to 1.8-fold), dry weight (1.4 to 2.2-fold), and root-to-shoot ratio of *A. viridis* seedlings under Cd^{2+} stress. These findings highlight AF2 as a potent bioremediation agent and biofertilizer, capable of mitigating heavy metal toxicity while improving crop yields in contaminated agroecosystems.

Keywords: Heavy metal, Cadmium toxicity, Plant growth promotion, bioremediation, *Pseudomonas aeruginosa* strain AF2, *Amaranthus viridis* L., IAA, siderophore, ACC deaminase.

I. INTRODUCTION

Rapid industrial development and the strain of an ever-growing population have adversely affected agricultural soil health. The contamination of agricultural soils with heavy metals has resulted in a wide range of environmental issues and heightened ecological instability. Heavy metals, unlike organic pollutants, are non-biodegradable and persist in the environment for extended periods. The prevalence of these substances in the soil has detrimental effects on crop productivity, plant growth, and reproduction. Regular intake of vegetables contaminated with heavy metals also poses health risks to humans [1,2,3]. In addition to being released into nature through geogenic processes such as weathering, leaching, and volcanism, heavy metals are mostly released into the environment through the increased industrial activity, fossil fuel combustion, mining, smelting, incineration of municipal waste, the use of agrochemicals, and controversial agricultural practices [4,5].

Cadmium (Cd), a nonessential heavy metal, is highly toxic and carcinogenic in nature. It has a prolonged biological half-life and exhibits high mobility in soil environments. The concentration of cadmium in the earth's crust ranges from 0.1 to 0.5 mg kg^{-1}. Cd^{2+} is primarily found in nature in association with the sulphide ores of metals like zinc, lead, and copper [6]. Cd^{2+} is commonly used in batteries, metal electroplating, pigments, stabilizers for plastics, the energy sector, modern electronics, and the communication industry.

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The average global concentration of cadmium in agricultural soils is 0.36 mg/kg, with a range of 0.01-1 mg/kg. Cd^{2+} contamination of agricultural soil is mostly caused by atmospheric deposition, use of phosphate fertilizers and pesticides, irrigation with municipal waste and sewage sludge deposits. Plants and vegetables cultivated in contaminated soil readily absorb Cd^{2+}, which can enter the aerial parts of the plant, posing risks to human health and food safety. In plants, cadmium toxicity results in chlorosis of the leaves, reduced pigment synthesis and photosynthesis, browning of the root tips, wilting, senescence, inhibition of seed germination, and stunted overall plant growth [7,8].

Microbes play a vital role in the natural cycling of heavy metals and are crucial for decontaminating soil polluted with these metals. When exposed to high levels of toxic metals, microbes develop resistance and can help in bioremediation by employing various detoxification methods. Certain metal-resistant plant growth-promoting rhizobacteria (PGPR) can effectively aid in remediation efforts by mitigating metal-induced stress and enhancing plant growth. This eco-friendly approach is cost-effective and can involve bacteria such as *Ochrobactrum* sp. and *Bacillus* sp. [9], *Burkholderia* sp. SRB-1 [10], *Raoultella* sp. [11], *Klebsiella sp*. and *Lelliottia* sp. [12], *Leclercia adecarboxylata* HW04 [13], *Curtobacterium* sp. [14], and *Enterobacter* sp. [15], which exhibit plant growth-promoting traits and heavy metal resistance. These bacteria may resist heavy metals by either accumulating them within their cells, adsorb in cell wall or transforming them into less toxic forms. Despite limitations, including strains' selectivity for specific metals, environmental compatibility, and plant growth-promoting abilities, the demand for metal-resistant PGPR remains high in contemporary global bioremediation efforts [16].

In the present study, multiple bacterial isolates were screened for Cd^{2+} resistance, among which strain AF2 was found to be highly tolerant. In addition to its metal resistance, AF2 exhibited diverse PGP traits that significantly enhanced the growth of *Amaranthus viridis* L. seedlings under Cd^{2+} stress.

II. MATERIALS AND METHODS

A. Site Characterization and Physicochemical Properties of Soil

Rhizospheric soil samples were collected from cadmium-contaminated agricultural fields adjacent to an open municipal waste disposal site in Raiganj, Uttar Dinajpur, West Bengal, India (25°36'31.8"N 88°06'47.2"E), and stored at 4°C. Air-dried, pulverized soil samples were sieved through a 2 mm mesh. Following this, the soil samples were characterized by measuring pH, electrical conductivity (EC), Carbon content (C), Nitrogen (N), Potassium (K), Phosphate (P2O5), Sulphur (S) and trace elements, such as Iron (Fe), Copper (Cu), Zinc (Zn), Manganese (Mn), Boron (B). Cadmium content of the soil samples was determined using atomic absorption spectrometry (AAS, PinAAcle 900F, PerkinElmer) after digestion following the di-acid digestion method in 3:1 (v/v) HCl/HNO_3 (17).

B. Isolation and Selection of Cd-resistant PGPR

To isolate Cd^{2+}-resistant bacteria, 1 g of rhizospheric soil sample was added to 9 mL of sterile water and agitated at 150 rpm at 32°C for 2 h. Subsequently, 1mL aliquot of the suspension was serially diluted (up to 10^{-6}) and plated in Luria Bertani (LB) agar media [Composition: tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L), and agar (15 g/L); pH 7.5] supplemented with $CdCl_2$. H_2O (1 mM) by the standard pour plate method. These plates were then incubated at 32°C with shaking at 150 rpm for 72-96 hours. Colonies that were distinct in morphology and appeared after overnight incubation were selected and purified by streaking. The pure cultures were maintained on Miller Luria Bertani agar slants in the presence of 1 mM $CdCl_2$, sub-cultured every 15 days, and stored at a temperature of − 20 °C for future studies. For selection, isolates were screened for tolerance to Cd^{2+} using the agar dilution method [18] on LB agar plates supplemented with increasing concentrations of $CdCl_2 \cdot 2H_2O$ (ranging from 1 mM to 20 mM). Cd^{2+} tolerance was determined by the appearance of growth of bacteria after 72 hours of incubation at 32 ºC.

C. Molecular Identification of the Selected Bacterial Isolates

Genomic DNA of bacterial strain AF2 was extracted from pure cultures grown from single colonies using NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. Genomic DNA was used as the template with the universal forward primer 16S-F (5'-GGATGAGCCCGCGGCCTA-3) and 16S Reverse primer (5'-CGGTGTGTACAAGGCCCGG-3'). The amplified DNA was purified and sequenced at BioKart Ind Pvt. Ltd. (https://biokart.com/). The strain sequences were submitted to GenBank and compared with published sequences in the database using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Highly homologous sequences were selected and aligned using CLUSTAL X. A phylogenetic tree was constructed by MEGA 11 software using the neighbour-joining method to elucidate the phylogenetic position of the strain [19].

D. Morphological and Biochemical Characterization of the Selected Bacterial Isolates

The morphological and physio-biochemical characteristics of the AF2 strain were assessed to facilitate its identification. Activities for some enzymes, such as catalase, oxidase, amylase, caseinase, urease, gelatinase, DNase, and nitrate reductase, were tested according to standard protocols. Salt stress tolerance of the isolate was assessed by growing it in LB medium supplemented with varying concentrations of NaCl (0.5% to 10%) at 32°C with shaking at 120 rpm for 5

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days. The isolate was also incubated at temperatures ranging from 10°C to 50°C and at pH levels from 3 to 10 to determine the optimal growth conditions. Bacterial growth was monitored every 6 hours by measuring the absorbance of the culture at 610 nm. Colony characteristics were evaluated after isolates were grown on LB agar for 72 hours at 32°C [20, 21, 22]. Cellulase production was detected on an LB agar plate containing 1% CMC, flooded with 0.8% Congo red and 1M NaCl [23]. The carbohydrate utilization efficacy of the isolate was determined using the HiMedia carbohydrate utilization kit (KB009, HiMedia). For the chitinase assay, the isolate was spot-inoculated on colloidal chitin agar medium and incubated at 32 °C. A clear halo zone after 120 hours of incubation indicates chitinase activity [24]. To determine pectinase activity, the isolate was spot-inoculated onto pectinase screening agar. A clear hydrolysis zone around the bacterial growth indicates a positive result [25]. All assays were performed in triplicate.

E. SEM-EDAX Analysis

To detect any morphological change and Cd accumulation on the cell surface affected by Cd treatment, a scanning electron microscope with energy dispersive X-Ray analysis (SEM-EDAX, Model: Jeol 6390LA/ OXFORD XMX N, 0.5–30 kV) study was performed. For this, the strain was grown under control (without Cd^{+2}, 0 mM) and treated (1 mM Cd^{2+}) conditions. Bacterial cells for the SEM study were processed as described by Mandal et al. [26].

F. Effect of Cd on Bacterial Growth and Determination of Minimum Inhibitory Concentration (MIC)

A set of three 250 mL flasks, each containing 50 mL of LB broth, was sterilized and then supplemented with 1 mM and 5 mM $Cd2^{+}$. These were then inoculated with a freshly prepared inoculum and incubated in a shaking incubator at 37°C and 100 rpm. In one flask, no Cd^{2+} (control) was added. Growth of the selected bacterial isolates in LB broth was monitored at regular intervals by measuring the OD of the culture at 610 nm using a spectrophotometer. Growth response (O.D.) was plotted against incubation time to construct the growth curves of bacterial isolates with and without Cd. MIC is defined as the lowest concentration of a substance that prevents visible microbial growth. The MICs of Cd^{2+} and some other heavy metals, viz. Co^{2+}, Cr^{6+}, Zn^{2+}, and Ni2+ were determined for the selected bacterial strain on an LB agar plate and in liquid medium supplemented with each heavy metal. Cadmium chloride ($CdCl_2$. H_2O), cobalt chloride ($CoCl_2$. $6H_2O$), potassium dichromate ($K_2Cr_2O_7$), zinc sulphate ($ZnSO_4$. $7H_2O$) and nickel chloride ($NiCl_2$. $6H_2O$) were used for Cd^{2+}, Co^{2+}, Cr^{6+}, Zn^{2+}, and Ni^{2+} source, respectively. After inoculation, LB agar plates were incubated at 32 ± 2 °C for 96-120 hours, and LB liquid medium was incubated in a rotary shaker at 120 rpm at 32 ± 2 °C for 72-96 hours. The concentration at which the isolates failed to grow was then observed [27].

G. Screening for PGP Traits

To assess various plant growth-promoting traits, all bioassays were performed in triplicate. For the siderophore assay, Chrome Azurol S (CAS) agar plates amended with 0 or 1 mM Cd2+ were spot-inoculated and incubated for 5 days at 32 °C. The presence of a yellow-to-orange halo around bacterial growth indicated the production of siderophores [28]. Quantitatively, siderophore production was estimated according to Alexander and Zuberer [29] and expressed as a percentage siderophore unit (SU). The activity of ACC deaminase (1-aminocyclopropane-1-carboxylic acid deaminase) was assessed qualitatively and quantitatively. The qualitative detection was performed by streaking on DF (Dworkin and Foster) salts minimal medium containing 3 mM ACC, which served as the sole nitrogen source. Control plates included DF medium without ACC (negative control) and DF medium supplemented with 0.2% w/v (NH4)2SO4 (positive control). Quantitative estimation of ACC deaminase activity involved measuring the amount of α-ketobutyrate produced through enzymatic hydrolysis of ACC [30]. Potassium solubilization ability was detected on modified Aleksandrov medium amended with mica powder in the presence or absence of Cd (1mM). The appearance of a clear halo zone is considered evidence of potassium solubilization [31]. Nitrogen fixation ability of the isolates was determined by the appearance of luxuriant growth in Ashby's Mannitol Agar medium and Burk's N-free medium [32]. Inorganic phosphate solubilization ability was qualitatively screened by the appearance of a clear halo zone around the bacterial colony on the National Botanical Research Institute's Phosphate Medium (NBRIP) containing insoluble tricalcium phosphate. The halo zone was measured to determine the phosphate solubilization index (PSI). For quantification, bacterial isolates were cultured in liquid NBRIP broth medium and incubated at 32°C ± 2°C for 3 days. The solubilized phosphate levels were measured following the standard protocol of Nautiyal [33]. Zinc solubilization was detected on a modified Pikovskaya agar plate [34]. IAA production was evaluated according to the method of Bric et al. [35] over a 48-hour period, using a 6-hour interval, in LB broth supplemented with tryptophan (200 µg ml-1) with and without Cd (1 mM). IAA was quantified against a standard curve of pure IAA. Ammonia production was assessed using qualitative peptone assays, following the method described by Cappuccino and Sherman [36]. HCN production was detected according to the method of Bakker and Schippers [37].

H. Antibiotic Susceptibility Test

Antibiotic susceptibility was tested by the disk diffusion method, using 15 antibiotic disks (HiMedia Laboratories Ltd., Mumbai, India), on Mueller-Hinton (MH) plates, and the plates were incubated at 37 °C for 24 h [38]. The antibiotics used were: Azithromycin (15 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg), Levofloxacin

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(5 µg), and Rifampicin (5 µg). Zones of inhibition were measured after 24 h at 32°C. The isolate was classified as sensitive (+) and resistant (-) based on the size of the zone of bacterial growth inhibition.

I. Biofilm Formation

Biofilm formation was assessed using the Congo Red Agar (CRA) method. Brain heart infusion agar medium was prepared with 0.8 g/L Congo red and supplemented with Cd^{2+} (0 and 1 mM). A positive result was indicated by black colonies with a dry crystalline consistency [39]. Biofilm formation was quantified using the test tube assay under Cd2+ stress. The isolate was grown in trypticase soy broth (TSB) with 1% glucose for 72 h, with different concentrations of Cd^{2+} (0, 0.5, 1, 3, and 5 mM). The empty tubes were washed with phosphate buffer saline (PBS) (pH 7.3) and stained with 4% crystal violet solution after drying. The positive rings were dissolved in 7% ethanol, and absorbance was recorded at 600 nm [40].

J. *In vitro* Study for Plant Growth Under Cd^{2+} Stress and PGPR Treatment

Seeds of *Amaranthus viridis* L. (Amaranthaceae), locally known as Noteshak, were surface-sterilized in a 2% sodium hypochlorite solution for 10 minutes, followed by repeated rinsing in distilled water. Subsequently, the surface-sterilized seeds were transferred to standard Petri dishes lined with two layers of filter paper, each moistened with one of the following Cd^{2+} concentrations (0, 0.05, 0.10, 0.25, 0.5, or 1 mM). The dishes were then placed in a growth chamber and incubated at 32 ± 2 °C with 65% relative humidity under constant darkness for 72 hours. Another replicate of the same experiment was established with seeds inoculated with an overnight-grown bacterial suspension (OD_{610} = 0.5). The growth parameters, including root length, shoot length, fresh weight, dry weight, and germination %, were recorded after 3 days. Germination % was calculated using the formula:
Germination % = Number of seeds germinated/Total number of seeds × 100 [41,42]

K. Statistical Analysis

All experiments were performed in triplicate. Standard error (SE) was calculated from the mean values presented as ± in the tables and error bars in the figures. Differences between groups were determined by one-way ANOVA or two-way ANOVA, followed by Tukey's test as a post hoc test. All statistical analyses were performed using SPSS (version 21.0) and Microsoft Excel. Similar lowercase letters above the bars indicate non-significant results at $p \leq 0.05$.

III. Results And Discussion

A. Site Characterization and Physicochemical Properties of Soil

Chemical and elemental analyses of rhizospheric soil from agricultural sites indicated a slightly acidic pH (5.37). As shown in Table I, the samples contained essential macro- and micronutrients, with prominent levels of nitrogen (N), phosphorus (P), and potassium (K). Among the detected elements, iron (Fe) reached the highest concentration, followed by sulfur (S). Additionally, the analysis confirmed the presence of cadmium alongside the primary nutrient profile. The soil organic carbon concentration was 0.76%, which is below the commonly used threshold of 2% [43]. The zinc concentration in soils (0.51 mg kg^{-1}) was found to be slightly below the critical threshold of 0.6 mg kg^{-1} [44]. The results revealed that the Cd concentration in the soil was above the screening level (1.4 mg kg^{-1}) and response level (13 mg kg^{-1}) prescribed in the 1st edition of *Guidance Document for Assessment and Remediation of Contaminated Sites in India, 2015*. An antagonistic relationship exists between Zn^{2+} and Cd^{2+} stress, which is significantly influenced by soil organic carbon (SOC) concentrations. SOC directly influences the mobility and bioavailability of these metals through sequestration and immobilization processes. Zn^{2+} and Cd^{2+} share similar physicochemical properties and compete for the same transmembrane transport pathways, such as those in the ZIP (ZRT/IRT-like protein) and NRAMP (Natural Resistance-Associated Macrophage Protein) families [45.46].

TABLE I Analysis of Physicochemical Properties of Soil

Site	pH	E.C. (ds/m)	% of C	N (Kg/ha)	P_2O_5 (Kg/ha)	K_2O (Kg/ha)	Zn (mg kg^{-1})	B (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})	S (mg kg^{-1})	Cd (mg kg^{-1})
25°36' 31.8"N 88°06'47. 2"E	5.37 ± 0.18	0.43 ± 0.02	0.76 ± 0.14	449.3 ± 6.36	236 ± 2.65	225 ± 2.89	0.51 ± 0.08	0.57 ± 0.09	45.17 ± 1.61	17.23 ± 0.54	1.03 ± 0.15	40.1 ± 1.87	22.25 ± 0.12
Note. Data are the mean of three replications ± SE													

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B. Isolation and Selection of Cd^{2+}-resistant PGPR

Microorganisms are adversely affected in Cd-contaminated environments due to the metal's extreme toxicity. However, under natural selection pressure, some species develop adaptive resistance. Numerous indigenous microorganisms have been isolated from heavy metal–polluted sites, exhibiting remarkable tolerance, particularly to cadmium. These phylogenetically distinct organisms play a crucial role in mitigating environmental pollution, crop productivity, and the resulting risks to human health [47]. The isolation of Cd^{2+}-resistant PGPR from contaminated rhizospheric soils has been reported by Pramanik et al. [3], Liu et al. [19], and Madhogaria et al. [48]. In the present study, a total of 11 isolates were obtained from soil samples, exhibiting varying degrees of Cd^{2+} resistance. The results revealed that two bacterial isolates grew luxuriantly on LB plates supplemented with 10 mM or more $CdCl_2$ in H_2O after 72 hours of incubation at 32 °C. Among them, AF2 was selected for further experimental studies due to its relatively high Cd^{2+} resistance.

C. Characterization and Phylogenetic Analysis of the Strain AF2

Primary characterization of *P. aeruginosa* was based on its morphological, physiological, and biochemical traits. Strain AF2 was characterized as a Gram-negative, short rod-shaped bacterium forming smooth, circular, glossy, convex, greenish-blue colonies (Fig. 1b), with optimum growth at 32-37°C (≤42°C) and pH 6-7.5. The morphological, biochemical, and physiological results are detailed in Tables II and III. The morphological and biochemical profile of strain AF2 closely matches the established characteristics of *Pseudomonas aeruginosa*. The observation of blue-green pigmentation is a primary diagnostic trait of this species, typically attributed to the production of pyocyanin and pyoverdine. The ability of the strain to grow at 42°C and on MacConkey agar further distinguishes it from other *Pseudomonas* species [49, 50, 51]. The biochemical versatility of AF2, particularly its ability to produce extracellular enzymes such as amylase and gelatinase, suggests significant metabolic adaptability. These phenotypic findings were conclusively validated by 16S rRNA gene sequencing, which revealed 100% similarity with *Pseudomonas aeruginosa* strains BQ26 and NCTC 13628. This multiphasic approach confirms that strain AF2 is *P. aeruginosa*. The 16S rRNA sequencing data have been deposited in GenBank with accession numbers OR539293. A phylogenetic analysis of strain AF2 also revealed its homology with other *Pseudomonas* spp. strains, as shown in Fig. 1.

TABLE II MORPHOLOGICAL CHARACTERISTICS OF STRAIN AF2

Strain	Gram reaction	Cell shape	Size (µm)	Endospore	Motility	Margin	Colour	Pigment	Opacity
Pseudomonas aeruginosa strain AF2	-	Short rod	1-1.5	-	+	Entire	Greenish blue	Fluorescent greenish blue pigment	Opaque
Note. "+" indicates positive; "–" indicates negative									

TABLE III PHYSIOLOGICAL AND BIOCHEMICAL PROFILE OF STRAIN AF2

Physiological and biochemical characteristics	Results
Lactose, Maltose, Fructose, Raffinose, Trehalose, Melibiose, Sucrose, Inulin, Sodium gluconate, Glycerol, Salicin, Inositol, Sorbitol, Mannitol, Adonitol, Arabitol, Erythritol, Cellobiose, Melezitose, alpha-Methyl-D-Mannoside, Xylitol, o-Nitrophenyl-β-D-galactopyranoside (ONPG), Sorbose	-
Xylose, Dextrose, Galactose, L-Arabinose, Mannose, Rhamnose, Esculin, D-Arabinose, Citrate, Malonate, Dulcitol, alpha-Methyl-D-glucoside	+
Growth on MacConkey agar	+
Nitrate Reduction test	+
Indole test, Methyl Red (MR) test, Voges-Proskauer (VP) test	-
Caseinase, Amylase, Oxidase, Catalase, Chitinase, Gelatinase	+
Cellulase, Pectinase, Urease, DNase	-
Optimum pH	6 -7.5
Optimum Temperature	32-37 °C (≤ 42 °C, No growth at 4 °C
NaCl	≤ 2%

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Note. "+" indicates positive; "–" indicates negative

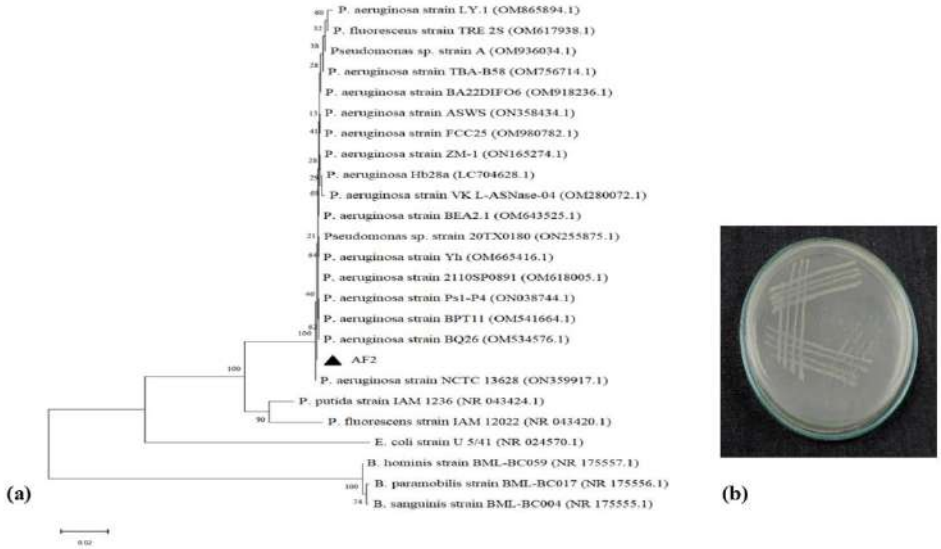

Fig. 1 (a) Based on the 16S rRNA gene sequence, a neighbour-joining phylogenetic tree was constructed with a bootstrap value of 1000, demonstrating the phylogenetic position of the isolated Cd-resistant strain. The 16S rRNA gene sequences of closely related species were obtained from the NCBI GenBank database. The scale bar represents 0.02 substitutions per nucleotide position. (b) Colony morphology of the AF2 strain (GenBank Accession numbers OR539293)

D. SEM-EDAX Study

The cellular morphology of strain AF2, grown with and without Cd^{2+}, was analyzed using scanning electron microscopy (SEM) at 6,000x magnification. Control cells (0 mM) exhibited a typical short rod shape with a smooth surface (Fig 2a). In contrast, cells exposed to 1 mM Cd^{2+} displayed a rough surface characterized by irregular, white crystalline deposits, hypothesized to be sequestered cadmium (Fig 2c). Exposure to 1 mM Cd^{2+} induced mild morphological distortions, including surface roughening and cellular shrinkage. EDAX analysis confirmed the biosorption of Cd^{2+} within the cell wall (Fig 2d). The detection of two distinct absorption peaks further suggests that AF2 utilizes multiple binding states for Cd2+ biosorption, affirming its efficiency in Cd2+ removal. In the present study, SEM-EDAX analysis revealed the ability of AF2 strain to adsorb Cd2+ on cell wall. The lack of major cell wall damage after exposure to 1 mM Cd2+ suggests a high level of metal tolerance. These findings align with previous research demonstrating the biosorption capabilities of *P. aeruginosa* strains and other bacterial cells. Bacteria exhibit variations in their capacities for and mechanisms of Cd2+ adsorption. Most studies indicate that functional groups such as hydroxyl, carboxyl, and amino groups located on bacterial cell surfaces are crucial for binding with Cd^{2+} and other heavy metal ions [52].

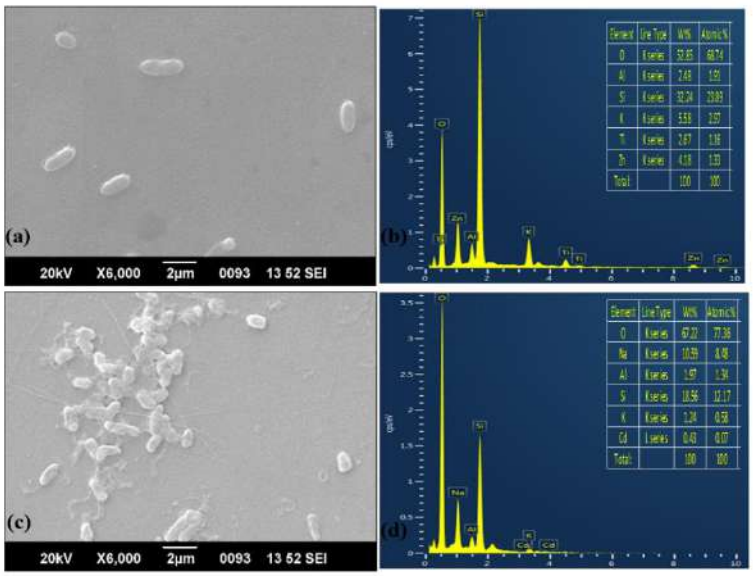

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Fig. 2 SEM-EDAX analysis of strain AF2. (a) SEM image and (b) EDAX study in the absence of Cd^{2+}; and (c) SEM image and (d) EDAX study under 1 mM Cd^{2+} stress

E. Effect of Cd on Bacterial Growth and Determination of MIC

The effect of Cd^{2+} on the growth of strain AF2 was assessed in the presence of 1 mM and 5 mM Cd^{2+}, as well as in its absence. In the control group, strain AF2 entered the log phase and reached the stationary phase significantly faster than in the media supplemented with Cd^{2+}. Moreover, media supplemented with 1 mM Cd^{2+} notably reduced overall bacterial growth. This effect was more pronounced with 5 mM Cd^{2+}, resulting in a further delay and reduction in growth (Fig. 3a). The MIC of Cd^{2+} for the AF2 isolate was 16 mM in LB agar and 9 mM in LB broth at pH 7.2. Additionally, AF2 exhibited resistance to other heavy metals, including Co^{2+}, Cr^{6+}, Zn^{2+}, and Ni^{2+}, with MICs of 3 mM, 2 mM, 20 mM, and 6 mM, respectively, in LB agar medium (Fig. 3b). These results indicate that **Cd^{2+}** inhibited the growth of strain AF2 in a dose-dependent manner compared to the control group. This dose-dependent inhibition indicates that Cd^{2+} is toxic at these concentrations, even when survival mechanisms are intact. The delay in achieving maximum growth at higher Cd^{2+} concentrations suggests the bacteria may require a longer lag phase for adaptation, or that the toxic effects slow fundamental metabolic processes and cell division [53]. The strain *P. aeruginosa* AF2 showed the ability to grow in LB broth supplemented with a MIC of 9 mM Cd^{2+}. AF2 strain showed resistance particularly to high concentrations of Cd^{2+} and Zn^{2+}, less so to Ni^{2+}, and least tolerant to heavy metals like Co^{2+} and Cr^{6+}. Several *Pseudomonas* sp. have previously been reported to be resistant to Cd^{2+} and/or Zn^{2+} [54].

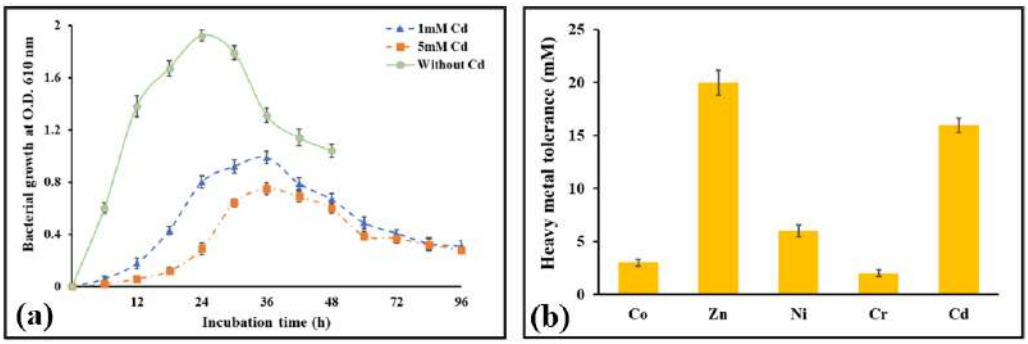

Fig. 3 (a) Effect of Cd^{+2} (0, 1, and 5 mM) on bacterial growth, and (b) multi-heavy metal-resistance on LB agar plate

F. PGP Traits and Effect of Cd^{+2}

Cd^{+2} tolerant strain AF2 was screened for its PGP properties, both qualitatively and quantitatively, with or without Cd+2, as detailed in Table IV, and Figs. 4 and 5. ACC deaminase activity of the AF2 strain was measured over a period of 48 hours (Fig. 5a). This enzyme activity was 27.1% higher after 48 hours than that measured at 24 hours of culture in the absence of Cd^{+2}. The enzyme activity declined significantly at 24 hour (69.52%) and 48 hour (39.1%) after 1mM Cd^{+2} treatment. IAA production by Strain AF2 gradually increased with bacterial growth, and the highest concentration of IAA was obtained from 42 h culture in the absence of Cd^{+2}. However, significant decline in IAA production was noticed in presence of Cd^{+2}. 1 mM Cd^{+2} decreased the concentration of this plant growth regulator by 35.1% after 48 h (Fig. 5b). Strain AF2 has high siderophore production with SU values 81.62%. SU values decreased in Cd^{+2} amended medium by 18.85% (Fig. 5c). The appearance of clear halo zone on NBRIP agar medium showed the ability of the strain AF2 to solubilize the inorganic phosphate in presence or absence of Cd^{+2} with PSI 3.08 and 2.17, respectively (Table IV). Also, the ability of AF2 to solubilize inorganic phosphate was measured at 87.81 µg mL^{-1} and 51.42 µg mL^{-1} respectively, in Cd^{+2} treated and untreated NBRIP liquid medium respectively with a decline of 41.45% (Fig 5d). The capacity to solubilize Zn was also decreased in similar manner. Zn solubilization index (ZSI) decreased from 2.89 in Cd^{+2} (0 mM) medium to 1.5 in Cd^{+2} (1mM) amended medium. However, AF2 strain showed negative results for K solubilization. In addition, the strain AF2 was tested to be positive for nitrogen fixation, ammonia production, and HCN production.

The presence of multiple PGP traits in Strain AF2 confirms its potential to enhance plant development under Cd^{2+} stress. Despite the relative abundance of phosphorus (P) in agricultural soils, its bioavailability is severely limited as it primarily exists in insoluble mineral and organic forms including apatite, phytate, strengite, and variscite, which are largely inaccessible for direct plant uptake [55]. The strain AF2 demonstrated the ability to convert these insoluble phosphate compounds into soluble forms. This transformation is likely mediated by mechanisms such as acidification, enzymolysis, chelation, and organic acid production. Solubilized phosphate, mainly HPO_4^{2-} and $H_2PO_4^-$, can facilitate the immobilization of heavy metals in the rhizosphere, thereby reducing their bioavailability and uptake [56]. In this study, the phosphate-solubilizing capacity of strain AF2 was effective under Cd^{2+} stress. This finding is consistent with reports on *Pseudomonas sp.* K32, which similarly promotes P-solubilization under Cd^{2+} toxicity [57]. Bacterial IAA promotes cell division, tissue differentiation, and the initiation of lateral and adventitious roots, thereby expanding the root surface

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area and significantly enhancing mineral absorption and nutrient uptake [58]. Although AF2 showed reduced IAA under Cd^{2+} exposure, consistent with *Enterobacter* strains, the secretion was sufficient to mitigate oxidative damage and sustain seedling growth. This hormonal buffering maintains homeostasis in crops such as rice and maize. Studies on Cd^{2+}-resistant rhizobacteria, including *Burkholderia* and *Pseudomonas*, confirm that microbially derived IAA enhances nutrient acquisition and biomass accumulation under metal toxicity, countering growth inhibition [3, 59]. ACC deaminase–producing strains lower ethylene levels by cleaving ACC, the immediate precursor to ethylene that would otherwise inhibit root elongation and accelerate senescence [60]. In this study, the selected AF2 strain exhibited significant ACC deaminase activity, suggesting its primary role in alleviating Cd^{2+} stress by modulating ethylene homeostasis [61]. Recent findings confirm that PGPR strains possessing ACC deaminase activity, such as *Pseudomonas* and *Bacillus* species, significantly enhance biomass and photosynthetic pigment stability in contaminated soils by maintaining sub-inhibitory ethylene concentrations [62, 63]. PGPR-secreted siderophores, low molecular-weight compounds, are high-affinity ligands that selectively chelate ferric iron (Fe^{3+}), significantly enhancing iron bioavailability in the rhizosphere. Recent findings confirm siderophores mitigate Cd^{2+} toxicity by forming stable extracellular complexes that reduce free Cd^{2+} ions available for root uptake. Thus, the siderophore production of strain AF2 serves as a dual-action strategy for nutritional support and heavy metal exclusion [64]. Recent studies reported that N_2-fixing PGPR reshapes the rhizosphere to improve nitrogen use efficiency, effectively countering metal-induced growth inhibition [65]. Under Cd^{2+} stress, biological N_2 fixation is thus critical for maintaining photosynthetic activity and biomass, as seen in Cd^{2+}-tolerant diazotrophs like *Klebsiella* and *Pseudomonas* [66]. In metal-contaminated environments, HCN-producing strains offer the dual advantage of soil-borne pathogen suppression through the inhibition of cytochrome c oxidase, and growth promotion, thereby enhancing the overall fitness of crops under chemical stress [67,68]. Our study confirmed the HCN-producing ability of AF2. Increased Zn^{2+} bioavailability in the rhizosphere allows Zn^{2+} to compete with Cd^{2+} for entry into plant roots, effectively reducing Cd^{2+} uptake and translocation. Bacterial solubilization of Zn^{2+}, as seen in our study, enhances the plant's enzymatic antioxidant system, mitigating Cd^{2+}-induced oxidative damage. Zinc-solubilizing bacteria consortia (*ZM27* and *AN31)* can reduce Cd bioaccumulation by up to 86% while significantly increasing chlorophyll content and grain Zn^{2+} levels [69]. Recent studies emphasize that ammonia-producing strains, such as *Bacillus* sp. and *Pseudomonas* sp., can maintain growth in soils contaminated with up to 100 mg/kg of Cd, effectively replacing chemical nitrogen fertilizers while reducing metal bioavailability. Ammonia-producing, Cd^{2+}-resistant PGPR act as vital nitrogen sources, improving plant vigor and photosynthetic efficiency under metal pressure [70]. Biofilm formation and the production of extracellular polymeric substances (EPS) represent key adaptive strategies that bacteria employ to withstand heavy metal stress. Biofilms function as both physical and chemical barriers, with anionic functional groups such as carboxyl and hydroxyl playing a crucial role in sequestering and immobilizing metal ions, thereby reducing their bioavailability and toxicity. Previous studies have demonstrated that biofilm formation is enhanced at lower cadmium concentrations, up to 0.75 mM in *Pseudomonas aeruginosa* strain CD3 [55] and 2 mM in *Bacillus subtilis* strain 1JN2 [71]. However, at higher concentrations, biofilm formation declines. Consistent with these findings, our study revealed maximal biofilm formation at 1 mM Cd^{2+}, followed by a sharp reduction at 3 mM and 5 mM.

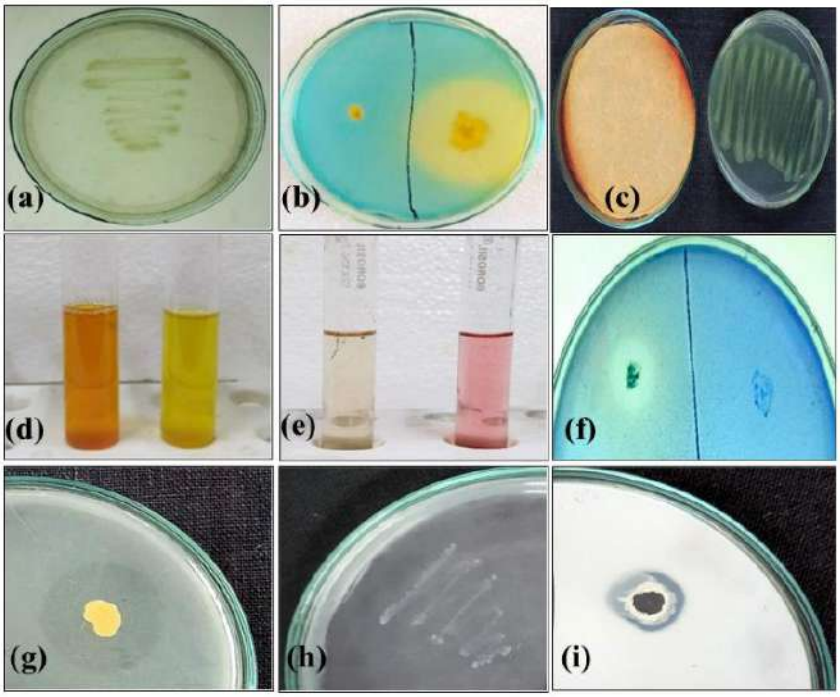

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Fig. 4 PGP traits (a) ACCD activity, (b) Siderophore production, (c) HCN production, (d) Ammonia production, (e) IAA production, (f) Phosphate solubilization, (g) Zn solubilization, (h) Growth on Burk's N_2-free media, and (i) Growth on Ashby's N2-free media

TABLE IV SCREENING FOR PGP TRAITS

HCN production		K Solubilization		Ammonia production		PSI* (λ/λ_0)		Zn Solubilization (ZSI)		Growth on N_2-free media			
										Burk's medium		Ashby's medium	
Cd (0)	Cd (1mM)	Cd (0)	Cd (1mM)	Cd (0)	Cd (1mM)	Cd (0)	Cd (1mM)	Cd (0)	Cd (1mM)	Cd (0)	Cd (1mM)	Cd (0)	Cd (1mM)
+	+	-	-	+	+	3.08 ±0.12	2.17 ±0.08	2.8 ±0.06	1.5 ±0.04	+	+	+	+

Note. "+" indicates positive; "–" indicates negative. Data are the mean of three replications ± SE

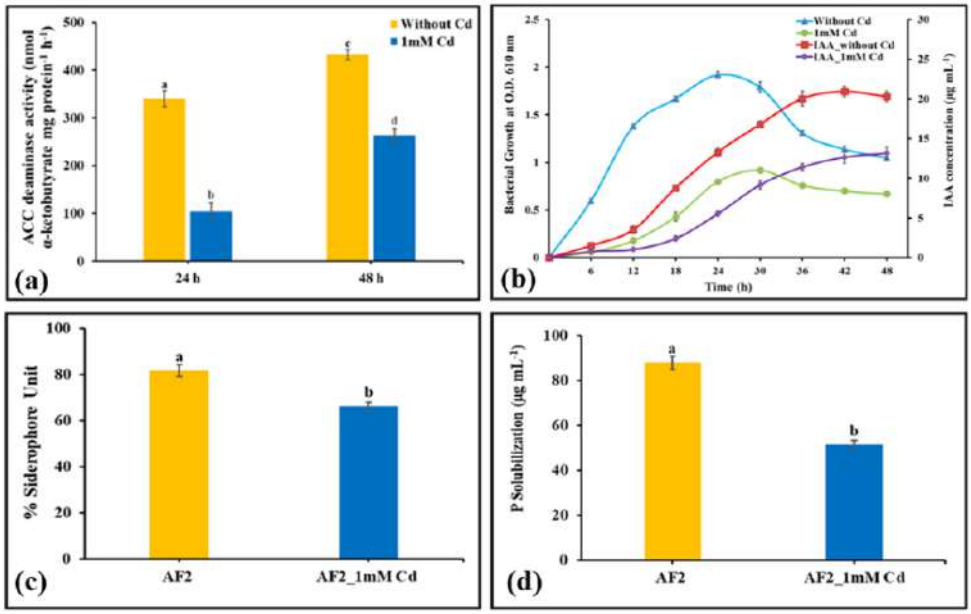

Fig. 5 Quantitative estimation of (a) ACCD activity, (b) IAA production with growth, (c) siderophore production, and (d) phosphate solubilization in the presence and absence of Cd^{+2}

G. **Antibiotic Susceptibility**

In the antibiotic sensitivity test, AF2 was susceptible to chloramphenicol (30 µg), ciprofloxacin (5 µg), and levofloxacin (5 µg). The strain AF2 was found to resist oxacillin (1 µg), azithromycin (15 µg), erythromycin (15 µg), and rifampicin (25 µg), as shown in Table V and Fig. 6. The strain AF2 also showed high resistance to antibiotics, which is consistent with previous studies [49, 71]. Many heavy metal-resistant bacteria exhibit multi-antibiotic resistance. This dual resistance typically arises through cross-resistance (a shared physiological mechanism) or co-resistance (genetically linked traits) [72, 73].

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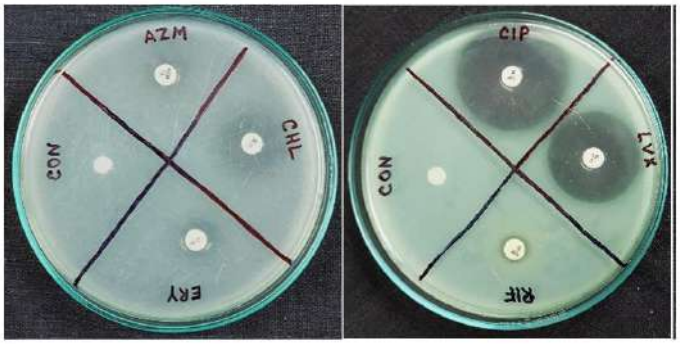

Fig. 6 Antibiotic susceptibility test for strain AF2

TABLE V ANTIBIOTIC SUSCEPTIBILITY OF STRAIN AF2

Mode of Action	Group	Antibiotic	Symbol	Content (µg)	Sensitivity
Protein synthesis (50S subunit) inhibitor	Macrolides	Azithromycin	AZM	15	-
	Macrolides	Erythromycin	ERY	15	-
	Chloramphenicol	Chloramphenicol	CHL	30	+
DNA synthesis inhibitor	Fluoroquinolones	Ciprofloxacin	CIP	5	+
	Quinolones	Levofloxacin	LVX	5	+
RNA synthesis inhibitor	Rifampin	Rifampicin	RIF	5	-
Note: "+" indicates sensitive; "±" indicates intermediate; "–" indicates resistant.					

H. Biofilm Formation

The Congo red agar qualitative test confirmed biofilm production by strain AF2, as evidenced by black colonies with a dry, crystalline texture. A quantitative assay of biofilm formation in the presence of varying Cd^{2+} concentrations showed that the highest biofilm production by bacterial strain AF2 was observed at 1 mM Cd^{2+}, as indicated by OD_{600} values. However, a significant reduction in biofilm levels was observed when exposed to elevated Cd^{+2} concentrations (3 mM and 5 mM), as shown in Fig. 7. Biofilm formation represents a key adaptive strategy that bacteria employ to withstand heavy metal stress. Biofilms function as both physical and chemical barriers, with anionic functional groups such as carboxyl and hydroxyl playing a crucial role in sequestering and immobilizing metal ions, thereby reducing their bioavailability and toxicity. Previous studies have reported that biofilm formation is enhanced at lower cadmium concentrations, up to 0.75 mM in *Pseudomonas aeruginosa* strain CD3 [53] and 2 mM in *Bacillus subtilis* strain 1JN2 [71]. However, at higher Cd^{2+} concentrations, biofilm formation declines. Consistent with these findings, our study revealed maximal biofilm formation at 1 mM Cd, followed by a sharp reduction at 3 mM and 5 mM.

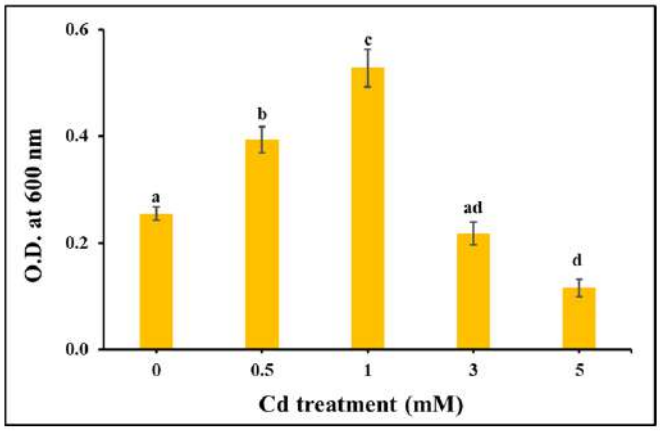

Fig. 7 Biofilm formation at different Cd^{2+} concentrations. Different lowercase letters indicate significant differences relative to the control according to the Tukey HSD test at $p < 0.05$. The error bars in the figures represent the standard error from the mean.

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I. *In vitro* plant growth promotion by AF2 strain under different Cd^{2+} stress conditions

The PGP activity of strain AF2 in *A. viridis* seedlings under *in vitro* Cd^{2+} stress was evident across multiple growth parameters. Shoot length increased 1.5-fold at 0.05 mM, 2.5-fold at 0.10 mM, 2.1-fold at 0.25 mM, 2.0-fold at 0.50 mM, and 3.3-fold at 1.00 mM in AF2-treated seedlings compared to untreated controls (Fig. 8a,b). At 0.05 mM and 0.10 mM, AF2-treated seedlings exhibited shoot lengths comparable to or exceeding those of control plants grown without Cd^{2+}, whereas at higher concentrations of 0.25 mM and 1 mM, the positive effect was less pronounced. In contrast, AF2 significantly enhanced root length across all Cd^{2+} treatments, counteracting the severe shortening observed in uninoculated seedlings. Root length increased 2.4-fold at 0.05 mM, 3.0-fold at 0.10 mM, 3.1-fold at 0.25 mM, 3.0-fold at 0.50 mM, and 30-fold at 1.00 mM compared to controls (Fig. 8a,b). Under Cd^{2+} stress, germination percentage in non-bacterized seeds declined sharply, reaching only 8.89% at 1.00 mM, whereas AF2 treatment maintained significantly higher germination, with increases up to 8-fold. Root-to-shoot ratio in uninoculated seedlings declined markedly at 0.50–1.00 mM Cd^{2+}, reflecting disproportionate root damage. In AF2-treated plants, the ratio remained comparable to controls at 0 mM and, although reduced at higher concentrations, consistently exceeded that of untreated seedlings (Fig. 8c). AF2 inoculation also significantly ($p \leq 0.05$) improved fresh and dry weight across Cd^{2+} treatments, with increases ranging from 1.3–1.8-fold and 1.4–2.2-fold, respectively (Fig. 8d). The present study demonstrated that Cd^{2+} exposure markedly inhibited the growth of *A. viridis* seedlings, as reflected in reduced root and shoot length, germination percentage, and overall biomass. This decline in growth under cadmium stress may be attributed to reduced water potential, nutrient imbalance, and disruption of proton pump activity, collectively impairing cell division and elongation [74, 75]. The reduction in biomass is further explained by cadmium's adverse effects on mineral uptake, photosynthesis, and chlorophyll biosynthesis. Since Cd is initially absorbed by the roots, it subsequently enters the plant system via the apoplastic pathway, where it interferes with metabolic processes and ultimately disrupts overall physiology [76, 77]. These findings are consistent with those of Khanna et al. [78], who reported that Cd treatment in *Lycopersicon esculentum* reduced root and shoot elongation and biomass. Similar observations have been documented in *Triticum aestivum* L. [79] and *Oryza sativa* L. [80]. Importantly, in our study, the strain AF2 exhibited dual functionality, combining PGP traits with Cd^{2+} biosorption. This synergistic effect significantly enhanced germination and early growth of *A. viridis* seedlings under Cd^{2+} stress *in vitro*. The elevated germination rates suggest that AF2 supports critical initial life stages even under environmental stress. The consistently higher root/shoot ratio observed with AF2 treatment may indicate a physiological adaptation, whereby the PGPR-treated plant develops more roots to anchor and potentially compartmentalize heavy metals away from edible or sensitive shoot tissues. Stimulation of growth parameters by PGPR treatment has also been reported by Chatterjee et al. [81] in *Capsicum annuum* L. Madhogaria et al. [48] also reported similar results in *Vigna radiata* L. under Cd stress using *Pseudomonas geniculata*. In our study, the observed increase in growth parameters is primarily attributed to the PGP traits of the AF2 strain, which mitigated Cd^{2+} toxicity.

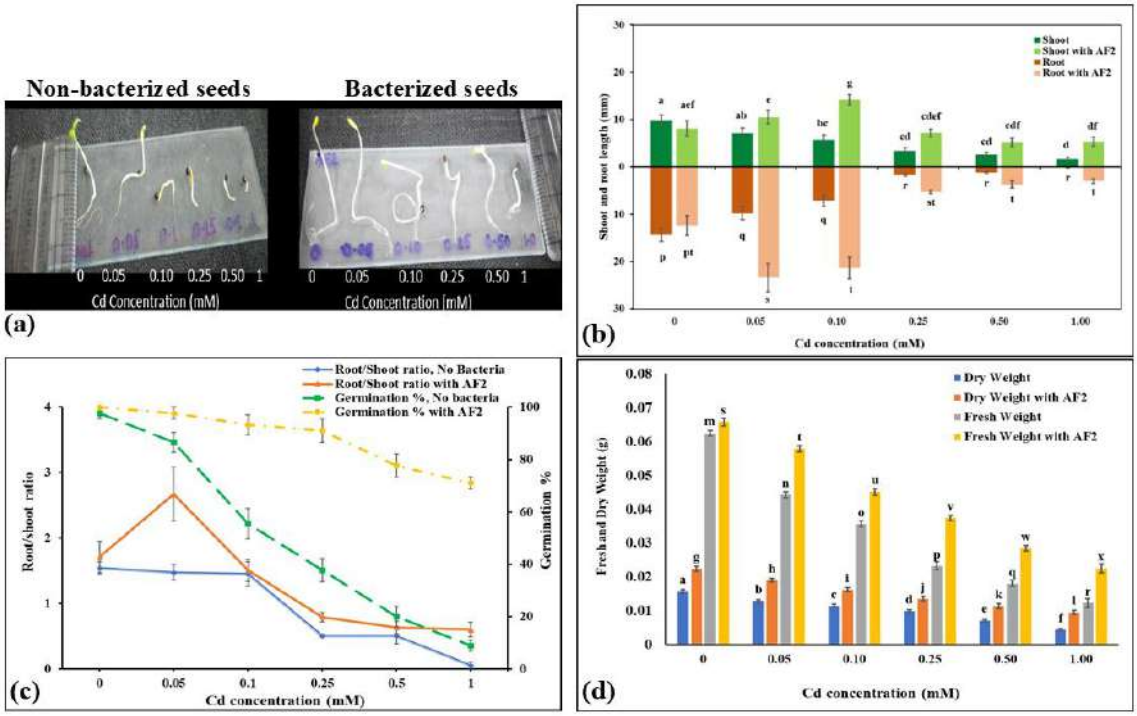

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Fig. 8 Effect of different doses of Cd^{2+} on (a) non-bacterized seeds and bacterized seeds in *in vitro* experiment, (b) shoot and root length, (c) root-to-shoot ratio and germination %, and (d) fresh and dry weight

IV. CONCLUSION

A Gram-negative, cadmium-resistant bacterial strain (AF2) was isolated from rhizospheric soil irrigated with municipal wastewater and identified as *Pseudomonas aeruginosa*. The findings of this study indicate that strain AF2 exhibits multi-heavy metal resistance and possesses key plant growth-promoting (PGP) traits, enabling enhanced seedling growth of *Amaranthus viridis* L. under cadmium stress. Remarkably, AF2 maintained the production of diverse PGP biomolecules even under cadmium exposure, suggesting that these traits contributed significantly to sustaining plant growth and physiological functioning. In addition, AF2 displayed cadmium biosorption ability at the cell wall level, highlighting an important mechanism for heavy metal stress management. Collectively, these results suggest that inoculation with cadmium-resistant bacterial strains such as AF2 may serve as an effective strategy for remediating cadmium-contaminated agricultural soils. The capacity of AF2 to enhance rice seedling growth under cadmium exposure further underscores its promise as a biofertilizer and plant growth promoter in heavy metal-stressed agroecosystems.

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