ISOLATION AND CHARACTERIZATION OF CADMIUM-RESISTANT BACTERIA AND ITS POTENTIAL IN PROMOTING TOMATO GROWTH

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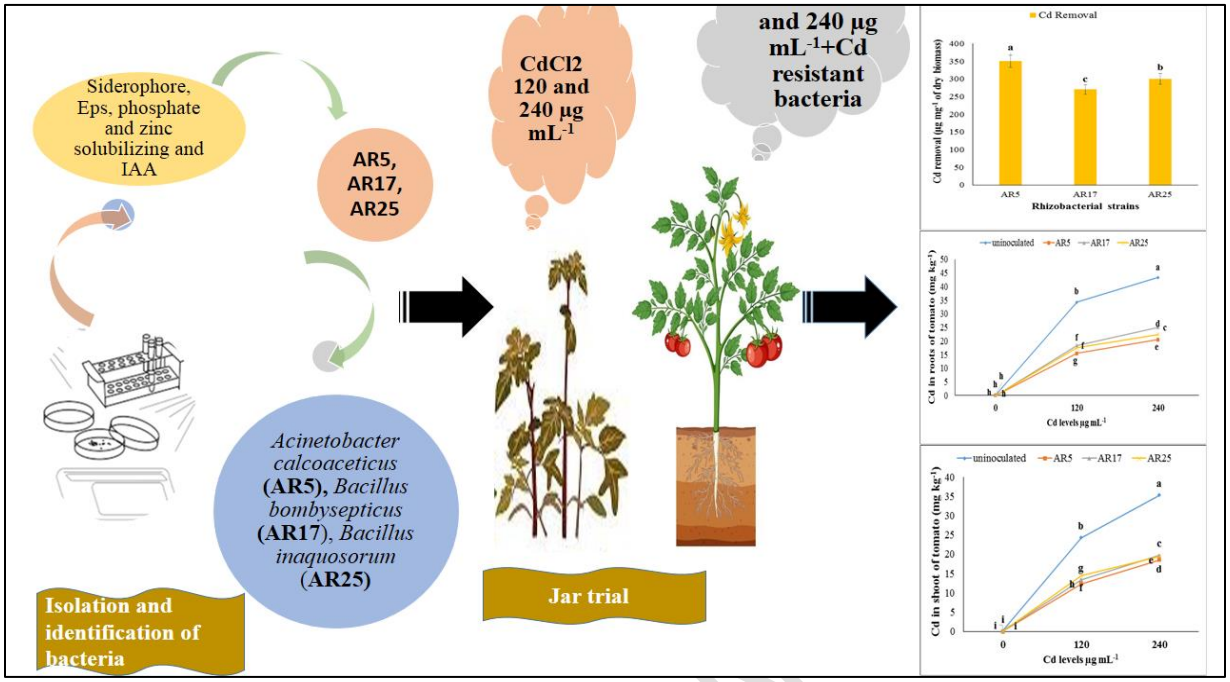
Graphical Abstract



ABSTRACT

This study primarily focuses on the isolation and characterization of bacterial strains resistant to cadmium (Cd) and evaluates their role in promoting plant growth under varying Cd concentrations (0, 120, and 240 μg mL^{-1}). A total of 45 Cd-resistant bacterial strains were isolated from wastewater-irrigated fields, of which 16 isolates demonstrating consistent growth were selected for further analysis. Among them, three highly efficient strains i.e. AR5, AR17, and AR25 were identified based on their ability to produce siderophores and exopolysaccharides (EPS), along with additional plant growth-promoting (PGP) traits. These selected strains were tested in a jar trial, where their inoculation significantly enhanced shoot length (21%), root length (20%), shoot dry weight (30%), and root dry weight (33%) at 120 μg mL^{-1} Cd compared to the uninoculated control. Additionally, bacterial inoculation reduced Cd accumulation in both shoots and roots of tomato plants. Based on 16S rRNA gene sequencing, the three strains were identified as *Acinetobacter calcoaceticus* (AR5), *Bacillus bombysepticus* (AR17), and *Bacillus inaquosorum* (AR25). The findings suggest that these Cd-resistant bacteria significantly improve tomato growth under Cd stress and have the potential to be further explored in controlled and field conditions for developing biofertilizers to enhance vegetable production in Cd-contaminated soils.

Keywords: Cadmium, Biofertilizers, *Bacillus*, Siderophore, EPS, Cd-resistant bacteria

INTRODUCTION

Agricultural production faces a growing threat from cadmium (Cd), a potent teratogen, mutagen, and carcinogen, whose environmental prevalence is increasingly magnified by smelting, industrial waste disposal, and phosphate fertilizers application (Guo et al. 2020; Wei et al. 2018). Cadmium (Cd) is an extremely hazardous heavy metal and a prevalent environmental pollutant with detrimental consequences on both plants and human health (Suhani et al., 2021; Zhang et al., 2019). For instance, plants cultivated in Cd-contaminated soil effectively uptake cadmium through their roots and accumulate it in edible parts such as fruits or seeds (Rabêlo et al., 2020). Cadmium toxicity manifests in a multitude of physiological and biochemical dysfunctions, encompassing diminished nutrient availability, impaired photosynthesis, compromised antioxidant defence systems, disrupted stomatal function, altered protein synthesis, inhibited root development, reduced water uptake and transport, membrane integrity loss, compromised cellular osmoregulation, culminating in decreased yield and crop quality (Hussain et al., 2021; Huybrechts et al., 2020; Okem et al., 2016). Bioaccumulation of heavy metals within the food leads to potential human exposure and associated health risks (Guo et al. 2018; Sghayar et al. 2015).

To tackle the challenge, there has been an emergent interest in devising reliable and eco-friendly methods for the remediation of polluted areas. Prominent among these is the utilization of plant growth-promoting rhizobacteria (PGPR), which, through their multifaceted mechanisms of action, offer a cost-effective and environmentally sustainable approach to mitigate Cd stress (Suhani et al., 2021). These PGPR strains possess the potential to enhance plant nutrient acquisition through diverse mechanisms, including atmospheric nitrogen fixation, exopolysaccharides (EPS) production, phosphate solubilization, ACC-deaminase activity, catalase activity, oxidase activity, antioxidant defence activation and siderophore production (Chen et al.,

2017). The significance of cadmium-resistant bacteria lies in their unique ability to tolerate elevated concentrations of Cd and facilitate its binding in the soil with EPS, ultimately reducing its bioavailability and mitigating its toxic effects on plants (Suhani et al., 2021; Saeed et al., 2024). They do so by utilizing efflux pumps to efflux Cd ions from cells, by using metallothioneins (metal binding proteins), and certain bacteria possess detoxifying enzymes that catalyze toxic Cd ions into less toxic compounds (Luo et al., 2020). Rhizosphere bacteria immobilize cadmium through the extracellular production of organic compounds and exopolysaccharides, primarily to facilitate surface adhesion, self-protection, and water retention (Sayyed et al., 2011). Extracellular polymeric substances, composed primarily of proteins, polysaccharides, and humic substances, exhibit cadmium-binding capabilities and their conversion to less toxic Cd compounds, which help in metal detoxification (Sharma et al., 2016).

Phosphorus is also helpful in mitigating cadmium (Cd) toxicity by immobilizing Cd within the plant cell wall and forming Cd-phosphate complexes, thereby reducing its uptake and translocation to the upper plant parts. Supplementation with phosphorus in Cd-stressed maize plants enhanced chlorophyll content and resulted in the accumulation of phosphate deposits within vacuoles and on root surfaces, indicating Cd complexation (Jiang et al., 2007). Nitrogen enrichment is associated with augmented cadmium tolerance in plants, potentially mediated by upregulating Rubisco activity and subsequent enhancement of photosynthetic capacity (Jalloh et al., 2009). Adequate nitrogen availability is crucial for optimal cadmium tolerance in plants, as numerous cadmium defense mechanisms rely on nitrogen-rich metabolites like glutathione and phytochelatins (Walker et al., 2003). Cadmium ions (Cd^{2+}) exhibit chemical similarities to essential elements like iron (Fe^{2+}), magnesium (Mg^{2+}), calcium (Ca^{2+}), and zinc (Zn^{2+}), leading to competitive uptake in plants and subsequent mineral deficiencies. However, the inoculation with

root-associated bacteria can augment the accumulation of essential elements (phosphorus, magnesium, calcium, sulfur, iron, and manganese) within both root and shoot tissues of barley seedlings subjected to cadmium stress (Belimov and Dietz, 2000).

Siderophores are low molecular weight chelators produced by both microorganisms and plants and exhibit a high affinity for ferric ions, facilitating their acquisition under iron-limiting conditions (Sharma et al., 2016). Siderophore production is a prevalent feature amongst plant-associated rhizobacteria, critical for iron acquisition and subsequent plant growth promotion (Desai and Archana, 2011). Enhanced iron availability diminishes cadmium uptake, thereby conferring cadmium tolerance and promoting plant growth (Sharma et al., 2016).

Cadmium is a potent inducer of ethylene biosynthesis in plants, leading to stunted elongation of roots (Sun and Guo, 2013). The principal approach through which plant growth-promoting rhizobacteria (PGPR) alleviates Cd stress involves the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCD), which hydrolyze ACC, the immediate ethylene precursor, effectively mitigating its negative effects on root development (Glick, 2005). Inoculation with ACCD-producing, metal-tolerant plant growth-promoting rhizobacteria facilitated growth enhancement in Indian mustard (*Brassica juncea*) and canola (*Brassica napus*) cultivated on cadmium-polluted areas (Belimov et al., 2001).

Solanum lycopersicum L., commonly known as tomato, is a widely cultivated vegetable crop of significant economic importance. Exposure to cadmium (Cd) has been shown to reduce plant growth and yield, correlating with a marked increase in Cd accumulation in mature leaves, which play a crucial role in sugar production and storage (Hediji et al., 2010). High concentrations of heavy metals in the soil severely impede tomato growth and fruit quality, as the crop is highly susceptible to heavy metal-induced stress (Hussain et al., 2019). Research led by Zhao et al. (2016)

demonstrated that cadmium chloride ($CdCl_2$) application significantly increased Cd accumulation in both the roots and leaves of the spr2 tomato variety. Across all Cd treatment levels, root tissues exhibited a tenfold increase in cadmium concentration compared to leaf tissues, leading to restricted growth. Additionally, a study by Khan et al. (2016) highlighted that Cd toxicity reduces the availability of essential nutrients such as manganese, zinc, copper, iron, and calcium in tomato seedlings. Cadmium exposure also significantly impacts flower production, ultimately affecting fruit yield. Notably, tomato plants subjected to 100 mM Cd in a nutrient solution for 90 days failed to produce any fruit (Hediji et al., 2010).

Current research aims to isolate and characterize cadmium-resistant bacteria from heavy metal-contaminated fields. The objectives encompass the identification of novel bacterial strains and their characterization regarding their potential to promote plant growth under cadmium stress and to investigate Cd uptake by plants and the substantial impact of Cd-resistant bacteria on the growth and development of tomato plants under Cd stress. Unlike previous research that primarily focuses on the negative impacts of Cd stress, this study highlights the potential of beneficial rhizosphere bacteria to enhance plant growth while reducing Cd uptake.

MATERIALS AND METHODS

Soil Sample Collection

Soil samples were collected in sterilized polythene bags from wastewater-irrigated vegetable fields in the research area, Department of Soil Science, The Islamia University of Bahawalpur (GPS coordinates 29.4125° N, 71.6828° E). Samples were stored at 4˚C till further analysis. The soil samples were air dried and passed through a sieve of 2 mm mesh size. Bacterial isolation was carried out within 24 hours of sample collection.

Isolation of Cadmium resistant bacteria

Isolation of Cd-resistant bacteria was carried out through serial dilution (Aneja, 2003) of soil samples and inoculated on modified Luria Bertani agar media spiked with $CdCl_2$ at 1200 µg Cd mL^{-1} in agar. Agar plates were incubated at 32±1°C and after 48 hours of incubation, plates with maximum growth were chosen. For additional testing, purified colonies were kept as pure isolates in slants containing 1200 µg mL^{-1} of Cd^{2+} (Pramanik et al., 2017; Marzan et al., 2017).

Isolates were inoculated in broth culture amended with 1200 µg mL^{-1} of Cd^{2+} and incubated at 30±1 ˚C for three days in a shaking incubator for daily measurements of the optical density (OD_{600}) on Spectrophotometer (Model G6860A, Agilent Technologies Cary 60 UV-Vis, Australia) at 600nm wavelength was used to measure the growth (optical density) of the isolates in three replications. Strains with higher OD values were selected for further studies. (Neethu et al., 2015).

Siderophore Production

Isolated cultures were assessed for their ability to produce siderophores under cadmium stress. The assay was carried out according to the procedure mentioned by Louden *et al.* (2011). Selected isolates were spot inoculated on CAS (chrome azurol S) agar media petri plates and incubated for 5 days at 30± 2˚C. The formation of an orange halo zone around the colonies shows siderophore production.

Exopolysaccharides Production

Bioassay was carried out to determine the capacity of selected isolates to produce exopolysaccharides following the Tallgren *et al.*, (1999) method. The selected bacterial isolates were cultured for 7 days at 28 °C on ATCC No. 14 medium (Monopotassium phosphate 0.2 g, Dipotassium phosphate 0.8 g, Sodium molybdate 1.0 mg, yeast extract 0.5 g, Calcium Sulfate Dihydrate 0.1 g, Magnesium Sulfate 0.2 g, Iron chloride1.0 mg, Agar 15.0 g, sucrose 20.0 g per

litre) diluted with deionized water and pH was maintained at 7.2 at different levels of Cd i.e., 0, 500, 1000, and 1500 µg mL^{-1} (Nazli et al., 2020). Isolates that form thick slime (*mucoid*) around colonies show Exopolysaccharide production.

Biochemical characterization of PGPR

The potential of isolates to produce auxins with and without L-tryptophan was observed according to the procedure suggested by Bric *et al.* (1991). Spectrophotometer was used to measure the intensity of color in samples as well as in standards at 535 nm. Bioassay for the solubilization of phosphate was carried out following the method of Verma *et al.* (2001). Pikovskaya's medium enriched with inorganic phosphate was used to spot-inoculate the bacterial strains. Bacterial isolates possessing clear halo zones around their colonies indicate solubilization of the phosphate. Aleksandrov's protocol was followed to execute the solubilization of potassium (Aleksandrov *et al.*, 1967). Bacterial strains were streaked on Aleksandrov media enriched with mica powder. A clear halo zone around bacterial colonies represents the potassium-solubilizing ability of isolates. For the zinc solubilization assay of rhizobacterial strains, Fasim *et al.* (2002) procedure was followed. Zinc oxide was used as an inorganic source in the medium prepared to check the Zn solubilization of strains (Pikovskaya, 1948). The appearance of clear halo zones around the colonies showed Zn solubilization. The ability of rhizobacterial strains to produce ammonia was evaluated by the procedure described by Dye (1962) using Nessler's reagent. Production of hydrogen cyanide (HCN) was checked on 1% picric acid dipped filter papers on DF minimal salt medium using the method given by Lorck (1948). Catalase activity was analyzed by the appearance of bubbling upon the addition of 1 drop of hydrogen peroxide (30%) on fresh microbial colonies smeared on a glass slide (Cappuccino and Sherman 2013). Oxidase activity was analyzed by the following method as suggested by (Steel, 1961). The immediate appearance of the

dark purple color of the 1% Kovac's reagent-dipped filter paper after rubbing fresh colonies on the filter paper represents positive oxidation activity. The capability of the chosen rhizobacterial isolates to colonize tomato seeds was examined by using the conventional procedure of root colonization suggested by Simons *et al.* (1996). Each assay was replicated three times. The most efficient Cd-resistant plant growth-promoting isolates were selected based on their plant growth-promoting characteristics and their root colonizing potential for further studies.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) was used to screen the bacteria based on their resistance to Cd. Pure bacterial strains were streaked on LB agar media amended with different concentrations of Cd. The source used for Cd stress was $CdCl_2$. The starting concentration of Cd in media was 50 mg L^{-1}. The concentration gradually elevated until it prevented the visible proliferation of bacterial colonies at 30°C for 48 hours (Haq et al., 1999). Plates without the addition of Cd were also placed as a control. The lowest Cd concentration that inhibited clear growth of colonies was considered MIC (Vela-Cano et al., 2014).

Effect of Cd on Rhizobacterial Isolates in Broth Culture

The effect of Cd on rhizobacterial isolates in broth culture was determined following the method of Andrews (2001). Concentrations of Cd 0, 400, 800, 1200 and 1600 µg mL^{-1} based on the MIC were amended in the culture using $CdCl_2$, and each treatment was replicated three times, and test tubes were incubated in a shaking incubator (100 rpm) at 30ºC for seventy-two hours. Proliferation of bacteria, i.e. optical density (OD) in broth, was evaluated at 600 nm wavelength using a spectrophotometer from twenty-four to seventy-two-hour intervals (Raja *et al.,* 2006).

Quantitative Cd Accumulation by Rhizobacterial Strains

Selected isolates were grown in LB broth devoid of Cd and placed in an incubator at 30 ˚C for seven days. Upon completion of seven days, samples were centrifuged at 12000 rpm for twenty minutes to harvest the rhizobacterial cultures from the pallets. Saline buffer was used to wash the pallets twice. Resuspend the pallets in broth modified with 1200 µg mL^{-1} of $CdCl_2$, and the temperature was adjusted to 28 ºC for three hours, and the process of centrifugation and cell harvesting was repeated as mentioned before. Residual Cd in the supernatant was measured using an Atomic Absorption Spectrophotometer (Model 240 FS, Agilent Technologies, USA) (Vela-Cano et al., 2014). The dry weight of bacterial pallets was also calculated. Each treatment was replicated three times. Accumulation of metal by bacterial strains was calculated by the equation proposed by Zafar *et al*. (2007) as follows:

$$\text{Metal removal} = \frac{C0-Ct}{M} \text{V} \ldots\ldots\ldots\ldots\ldots (1)$$

Where,

C0 Initial concentration of metal (mg/mL)

Ct Final concentration of metal after incubation (mg/mL)

M Dry mass (g) of bacterial pallet

V Total volume of culture (L)

Jar Trial

Depending on the *in vitro* characterization of PGPR strains, cadmium-resistant bacteria were selected regarding their plant growth-stimulating characteristics under axenic conditions. The surface disinfected tomato seeds (7% sodium hypochlorite solution and 70% ethanol) were inoculated with sterilized bacterial isolates by soaking in respective broth for 20-30 minutes before sowing. Cadmium was applied in three levels (0, 120 and 240 µg mL^{-1}) by spiking the soil with $CdCl_2$ as a source of Cd in Hoagland solution. Inoculated seeds were sown in autoclaved sand-

filled jars amended with different levels of Cd in a completely randomized design (CRD) with factorial settings and three replications. Sterilized Hoagland solution half strength (25 ml) was used as a source of irrigation and nutrients for control (Ahmad *et al*., 2016). Hoagland for stressed treatment was amended with 120 and 240 µg mL^{-1} of Cd^{2+}. Jars were placed in the growth room at 28 ±1 ºC with 10 hours of light and 14 hours of dark period of 15 ±1 ºC. The data regarding tomato plants was taken after 30 days of sowing.

Analysis of Cd in tomato seedlings

After 30 days, tomato plants were harvested, washed, and air-dried in the shade, after drying plants were placed in an oven at 65 ºC overnight. The oven-dried samples were then ground using a mechanical grinder into fine powder. Samples (0.2 g) were then wet digested by adding HNO_3 and $HClO_4$ in a 3:1 (v/v) ratio and used to analyze Cd concentration in plants using an atomic absorption spectrophotometer.

Identification of Cadmium resistant strains

Cd-resistant rhizobacterial strains used in the jar trial were identified through 16S rRNA amplification and sequencing following the method of Hussain et al. (2011). Bacterial DNA was extracted using PBS buffer and proteinase-K enzyme based on Mahuku's (2000) protocol, and quantification was performed using a Nanodrop before being sent to Macrogen (Seoul, Korea) for sequencing. The partial 16S rRNA gene sequences were analyzed using the NCBI BlastN tool. Phylogenetic analysis and molecular assessments were conducted using MEGA version 7 software following the approach of Roohi et al. (2012). Evolutionary relationships were inferred using the neighbor-joining method (Saitou and Nei, 1987), while evolutionary distances were determined through the maximum composite likelihood method (Tamura et al., 2004).

Statistical Analysis

The data was computed statistically by using Steel *et al*. (1997)'s method through two-way analysis of variance techniques (ANOVA) to analyze the significance of the data and treatment means were compared through Tuckey's test (HSD) to check the differences among treatments (Duncan, 1955).

RESULTS

Bacterial isolation and Screening against exopolysaccharides (EPS) and Siderophore production

Forty-five bacterial strains were isolated from contaminated soil irrigated with sewage water and coded as AR1-AR45. The isolate's ability to tolerate Cd was validated by cultivating them in liquid culture supplemented with Cd at 1200 µg mL^{-1} using $CdCl_2$. Results in table 1 revealed that all the examined isolates were capable of thriving in Cd-amended broth to varying extents (Table-1). Out of forty-five, sixteen bacterial isolates with maximum growth (OD value) in the presence of Cd in the LB media were further analyzed to assess their EPS and siderophore production. Out of sixteen, all isolates exhibit mucoid growth except AR40. However, AR5, AR17, AR25 and AR45 showed maximum EPS production (Table-2). In case of siderophore 5 isolates (AR5, AR10, AR17, AR25 and AR37) showed maximum siderophore production but AR20, AR35 and AR40 did not show any growth (Table-2).

Screening of Rhizobacterial isolates for their Plant Growth Promoting Properties

Results in Table 2 represent that rhizobacterial isolates AR5, AR10, AR17, AR25 and AR37 can effectively solubilize the Zinc, as indicated by colonies growth and the presence of clearing zone around them. In contrast, AR30, AR40, and AR43 did not display a clear zone around the colony. For the phosphate solubilization bioassay, the Cd-resistant strains AR2, AR20, AR30, AR40, and AR43 failed to produce a clear halo zone around colonies on an agar plate. All Cd-

resistant strains except AR1, AR5, AR10, and AR35 showed the ability to produce HCN (Table 3). For ammonia production, AR1, AR5, AR8, AR10, AR17, AR23, AR25, and AR37 were found positive. Among Cd-resistant strains for the assessment of catalase, all strains were positive for catalase production except AR1, AR8, AR23, AR30, AR35, AR42, and AR45. For oxidase production AR1, AR8, AR17, AR23, AR25, AR35, AR42 and AR45 performed best.

Findings (Table 3) demonstrated that all the examined strains possess the potential for the colonization of tomato roots. However, population density varies among these strains. Notably, strain AR5 (4.14×10^6) displayed the maximum extent of root colonization, succeeded by AR25 (3.61×10^6) and AR17 (3.46×10^6), respectively.

Selected Cd-resistant strains were also subjected to the synthesis of Indole acetic acid. Findings (Table 4) showed that all the examined isolates can produce IAA but the quantity varied between conditions with and without L-tryptophan (L-TRP). With L-TRP the maximum IAA production was observed in AR5 (14.6 mg IAA L^{-1}) followed by AR25 (13.34 mg IAA L^{-1}) and AR17 (11.38 mg IAA L^{-1}). Without L-TRP, IAA production ranges between 0.87 to 7.21 mg IAA L^{-1}.

Quantifying the Cd Effect on Growth, Siderophore and EPS Production Ability of Rhizobacterial Isolates

Cadmium-resistant isolates with siderophore, EPS and PGP characteristics were inoculated under varying degrees of induced stress. The growth, quantitative siderophore and EPS production by these strains were observed for 24 to 72 h. The data presented in Figure 1 indicates that increasing levels of Cd stress had a significant adverse impact on the proliferation of isolates. Additionally, increased Cd levels led to a reduction in the duration of the logarithmic growth phase, consequently causing an earlier entry into the stationary phase. The isolates exhibited varying

levels of Cd stress tolerance, though the differences in growth were not significant. An exception was AR5, which demonstrated significantly higher growth compared to AR17 at a Cd stress level of 1000 µg mL^{-1}.

The synthesis of siderophore and EPS by the selected strains exhibited an increase up to an initial forty-eight hours of growth, followed by a decline in production of both siderophore and EPS among all strains after 72h of inoculation (Figures 2 and 3). However, the variations in EPS production capability were evident among the strains. The strain AR25 showed maximum production of EPS (90.28 µg mL^{-1}) after 48 h of incubation at a lower level of Cd, specifically 500 µg mL^{-1}. Strain AR5 exhibited maximum EPS production when exposed to Cd stress at almost all levels. Substantial reduction in the production of EPS capacity of all isolates was evident at the maximum concentration of Cd after twenty-four, forty-eight, and seventy-two hours of incubation. Moreover, the strains exhibited substantial variability in their capacity to produce siderophore both in control and under Cd stress. The maximum siderophore production was 62 % by AR5 strain, followed by AR17 (58 %), and AR 25 (55 %) after 48 h of growth under lower Cd level (Figure 3), while the minimum siderophore production was recorded at 1500 µg mL^{-1} Cd stress after 72 h of growth which was 32, 20, and 26% by the AR5, AR17, and AR25, respectively.

Determination of Minimum Inhibitory Concentration of Cd (MIC)

The susceptibility of the chosen strains, AR5, AR17, and AR25 to Cd was examined, and their minimum inhibitory concentration (MIC) values were calculated. The collected data (Table 5) indicated that AR5 exhibited the maximum resistance against Cd. Specifically, the MIC values for Cd were measured at 1900, 1800 and 1700 µg mL^{-1} of Cd. The MIC values for an initial Cd concentration of 1200 µgmL^{-1} were used to measure the acquired Cd content by each strain.

Effectiveness of Cd Resistant Bacteria under Control Conditions

The selected Cd-resistant strains that produce siderophore and EPS and have PGP characters, were assessed for their potential to promote tomato plant growth under axenic conditions. Three different levels of Cd (0, 120 and 240 µg mL^{-1}) using $CdCl_2$ were maintained for the jar experiment. Apparently, Cd stress reduced the tomato plant growth but inoculation with Cd-resistant bacteria had a positive effect on tomato growth. Data collected for the Cd accumulation by the strain (Figure 5) indicated the ability of strains to accumulate/remove Cd. AR5 showed the maximum amount of Cd accumulation followed by AR25 and AR17.

Identification of bacterial isolates

The morphological and biochemical characteristics of these strains were investigated, revealing that AR5 has a gram-negative cell wall, rounded to rod-shaped colonies and AR17 and AR25 have gram-positive cell walls, rod-shaped bacteria that form smooth, white-coloured colonies. Furthermore, the strain's 16SrRNA partial sequence, designated as AR5, AR17, and AR25 were deposited in Gene Bank under accession numbers OR592656, OR592657, and OR592658, respectively. Blastin analysis and 16rRNA sequence analysis (Figure 4) showed that AR5 belongs to the *Acinetobacter* genus, for AR17, and AR25's closest similarity to the *Bacillus* genus. Notably, AR17 was closely related to *Bacillus bombysepticus* AR25 was found to be *Bacillus inaquosorum* and AR5 was identified as *Acinetobacter calcoaceticus.*

Effect of Cadmium resistant bacterial strain on plant growth attributes of *Solanum lycopersicum* (L.) under Cd stress

The effects of Cd on tomato plant height, root length, shoot dry weight, and root dry weight are presented in Figures (6 A, B, C and D). Plant height was negatively affected due to Cd stress at both levels. Substantial improvement in plant height was noted under Cd-resistant bacterial inoculation. Results (Figure 6A) indicated that the application of AR5 improved the plant height

up to 31% and 21% at 120 and 240 µg mL^{-1} Cd in comparison to its respective control. There was no significant difference between AR17 and AR25 performance at both stress levels.

The effect of three strains on the root length of tomato with or without Cd is shown in Figure 6B. Cadmium stress significantly reduced the root lengths, but plants inoculated with Cd-resistant bacteria were able to increase root lengths as compared to their respective control. Maximum reduction (39%) in root length was observed in uninoculated control at 240 µg mL^{-1}. Strain AR5 inoculation improved to 36%, followed by AR25 with 30% increase in root length at 120 µg mL^{-1}.

Maximum reduction in dry weight of tomato shoot (Figure 6C) was recorded at 240 µg mL^{-1} (50%) but the inoculation with AR5 improved the tomato dry weight by 35% followed by AR25 with 29% more dry weight in comparison to control.

In the case of Cd, enhancing Cd concentration from 120 to 240 µg mL^{-1}, a steady reduction in the dry weight of the root was noted (Figure 6D). Application of Cd-resistant bacterial strain increased the root dry weight at all levels of Cd. AR5 increased the dry root weight to 33% followed by AR25 and AR17.

Accumulation of Cd by tomato seedlings

Results regarding Cd uptake by tomato plants are presented in (Figures 6 E and F). Increasing the concentration of Cd, increased the Cd uptake by roots and ultimately shoots. Maximum uptake was observed at 240 µg mL^{-1} both in roots and in shoots of tomato plants. Inoculation of Cd resistant strain (AR5) reduced the uptake of Cd in roots by 47% and 40% at 120 and 240 µgmL^{-1}, respectively, as compared to control and the same trend was followed for the shoots by strains. For the shoot, metal uptake was low in comparison to the roots of tomato plants.

Application of Cd resistant strain (AR5) decreased the uptake of Cd by 33% followed by AR25 leading to a 30 % reduction in Cd uptake at 120 µgmL^{-1}.

Table 1 Rhizobacterial isolates' growth (OD600) on LB media, supplemented with Cd at 1200 µg ml^{-1}.

Rhizobacterial isolates	OD value	Rhizobacterial isolates	OD value	Rhizobacterial isolates	OD value
AR1	1.22± 0.02	AR16	0.88± 0.01	AR31	0.83± 0.02
AR2	0.96± 0.03	AR17	1.51± 0.03	AR32	1.07± 0.02
AR3	0.91± 0.03	AR18	1.04± 0.02	AR33	0.56± 0.04
AR4	0.81± 0.05	AR19	0.92± 0.04	AR34	0.67±0.03
AR5	1.62± 0.03	AR20	1.01± 0.02	AR35	1.01±0.02
AR6	0.85± 0.04	AR21	0.88± 0.05	AR36	0.43±0.03
AR7	1.03± 0.02	AR22	1.05± 0.05	AR37	1.15±0.03
AR8	1.17± 0.04	AR23	1.06± 0.03	AR38	0.39±0.05
AR9	.11± 0.03	AR24	0.97± 0.04	AR39	0.61±0.05
AR10	1.25± 0.05	AR25	1.31± 0.03	AR40	0.58±0.01
AR11	1.03± 0.05	AR26	1.02± 0.02	AR41	0.23±0.02
AR12	1.01± 0.01	AR27	1.06± 0.02	AR42	1.13±0.03
AR13	1.02± 0.02	AR28	0.77± 0.03	AR43	0.34±0.07
AR14	1.12± 0.03	AR29	0.89± 0.04	AR44	0.42± 0.05
AR15	1.03± 0.03	AR30	1.23± 0.02	AR45	0.51± 0.03

The values expressed within columns represent the mean of three repetitions ± standard error. Means within columns have the same letter are not significantly different ($p<0.05$) according to Tuckey's test.

Table 2: Screening of rhizobacterial isolates for plant growth-promoting (PGP) characteristics

Isolates	EPS Production	Siderophore Production	Zinc Solubilization	Phosphate Solubilization
AR1	++	++	+	+
AR2	+	+	+	-
AR5	+++	+++	++	++
AR8	++	+	+	+
AR10	++	+++	+	+
AR17	+++	+++	++	+++
AR20	+	-	+	-
AR23	++	+	+	+
AR25	+++	+++	++	++
AR30	+	+	-	-
AR35	+	-	+	+
AR37	++	+++	+	+
AR40	-	-	-	-
AR42	++	++	+	+
AR43	+	+	-	-
AR45	+++	+	++	++

(+++)= maximum growth, (++) = medium growth, (+) = growth, (-) = no growth

Table 3: Screening of rhizobacterial isolates for plant growth-promoting (PGP) characteristics

Isolates	HCN Production	Ammonia Production	Root colonization	Catalase Production	Oxidase Production
AR1	-	+	2.21×10^{6} jk	-	+
AR2	+	-	2.46×10^{6} i	+	-
AR5	-	+	4.14×10^{6} a	++	-
AR8	+	+	3.41×10^{6} d	-	+
AR10	-	+	2.18×10^{6} k	+	-
AR17	++	+	3.46×10^{6} c	+	++
AR20	+	-	1.72×10^{6} m	+	-
AR23	+	+	1.83×10^{6} l	-	+
AR25	+++	+	3.61×10^{6} b	++	+
AR30	+	-	3.21×10^{6} e	-	-
AR35	-	-	1.18×10^{6} n	-	+
AR37	+	+	2.59×10^{6} h	+	-
AR40	+	-	3.16×10^{6} f	+	-
AR42	+	-	2.23×10^{6} j	-	+
AR43	+	-	2.41×10^{6} i	+	-
AR45	+	-	3.11×10^{6} g	-	+

HCN= Hydrogen cyanide

(+++) = maximum growth, (++) = medium growth, (+) = growth, (-) = no growth.

The values expressed within columns represent the mean of three repetitions ± standard error. Means within columns have the same letter are not significantly different ($p<0.05$) according to Tuckey's test.

Table 4: Production of Indole-3-Acetic Acid by rhizobacterial strains with and without L-Tryptophan

Isolates	IAA Production mg/mL		Isolates	IAA Production mg/mL	
	With L-TLP	Without L-TLP		With L-TLP	Without L-TLP
AR1	9.79± 0.08 **d**	1.43± 0.15 **i**	AR27	7.87± 0. 38 **k**	2.19± 0.11**f**
AR2	8.51± 0.18 **j**	5.51± 0.06 **b**	AR30	9.16± 1.41**g**	4.31± 0.14 **c**
AR5	14.64± 0.14 **a**	7.21± 0.11 **a**	AR35	5.73± 0.53 **n**	3.18± 0.19 **d**
AR8	9.51± 0.03**e**	0.98± 0.12 **k**	AR37	7.53± 0.48 **l**	0.97± 0.17 kl
AR10	9.21± 0.06 **g**	2.11± 0.04 **f**	AR40	6.37± 0.43 **m**	2.43± 0.16 **e**
AR17	11.38± 0.04 **c**	3.21± 0.06 **d**	AR42	9.38± 0.77 **f**	1.91± 0.17 **g**
AR23	9.01± 1.33 **h**	3.13± 0.04 **d**	AR43	5.29± 0.51 **o**	0.87± 0.07 **l**
AR25	13.34± 1.53 **b**	1.18± 0.05 **j**	AR45	8.84± 0.05 **i**	1.79± 0.13 **h**

The values expressed within columns represent the mean of three repetitions ± standard error. Means within columns have the same letter are not significantly different ($p<0.05$) according to Tuckey's test.

Table 5: Minimum Inhibitory Concentration of Cd (MIC) of Rhizobacterial isolate

Rhizobacterial isolate	Strain name	Minimum inhibitory concentration (MIC) µg mL^{-1}
Acinetobacter calcoaceticus	AR5	1900
Bacillus bombysepticus	AR17	1800
Bacillus inaqusorum	AR25	1700

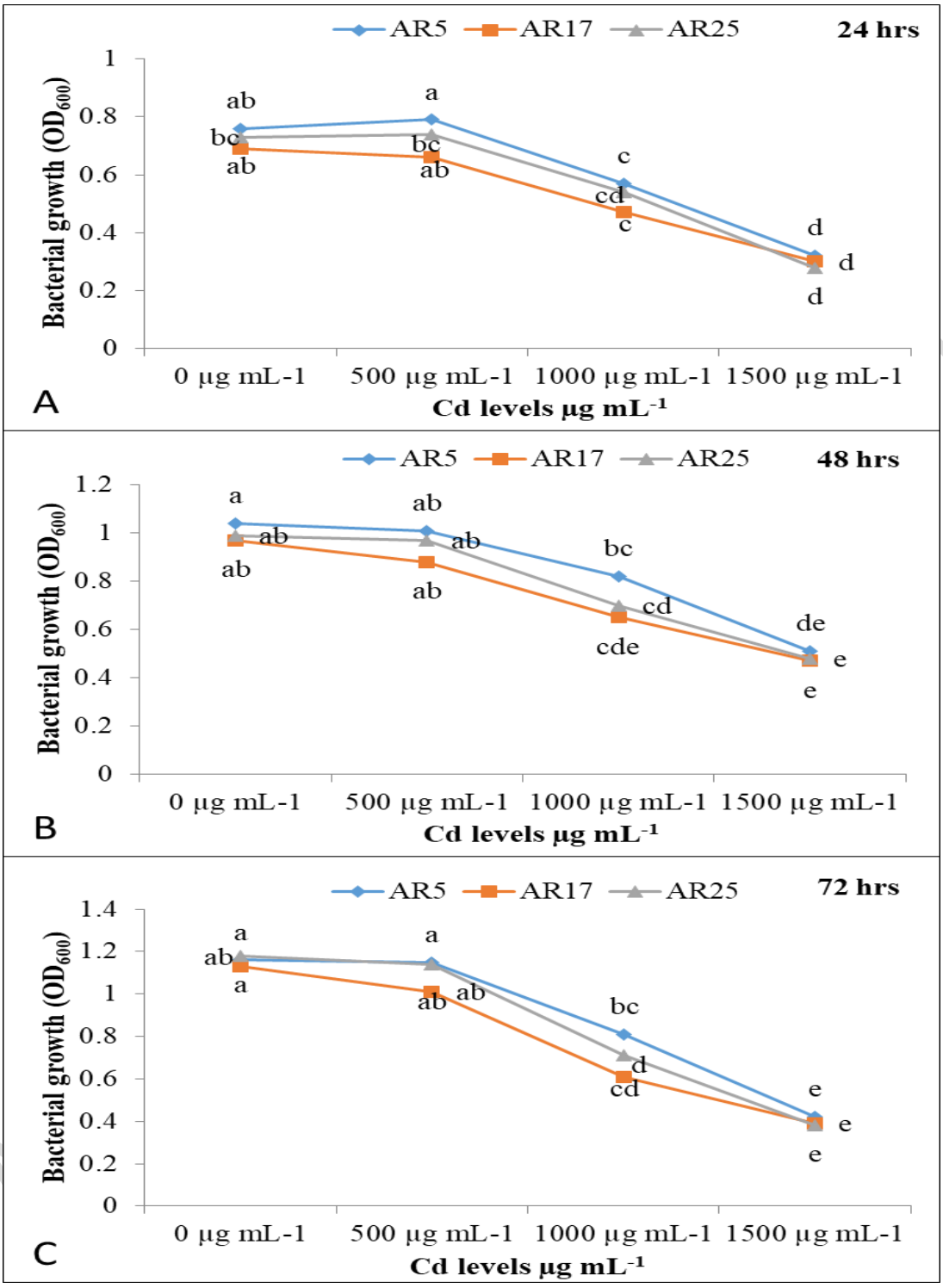


Figure 1: Effect of different cadmium concentration on bacterial growth (OD_{600}) over different time periods in broth culture. The lines represent the mean of three repetitions. Lines marked by different letters differ significantly ($p < 0.05$) as revealed by the Tuckey's test.

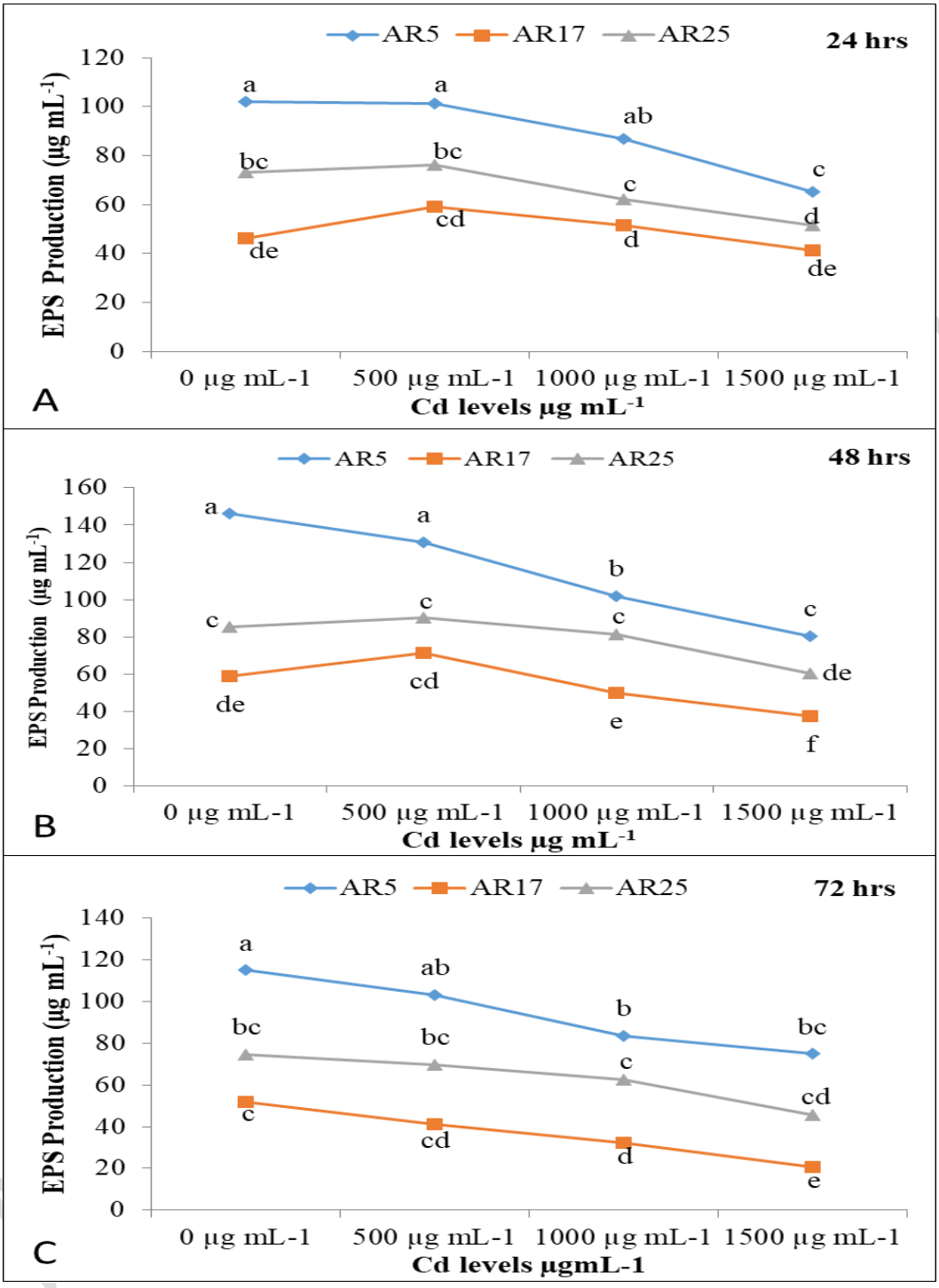


Figure 2: Effect of different cadmium concentration on EPS (µg mL^{-1}) production of rhizobacterial isolates over different time period in broth culture. The values expressed within columns represent the mean of three repetitions ± standard deviation. Bars marked by different letters differ significantly ($p < 0.05$) as revealed by the Tuckey's test.

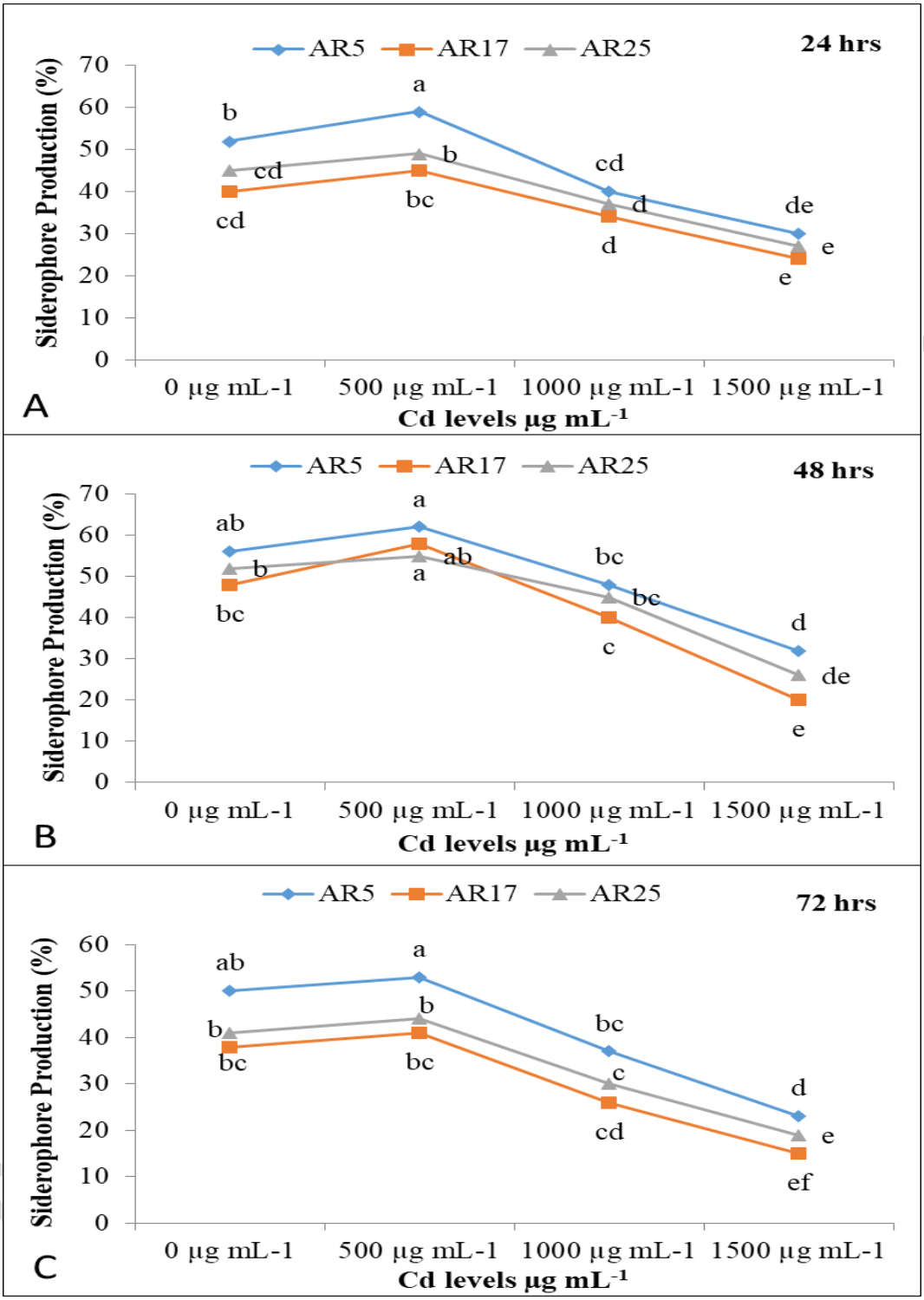


Figure 3: Effect of different cadmium concentrations on siderophore (%) production of rhizobacterial isolates over different time periods in broth culture. The values expressed within columns represent the mean of three repetitions ± standard deviation. Bars marked by different letters differ significantly ($p < 0.05$) as revealed by the Tuckey's test.

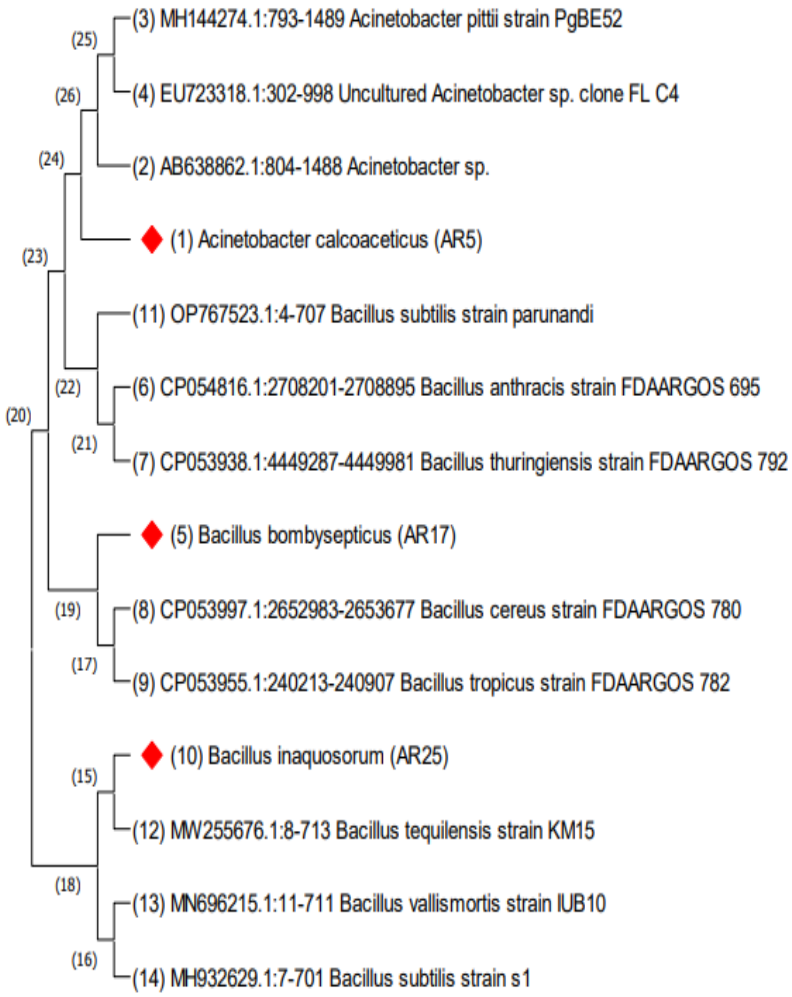


Figure 4: Neighbor-joining phylogenetic tree of *Acinetobacter calcoaceticus* AR5, *Bacillus bombysepticus* AR17 and *Bacillus inaquosorum* AR25 found in GenBank database.

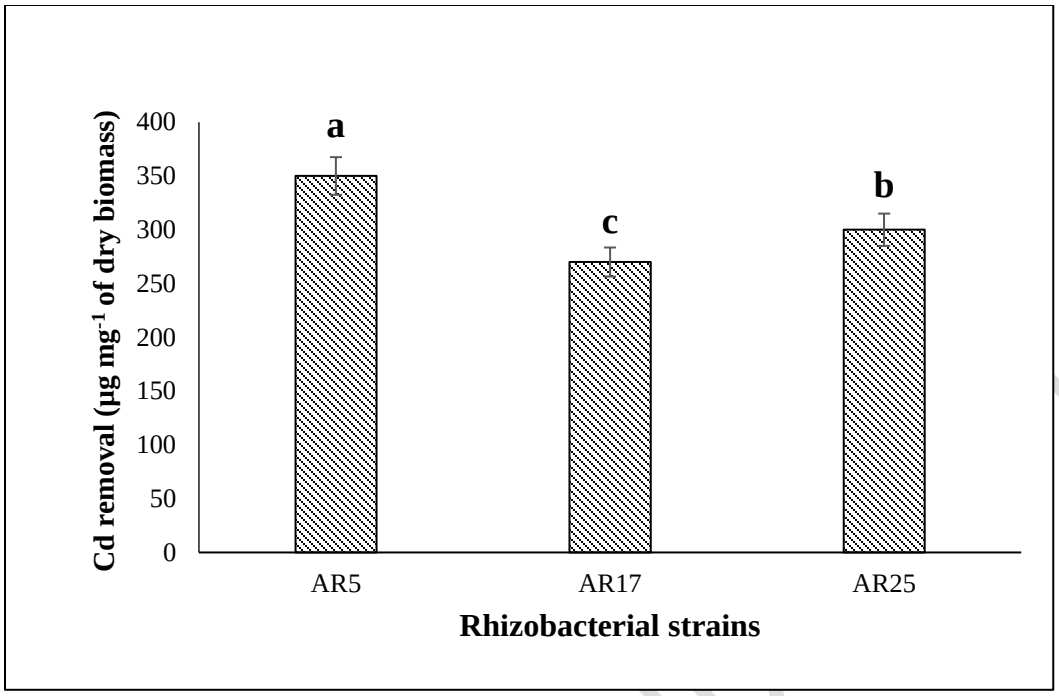


Figure 5: Quantity of Cd accumulation by rhizobacterial isolates. The bars represent the mean of three repetitions ± standard error. Bars marked by different letters differ significantly ($p < 0.05$) as revealed by the Tuckey's test.

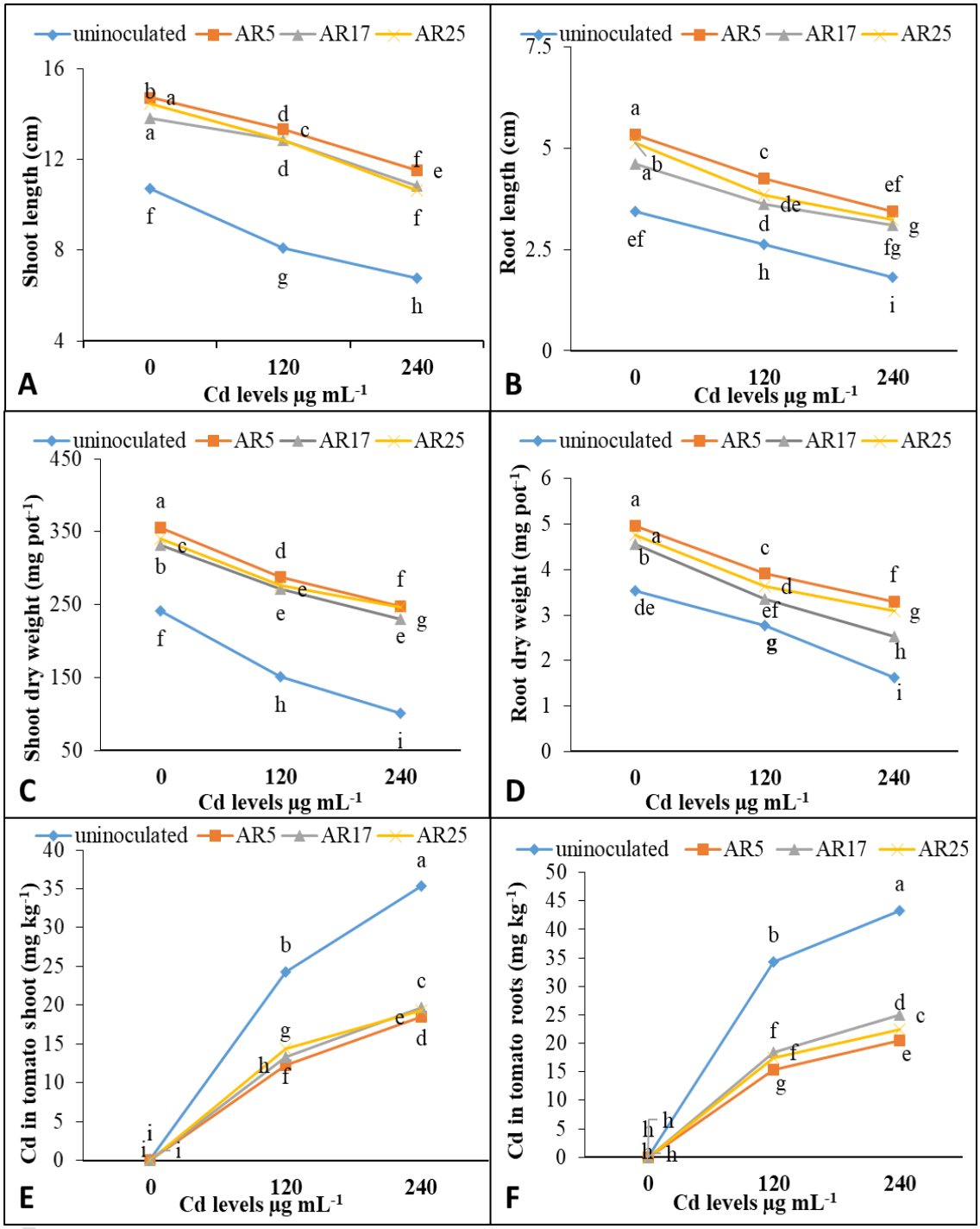


Figure 6: Effect of potential rhizobacterial strains on (A)shoot length, (B) root length, (C) shoot dry weight, (D) root dry weight, (E) Cd in tomato shoot and (F) Cd in tomato root under different stress levels of Cd. Lines marked by different letters differ significantly ($p < 0.05$) as revealed by the Tuckey's test.

DISCUSSION

The present investigation described the role of Cd-resistant rhizobacteria in tomato growth promotion under metal stress, especially cadmium (Cd).

Isolation, characterization and identification of strains

A total of 45 Cd-resistant strains were isolated on LB medium amended with $CdCl_2$ similar to our previous study of Shahid et al. (2023) and characterized for exopolysaccharides (EPS), siderophore, and other PGP characters. Out of 45 isolates, only 16 were efficient producers of siderophores and EPS. Three potential rhizobacterial isolates with maximum Cd tolerance were further characterized for their morphological and biochemical plant growth-promoting characteristics as mentioned by Wang et al. (2022a). The results depicted that these strains were siderophore, EPS, HCN and IAA producers along with P-solubilization, Zn-solubilization, catalase and oxidase activities which make them suitable candidates for their use in crop production especially tomato. Similar characteristics of Cd-tolerant bacteria were also studied by different researchers like EPS by Zhang et al. (2024), siderophore production by Wang et al. (2022b), IAA production by Rolón-Cárdenas et al. (2022), minerals (P and Zn) solubilization by Ammar et al. (2024) and enzymatic activities by Jabeen et al. (2024). Three potential isolates were identified as *Acinetobacter calcoaceticus* (AR5), *Bacillus bombysepticus* (AR17), and *Bacillus inaquosorum* (AR25) for tomato plant growth promotion. Bravo and Braissant (2022) described different genera of bacteria (*Bacillus, Pseudomonas, Acinetobacter, Acetobacter, Enterobacter, Brevundimonas* etc.) as potential meta tolerant, especially cadmium. Liang et al. (2025) described the role of *Bacillus cereus* as Cd tolerant bacteria for the bioremediation of contaminated soils.

Isolated strains were characterized for their plant growth-promoting characters like zinc and phosphate solubilization, IAA, EPS, siderophore, and HCN production and the results were at

par with Ahmad et al. (2018). *Acinetobacter* strains in this study showed remarkably higher phosphorus solubilization than previously reported *Pseudomonas* and *Serratia* species (Suresh et al., 2010). Proliferation of IAA-producing bacterial populations within the rhizosphere, accompanied by the secretion of IAA, could potentially stimulate root development (rhizogenesis) and better colonization by PGPR (Rokhbakhsh-Zamin et al., 2011; Dar et al., 2022). In our study, Cd-resistant rhizobacterial isolates AR5, AR17, and AR25 were able to solubilize both zinc and phosphate. The mobilization of zinc and phosphate minerals by microorganisms enhances nutrient accessibility, while catalase and oxidase enzymes contribute to the regulation of oxidative stress in tomatoes (Vázquez-Hernández et al., 2021; Kumar et al., 2020; Sudmoon et al., 2024). Furthermore, the strains showed significant variation in their ability to generate siderophore under both normal and Cd-stressed conditions. Siderophores make a complex with cadmium and various other metals, thereby diminishing their availability and mitigating their toxicity (Neilands, 1981; Shahid et al., 2024).

Cd-tolerance/Cd-resistance by bacteria

The identified strains showed differential potential in their minimum inhibition concentration of Cd in growth medium ranging from 1700-1900 µg mL^{-1}. The results of MIC are in line with Zeng et al. (2009) and Nath et al. (2014) who isolated Cd-tolerant strains from *P. aeruginosa*, E1, and *P. aeruginosa*, SN1 and SN3 with MICs of 2092.72, 1700 and 1800 µg mL^{-1}, respectively. The increased tolerance to higher Cd concentrations in these strains might be due to various mechanisms, such as intracellular/extracellular sequestration, exclusion, detoxification, and ATP-mediated efflux of Cd from cellular components (Schwager et al., 2012; Gadd et al., 2004). Moreover, the Cd removal by bacteria from the growth medium also provides the adaptive mechanism of these bacteria to cope with higher Cd concentrations. Ali et al. (2022) described that

483 the Bacillus strains might tolerate Cd metal by biosorption of Cd on the bacterial cell wall might
484 be due to the presence of functional groups i.e. $COOH^-$, NH_2, OH^-, SO_3 and C-N groups. Another
485 reason of the biosorption of Cd on bacterial cell walls might be the production of EPS which also
486 bind Cd by ino exchange mechanism in the presence of different functional groups (Mathivanan
487 et al., 2023).

488 **Bacterial strain and plant growth under Cd stress**

489 The results of our investigation depicted that cadmium stress negatively affects tomato
490 growth, but the application of the Cd-tolerant bacteria helps plants to cope with the toxic effects
491 of Cd as compared to the control. Previous studies also witnessed that contamination of the growth
492 medium (soil) with any metal hinders plant growth (Naveed et al., 2020). Cadmium stress
493 decreases plant growth, it might be due to a reduction in photosynthetic activity, disruption in the
494 membrane's structure and disturbance of nutrition (Nas and Ali, 2018; Manisha et al., 2019).

495 However, the application of Cd-resistant bacterial strains revealed a significant
496 improvement in plant biomass, root length, and nutrient uptake and effectively reduced Cd uptake
497 by tomato plants exposed to high Cd concentration in soil. This improvement might be due to a
498 direct correlation between the cadmium detoxification abilities of these bacteria and their positive
499 influence on plant growth parameters and soil carbon (Shi et al., 2020; Tong et al., 2024).
500 Mukherjee in their study observed that the *Halomonas* sp. EXo1 improved plant growth by
501 lowering metal accumulation. This reduction was attributed mainly to EPS production by EXo1
502 which sequesters metal ions either by binding them or by biosorption on microbial surfaces
503 (Mukherjee et al., 2019). Inoculation with AR5, AR 17 and AR25 positively improved the total
504 dry biomass of tomato. This growth improvement might be linked with the reduced Cd uptake by

the bacterial modifications in the rhizosphere, biosorption on their surface or bio stabilization in the soil (Sarwar et al., 2023).

Another reason for tomato growth improvement might be the ability of these strains to possess plant growth-promoting traits i.e. mineral solubilization, IAA production, siderophore production, catalase, oxidase and ACC-deaminase activity (Dar et al., 2024). The isolated strains exhibited not only resistance to cadmium but also demonstrated plant growth-promoting traits, indicating a dual benefit in the context of sustainable agriculture (Sun et al., 2020; Zeng et al., 2025). The results of our findings are in line with the findings of Sarwar et al. (2023) who also described plant growth-promoting traits as a possible reason for growth improvements in tomatoes.

CONCLUSION

In conclusion, strain AR5 demonstrates the ability to stabilize heavy metals in contaminated soils, contributing to reduced cadmium accumulation in the edible parts of crops. By exploring the interactions between these bacteria and plants, this study offers valuable insights into crop production on cadmium-contaminated soils. Furthermore, it highlights the potential for developing sustainable and effective bioremediation strategies, addressing both environmental and agricultural challenges.

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Data Availability Statement

The data presented in this study is available on request from the first corresponding author.

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