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# Research Article
# Cadmium-resistant *Ralstonia mannitolilytica* Relieved Cadmium Toxicity in Mustard Plant Through Root Colonization and Growth Promoting Activity

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## Abstract

**Background:** Increasing concentration of toxic metals like cadmium (Cd) in the environment due to anthropogenic activities is one the serious global concern since these metals accumulate in soil and are often 'biologically magnified' and enter into the human body through food chain, leading to several diseases. Application of metal resistant plant growth promoting bacteria decreases metal concentration in the edible plants influencing their growth. **Methodology:** A Cd tolerant (500 mg $L^{-1}$) bacterial strain was isolated from an industrial waste collected at Kalyani, West Bengal, India and identified as *Ralstonia mannitolilytica* KUCd7 on the basis of 16S rDNA sequence analysis and phenotypic characterizations. **Results:** The isolate also showed tolerance to other metals including chromium (150 mg $L^{-1}$), nickel (175 mg $L^{-1}$) and zinc (200 mg $L^{-1}$). About 28°C temperature, pH 7 and glucose as carbon source were most favourable conditions for its growth and generation time was calculated as 40 min 15 sec. When it was grown in medium supplemented with Cd (100 mg $L^{-1}$) it could remove 83.04% Cd from the medium and accumulated Cd in cells (44.05 mg $g^{-1}$ of the dry weight). Regarding plant growth promoting properties, it solubilized insoluble inorganic phosphate (418.83 mg $L^{-1}$), produced hydroxamate type of siderophore (84.23 μM) and synthesized 1-aminocyclopropane-1-carboxylate (ACC) deaminase to break ACC into α-ketobutyrate (0.627 μmol $mg^{-1}$ $h^{-1}$). All these attributes might cumulatively promote growth of mustard (*Brassica* sp.) plant when the isolate was applied as a seed inoculant and reduce Cd uptake (71.4% in shoot and 64.8% in root) by the plant. **Conclusion:** So, *Ralstonia mannitolilytica* KUCd7 could be utilized for formulation of the biofertilizer in Cd contaminated soil in future.

Key words: Bioremediation, cadmium, mustard, plant growth promotion, *Ralstonia mannitolilytica*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Contamination of agronomic soil with heavy metals due to indiscriminate release of industrial effluents is becoming one of the most severe global threats since the metals are not degraded biologically and persist in the environment indefinitely. Cadmium (Cd) is a nonessential highly toxic heavy metal. It is released into the environment by activities of mining and smelting, deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge and fossil fuels burning[1]. Soil acidity increases its mobilization and uptake by crops. Plants exposed to Cd exhibit toxic effects like inhibition of root formation, the main root became brown, rigid, twisted, mucilaginous and decomposed, reduction of root and shoot elongation, rolling of leaves[2]. Modifications of chloroplast ultrastructure, lowering of chlorophyll contents resulting chlorosis and restricted activity of photosynthesis are the major toxic effects of Cd on foliage. In planta, Cd gets accumulated in the edible portions and enters into food chain. In human, Cd exposure results in damage to lungs, gastrointestinal tract, kidneys and nervous system[3]. The Cd is known to bind with essential respiratory enzymes causing oxidative stress and cancer[4,5]. In Japan, itai-itai disease occurred due to high Cd concentration in silver mine waste water, causing skeleton deformation and spontaneous fractures[6]. Strategies should be developed to inhibit cadmium uptake for limiting long term toxicity since it has an extremely long half-life of 30 years in humans[7].

Metal-resistant Plant Growth Promoting Bacteria (PGPB) mitigate metal toxicity in soil and in plant via sequestration and intracellular accumulation of metal ions and improve competitiveness and responses of plant to stress factors[8]. These organisms actively colonize rhizosphere and rhizoplane and promote plant growth by fixing atmospheric nitrogen, producing siderophore that chelates iron to make it available to plant root, solubilizing minerals such as phosphorus, synthesizing plant growth regulators such as indole acetic acid, lowering the level of ethylene in plants by production of enzyme, 1-amino cyclopropane-1-carboxylate (ACC) deaminase and reducing the deleterious effects of plant pathogens on crop yield by producing antimicrobial compounds and other mechanisms. Deficiencies of Fe and P in plants is induced by Cd[9,10] and metal-resistant PGPB may overcome this demand by supplementing Fe and P by producing siderophore and solubilizing phosphate, respectively and promote growth in normal as well as metal stressed conditions. It may therefore, be advisable for growers to inoculate plants with such microbial inoculants in order to increase plant biomass and thereby stabilize, rejuvenate, restore and remediate heavy metal polluted soils.

In this context the aim of this study is to isolate and identify Cd resistant bacteria from heavy metal contaminated site, investigate its Cd removal property, explore its plant growth promoting attributes such as sideophore production, phosphate solubilization, ACC deaminase activity and also evaluate its efficacy of Cd bioremediation as well as growth promotion of mustard plant in both normal and Cd stressed conditions so that it could be utilized in biofertilizer formulation for the Cd contaminated agricultural soil in future.

## MATERIALS AND METHODS

**Isolation and selection of cadmium tolerant bacteria:** Soil sample collected from an industrial waste contaminated with various heavy metals at Kalyani (N 22°59', E 88°26'), West Bengal, India was screened for isolation of bacteria by dilution plate technique on Glucose Peptone (GP) agar medium (peptone 1%, NaCl 0.5%, glucose 0.8%, agar 2%, pH 7) supplemented with Cd (100 mg $L^{-1}$) prepared from cadmium chloride monohydrate (≥98%, Merck). The colonies which appeared within 5 days of incubation at 28°C were further tested for higher Cd tolerance (200-500 mg $L^{-1}$) and the isolate which could tolerate highest Cd concentration was selected for further studies.

**Identification of the isolate:** The promising isolate was identified based on morphological, biochemical characterizations and 16S rDNA sequence analysis. Colony morphology was studied by growing the isolate on Glucose Minimal (GM) medium at 28°C for 3 days and the colony colour was observed on the medium containing 2, 3, 5 triphenyl tetrazolium chloride. Cell morphology was studied using scanning electron microscope. Gram reaction, endospore staining and motility test were performed[11]. Biochemical tests were performed in accordance with Bergey's Manual of Systematic Bacteriology[12]. Test for acid production was performed using minimal medium supplemented with different types of carbohydrates at a concentration of 1% (w/v) and alkalinization of organic acids was carried out using Simmon's agar medium.

Genomic DNA was extracted using the methods described by Sambrook and Russell[13]. The 16S rDNA gene was amplified using bacterial specific forward and reverse primer set, 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-TACGGTTACCTTGTTACGACTT-3', respectively. The amplifications were performed using Perkin-Elmer PCR system. The PCR product was purified and sequenced by ABI PRISM 377 automated DNA sequence (Perkin-Elmer,

Applied Biosystem, Inc.). Similarity searches of the sequence obtained were performed using nucleotide BLAST function at NCBI[14]. Phylogenetic relationship was analysed using the software MEGA5[15]. Multiple sequences alignment was carried using a CLUSTALW and the evolutionary history was inferred using the neighbour-joining method[16].

**Optimization of growth conditions and growth kinetics study:** Growth temperature was optimized by incubating 1% culture of the isolate at different temperature (10, 20, 28, 37 and 45°C) and for optimization of growth pH same amount of inoculum was incubated at variable pH (4, 5, 6, 7, 8, 9 and 10) in Glucose Minimal (GM) medium [(g $L^{-1}$): $K_2HPO_4$ 3, $Na_2HPO_4$ 6, NaCl 5, $NH_4Cl$ 2, $MgSO_4$ 0.1, glucose 8]. To assess the most favourable carbon source, 1% culture was incubated at 28°C in minimal medium supplemented with different carbon sources (glucose, lactose, sucrose, mannitol and sorbitol) individually at a concentration of 0.8%. Growth was examined by measurement of optical density at 600 nm of the turbidity of culture after 96 h. Growth kinetics of the isolate was studied by incubating 1% culture in GM medium at optimized growth conditions and measuring the turbidity of culture (OD at 600 nm) at interval up to 96 h.

**Multi-metal and antibiotics tolerance study:** Tolerance to heavy metals was determined by observing growth of the isolate with increasing concentrations of the metals (Cd, Cr, Ni and Zn) on GP agar medium. Metals were supplemented in the media from stock solutions prepared from salt of the metals, viz., cadmium chloride monohydrate (≥98%, Merck), potassium chromate (99.5%, Merck), nickel chloride hexahydrate (≥97%, Merck) and zinc sulphate heptahydrate (99.5%, Himedia) individually in deionized water. These solutions were sterilized through milipore filter of 0.22 µm pore size. Antibiotic resistance property was tested by paper disc assay using the antibiotics viz., ampicillin, chloramphenicol, rifampicin, streptomycin and tetracycline, each at a concentration of 50 µg $mL^{-1}$.

**Assay of cadmium removal at different growth phases:** Isolate was grown in Glucose Peptone Water (GPW) in absence (control) and presence of Cd (100 mg $L^{-1}$) at 28°C for 5 days on 120 rpm and the growth responses were determined at 24 h interval by observing OD at 600 nm. To study the Cd removal efficacy of the isolate, supernatant from both control and treated sets collected at interval were digested with concentrated nitric acid (69%, Merck) at 80°C and Cd concentration was analysed using atomic absorption spectrophotometer (Spectra AA-240, Agilent) with an air-acetylene flame and a digital readout system at a wave length of 228.8 nm as per recommendation. Detection limit of the instrument for Cd was 0.01 mg $L^{-1}$. The instrument was calibrated using standard solution of Cd prepared from cadmium chloride monohydrate (≥98% Cd, Merck). Percentage of Cd removal was calculated using the formula:

$$\frac{C_0 - C_1}{C_0} \times 100$$

Where:

$C_0$ = Cd concentration of supernatant of control

$C_1$ = Cd concentration of supernatant of inoculated set

The Cd accumulation in bacterial cell was determined from acid digested cells collected after 96 h of incubation. It was determined that all the measured concentrations of the metal were 99±1% of the nominal concentrations.

**Characterization of *in vitro* plant growth promoting properties**

**Qualitative assay:** The promising Cd tolerant isolate was tested for its ability to produce ACC deaminase for growth in presence of ACC as sole nitrogen source. Siderophore production was assayed by formation of orange halo on Chrome Azurol Sulfone (CAS) plate. Indole acetic acid production was tested using Salkowski reagent[17]. The HCN production was detected by growing the organism on glycine supplemented medium and development of brown colour of the filter paper soaked with $Na_2CO_3$ and picric acid solution. Production of $NH_3$ was tested using Nessler's reagent. Solubilization of inorganic phosphate was assayed by formation of halo zone on Pikovskaya's agar medium. Isolate positive for qualitative tests were further studied for quantitative estimation.

**Assay for 1-aminocyclopropane-1-carboxylate deaminase activity:** The enzyme activity in the cell free extract of the bacterial isolate was estimated by measuring the amount of α-ketobutyrate (αKB) generated by the hydrolysis of ACC[18]. The amount of αKB was quantified spectrophotometrically by reacting with 2,4-dinitrophenyl hydrazine reagent and plotting it against standard curve of αKB. The protein concentration in cell suspensions was determined using Folin-Ciocalteau reagent with bovine serum albumin as a standard.

**Assay for siderophore production:** Siderophore production in GM medium was determined as described by Schwyn

and Neilands[19] using Chrome Azurol Sulfone (CAS) assay with desferal (Sigma, USA) as a standard. Chemical nature of the siderophore was detected according to Csaky[20] for hydroxamate type and Arnow[21] for catechol type with hydroxylamine hydrochloride and 2,3-dihydroxybenzoic acid as standards, respectively.

**Assay for phosphate solubilization:** Phosphate solubilizing activity was examined by determining the solubilization index [the ratio of the total diameter (colony+halo zone) to the colony diameter][22] after inoculating the isolate on Pikovskaya's agar medium and incubated for 5 days at 28°C. Production of soluble phosphate from inorganic insoluble form in Pikovskaya's broth was carried out by growing the isolate in the medium for 5 days at 28°C and soluble phosphate was estimated using molybdate vanadate ammonium reagent and $KH_2PO_4$ as a standard[23].

**Study of phosphate solubilization and siderophore production in presence of cadmium:** Isolate was grown on Pikovskaya's agar medium supplemented with increasing concentrations (0, 1, 5 and 10 mg $L^{-1}$) of Cd. Colony diameter and solubilization indices were measured at each Cd concentration after 5 days. Simultaneously, isolate was cultivated in GM medium with increasing concentrations (0, 1, 5 and 10 mg $L^{-1}$) of Cd. After 96 h of incubation at 28°C CAS assay was performed using the culture supernatant to measure the siderophore production in each set.

**Bioassay for plant growth promotion:** Surface-sterilized mustard seeds were soaked either with sterilized water or with bacterial suspension (6 log CFU $mL^{-1}$) overnight at 4°C in dark and sown in sterilized soil. Four sets of treatment were prepared of which two were inoculated with the isolate. The Cd stress was created in one inoculated and one uninoculated sets following irrigation of Cd supplemented water to the soil so that final Cd concentration in the soil would be 10 mg $kg^{-1}$. The concentration of choice for the metal was higher than maximum permeable limit of Cd in the soil but would not have a severe effect on the test plant. Five plants were considered for each set. The plants were harvested after 45 days and measurement of growth parameters was done prior to dry weight estimation. Chlorophyll in leaves was extracted with acetone and measured using the method according to Arnon[24]. To study rhizosphere colonization, soil sample adhering to the roots was taken and the bacterial population was determined by dilution plating on GP agar medium supplemented with Cd (100 mg $L^{-1}$) or without Cd for the assessment of contaminants, if any. The colonies that appeared on the medium supplemented with Cd were also identified on the basis of colony morphology and other characteristics.

**Cadmium estimation in plants:** For estimating total Cd in the plant parts like root and shoot, samples were vigorously washed with 0.01 M EDTA solution and then in distilled water to remove any nonspecifically bound Cd and were digested in a mixture of concentrated $HNO_3$ and $HClO_4$ (4:1, v/v). Total Cd content in the digest was determined by atomic absorption spectrometer. Transfer Factor (TF) and translocation factor (TLF) for Cd were measured as follows[25]:

TF = Root Cd concentration/Soil Cd concentration
TLF = Shoot Cd concentration/Root Cd concentration

**Statistical analysis:** Descriptive statistical analysis of data were performed for each experiment. For pot experiment, data of each set were subjected to statistically analyzed by one way ANOVA ($p = 0.05$) using 'Microsoft office excel 2013' software, considering each set as one treatment with five replications including respective control set. The obtained F-values were compared for variance and respective critical difference values (CD, $t_{0.05}$) were calculated to test the hypotheses.

## RESULTS

**Isolation and identification of cadmium tolerant isolate:** In the primary screening with Cd concentration (100 mg $L^{-1}$) a number of Cd-tolerant bacterial colonies were obtained. As a consequence of the increasing dose of Cd, most of these failed to survive. Only one isolate, designated as KUCd7 could tolerate the highest Cd concentration (500 mg $L^{-1}$) and was selected for further studies.

The isolate, KUCd7 was found to be Gram negative, aerobic, non-spore-forming, motile, rod shaped, positive for catalase, oxidase and arginine dihydrolase, produce acid from L-arabinose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, trehalose and D-xylose; also alkalize acetate, citrate and tartrate (Table 1). Colony took pink colour when grown on medium containing 2, 3, 5-triphenyl tetrazolium chloride. All the phenotypic characterization has suggested that KUCd7 belongs to the genus *Ralstonia*.

About ~1.5 kb amplified 16S rDNA region of the isolate was observed on agarose gel and from this a stretch of

Table 1: Morphological and biochemical characteristics of *Ralstonia mannitolilytica* KUCd7

| Tests | Properties |
|---|---|
| Colony morphology on GM agar medium after growth at 28°C for 3 days | Circular, entire, flat, smooth, opaque, white, 5 mm in diameter |
| Colour of colony in presence of 2, 3, 5 triphenyl tetrazolium chloride | Pink |
| Bacterial cell shape, size | Rod (3.74×0.72 μm) |
| Gram reaction | Gram negative |
| Motility | + |
| Spore | - |
| Growth on MacConkey | + (NLF) |
| Indole test | - |
| Methyl red test | - |
| Voges Proskauer test | - |
| Casein test | - |
| Nitrate reduction | - |
| Catalase/Oxidase | +/+ |
| Arginine dihydrolase | + |
| $H_2S$ production | - |
| **Hydrolysis of** | |
| Starch | - |
| Tween 80 | + |
| Esculine | - |
| Urea | - |
| Gelatin | - |
| **Production of acid from** | |
| L-Arabinose | + |
| Fructose | + |
| Galactose | + |
| Glucose | + |
| Inositol | - |
| Lactose | + |
| Maltose | + |
| Mannitol | + |
| Mannose | + |
| Raffinose | + |
| Rhamnose | + |
| Salicin | + |
| Sucrose | - |
| Trehalose | + |
| D-xylose | + |
| **Alkalinization of** | |
| Acetate | + |
| Citrate | + |
| Oxalate | - |
| Tartrate | + |

1323 bp had been sequenced. Homology searches for the partial rDNA sequence of KUCd7 through nucleotide BLAST algorithm at NCBI database showed maximum identity (99%) with the rDNA sequence of *Ralstonia mannitolilytica* CSIB3-10 and these belong to the same evolutionary branch in the neighbor-joining phylogenetic tree (Fig. 1). The sequence has been submitted to GenBank and allotted an accession number KF242535.

**Optimization of growth conditions and growth kinetics study:** Though *R. mannitolilytica* KUCd7 can able to grow at variable range of temperature (20-45°C) and pH (5-9), 28°C temperature and pH 7 were considered as the optimal for maximum growth of KUCd7 in GM medium (Fig. 2a, 2b). Glucose was found to be the most favourable carbon source followed by mannitol for growth of KUCd7 (Fig. 2c). The growth kinetics study of *R. mannitolilytica* KUCd7 in GM medium under optimum conditions revealed that after 6 h of lag phase a sharp increase in growth response was observed (Fig. 2d). Maximum growth (OD 2.4) was observed at 32 h and almost remained constant up to 72 h. Finally, slight decrease in OD of cell biomass was observed at 96 h may be due to autolysis of some cells. Generation time of *R. mannitolilytica* KUCd7 was calculated as 40 min 15 sec that implies it was a fast growing bacterium.

**Tolerance to heavy metals and antibiotics:** The isolate, KUCd7 showed tolerance to cadmium up to 500 mg $L^{-1}$,

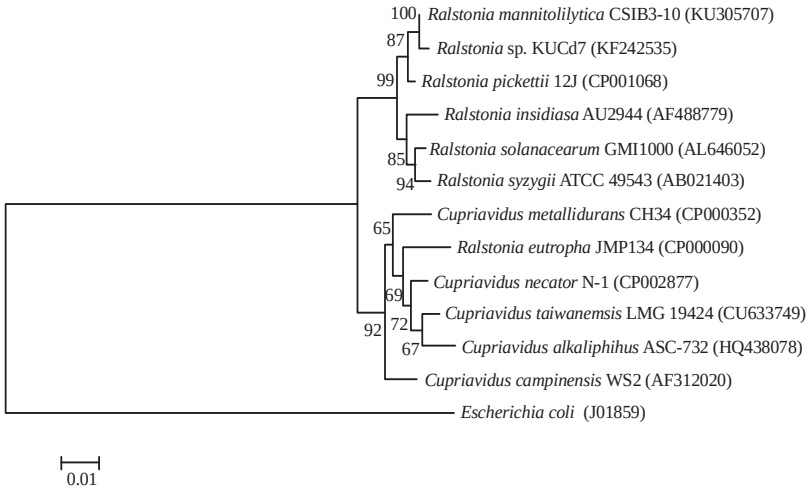


Fig. 1: Phylogenetic tree based on 16S rDNA gene sequence prepared by neighbor-joining method showing relationship of *R. mannitolilytica* KUCd7 with the closest representatives. The numbers at the nodes are percentages that indicate the levels of bootstrap support (n = 1,000 re-samplings). Except for the sequence determined in this study, all 16S rDNA sequences were retrieved from GenBank

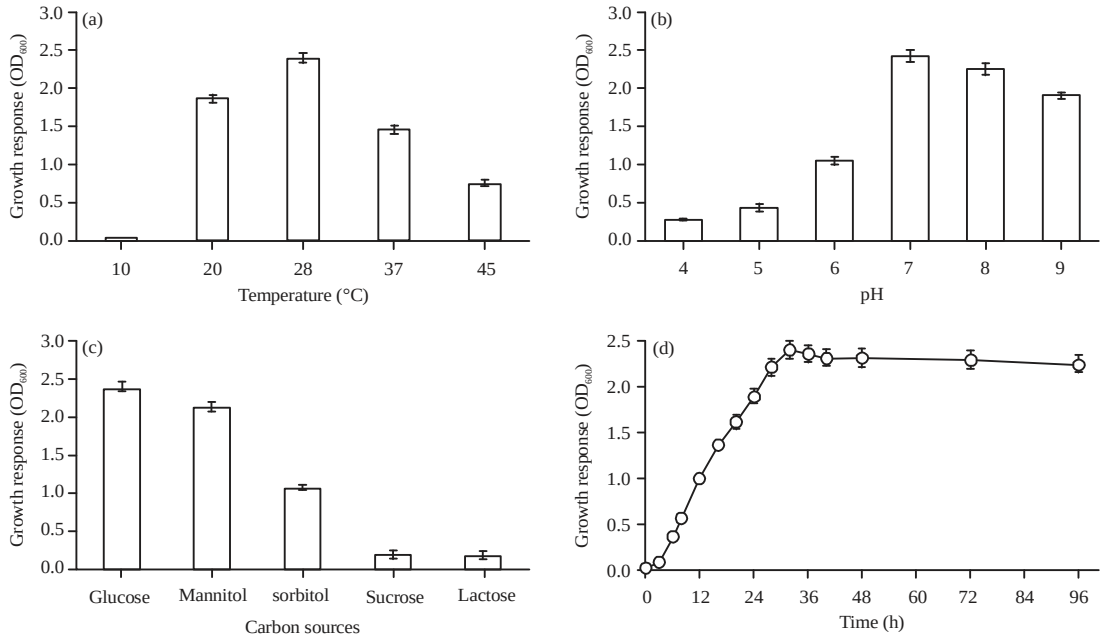


Fig. 2(a-d): (a) Optimization of temperature, (b) pH, (c) Favourable carbon source for *Ralstonia* sp. KUCd7 and (d) Growth kinetics of *R. mannitolilytica* KUCd7 in optimized growth conditions. Bars represent Mean±SE

chromium up to 150 mg $L^{-1}$, nickel up to175 mg $L^{-1}$ and zinc up to 200 mg $L^{-1}$ (Table 2). Thus, the order of tolerance regarding the metal concentration was Cd>Zn>Ni>Cr. The isolate was found to resistant against ampicillin and tetracycline (Table 2).

***In vitro* cadmium bioremediation activity:** In glucose peptone water medium supplemented with Cd (100 mg $L^{-1}$), considerable increase in the cell growth was observed after 24 h of incubation (Fig. 3). The cadmium removal efficiency of the organism as determined by atomic absorption spectroscopy was found to increase with time during active growth phase. After 96 h of incubation 83.04% Cd removal was also observed when the cell population had reached its maxima (2.21 OD at 600 nm). At 120 h slight increase in Cd removal (83.8%) was observed and Cd accumulation was

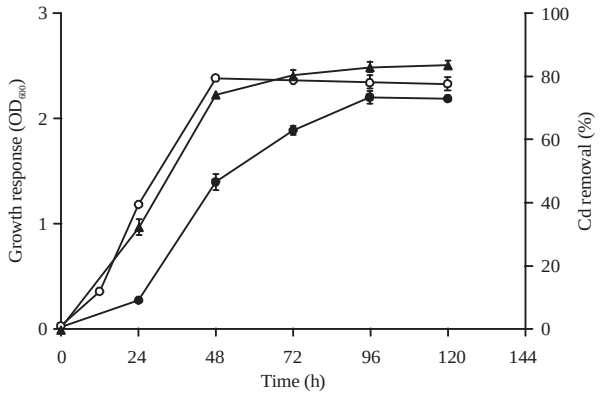


Fig. 3: Growth response in absence (opened circle) and presence (filled circle) of cadmium and cadmium removal (filled triangle) by *R. mannitolilytica* KUCd7 in glucose peptone water medium supplemented with Cd. Bars represent Mean±SE

Table 2: Antibiotic and heavy metal resistance property of *Ralstonia mannitolilytica* KUCd7

| | | |
|---|---|---|
| Metals | Cd | 500 mg $L^{-1}$ |
| | Cr | 150 mg $L^{-1}$ |
| | Ni | 175 mg $L^{-1}$ |
| | Zn | 200 mg $L^{-1}$ |
| Antibiotics (50 μg $mL^{-1}$) | Ampicillin | + |
| | Chloramphenicol | - |
| | Tetracycline | + |
| | Streptomycin | - |
| | Rifampicin | - |

+Stands for resistance and -Stands for sensitive

measured 44.05 mg $g^{-1}$ of the dry weight of cells of KUCd7 (Fig. 3). Final cell biomass of KUCd7 produced at the end of growth were almost similar in presence and absence of Cd, although in presence of Cd growth response was slow and took more time to reach to its maxima (Fig. 3).

**Characterization of *in vitro* plant growth promoting properties:** *Ralstonia mannitolilytica* KUCd7 developed distinct colony on ACC supplemented medium and could utilize ACC as a sole nitrogen source. The ACC deaminase mediates conversion of ACC to α-ketobutyrate. The ACC deaminase activity of KUCd7 was expressed by production of substantial amount of αKB (0.627 μmol $mg^{-1}$ $h^{-1}$). The KUCd7 showed halo zone around its colony on Pikovskaya's agar medium indicating its ability to solubilize insoluble tricalcium phosphate [$Ca_3(PO_4)_2$]. Solubilization Index (SI) was measured as 1.8. In liquid PKV medium concentration of soluble phosphates released by KUCd7 was quantified as 418.83 mg $L^{-1}$. The isolate produced an orange halo zone around its colony on CAS agar plate with the diameter of 1.75 cm. Quantitative estimation revealed that the isolate

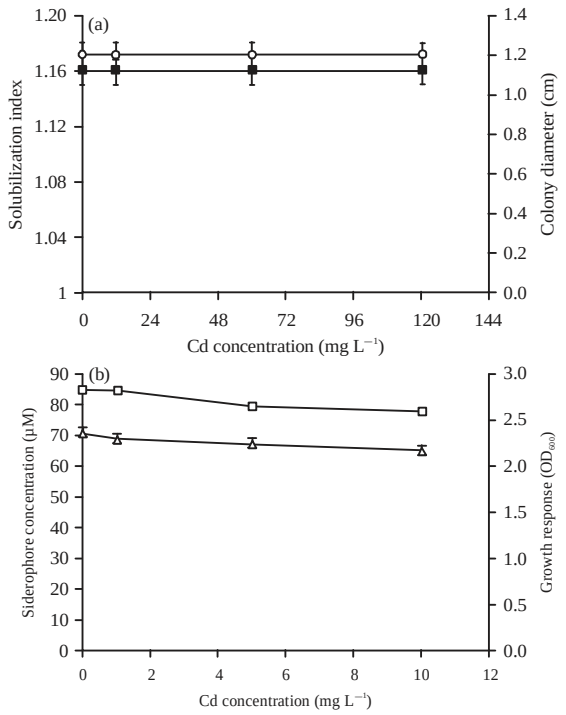


Fig. 4(a-b): Effects of Cd concentrations up to 10 mg $L^{-1}$ on (a) Phosphate solubilization index (filled square) along with colony size (opened circle) of KUCd7 and (b) Siderophore production (opened triangle) along with growth response (opened square) of KUCd7

produced siderophore (84.23 μM) during late log phase. Chemical nature of the siderophore was hydroxamate type as ascertained by Csaky's assay only. The isolate was found to be negative for production of IAA, HCN and $NH_3$.

**Effect of cadmium on phosphate solubilization and siderophore production:** With the increase of Cd up to 10 mg $L^{-1}$ colony size and phosphate solubilization indices were almost remained unchanged (Fig. 4a). No considerable change was also observed in growth and siderophore production of KUCd7 with the variation of Cd concentration at that same range (Fig. 4b). So it can be concluded that Cd concentration up to 10 mg $L^{-1}$ did not affect phosphate solubilization and siderophore production ability of *R. mannitolilytica* KUCd7.

**Effects of *R. mannitolilytica* KUCd7 on mustard plant under normal and Cd stressed conditions:** The isolate successfully colonized in the rhizosphere soil of mustard plant (*Brassica* sp.) grown both in normal and Cd enriched soils, though KUCd7 persisted in the rhizosphere comparatively

better in normal (7.093 log CFU $g^{-1}$ of soil) than Cd contaminated condition (6.856 log CFU $g^{-1}$ of soil). Table 3 shows the effect of the application of KUCd7 in root and shoot elongation, increase in chlorophyll content and dry weight of *Brassica* plants under normal and Cd stress conditions. All the growth parameters like shoot and root length, chlorophyll content and plant dry weight decreased by 34.34, 17.21, 66.18 and 21.05%, respectively due to Cd stress conditions in the absence of KUCd7. Tran and Popova[2] also reported same kind of effect in plants due to Cd toxicity. In contrast, in absence of Cd stress, considerable increase in shoot and root length, chlorophyll content and plant dry weight by 32.32, 17.88, 1.07 and 30.26%, respectively was observed due to application of KUCd7. This data confirmed the plant growth promoting activity of the isolate. However, in presence of Cd stress, inoculation with KUCd7, increased shoot and root length, chlorophyll content and plant dry weight by 48.46, 17.6, 36.5 and 25%, respectively in compare to Cd stressed uninoculated condition. Application of KUCd7 decreased accumulation of Cd in root by 64.8% (26.73 μg $g^{-1}$) in compare to uninoculated set (75.93 μg $g^{-1}$) and in shoot by 71.4% (16.83 μg $g^{-1}$) also in compare to uninoculated set (58.85 μg $g^{-1}$) (Table 3). Bacterial application also influenced both transfer and translocation factors of mustard plant. Transfer factor was reduced by 64.8% and translocation factor was reduced by 18.76% in presence of KUCd7 (Fig. 5).

## DISCUSSION

In response to heavy metal challenge, metal-tolerant bacteria adapt various mechanisms through which they could survive in these habitats. If these bacteria become beneficial to economically important plants, promoting their growth and reducing metal uptake in the plants, then the bacteria could be applied for their potential role in the bioremediation of contaminated sites[26]. In the present study, a number of Cd tolerant bacteria were isolated from an industrial waste sample contaminated with heavy metals at Kalyani, West Bengal, India. Among these isolates KUCd7 was screened as the most promising strain due to its highest Cd tolerance ability and identified as *Ralstonia mannitolilytica* based on 16S rDNA sequence analysis and phenotypic characterization (Table 1). The genus *Ralstonia*[27] had been divided into two distinct sublineages with a 16S rDNA sequence dissimilarity of >4%[28]. The *Ralstonia eutropha* lineage comprised of *R. basilensis*, *R. campinensis*, *R. eutropha*, *R. gilardii*, *R. metallidurans*, *R. oxalatica*, *R. paucula*, *R. respiraculi* and *R. taiwanensis*. The *Ralstonia pickettii* lineage (genus *Ralstonia sensu stricto*) comprised of *R. insidiosa*, *R. mannitolilytica*, *R. pickettii*, *R. solanacearum* and *R. syzygii*. In addition, metal-resistant *Ralstonia* species mainly belonging to *R. eutropha* lineage were later classified

Table 3: Effect of *Ralstonia mannitolilytica* KUCd7 on mustard plant under normal and Cd stressed conditions

| | Treatments | | | | |
|---|---|---|---|---|---|
| Parameters | Control (untreated plant) | With Cd stress | With KUCd7 | Cd stress+KUCd7 | Critical difference at 5% significance level |
| Shoot length (cm) | 19.8 (±1.58) | 13 (±0.92) | 26.2 (±1.33) | 19.3 (±0.93) | 3.960 |
| Root length (cm) | 15.1 (±0.8) | 12.5 (±0.84) | 17.8 (±0.86) | 14.7 (±0.74) | 4.050 |
| Chlorophyll content (mg $g^{-1}$) | 2.72 (±0.043) | 0.92 (±0.045) | 2.75 (±0.045) | 1.26 (±0.067) | 0.165 |
| Dry weight of plant (g) | 0.228 (±0.011) | 0.18 (±0.007) | 0.297 (±0.004) | 0.225 (±0.004) | 0.025 |
| Cd in shoot (μg $g^{-1}$) | BDL | 58.85 (±3.17) | BDL | 16.83 (±1.37) | 6.830 |
| Cd in root (μg $g^{-1}$) | BDL | 75.93 (±2.29) | BDL | 26.73 (±3.2) | 7.640 |

BDL: Below detection level, Data is the mean of three replications with ±SE in parenthesis, Critical difference ($p<0.05$) extracted from analysis of variances

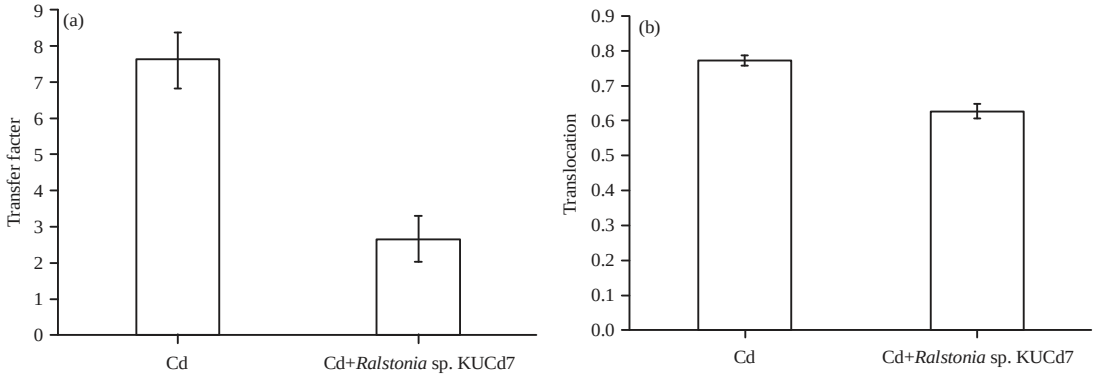


Fig. 5(a-b): Effects of KUCd7 on Cd (a) Transfer and (b) Translocation factors of mustard plant grown in 10 mg $kg^{-1}$ of cadmium amended soil

under the genus *Cupriavidus*[29,30]. In the phylogenetic tree prepared by neighbor-joining method, the isolate KUCd7 has placed to the same evolutionary branch with *Ralstonia mannitolilytica* strain CSIB3-10 and belonged in different branch from *Cupriavidus* strains (Fig. 1). Although, metal resistance has been observed in *Ralstonia* and *Cupriavidus* spp.[31] to the best of our knowledge, this is the first research report elucidating the role of a heavy metal-resistant *Ralstonia mannitolilytica* in Cd bioremediation in the medium and in the mustard plant with its concurrent promotion of growth in normal and Cd stressed conditions. Chovanova *et al*.[32] characterized eight CD resistant bacteria belonging to the genera *Alcaligenes*, *Comamonas*, *Klebsiella*, *Pseudomonas* and *Serratia* from high cadmium (7.5 µg g) containing sewage sludge sample. Occurrence of metal resistant property in different bacteria might be due to horizontal transfer of genes responsible for the character[33].

The isolate, KUCd7 also showed increased tolerance to other metals including Cr (150 mg $L^{-1}$), Ni (175 mg $L^{-1}$) and Zn (200 mg $L^{-1}$) (Table 2). This multi-metal tolerance property might play a significant role for survival of the organism and to perform as an effective Cd bioremediating agent in an environment contaminated with Cd and other heavy metals like Cr, Ni and Zn.

Optimization of growth conditions is an important criterion for mass production of the isolate. As the isolate, KUCd7 was isolated from soil optimum temperature and pH for growth was found to be 28°C and pH 7 (Fig. 2a, b). Fast growing character of the isolate is also useful for its rapid cultivation.

Metabolic conditions of bacterial cell vary with changing growth phase that can influence metal uptake and its removal from the environment. To study the relation, metal removal efficacy was investigated at different growth phases of KUCd7 (Fig. 3). The Cd removal was dependent on an active cell population since uptake of the metal across cell membrane was an energy requiring process[34]. The cadmium removal efficiency of KUCd7 was found to support the view as Cd removal by the isolate has been increased sharply throughout the exponential growth phase. After achieving late log phase, a steady removal of Cd was observed independent of viable cell mass increment. Bacterial growth reached to its stationary phase with the maximum cell population (2.21 OD at 600 nm) after 96 h which also affected the Cd removal to reach 83.04%. Slight increase in Cd removal (83.8%) at 120 h was observed might be due to adsorption of the Cd on the surface of viable and non-viable cell mass. The Cd removal varied in different organisms, for example, 62% by *Pseudomonas aeruginosa* BC15[35], 82.7% by *Escherichia coli*[36] and 93% by *Klebsiella pneumoniae* CBL-1[37]. The Cd accumulation was measured as 44.05 mg $g^{-1}$ of the dry weight of cells of KUCd7. Similar kind of results was also found in case of *Pseudomonas stutzeri* KCCM 34719 which absorbed 43.5 mg Cd $g^{-1}$ of cell[38], while *Streptomyces* sp. The F4 absorbed 42.7 mg $g^{-1}$ of dry cell mass[39].

Bacteria having ACC deaminase activity are beneficial to plant growth by lowering the level of ethylene and protect plants from damage[18]. *Ralstonia* KUCd7 developed distinct colony on ACC supplemented medium and could utilize ACC as a sole nitrogen source using the enzyme, ACC deaminase that mediated conversion of ACC to α-ketobutyrate. The ACC deaminase activity of KUCd7 was expressed by production of substantial amount of αKB (0.627 µmol αKB $mg^{-1}$ $h^{-1}$). *Variovorax paradoxus* 5C-2 showed ACC deaminase activity of 4.2±0.2 µmol αKB $mg^{-1}$ $h^{-1}$ and different strains of *Rhizobium leguminosarum* bv. *viciae* showed varied range of ACC deaminase activity from 0.43-1.06 µmol αKB $mg^{-1}$ $h^{-1}$ [40,41].

Iron is an essential element of all living organisms but under aerobic condition and at biological pH, it remains insoluble and scarcely available. To face the challenge, microorganisms secrete siderophore to chelate ferric iron for their use. Siderophores were also thought to facilitate biocontrol by sequestering iron from the environment, thus limiting growth of pathogens and facilitating plant disease control. Iron supplied by microbial siderophores such as ferrioxamine B, rhodotorulic acid and agrobactin, was utilized by certain plants, however, the proper mechanism of iron uptake was indistinct[42]. *Ralstonia mannitolilytica* KUCd7 produced an orange halo zone around its colony on CAS agar plate with the diameter of 1.75 cm. *Sinorhizobium meliloti* showed positive CAS assay with zone width of halo 1.7±0.3 cm[43] almost similar with KUCd7. The KUCd7 produced much higher concentration of siderophore (84.23 µM) than *Pseudomonas aeruginosa* KUCd1 (19.33 µM siderophore)[44]. Chemical nature of the siderophore was hydroxamate type as ascertained by Casky's assay only. This type of siderophore was reported to immobilize the heavy metals in soil[45].

Phosphorus (P) is one of the key nutrients for plant growth and development. It exists as organic and inorganic forms in soils. Insufficiency of available phosphates due to low solubilities of common phosphates such as $Ca_3(PO_4)_2$, hydroxyapatite and aluminum phosphate, in soil considered as one of the important growth-limiting environmental factors for plants[23]. Bacteria may supply this nutrient to plant by liberating P from insoluble phosphates (both organic and inorganic) compounds. Most organic phosphorous compounds were mineralized by production of enzymes,

such as phosphatase, phytase, phosphonoacetate hydrolase, D-α-glycerophosphatase, C-P lyase by rhizobacteria. Solubilization of inorganic phosphates, such as tricalcium phosphate was mainly facilitate by production of organic acids, such as gluconic acid, 2-ketogluconic acid, acetic acids, glycolic acid, oxalic acid, malonic acid, succinic acid, citric acid and propionic acid by soil microorganisms and corresponding decrease in pH[46]. *Ralstonia* KUCd7 showed halo zone around its colony on Pikovskaya's agar medium and its solubilization index [SI] was measured as 1.8. Solubilization indexes measured for three strains of *Pseudomonas* spp. designated[47] as KUCd2, KUCd3 and KUCd4 were 2.5, 1.8 and 2.2 among which KUCd3 has almost similar SI to *R. mannitolilytica* KUCd7. In liquid PKV medium concentration of soluble phosphates released by KUCd7 was quantified as 418.83 mg $L^{-1}$ which was almost similar to *P. fluorescens* B16 (427.7 mg $L^{-1}$)[23].

Phosphate solubilisation and siderophore production ability of *R. mannitolilytica* KUCd7 have not been affected by Cd concentration up to 10 mg $L^{-1}$. In *Pseudomonas aeruginosa* increased siderophore production was reported with increase of Cd concentration upto 1.75 mM[44].

Application of *R. mannitolilytica* KUCd7 reduced accumulation of Cd in root by 64.8% and in shoot by 71.4%. This Cd bioremediating trait of KUCd7 is better than *Pseudomonas aeruginosa* that decreased 52.44 and 36.89% of Cd uptake in roots and shoots of mustard plant, respectively[44]. In the presence of high levels of metals most plants synthesize ethylene and also become severely iron depleted. Utilization of ACC was the best, among several possible mechanisms through which the metal resistant rhizosphere bacteria protect the host plants from metal toxicity[48]. *Variovorax paradoxus* strains 2C-1, 2P-4, 3C-5 and 5C-2 having high level of ACC deaminase activity (6.2-9.3 μmol αKB $mg^{-1}$ $h^{-1}$) showed maximum root length-promoting effect on Cd-treated *Brassica juncea* seedlings, whereas *Ralstonia* sp. 2P2 had also contributed in root elongation mechanism of Cd-treated *B. juncea* seedlings significantly[49]. In presence of ACC deaminse producing *Ralstonia* KUCd7 significant level of root elongation of mustard plant was observed irrespective of Cd treated and untreated conditions. The Cd was reported to interfere with the uptake of several elements including P by plant[50]. The KUCd7 with its phosphate solubilization capacity might compensate phosphate deficiency and stimulate plant growth by influencing availability of this micronutrient. The Cd also inhibited the chelating process of iron and the uptake of the element[51]. Siderophore produced by KUCd7 might solve the problem by supplying iron in the form of Fe-siderophore complex in iron deficient condition. Sinha and Mukherjee[44] reported that in presence of siderophore producing Cd resistant *Pseudomonas aeruginosa* supply of iron in mustard plant increased in Cd amended soil. Transfer factor was reduced by 64.8% and translocation factor was reduced by 18.76% in presence of KUCd7 (Fig. 5). According to Tran and Popova[2] roots of plants retained greater portion of cadmium taken up by plant, though a portion was translocated to the aerial parts of the plant also. Findings of this experiment regarding distribution of accumulated Cd in plant parts also support their view.

## CONCLUSION

Therefore, successful rhizosphere colonization and cumulative effects of phosphate solubilization, siderophore production and ACC deaminase activity of *R. mannitolilytica* KUCd7 might actively contribute to the growth promotion of mustard plant both in normal and Cd stressed conditions. It also decreased Cd stress in the plant by reducing Cd mobilization and accumulation from rhizospheric soil. So, we can consider *R. mannitolilytica* KUCd7 as a potential plant growth promoting and Cd bioremediating agent, although further exploration is required to study its magnitude of growth promoting feature and Cd bioremediation efficacy on other crops and under variable field conditions at Cd contaminated sites.

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