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IN-VITRO CADMIUM BIOREMEDIATION POTENTIALITY OF *RALSTONIA MANNITOLILYTICA* KUCD7 AND ITS APPLICATION TO REMOVE THE METAL FROM SEWAGE WATER

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ABSTRACT

Microorganisms play vital roles to decide environmental fate of toxic metals such as cadmium (Cd) with an array of mechanisms, including bioaccumulation of the metal extracting it from the contaminated site. Therefore this can be exploited as potential Cd bioremediation method. The present study investigated the influence of variable physiological factors such as temperature, pH, and salinity on the Cd removal activity of a Gram negative cadmium resistant bacterium, *Ralstonia mannitolilytica* KUCd7. Temperature 28°C, pH 7 and 0.5% NaCl concentration of the medium favoured the maximum amount of bacterial growth, and facilitated the highest level (83.04%) of Cd removal activity after 96 h of incubation. Increasing initial concentration of Cd (10-100 mg/L) declined the metal removal efficiency without altering the bacterial growth, considerably. *R. mannitolilytica* KUCd7, also removed zinc (Zn), nickel (Ni) and chromium (Cr) from medium in presence of Cd. But other metals showed antagonistic effects on its Cd removal efficiency. Transmission electron microscopy (TEM) of ultra-thin section of Cd treated cells of the organism along with energy dispersive X-ray spectroscopy (EDX) revealed that intercellular accumulation of Cd in the cell peripheral region. Cell fractionation study strongly supported the finding as Cd was found to be localized mainly in periplasm (47.77%) and membrane (36.28%) fraction. *R. mannitolilytica* KUCd7 also removed up to 78.18% of Cd from 100 mg/L of Cd amended sewage water at *in-vitro* condition. Thus, Cd bioremediation process of *R. mannitolilytica*

KUCd7 greatly relies on bioaccumulation mechanism and optimized physiological conditions, for achieving maximum bioremediation efficacy, which may offer a suitable tool for Cd bioremediation from contaminated sewage water.

Keywords: Bioremediation, cadmium, Atomic absorption spectroscopy, Transmission electron microscopy, energy dispersive X-ray spectroscopy, cell fractionation, sewage

INTRODUCTION

Cadmium (Cd) is a heavy metal pollutant which is non-essential for biological system and highly toxic even at a relatively low concentration. According to World Health Organization (WHO) the highest permissible level of cadmium in drinking water is 0.003 mg/L **[1]**. Anthropogenic activities such as industrial waste disposal from plastic manufacturing, galvanizing plating or pigments industries, Cd/Ni battery factories, fertilizer application and sewage sludge disposals on land have led to the accumulation of Cd in soil and it's leaching under certain soil and environmental conditions **[2]**. Its high solubility in water increases its bioavailability and rapid uptake by plant with lowering soil pH **[3]**. Soil contamination of Cd can decrease crop yields by inhibiting photosynthesis, respiration, nitrogen metabolism and by reducing water and essential mineral uptake **[4]**. After entering into the food chain through dietary intake of plant-derived food and beverages inflicting long-term effects on human health including kidney damage, high blood pressure, and bone fractures **[5, 6]**. The problem is intensified by the fact that cadmium has a very long biological half-life (30 years in the kidneys). Cadmium induces DNA damage and its long-term mutagenic and carcinogenic effects has been well recognised. In Japan, itai-itai disease occurred due to consumption of rice cultivated in a field irrigated with highly Cd contaminated silver mine waste water, and causing pain, skeletal deformation and spontaneous fractures **[7]**. It is acknowledged as a priority pollutant by the US Environmental Protection Agency (USEPA) **[8]**. The European Chemicals Agency (ECHA) has placed Cd^{2+} on the Candidate List of Substances of Very High Concern for Authorisation (SVHC).

Since the quality of life is inseparably related to the overall quality of the environment, attention has been focused on controlling the heavy metal contaminants such as cadmium that threaten the ecosystem and restore the environmental health. Parallel advancement in science and technologies to industrial revolution has facilitated to discover and exploit the inherent traits of natural biological resources to overawe the heavy metal pollution. The advent of bioremediation technology of heavy metal has provided an alternative to conventional methods as it becomes more economical and eco-friendly process which mainly exploit resistant mechanisms of microorganisms against certain metal pollutants for pollution control. Many bacteria evolved within environmental stress became tolerant to high concentrations of Cd. Genetic determinants for their Cd resistant can be plasmid borne or chromosomal. Making the microbial cells impermeable to Cd^{2+}, cell surface sorption through passive physicochemical interactions between Cd^{2+} and the functional groups of the cell wall, extracellular precipitation of Cd^{2+} into sulfide or phosphate minerals, exopolysaccharides or capsular materials dependent binding of Cd^{2+}, Cd^{2+} uptake followed by detoxification through active efflux by RND driven systems (e.g., Czc) or P-type ATPase pump (e.g., CadA), intracellular sequestration as insoluble form (cell bound phosphate or sulfide salt) or Cd^{2+} binding by thiol group enriched peptides and metallothionein proteins are the basic mechanisms for developing resistance to cadmium by microorganisms **[1, 9]**. These microbial mechanisms influence metal mobility and bioavailability, which are the fundamental components of biogeochemical cycles of metals and other elements including carbon, nitrogen, sulfur and phosphorus as well, with additional implications for plant productivity and human health **[10]**.

Though Cd causes reduced growth, long log phases, lower cell densities, and even bacterial death **[1]** some bacteria were adapted to develop resistance against this toxic element. *Ralstonia mannitolilytica* KUCd7 was reported as such a Cd resistant bacterium and Cd bioremediation agent which successfully relieved Cd toxicity from mustard plant grown in Cd amended soil and promoted plant growth **[11]**. *In-vitro* studies also supported the view as it could remove up to 83.8% of Cd from culture medium after 120 h of incubation. On this background the aim of this investigation is to characterize the bioremediation mechanism of *Ralstonia mannitolilytica* KUCd7 in more detail, assess the influence of physiological factors and other heavy metals on its Cd bioremediation efficiency. Finally, *in-vitro* evaluation of its Cd bioremediation efficacy from artificially Cd contaminated sewage water, commonly used for irrigation in agricultural field, was also performed.

MATERIALS AND METHODS

Inoculum, culture medium, growth and metal removal estimation:

Cadmium resistant (500 mg/L) *Ralstonia mannitolilytica* KUCd7 was grown in glucose peptone (GP) medium [(g/L): Peptone 10, NaCl 5, Glucose 8; pH 7±0.2] and from this 1% late log phase culture was used as initial inoculum for inoculating the metal supplemented broth. After 96 h of incubation at variable conditions according to the need of the experiment (described in following sections) growth was monitored by measuring the turbidity [optical density (OD)] of the culture at 600 nm through UV visible spectrophotometer (CECIL CE7200). It was then centrifuged to separate the cell and the culture supernatant was digested with nitric acid (69%, Merck) at 80°C and metal concentration in cell free supernatants were analysed using atomic absorption spectrophotometer (AAS) (Spectra AA-240, Agilent) with an air-acetylene flame and a digital readout system at a wave length of 228.8 nm, 232 nm, 213.9 nm and 357.9 nm as per recommendation for the measurement of Cd, Ni, Zn and Cr concentration, respectively. Detection limit of the instrument for Cd, Ni, Zn and Cr were 0.02 mg/L, 0.1 mg/L, 0.01 mg/L and 0.06 mg/L respectively. The instrument was calibrated using standard solutions of cadmium, nickel, zink and chromium prepared from 1 g/L stock solution of cadmium chloride monohydrate (≥ 98%, Merck), nickel chloride hexahydrate (≥ 97.0%, Merck), zinc sulphate heptahydrate (≥ 99.0%, Himedia) and potasium chromate (≥ 99.5%, Merck), respectively.

Percentage (%) of metal removal activity was calculated using the formula: $[(C_0 - C_1)/C_0] \times 100$..... (1); where, C_0 and C_1 = initial and final metal concentrations (mg/L) of the cell free supernatant. It was determined that all the measured concentrations of the metal were 99±1% of the nominal concentrations.

Effects of physiological factors on growth and Cd removal activity:

Effect of temperature: Four sets of GP medium supplemented with Cd (100 mg/L) were inoculated with *R. mannitolilytica* KUCd7 and incubated for 96 h in a shaker (160 rpm) at four different temperatures, *viz.*, 10°C, 28°C, 37°C and 45°C. Culturing and monitoring the growth responses of the organism were done following the process discussed earlier. Cd removal activity by *R. mannitolilytica* KUCd7 were calculated according to the formula (1) described previously.

Effect of pH: Six sets of the GP medium supplemented with Cd (100 mg/L) were prepared adjusting the initial pH of the medium at 4 to 9 using 1 N HCl or 1 N

NaOH. Each set was inoculated and incubated at 28°C in a shaker (160 rpm) for 96 h. Culturing and monitoring the growth responses of *R. mannitolilytica* KUCd7 was done following the process discussed earlier. Cd removal activity by KUCd7 in each set of treatment were calculated according to the formula (1) described previously.

Effect of NaCl: Five sets of Cd (100 mg/L) supplemented GP medium containing 0%, 0.5%, 1% 2% and 4% of NaCl were prepared, inoculated and incubated at 28°C in a shaker (160 rpm) for 96 h. Culturing and monitoring the growth responses of *R. mannitolilytica* KUCd7 was done following the process discussed earlier. Cd removal activity by KUCd7 in each set of treatment were calculated according to the formula (1) described previously.

Effects of initial Cd concentrations on Cd removal activity: Five sets of GP medium (pH 7±0.2), supplemented with variable concentrations of Cd (10 mg/L, 25 mg/L, 50 mg/L, 75 mg/L and 100 mg/L) were inoculated and incubated at 28°C for 96 h in a shaker (160 rpm). Culturing and monitoring the growth responses of *R. mannitolilytica* KUCd7 was done following the process discussed earlier. Cd removal activity by KUCd7 in each set of treatment were calculated according to the formula (1) described previously.

Multi-metal removal study:

It was reported previously that Cd (500 mg/L) resistant *R. mannitolilytica* KUCd7 could also tolerate other heavy metals including Ni (175 mg/L), Zn (200 mg/L) and Cr (150 mg/L). Therefore, these metal were selected to assess their effect on metal removal abilities of the organism in multi-metal system. 1% culture was inoculated in GP medium (pH 7±0.2) supplemented with equal concentrations (50 mg/L) of Cd and any one of the metals (Ni or Zn or Cr). To study the cumulative effect of all the four metals, the culture was inoculated in medium supplemented with equal concentrations (25 mg/L) of all the four metals including Cd so that the total metal concentration would be equivalent to 100 mg/L of Cd. Set of positive control was prepared by growing KUCd7 separately in GP medium supplemented with Cd (100 mg/L) to compare the metal removal activity with multi-metal treated sets. Sets of negative controls were also prepared in which GP medium were treated with same concentration of metals to its corresponding set of metal combination and kept as uninoculated combination. The inoculated sets were incubated at 28°C in a shaker (160 rpm) for 96 h. Culture in each conical flask then centrifuged at 10000 rpm for 5 min. The metal treated cell free supernatants were acid digested and the metal concentrations in the samples were analysed using AAS. Metal removal activity was calculated according to the formula (1) described previously.

Transmission electron microscopy (TEM) and detection of Cd through energy dispersive X-ray spectroscopy (EDX):

To detect any intracellular change as well as intracellular accumulation of Cd by KUCd7 after Cd treatment, TEM-EDX was carried out. For this purpose KUCd7 was grown in GP medium either treated or untreated with Cd (100 mg/L) up to late log phase, harvested through centrifugation at 5000 rpm for 10 min at 4°C and washed several times with Na-phosphate buffer (0.1 M, pH 7.4), then fixed in 2.5% glutaraldehyde in the same buffer for 3 h at 4°C, followed by treatment with 1% osmium tetraoxide for 1 h. Cells were dehydrated through a gradient of ethanol series [30, 50, 70, 90 and 100% (v/v); 30 min at each step]. Fixed dehydrated cells were treated with propylene oxide and embedded in epoxy resin. Ultrathin sections cut by an ultramicrotome were loaded in nickel grid. The micrograph of the samples were documented along with elemental analysis using transmission electron microscope (TEM) attached with energy dispersive X-ray spectroscopy (EDX) (FEI Tecnai G^2 F20 S-TWIN).

Cell fractionation study:

To localize the site of intracellular Cd accumulation in the bacterial strain KUCd7, it was grown in GP medium supplemented with Cd (100 mg/L) and incubated at 28°C for 96 h in a shaker (160 rpm). Cells were harvested through centrifugation at 10000 rpm for 5 min and were subjected to fractionation to obtain cytoplasmic, periplasmic and total membrane fractions **[12]**. Equal volume of (40 mL) Tris-HCl buffer (pH 7.1, 0.2 M) and sucrose (40%, w/v) were used to suspend Cd treated cell pellets (1g wet wt.) and kept for 30 min for equilibration purpose. Then the suspension was centrifuged and the cell pellets quickly suspended in 80 mL $MgCl_2$ (0.5 mM) in an ice bath for releasing the periplasmic fraction. The suspension was centrifuged and the post-shock cells (pellets) were disrupted through sonication at different time intervals (30-150 sec) and at a frequency of 25 kHz and these homogenates were centrifuged at 90000 g for 1 h to separate the cytosol (present in supernatant) from total membrane components (pellets). The amounts of Cd accumulated in the cellular compartments were quantified by measuring the concentration of Cd in these subcellular fractions through ASS as described previously. Protein content of each subcellular fraction was estimated to calculate the amount of Cd accumulated per unit of protein present in each compartment of bacterial cell. Cd accumulation in each subcellular fraction was compared with total Cd accumulation per unit of total protein of the bacterial cell. Protein content of the bacterial cell was measured by Lowry's method **[13].**

Study of Cd removal from sewage water: KUCd7 was grown in GP medium upto mid log phase. Sewage water sample was collected from sewage treatment plant of Kalyani Municipality, Nadia, West Bengal, India. Temperature and pH of the sewage was monitored. The sample was filled in an airtight bottle. The dissolved oxygen (DO) content of the sample was determined before and after five days of incubation at 28°C using modified Winkler's method following the procedure laid down in American Public Health Association (APHA) **[14]** and the biological oxygen demand (BOD) was calculated from the difference between initial and final DO. 15% mid log phase culture was inoculated in 10 mL of sewage water kept in sterile conical flasks **[15]**. Same treatment was also done with 10 mL of distilled water. Uninoculated sewage water was kept as control. Sewage water sample and distilled water sample used in this experiment were supplemented with Cd (100 mg/L). After 4 days and 8 days of incubation in a shaker (160 rpm) at 28°C samples were centrifuged at 10000 rpm for 5 min. After acid digestion, concentration of Cd in all the cell free supernatants of control and inoculated sets was measured through AAS and percentage of Cd removal was calculated using the formula (1) mentioned earlier.

RESULTS

Effects of physiological factors on growth and Cd removal activity

Effect of temperature: KUCd7 could not grow at 10°C. At 20°C its growth OD was measured as 1.26 in GP medium supplemented with Cd (100 mg/L) and at that time it could remove 74.4% Cd from the medium. 28°C was found to be its optimum growth temperature, at which its growth was maximum (OD 2.21) and Cd removal was highest (83.04%). With the increase of temperature (37°C and 45°C) its growth and Cd removal activity were found to be decrease (Fig.1a).

Effect of pH: Initial pH of the culture medium was found to be a decisive factor for the growth as well as the Cd removal activity of the isolate when it had been grown separately in Cd supplemented medium adjusting the initial pH ranging from 4 to 9. Maximum growth of KUCd7 (OD 2.21) occurred at neutral pH (pH 7) which also resulted in highest removal of Cd by by KUCd7 (83.04%) (Fig.1b). Both acidic and alkaline conditions affected the bacterial cell mass generation and reduced Cd removal activity of the isolates.

Effects of NaCl: Salinity of the medium was reflected as a regulatory factor for growth and Cd removal by the isolate. In presence of 0.5% NaCl, highest Cd removal by KUCd7 (83.04%) has been observed, because at this condition it showed highest growth. With increase of the salinity of the medium, growth response and Cd removal activity of KUCd7 declined and moderate growth (OD 1.53) and Cd removal (53.12%) activity were observed at 4% NaCl concentration (Fig. 1c).

Effects of initial Cd concentrations: When KUCd7 was grown in GP medium supplemented with increasing concentrations of Cd from 10-100mg/L, Cd removal activity of KUCd7 declined gradually (Fig.1d). No significant change in growth response of KUCd7 was detected with increasing stress of Cd.

Multi-metal removal study:

From Ni and Cd supplemented medium KUCd7 could remove 33.03% Ni and 68.56% Cd. From Zn and Cd amended medium it could remove 58.75% Zn and 60.65% Cd. From Cr and Cd added medium it could remove 34.33% Cr and 69.62% Cd. In absence of other heavy metal KUCd7 could remove 83.04% Cd from the medium. Thus, Cd removal by KUCd7 reduced 14.48%, 22.39% and 13.42% due to the individual presence of Ni, Zn and Cr in compare to the condition when Cd was the only supplemented metal in the medium and interference of Zn was maximum for Cd removal by the KUCd7 (Fig.2). When the isolate, KUCd7 was exposed to equal concentrations of all the four metals (25 mg/L each) collectively, the order of metal removal was Cd (39.58%) > Zn (36.72%) > Cr (27.76%) > Ni (26.04%). Thus, Cd removal by KUCd7 was decreased 43.46% due to the cumulative effects of other three metals present in medium.

Transmission electron microscopy with EDX analysis:

Transmission electron micrographs of ultrathin sections of the cells of KUCd7 grown under Cd supplemented medium showed intracellular electron dense spots mostly visible at peripheral region of the cytoplasm, periplasm and membrane portion (Fig. 3a). Such spots were, however, absent in Cd untreated cells (Fig.4a). EDX analysis of the electron dense regions of the cells showed distinct peaks for Cd and its relative amount (%) confirming the presence of intracellular Cd within Cd treated cells (Figs.3b). No peak of Cd was detected within Cd untreated cells (Figs. 4b). However, some unrelated peaks which were also found in this analysis were actually attributed by the elements presented in buffer solutions and metallic grid etc.

Intracellular compartmentalization of accumulated Cd:

Amount of accumulated Cd, its concentration and percentage in subcellular fractions are shown in Table 1 and Fig. 5. In KUCd7 highest accumulation of Cd was found in periplasm (33.25 µg/mg; 47.77%), followed by membrane fraction (25.25 µg/mg; 36.28%) and cytosol (11.08 µg/mg; 15.92%).

Cd bioremediation from sewage water:

Attempt had been made to study the Cd bioremediation ability of KUCd7 in naturally polluted aquatic environment (Fig. 6). When the distilled water supplemented with Cd (100 mg/L) was treated with 15% mid log phase culture of KUCd7 47.27% and 59.09% removal of Cd were observed on 4th and 8th day, respectively. When the sewage water (temperature 28°C, pH 7.2 and BOD 1.23±0.04 mg/L) was supplemented with Cd (100 mg/L) without any additional bacterial inoculation, 23.64% and 36.86% of Cd were removed on 4th and 8th day, respectively due to activities of native microflora present in the sewage water. Other abiotic impurities of sewage might also play an important role in this Cd removal process. Treatment of Cd (100 mg/L) supplemented sewage water with KUCd7 removed 62.73% and 78.18% of Cd on 4th and 8th day, respectively. These results indicated the natural microflora present in the sewage water could not inhibit the Cd removal activity of KUCd7 in the experimental conditions and vice versa. Instead, Cd removal activity became maximum due to cumulative effort of natural microflora and KUCd7. Irrespective of treatment regime Cd removal increased along with number of days of treatments.

Table 1: Accumulation of Cd in subcellular fractions of *R. mannitolilytica* KUCd7

Cellular Fractions	Total Cd content (mg)	Concentration of Cd (µg/mg of protein)
Cytosol	**1.334±0.16**	**11.28±1.08**
Periplasm	**4.002±0.11**	**33.84±1.12**
Membrane	**3.039±0.13**	**25.7±1.96**
Whole cell	**8.378±0.08**	**69.61±1.56**

Statistical analysis: Data are the mean of five replications with standard error

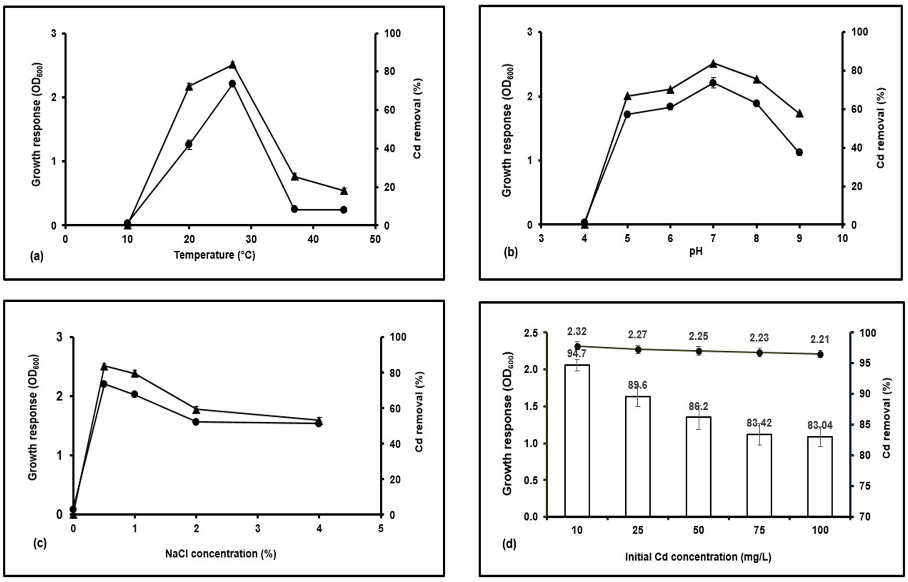


Figure 1: Effect of (a) temperature, (b) pH and (c) NaCl concentration on the growth (closed circle), Cd removal activity (closed triangle) and (d) effect of initial Cd concentration on growth (closed circle) and Cd removal activity (pillar) of *R. mannitolilytica* KUCd7 in GP medium supplemented with Cd (100 mg/L) after 96 h. Bars represent Mean ±SE.

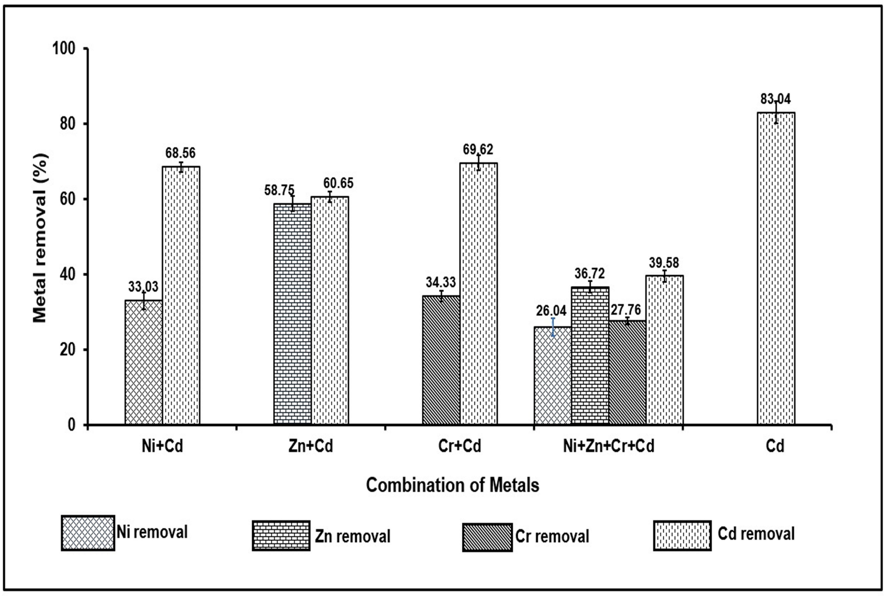
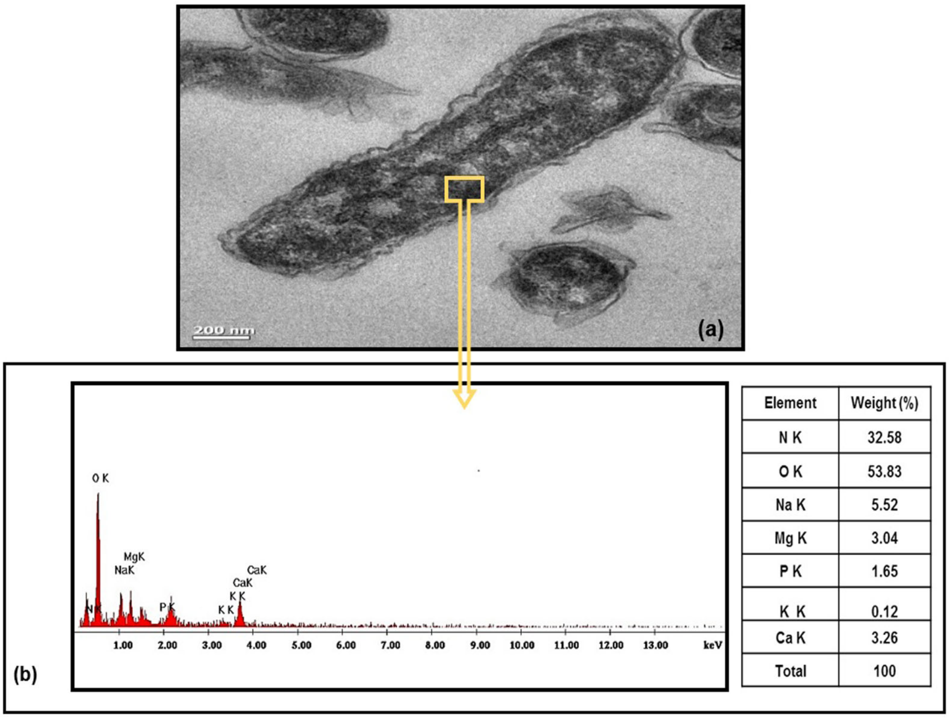
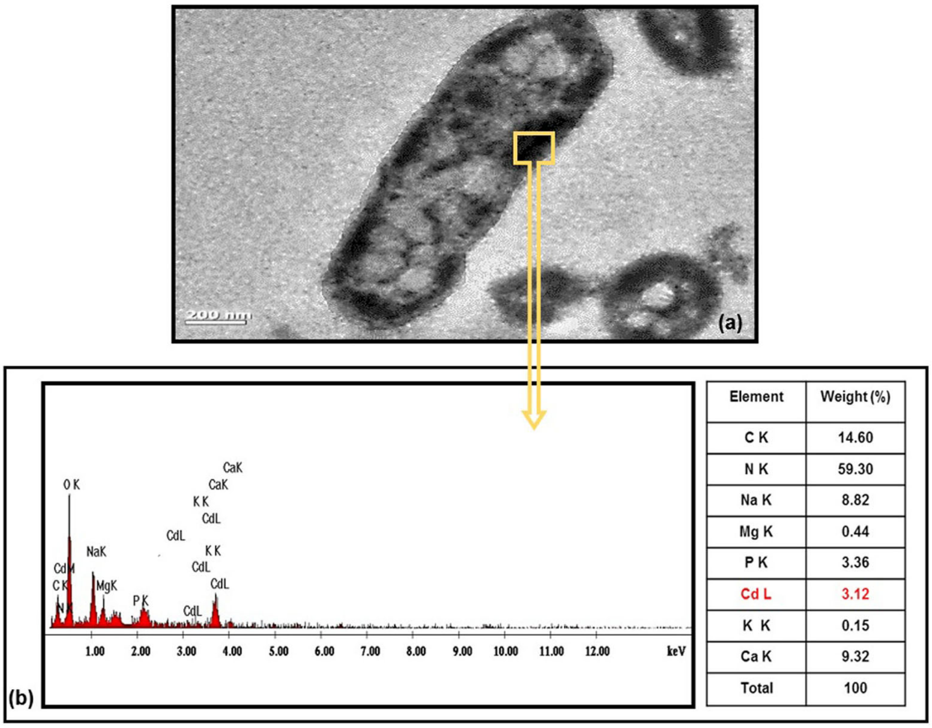


Figure 2: Removal of Cd, Ni, Zn and Cr from the metals supplemented GP medium when metals were present in different binary combination with Cd, all together and in solitary presence of Cd. Bars represent Mean ±SE.



Element	Weight (%)
N K	32.58
O K	53.83
Na K	5.52
Mg K	3.04
P K	1.65
K K	0.12
Ca K	3.26
Total	100

Figure 3: Transmission electron micrograph of (a) ultrathin sections of the *Ralstonia manitolilytica* KUCd7 grown under Cd free condition and (b) elemental analysis through EDX.



Element	Weight (%)
C K	14.60
N K	59.30
Na K	8.82
Mg K	0.44
P K	3.36
Cd L	3.12
K K	0.15
Ca K	9.32
Total	100

Figure 4: Transmission electron micrograph of (a) ultrathin sections of the *Ralstonia manitolilytica* KUCd7 grown under 100 mg/L of Cd treated condition and (b) elemental analysis through EDX.

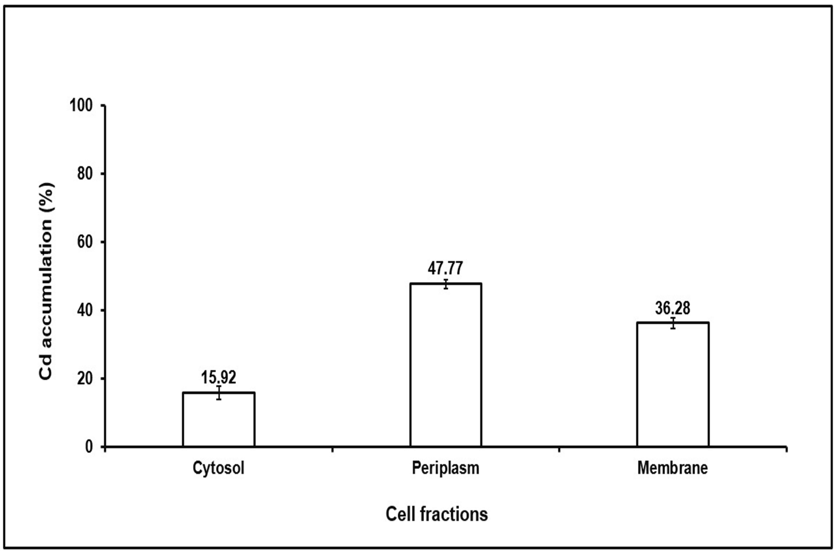


Figure 5: Distribution of accumulated Cd within cell fractions of KUCd7. Bars represent Mean ±SE.

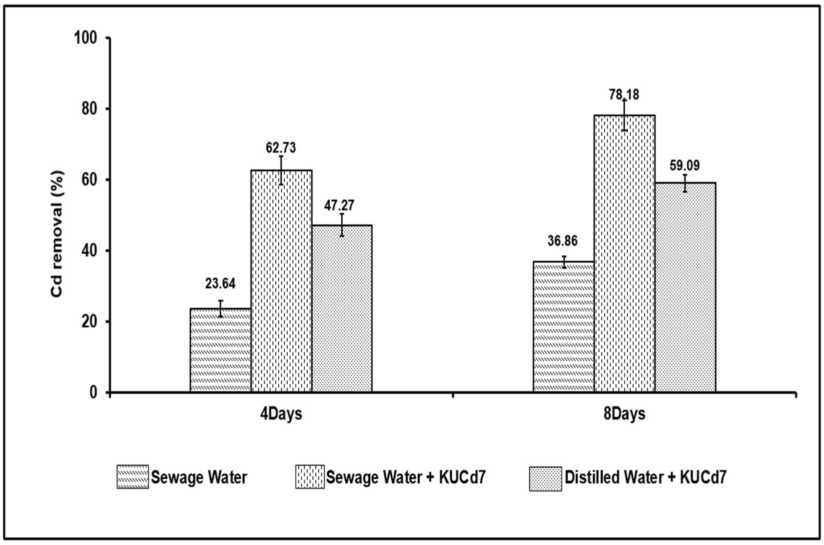


Figure 6: Comparative study of Cd removal from sewage and distilled water by *R. mannitolilytica* KUCd7 at two different times. Bars represent Mean ±SE.

DISCUSSION

Physiological parameters such as temperature, pH, salinity and initial metal concentration are important criteria for determining efficacy of a bioremediating agent. When the Cd removal activities by *R. mannitolilytica* KUCd7 were measured at variable temperature, pH, and NaCl concentrations, it was found that the Cd removal varied accordingly along the line of growth and optimum conditions for maximum growth of the organism was necessary for their highest Cd removal activity (Fig.1). Thus, biomass was an important factor for Cd accumulation. In *Ochrobactrum* sp. highest Cd removal activity was observed at its maximum cell population **[16]**. With the variation of temperature, growth as well as Cd removal activity of KUCd7 differed. But the highest amount of Cd removal (83.04%) was observed at 28°C which also favoured the maximum growth of KUCd7. At acidic condition (pH 5) yield of the bacterial cell mass and Cd removal activity of KUCd7 were poor. Low metal uptake at acidic pH range could be attributed to the higher solubility of the cations along with the predominantly protonated state of cellular binding groups and competition rendered by H^+ ions. Neutral pH was found to be the most favourable condition for growth of KUCd7 and Cd removal activity as well. Growth of the KUCd7 and its Cd removal activity declined gradually when medium became more alkaline. Though 0.5% NaCl concentration was most suitable for growth and Cd removal activity of KUCd7, KUCd7 could also tolerate as high as 4% NaCl concentration. NaCl played a critical role in cadmium accumulation in *Klebsiella planticola* **[15]**. Tolerance to high concentration of NaCl by *R. mannitolilytica* KUCd7 might be associated with its high cadmium resistance and accumulation potentiality.

With the increase of initial Cd concentration in the medium from 10-100 mg/L, growth of KUCd7 unaltered (Fig. 1d). This suggests that KUCd7 is less vulnerable to the toxic effect of Cd. Highest Cd removal activity was observed at lowest Cd concentration (10 mg/L). With the increase of initial Cd concentration Cd removal activity of KUCd7 declined gradually. As the bacterial cell populations were almost equal throughout the concentration range of Cd treatment, the number of sites for Cd accumulation in cell mass were nearly constant also, irrespective of initial Cd concentration. It implies that whatever the initial Cd concentration would be only a specific amount of Cd could be possible to accumulate or remove and excess Cd would persist in the medium. As a result, the amount of this residual Cd increased with the increase of initial Cd concentration and percentage of Cd removal activity decreased gradually.

Removal of the heavy metals by *R. mannitolilytica* KUCd7 was lower in multiple metal system solutions than in single metal system solutions (Fig. 2). It was found that in a binary metal system (two metal co-exist in same concentration, 50 mg/L), Cd removal by KUCd7 was always lower in either presence of Ni and Zn as compared to Cd treated (100 mg/L) single metal system. Competition between different bivalent ions for metal transporter and binding sites may be the probable reason for reduction of individual metal uptake and binding capacity. Nies and Silver **[17]** reported a transport system with low substrate specificity in *Alcaligenes eutrophus* responsible for uptake of divalent metal cations and Zn^{2+}, Cd^{2+}, and Ni^{2+} competed with each other for transport by this system. They also assumed that this kind of broad-substrate-range divalent-cation transporter system was common in prokaryotes, although additional transport systems with higher ion selectivity might exist. Moreover, Zn and Cd belong to the same group [Group 12 (IIb)] in periodic table and have structural similarity. Metallothionein like SmtA of *Synechococcus* PCC 7942 can bind to both Zn^{2+} and Cd^{2+} **[18, 19]**. This strongly supports the chances of competition within divalent metals for metal binding sites. Kang et al. **[20]** reported that Cr^{3+} could reduce the uptake of divalent ions such as Co^{2+} or Ni^{2+} by *Pseudomonas aeruginosa*. In presence of Cr^{3+}, Cd^{2+} removal by KUCd7 from medium was also reduced. When Ni^{2+}, Zn^{2+}, Cr^{3+}, and Cd^{2+} were present in equal concentration (25 mg/L) in the culture medium their cumulative competitive effect was stronger on Cd removal activity by KUCd7. As a result, Cd^{2+} removal activity reduced drastically. Antagonism of Zn was highest during Cd removal by KUCd7. Chaudhary et al. **[21]** observed that Cd^{2+} uptake was most sensitive to any competing ion such as Ni and Zn. The order of preferential metal removal by KUCd7 was Cd > Zn > Cr > Ni.

The cadmium resistance mechanisms take several forms, such as extracellular precipitation and exclusion, binding to the cell surface and intracellular sequestration **[22]**. Cadmium was reported to be accumulated intracellularly as CdS as in *Pseudomonas aeruginosa* KUCd1 **[23]** or as phosphate and silicate compounds as in *Bacillus* sp. ML1-2 **[1]**. TEM and EDX studies showed that cells of *R. mannitolilytica* KUCd7 can immobilize discrete aggregates of the Cd compounds in the form of electron dense area when the cells were grown in presence of Cd. The accumulation sites were mainly confined within the periplasmic and membrane regions surrounding the cell and a few spots (Cd accumulation sites) were noticed in the cytoplasm (Fig.3). These kinds of intracellular Cd^{2+} accumulated sites were also reported from other bacteria including *Rhodobacter sphaeroides* **[24]**, *Pseudomonas putida* 62BN **[2]** and sulfate reducing bacteria such as *Desulfovibrio desulfuricans* DSM 1926 and

Desulfococcus multivorans DSM 2059 **[25]**.

The distribution of Cd in the subcellular fractions of *R. mannitolilytica* KUCd7 revealed that the cell envelope region containing the membrane and periplasm fraction, accumulated maximum proportion of the total accumulated Cd, with the periplasm being the major sink within the cells (Table 1, Fig. 5). The cytoplasm accounted for low amount of Cd deposition making up to 15.92% of the total accumulated Cd by KUCd7. Cell compartmentalization study supported TEM observation that major accumulation of Cd was found to be localized at cell envelope (membrane and periplasm) region in the organism. These results corroborated the earlier findings on cell envelope-associated accumulation of Cd in *Pseudomonas aeruginosa* **[23]**, *Rhodobacter sphaeroides* **[24]**, *Escherichia coli* **[26]** and *P. stutzeri* **[27]**. However, in *Massilia* sp. III–116-18, *Pseudomonas* sp. IV-111-14, and *Bacillus* sp. ML1-2, the amounts of Cd^{2+} deposition measured in the cytosol were significantly higher compared to those measured in the peripheral parts of the cells **[1]**. Both periplasmic and cytoplasmic sequestrations of Cd might be due to expression of metallothionein in *E. coli* **[28]**. So it can be inferred that peripheral sequestration of toxic heavy metals might have evolved as an effective strategy for the survival in metal contaminated environment by preventing access of the toxic metal to the cytoplasm and developing metal resistance property **[12].**

R. mannitolilytica KUCd7 was evaluated for its ability to remove Cd from naturally polluted aquatic environment by treating it in distilled water and sewage water supplemented with Cd (100 mg/L) (Fig. 6). The percentage of Cd removal from the distilled water was lower than that from sewage water by KUCd7. The sewage water contained a variety of Cd resistant natural microflora that contribute in additional removal of Cd. This microflora alone could able to remove only 36.86% of Cd from the sewage water, but they in association with KUCd7 increased Cd removal 41.32% after 8 days. This indicates that KUCd7 could coexist with natural microflora of sewage water, and their cumulative efforts showed better Cd removal efficacy. Cd removal by the organism in sewage water occurred in a time dependent manner and removal at 8th day was higher than that at 4th day. Moreover, after 4 days of incubation, it was observed that Cd removal in sewage water (62.73%) was also lower than observed in nutrient supplemented culture medium (83.04%) that encourages the confluent growth of culture. Nutrient limited condition of mixed culture population may be the restraining factor for growth of the organism which caused delayed and reduced Cd removal activity compare to nutrient enriched culture media. Irrespective of treatment regime Cd removal increased along with number of days of treatments. After 8 days of incubation, 78.18% cadmium were removed from KUCd7 treated sewage water. Shamim and Rehman **[29]** also reported that in the presence of *Kleibsiella pneumoniae* 44% and 76% cadmium were removed from the industrial wastewater supplemented with Cd (100 mg/L) after 4 and 8 days of incubation.

CONCLUSION

From the present study it can be inferred that physiological parameters including temperature, pH, and salinity played significant role for determining the cadmium bioremediation potential of Cd resistant *Ralstonia mannitolilytica* KUCd7 and optimized conditions favoured bacterial growth which in turn triggered maximum bioremediation efficacy. In a multi-metal system each metal showed antagonistic effect on Cd removal activity as a result of which Cd removal declines in presence of other heavy metals. It was evident from TEM-EDX analysis and cell fractionation study intracellular Cd bioaccumulation by *Ralstonia mannitolilytica* KUCd7 has certain role in Cd bioremediation process. Finally, the effective application of *Ralstonia mannitolilytica* KUCd7 revealed that it can be a useful tool for bioremediation of Cd from sewage water.

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