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Role of *Pseudomonas stutzeri* MTCC101 in Cadmium Bioremediation

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ABSTRACT

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Gram-negative bacteria like *Pseudomonas* are well reported to play a significant role in detoxification of various heavy metals like cadmium. The possible way to remove cadmium from the environment is to immobilize the metal within the system. *Pseudomonas stutzeri* MTCC 101, a highly cadmium tolerant strain harbors a mega plasmid (~15kb) which is likely responsible for such metal tolerance. However MTCC 101 showed conspicuous morphological abnormalities as revealed by SEM-EDX analysis. Focus was given to determine the biosorption potential of the organism. Optimization of biosorption potential of MTCC 101 showed that the mid log phase cells could accumulate maximum Cd^{2+} at neutral pH when cell-metal mixtures were incubated for about 30 hr at 37°C. MTCC 101 produces profuse EPS under metal stress which is likely to contribute to biosorption potential of the organism. Hence, the metal sorption potential of *P. stutzeri* could be effectively utilized in the bioremediation of cadmium.

Introduction

Cadmium is a naturally occurring element. Now a days this soft, silver-white metal used as an electrode component in alkaline batteries and also used in pigments, coatings, platings, stabilization of plastic (Fernando et al. 2013). It binds to thiol group (Edwards et al. 2013) resulting in protein denaturation; interacts with calcium and zinc metabolism affecting the skeletal system. Bioremediation processes are very attractive in comparison to physicochemical methods such as electrochemical treatment,

ion exchange, precipitation, and reverse osmosis because they can have lower cost and higher efficiency at low metal concentrations (Gadd 1993; Chatterjee et al. 2012). Such processes are of industrial interest, since the removal of potentially hazardous heavy metal from industrial effluents by microbial biomass can lead to detoxification and also to recovery of valuable elements (Brierley et al. 1985). Microorganisms are efficient in doing both the biotransformation and could adsorb cadmium on their cell surface

(Watcharamusika et al. 2008; Deb et al. 2013). Among them Gram negative soil bacteria *Pseudomonas* has widely been studied for their well adapted metal resistance properties (Singh et al. 2010; Deb et al. 2013). In this work, attempts have been made to elucidate the cadmium resistance potential of *Pseudomonas stutzeri* (MTCC101) and determine the optimal biosorption capacity of the organism.

Materials and Methods

Bacterial Strains

The strains of bacteria used in our study were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India. The strains are: a) *Pseudomonas stutzeri* (MTCC101) b) *E.coli* (MTCC390), a plasmid less strain.

Chemicals

Luria Bertani broth, Luria Bertani agar, Cadmium chloride dried (anhydrous), Tris base, EDTA Na₂ salt, NaOH, Dextrose, 10% SDS, Potassium acetate, Agarose low EEO, Ethanol, Phenol, Chloroform, Isopropanol, RNase A, Lysozyme, Loading dye 5X, EtBr, HCL, Glacial acetic acid, CaCl₂, 2% HNO₃, H₂SO₄. All chemicals used were of analytical grade and purchased from Himedia, SRL, and Merck.

Growth of *Pseudomonas stutzeri* (MTCC 101)

Degree of resistance of MTCC 101 was evaluated in LB containing 100 to 800µg/ml cadmium. Growth of MTCC 101 in LB was determined by measuring the optical density at 540 nm using the un-inoculated broth as blank. Relative growth of MTCC 101 was expressed as the percentage of those obtained in untreated control which was

taken as 100%.

Isolation of Plasmid from MTCC 101 for Transformation of Plasmidless *E. coli* MTCC 390

Plasmid was isolated from MTCC 101 by alkaline lysis method; the concentration of plasmid determined by UV-VIS Spectrophotometer 118 (Systronics); electrophoresed in 0.8-1% agarose, visualized under UV Transilluminator with filter (312 nm). A plasmidless strain *E. coli* MTCC 390 was transformed by CaCl₂ method (Malik and Jaiswal 2000) and transformants thus obtained were screened for their cadmium tolerance in LA supplemented with 100 & 200µg/ml of cadmium.

Growth Acclimatization

Transformed MTCC 390 cells were initially grown in Luria Broth supplemented with 100µg/ml Cd⁺ overnight at 37°C, shaking and OD at 540nm determined. Cells were then passaged from the overnight grown culture into LB supplemented with higher Cd²⁺ concentration upto 700µg/ml to compare the tolerance level of the transformed cells to that of the parent culture.

Determination of Highest Sublethal Concentration of Curing Agents

Acridine orange, Sodium dodecyl sulphate (SDS), elevated temperature, mitomycin-C were used to cure the plasmid. Sublethal doses of these agents were determined to select optimum conditions for curing. The growth of strain MTCC 101 was tested in Luria broth, separately containing acridine orange (5, 10, 20, 40µg/ml), SDS (2, 4, 6, 8 & 10%), elevated temperature (25°C, 30°C, 37°C, 40°C, 45°C, 50°C), mitomycin-C (5,

10, 15, 20, 25, 50, 75, 100µg/ml). For this purpose sterilized solutions of curing agents were added to luria broth (5 ml). Freshly grown cultures were used to inoculate luria broth supplemented with the curing agents. MTCC 101 inoculated luria broth without curing agent served as control. The tubes were incubated in a shaker at 37°C and 120 rpm for 24-48 hrs. The cells growing in presence of different concentration of curing agents were enumerated on luria agar plates.

Curing of Plasmid

Plasmid curing trials were carried out by using acridine orange, mitomycinC, SDS, elevated temperature at their highest sublethal doses i.e 40µg/ml, 100µg/ml, 10%, 50°C respectively, in luria broth . Luria agar supplemented with cadmium chloride (100µg/ml & 200µg/ml) and ampicillin (10mcg) impregnated disc were used as markers in the curing experiment. Viability of the cells was confirmed on luria agar plates and cured cells were selected by replica plating. Loss of plasmid from the cured cells was further confirmed.

Biosorption of Cadmium by *Pseudomonas stutzeri* (MTCC 101)

Luria broth supplemented with varying concentration of Cd²⁺ (100, 200 and 400µg/ml) was inoculated with MTCC 101 incubated for 24h at 37°C, shaking. The culture was then centrifuged at 8000 rpm for 10 min at room temperature. The oven-dried (+/- 80°C) cell pellet was subjected to acid digestion using standard method (Basu and Paul 2008). Amount of cadmium (%) present in the cell-pellet & culture supernatant was estimated by AAS (Agilent spectra). Optimization of biosorption potential of the MTCC 101 was hence determined in relation to cell age, metal concentration, period of incubation, pH and

temperature by incubating the cell-metal mixture under varying condition.

EPS Production and its Cd Biosorption Potential

Extracellular polymeric substances (EPS) produced by MTCC 101 was extracted from mid log phase culture (10hr old) using standard method (Chatterjee et al. 2012). Briefly cells grown in presence of varying concentration of Cd (100-800µg/ml) and without the salt (as control) were centrifuged at 7000 rpm for 10 min; EPS was extracted from the pellets and the amount of polysaccharide, protein, DNA, RNA were measured by standard methods. Additionally the extracted EPS was incubated in varying cadmium concentration (200, 400, 600, 800, 1000 µg/ml) at 37°C, pH 7 for 30 hr to determine the metal accumulation by AAS.

SEM-EDX Analysis for Cadmium Accumulation by MTCC 101

Cells obtained from 10 ml overnight grown broth cultures of MTCC 101 in LB containing Cd (0, 400 and 800 µg/ml) were subjected to SEM (FEI QUANTA 200), EDX (ED3 Hindhivac direct drive rotary vacuum pump) analysis after requisite processing for visualization of metal biosorption (Jaysankar et al. 2008). Also interference of other heavy metals (Mn, Ni, Co, Cu, Cr, Zn, Hg) along with cadmium on growth and surface accumulation on bacterial cells were also studied.

Results and Discussion

Relative Growth of MTCC 101

The relative growth of MTCC 101 revealed that after 24hrs of incubation there was drastic reduction in growth with increased cadmium concentration (Table 1). It

indicates that apparently the organism was not a very Cd tolerant strain. However on prolonged incubation for 48-72 hour the organism got acclimatized to the metal stress and was able to tolerate and grow showing more than 50% relative growth at the highest concentration of Cd (800µg/ml).

Isolation of Plasmid from MTCC 101 for Transformation of Plasmidless *E. coli* MTCC 390 and Growth Acclimatization

Transformed colonies were observed on Cd (upto 150µg/ml) incorporated LA plates. The transformation efficiency of MTCC 390 was 300 cfu/ng of DNA. MTCC 101 harbours a megaplasmid (~15 kb) which upon transformation into MTCC 390 (originally plasmidless strain) yielded transformants having comparable cadmium tolerance (Fig. 1 & 2). The bacterial resistance to antibiotics and heavy metals are the common phenotypic characters, which are often plasmid encoded (Trevors 1985).

Curing of Megaplasmid

Acridine orange, SDS, elevated temperature, mitomycinC were separately tried for curing of megaplasmid. Highest sublethal dose of the curing agent was determined. Among them mitomycin C alone appeared to be effective in curing the plasmid. Cells were not viable at above 50µg/ml mitomycin C concentration. So, 50µg/ml was the highest sublethal concentration of mitomycin C used. MitomycinC treated (50µg/ml) cells showed loss of cadmium as well as ampicillin resistance property whereas untreated cells were resistant to cadmium chloride (100 to 800µg/ml) as well as ampicillin (10mcg) (Fig. 3 a & b) indicating that the gene (s) conferring cadmium and antibiotic resistance is plasmid mediated. This finding corroborates with *Pseudomonas* strain WP19 which was resistant to Cu, Ni, Cd and Cr and having plasmids of 20.8,

19.6, 8.0 and 4.7 kb approximately (Malik and Jaiswal 2000, Ünaldi et al. 2003)

Biosorption of Cadmium by *Pseudomonas stutzeri* (MTCC 101)

Microorganisms are often known to produce extracellular polymeric substance (EPS) localized at or outside the cell surface & are composed of high molecular weight organic macromolecules such as polysaccharides, proteins, nucleic acids, and phospholipids (Wingender et al. 1999) From the Atomic Absorption Spectroscopy studies it is evident that MTCC 101 when grown in presence of increasing concentration of cadmium could accumulate the metal maximally upto a certain level (400µg/ml) beyond which it could not accumulate even on increasing the incubation period (Fig. 4). At 400µg/ml Cd²⁺, the amount of cadmium in the cell pellet versus supernatant was around 50-50% and this was true for 24h incubation. Hence all further studies were done using 400µg/ml. Cells at different growth phases were harvested & cell-metal mixture were incubated for varying time at varying pH. It was found that mid log phase cells (Fig. 5a) could accumulate maximum Cd²⁺ at neutral pH (Fig. 5c) and 37°C (Fig. 5d) when the mixtures were incubated for about 30h (Fig. 5b).

Such biosorption property of the organism may be attributed partially via the extracellular polymeric substance (EPS) production in addition to the cell membrane associated metal accumulation as reported earlier (Deb et al. 2013). Metal resistant bacterial strains have been found to produce high amount of exopolymer during growth in presence of heavy metals. As shown in Fig. 5 MTCC 101 was capable to produce wide range of polysaccharide & protein with increasing concentration of cadmium (Fig. 6a).

Table.1 Relative Growth of MTCC 101 in LB Supplemented with Varying Concentration of Cadmium (0 to 800µg/ml) for 24, 48 & 72 Hours

MTCC 101 grown in LB supplemented with cadmium (µg/ml)	Relative growth (%)		
	24 hour	48 hour	72 hour
1. 100	77.52	77.34	77.34
2. 200	73.03	73.42	73.42
3. 300	66.29	69.06	69.06
4. 400	66.29	67.40	67.40
5. 500	6.74	57.45	66.85
6. 600	2.80	30.38	65.74
7. 700	0.00	19.33	57.45
8. 800	0.00	14.36	55.24

All values represent the average of triplicates

Fig. 1

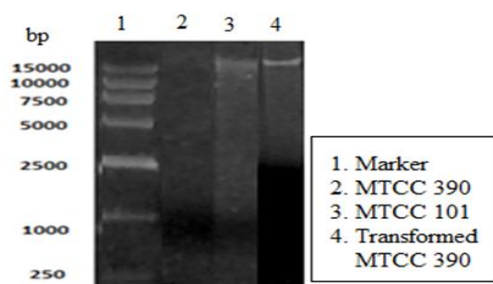


Fig. 2

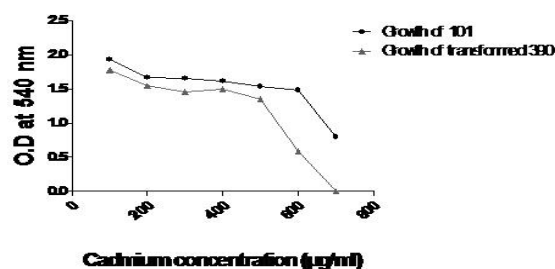


Fig. 3

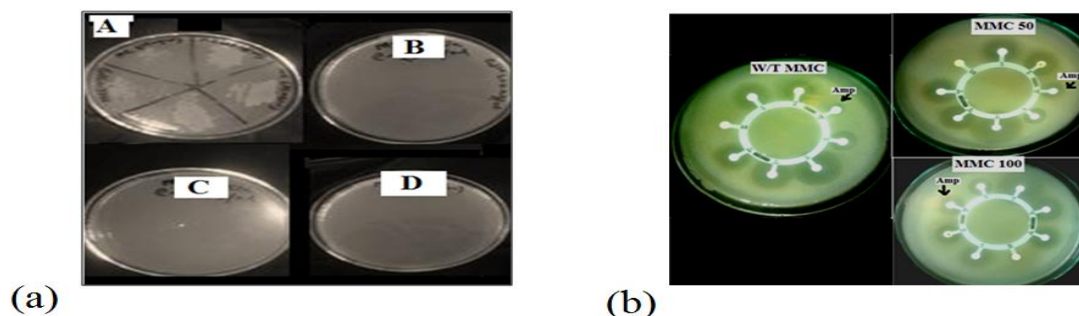


Fig.1 Detection of Plasmid in MTCC 101, MTCC 390 and Transformed MTCC 390; **Fig.2** Comparison of Growth of MTCC 101 and Transformed MTCC 390 in Cd²⁺ Supplemented LB; **Fig.3a** Growth of *P. stutzeri* (MTCC 101) in absence (A) and presence (B, C, D) of Cd after plasmid curing with Mitomycin C (MMC): *P. stutzeri* after treatment with MMC was streaked on Luria agar (A), Luria agar supplemented with cadmium (B: MMC 50, C: MMC 75, D: MMC 100 µg/ml). After curing bacteria showed reduced (<100µg/ml) tolerance to the metal. (b) Variation in antibiotic sensitivity profile of *P. stutzeri* by treatment with Mitomycin C: *P. stutzeri* originally resistant to ampicillin (Octadiscs GI minus, Himedia) after mitomycin C treatment showed exhibited result;

Fig. 4

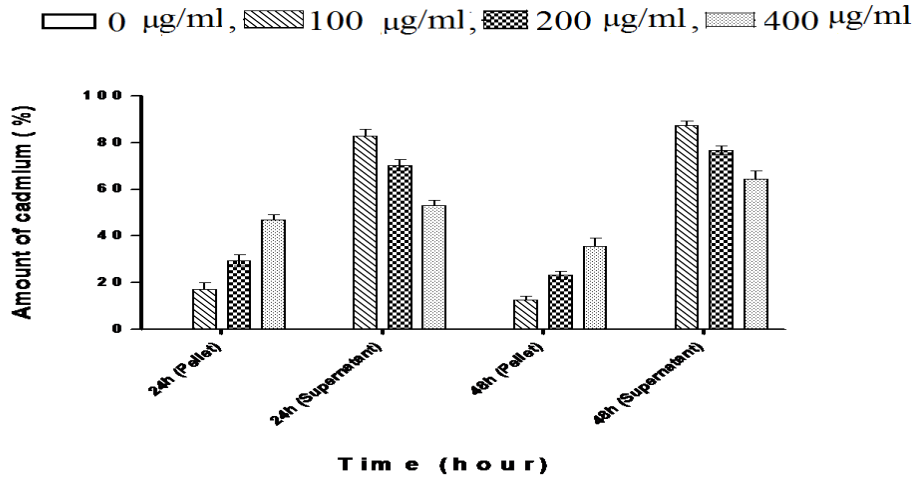


Fig.4 Accumulation of Cadmium by MTCC 101 Grown in Metal Supplemented LB

Fig. 5

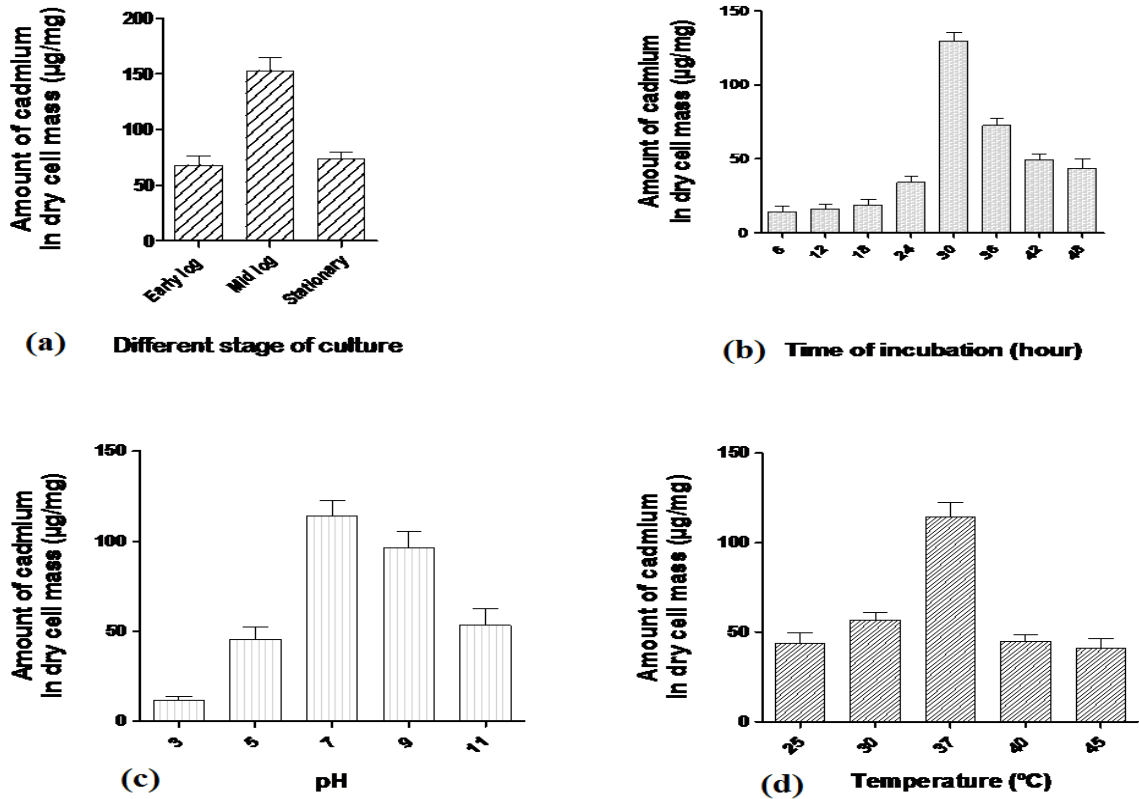


Fig.5 Optimization of Conditions for Cadmium Accumulation by MTCC 101. (a) Different Stage of culture (b) Varying Time (c) Varying pH (d) Varying Temperature

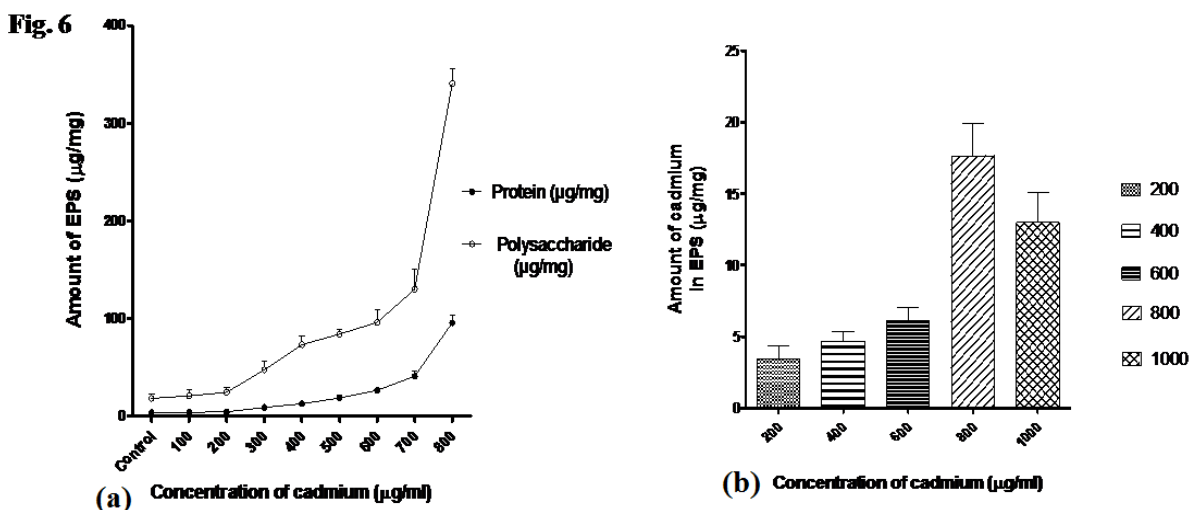
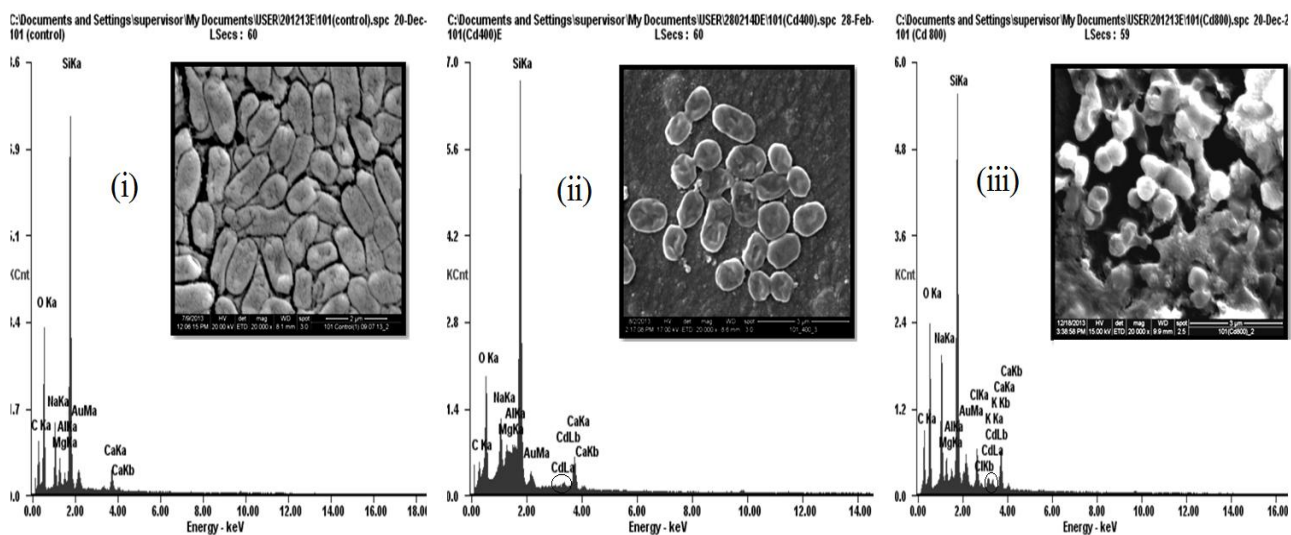


Fig.6 EPS Production and Biosorption of Cadmium by MTCC 101. (a) Cell Bound EPS in MTCC 101 under mMetal Stress (b) Biosorption of Cadmium by EPS Produced by MTCC 101



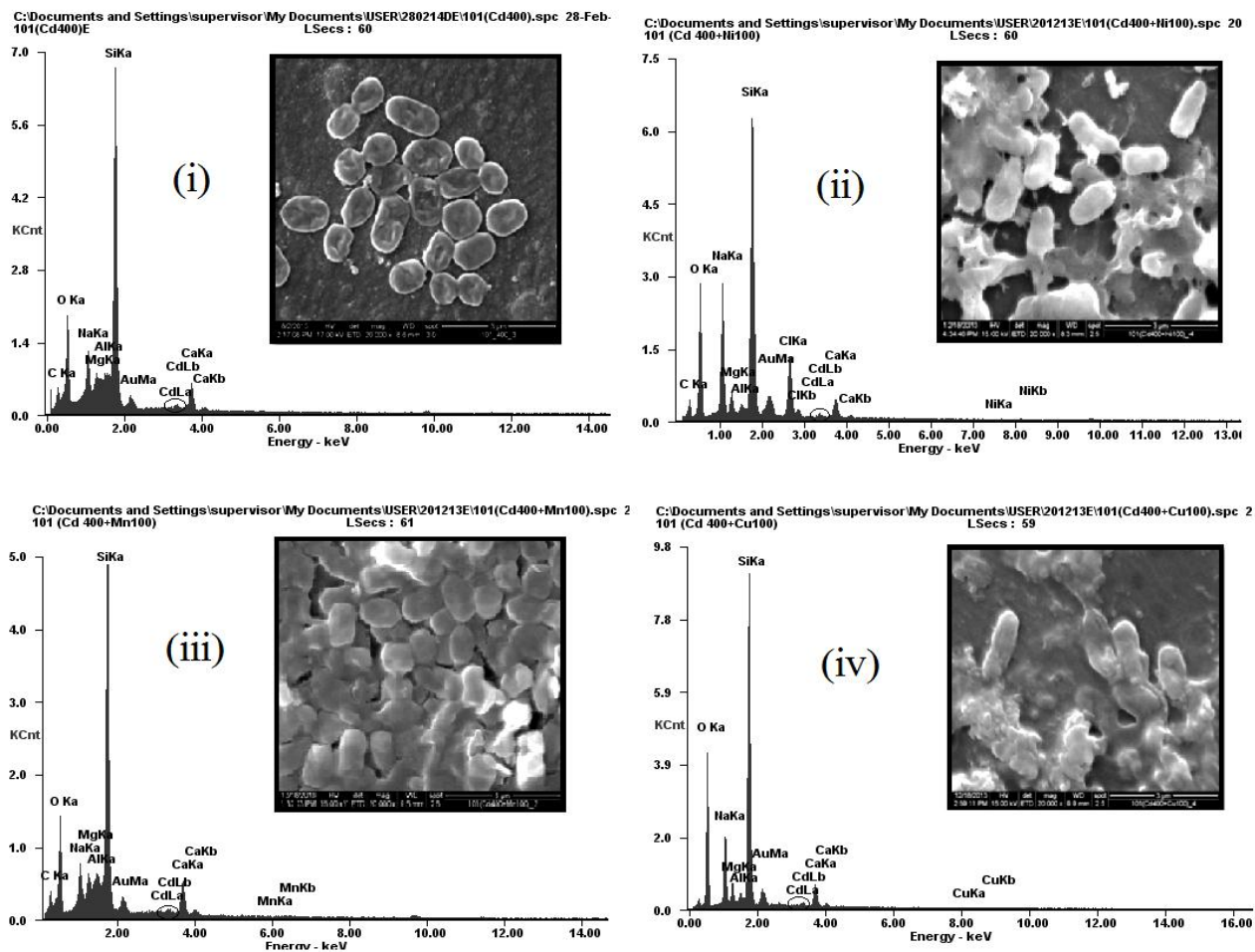


Fig.8 Interference of Ni, Co and Mn with Cadmium on MTCC 101. SEM analysis (20000X; inset) and EDX of MTCC 101 cells treated with cadmium alone [(i) 400µg/ml] and cadmium (400µg/ml) with nickel [(ii) 100µg/ml], manganese [(iii) 100µg/ml] and copper [(iv) 100µg/ml]

The EPS after extraction by standard method (Chatterjee et al. 2012) was incubated in cadmium chloride solution of varying concentration (200-1000µg/ml). Pellets were washed twice in PBS, acid digested by standard method and subjected to AAS for analysis of cadmium. It was found that EPS alone could absorb maximum cadmium (17µg/mg) when 800µg/ml concentration of the metal was used (Fig. 6b) which was not comparable to that of previous reports (Kazy et al. 2002). Although metals get bound to the carbohydrate or protein moiety of the

EPS remaining outside the bacterial cell (Gutnick and Bach 2000), since the EPS produced by MTCC 101 upon analysis showed more of carbohydrate than protein content probably extracellular polymeric substances was not efficient in chelating.

SEM-EDX Analysis for Cadmium Accumulation by MTCC 101

Cadmium induced morphological variation & surface accumulation of metal by MTCC 101 as is evidenced by SEM-EDX analysis.

Unlike the cells grown in normal growth medium [Fig. 7(i)] presence of Cd at concentration 400µg/ml [Fig. 7(ii)] & 800µg/ml [Fig. 7(iii)] in the medium resulted in abnormality in cell morphology suggesting toxic effect of the metal on the bacterial cells. In EDX spectrum Cd-L peak in the Cd 400 µg/ml & 800 µg/ml respectively confirmed the presence of cadmium [Fig. 7(ii) and (iii)]. Additionally, peaks for other essential metals like O-K, Na-K, Cl-K, Au-M, Mg-K were also found, which were already present in the culture media and were part of the cell surface components. Bacteria grown in medium without Cd showed no EDX signals [Fig. 7(i)].

Interference of other heavy metals like Ni [Fig. 8(ii)], Mn [Fig. 8(iii)], Co [Fig. 8(iv)], etc. with Cd affecting growth of MTCC 101 demonstrated varying results. In presence of Mn, Ni, Co (100µg/ml) along with Cd (400µg/ml) MTCC 101 although SEM-EDAX data shows distorted cell morphology when grown in medium containing Cd (400µg/ml) with Ni (100µg/ml) & Cd 400µg/ml with Co 100µg/ml. However in presence of cadmium 400µg/ml with Mn 100µg/ml the cells did not exhibit any notable distortion in morphology as compared to those when grown in Cd alone was similar to those reported by others as analysed by SEM-EDX (Wong et al. 1978). Studies have shown that in *Pseudomonas putida*, presence of cadmium and nickel salts in growth medium resulted in reduction of cellular morphology to thin short rods. The resistance to cadmium and nickel may depend upon the membrane alteration which either prevent metal efflux or might produce efflux once the metal enters into the cell (Chakravarty et al. 2007).

In conclusion, *P. stutzeri* MTCC 101 was

found to be a highly cadmium tolerant (plasmid borne strain) strain producing profuse EPS under metal stress which is likely to contribute to biosorption potential of the organism. Hence further elucidation of this tolerance potential by genomic and proteomic studies shall help one to dissect the bioremediation strategies.

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