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Removal of Cadmium by *Halobacterium* strain R1 MTCC 3265 from saline and non-saline econiches

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Haloarchaeon *Halobacterium* strain R1 MTCC 3265 is an attractive candidate for use in bioremediation due to its resistance to 4 mM cadmium (Cd) in nutrient rich medium NTYE (25% NaCl Tryptone Yeast extract) and up to 2 mM Cd in synthetic medium NGSM (20% NaCl glucose synthetic medium) containing 0.2% glucose as sole source of carbon. The cells accumulated a maximum of 18 ppm of Cd on the 5th day of growth. In line with its true haloarchaeal nature the cells of *Halobacterium* strain R1 lysed on suspension in distilled water or media containing low NaCl concentrations. As such this strain cannot be used for the removal of metal from non-saline environments. However to overcome this problem cells were immobilised in calcium alginate. The immobilized cells were viable and could efficiently remove up to 63% Cd from deionized water.

[Keywords: *Halobacterium* strain R1 MTCC 3265, heavy metal, cadmium, sorption, immobilization, bioremediation]

Introduction

Metal pollution is on the rise in the estuarine econiches, due to surface water traffic of barges, trawlers, passenger ferries, etc. as well as release of effluents from industries dealing with pesticides, fertilizers etc. Thus the estuarine network is exposed to a constant anthropogenic flux. Cadmium occurs naturally in ores of zinc, copper and lead[1]. It is used in nuclear fission reactions, nickel-cadmium batteries, electroplating, fertilizers, paint industry and also in nuclear fission reactions. As a result of such anthropogenic activity, cadmium is a major pollutant in the environment. Cadmium (Cd) has no metabolic role in animals, plants or microrganisms. Cadmium and other chemical derivatives of cadmium are not biodegradable and therefore they are accumulated in the ecosystem. Their high solubility in water enhances further accumulation in the environment. It enters the food web through plants and microorganisms. Cadmium gets accumulated mainly in kidneys and liver and induces the production of ROS (Reactive Oxygen Species) thereby promoting mutations in DNA[2]. Cadmium is also reported to cause pulmonary edema, hemorrhage, fulminate hepatitis, testicular injury, nephrotoxicity, osteotoxicity, immunotoxicity and tumor[3,4]. It contributes to the cadmium-induced zinc deficiency, whenever it replaces zinc in metallo-enzymes.

Tolerance of microorganisms to metals has been widely studied using eubacteria[5-7]. Studies on tolerance/resistance of halophilic bacteria/archaea are limited and just gaining momentum[8-18].

Archaea are reported to exhibit resistance to cadmium at different concentration levels for example, the thermoacidophilic crenarchaeon *Sulfolobus metallicus* the hyperthermophilic radioresistant archaeon *Thermococcus gammatolerans*, and the haloarchaeon *Halobacterium* strain R1 MTCC 3265 is resistant to 4mM Cd, 2mM /3mM Cd and 4mM Cd respectively[19,20].

The present paper communicates resistance and accumulation of cadmium by growing cells of *Halobacterium* strain R1, discusses entrapment of whole cells with calcium

alginate and their use in removal of cadmium from aqueous and saline systems.

Materials and Methods

Halobacterium strain R1 was grown in NaCl tryptone, yeast extract media (NTYE) media containing 25% (w/v) crude salt/NaCl[21]. The culture was maintained on NTYE agar slopes/plates at room temperature (28-30°C). Cadmium chloride ($CdCl_2$) salt of cadmium heavy metal was used and the metal tolerance/resistance studies were conducted using NTYE and mineral salt media (NaCl Glucose Synthetic Medium) containing 20% (w/v) NaCl and supplemented with 0.2% (w/v) glucose[22,23].

The growth kinetics and Cd tolerance studies were done in NGSM. Final Cd metal concentration in the media was 1 mM. Individual flasks were then inoculated with 3 day old haloarchaeal culture grown in the same medium devoid of Cd metal (control). Flasks, were incubated at RT on a rotary shaker at 160 rpm. Absorbance of culture broth from each flask was monitored at 600 nm every 24 h using a spectrophotometer (Shimadzu UV- 240, Japan) against uninoculated medium maintained at identical conditions. Control culture flask without metal was also maintained.

Cadmium was estimated in the cells by the dithizone method[24]. Five ml of the culture was centrifuged at 8000 rpm for 10 min. The cell pellets obtained were mixed with 2 ml of deionised water and vortexed for 2 min. This was taken in a 100 ml separating funnel and diluted with 8 ml of deionised water. 5 ml of 25% (v/v) potassium sodium tartarate was added dropwise till the red colour of the indicator turned to yellowish orange. To this 2 ml of NaOH–potassium cyanide solution was added, followed by 1 ml of hydroxylammonium chloride solution and 10 ml of dithizone solution. The reagents were added in the same order and mixed thoroughly after each addition. The separating funnel was allowed to stand at R.T. for phase separation. The solvent phase was transferred into a glass cuvette (3 ml) and measured at 530 nm against a blank of deionised water treated in the same manner. The values obtained were compared with standard curve of Cd (stock of 1 µg/ml Cd).

A modified dithizone method was formulated and employed for visual estimation of cadmium. Cell pellet was mixed with 1 ml of distilled water, vortexed for 2 min to obtain a lysate. To 0.1 ml of this lysate, 100 µl of NaOH and 200 µl of dithizone (0.1% in methanol) and 100 µl of formaldehyde was added and observed for visual colour. A magenta pink coloration indicated the presence of cadmium.

Cells of *Halobacterium* strain R1 were immobilized in calcium alginate. For this the culture growing in the absence of Cd was harvested on the 4th day of growth (A_{600}=1.5) by centrifuging at 8000 rpm for 10 min to obtain cell pellet. The cell pellet was washed once in NSM and resuspended in NSM to a final absorbance of 4.0. 40 ml resting cells were suspended in NSM to a final absorbance of 4.0, were taken in a 500 ml beaker. This suspension was then mixed with equal volume of 4% sodium alginate slurry prepared in deionised water and kept at 15°C for 2-3 h to dissociate the entrapped air bubbles. This mixture was extruded drop wise, through a nozzle of 15 ml syringe, into ice cold 0.2 M calcium chloride and kept overnight in the refrigerator at 15°C to from beads of calcium alginate. Beads were washed with cold deionised water, and stored in the refrigerator at 15°C. Calcium alginate entrapped cells of approximately 14 g wet weight were suspended in 100 ml of 1 mM Cd solution prepared in deionised water in Erlenmeyer flasks of 250 ml capacity. The flasks were kept on a rotary shaker at 150 rpm and the suspension fluid was estimated for metal at intervals of 8 h by the dithizone method as described above.

Results and Discussion

Salinity being an inherent characteristic of solar salterns and estuarine econiches, the haloarchaea thriving in such habitats represents one of the dominant microflora and hence possibly play a major role in the geochemical cycles of the estuaries[25,26].

As estuarine environment undergoes transient phases of nutrient high and limitation, we attempted to study the response of *Halobacterium* strain R1 in nutrient rich NTYE medium and synthetic medium with 0.2% glucose as sole source of carbon.

Halobacterium strain R1 grew with a bright orange pigment in NTYE and a mauve pigmentation in NGSM medium. The pigments were characterized using UV-visible spectrophotometer and were identified as carotenoids typical of halophilic archaea with a main peak at 492nm and two shoulder peaks[21]. Scanning electron microscopy revealed cup shaped morphology characteristic of the genus *Halobacterium* strain R1 (Fig. 1).

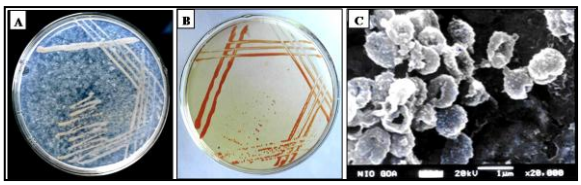

Fig.1-*Halobacterium* strain R1 growing on A) NGSM agar B) NTYE agar, C) Scanning electron micrograph

The culture utilized a wide range of carbon substrates and showed resistant to cadmium up to 4 mM in NTYE and 2 mM in NGSM. Toxicity of Cd to the growth of archaeon *Halobacterium* strain R1 in NGSM was observed to be doubled as compared to that in complex NTYE medium and showed a corresponding 50% decline in the maximum tolerance level (MTC). The minimum inhibitory concentration (MIC) of Cd was observed to be 5 mM in NTYE and 2.5 mM in NGSM, above the respective Cd concentration the growth was abolished totally. This denotes that the MIC is dependent on the type of media used and is in agreement with Lagorce et al 2012 and Ramamoorthy & Kushner, 1975[19,27]. The MIC of Cd for our archaeon *Halobacterium* strain R1 was up to 5 mM in nutrient rich NTYE medium as compared to the report by Nieto et al (1987) which was 2.5 mM for *Haloferax mediterranei* ATCC 33500[9].

In presence of Cd, the exponential phase began after a lag of 20 h. The dividing cells began accumulating up to 2 ppm Cd on the third day of growth, which increased progressively to 18 ppm on the 5th day of growth and remained constant thereafter (Fig. 2). A decrease in pH was observed in the growth from pH 7 to pH 5.2.

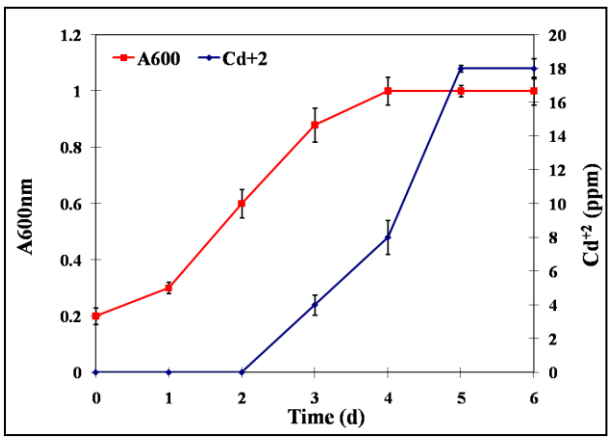

Fig.2-Accumulation of Cd by cells of *Halobacterium* strain R1 during growth in 1 mM Cd

When grown in NGSM containing 1mM Cd, adjusted to varying pH, the archaeon accumulated 12 ppm of Cd at pH 5, 18 ppm at pH 6 & 7 and 16 ppm of Cd at pH 8. NaCl concentration in the growth medium also affected the accumulation of Cd by *Halobacterium* strain R1. At 15, 20, 25 and 30% NaCl concentration, the culture accumulated 18, 18, 20 and 23 ppm of Cd, respectively.

Accumulation of cadmium in cells was estimated using the dithizone method. However, cadmium could not be estimated in the supernatants by either Atomic absorption spectroscopy (AAS) or the Dithizone method due to interference of high NaCl concentration. When the cells were treated using the modified dithizone method, cells grown in the presence of Cd showed a magenta pink coloration while those grown in the absence of Cd showed a yellowish coloration. Acetone extracts of the archaeon grown in NTYE, NGSM and NGSM containing 1 mM Cd were also treated using this method. The magenta pink coloration occurred only in the acetone extract of the archaeon when grown in NGSM containing 1mM Cd .

Halobacterium strain R1 in keeping with their true haloarchaeal nature lysed on suspension in distilled water, therefore it cannot be used for the removal of metal from non-saline environments. Cell immobilization was thus attempted. Cells of *Halobacterium* strain R1 MTCC 3265 were immobilized using calcium alginate. The cells entrapped in sodium alginate yielded pinkish beads and were viable (Fig. 3). Suspension of these beads in 0.2 M calcium chloride solution and keeping for 12-24 h in the refrigerator resulted in hardening of the beads.

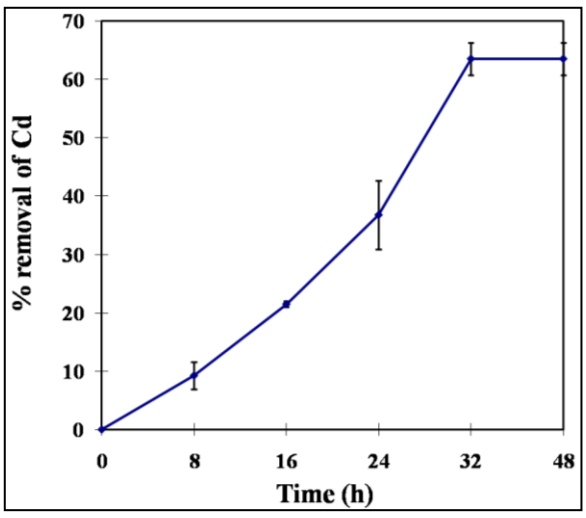

Fig.3-Removal of cadmium by immobilised cells of *Halobacterium* strain R1

Removal of cadmium was linear up to 32 h with respect to time and remained unchanged on further incubation of 48 h. Cells entrapped in calcium alginate showed a 63% removal of Cd from deionised water (Fig. 3). Beads of the haloarchaeon on suspension in 1 mM Cd for 8 h and treated with the modified dithizone reagent stained pink while a magenta pink coloration was observed on suspension of the beads in Cd for 24 h (Fig. 4).

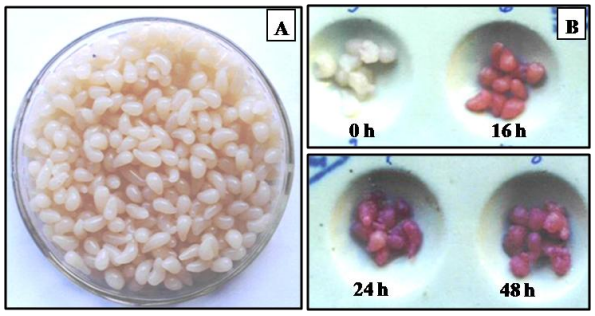

Fig.4-A) Cells of *Halobacterium* strain R1 immobilised in calcium alginate. B. Magenta pink cadmium dithizonate complexation by immobilised cells suspended in 1mM Cd solution for various time periods.

Suspension of the immobilized cells in 25% NaCl led to the disintegration of the beads whereas the same on suspension in water remained intact. This could be a result of the disintegration of the calcium alginate matrix in high salinity. On suspension in water, the calcium alginate matrix probably protects the cell from the stressful environment (lack of NaCl in the surrounding medium).

Conclusions

In the present study, we have reported cadmium bioremediation potential of extremely halophilic archaeon, *Halobacterium* strain R1 MTCC 3265. The culture could resist and grow in presence of up to 4 mM Cd in complex medium containing 25% NaCl, with maximum intracellular Cd accumulation of up to 18 ppm. This shows the potential of the strain to be exploited to sorb Cd from hypersaline econiches. Also, metal sorbtion potential of the ca-alginate immobilized archaeal cells was successfully explored, so as to employ the strain for bioremediation of non-saline environments. To the best of our knowledge, this is the first report on Cd sorbtion potential of *Halobacterium* strain R1, which makes it an attractive candidate for use in bioremediation.

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