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Evaluation of remediation in heavy metal tolerance and removal by *Comamonas acidovorans* MTCC 3364

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Abstract: *Comamonas acidovorans has vital role in degradation of natural as well as complex organic compounds. Comamonas acidovorans MTCC 3364 is mainly used for bioconversion of different steroids but now it is a novel approach on bioremediation. In heavy metals hexavalent chromium, mercury and lead is very toxic and carcinogenic for human health. Organism can tolerate heavy metals like hexavalent chromium, mercury, lead and aluminium with high efficiency. Removal of hexavalent chromium is major problem to textile as well as different industries. Comamonas acidovorans MTCC 3364 removed 99% of the hexavalent chromium from the medium and it can tolerate up to 600 ppm of chromium and 200 ppm of mercury in solidified medium. This organism shows high tolerance against salt i.e. it can tolerate up to 10% of salt. Chromium removal was also observed by using biosorption studies and MIC method. This bacteria increases pH during removal of chromium and makes chromium oxide which is trivalent chromium; it is a non-toxic compound. High salt tolerance, heavy metal tolerance and removal of hexavalent chromium make applicability in the treatment of waste water technology and treatment of industrial effluent which contain high amount of salt and heavy metals.*

Keywords: *antibiotic resistance, Comamonas acidovorans MTCC 3364, heavy metal tolerance, hexavalent chromium removal, salt tolerance*

I. Introduction

Heavy metals such as mercury, chromium, lead and arsenic are toxic metals that have no known vital or beneficial effect on organisms. Heavy metal toxicity can result in damaged or reduced mental and central nervous function, lower energy levels, and damage to blood composition, lungs, kidneys, liver, and other vital organs. Long-term exposure may result in slowly progressing physical, muscular, and neurological degenerative processes. Extensive application of chromium in industries particularly metal finishing industries, petroleum refining, leather tanning, iron and steel industries, inorganic chemical production, textile manufacturing and pulp production industries[1-2] leads to the formation of chromium-contaminated soil and ground water which pose a serious threat to living organism particularly to human health [3-4]. Chromium is a potent pollutant which is mutagenic, carcinogenic and teratogenic in humans, animals and plans [5-7].

Mercury-contaminated soils and sediments are commonly remediated by soil excavation, relocation, and burial; soil washing with halogenated substances; heating soil to high temperature. In general, treatment of effluents includes physiochemical methods such as filtration, specific coagulation, use of activated carbon and chemical flocculation [8]. However, a majority of these technologies are costly to implement and cause further disturbance to the already damaged environment [9]. Biological treatment methods using different bacteria, fungi and plants have been widely studies. Biological reduction of hexavalent chromium to and its precipitation into immobile trivalent by chromium resistant microbes is considered to be an effective method for detoxification of chromium-contaminated environments and have a potential use in bioremediation. Reduction of chromium has been demonstrated in various bacterial species including *Escherichia coli* [10], *Pseudomonas putida* [11], *Desulfovibrio* sp. [12], *Bacillus* sp.[13] and *Arthrobacter* sp. [14] and actinomycetes [15]. The response of microorganisms towards toxic heavy metals is of importance in view of their interest in the reclamation of polluted sites. Several studies have indicated a co-relation between antibiotic resistance and heavy metal resistance [16-17].

Comamonas acidovorans is belonging from *Comamonadaceae* family of the β-proteobacteria. It has vital role in degradation of natural as well as complex organic compounds like 4-nitrobenzoate and cocaine [18-19]. *Comamonas acidovorans* MTCC 3364 has been routinely reported for bioconversion of different steroids like progesterone, testosterone and cholesterol [20-21]. The main purpose of study was to investigate efficacy of heavy metal tolerance and effective removal of hexavalent chromium by *Comamonas acidovorans* MTCC 3364. Efficient hexavalent chromium removal was also observed by using different parameters like time of addition, split dose, concentration of chromium and pH of the medium was also observed. Conversion of hexavalent to trivalent chromium was also observed and biosorption mechanism was studied by using free live and dead cells of an organism. Co-relation between chromium resistant bacteria and β-lactam antibiotics was also studied.

II. Materials And Methods

2.1 Chemical and reagents

Nutrient broth, nutrient agar, ampicillin (10 mcg) and penicillin G (10 units) discs were purchased from Sigma Pvt. Ltd. Diphenyl carbazide, sodium chloride, potassium dichromate, potassium chromate, mercury chloride, silver nitrate, lead nitrate, aluminium chloride and stannous chloride was purchased from Merck Specialities Pvt. Ltd. Acetone and concentrated sulphuric acid were purchased from standard Indian suppliers. All chemicals are highly pure and analytical graded.

2.2 Micro-organisms and culture conditions

Comamonas acidovorans MTCC 3364 was purchased from Microbial Type Culture Collections, Institute of Microbial Technology, Chandigarh, India. The pure culture was maintained on nutrient agar slants and stored at 4^{o}C. The organism was sub-cultured every month. Heavy metal tolerance studies and chromium removal studies were performed using nutrient broth medium (13 g/L).

2.3 Maximum Tolerance Concentration studies of heavy metals

Different heavy metals were used to study maximum efficacy of heavy metal tolerance of an organism. All metals had been grown along with nutrient medium by varying different concentration of heavy metals like chromium, mercury, lead, silver, stannous and aluminium. 20 ml of nutrient broth (13 gm/lit) were autoclaved, cooled and then inoculated with 100 µl of previous grown culture. Flasks were incubated on rotary shaker at 37^{o}C for overnight. Next day, 100 to 500 ppm of sterilized heavy metals was added and observed after 24 hours.

2.4 Chromium removal studies

20 ml of nutrient broth (13 gm/lit) were autoclaved, cooled and then inoculated with 100 µl of previous grown culture. Flasks were incubated on rotary shaker at 37^{o}C for overnight. Next day, 100 to 500 ppm of sterilized chromium ($K_2Cr_2O_7$ and K_2CrO_4) was added to the flasks and incubated for 72 hours at 37^{o}C in shaking condition. Samples were withdrawn and OD was determined for chromium at interval of 24 hours using diphenyl carbazide method.

2.5 Effect of time of addition of Chromium with split dose

Following variants for chromium addition were selected: [1] Flask containing 20 ml of nutrient broth and 100 ppm of chromium were autoclaved, cooled and then inoculated with 100 µl of *Comamonas acidovorans* MTCC 3364 culture. [2] Flask containing 20 ml of overnight grown culture and then 100 ppm of chromium was added. Both flasks were incubated for 48 hours at 37^{o}C at shaking condition. Samples were withdrawn and OD was determined for chromium at interval of 24 hours by using DPC method. 200 ppm of Chromium was again added after 48 hours to observe split dose studies and OD was again determined at interval of 24 hours by using DPC method.

2.6 pH observation studies

100 ml of nutrient broth was taken and pH observed, autoclaved it and inoculated 100 ml of culture of organism. 5 ml of sample was withdrawn aseptically, pH observed and flasks were again incubated at 37^{o}C at shaking condition. Next day, 100 ppm of chromium ($K_2Cr_2O_7$ & K_2CrO_4) added, sample was collected again and pH observed for 96 hours.

2.7 Chromium removal studies using free cells

20 ml of Culture was transferred into centrifuge tube aseptically and centrifuge at 10,000 rpm for 10 minutes. Cells harvested and suspended in phosphate buffer (pH 6.85). 100 ppm, 200 ppm and 300 ppm of chromium ($K_2Cr_2O_7$ and K_2CrO_4) was added and incubated for 24 hours at 37^{o}C at shaking condition. Chromium estimated at interval of 2 hours and calculated % chromium removal. Biosorption was also studied using Energy Dispersive Analysis of X-Ray (EDAX).

2.8 Chromium removal studies using dead cells

20 ml of culture was transferred it into centrifuge tube aseptically. Centrifuge at 10,000 rpm for 10 minutes. Cells were harvested, resuspended in phosphate buffer (pH 6.85) and autoclaved for 15 minute at 121^{o}C. 100 ppm of chromium was added and incubated for 24 hours at 37^{o}C at shaking condition. Chromium estimated at interval of 2 hours and calculated % chromium removal.

2.9 Chromium, mercury and salt tolerance studies on solidified medium

Plates containing nutrient agar with 100-500 ppm of Chromium ($K_2Cr_2O_7$& K_2CrO_4) and 50-250 ppm of mercury ($HgCl_2$) were prepared respectively and plates containing nutrient agar with 0.5 % to 20 % of

sodium were also prepared. 100 µl of organism was poured in each plate, solidified it and incubated for 24 hours at 37^{o}C. Next day results were observed.

2.10 Effect of β-lactam antibiotics on Chromium resistant *Comamonas acidovorans* MTCC 3364

Nutrient broth containing 200 ppm of chromium along with 100 µl of culture was prepared and incubated for 24 hour at 37^{o}C, 120 rpm rotary shaker. Next day Nutrient agar plates were prepared and chromium resistant *Comamonas acidovorans* MTCC 3364 culture was spread on nutrient agar plates. Antibiotic discs (ampicillin and penicillin G) were kept on the centre of the plates. All the plates were incubated overnight at 37^{o}C. Negative control (without chromium) plates were also prepared for comparative study.

III. Result And Discussions

3.1 Maximum Tolerance Concentration studies of heavy metals

Comamonas acidovorans MTCC 3364 could efficiently tolerate up to 600 ppm of hexavalent chromium, 200 ppm of mercury, 500 ppm of lead, 400 ppm of silver, 350 ppm of stannous and 500 ppm of aluminium. This data showed that *Comamonas acidovorans* MTCC 3364 has high tolerance efficacy in terms of heavy metals specially it tolerated hexavalent chromium, lead and aluminium with highest efficacy. Highest tolerance might be involved in the organism either presence of cross-resistance with heavy metals or presence of plasmid [17].

3.2 Chromium Removal studies

The results of chromium removal by *Comamonas acidovorans* MTCC 3364 have been. Organism removed 99.40% of 100 ppm K_2CrO_4within 48 hours and 98.70% of 100 ppm $K_2Cr_2O_7$. Cells were also removed 98% of 200 ppm of chromium efficiently. It removed chromium up to 400 ppm. Woo Chul Bae showed that *E.coli* removed chromium 40 ppm within 20 hours whereas *Comamonas* removed 100 ppm efficiently within 48 hours suggesting the suitability of the organism for treatment of Industrial effluent containing large amount of chromium at 37 ^{o}C and pH 7.0±0.02 [2].

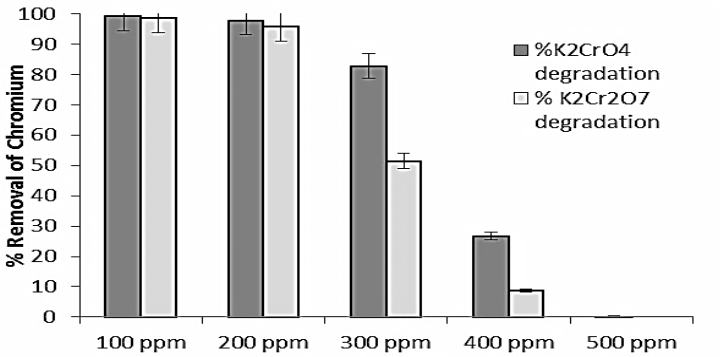

Fig. 1 Chromium Removal by *Comamonas acidovorans* MTCC 3364

3.3 Effect of time of addition of Chromium with split dose

To study the effect of chromium on growth and decolorization, chromium was added to the reaction medium either during inoculation or after overnight growth. Removal of chromium was followed after 24 hrs. The results have been shown in Fig. 2 and 3 which clearly depict that organisms removed efficiently chromium when chromium was added before growth. It showed efficiently removal of chromium even after split dose. Medium showed green color after 72-96 hours after inoculation of organisms. This indicated that chromium (+6) was converted into chromium oxide; which has significant importance in textile, paper and other industries [22].

3.4 pH observation studies

The pH change in the medium was suspected to be the cause of change in color of the medium and was confirmed by measuring the pH of colored medium which was found to be between 8 and 9. It is a major application from the point of view of bioremediation and industrial aspects in that hexavalent chromium converted into trivalent chromium only if alkaline condition is possible so removal of chromium was beneficial. K.Poornima et al also reported pH studies during removal of chromium [22] [25].

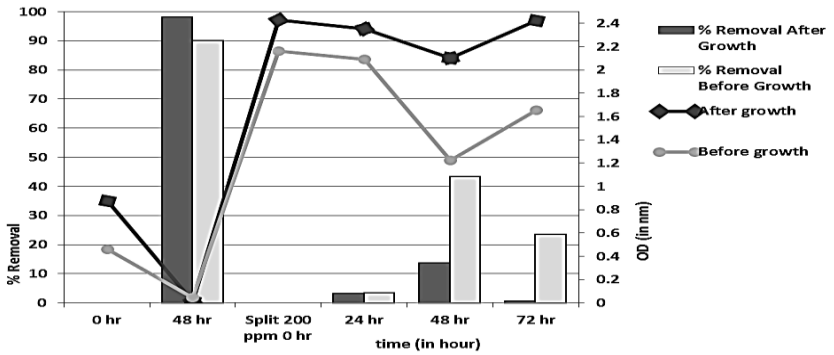

Fig. 2 Effect of time of addition of $K_2Cr_2O_7$ with split dose

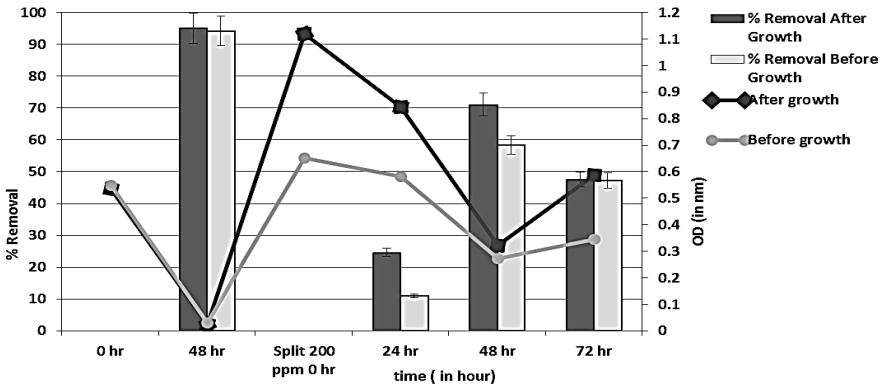

Fig. 3: Effect of chromium time of addition of K_2CrO_4 with split dose

Table 1 pH observation studies during removal of chromium

Condition	Nutrient broth (Control)	Nutrient broth with $K_2Cr_2O_7$	Nutrient broth with K_2CrO_4
Before autoclaving	6.85	7.03	7.15
After autoclaving and inoculation	6.89	7.17	7.35
24 hour	6.92	7.98	8.24
48 hour	6.95	8.27	8.52
72 hour	6.98	8.86	8.99
96 hour	7.00	9.08	9.32

3.5 Chromium removal studies using free cells

In order to test the reusability and importance of metabolic activity of cells for chromium removal, the cells of the organism were harvested from overnight grown culture and incubated with the chromium in buffered medium. The results of free cells for chromium removal are depicted in Fig. 4 (A). Free cells showed almost half of the chromium removal by biosorption. Chromium was removed almost 50-56% by biosorption studies so it is indicative that metabolic function of cells in not mandatory for chromium removal by this organism. P. Saranraj et al also suggested that biosorption also occurred by live and dead cells of *Enterococcus casseliflavus* [23].

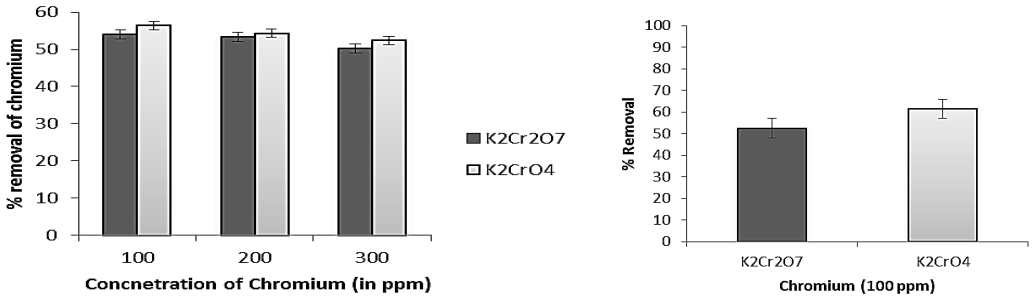

Fig. 4 Chromium removal using free (A) live cells (100-300 ppm) (B) dead cells (100 ppm) (Biosorption studies)

3.6 Chromium removal studies using dead cells

Chromium removal studies also cross checked with the biosorption studies. Dead cells also showed 52-61% of removal of chromium which highlighted in Fig. 4 (B) that presented the role of biosorption in contributing towards removal of metals by *Comamonas acidovorans* MTCC 3364 [23]. EDAX analysis of free cells suspended in buffer containing 100 ppm of chromium, which was detected under Energy Dispersive X-ray analyser, showed a peak of chromium in EDAX graph as shown in Fig. 5 [25].

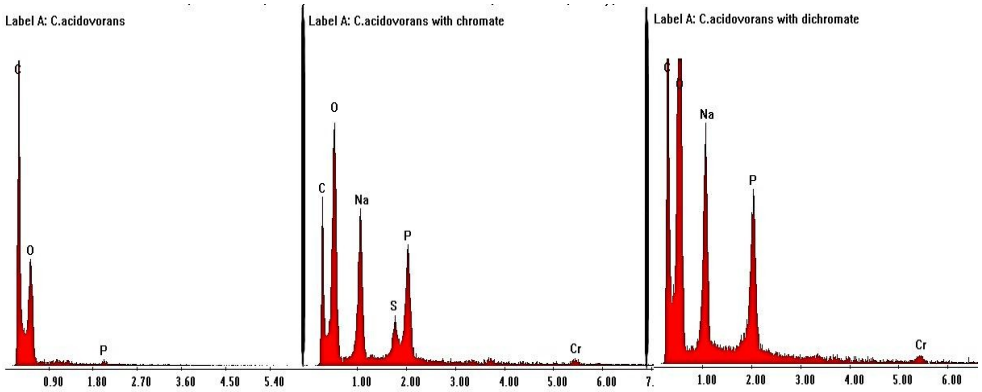

Fig. 5 EDAX spectrums of (A) culture of *Comamonas acidovorans* MTCC 3364 (control), (B) cells incubated with potassium chromate and (C) cells incubated with potassium dichromate

3.7 Chromium studies on solidified medium

Growth was observed on solidified medium showed that organism can tolerate up to 600 ppm of chromium in solidified medium but it showed some spotted colony on 700 ppm containing chromium plates and up to 200 ppm of mercury. *Comamonas acidovorans* MTCC 3364could also tolerate 10% of salt in the medium. This data depicts that some detergent or textile industries contained high amount of salt or alkalinity in their effluents so this organism could also survive up to 10% of salt concentration. It is significant in waste water technology and textile industries that contained high concentration of heavy metals and high amount of salts so it can tolerate high concentration of salt along with chromium and simultaneously mercury.

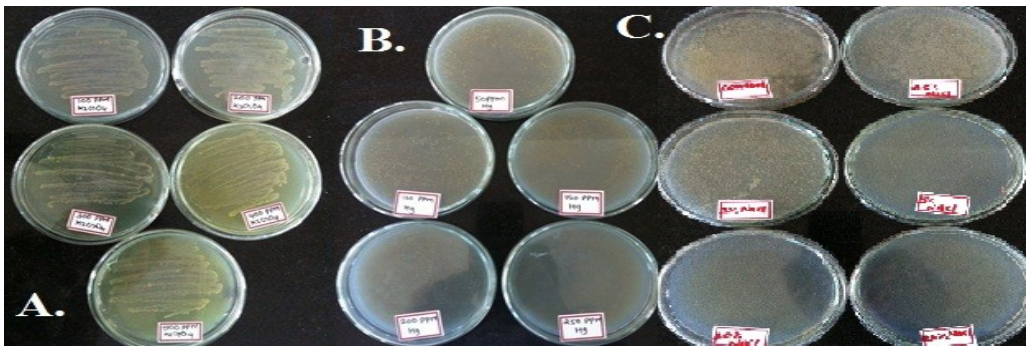

Fig. 6 Growth of *Comamonas acidovorans* MTCC 3364 on solidified medium contained (A) chromium up to 500 ppm (B) mercury up to 250 ppm (C) salt concentration up to 20%.

3.8 Effect of various antibiotics on Chromium resistant *Comamonas acidovorans* MTCC 3364

Relation between antibiotics and heavy metals is significant prospectus for industrial purpose. *Comamonas acidovorans* MTCC 3364 showed tolerance for the chromium at higher concentration. It directly affected on antibiotic resistance on normal *Comamonas acidovorans* MTCC 3364 cells. Chromium resistant cells showed less sensitivity against antibiotics. It is showed in Fig. 7.

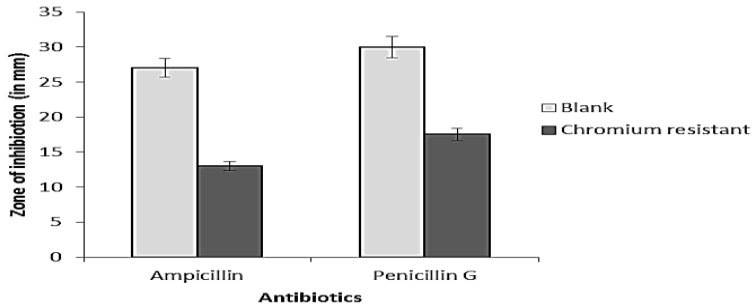

Fig. 7 Comparison of β-lactam antibiotics between chromium resistant bacteria and control (without any metal)

IV. Conclusion

Comamonas acidovorans MTCC 3364 could tolerate heavy metals with high efficiency. The strain could be efficiently applied for removal of high concentration of chromium from the industrial effluents. Biosorption and chemical reduction have been indicated to play role in chromium removal by the strain. pH observation studies and EDAX analysis revealed that hexavalent chromium is converted in to less toxic and useful compound chromium oxide which is trivalent. Organism showed high resistant activity against β-lactam antibiotics when chromium is present in medium. Thus, *Comamonas acidovorans* MTCC significant in waste water technology and textile industries that contained high concentration of heavy metals and high amount of salts.

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