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Original Article

Reduction of Chromium-VI by Chromium Resistant Lactobacilli: A Prospective Bacterium for Bioremediation

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ABSTRACT

Chromium is a toxic heavy metal, which primarily exists in two inorganic forms, Cr (VI) and Cr (III). Highly soluble hexavalent chromium is carcinogenic due to its oxidizing nature. It is well established that the intestinal bacteria including Lactobacilli have regulatory effect on intestinal homeostasis and a breakdown in the relationship between intestinal cells and bacteria results in the manifestation of gastrointestinal (GI) disorders. In this study Cr (VI) resistance was developed in Lactobacillus strains and the reduction of Cr (VI) was evaluated. All resistant strains showed similarities with their respective normal strains and did not acquire resistance to various antibiotics. A complete bacterial reduction of 32ppm Cr (VI) was observed within 6 to 8 hours. The presence of chromate reducing enzyme have also been established following the partial purification (2 to 5 fold) and characterization of chromate reductase in Lactobacillus strains. The chromate reductase of our strains showed optimum activity at pH 6.0 and 30^{0}C. To our knowledge; these strains are fast in Cr (VI) reduction than any other known bacteria. The results suggest that chromate- resistant Lactobacillus strains would be useful for chromium detoxification from GI-tract as well as for bioremediation of hexavalent chromium from contaminated environment.

Key words: Bioremediation, chromate reductase, hexavalent chromium, lactobacilli

INTRODUCTION

Chromium is one of the most toxic chemical compounds because of its increased level in the environment as a result of metallurgies refractory, chemical and tannery industries as well as by agricultural practices. It has become one of the most abundant pollutants in aquatic and terrestrial ecosystems.[1] Hexavalent Cr (VI) and trivalent Cr (III) are two stable chromium oxidation states found in nature.

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As an analogue of sulfate, chromate can enter bacterial and mammalian cells readily via sulfate transport systems.[2] Cr (III), which is less toxic and less soluble than Cr (VI), is readily being converted into Cr (VI) under natural conditions through various oxidation processes and this oxidized Cr (VI) reacts with nucleic acids and other cell components to produce mutagenic and carcinogenic effects on biological systems.[3] However, Cr (III) is considered to be a trace element essential for the proper functioning of living organisms.[4,5]

To check the chromium exposure into environment there are various treatment options, however, they are energy expensive and less successful due to their high running cost. In this context, biotransformation of Cr(VI) to Cr(III) by bacteria offers a viable, economically safe and sustainable alternative.[6] Biosorption, bioaccumulation and enzymatic oxidation/reduction are the processes by

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which the microorganisms interact with the toxic metals. The enzymatic reduction of Cr (VI) involves membrane bound chromate reductase during anaerobic respiration or a soluble cytosolic chromate reductase under aerobic conditions and activity of which is enhanced by NADH or glutathione as enzyme co-factors.[7] In this whole process chromate acts as the terminal electron acceptor.[8] Furthermore, chromium ingestion through drinking water and food causing its toxic insult leading to severe diseases, including gastrointestinal (GI)- disorders, are common environmental-related problems. Mammalian intestinal bacteria including the predominant Lactobacilli have regulatory effect on intestinal homeostasis, and a breakdown in the relationship between intestinal epithelial cells and bacteria results in the manifestation of GI-disorders.[9] Lactobacilli are also known to provide specific health benefits as probiotics when consumed as a food component or supplement. In the present study chromium resistance was developed in Lactobacillus strains and the chromate reducing enzyme chromate reductase was partially purified and characterized. Furthermore, in order to ascertain their utility in detoxification and bioremediation of chromium contaminated environment, Cr- (VI) reduction properties have been evaluated.

MATERIALS AND METHODS

Organism

Pure cultures of Lactobacillus strains namely *Lactobacillus acidophilus* (MTCC 447), *Lactobacillus rhamnosus* (MTCC 1408) *and Lactobacillus casei* (MTCC 1423) were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained in the laboratory. From normal rat cecum, mixed population of *Lactobacillus* sp. was isolated, identified and pure cultures prepared.[10] *L.acidophilus*, *L. rhamnosus, L. casei* and mixed population of *Lactobacillus* sp. were cultivated in MRS broth (HiMedia). The minimal inhibitory concentration (MIC) of Cr(VI) (potassium dichromate from Ranbaxy Laboratories Limited) was determined by the standard broth agar dilution method. Viability was tested by colony forming unit (CFU) on agar plates.

Development of Cr(VI) resistant mutant strains

Lactobacillus strains were grown in Lactobacillus MRS Broth at 37° C for 18 h, and approximately 5 × 10^7 CFU were inoculated into a series of tubes containing 9.9 ml of MRS broth with chromium concentrations (1 mg/L, 2 mg/L, 4 mg/L, 8 mg/L, 16 mg/L and 32 mg/L) consisting of doubling dilutions below and above the MIC. After incubation, aliquots from the tube nearest the MIC, which had the same turbidity as the chromium free control, were used following a 1:100 dilution to inoculate a second set of tubes containing the MRS broth with chromium. After overnight incubation, the bacteria were transferred again and 10-15 serial passages were carried out. Likewise, each strain was then cultivated in doubling concentrations of chromium. The glycerol stocks of chromium-resistant mutant strains were kept frozen at – 40° C and time to time sub cultured to assess the stability of resistance.

Growth phase studies

Growth patterns of parent strains and Cr (VI)-resistant mutant strains of Lactobacilli were measured by growing approximately equal number of cells (2 × 10^6 cells) in MRS broth at different time intervals up to 30 h using turbidimetry at 610 nm. In case of resistant strain, growth measurements were carried out in the absence and presence of chromium in the media.

Antibiotic susceptibility test

Normal parent and mutant strains were tested for antibiotic sensitivity following the National Committee for Clinical Laboratory (NCCL) standard disk diffusion method. The following antibiotic disks from HiMedia, India were used: Amoxycillin (25 μg), Chloramphenicol (25 μg), Ciprofloxacin (10 μg), Gentamicin (10 μg), Kanamycin (30 μg), Norfloxacin (10 μg), Novabiocin (30 μg), Erythromycin (10 μg), Streptomycin (10 μg) and Teicoplanin (30 μg).

Tolerance to other metals

Heavy metal tolerance was tested by freshly grown cultures of 32 ppm Cr(VI)-resistant *L. acidophilus, L. rhamnosus, L. casei* and mixed rat gut Lactobacilli inoculated aseptically on Lactobacillus MRS agar plates supplemented individually with other heavy metals. The metal salts used were $HgCl_2$, $Cd(CH_3COO)_2$ and $Pb(NO_3)_2$. The metal ion concentration tested ranged from 1.36 to 2.4 mg/mL.

Enzyme assays and biochemical parameters

Dehydrogenase (DHA) and esterase activity (EA) tests were carried out as described by Liu[11] and Obst and Holzapfel-Pschorn,[12] respectively. Bacterial cell membrane was prepared as described by Kumar and Upreti.[13] Alkaline phosphatase (EC 3.1.3.1) was determined according to Weiser[14] and Ca^{2+}- Mg^{2+}-ATPase (EC 3.6.1.3.) as described by Hidalgo *et al*.[15] Enzyme units were defined as micromoles of product formed or liberated per minute under the assay conditions. Specific activity was expressed as units per milligram of protein. Protein was determined according to Lowry *et al*.[16] using bovine serum albumin as a standard. Carbohydrate (total hexoses) and sialic acid were, respectively, estimated according to Roe[17] and Warren.[18] Total lipid was extracted according to Folch *et al*.[19] Phospholipids were quantified following digestion with 70% perchloric acid and estimated according to the method of Wagner *et al*.[20]

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Determination of Cr (VI) reduction by Lactobacillus strains

For the measurement of Cr (VI) reduction 32ppm Cr-resistant *L. acidophilus, L. rhamnosus, L. casei* and *Lactobacillus* sp. were grown in MRS Broth for overnight at 37° C. After taking O.D. (610 nm) of all cultures of *Lactobacillus* strains, equal numbers of cells from each strains (2 mL) were inoculated in 10 mL of fresh MRS broth containing 32 mg/L of Cr (VI). O.D. was taken after 30 mins, 1, 2, 4, 6, 8 and 10 h and simultaneously 1 ml sample was taken at the same time interval. Cells were harvested (8000 rpm for 15 mins. in cold conditions) and obtained supernatant was used for chromium estimation. Chromium estimation was also carried out in MRS media containing 32 mg/L Cr (VI) at the same time intervals. The hexavalent chromium in the culture supernatants was measured using 1, 5- diphenylcarbazide (DPC) regent as described by American Public Health Association.[21] Total chromium was measured using atomic absorbance spectrophotometry after acid digestion of the sample.

Partial purification and characterization of chromate reductase

Cells from culture were harvested by centrifugation at 8000 rpm and re-suspended in 50 mM potassium phosphate buffer of pH 6 (1.0 g cells wet wt in 6 mL buffer). The cells were lysed by sonication (5 cycles of 40 s on and 40 s off at 175 W), centrifuged (15000 rpm for 30 min at 4° C) and the cell-free extract (CFE) was collected. The CFE was fractionated with ammonium sulfate corresponding to 0-30% (w/v) with constant stirring for 1h in cold condition. It was then centrifuged and supernatant was collected. Supernatant was further fractionated to 30 - 40% (w/v) salt concentration and the precipitate was re-dissolved in phosphate buffer. The sample was dialyzed against the phosphate buffer for overnight and chromate reductase was assayed.

Chromate reductase activity was assayed using NADH as an electron donor. The reaction mixture contained 0.1 mL of partially purified enzyme preparation, 0.1 mM NADH and 3.4 μM Cr (VI) in 50 mM potassium phosphate buffer (pH 6.0) in a total volume of 1.0 mL. Assay mixtures comprising of similar composition given above except enzyme or NADH were used as respective controls. The assay mixtures were incubated at 30° C for 30 min. The amount of hexavalent chromium was measured using 1, 5-(DPC) reagent. One unit of enzyme is defined as the amount that catalyzes the conversion of 1 μ M of substrate Cr (VI) per minute per mg protein.

To check the pH stability, chromate reductase activity was measured at different pH using various buffers (50 mM sodium acetate, pH 4–5.5; 50 mM sodium phosphate, pH 5.5–8 and 50 mM sodium carbonate, pH 8–10). Effect of temperature was studied by measuring enzyme activity between 20° C and 60° C at pH 6.0. For thermal stability, enzyme was incubated at 10–60° C and at pH 6.0 for 30 and 60 min, cooled in ice bath and the residual enzyme activity was measured.

RESULTS AND DISCUSSION

Sub-culturing of all tested Cr-resistant Lactobacillus strains (mutant strains) in the presence of chromium gave increased MICs, with MICs rising from 2.0 – 8.0 mg/L to 8.0 – 64 mg/L after 6 to 15 sub-cultures. All the normal parent Lactobacilli strains did not grow when grown in presence of higher than MIC of Cr (VI) in the media. We have earlier shown that chromium- resistant bacteria followed entire growth phase patterns similar to that of respective normal parent strains when grown either in the absence or in the presence of chromium (up to 32 mg/L) in the media. Simultaneously, similarities in various biochemical parameters in the evaluation of toxicity have also been shown between Cr-resistant Lactobacillus strains and their respective normal strains.[22]

To investigate if these Cr-resistant Lactobacillus strains also developed the resistance against antibiotics, the antibiotic sensitivity test of normal and respective resistant bacteria was carried out. It was observed that Cr-resistant Lactobacillus strains did not acquire resistance against various antibiotics [Figure 1]. Similar findings were found in case of chromium- resistant bacteria which are isolated from tannery effluents.[23] Heavy metal resistance in a number of different bacteria is known to be present together with antibiotic resistances. Under environmental conditions of metal stress, such metal and antibiotic resistant population adopts faster by the spread of R-factors than by mutation and natural selection.[24,25] Non-acquirement of resistance against various antibiotics by our Cr- resistant bacteria indicates that the chronological chronic exposure

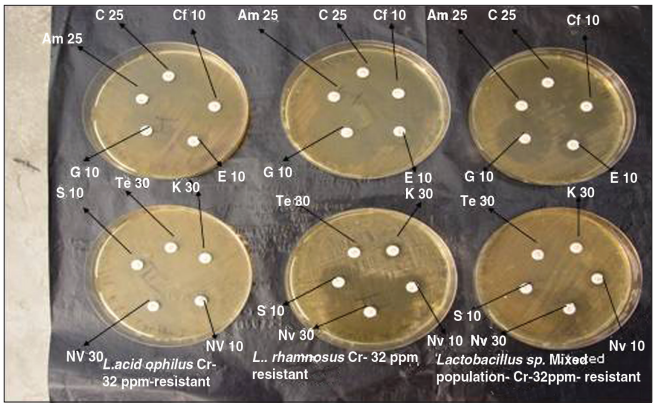


Figure 1: Antibiotic sensitivity test of Cr (VI)- resistant Lactobacilli strains by disk diffusion method. Am=Amoxycillin: C=Chloramphenicol; Cf=Ciprofloxacin; E=Erythromycin; G=Gentamicin; K=kanamycin; Nu=Novobiocin; Nc=Norfloxacin; Te=Teicoplanin

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of Cr (VI) did not influence the occurrence of antibiotic resistance. These strains were also found to be sensitive to Hg^{+2}, Cd^{+2}, and Pb^{+2} (data not shown).

Time course of Cr (VI) reduction during the growth of Cr-resistant Lactobacillus strains is shown in Figures 2 and 3. Cr-resistant *L. acidophilus, L. rhamnosus, L. casei* and mixed rat gut *Lactobacillus* sp. showed 15 to 30 % reduction of Cr (VI) in 30 minutes and 100% reduction were observed during 6 to 8 hour. Atomic absorption showed negligible concentration of chromium inside the cells. These strains were able to reduce 32 ppm Cr-VI absolutely during 6-8 hours of time interval, *L. acidophilus* being the fastest among the three individual strains. Reduction of Cr (VI) to Cr (III) has been reported in many bacterial types taking 2–9 days.[26-29] Whereas, in the case of *Bacillus* sp. RE, more than 95% of Cr (VI) reduction at 24 h with 10 μg Cr(VI)/mL have been reported.[7] The prolonged period for chromium removal may lead to accumulation of metal in the environment. Thus, Cr (VI) resistant Lactobacillus being the fastest in chromate reduction would be a better choice of bacteria. During the growth in medium containing Cr (VI), the concentration of total chromium remained constant in the medium irrespective of the initial chromate concentration, which indicated possible existence of efflux pump and the absence of chromium bio-accumulation. These observations corroborate with the findings reported by Elangovan *et al*.[7] Cr (VI) reduction and its subsequent efflux in the form of Cr (III) is an important step in bioremediation of chromium from the environment. MRS media containing 32 mg/L Cr (VI) (in absence of Lactobacillus strains) showed negligible reduction in 30 minutes and 34% reduction by the end of 10 hours. This reduction may be due to the presence of thiol and/or other sulfhydril group containing components in the media that acted as electron donors similar to NADH.[30,31] Instead of MRS media, when Minimum essential mineral (MEM) media containing 32 mg/L Cr (VI) was used, it did not reveal Cr (VI) reduction as there are no such reducing components present in MEM media.

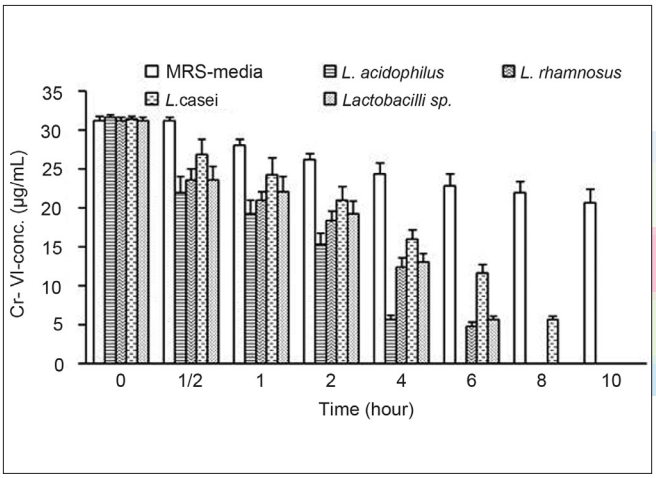


Figure 2: Reduction of Cr-VI by MRS-media (containing 32 µg/ml Cr- VI) and by Cr- resistant Lactobacillus strains. Values are mean ± s.d. from three experiments

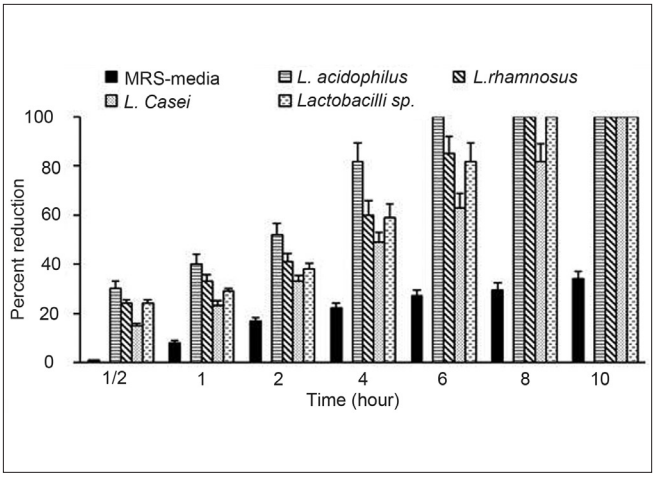


Figure 3: Percent reduction of chromium- VI by MRS-media (containing 32 µg/ml Cr- VI) and by Cr-resistant Lactobacillus strains. Values are mean ± s.d. from three experiments

We have partially purified and characterized the chromate reductase enzyme from the three Cr (VI) resistant *Lactobacilli* strains, which was responsible for the reduction of toxic Cr (VI) without accumulating chromium inside the cell. Respective normal (wild) strains did not show chromate reductase activity. In comparison to cell extract (crude enzyme), partially purified enzyme of 32 mg/L Cr (VI)-resistant *L. acidophilus* showed 3-fold purification. Respective fold purification was 6 and 2 fold for *L. rhamnosus* and *L. casei*. Partially purified enzyme when assayed in presence of NADH (as an electron donor); *L. acidophilus, L. rhamnosus, L. casei* showed 38%, 24% and 17% increase, respectively in the specific activity [Table 1]. Fractionation and assay of the cell-free extract of Lactobacillus strains revealed that a soluble NADH-dependent enzyme is responsible for the catalytic reduction of Cr (VI). Similar findings have also been reported in *A. crystallopoietes* ES 32, *Pseudomonas putida*, *Pseudomonas sp.* and *Bacillus* sp.[3,7,32,33]

Table 1: Partial purification of chromate reductase from 32ppm Cr-VI -resistant Lactobacillus strains

Strains	Sp. activity (Units/mg protein)		
	Cell extract	Partially purified	Fold purification
Cr-VI-resistant			
L.acidophilus (- NADH)	0.24	0.75	3
L.acidophilus (+ NADH)	0.34	1.04	3
L.rhamnosus (- NADH)	0.25	1.50	6
L.rhamnosus (+ NADH)	0.36	1.86	5
L.casei (- NADH)	0.87	1.76	2
L.casei (+ NADH)	1.52	2.07	1.3

One unit is defined as the amount of enzyme that catalyzes the conversion of one micromole of substrate (chromium) per minute.

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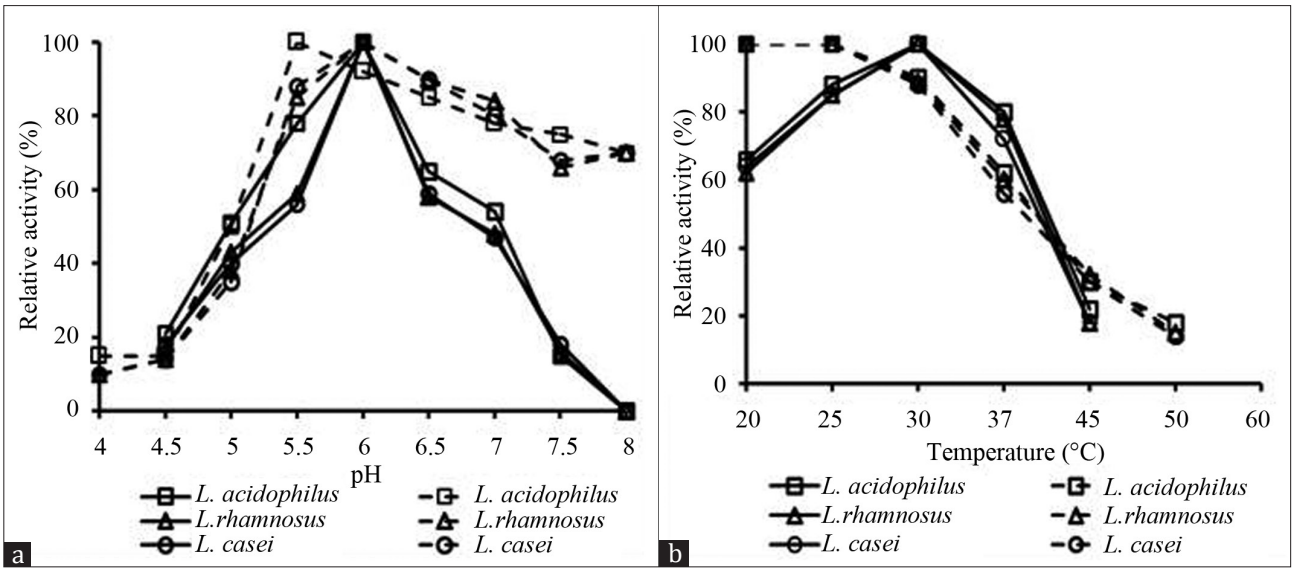


Figure 4: Effect of (a) pH and (b) temperature on chromate reductase activity (____) and stability (-----) of Cr-resistant Lactobacillus strains (sp. act. *L.acidophilus* 1.04 U /mg; *L.rhamnosus* 1.86 U /mg; *L.casei* 2.07 U/ mg).

Chromate reductase activity in all three strains of Lactobacillus was observed in a narrow pH range with an optimum at pH 6.0 [Figure 4a]. Acidic pH drastically decreased the enzyme stability while alkaline pH slightly reduced the stability with retention of around 75% residual activity. At the optimum pH of 6.0, maximum chromium reductase activity was found at 30° C [Figure 4b]. Activity at 25° C was 80% of the optimal activity and at 20° C, observed activity was 60% of the optimum. A significant decrease in activity was observed above 30° C. At optimum pH of 6.0, chromate reductase was most stable below 30° C, which is its optimum temperature for activity. Both the activity and stability of chromate reductase was dependent on pH. In general, for bacterial chromate reductases the reported optimum pH and temperature are pH 5–9 and 30° C, respectively.[7,34,35]

In conclusion, our studies reveal that sequential sub culturing of Lactobacillus strains in hexavalent chromium led to the development of resistance of chromium. The resistant strains showed good chromium tolerance and Cr (VI)-reduction through soluble enzyme. It did not accumulate chromium inside the cell, hence can play important role in the detoxification of chromium in gastrointestinal tract. In addition, Lactobacilli being a friendly and non-pathogenic group of bacteria, the chromium resistant Lactobacillus can be a better choice in comparison to other chromium-resistant bacteria for bioremediation of chromium-contaminated environment.

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