

Bioadsorption of Chromium Resistant *Enterococcus casseliflavus* Isolated from Tannery Effluents

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Abstract

Bioadsorption, bioaccumulation and enzymatic reduction are the processes by which the microorganisms interact with the toxic metals, enabling their removal or recovery. In the present study, a bacterial strain was isolated from tannery effluent and identified as *Enterococcus casseliflavus*. It showed a high level resistance of 800 µg/ml chromium. The minimal inhibitory concentration of chromium was found to be 512 µg/ml of potassium dichromate in Nutrient broth medium. The chromium adsorption was more significant by the live cells than killed cells at different time intervals. It was observed that, the inoculation of *Enterococcus casseliflavus* reduced the BOD and COD values of tannery effluent. The maximum adsorption of chromium was at a temperature of 35°C to 45°C and at a pH of 7.0 to 7.5.

1. Introduction

Chromium, a priority pollutant is well known for its mutagenicity, carcinogenicity and teratogenicity in humans, experimental animals and plants. Extensive use of chromium in industries such as leather tanning, stainless-steel production, electroplating and wood preservatives have resulted in chromium contaminated soil and ground water at production sites (Turick *et al.*, 1996) which pose a serious threat to human health. The hexavalent chromium compounds are comparatively more toxic than trivalent chromium compounds due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids (Sultan and Hasnain, 2005). Accordingly, chromium and its compounds are placed on the priority list of toxic chemicals by US EPA (Urvashi Thacker and Rasesh Parikh, 2007). A maximum acceptable concentration of 0.05 mg/l for hexavalent chromium in drinking water has been established on basis of health considerations. However, its high toxicity, mutagenicity and carcinogenicity renders it hazardous even at very low concentration.

Medical research has further confirmed that hexavalent chromium exposure is associated with increased human lung cancer risk. The presence of sulphide and chromium in tannery effluent affects plant life and soil fertility. Then the chromates were absorbed in soils and low pH favored their reduction in effluents. Chromium level was found to increase with increase in the concentration of the tannery effluent. Chromium associated pollution is of increasing concern nowadays. Conventional methods for treatment of toxic chromate include chemical reduction followed by precipitation, ion

exchange, and adsorption on activated coal, alum, kaolinite and ash which require large amounts of chemicals and energy and therefore are unsuitable (Camargo *et al.*, 2003).

Several reports have indicated biological reduction of hexavalent chromium by microorganisms, both aerobes and anaerobes. Biological reduction of hexavalent chromium usually occurs at a neutral pH range and generates an insignificant quantity of chemical sludge as well as offers potential cost-effective remediation strategy (Mahesh *et al.*, 2002)

Bioadsorption is the simple uptake of the constituents of the effluent by the organisms without any usage in its metabolism. A number of chromium resistant microorganisms have been reported to detoxify hexavalent chromium, which include *Pseudomonas* sp., *Microbacterium* sp., *Desulfovibrio* sp., *Enterobacter* sp., *Escherichia coli*, *Shewanella alga*, *Bacillus* sp., etc., where the method of detoxification is periplasmic biosorption, intracellular bioaccumulation and biotransformation through direct enzymatic reaction or indirectly with metabolites (Camargo *et al.*, 2003). Some of the fungi that remove chromium from tannery wastes are *Aspergillus*, *Nostoc*, *Cyanobacteria*, etc. Many microbes by cellular activities and their products significantly contribute in these biogeochemical cycles. Biotechnological approaches to the abatement of toxic metal pollution consist of selectively using and enhancing these natural processes to treat particular wastes. The processes by which the microorganisms interact with the toxic metals enabling their removal/and recovery are biosorption, bioaccumulation and enzymatic reduction.

Hexavalent chromium is readily immobilized in soils by adsorption, reduction, and precipitation processes, with only a fraction of the total chromium concentrations available for plant uptake. When taken up by plants, >99% of the absorbed chromium is retained in the roots where it is reduced to trivalent chromium species in a short time. Phytotoxic levels of chromium in most plants seem to limit its accumulation in the food chain. Because most plants have low chromium concentrations, even when grown on chromium rich soils, the food chain is well protected against chromium toxicity. In regions, where hexavalent chromium contamination of the environment represent a major area of concern, the use of chromium-hyper-accumulator plant species or chromium-reducing microorganisms may represent a cost efficient and highly effective technology for the removal and detoxification of the toxic forms of chromium. Chrome recovery is an indirect way of recycling chrome in leather production.

Recent developments in biology have provided new tools and approaches for monitoring the environment and engineering organisms with the capacity to degrade environmental pollutants. Different types of organisms can be bioremediation agents. Considering the enormous biodiversity and biological wealth in this country, basic research and modern biology, product oriented research for new generation of vaccines, diagnostics, recombinant products and other bio products specially the eco-friendly technologies would be the priority agenda for future. The present study is focused on isolation, identification of chromium resistant bacteria and its ability to remove chromium contaminants in tannery effluents.

2. Materials and Methods

Sample collection

Tannery effluents were collected from leather industry at Ambur in Vellore district. The sample was collected after the primary treatment in leather industry.

Isolation and identification of chromium resistant bacteria from Tannery effluent

The tannery effluent sample was taken and diluted in water and was isolated by Serial dilution technique followed by Spread plate method. The isolated colony was sub-cultured in Minimal salt agar medium supplemented with hexavalent chromium 100µg/ml (hexavalent chromium was supplemented in the form of potassium dichromate) and also in Nutrient agar with Potassium dichromate medium 50mg/100ml, and incubated at 37°C for 2 days. Identification of the bacterial isolates was carried out by the routine

bacteriological methods i.e., By the colony morphology, preliminary tests like Gram staining, capsule staining, endospore staining, motility, catalase and oxidase, plating on selective medias like Bile esculin agar, Colombia blood agar and Nutrient agar with 7.5% NaCl and performing biochemical tests.

Evaluation of chromium tolerance

The isolated bacterium (*Enterococcus casseliflavus*) was tested for their resistance to chromate both by Agar dilution method and Broth dilution method.

Agar dilution method

The test organism inoculating into nutrient broth and incubated at 37°C in a shaker incubator at 150 rpm till the concentration of the test organisms matched with 0.5 McFarland standards. In this method, *Enterococcus casseliflavus* was aseptically streaked into freshly prepared Nutrient agar plates amended with hexavalent chromium as dichromate at various concentrations ranging from 100 – 1500 µg/ml. Plates were then incubated at 37°C for 24 hours.

Broth dilution method

The test organism was inoculated into nutrient broth and incubated at 37°C in a shaker incubator at 150 rpm till the concentration of the test organisms matched with 0.5 McFarland standard. A stock solution of chromium was prepared (50 mg/100ml). Two fold dilutions of this solution were prepared in Nutrient broth medium. A standard suspension of *Enterococcus casseliflavus* was inoculated onto the medium with one control chromium solution free medium. The inoculated medium was then incubated at 37°C for 24 hours.

Bioadsorption studies

Estimation of standard chromium: (Diphenyl carbazide method)

A standard curve for the reaction between chromium and its binding dye, 1,5 – diphenyl carbazide was added to determine the chromium concentration. The diphenyl carbazide forms a pink complex with hexavalent chromium but not with trivalent chromium. (Young Hak *et al.*, 2003). 50 mg of potassium dichromate was dissolved in 100 ml of sterile distilled water, in standard flask. From this standard, 0.02, 0.04, 0.06, 0.08 and 0.1 ml of chromium was added into different tubes, then the volume was made upto 2 ml. 2 ml of distilled water was taken as blank. To all the above tubes, 0.04 µl of diphenyl carbazide and one drop of sulphuric acid were added and the residual hexavalent chromium content was determined at 540nm in colorimeter.

Chromium uptake

24 hours old culture of *Enterococcus casseliflavus* was prepared in Nutrient broth. To 50 ml of Nutrient broth, 50 µg/ml of potassium dichromate was added and sterilized. Similarly another set was prepared. To the first set, 10 ml of overnight broth culture *i.e.*, live cells were added. To the second set, 10 ml of 24 hours culture was autoclaved at 121°C for 15 minutes *i.e.*, killed cells was added. From above, 10 ml of samples were removed at different time intervals and centrifuged at 8000 rpm for 20 minutes. Then the pellet obtained was homogenized in 2 ml of phosphate buffer. To this 0.04 µl of diphenyl carbazide and one drop of sulphuric acid was added to the homogenate. Chromium uptake was estimated using diphenyl carbazide method at 540 nm in colorimeter.

Chromium reduction

Reduction of chromium was determined by growing the *Enterococcus casseliflavus* in Nutrient broth supplemented with hexavalent chromium as dichromate at a concentration of 50 µg/ml. Cells were grown on a shaker incubator (150 rpm) for 24 hours. Supernatant obtained after centrifugation was used for chromate reductase assay. Chromate reductase activity was estimated as the decrease in chromium concentration with time using hexavalent chromium specific colorimetric reagent 1,5-diphenyl carbazide (DPC) 0.5% (vol/vol) prepared in acetone. To 1 ml of supernatant, 1 ml of phosphate buffer (pH 7.2) with chromium was added and incubated for 1 hour. After incubation, 1 drop of 0.1 M sulphuric acid and 0.04 µl of 1,5 diphenyl carbazide was added. The readings were taken in colorimeter (540nm).

Effect of pH on chromium removal in Tannery effluents

The influence of pH on chromium removal was assessed with the tannery effluent. The pH of the tannery effluent was adjusted to 4.5, 7 and 11.6 using 1 N HCl and 1 N NaOH, and then sterilized by autoclaving at 121°C for 15 minutes. Then

Enterococcus casseliflavus was inoculated in the above sample and incubated in shaker incubator at 150 rpm for 24 hours. Chromium removal was assessed by measuring the tannery effluent with 1,5 – diphenyl carbazide at 540nm by colorimeter.

Effect of temperature on chromium removal in Tannery effluents

The influence of temperature on chromium removal was assessed with the tannery effluent. The culture (*Enterococcus casseliflavus*) was inoculated in tannery effluent and incubated at different temperatures (37°C, 45°C, 65°C and 75°C). Chromium removal was assessed by measuring the tannery effluent with 1,5 – diphenyl carbazide at 540nm by colorimeter.

Physico-chemical analysis of tannery effluent

Physico-chemical analysis of tannery effluent was analyzed before and after treatment.

3. Results and Discussion

Isolation and identification of chromium resistant bacteria from Tannery effluent

Small and yellow coloured colonies were found. The margin was smooth and circular form. The chromium resistant bacterium from tannery effluent was identified by preliminary tests, cultivating on selective media and standard biochemical tests. Based on the Conventional Biochemical tests, the organism was identified as *Enterococcus casseliflavus*. (Bergey's 9th edition). The results were shown in Table-1. In this study, a chromium resistant bacterium was isolated from tannery effluents in minimal salt agar medium supplemented with potassium dichromate. This was identified as *Enterococcus casseliflavus* using Biochemical methods. Muhammad *et al.*, (2004) and Urvashi Thacker *et al.*, (2006) has done the isolation of chromium resistant bacteria from tannery effluents and identified as *Brevibacterium* sp. and *Brucella* sp. respectively.

Table-1. Identification of chromium resistant bacteria from Tannery effluent

S.no	Characteristics	Results
1	Gram Staining	Gram Positive cocci
2	Motility	Motile
3	Catalase	Negative
4	Oxidase	Negative
5	Voges Proskauer	Positive
6	Carbohydrate fermentation	
	Glucose	Positive
	Lactose	Positive
	Sucrose	Positive
	Mannitol	Positive
	Glycerol	Negative
7	Arginine hydrolysis	Positive
8	H ₂ S production	Negative
9	Yellow pigmented colonies in Nutrient agar	Positive
10	Growth in Nutrient agar with 7.5% NaCl	Positive
11	Growth in Nutrient agar at 45°C	Positive
12	Growth in Nutrient agar at 50°C	Negative
13	Growth in Bile esculin agar	Positive
14	Growth in Columbia blood agar	Positive

Evaluation of chromium tolerance

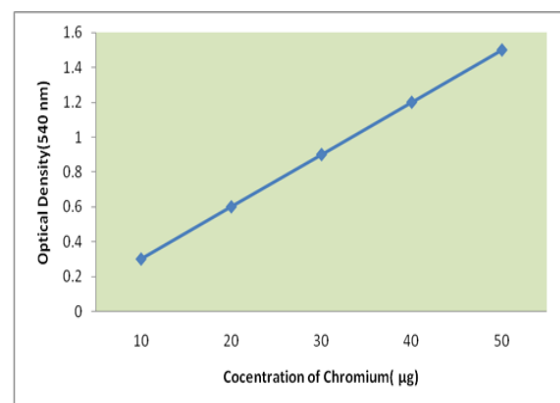
In Agar dilution method, the bacterial isolate (*Enterococcus casseliflavus*) was resistant to hexavalent chromium level at 800 µg/ml. Above 800 µg/ml of chromium, the growth was inhibited. In Broth dilution method, the bacterial isolate (*Enterococcus casseliflavus*) was resistant to hexavalent chromium level at 512 µg/ml. Growth was inhibited at above 512 µg/ml of hexavalent chromium level.

In this work, chromium resistant *Enterococcus casseliflavus* isolated from effluent of tanneries could resist upto 800 µg/ml of chromium by agar dilution method. *Enterococcus casseliflavus* has showed minimal inhibitory concentration upto 512 µg/ml of chromium by broth dilution method. Shahida Hasnain *et al.*, (2007) found that *Brevibacterium* sp. showed high level – resistance of chromium upto 40 mg/ml by Agar dilution method. Thiruneelakantan Srinath *et al.*, (2001). Showed that several facultative anaerobes namely *Aeromonas* and *Micrococcus* has minimal inhibitory concentration of >400 µg/ml in the medium. Megharaj and Naidu, (2003) examined that *Bacillus* sp. tolerated hexavalent chromium at 100 mg/ml on a minimal medium supplemented with 0.5% glucose.

Bioadsorption studies

In this study, the standard chromium was estimated by Diphenyl carbazide method and the results were shown in Fig.1.

Figure-1. Estimation of standard chromium

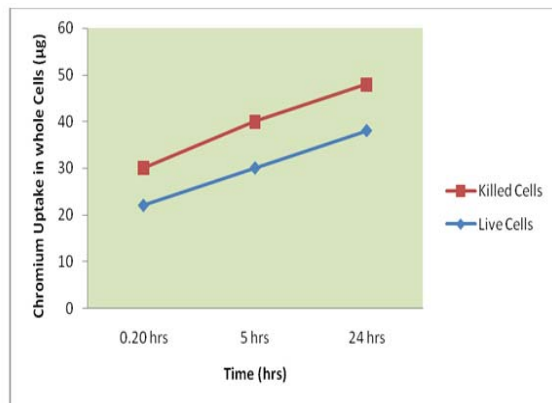


Bioadsorption studies were done to test the ability of cells (both live and dead cells) to accumulate chromium at different time intervals 15 minutes, 5 and 24 hours. The results were shown in Figure-2. The living cells accumulated 22, 30 and 38 µg/ml of chromium and the killed cells accumulated 8, 10 and 10 µg/ml of chromium. The rate of chromium accumulation was rapid. It showed that the living cells exhibited better chromium uptake compared to heat killed cells. The ability of *Enterococcus casseliflavus* to reduce chromium at different time intervals 15, 30 and 60 minutes was 19, 18 and 18 µg/ml of chromium.

Enterococcus casseliflavus has not shown significant chromium reduction in the supernatants.

Kader et al., (2007) showed that the rate of chromium accumulation by active cells was also faster compared to chromium reduction. The maximum amount of chromium accumulated by consortia was 6.4 mg/L and the reduction of chromium was 1.8 mg/L. Shahida Hasnain et al., (2007) found that *Brevibacterium* sp. living cells pellet showed higher chromium uptake than killed cells pellet when time intervals increased.

Figure-2. Estimation of Chromium Uptake

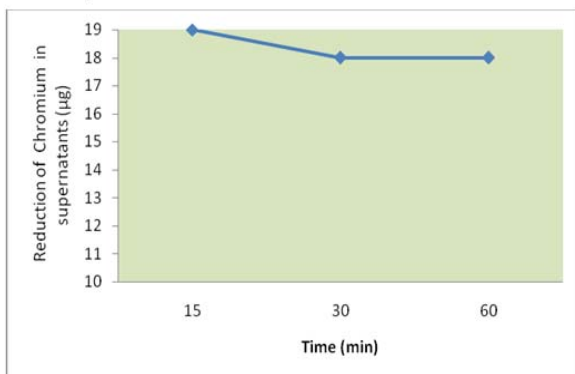


Chromium reduction

The ability of *Enterococcus casseliflavus* to reduce chromium at different time intervals 15, 30 and 60 minutes was 19, 18 and 18 µg/ml of chromium and the chromium reduction is shown in Fig-3. *Enterococcus casseliflavus* has not shown significant chromium reduction in the supernatants.

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Figure-3. Estimation of Chromium reduction

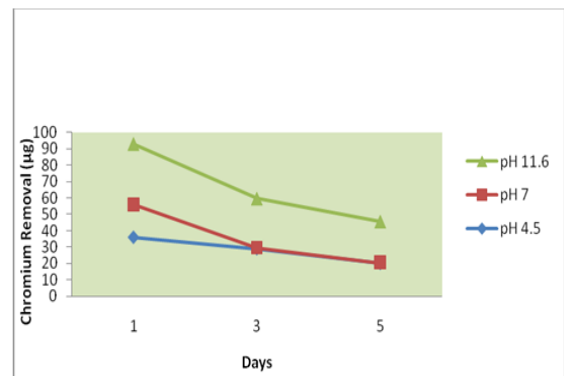


Effect of pH on chromium removal

In this study, the pH of the effluent was brought down from 11.6 to 7 and the removal of chromium content level by *Enterococcus casseliflavus* was high within 5 days at pH 7 than pH 4.5 and pH 11.6 in tannery effluents and the results were shown in Fig-4.

Laxman and More ,(2002) identified that maximum chromium reduction occurred by *Streptomyces griseus* in pH range of 6 - 7. Vidal et al., (2004) reported that hexavalent chromium reduction by *Enterobacter cloacae* occurred at pH 6.5 to 8.5.

Figure-4. Effect of pH on chromium removal

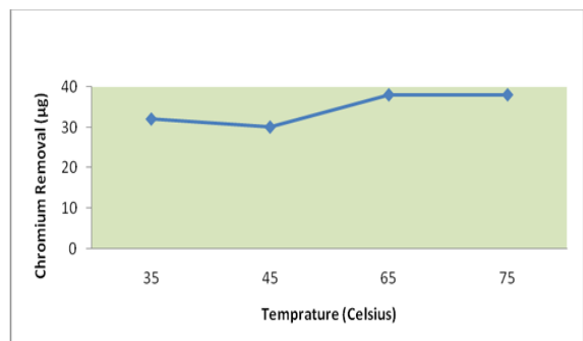


Effect of temperature on chromium removal

The chromium uptake was higher at temperature range between 35°C to 45°C and the results were shown in Fig-5. Temperature is an important factor for bacterial growth and will affect enzymatic reactions necessary for hexavalent chromium reduction.

Laxman and More, (2002) found that effect of temperature on chromium reduction occurred by *Streptomyces griseus* was higher at 37° and 50°C than at 28°C upto 24 hours but after 48 hours reduction was maximum at 28°C. Camargo et al., (2003) observed that maximum chromium reduction occur at 30°C by *Bacillus* sp.

Figure-5. Effect of temperature on chromium removal



Physico-chemical analysis of tannery effluent

Physico-chemical analysis of tannery effluent was analyzed before and after treatment. After the inoculation of *Enterococcus casseliflavus* in tannery effluents, the pH of the tannery effluents was brought down from 11.6 to 7. The BOD mg/lit of the tannery effluents was reduced from 8000 to 6400. The COD mg/lit of the tannery effluents was reduced from 5000 to 2500. Chromium content level was reduced from 38 µg- 25 µg/ml.

Duangporn Kantachote et al., (2005) identified the reduction of biological oxygen demand in tannery effluents from 4967 mg/L to 1010 mg/ml after inoculation of *Rhodospseudomonas blastica*. He also found the reduction of chemical oxygen demand from 7328 mg/L to 3371 mg/L in tannery effluents.

4. Conclusion

The chromium uptake from tannery effluents was significantly found in live pellet cells than killed pellet cells of *Enterococcus casseliflavus*. Irrespective of the pH of the the tannery effluent, *Enterococcus casseliflavus* has shown significant chromium adsorption by bringing down the pH of tannery effluent to neutral pH 7. Application of traditional wastewater treatment requires enormous cost and continuous input of chemicals which becomes uneconomical and causes further environmental damage. Hence easy effective economical and eco-friendly techniques such as utilization of bacteria can be applied for fine tuning of wastewater treatment.

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