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Bioadsorption of Chromium by *Penicillium chrysogenum* and *Aspergillus niger* Isolated from Tannery Effluent

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Abstract: In the present study, fungal strains were isolated from tannery effluent which showed the chromium resistant activity and were identified as *Penicillium chrysogenum* and *Aspergillus niger*. *Penicillium chrysogenum* showed very high level chromium resistance level of 800 µg/ml by Agar dilution method when compared to *Aspergillus niger*. The minimal inhibitory concentration of chromium was found to be 512 µg/ml of potassium dichromate in Sabouraud's dextrose broth medium. The chromium uptake was significantly high in live pellet cells than killed pellet cells at 540 nm in different time intervals. Removal of chromium by *Penicillium chrysogenum* in tannery effluents was observed high when compared to *Aspergillus niger*.

Key words: Tannery Effluent · Chromium · *Penicillium chrysogenum* And *Aspergillus niger*

INTRODUCTION

Tanning industries worldwide generate approximately 40 million of waste containing chromium (Cr) every year [1]. With inadequate regulatory guidelines, wastes are largely disposed on land and water bodies throughout the world. Studies show that tannery waste disposals have led to severe contamination of productive agricultural land in Bangladesh [2], India [3] and in Australia [4]. Chromium is frequently one of the more toxic elements present in tannery waste. High chromium concentrations ranging from 1 to 50g/kg were reported in soils surrounding tannery waste disposal sites in India, with hexavalent chromium present in groundwater at these sites [5].

Chromium and nickel are released into the environment by a large number of processes such as electroplating, leather tanning, wood preservation, pulp processing, steel manufacturing, etc. and the concentration levels of chromium and nickel in the environment widely varies. These two metals are of major concern because of their large usages in developing countries and their non degradability nature. Hexavalent chromium is highly soluble in water and carcinogenic to human. Ni(II) is more toxic and carcinogenic metal when compared with Ni(IV). Due to their toxic effects on living systems stringent limits have been stipulated for the discharge of chromium and nickel into the environment. According to ISI: Bureau of Indian Standard (BIS) the industrial effluent permissible discharge level of Cr(VI) and Ni(II) into inland water is 0.1 and 3.0 mg L^{-1}, respectively [4, 5].

Elimination of heavy metals from industrial wastewater is important for preserving the quality of aquatic systems, streams and ground waters. Contaminated waters are generally cleaned by precipitation of a metallic oxyhydroxide sludge or by ion exchange with synthetic resins. Various types of nonliving biomass, bacteria, filamentous fungi, algae and higher plants can be profitably used in alternative metal removal processes because of their low cost and the high ion exchange capacity of cell walls [6].

Filamentous fungi can be profitably used in processes for heavy metals removal from wastewater due to their low cost and the high ion exchange capacity of their cell walls. This property arises from the large density of functional groups present in the cell wall (carboxyl, hydroxyl, amine, phosphoryl, sulfhydryl), creating a negatively charged surface [7]. The sorption properties of cell have been widely studied by solution chemistry [8], but the chemical nature of complexing groups is not known. In the present work, the nature of Pb binding sites

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on the cell walls of the filamentous fungus *Penicillium chrysogenum* was investigated at the macroscopic level by sorption isotherm and at the molecular level by extended X-ray absorption free structure (EXAFS) spectroscopy by varying the metal concentration by two orders of magnitude, down to 4.8 10 .3 m mol Ph/g.

Bio sorption by fungi as an alternative treatment option for wastewater containing heavy metal has been reviewed by Kapoor and Viraghavan [9] and Modak *et al*. [10]. Any fungi can tolerate high concentration of potentially toxic metals and with other microbes; this may be correlated with decreased intracellular uptake or impermeability. A close relation between toxicity and intracellular uptake has been shown for Cu^{2+}, Cd^{2+}, Co^{2+} and Zn^{2+} in yeast *Saccharomyces cerevisiae* [11].

The cell walls of the filamentous fungus *Penicillium chrysogenum*, a byproduct of the pharmaceutical industry, are examined for their Zn and Pb complexing capability. The elevated sorption rate of the cell wall, with respect to a mineral surface for example, arises from its composite structure. The skeleton of *Penicillium chrysogenum* is mostly composed of chitin and glucan chains, which are cemented by proteins, lipids, pigments and other polysaccharides. Therefore, a large variety of functional groups exist (including carboxyl, hydroxyl, phosphoryl, amine and sulfhydryl groups) creating a negatively charged surface. However, these are all amphoteric, so the pH regulates the charge [12].

The identity of Zn and Pb complexing sites of *Penicillium chrysogenum* cell walls was investigated by varying the metal concentration by 3 (Zn) and 2 (Pb) orders of magnitude, from the micromolar to millimolar range. Chromium, a priority pollutant is well known for its mutagenicity [13], carcinogenicity [14] and teratogenicity [15] in humans, experimental animals [16] and plants [17]. 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 [18, 19] 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 [20, 21]. Accordingly, chromium and its compounds are placed on the priority list of toxic chemicals by US EPA [22]. 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 render it hazardous even at very low concentration.

MATERIALS AND METHODS

Sample Collection: Tannery effluents were collected from the Leather industry at Ambur in Vellore district. The sample was collected after the primary treatment in Leather industry.

Isolation of Chromium Resistant *Penicillium chrysogenum* and *Aspergillus Niger* from Tannery Effluent: The tannery effluent sample was taken and diluted in water by serial dilution technique followed by pour plate method. The isolated colonies were sub-cultured in Minimal salt agar medium supplemented with hexavalent chromium 100µ g/ml (hexavalent chromium was supplemented in the form of potassium dichromate) and also in Sabouraud's Dextrose Agar with potassium dichromate medium 50 mg/100 ml and incubated at room temperature for 3 days. Well grown fungal colonies were maintained on Sabouraud dextrose agar slants and stored at 4°C.

Identification of Chromium Resistant *Penicillium Chrysogenum* and *Aspergillus Niger* from Tannery Effluent: Identification of the fungal isolates was carried out by the routine mycological methods i.e.

- By Lactophenol cotton blue staining
- Plating on Sabouraud's dextrose agar medium.

Evaluation of Chromium Tolerance: The isolated fungal isolates (*Penicillium chrysogenum* and *Aspergillus niger*) were tested for their resistance to chromate both by Agar dilution method and Broth dilution method.

Agar Dilution Method: The test organisms were inoculating into Sabouraud's dextrose broth and incubated at room temperature in a shaker incubator at 150 rpm till the concentration of the test organisms matched with 0.5 McFarland standards. In this method, *Penicillium chrysogenum* and *Aspergillus niger* were aseptically streaked into freshly prepared Sabouraud's dextrose agar plates amended with hexavalent chromium as dichromate at various concentration ranging from 100 – 1500 µg/ml. Plates were then incubated at room temperature for 3 days.

Broth Dilution Method: The test organisms were inoculated into Sabouraud's dextrose broth and incubated at room temperature in a shaker incubator at 150 rpm till the concentration of the test organisms matched with 0.5 McFarland standards. A stock solution of chromium was prepared (50 mg/100 ml). Two fold dilutions of this solution were prepared in Sabouraud's dextrose broth. A standard suspension of *Penicillium chrysogenum* and *Aspergillus niger* was inoculated onto the medium with one control chromium solution free medium. The inoculated medium was then incubated at room temperature for 3 days.

Bio Adsorption 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. 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 540 nm in colorimeter.

Chromium Uptake: The 24 hours old cultures of *Penicillium chrysogenum* and *Aspergillus niger* was prepared in Sabouraud's dextrose broth. To 50 ml of Sabouraud's dextrose 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 3 days 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 *Penicillium chrysogenum* and *Aspergillus niger* in Sabouraud's dextrose 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 1ml of supernatant, 1ml of phosphate buffer (pH 7.2) with chromium was added and incubated for 1 hour. After incubation, 1 drop of 0.1M 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 Effluent: 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 1N HCl and 1N NaOH, then sterilized it. Then *Penicillium chrysogenum* and *Aspergillus niger* were 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 Effluent: The influence of temperature on chromium removal was assessed with the tannery effluent. The cultures (*Penicillium chrysogenum* and *Aspergillus niger*) 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.

RESULTS

Isolation and Identification of Chromium Resistant *Penicillium Chrysogenum* and *Aspergillus Niger* from Tannery Effluent

Penicillium Chrysogenum

Microscopic Examination: Septate hyphae with branched or unbranched conidiophores which have secondary branches known as medulla were detected. On the medulla flask shaped sterigmata were arranged that beard unbranched chains of round conidia. Entire structure formed a brush border.

Colony Morphology on SDA Plate: Colony surface at first appeared white then became powdery bluish green with a white border. Some species differed in gross appearance. Reverse side was white.

From these above results, the fungal isolate was identified as *Penicillium chrysogenum*.

Aspergillus Niger

Microscopic Examination: Conidiophore stipes smooth-walled, hyaline or pigmented was detected. Vesicles sub-spherical, conidial heads radiate were seen. Conidiogenous cells biseriate were also seen. Medulla appeared twice as long as the phialides. Conidia brown, ornamented with warts and ridges were shown. Hyphae were septate.

Colony Morphology on SDA Plate: Colonies were black, consisting of a dense felt of conidiophores.

From these above results, the fungal isolate was identified as *Aspergillus niger*.

Evaluation of Chromium Tolerance: In Agar dilution method, the bacterial isolate (*Penicillium chrysogenum* and *Aspergillus niger*) 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 (*Penicillium chrysogenum* and *Aspergillus niger*) was resistant to hexavalent chromium level at 512 µg/ml. Growth was inhibited above 512 µg/ml of hexavalent chromium level.

Bioadsorption Studies: In this study, the standard chromium was estimated by Diphenyl carbazide method and the results were shown in Table -1.

Chromium Uptake: 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 Table-2 and Table -3. 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 *Penicillium chrysogenum* and *Aspergillus niger* to reduce chromium at different time intervals 15, 30 and 60 minutes was 19, 18 and 17 µg/ml of chromium. The isolates *Penicillium chrysogenum* and *Aspergillus niger* has not shown significant chromium reduction in the supernatants.

Chromium Reduction: The ability of *Penicillium chrysogenum* and *Aspergillus niger* to reduce chromium at different time intervals 15, 30 and 60 minutes was 19, 18 and18 µg/ml of chromium and the chromium reduction is shown in Table – 4 and Table-5. *Penicillium chrysogenum* and *Aspergillus niger* has not shown significant chromium reduction in the supernatants.

Table 1: Estimation of Standard Chromium (Diphenyl Carbazide Method)

Concentration of Chromium (in µg)	Optical Density (in nm)
10	0.30
20	0.60
30	0.90
40	1.20
50	1.50

Table 2: Estimation of chromium uptake *Penicillium chrysogenum*

	Chromium uptake in whole cells (µg)	
Time (in hours)	Live pellet	Killed pellet
15 minutes	22	8
5	30	10
24	38	10

Table 3: Estimation of chromium uptake by *Aspergillus niger*

	Chromium uptake in whole cells (µg)	
Time (in hours)	Live pellet	Killed pellet
15 minutes	17	3
5	25	5
24	33	5

Table 4: Estimation of chromium reduction by *Penicillium chrysogenum*

Time (Minutes)	Reduction of Chromium Level in Supernatant (µg)
15	19
30	18
60	18

Table 5: Estimation of chromium reduction by *Aspergillus niger*

Time (Minutes)	Reduction of Chromium Level in Supernatant (µg)
15	14
30	13
60	13

Table 6: Effect of pH on chromium removal by *Penicillium chrysogenum*

	Chromium Removal (µg)		
Days	pH 4.5	pH 7	pH 11.6
1	36	20	37
3	30	0.5	30
5	20	0.4	25

Effect of pH on Chromium Removal: The pH of the effluent was brought down from 11.6 to 7 and the removal of chromium content level by *Penicillium chrysogenum* and *Aspergillus niger* 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 Table-6 and Table-7.

Table 7: Effect of pH on chromium removal by *Aspergillus niger*

	Chromium Removal (µg)		
Days	pH 4.5	pH 7	pH 11.6
1	35	15	32
3	25	0.3	25
5	15	0.2	20

Table 8: Effect of temperature on chromium removal by *Penicillium chrysogenum*

Temperature (°c)	Chromium Removal (µg)
35	32
45	30
65	38
75	38

Table 9: Effect of temperature on chromium removal by *Aspergillus niger*

Temperature (°c)	Chromium Removal (µg)
35	27
45	25
65	33
75	33

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 Table-8 and Table-9 Temperature is an important factor for bacterial growth and will affect enzymatic reactions necessary for hexavalent chromium reduction.

DISCUSSION

Biosorption is an innovative technology aimed at the removal of toxic metals from polluted streams by using inactive and dead biomasses. Metals entrapment is due to chemico-physical interactions with active groups present on the cell wall: carboxylic, phosphate, sulfate, amino, amide and hydroxyl groups are the most commonly found, according to the biosorbent nature [23]. Considering its mechanism, biosorption is affected by several factors such as pH, simultaneous presence of other metals, kind of biosorbent material. In any case, the development of a model in agreement with experimental data is fundamental in order to simulate and predict biosorption courses.

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 bio-sorption, 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.

Biotechnological exploitation of biosorption technology for removal of heavy metals depends on the efficiency of the regeneration of biosorbent after metal desorption. Therefore non-destructive recovery by mild and cheap desorbing agents is desirable for regeneration of biomass for use in multiple cycles [24].

Living and dead cells of fungi are able to remove heavy metals ions from aqueous solutions [25] and the use of dead cells seems to be more advantages than using living cells [26]. In multi components systems (biosorbents), biosorption of heavy metal ions depends not only on the specific surface properties of the biomass and physicochemical parameters of the solution, say, temperature, pH, initial metal-ion concentration and biomass concentration, but also on features of those components, as well as, the cultural conditions of the organisms. The efficiency of biosorption can be increased by different physical and chemical pretreatments of the microbial biomass [27, 28].

In this present study, a chromium resistant bacterium was isolated from tannery effluents in Minimal salt agar medium supplemented with potassium dichromate. This was identified as *Penicillium chrysogenum* and *Aspergillus niger* using Lacto phenol cotton blue staining and plating on Sabouraud's Dextrose agar.

Faisal and Hasnain [29] and Thacker and Parikh [30] isolated chromium resistant bacteria from tannery effluents which were identified as *Brevibacterium* sp. and *Brucella* sp. respectively. In this work, chromium resistant *Penicillium chrysogenum* and *Aspergillus niger* isolated from effluent of tanneries could resist upto 800 µg/ml of chromium by agar dilution method. *Penicillium*

chrysogenum and *Aspergillus niger* has showed minimal inhibitory concentration upto 512 µg/ml of chromium by broth dilution method.

Shahida Hasnain *et al*. [31] found that *Brevibacterium* sp. showed high level – resistance of chromium upto 40 mg/ml by agar dilution method. Thiruneelakantan Srinath *et al*. [32]. Showed that several facultative anaerobes namely *Aeromonas* and *Micrococcus* has minimal inhibitory concentration of >400 µg/ml in the medium. Meghara *et al.* [33] examined that *Bacillus* sp. tolerated hexavalent chromium at 100 mg/ml on a minimal medium supplemented with 0.5% glucose.

In this study, bio adsorption studies were done by using *Penicillium chrysogenum* and *Aspergillus niger* 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 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 *Penicillium chrysogenum* and *Aspergillus niger* to reduce chromium at different time intervals 15, 30 and 60 minutes was 19, 18 and 18 µg/ml of chromium. *Penicillium chrysogenum* and *Aspergillus niger* did not show significant chromium reduction in the supernatants. Kapoor *et al.* [34] showed that the rate of chromium accumulation by active cells is 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.

Luef *et al*. [35] found that *Brevibacterium* sp. living cells pellet showed higher chromium uptake than killed cells pellet when time intervals increased. In this study, the pH of the effluent was brought down from 11.6 to 7 and the chromium uptake was significantly high within 5 days at pH 7. Also, the chromium uptake was higher at temperature range between 35°C to 45°C. Temperature is an important factor for bacterial growth and will affect enzymatic reactions necessary for hexavalent chromium reduction.

Kapoor *et al*. [36] identified that maximum chromium reduction occurred by *Streptomyces griseus* in pH range of 6 – 7. Wang *et al*., (1990) reported that hexavalent chromium reduction by *Enterobacter cloacae* occurred at pH 6.5 to 8.5. Juliette Lamberta and Mohammed Rakib [37] found that effect of temperature on chromium reduction occurred by *Streptomyces griseus* was higher at 37°C and 50°C than at 28°C upto 24 hours but after 48 hours reduction was maximum at 28°C. Bento *et al*. (2003) observed that maximum chromium reduction occur at 30°C by *Bacillus* sp.

Saranraj *et al*. [38] isolated a bacterial strain 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.

The chromium uptake from tannery effluents was found in live pellet cells than killed pellet cells of *Penicillium chrysogenum*. Irrespective of the pH of the tannery effluent, *Penicillium chrysogenum* has shown significant chromium adsorption by bringing down the pH of tannery effluent to neutral pH 7 when compared to *Aspergillus niger*. 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 ecofriendly techniques such as utilization of bacteria can be applied for fine tuning of wastewater treatment.

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