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IN VITRO REDUCTION OF HEXAVALENT CHROMIUM IN TANNERY EFFLUENT

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ABSTRACT
Throughout the last century, heavy metal contamination of the aqueous environment has gained much attention due to its significant potential health impact on the public. More than 1,70, 000 tonnes of Chromium waste were released annually in to the ecosystem. Chromium is one of the most toxic substances that have been released into the environment; it has become a serious health concern. So there is a need to develop new cost effective eco friendly method to combat the pollution due to Chromium. Certain types of microbes can retain or reduce relatively high quantities of metals by passive process known as bio sorption, bio reduction respectively. Ten predominant cultures of bacteria were isolated from the tannery effluent and three were found to be promising. The isolated strain showed a maximum reduction of Chromium VI (99 %), where the initial concentration of Cr VI was 100 mg/ l. The chromium reductase activity was assayed in cell filtrates as well as in the periplasmic proteins. Protein analysis of cell free extract from 100 mg spiked Chromium showed 27 KD, 22 KD proteins. Upon Chromium stress flavoproteins were produced in abnormal amounts by which the organism is able to reduce the hexavalent Chromium into trivalent form.

KEY WORDS: Tannery Effluent – Invitro reduction- Chromium- Bioremediation.

INTRODUCTION

In developing countries, industrial effluents are released directly or indirectly into the natural water resources without proper treatment, thus posing a serious threat to the environment (Shakoori *et al.,* 2000). Environmental pollution due to Chromium and its compounds is widespread because of their vast applications in industries. Tannery industries uses chromium compounds in the tanning process and produces spent chromium laden wastes in to the nearby places. The tanneries are considered as highly water intensive industries. Leather processing in a tannery generally comprises three categories: pretreatment of skin/hide (beam house operations), chrome or vegetable tanning of skin/hide (tanning operation) and finishing operations (Thanikaivelan *et al.,* 2004). Nearly 30 m^3 of wastewater is generated during processing of one tonne of raw skin/hide (Suthanthararajan *et al.,* 2004). These wastewater contain large quantities of chemical oxygen demand (COD), color, sodium sulphide, nitrate, chloride, chromium and suspended solids (SS) (Sharma and Malaviya, 2014), which hamper light penetration and decreases dissolved oxygen in the aquatic ecosystem. The release of Chromium becomes a serious health concern due to the fact that it is one of the most toxic substances that have been released into the environment (Gardea Torresdey *et al.,* 2000). The US Environmental protection agency designated Chromium as well as its compounds as one of the seventeen chemicals posing the greatest threat to human health (Marsh and McInerney, 2001). Chromium exists in several oxidation states (I–VI), more stable as Cr (III). Cr (VI) is the toxic form of the element which causes severe diarrhoea, ulcers, eye and skin irritation, kidney dysfunction and probably lung carcinoma (Malaviya and Singh, 2011). Soluble hexavalent Chromium species are extremely toxic and causes not only mutational effect, but also causes carcinogenic effects on Biological systems due to their strong oxidizing nature (Jeef Mclean and Beveridge, 2001 and Camargo *et al.,* 2003). At global level, chromium is known to be one of the inorganic contaminants polluting groundwater (Garg *et al.,* 2012).

Conventional methods for removing Chromium include reduction followed by precipitation, ion exchange and adsorption on coal, activated carbon, alum and fly ash, soil washing, soil flushing, solidification and stabilization etc. These methods requires high energy and not economical, hence not suitable for full scale remediation (Avudainayagam *et al.,* 2003). So there is a need to develop new cost effective eco friendly method to combat the pollution due to Chromium. Bioremediation uses living organisms particularly microorganisms to remove contaminants from the environment and transforming them into non toxic forms. The major advantages of bioremediation include low cost, minimized chemical usage and minimized sludge production, recycling of cell biomass and recovery of pollutants. Certain types of microbes can retain or reduce relatively high quantities of metals by passive process known as biosorbtion, bioreduction

respectively. In most contaminated sites biosorption is employed. However biosorption methods depend on efficiency of the free cells. Hence, an attempt was made to study the reduction of Chromium by microbial process, and stimulating the microbial growth through adjustment of pH, addition of nutrients and temperature.

MATERIALS & METHODS

Collection of samples

The tannery waste water and tannery effluent contaminated soils were collected from a leather processing small scale industry near Erode, Tamil Nadu. The combined effluent samples from processing units were collected and stored in cold room for further analyses. The samples were serially diluted upto 10^{-6} as per the method given by Camargo *et al*., 2003 and spread plated on Luria Bertani agar media and incubated at 37^0C. The colonies grown were isolated and purified by streak plate method.

Screening for Chromium tolerance

The isolated colonies were screened based on colony morphology, gram reaction, cell morphology, pigment production, oxidase test and gelatin hydrolysis (Gregory Luli *et al*., 1983). The chosen isolates were analyzed for its ability to grow in the presence of chromate, which is normally a toxic substance. Thus the isolated members were further grown in LB Agar media spiked with various concentration of Chromium to check the minimal inhibitory concentration (MIC). LB Agar media was prepared, sterilized and each flask were added with different concentrations of filter sterilized potassium dichromate ranging from 100 mg/l (100ppm) to 3g/l (3000ppm) and poured into the plates. The solidified media with Chromium was inoculated with chosen cultures by streak plate method. Then the inoculated plates were incubated at 37°C along with the control plates without Chromium. The growth was checked after 24 hrs, 48 hrs and 72 hrs.

Optimization of growth conditions for Chromium tolerance.

The chosen isolates were inoculated in sterile broth in the presence of varying concentrations of chromate, ranging from 100mg/l (100ppm) to 3g/l (3000ppm). The inoculated samples were kept in the incubator at 37^0C with shaking for 18 hrs. The turbidity was measured at 600 nm using spectrophotometer (Systronics) in all the flasks (Ganguli and Tripathi *et al*., 2002).

Effect of pH and temperature

The Chromium tolerant isolates were grown for pH and temperature optimization. The pH range chosen was 5, 6, 7, 8, 9 and 10 and the temperature range was 30°C, 35°C, 37°C and 42°C. The isolate TS4 was inoculated in the LB broth adjusted to this varied pH. The inoculated broth was then incubated at 37°C for pH and at chosen temperature for temperature optimization with shaking for 24hrs and turbidity was measured at 600 nm (Komori *et al.,* 1989).

Chromium reduction assay

After incubation, the cultures were centrifuged at 40°C for 10 min at 10,000 rpm and the remaining unutilized Cr (VI) was determined by the spectrophotometric method (Camargo *et al.,* 2003). The residual Chromium was assessed in cell free extracts (Pattanapipitpaisal *et al.,* 2001) and resting cells (Meharaj *et al*., 2003). The percent reduction was calculated by using the formula described by Murugesan and Vasanthy, 1996.

$$\% \text{ Reduction} = \frac{I_1 \quad a \quad - F \quad a}{\text{Initial absorbance}} x\ 100$$

The cell free extract as well as the bacterial pellet were used for the biochemical characterization of the Chromium reduction mechanism. The cell free extract subjected to dialysis, protein estimation, lyophilisation and SDS – PAGE (Ackerley *et al*., 2004)

RESULTS

Tannery effluent and effluent contaminated soils were collected from a leather processing industry and used for the isolation of microbes capable of reducing chromium. About 10 predominant cultures were isolated from both the samples which are contaminated with Chromium. The isolated cultures were subjected to several screening process to identify organisms as well as to stimulate the growth by optimizing the growth conditions (Table 1)

TABLE 1.Isolation of microbes from tannery effluent and effluent contaminated soil

Characteristics	Isolates									
	TS 1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	TS9	TS10
Gram Reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Motility	+	+	+	+	+	+	+	+	+	+
Pigment Production	++	+	+	+++	+	+	+	+	+	++
Oxidase	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+
Chromium tolerance (ppm)										
100	++	+	-	++	-	++	-	-	+	-
200	+	-	-	++	-	+	-	-	-	-

Among the isolated cultures, three were found to show tolerance to high chromium concentration, gram negative reaction, aerobic rods, motile and produced green water soluble pigments.

Chromium tolerance

The bacterial isolates were screened from the tannery effluent. The minimal inhibitory concentration was studied and the strain TS4 had shown maximum tolerance for Chromium up to 3000mg/l (Table 2). The growth of TS1 strain was inhibited after 500mg/l; whereas TS6 strain the growth was inhibited after 250mg/l concentration of Chromium. Thus for the optimization of the process the isolate TS4 was chosen.

TABLE 2 Chromium tolerance of different isolates

Culture	Minimal Inhibitory concentration of Chromium (in ppm)							
No	100	250	500	750	1000	1250	1500	3000
TS1	+	+	+	-	-	-	-	-
TS 4	+	+	+	+	+	+	+	+
TS 6	+	+	-	-	-	-	-	-

Effect of pH and temperature

In the present study, optimization of pH and temperature was done to obtain maximum tolerance and reduction to varied levels of Chromium. TS4 had shown maximum growth at pH 9 till 3000 mg /l of dichromate. There was completely no growth at pH 5 and 6; whereas in the case of pH 7 growth was slower and it was only upto 750mg/l of dichromate. Maximum growth was obtained only when the TS4 strain was incubated at 37°C and 42°C the growth was very slow (Figure 1 and 2).

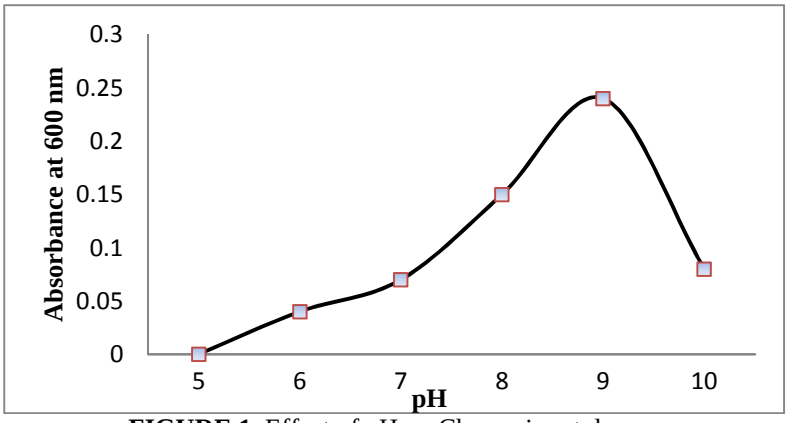

FIGURE 1. Effect of pH on Chromoium tolernce

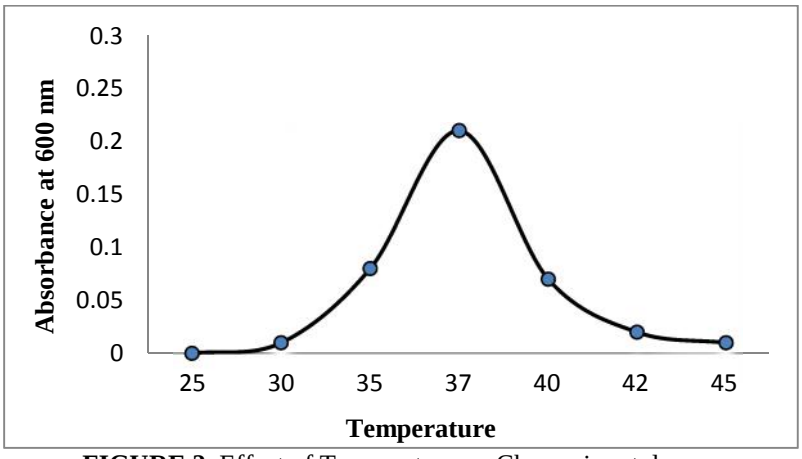

FIGURE 2. Effect of Temperature on Chromoium tolernce

Assay for Chromium reduction

The well grown TS4 cultures in LB broth spiked with different concentrations of Potassium Dichromate was used for the analysis of the residual Chromium. After 24 hrs, the cultures showed very little reduction whereas the reduction rate was remarkably high after 48 and 72 hrs (Table 3 and 4).

TABLE 3: Residual Chromium in the cell free extract

Initial Conc.of $K_2Cr_2O_7$	Optical Density before treatment	Optical Density after treatment	Per cent removal
100mg	2.199	0.008	99.63
250mg	2.292	0.025	98.90
500mg	2.293	0.155	93.21
750mg	2.299	0.264	88.51
1000mg	2.299	0.600	73.30
1250mg	2.299	1.230	46.07
1500mg	2.302	1.720	25.27
2000mg	2.032	1.840	19.77

The reduction percentage was high in the cell free extract of 72 hrs cultures, which was a remarkable result for the Chromium bioremediation, than the 24 hr and 48 hrs cultures. Assay of resting cells was done to prove the bacterial cells are the one participating in the Chromium reduction mechanism. In both resting cells and cell free extract the reduction was very much effective till 3000mg/l of $K_2Cr_2O_7$.

TABLE 4: Residual Chromium in the resting cell

Initial Conc.of $K_2Cr_2O_7$	Optical Density before treatment	Optical Density after treatment	Per cent removal
100mg	2.197	0.030	98.63
250mg	2.292	0.070	96.81
500mg	2.293	0.200	91.23
750mg	2.299	0.400	82.26
1000mg	2.299	0.800	64.88
1250mg	2.299	1.270	44.60
1500mg	2.299	1.860	18.90
2000mg	2.032	2.010	12.43

The TS4 inoculated LB broth spiked with Chromium always produced the green pigments in a terrific manner within 24 hrs, whereas in the Chromium less broth pigmentation was found only after a week. The samples were run in the 10% gel and the protein was compared with the maker protein to locate the dimeric flavoprotein. It was detected as 2 bands of 27 kd and 22 kd polypeptides.

DISCUSSION

About 10 predominant cultures were isolated from the tannery effluent and soil, contaminated with Chromium by spread plate method. The isolated cultures were subjected to several screening test to identify the organisms. Among the isolated cultures, three were found to produce green water soluble pigments. The bacterial isolates were screened from the tannery effluent with a view that the indigenous bacteria from contaminated environments may be minimizing the inhibitory effects of other compounds that may be present along with Cr (VI), because of their resistance power gained (Turick *et al.,* 1996). The minimal inhibitory concentration was studied and the strain TS4 had shown maximum tolerance for Chromium up to 3000mg/l. The growth of TS1 strain was inhibited after 500mg/l; whereas TS6 strain the growth was inhibited after 250mg/l concentration of Chromium. Thus for the optimization of the process the isolate TS4 was chosen.

In the present study, optimization of pH and temperature was done to obtain maximum tolerance and reduction to varied levels of Chromium. There was completely no growth at pH 5 and 6; whereas in the case of pH 7 growths was slower and it was only upto 750mg/l of dichromate. Maximum growth was obtained only when the TS4 strain was incubated at 37^{o}C and 42^{o}C the growth was very slow. Moreover the growth of TS4 strain was inhibited by the presence of even lower concentration of Chromium in theses temperatures. This was in accordance with the report of Komori *et al.,* 1989. This optimization was much helpful for the further treatment process because the chrome processed tannery effluent is having pH around 9.2 and also the optimal temperature is not a problem in a country like India. The results of the present study, suggested that controlling temperature would be critical for maintaining the bacterial process for chromate removal.

Assay for Chromium reduction

The well grown TS4 cultures in LB broth spiked with different concentrations of Potassium Dichromate was used for the analysis of the residual Chromium. The reduction percentage was high in the cell free extract of 72 hrs cultures, which was a remarkable result for the Chromium bioremediation, than the 24 hr and 48 hrs cultures. Assay of resting cells was done and in both resting cells and cell free extract the reduction was very much effective till 3000mg/l of $K_2Cr_2O_7$. The reduction was high for the concentration ranging from 100 mg/l to 1000mg/l, comparatively the reduction was less for the concentrations ranging from 1250 to 2000 mg/l. The result indicates a maximum reduction of Cr (VI), when the initial concentration of Cr (VI) was 100 mg/l (Gregory Luli *et al.,* 1983). It is important to note that the amount of Chromium in the tannery effluent is usually less than the amount of Chromium used in this study.

Chromium adversely affects the growth of microorganisms and interferes with nucleic acid synthesis (Shakoori *et al.,*

2000). Some bacteria have the ability to detoxify chromate and dichromate. Different types of chromate reductase found in different bacteria under free and immobilized conditions and their applications in removal of hexavalent chromium was reported by Thatoi *et al.*, 2014. Wang *et al.*, 1989, Henry and Ehrlich 1994, Pattanapipitpaisal *et al.*, 2001 and Ganguli *et al.*, 2002 reported many *Pseudomonas* sp. tolerant to toxic crómate. Jesus Camos *et al.*, 1995 isolated *Bacillus* strain tolerant to Chromium. Chromium reduction proceeds on the cell surface, outside the cell, directly through chromate reductase enzymes or indirectly through metabolic reduction. The uptake of chromium involves biosorption, followed by bioaccumulation. Choosing an appropriate strategy for Chromium is important to understand the key mechanisms involved in resistance and removal of chromium (Joutey *et al.*, 2015, Sivakumar, 2016).

The chromium reductase activity in the cell free extracts showed the Intracellular reduction of Cr (VI) (Batool *et al* 2012, Thatoi *et al*., 2014 and Joutey *et al.*, 2015). Ejechi and Akpomie (2016) reported that two-stage sequential treatment process may be of potential cost saving process for removal of chromium from industrial wastes. Sharma and Malaviya, 2016 reported that *Aspergillus flavus* SPFT2 isolated from untreated tannery effluent wastewater exhibited minimum inhibitory concentration (MIC) for Cr (VI) as 500 ppm. The TS4 inoculated LB broth spiked with Chromium always produced the green pigments in a terrific manner within 24 hrs; whereas in the Chromium less broth pigmentation was found only after a week. Ackerley *et al.*, (2004a) found out the soluble protein always linked with Chromium reduction. Thus in the present study, the preliminary characterization of the flavoprotein was done.

Throughout the growth of the isolate TS4, upon Chromium stress enormous amount of pigment production had been seen. Along with the pigment, precipitates were also dispersed in the media (Wang *et al*.,1990). Jeef Mclean and Beveridge, 2001 reported that bacteria are excellent nucleation sites and the Cr (III) formed is free to bind these sites. He also observed Chromium reductase activity was observed in cell filtrates as well as in the periplasmic proteins. In the present study the reductase activity has been found in both cell free extracts as well as in the resting cells of isolate TS4, so the study was extended to characterize the soluble proteins which functions as Chromium reductase in the *Pseudomonas* sp (Ackerely, 2004a). An indigenous chromium-reducing bacterial strain, Rb-2, isolated from a tannery water sample, was identified as *Ochrobactrum intermedium*, on the basis of 16S rRNA gene sequencing and the Intracellular reduction of Cr(VI) was proved by reductase assay using cell-free extract (Batool *et al.*, 2012 , Hora and Shetty, 2015)

So the present study confirms that upon Chromium stress flavoproteins were produced in abnormal amount by which the organism is able to reduce the hexavalent Chromium in to trivalent form. The isolate TS4 have developed a highly complex set of strategies to deal with toxic Chromium at high concentrations through enzymatic processes. By producing a soluble flavoprotien that can catalyze the reduction of Cr (VI) to Cr (III), it can detoxify Chromium contaminated environment, hence a better candidate for bioremediation strategy.

RECOMMENDATIONS

There is a possibility for getting microbial cultures capable of converting trivalent chromium to Heaxavalent, which doesn't cause any side effects. In the present study the isolate TS 04 showed maximum chromium reduction, which could be utilized commercially after substantial studies to determine the bioremediation potential.

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