

Bioremediation of Toxic Heavy Metal Chromium in Tannery Effluent Using Bacteria

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Abstract: Tanning industries worldwide generate approximately 40 million of waste containing chromium (Cr) every year. With inadequate regulatory guidelines, wastes were largely disposed on land and water bodies throughout the world. Cr (VI) is toxic, carcinogenic and mutagenic to animals as well as humans and is associated with decreased plant growth and changes in plant morphology. They cause physical discomfort and sometimes life-threatening illness including irreversible damage to vital body system. In this study three bacterial isolated from tannery effluent named as Z3, KS1 and KHL1. The three bacterial isolates optimized based on pH, Inoculum and temperature to calculate the chromium reduction. This study proved the chromium concentration reduced in tannery effluents using this bacterial isolates.

Key words: Tannery • Effluent • Heavy Metals and Bacteria

INTRODUCTION

Biodegradation is one of the biological processes facilitating the chemical changes of pollutants by microorganisms present in the polluted environment. Microorganisms are involved in the removal of toxic wastes, either in the environment or in controlled treatment systems. Tannery effluents are a major source of aquatic pollution in India with chemical oxygen demand (COD), biological oxygen demand (BOD) and hexavalent chromium. Nearly 80% of the tanneries in India are engaged in the chrome tanning process [1]. The tannery waste primarily consists of chromium and protein. Long term disposal of tannery wastes has resulted in extensive contamination of agricultural land and water sources in many parts of the India. There are more than 2500 tanneries in the country and nearly 80% of the tanneries are engaged in the chrome tanning process.

Chromium (Cr) is a transition metal present in group VI-B of the periodic table. Chromium is important metal due to its high corrosion, resistance and hardness. It is used extensively in manufacturing of stainless steel. Chromium contamination of the environment is of concern because of the mobility and toxicity of Cr (VI). Chromium, a transition metal, is one of the major sources of environmental pollution. It is discharged into the

environment through the disposal of wastes from industries like leather tanning, metallurgical and metal finishing, textiles and ceramics, pigment and wood preservatives, photographic sensitizer manufacturing etc. [2].

In 1977, the first reported bacterial strains, *Pseudomonas*, were isolated from chromate (CrO_4^{2-}) contaminated sewage sludge by Russian scientists N.A. Romanenko and V. Korenkov. Since 1977, several other CrO₄²⁻ reducing strains have been reported, including other strains such as *B. cereus*, *B. subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas ambigua*, *Pseudomonas fluorescens*, *E. coli*, *Achromobacter eurydice*, *Micrococcus roseus*, *Enterobacter cloacae*, *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* [3]. Jonnalagadda *et al.* [4] reported biological removal of carcinogenic chromium (VI) using mixed *Pseudomonas* strains. In this study an aerobic reduction of Cr (VI) to Cr (III) by employing mixed *Pseudomonas* cultures isolated from a marshy land has been reported.

MATERIALS AND METHODS

Sample Collection: Chromium tolerant microorganisms were collected from tannery effluents located in Ranipet, Vellore district, Tamil Nadu, India.

Isolation and Identification of Chromium Resistant

Organism: The tannery samples were serially diluted upto 10^{-7} in distilled water using sterile pipettes and plates in Cr free Nutrient agar plates. 0.1 ml of suspension was pipetted out and spread using L-rod into the Nutrient agar medium in sterile petriplates. The plates were incubated at 37°C for 24 hrs for the development of colonies. The colony characteristics were observed. The colonies were identified by Gram staining and based on biochemical characters such as Indole, Methyl red test, Voges-Proskauer test, Citrate utilization test, Urease test, Oxidase test and Catalase test.

Optimization of Inoculum Volume: It is essential to determine the volume of the inoculums in order to achieve maximum Cr (VI) reduction in liquid medium. To 21 conical flasks, 0.65 gram of nutrient broth is dissolved in 50 ml of distilled water. These conical flasks were sterilized in an autoclave at 121°C for 15 minutes at 15lbs pressure. After this 100 µl of $K_2Cr_2O_7$ is [70 mg/l of Cr (VI)] added to the conical flasks. To these flasks 1ml of the three isolates were added to three flasks individually. Then, the isolates were grown at various temperatures with hexavalent chromium (As $K_2Cr_2O_7$) at 25°C, 30°C and 37°C. To optimize the inoculums size, different volume of inoculums like 1, 2 and 3 ml of inoculums volume with fixed concentration of 70mg/l of Cr (VI) (as $K_2Cr_2O_7$). Controls experiments without organisms were also performed with same nutrient broth containing 70 mg/l concentration Cr (VI) as $K_2Cr_2O_7$ to ensure that removal was due to the microorganisms and not due to any other abiotic reasons or precipitation.

Measurement of Cell Density: After regular intervals of time, the cell density is measured to analyze the growth of microorganisms at different time and temperature respectively. After 24 hours the chromium inoculated cell culture and control is removed from the incubator. From the removed culture and control, 2ml was added to the Eppendorf tubes. Then, the cell density of the culture is measured by taking OD of the culture at 620 nm using a spectrophotometer. The same cell density was quantified at various time intervals of 24, 48, 72, 96, 120 and 144 hours respectively.

Diphenylcarbazide Test: In the test, there is an organo metallic reaction between the chromium ions and diphenylcarbazide. This leads to the formation of intense colored complex. The complex is measured quantitatively by its visible absorbance at 540 nm. After the measurement of cell density at 540 nm, the microbial cell

culture in Eppendorf is kept in a centrifuge at 8000 rpm for 15 minutes. After centrifugation 200 µl of the supernatant was added to standard flask. About half of the flask is filled with distilled water for dilution of the sample. 250 µl of the ortho-phosphoric acid was added after the addition of distilled water in the standard flask. Then, distilled water was added to the marked level. After the addition of distilled water 2 ml of diphenylcarbazide was added and mixed well. This leads to the formation of red-violet color due to organo metallic reaction between the Cr (VI) and diphenylcarbazide. Then, the concentration of chromium is determined quantitatively by taking OD at 540 nm. The same procedure for the estimation of chromium is done at regular time intervals of 48, 72, 96, 120 and 144 hours, respectively.

Optimization of Temperature: After the isolation of microorganisms they are grown at various temperatures with $K_2Cr_2O_7$ at 27°C, 30°C and 37°C to optimize the temperature. To 21 conical flasks, 0.65 g of nutrient broth is dissolved in 50ml of distilled water These conical flasks were sterilized in an autoclave at 121°C for 15 minutes at 15lbs pressure. After this 100 µl of $K_2Cr_2O_7$ is [70mg/l of Cr (VI)] added to the conical flasks. To these flasks 3 ml of the three isolates were added to three flasks individually. Then, they were incubated at three different temperatures 27°C, 30°C and 37°C under continuous shaking.

Optimization of pH: After the optimization of temperature, time and pH is optimized. Nutrient broth containing 0.65 gram of nutrient broth and 50 ml of distilled water was prepared. 0.1N of NaOH and HCl were added in various concentrations to attain various pH of 5, 7 and 9. The pH was determined by using various strips to check the pH. After getting appropriate pH they were autoclaved. 70 mg/l concentration of hexavalent chromium (As $K_2Cr_2O_7$) and 3 ml culture of Z3, KS1 and KHL2 was added to conical flasks containing nutrient broth. Then, they were incubated in a 37°C and 30°C shaker. Cell density was quantified at various time intervals of 24, 48, 72, 96, 120 and 144 hours, respectively. After the centrifugation of sample, the concentration of chromium is estimated quantitatively by diphenylcarbazide test.

Effect of Medium: The effect of different medium sources for the degradation of Cr (VI) was studied by supplementing the medium. Z3, KS1 and KHL2 culture were inoculated in respective broth at the optimized concentrations of $K_2Cr_2O_7$ and kept for incubation at respective temperature. Cell density and Cr (VI) reduction were estimated.

Minimum Inhibitory Concentration: MIC in microbiology is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Plates containing nutrient agar medium supplemented with different concentrations (25 and 50µl) of K₂Cr₂O₇. Then, the plates were inoculated with 50 µl of a fresh overnight culture grown in nutrient broth medium. All plates were incubated with shaking at 37°C for 24 hours. The growth of bacteria was monitored by colony counting method.

Antibiotic Disk Sensitivity Test: To determine the antibiotic sensitivity of the chromate resistant bacterial strain to 6 different antibiotics-Penicillin-G (10 unit), Methicillin (5 mcg), Vancomycin (30 mcg) Oxacillin (1 mcg), Erthromycin (15 mcg) and Ampicillin (10 mcg). Muller Hinton Agar was prepared and boiled until the entire agar was melted. About 20 ml of molten agar was transferred to each sterilized petridishes. Antibiotic-impregnated discs were placed on the freshly prepared agar plates and incubated at 37°C for 24 hours. The diameter of the inhibition zones was measured to the nearest cm and the isolate is classified as resistant, intermediate and susceptible following the standard antibiotic disc sensitivity testing method.

RESULTS AND DISCUSSION

The colony morphology of the three screened organisms viz., Z3, KS1 and KHL2 were analyzed and the results were furnished in Table 1. The microbe Z3 was large, white, irregular, pulvinate and undulate. The characteristic of the KS1 was noticed as large, yellow, irregular, umbonate and undulate. The colony morphology of KHL2 was circular, yellow, entire, rough and dry.

Table 1: Colony morphology of screened organisms

S. No.	Microorganisms	Colony morphology
1	Z3	Large, white, irregular, pulvinate, undulate
2	KS1	Large, yellow, irregular, umbonate, undulate
3	KHL2	Circular, yellow, entire, rough, dry

Among the colony morphology of screened organisms, Z3, KS1 and KHL2 cultures of growth period was analyzed. The three isolates showed growth curve for eight days (Table 2).

Based on Gram staining and biochemical characters the Z3 was proved by *Staphylococcus aureus* and KS1 was confirmed by *Escherichia coli* and KHL2 was confirmed by *Pseudomonas aeruginosa*.

Optimization of the Inoculum Volume: The isolated microorganisms were grown at inoculum volume of 1, 2 and 3 ml with fixed Cr (VI) concentration of 70 mg/l at temperature 37°C (Table 4 to Table 6). Lowest Cr (VI) reduction (70 mg/l as K₂Cr₂O₇) was observed with 1 ml inoculum volume (Table 4), whereas highest Cr (VI) reduction was observed at 3 ml inoculum volume. In 3ml inoculum highest chromium reduction was recorded 0.025 mg/concentration in *Pseudomonas aeruginosa* (Table 6).

Effect of inoculum volume for 1ml at various temperatures on chromium reduction at 70mg/l concentration, pH 7 at various time periods 24,48,72,120, 144, 168, 192 the chromium reduction was noticed at 0.030 mg/concentration in 192 hrs in Z3 (*Staphylococcus aureus*). Z3 strain noticed greatest chromium reduction compared to other strains.

In inoculums 2ml at various temperature on chromium reduction in various time intervals the KHL2-*Pseudomonas aeruginosa* was recored highest chromium reduction 0.027mg/ concentration compared to other strains.

Table 2: Growth characteristics of organisms isolated from tannery sludge

S. No	Microorganisms	Time (hrs)							
		24	48	72	96	120	144	168	192
1	Z3	1.907	2.201	2.545	2.587	2.391	2.081	1.995	2.032
2	KS1	1.568	2.012	2.368	2.507	2.107	1.848	1.261	1.134
3	KHL2	2.149	2.531	2.602	2.658	2.254	1.675	1.009	0.957

Table 3: Identification of organisms through Gram Staining and IMVIC Test

S. No.	Microorganisms	Gram Staining	Indole	MR	VP	Citrate	Catalase	Oxidase	Urease
1	Z3	+	-	-	+	+	+	+	+
2	KS1	-	-	-	+	+	+	+	+
3	KHL2	-	-	-	+	+	+	+	+

Z3-*Staphylococcus aureus*; KS1-*Escherichia coli*; KHL2-*Pseudomonas aeruginosa*.

Table 4 Effect of inoculum volume for 1 ml at various temperatures on chromium reduction at 70 mg/l concentration, pH 7 (Cr (VI) reduction at 37°C)

S.No	Microorganisms	Time (h)							
		24	48	72	96	120	144	168	192
1	Z3	0.071	0.068	0.058	0.055	0.042	0.038	0.036	0.030
2	KS1	0.079	0.065	0.067	0.065	0.063	0.050	0.042	0.038
3	KHL2	0.069	0.062	0.056	0.052	0.048	0.043	0.037	0.032

Z3-*Staphylococcus aureus*, KS1-*Escherichia coli*, KHL2-*Pseudomonas aeruginosa*.

Table 5: Effect of inoculum volume for 2 ml at various temperatures on chromium reduction at 70 mg/l concentration, pH 7 (Cr (VI) reduction at 37°C)

S.No.	Microorganisms	Time (h)							
		24	48	72	96	120	144	168	192
1	Z3	0.101	0.073	0.070	0.063	0.060	0.055	0.049	0.035
2	KS1	0.073	0.067	0.060	0.054	0.051	0.048	0.039	0.031
3	KHL2	0.069	0.071	0.055	0.051	0.046	0.039	0.032	0.027

Z3-*Staphylococcus aureus*; KS1-*Escherichia coli*; KHL2-*Pseudomonas aeruginosa*.

Table 6: Effect of inoculum volume for 3 ml at various temperatures on chromium reduction at 70 mg/l concentration, pH 7 (Cr (VI) reduction at 37°C)

S. No	Microorganisms	Time (h)							
		24	48	72	96	120	144	168	192
1	Z3	0.118	0.065	0.060	0.057	0.055	0.048	0.042	0.033
2	KS1	0.076	0.066	0.062	0.060	0.058	0.052	0.047	0.039
3	KHL2	0.067	0.062	0.050	0.047	0.043	0.036	0.030	0.025

Z3-*Staphylococcus aureus*; KS1-*Escherichia coli*; KHL2-*Pseudomonas aeruginosa*.

Table 7: Effect of pH on chromium reduction at 70 mg/l concentration (pH 5 at 37°C)

S. No.	Microorganisms	Time (h)					
		24	48	72	96	120	144
1	Z3	0.068	0.040	0.031	0.026	0.019	0.015
2	KS1	0.065	0.060	0.052	0.045	0.042	0.037
3	KHL2	0.069	0.061	0.055	0.049	0.044	0.035

Z3-*Staphylococcus aureus*; KS1-*Escherichia coli*; KHL2-*Pseudomonas aeruginosa*.

Table 8: Effect of pH on chromium reduction at 70 mg/l concentration, pH 7 (pH 7 at 37°C)

S. No	Microorganisms	Time (h)					
		24	48	72	96	120	144
1	Z3	0.066	0.054	0.049	0.045	0.038	0.029
2	KS1	0.061	0.057	0.050	0.042	0.037	0.030
3	KHL2	0.069	0.065	0.062	0.058	0.050	0.039

Z3-*Staphylococcus aureus*; KS1-*Escherichia coli*; KHL2-*Pseudomonas aeruginosa*.

At 2 ml inoculum volume no significant variation in cell growth was observed. This may be due to over populated culture and fixed amount of nutrient with which the organisms starts liberating proteolytic enzyme, enhancing self consumption [5]. So fixed amount of inoculums were optimized and 3ml of inoculum volume gave the best result than other size of inoculum.

The pH 5 and 9 for Z3 isolate found to give high percentage of Cr (VI) reduction, whereas for Z3, pH5 gave the better result compared to other pH level. Effect of pH on chromium reduction in pH 5 at various time intervals in 144hr the Z3 highest value 0.015 mg/concentration compared other strains. But the highest Cr (VI) reduction was achieved at the pH 7 (Table 7 to Table 9).

Table 9: Effect of pH on chromium reduction at 70 mg/l concentration, pH 7 (pH 9 at 37°C)

S. No	Microorganisms	Time (h)					
		24	48	72	96	120	144
1	Z3	0.073	0.069	0.059	0.055	0.049	0.035
2	KS1	0.088	0.077	0.064	0.057	0.048	0.039
3	KHL2	0.085	0.074	0.068	0.062	0.056	0.049

Z3-Staphylococcus aureus; KS1-Escherichia coli; KHL2-Pseudomonas aeruginosa.

Table 10: Effect of temperature on chromium reduction at 70 mg/l concentration, pH 7 (At 25°C)

S. No	Microorganisms	Time (h)					
		24	48	72	96	120	144
1	Z3	0.082	0.060	0.053	0.049	0.042	0.033
2	KS1	0.087	0.067	0.058	0.055	0.047	0.037
3	KHL2	0.086	0.067	0.054	0.050	0.045	0.030

Z3-Staphylococcus aureus; KS1-Escherichia coli; KHL2-Pseudomonas aeruginosa.

Table 11: Effect of temperature on chromium reduction at 70 mg/l concentration, pH 7 (At 30°C)

S. No.	Microorganisms	Time (h)					
		24	48	72	96	120	144
1	Z3	0.075	0.066	0.059	0.051	0.047	0.035
2	KS1	0.078	0.062	0.055	0.048	0.041	0.031
3	KHL2	0.073	0.054	0.046	0.038	0.029	0.019

Z3-Staphylococcus aureus; KS1-Escherichia coli; KHL2-Pseudomonas aeruginosa.

Table-12: Effect of temperature on chromium reduction at 70 mg/l concentration, pH 7 (At 37°C)

S. No.	Microorganisms	Time (h)					
		24	48	72	96	120	144
1	Z3	0.060	0.048	0.040	0.032	0.025	0.018
2	KS1	0.074	0.058	0.049	0.041	0.038	0.029
3	KHL2	0.069	0.055	0.050	0.040	0.031	0.021

Z3-Staphylococcus aureus; KS1-Escherichia coli; KHL2-Pseudomonas aeruginosa.

The optimization of chromium reduction using overnight grown cultures of three isolates was studied pH 7 with various concentration of hexavalent chromium (As $K_2Cr_2O_7$). Good reduction of hexavalent chromium was observed at 70 mg/l Cr (VI) reduction concentration in the temperature range at 37°C for Z3 and KHL2 when compared to 30°C and 25°C, respectively (Table 10 to 12).

In 25°C the highest chromium reduction was noticed at 0.030 mg/concentration in KHL2 (*Pseudomonas aeruginosa*) compared to Z3 and KS1.

Effect of temperature at 30°C in pH7 the least chromium reduction was noticed in 144 hrs was Z3 and highest chromium concentration was noticed in KHL2.

Saranraj *et al.* [6] 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 [7].

Table 13: Effect of different medium on chromium reduction (%) at 70 mg/l concentration, pH 7 (Z3 at 37°C)

S. No.	Medium	Time (h)		
		24	48	72
1	Luria Bertani	30.78	35.65	42.62
2	Peptone yeast extract	13.10	18.12	26.11
3	Minimal mineral medium	1.76	7.24	19.98
4	M9-minimal salt medium	5.74	8.321	17.82
5	Nutrient broth	32.58	37.95	45.72

Z3-Staphylococcus aureus; KS1-Escherichia coli; KHL2-Pseudomonas aeruginosa.

Table 14: Effect of different medium on chromium reduction (%) at 70 mg/l concentration, pH 7 (KS1 at 37°C)

S. No.	Medium	Time (h)		
		24	48	72
1	Luria Bertani	18.72	22.48	29.91
2	Peptone Yeast Extract	15.35	27.25	34.15
3	Minimal Mineral Medium	8.56	15.25	23.87
4	M9-Minimal Salt Medium	9.41	17.69	19.25
5	Nutrient broth	33.98	36.65	44.72

Z3-Staphylococcus aureus; KS1-Escherichia coli; KHL2-Pseudomonas aeruginosa.

Table 15: Effect of different medium on chromium reduction (%) at 70 mg/l concentration, pH 7 (KHL2 at 37°C)

S. No.	Medium	Time (h)		
		24	48	72
1	Luria Bertani	16.4	21.89	28.52
2	Peptone Yeast Extract	13.54	25.98	31.5
3	Minimal Mineral Medium	15.25	14.23	20.01
4	M9-Minimal Salt Medium	8.61	15.25	18.63
5	Nutrient broth	20.72	25.18	30.81

Z3-Staphylococcus aureus; KS1-Escherichia coli; KHL2-Pseudomonas aeruginosa.

Table 16: Antibiotic sensitivity profile of chromium resistant bacteria

S. No.	Antibiotics	Diameter of zone inhibition (cm)		
		Z3	KS1	KHL2
1	Ampicillin	2.8	-	2.1
2	Oxacillin	2.6	1.9	2.3
3	Vancomycin	1.8	3.7	2.7
4	Penicillin	2	1	1.6
5	Methicillin	1.7	1.6	1.4
6	Erythromycin	2	1.5	2.2

Z3-Staphylococcus aureus; KS1-Escherichia coli; KHL2-Pseudomonas aeruginosa.

The effect of four different mediums on chromium reduction was carried out for the organisms Z3, KS1 and KHL2. Among which, Luria Bertani medium gave high percentage of Cr (VI) reduction (Table 13 to Table 15).

The three isolates tested for its minimum inhibitory concentration for both the level of chromium (50 and 100 mg/l as $K_2Cr_2O_7$) showed minimum level of resistance, whereas KS1 alone showed slight resistant to (100 mg/l as $K_2Cr_2O_7$). The chromate resistant isolates was tested

for their sensitivity to 6 commonly used antibiotics such as Penicillin-G, Methicillin, Vancomycin, Oxacillin, Erythromycin and Ampicillin to access its degree of sensitivity. Three isolates appeared to be most susceptible being inhibited by all antibiotics. The strain KS1 and KHL2 was resistant to vancomycin and Z3 was resistant to Ampicillin and Oxacillin. The isolates showed intermediate response to all other antibiotics as described in Table 16.

CONCLUSION

The study concludes that organisms isolated from the sludge of tannery industrial area were capable of biosorbing more than 60% of chromium (VI) from liquid medium. The study indicated that biosorbing took place during the exponential growth phase. The bacterial isolates *Staphylococcus aureus* and *Escherichia coli* can be exploited for bioremediation of Cr (VI) containing wastes, since it seems to have the potential to reduce the toxic hexavalent form of chromium to its nontoxic trivalent form.

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