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Recovery of Chromium from the Tannery Wastewater by Use of Bacillus Subtilis in Gujranwala, Pakistan

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***Abstract**:Tanneries discharge wastes without any treatments on the vast areas of vacant land around them. Untreated effluents contain toxic materials like hexavalent chromium and sulphides which accumulate in soils and cause many health hazards. Hexavalent chromium is toxic and carcinogenic and is being extensively used in the tanning industry of Pakistan. The tannery wastewater samples were collected from two tanneries in Gujranwala, Pakistan. Chromium in these samples was determined using fractionation technique, spectrophotometry and MIBK extraction procedure. These samples were analyzed to recover chromium and chrome cake was purified using chemical treatment which can be used in recycling process. Enzyme was used to recover chromium from the samples. Two strains S_1 and S_2 of the Bacillus subtilis (isolated and identified from soil and tannery wastewater respectively) were used for enzymatic processing. Isolates were screened for extracellular protease activity. The strain S_2 of the Bacillus subtilis showed maximum zones of hydrolysis (2.3cm) and proteolytic activity of 107 PU/ml at 65^0C temperature, 150 rpm agitation speed and7.5 pH on Shake Flask Fermentation. The One-step and the Two-step methods were employed for chromium recovery using the S_2 strain of the Bacillus subtilis. From the One-step method 96% chromium was recovered from sample-A and 92% from the sample-B. From the Two-step method of chromium recovery 98% of chromium was recovered from the sample-A and 97% from the sample-B. This paper evaluates the alternative treatment options used to treat, recover or recycle chromium from the waste water in order to minimize the environmental pollution.*

***Key words:** Bacillus subtilis, Chromium, One Step and Two Step Methods, Pakistan, tannery wastewater.*

I. Introduction

Environmental pollution has been a foremost irritation to industrial development. Chemical-based industries are the prime targets of the environmentalists for their battle against pollution**.** The leather sector of Pakistan is one of the oldest sector. It has an established share in world market for tanned leather, leather garments and leather gloves. At present there are 720 tanneries in Pakistan which generated the exports of US $ 01 billion in year 2008-2009. The wastewater as result of tanning process is an important source adding Cr pollutent to the environment which causes many health hazards to all sort of life. The pressure by the environment protection agencies is so that it has become a common occurrence that the tanneries are forced to close down not only in developed countries but also in the developing countries. Chromium (III) salts are the most widely used chemicals for tanning processes, but 60% - 70% of total chromium salts reacts with the hides. In the other words, about 30%-40% of the chromium amount remains in the solids and liquid wastes (especially spent tanning solutions). One ton of wet salted hide yields only 200 kg of leather, but more than $50m^3$ of waste water[1&5]. Therefore, the removal and recovery of the chromium content of these wastewaters is necessary for environmental protection and economic reasons. Since the untreated effluents contain toxic materials like chromium and sulphides which accumulate in soils and cause many health hazards. For instance, according to an official report of the Environmental Protection Department Punjab (1997), the drinking water supplied by the municipality in Kasur (Pakistan) was found polluted with a high level of chromium. The diseases found, among the workers of tanning industry and residents of Kasur, were skin irritation, diarrhea, heart burning, respiratory tract infection, severe cough, fever and loss of eyesight. Lung cancer, high blood pressure, and kidney failure were the reported causes of death in many cases [36]. National Toxicology Program (NTP) study, inspected and observed the mid-term toxicity of chromium VI to rats and mice. The test animals were injected sodium dichromate in their drinking water for 3 months, and as result focal ulceration, metaplasia, and hyperplasia of the glandular stomach were found on both rats and mice. histiocytic infiltration of liver, duodenum, and pancreatic lymph nodes was also detected. Rats also showed an increase in lung and spleen weight and in macrophage activity [25]. Thus leather industry is pressurized to look for cleaner options. The effluents of these industries contain chromium at concentrations ranging from tenths to hundreds of milligrams per liter [6]. The current activity indicates that the trend is more towards design and utilization of cleaner and safer technologies

like the enzymatic process [3 & 31]. Chromium (III) salts are efficiently used as tanning agents in the leather industry. When waste is disposed off on soil, the risk of potential oxidation of chromium (III) to the hazardous hexavalent state exists in the presence of manganese (IV) oxide [20]. Among the two species Cr6+ is more toxic than Cr3+ [28] and is a serious environmental pollutant due to its wide use in different industries. It also effects the seed germination, seedling growth, pigments and enzymes content [10]. The removal of chromates from industrial wastewater effluents through Rhizopus oryzae indicates a feasible, economical technique for their removal [29].

Heavy metal removals from waste streams are not cost effective [9] and hence biological approach has been considered as an alternative remediation for heavy metal contamination. Recently microbial systems like fungus, bacteria and algae have been successfully used as adsorbing agents for removal of heavy metals [18, 19, 21 & 34]. Microbial populations in metal polluted environments adapt to toxic concentrations of heavy metals and become metal resistant [22]. Different species of *Aspergillus*, *Pseudomonas*, *Sporophyticus*, *Bacillus*, *Phanerochaete*, etc., have been reported as efficient chromium and nickel reducers [8 & 39]. Microorganisms play a significant role in bioremediation of heavy metal contaminated soil and waste water [27].

In a study conducted [7] in Pakistan has used different *Bacillus* sp., and *Aspergillus niger* isolates chosen after determination of maximum resistivity level against hexavalent chromium, were further accessed for their Cr6+ biosorptive capability for metal removal from aqueous solution. The present study was conducted with two strains of the *Bacillus subtilis* to test the efficiency of the enzyme in the recovery of chromium from the wastewater collected from two tanneries of the Pakistan which is not just affordable but also environment friendly.

II. Materials and Methods

2.1 Samples:

The two samples, one dark bluish green in color (A) from the tanning stage and the other light yellow (B) with a pungent odor from the last stage (after the formation of crust leather), were collected from the tanneries in Gujranawala, Pakistan.

2.2 Stages of study:

The study was divided in two steps

2.2.1 Determination of chromium in the samples

2.2.2 Recovery of Chromium from waste water

2.3 Reagents:

Distilled water, MIBK (methyl iso-butyl ketone), $K_2Cr_2O_7$, K_2CrO_4, KH_2PO_4, H_2SO_4, CH_3COOH, NH_4Cl, $Na_4P_2O_7$, $NH_2OH{\times}HCl$, HNO_3, $HNO_3{\times}HCLO_4$, $Na_2S_2O_4$, Phosphoric acid, 1,5 diphenyl carbazide, membrane filters of pore size 0.01, 0.45, 0.1, 0.05, 0.2, μm and Whatman # 1.

2.2.1 Determination of Total Water-Soluble Cr and Cr (Vl) in Wastewater:

Potassium dichromate ($K_2Cr_2O_7$) was used as standard Cr determinant. Two ml each of the sample (A and B) were shaken with 20 ml distilled water and 20 ml potassium dihydrogen phosphate (KH_2PO_4, 0.015 mol/ dm^{-3}) for 8 hours in the classical shaker and for 5 minutes in the ultrasonic homiginizer. The samples were centrifuged at 10,000 rpm for 20 minutes at 25°C and filtered through membrane filters of pore size (0.2 – 0.01 μm). The concentrations of total water-soluble chromium were determined on UV spectrophotometer. Influence of shaking time (1, 2, 4 and 8 hours) on chromium concentration was also noted and compared with ultrasonic agitation.

Spectrophotometry:

Sample preparation:

Ten ml each of the sample was dissolved in 100 ml K_2HPO_4 and shaken for three hours. The pH of the samples was measured. The extract was then filtered through 0.45 μm membrane filter paper.

Procedure:

Tannery effluents were not diluted in one experiment and one experiment was conducted without 1,5 diphenyl carbazide solution. Ten ml each of the sample (pH between 7.5 – 8.0) was mixed with 1 ml of 1,5 diphenyl carbazide solution and 1 ml of phosphoric acid solution and diluted up to 50 ml with distilled water. After 15 minutes, spectrophotometer readings were taken at 540 nm wavelengths against potassium chromate as standard. The same procedure was used, but with exception that the samples were not diluted in one experiment and one experiment was conducted without 1,5 diphenyl carbazide due to its interference effect on coloured species.

Extraction of Cr (Vi) –HCl complex with MIBK (methyl iso-butyl ketone):

Chromium (VI) forms the complex $HCrO_3Cl$ with HCl, which may be extracted with MIBK. The complex stability is affected by temperature and therefore cooling to 277K is required to assure its stability. The following procedure was used:

Forty ml of the sample-A, 4.0 ml of concentrated HCl and 5.0 ml of MIBK (Methyl iso-butyl ketone) were added. The mixture was shaken for one minute and the phases were allowed to separate. Before applying the MIBK (Methyl iso-butyl ketone) extraction procedure all the samples, reagents and laboratory ware were cooled to the same temperature (277K). Cr (VI) was determined immediately in the organic phase on UV spectrophotometer. One ml of L-ascorbic acid was added to compensate for possible interference due to Cr (III). The same process was employed to the sample-B.

Fractionation and Determination of Cr (Vl):

Fractionation of chromium was performed by various sequential extraction procedures. Modified method of Taylor et al. (1998) was followed.

Determination of Hexavalent Chromium:

Potassium chromate (K_2CrO_4) was used as standard chromium (VI) determinant. Modified method of Ramesh Kumar and Riyazuddin (2009) was used.

2.2.2 Recovery of Chromium Using the Enzyme

Bacillus subtilis used for the recovery of chromium was isolated from the wastewater samples along with other microbes. It was identified and checked for its proteolytic activity.

Organism:

The two strains of the *Bacillus subtilis* were used in this study to recover the hexavalent chromium from tannery effluents. Strain S_1 was isolated from tannery waste water (Sample B) and strain S_2 was isolated from the soil near by the laboratory. The isolation and identification of *Bacillus subtilis* was carried through biochemical tests described in an earlier study [14]. The two bacterial strains were routinely maintained on nutrient agar medium.

Materials:

Tween- 20 (a non-ionic surfactant), NaOH, MgO, H_2SO_4, enzyme extract, waste water samples

Production of the Enzyme:

Screening test for proteolytic activity was performed on both the strains of the *Bacillus subtilis* using sample plate technique in which zones of protein hydrolysis around the bacterial colonies were observed and the proteolytic activity was correlated with the zones of protein hydrolysis. Luria Casein Agar plates (1%) were used for this purpose. Sterilized plates of 1 % Luria Casein Agar were inoculated with the bacterial cultures using sterilized needle. After 24 hours of inoculation at 37°C, clear zone of hydrolysis were formed around each bacterial colony. The plates were flooded with 10% glacial acetic acid, which made the zones around the bacterial colonies more prominent. After 15 minutes the diameter of each zone of hydrolysis was measured.

Inoculum Preparation:

Glucose minimal medium (250 ml) was poured in three 1000 ml Erlenmeyer flasks; pH of the medium was adjusted at 7.0 by using 0.1 N sodium hydroxide and 0.1 N acetic acid. The medium was then autoclaved at 121°C for 20 minutes. The medium was cooled and inoculated with 2 loops full of each culture (S_1 and S_2). These flasks were then placed in incubator shaker at 37°C for 24 hours at 120 rpm.

Fermentation for the Crude Enzyme Production:

The *Bacillus subtilis* did batch culturing in the Shake Flask for fermentation to produce extra cellular proteases. In two 2800 ml Fern batch flasks, 1000 ml of glucose minimal medium (pH 7.5) was poured and autoclaved at 121°C for 20 minutes. Both the inoculums S_1 & S_2 (100 ml each) were added in different flasks. These flasks were then placed in shaking incubator at 37°C and 150 rpm for 72 hours. The samples were collected after every 12 hours. The strain S_2 of the *Bacillus subtilis* that gave maximum proteolytic activity of 107 PU/ml was used for further studies

Enzyme Assay:

The method of Kunitz (1965) was followed for the measurement of proteolytic activity of the extra cellular proteases. One ml of the 1% casein solution in tris buffer (pH 8.5) was mixed with 1 ml of the crude enzyme extract (pH 8.5). The mixture was incubated at 40°C for 30 minutes. Then 3 ml of 0.3 M trichloroacetic

acid (TCA) was added and the tubes were placed in ice for 15 minutes. The precipitates were removed by centrifugation at 10,000 rpm at 4°C for 30 minutes. The standard was prepared in the same way except that 3 ml TCA was added before incubation. All assays were made in triplicate (One unit of activity is defined as that amount of the enzyme which releases 1 μg of tyrosine under assay conditions. (pH 8.5, temperature 40^{o}C and 30 minutes incubation period).

Application of the Enzyme:

Maximum enzyme production was carried out at all optimum conditions for 48 hours. The medium was then centrifuged at 10,000 rpm for 20 minutes at 4°C. The enzyme solution having 107 PU/ml proteolytic activity, used for the recovery of chromium from tannery wastewater. Both the samples (A & B) of the tannery wastewater were used for the chromium recovery.The tannery wastewater, along with tween-20 and 6.0 g MgO was reheated at 65°C for 2 hours. The enzyme extract was then added in it and allowed to settle for 3 hours. After the enzyme digestion (65°C for three hours), the sample was pumped hot in porcelain funnel using Whatman # 1 filter paper; the solution was allowed to settle overnight. The upper protein hydrolysate layer was separated and the lower chromium layer was filtered through Whatman # 1 filter paper. Protein layers were stored 4°C. The unwashed chrome cake was collected and stored at 4°C.

Recovery of Chromium from the One-Step Process:

Both the samples (A & B) of the tannery wastewater were used for the chromium recovery. The tannery wastewater, along with tween-20 and 6.0 g MgO was reheated at 65°C for 2 hours. The enzyme extract was then added in it and allowed to settle for 3 hours. After the enzyme digestion (65°C for three hours), the sample was pumped hot in porcelain funnel using Whatman # 1 filter paper; the solution was allowed to settle overnight. The upper protein hydrolysate lawyer was separated and the lower chromium layer was filtered through Whatman # 1 filter paper. Protein layers were stored 4°C. The unwashed chrome cake was collected and stored at 4°C.

Recovery of Chromium from the Two-Step Process:

In step one of the Two-step processes 100 ml each of the sample (A & B) was mixed with 0.2 ml Tween–20, 6.0 g MgO and 500 ml distilled water in 2 L Erlenmeyer flask each. These flasks were than put in the shaker for 6 hours at 70-72^{0}C. The samples were then centrifuged hot at 10,000 rpm for 20 minutes and the supernatant was filtered through Whatman # 1 filter paper. The chrome sludge (supernatant) and gelable protein (filtrate) were stored at 4 ^{0} C. In the second step, the chrome sludge was warmed to room temperature and hundred percent chrome sludge was added to 200 ml distilled water and than 0.1 ml non-ionic surfactant (Tween-20) 0.2 g alkali (MgO) was added to it. These flasks were shaken for 1.5 hours to obtain maximum solubility at 70-72^{0}C. The pH was adjusted with MgO to the optimal pH for the enzyme. The enzyme (50 ml) was added and the sample was again shaken for 3.5 hours at 70-72^{0}C. The solution was filtered hot through Whatman # 1 filter paper and the protein solution was stored at 4^{0}C. The chrome cake was air-dried.

Treatment of the Chrome Cake:

Air-dried chrome cake (1 g) was dissolved in 50 ml of 3.6 N (23%) sulfuric acid. The pH was less than 1.0. The pH of the solution was slowly raised to 1.85-2.00 with sodium hydroxide (50% w/w solution). A flocculated precipitate formed that coagulated, when the solution was heated for several minutes at 60^{o}C. The solution was allowed to stand overnight at ambient temperature and was than filtered. The residue was washed with 0.01 N sulfuric acid to remove trapped chromium. The residue was dried overnight at 60^{0}C and weighed; the percent residue was calculated. Percent moisture was calculated by heating at 105^{o}C for 17 hours. The residue was ash-dry at 600^{0}C in oven and percent ash was calculated.

III. Results

Determination of Total Water-Soluble Cr AND Cr (VI) in the Tanning Effluents:

Cr (VI) investigated in the samples was found to be 46 μg/ml in the sample-A and 68 μg/ml in the sample-B. Total water-soluble Cr in the sample-A was 50 μg/ml and 81 μg/ml in the sample-B. The influence of shaking time on the concentration of total water- soluble Cr and Cr (VI) was studied (Table-1).

Comparison between Classical Shaking and ultrasonic Agitation on Determination of Total Water-Soluble Cr and Cr (Vl) in the Samples:

The results are presented in Table-1. It is evident that the concentration of total water-soluble Cr and Cr (VI) are higher with classical shaking than with ultrasonic agitation.

Spectrophotometry

The results of spectrophotometer method compared to the MIBK (Methyl iso-butyl ketone) extraction method are presented in Table-2.

Fractionation of Chromium in Tannery Wastewater:

The tannery wastewater was fractionated to determine the distribution of chromium species between various compounds. The results are presented in Figure 1-a. It is evident from Figure-1 (standard graph) that tannery waste water contained high concentration of chromium.

Determination of Hexavalent Chromium:

The results presented in Table-3 shows the hexavalent chromium was present in quite an amount in tannery waste water.

Production of the Enzyme for Chromium Recovery:

Qualitative Test:

Ability of the two strains of the *Bacillus subtilis* (S_1 and S_2) to produce extra cellular protease enzyme was determined by measuring the diameter of zone of hydrolysis on 1% Luria Casein Agar plates around each colony. Table 4-a shows the results.

Shake flask fermentation of the *bacillus subtilis* for protease production:

The Shake Flask Fermentation was employed to check the production of protease enzyme by the *Bacillus subtilis* for 72 hours. The results are shown in Table 4-b.

Optimum Temperature and pH for the Enzyme Extract:

The proteolytic activity of the crude enzyme extract was stable upto 84% at 50^0C and then there was sharp decrease in proteolytic activity with further increase in temperature and it was 38% at 80^0C. Table 4-c shows the effect of pH on proteolytic activity and Table 4-d shows the effect of temperature on proteolytic activity**.** Table 4-e shows the optimum ph for maximum proteolytic activity .

Recovery of chromium from the One-step process:

Table 5-a shows the amount of chromium recovered from both samples using the one step method. Chemical composition of recovered chrome cake is shown in Table 5-b.

Recovery of chromium from the Two-step process:

Sample-A showed the maximum recovery. The results are shown in Table 6-a. The recovered chrome cake was analyzed for moisture and ash content (Table 6-b).

Treatment of Chrome Cake:

Table 5-b and Table 6-b shows the results of treated chrome cake. The chrome cake was also characterized for its residue; the result is shown in Table 7.

IV. Discussion

Cr (III) salts are efficiently used as tanning agents in the leather industry. When the waste is disposed off on soil, the risk of potential oxidation of some Cr (III) species to the hazardous hexavalent state exists in the presence of manganese (IV) oxide. Recovery of chromium is one of the options to make leather industry environmental friendly.

The samples were collected from two Tanneries in Gujranwala, Pakistan and were tested for the presence and recovery of chromium. Taking into account the large volume of wastewater containing very high chromium concentrations and the difficulties in finding proper disposal sites for the sludge produced, many countries have turned to "clean" technologies including chromium recovery and reuse. It was investigated that common basic compounds could precipitate Cr (III) but the settling characteristics of the precipitate formed by the reaction of MgO were superior due to its minimum sludge volume [38 & 12].

The parameters influencing the extraction efficiency of total water-soluble Cr and Cr (VI) were investigated in the tannery wastewater. Water and KH_2PO_4 solution (0.015 mol / dm^3) were used as extractants. The latter, being an electrolyte, adsorbs Cr (VI) from the samples. Membrane filters of 0.1 μm pore size were used for the efficient removal of Cr (VI), when using potassium dihydrogen phosphate as an extractant and 0.05 μm filters when using water as an extractant. The equilibrium of total water-soluble chromium between solid and liquid phases was achieved after two hours of shaking in the classical shaker with potassium dihydrogen phosphate as an extractant and after eight hours when water was used as an extractant. Five minutes of ultra sonic agitation was very convenient for the tanning effluents. In actual practice, 20 ml of extractant and 2.0 ml

of the sample were used for the determination of total water-soluble Cr and Cr (VI) (Table-1). The above used method was compared with the method used in study [11] which optimized the extraction procedure for determination of total water-soluble Cr and Cr (VI) and found that Cr extracted through classical shaking was in greater concentration than that by ultrasonic agitation.

Chromium was extracted with diphenyl carbazide [13, 35 & 37] in presence of other metals after separation by solid phase extraction. Amongst the various analytical techniques spectrophotometry has been widely used for determination of Cr (VI) in different sample matrices [17].

Table-2 shows that concentration of total chromium ranged from 280 to 364 mg/L and that the pH of both the samples was from 4.5 to 8.9. It is also evident from these results that results obtained by spectrophotometry are in general much higher than those obtained by the MIBK (Methyl iso-butyl ketone) extraction technique. The reason for this lies in the non-specific reaction of diphenyl carbazide reagent with the sample that resulted in the development of the coloured species, which gave non-specific absorption at 540 nm. The results 2.10 mg/L in case of the sample-A and 3.85 mg/L in case of the sample-B calculated from the five-fold diluted sample were even higher than those for non-diluted samples. On the basis of these observations it can be said that spectrophotometry over estimates the results for Cr (VI) in coloured watewater samples. Therefore, the results obtained are not reliable, even though it was recommended spectrophotometry in DIN 533314 method [17], standard for the analysis of Cr (VI) in tannery wastewater. Reliable results were obtained by MIBK (Methyl iso-butyl ketone) extraction technique, which was sensitive enough and was not liable to interference effects in the sample matrix analysed. MIBK extraction for determination of chromium from tannery polluted water is also used in other studies [23 & 24]. Therefore, the latter technique was found to be suitable for determination of Cr (VI) in the tannery wastewater.

It is evident from the results shown in Table 2-a that concentration of total chromium in the tannery waste was very high and the prevailing chromium fraction was hydroxylamine hydrochloride extractable. Second largest quantity of chromium was bound to organic molecules. Acetic acid extractable fraction contained the third largest quantity of chromium. Compared to these, substantially more chromium exists in the water-soluble potassium dihydrogen phosphate (0.015 mol/ dm^3), and exchangeable fractions, while nitric acid (1 mol /dm^3) extractable and mineral fractions were negligible. Fractionation and oxidation of chromium in the tannery waste and found that KH_2PO_4 (0.085 μg/g) contained less amount of chromium and hydroxylamine hydrochloride was the largest fraction extracted from the samples [16]. Isolates for potential of chromium removal in minimal salt medium containing different fractions was screened in another study [26].

Bacillus subtilis secretes proteolytic enzymes, which can be used in recovery of chromium. Culture broth (whole fermentation broth) along with bacterial mass was used for the biotreatment of the tannery effluents. In the samples *Bacillus subitilis* was confirmed by morphological and biochemical tests. The ability of the two *Bacillus subtilis* strains (S_1 & S_2) obtained from soil and the tannery wastewater were checked for the production of extracellular proteases on 1% Luria Casein Agar plates. Both strains showed proteolytic activity but the strain S_2 of the *Bacillus subtilis* isolated from the tannery wastewater showed maximum (2.3 cm) zone of hydrolysis (Table 4-a). In "Shake Flask Batch Culturing" the S_2 strain of the *Bacillus subtilis* showed maximum proteolytic activity activity of 107 PU/ml after 48 hours of incubation, so it was selected for further studies (Table 4-b).

Maximum proteolytic activity was achieved at initial pH 7.5. These results correlate with the observation [27 & 30] in which maximum proteolytic activity with an initial pH 7.6 and temperature 37^{o}C by the *Bacillus alcalophilus* sub spp. *Haldurans was obtained.* Thermostability of the crude enzyme extract was tested and observed that the enzyme was stable up to 70^{o}C after one hour of incubation. *E*nzymes play an important role in the tanning of hides and skins [15]. *A* process was established [28 & 29] that could help leather industry in solving the potentially difficult waste disposal problem, the chrome waste was treated with the alkaline protease enzymes at moderate temperature for a short period of time. The pH at which the reaction took place (8.3 to 8.5) prevented the chromium from going into the solution, thus averting the poisoning of the enzyme by chromium and enabling the recovery of chromium as Cr $(OH)_3$ by filtration. The resulting protein solution may have commercial use as a feed or a fertilizer. The isolated chrome cake may potentially be recycled into the tanning process by treatment with sulphuric acid. Broad specificity of the enzyme and its stability at high temperature in alkaline conditions helped this enzyme to be used in the biotreatment of the tannery wastes [3].

In the One-step process the tanning effluents were heated for 2 hours at 65^{o}C to achieve optimal solubility (Table 5-a). Composition of the chrome cake was studied. The chrome cake was tested for its moisture, ash and chromic oxide content (Table 5-b). Maximum recovery of chromium was obtained from the Two-Step Process at agitation speed of 150 rpm, temperature 72^{o}C and pH 8.5 (Table 6-a). The chrome cake was treated chemically using sulphuric acid. Then it was analysed for moisture and ash content (Table 6-b). The percent recovery of chromium in the sample-A was 98% and 97% in case of the sample-B.

V. Conclusion:

The present bench scale study offers a method to acquire pure chromium from chromium loaded waste of tanneries. Maximum amount of this (upto 98%) can be recovered from the tannery wastewater, for which microbiological methods can be employed. The chromium produced is cost effective. The recovered chromium can also be used as retanning agent as well as in cements and mortars. Use of proteolytic enzymes to recover chromium can help in controlling many diseases. Furthermore, the environment pollution because of chromium can be minimized.

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Table-1

Comparison between ultrasonic homogenization and classical shaking.

Sample	Shaking	Total water soluble Cr µg/ml 0.2µm filter.	0.05µm filter.	Cr(VI) µg/ml 0.05µm filter
A	C(2 hours)	183	50	46
	U(5min)	59	41	32
B	C(2 hours)	121	81	68
	U(5 min)	48	32	29

C. Classical Shaker. U. Ultrasonic homogenizer.

Table-2

Total Cr in tannery wastewater samples by spectrophotometric method.

Colour of samples	pH of samples	Total Cr in samples mg/L	Cr (VI) in samples by spectrophotometic method mg/L Non-diluted (10 ml)	diluted (50ml)	Cr (VI) in samplesbyMIBK Extraction procedure mg/L
Dark bluish green	4.5	364	1.98	2.10	0.351
Light yellow	8.9	280	1.05	3.85	0.198

Table - 3.

Determination of Hexavalent Chromium.

Sample	Shaking Time	pH after shaking	Cr (VI) µg/ml
A	24	5.6	0.101
B	24	7.3	0.054

Table 4-a.

Proteolytic activity shown by *Bacillus subtilis* by forming zones of hydrolysis.

Strains	Source	Zone of hydrolysis cm
S_1	Soil	1.9
S_2	Tannery wastewater	2.3

Table 4-b

Proteolytic activity of *Bacillus subtilis* in shake flask culture.

Bacterial Strain	Proteolytic Activity PU/ml 24 hours	48hours	72 hours
S_1	19	58	43
S_2	28	93	71

Table 4-c.

Effect of pH on Protease Production.

Initial pH	Final pH	Proteolytic activity PU/ml
7.0	9.0	97
7.5	8.8	107

Table 4-d.

Effect of Temperature on Protease Production

Temperature ^{0}C	Proteolytic Activity PU/ml	Stability (%)
4	122	100
30	110	94
40	101	89
50	97	85
60	85	79
70	40	65
80	15	38

Table- 4-e

Optimum pH for Maximum Proteolytic Activity

pH	Proteolytic Activity PU/ml
7	76
7.5	88
8	93
8.5	107
9	98
9.5	92
10	83

Table 5-a.

Amount of Chromium Extracted from One-Step Process

Samples	Amount of Cr. g	% Recovery
A	9.628	96.23
B	9.213	92.13

Table 5-b.

Chemical Composition of Chrome Cake

Parameter (%)	Sample A	Sample B
Moisture	31.5	33.5
Ash	4.99	5.38
Chrome oxide	9.63	9.21

Table 6-a.

Amount of Chromium extracted from Two-Step Process.

Samples	Amount of Cr (g)	% Recovery
A	9.81	98.1
B	9.74	97.4

Table 6-b.

Analytical Tests for Chrome Sludge Obtained from Two-Step Process.

Parameter (%)	Sample A (g)	Sample B (g)
Gel extraction step.		
Chrome sludge:	83.71	84.01
Moisture	20.10	21.21
Ash	32.1	37.00
Protein		
Hydrolysis step.		
Chrome cake:		
Moisture	89.31	87.42
Ash	34.58	35.52
Protein	50.96	52.14

Table 7.
Characterization of Residue from Treatment of Chrome Cake

Samples	Final pH	% Residue	% Ash
A	1.81	9.13	0.37
B	1.85	6.33	0.18

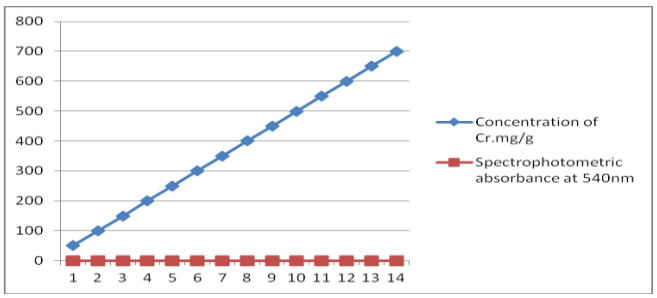

Figure 1. Standard graph.

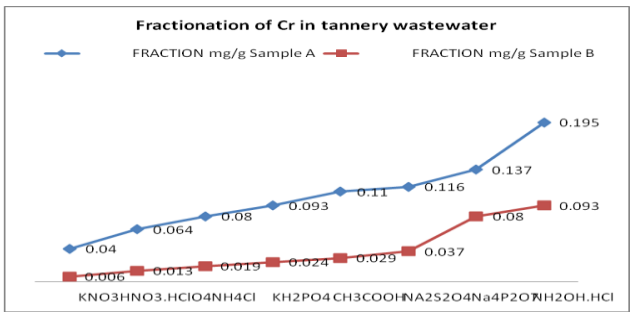

Fig: 1 a: Fractionation of chromium in tannery waste water.