CHAPTER 4

Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using *Bacillus subtilis* P13 and exploring its chromium resistance mechanisms

*For a successful technology, reality must take precedence over public relations, for Nature cannot be fooled.*

- Richard Feynman
4.1: Introduction

Leather processing, generates enormous solid and liquid wastes, obnoxious smell due to the degradation of protein from waste hide and the release of gases like H$_2$S, NH$_3$ and CO$_2$. Solid wastes generated are of two categories, chemical waste and high value protein-based waste from untanned and tanned sources (Kangaraj et al., 2006). Untanned wastes are mainly the fleshings, trimmings and are mainly used in glue/gelatine manufacture. The main composition of tanned waste is chromium and protein. Chrome shavings (CRS) are small, thin pieces of leather formed during shaving operation and constitute about 10% of the total weight of raw materials processed. It is estimated that 0.8 million tonnes of chrome shavings generated per year globally (Kanagaraj et al., 2001). Traditional practice of disposal is land filling, however the stringent restrictions are imposed on the disposal of chromium bearing waste in many parts of the world and also the presence of valuable protein necessitates search for other alternatives (Kangaraj et al., 2006).

Green chemistry as a concept represents the chemical processes where environmental pollutants can be replaced by ecofriendly alternatives. Eco-friendly options are reuse, by-product recovery, bioremediation etc (Rao et al., 2002). Chrome shavings can be used directly for the manufacture of bonded leather, leather boards, fibrous sheets grafted with acrylates, insulators and building material (Brown et al., 1998) and indirect use of tanned waste is basically the separation of chromium and protein. As a green route, chrome shavings are also used as a reductant for the preparation of basic chromium sulphate (BCS), commonly used tanning material, which otherwise is prepared by the reduction of chromium (VI) (sodium dichromate) in acid medium using molasses or sulphur dioxide as reductant. Combinations of alkali and enzymatic treatment are used to recover chromium from protein from CRS (Rao et al., 2002). Alkaline proteases like alcalase and combination of trypsin (0.05%) and esterase (0.05%) are also used in the preparation of soluble collagen hydrolysate called chrome cake (Marner et al., 1999). Trypsin attacks the peptide bond only where one amino acid is arginine or lysine, but esterase cleaves the bond between glutamine-histidine, serine-histidine, leucine-valine, leucine-tyrosine, and tyrosine and threonine. Gelatin was recovered from chrome shavings by treating with pepsin A and trypsin at pH 8.0 for a period of 6-24h at 70°C, where pepsin had
a mild effect on hydrolysis whereas trypsin was more effective. An alkaline protease is reported to completely digest the wet blue to gelable protein and the same formulated with casein and used for leather finishing (Cantera et al., 2000).

Katsifas et al., (2004) reported an Aspergillus carbonarius which could perform biodegradation of chrome shavings in solid-state fermentation experiments, where 97% liquefaction of the tannery waste was achieved and the liquid obtained from long-term experiments were used to recover chromium and proteinaceous liquid fertilizer and was used as animal feed additive. Proteinaceous products and recycled chromium were obtained from tannery waste using several chemical methods (Brown et al., 1996). Efforts to obtain chromium after the incineration of shavings did not result in a less toxic waste (Ferreira et al., 1999). Other approaches focused on the extraction of chromium from shavings or chromium oxidation with moisturized air (Tancous et al., 1981).

Microbial enzyme usage for CRS degradation demands a pretreatment, either alkali based denaturation or lime (5-6 %) treatment at 60-65 °C to denature the collagen (Kanagaraj et al., 2006). The use of microorganisms able to grow in highly concentrated chromium environments and transform the waste into an easily recycled byproduct offers a promising perspective for successful chromium recovery. The present chapter focuses on the development of a microbiological procedure using the hotspring isolate B. subtilis P13 for the biodegradation of chrome shavings, a solid waste generated during tanning process and the recovery of protease, a byproduct which has application in pre-tanning process.

4.2: Materials and Methods

4.2.1: Microorganism and growth conditions

Bacillus subtilis P13, an isolate from the sediment sample of hot spring at Vajreshwari, Mumbai, India was maintained on Luria-Bertanni (LB) agar, containing 1% skimmed milk. Inoculum was prepared by growing a colony of B. subtilis in LB broth up to 0.6 OD 600nm at 30° C. An aliquot amounting to 1-2% v/v of the above culture was added to the growth media. CRS containing media were autoclaved at 121 °C, 101 kPa for 15 min. Growth was monitored at regular intervals by spread plating on LB agar.
4.2.2: Microbial hydrolysis of chrome shavings

To understand their degradation pattern, 1% (w/v) CRS were added to LB and inoculated with \textit{B. subtilis} (0.6 OD culture). Degradation efficiency was checked based on weight reduction and microscopic observations. Smears were made from CRS collected during the study duration and observed under oil immersion light microscope (Olympus CX41). UV sterilized chrome shavings (1%) were added to another set of LB flask and experiment were performed in the similar manner to study whether initial thermal processing is required for the degradation of these proteins. Suitability of the organism to degrade chrome shavings (CRS) was studied by using heat and UV sterilized CRS in LB and production medium (OM) containing 1, 5 and 10% of chromeshavings. Protease production was assessed based caseinolytic activity and zymogram profiling, as described in section 2.213.

4.2.3: Microbial hydrolysis of chrome shavings from a solid substratum column

A glass column of 30 cm height was filled with CRS (3 g) and small silicon tubing was attached the bottom and closed with a stopper. Top portion of the column were plugged with cotton and the whole setup was autoclaved at 15 PSI/15 min. The column was saturated with sterile OM and inoculated with 2% wt/vol of 0.6 OD (600nm) culture. Initial height of the packed material in the column was noted and it was regularly monitored when incubated at 30°C for liquefaction of CRS, protease activity and reduction in the height of the column

4.2.4: Determination of Minimum Inhibitory Concentration of chromium

Freshly grown culture of 0.6 OD was inoculated into 10 ml media containing different concentrations of chromium. \textit{K}_2\text{CrO}_4 (10ppm – 50ppm) and \textit{CrCl}_3.6\text{H}_2\text{O} (100-500ppm) concentrations were used to study the tolerance of the culture to \textit{Cr} (VI) and \textit{Cr} (III) respectively. Optical density at 600nm was recorded after 24h growth at 30°C, 180 rpm, uninoculated LB having similar Cr concentration was used as blank. The minimal inhibitory concentration (MIC) of chromium was defined as the concentration at which no turbidity due to growth was observed in the broth (Luli et al., 1983).
4.2.5: Chromium estimation

Concentration of Cr (VI) from different experimental samples was estimated by the Diphenyl carbizide (DPC) method of (APHA, 1992, Camargo et al., 2003). Cr (III) estimation was carried out by the method of Sankalika et al., (2004). Chromium chloride hexahydrate equivalent to 1.0 mg of Cr (III) was subjected to heating in a muffle furnace in a silica crucible at 550°C for 6h and the ash was dissolved in 0.2 M H₂SO₄ (pH 1 ±0.5), to obtain a stock of 5 µg/ml of Cr (VI). From this stock solution, suitable aliquots were taken and 0.2 ml of DPC dye was added to each, allowed to stand for 5 min for full color development, volume was made up to 10 ml with distilled water to obtain a final concentration of the range 0.1- 0.8 µg/ml and the absorbance was measured at 544 nm. Cr (III) from culture pellet was estimated in similar manner after pre-weighing the pellet, where as 1ml of the culture supernatant was concentrated by heating in muffle furnace proceeded in similar manner.

4.2.6: Recovery of chromium from culture supernatant

Precipitation of chromium from 100ml of the culture supernatant was carried out by converting it to insoluble Cr (OH)₃ by raising the pH to 10.5 with the addition of 50% (w/v) NaOH, followed by the addition 1.16 g of MgO and simultaneous heating at 55°C for 2h with stirring, after which the sludge was left to settle at room temperature for 10 h and was then separated by filtration (Katsifas et al., 2004). To determine the chromium that can be recovered from culture pellet, pre-weighed cell pellet was resuspended in 10ml of sterile distilled water and subjected to above-mentioned treatment and estimated by the method of Sankalika et al. (2004).

4.2.7: Chemical Oxygen Demand (COD) by Open Reflux Method and Biological Oxygen Demand (BOD)

The chemical oxygen demand (COD) and biochemical oxygen demand (BOD) during 5 d were determined based on APHA methods (1992) from chromate-containing liquid phase. COD was defined as the concentration of the specified oxidant, which reacted with a sample under controlled conditions as mg/l of O₂, the quantity of oxidant consumed is expressed in terms of its oxygen equivalence. BOD was estimated and is defined as the measure of the amount of O₂ necessary for the decomposition or stabilization of a given amount of waste and is expressed quantitatively as parts of O₂ per million parts of the material under study.
Chapter 4: Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using *Bacillus subtilis* P13 and exploring its chromium resistance mechanisms

### 4.2.8: Estimation of Biosorption efficiency of Chromium by microbial biomass

*B. subtilis* P13 was inoculated into 100 ml LB in 500 ml conical flasks and incubated on a shaker at 150 rpm for 24 h at 30°C. The cells were grown to late exponential phase, harvested by centrifugation at 12,000 xg for 15 min at 4 °C and washed three times with deionized water. Cell suspension for the biosorption assay potential of live bacteria was prepared by resuspending the cell pellet in deionized water. Biomass concentrations in cell suspensions were determined by drying an aliquot in a pre-weighed aluminum foil container to constant weight at 80 °C (Puranik and Paknikar, 1999).

Cr (VI) (30mg/l) and Cr (III) (250mg/l) solutions were adjusted to pH 4.0 with 0.1 M sodium hydroxide and 0.1M nitric acid. About 50 mg dry weight of the living cells was added in triplicates to 25 ml chromium solution in 150 ml conical flasks. The flasks were shaken at 150 rpm at 30°C for 1 h. Metal-free and biosorbent-free solutions were prepared as controls (Srinath et al., 2002). The cells were harvested by centrifugation at 12,000 xg for 10 min at 4 °C and washed twice with sterile distilled water. The biomass was then dried at 80 °C in an oven and weighed and digested with the acid mixture as mentioned in section 4.2.10. The amount of chromium biosorbed was calculated as mg Cr/g dry weight.

### 4.2.9: Bioaccumulation of Chromium by *B. subtilis* P13

*B. subtilis* P13 grown to 0.6 OD and 1% of culture suspension was inoculated into 100ml LB containing 30 ppm Cr (VI) and 250 ppm of Cr (III) and incubated at 30°C for 24 h shaking at 150 rpm. After 24 h, bioaccumulated chromium was estimated by harvesting and processing the cells by digesting the samples with a mixture of concentrated nitric (six parts) and perchloric (one part) (Srinath et al., 2002). The amount of chromium accumulated was calculated as mg Cr/g dry weight. Uninoculated LB containing the appropriate concentration of Cr (VI) was used as a blank. All the tests were performed in triplicates. To understand the amount Cr(III) accumulated in the system, dry weight of the pellet was noted and subjected to thermal oxidation by heating in a muffle furnace in a silica crucible at 550°C for 6h (Bose et al., 2005) and proceeded as mentioned in section 4.2.10.
4.2.10: Chromate reductase assay

The bacterial culture was grown in 50 mL LB broth for 24 h at 30°C with and without Cr (VI) (10ppm). Chromate reductase activity was checked from three fractions viz, extracellular, cell bound and intracellular. Extracellular activity was checked from the culture supernatant of culture spun at 12,000xg for 5 min. Pellets recovered were washed twice with 10mM potassium phosphate buffer, pH 7.0 and cell bound activity was checked after resuspending the same in 1mL of 50mM phosphate buffer. To study the intracellular activity, cells were harvested by centrifugation at 12,000 x g for 15 min. Pellets were washed twice with 10 mM potassium phosphate buffer (pH 7.0) and were suspended in 3 mL of 50mM potassium phosphate buffer. Cells were disrupted by sonication for 5 min in cold condition. The resultant homogenate was centrifuged at 12,000 x g for 30 min at 4°C; the supernatant was used as a crude extract (Rehman et al. 2008).

Chromate reductase activity of all the three fractions viz, cell free extract (CFE), cell bound and extracellular fractions were assayed following the procedure of Camargo et al., (2003). The reaction mixture (1.0 ml) contained 0.6 ml of 1mM K_2CrO_4 and 0.3ml of 100mM Phosphate buffer pH 7. A n aliquot of 0.1 ml of CFE was added as the enzyme to initiate the reaction. Reduction of Cr (VI) was measured by estimating the decrease in Cr (VI) in the reaction mixture after 30 min of incubation at 30°C. Chromate reductase activity was defined as the amount of enzyme that reduced 1.0µM Cr (VI) per min at 30°C and the unit is expressed in terms of mg protein. Cr (VI) was quantified clorimetrically using 1, 5-diphenylcarbazide (DPC) as the complexing reagent as mentioned in section 4.2.5.

4.3: Results

4.3.1: Growth and protease production by *B. subtilis* P13 in presence of CRS

Growth profile and protease activity of *B. subtilis* P13 were compared in two different media LB and PM using CRS as a protein source and also in combination with SBM (1:1). Considering optimized medium (OM) as the control, growth profile appeared to be similar in all the combinations studied (Fig. 4.1a). Bacterial count was slightly less in the media containing only CRS compared to other combinations of SBM and CRS. CRS might not be a preferred protein source due to the fact that it is a collagen rich leather waste having chromium Cr (III), compared to SBM which
is a rich source of easily assimilable protein. Although growth was similar in all media, there was a significant difference in the protease activity in the media studied. Maximum activity of 2.4U/ml was observed from production medium with SBM after 24h. Optimized medium (OM) containing SBM and CRS (1:1) and also PM containing 1% CRS showed activity of 2.05 and 1.8 U/ml were obtained after 24h (Fig. 4.1b) and was 2.14 and 10 times higher than the respective basal media. Another significant difference observed was that in PM irrespective of the protein sources maximum activity was observed after 18 h. LB being a richer medium might be the reason for prolonged log phase.

![Graph](image)

**Fig. 4.1:** Growth and protease production by *B. subtilis* P13 in medium containing SBM and CRS as protein source.  

a: Growth profile in OM and LB; b: Protease activity profile in OM and LB;

### 4.3.2: Hydrolysis of chrome shavings by *B. subtilis* P13

About 90% weight reduction was achieved in 24h, whereas degradation efficiency was found to be about 56-60% with 5% CRS in 24h and increased to 90% after 48h. In the case of 10% CRS containing media, degradation was even slower and weight reduction of 18-20% and 30-40% were attained at 24 and 48h respectively (Fig. 4.2). The different stages of degradation in OM containing 1% CRS is depicted in **Fig. 4.3a.** Wherever 90% weight reduction was achieved, there was complete liquefaction of the waste and whole medium had taken up bluish tinge of chromium (Fig. 4.3b). When same experiment was repeated using UV-sterilized and non-sterilized CRS, no degradation was observed. Comparable observations were reported from the studies carried out using *A. carbonicus,* where they reported that double sterilization at 15PSI at 15 min. was essential for the degradation. Oil
immersion micrograph (Fig. 4.4) also clearly indicates the disintegration finally resulting in finer particles of the CRS.

Fig. 4.2: Hydrolysis of chromeshavings by \textit{B. subtilis} P13 with respect to weight reduction

a. Different sequences in the hydrolysis of CRS by \textit{B. subtilis} P13
b. Complete hydrolysis of 1% CRS leaving a bluish tinge to the supernatant after 24h-Flask1; Flask2- control containing OM +1%CRS, at 0\textsuperscript{th} h.

Fig. 4.3: Liquefaction of CRS by \textit{B. subtilis} P13.
Chapter 4: Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using *Bacillus subtilis* P13 and exploring its chromium resistance mechanisms

Fig. 4.4: Oil immersion micrograph of chrome shavings during different stages of hydrolysis

4.3.3: Chromium tolerance of *B. subtilis* P13

Since *B. subtilis* P13 could degrade CRS, it was of interest to study its tolerance to Cr. The bacterium could tolerate 35 ppm of Cr (VI) and 350 ppm of Cr III (Fig. 4.5). No visual morphological changes were observed on the plate after growing in presence of Cr. Growth had reduced drastically after a concentration of 25 ppm of Cr (VI) and became negligible at 35 ppm concentration. But in the case of Cr (III), the decrement in growth rate was more or less linear and could grow up to 250 ppm, decreased to 4% at 300, becomes negligible at 350 ppm (Fig. 4.5).

4.5: Effect of Chromium [Cr(III) and Cr(VI)] on growth (24h) of *B. subtilis* P13
4.3.4: Biosorption of chromium by *B. subtilis* P13

Biosorption efficiency of 12 and 24 h old live biomass (50mg dry weight) when studied separately for Cr (III) and Cr(VI) showed that the biosorption efficiency was better with 24 h old culture (**Fig. 4.6 a & b**). Maximum biosorption of 20µg/g was observed in the case of Cr (VI) and 350µg/g for Cr (III) when provided with 30 ppm and 250 ppm of Cr (VI) and Cr (III) respectively. Srinath et al., 2002 reported two *Bacillus* strains showing biosorption of 10mg/ml, when 100 ppm of chromium was provided. Better biosorption efficiency shown by 24h old culture can be attributed to the extrapolymeric substances produced by *B. subtilis* P13.

![Biosorption of Cr](image)

**Fig. 4.6: Biosorption of Cr by *B. subtilis* P13**

a: Biosorption of Cr(III) and b: Biosorption of Cr(VI)

4.3.5: Bioaccumulation of Chromium by *B. subtilis* P13

Bioaccumulation efficiency of *B. subtilis* P13 was evaluated separately using 12 and 24h old cultures and both the cases an increment of 6-10% in bioaccumulation shown by *B. subtilis* P13 after 24h. Residual Cr (VI) concentration in the supernatant was 5±1.09, 8± 2.143 and 19.8±0.962µg/ml in the case of 10, 20 and 30 ppm respectively. At higher concentration, residual Cr were more in supernatant and similar observations are already reported (Srinath et al., 2002) In the case of Cr (III) also maximum bioaccumulation were shown after 24h, leaving a residual Cr(III) of 40±0.894; 52±2.18; 76± 1.60 and 99± 0.921 µg/ml respectively when the concentration of 100, 150, 200 and 250 ppm of Cr(III) were studied (**Fig. 4.7a&b**).

Better bioaccumulation efficiency shown with respect to Cr (III) might be the attributed to the less toxicity of Cr (III) compared to Cr (VI). Better accumulation at lower concentration might be due to better growth rate.
Chapter 4: Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using *Bacillus subtilis* P13 and exploring its chromium resistance mechanisms

### 4.3.6: Chromium reductase activity of *B. subtilis* P13

Cr (VI) reduction experiments were conducted using cell free extract (CFE), cellbound (CB) and extracellular enzymes of *B. subtilis* P13, to understand the component responsible for biotransformation of Cr(VI). The specific Cr (VI) reduction activity obtained for CFE at 30 °C and a pH of 7.0 was 11.6/mg protein/min, whereas cell bound and extracellular reductase activity were about 4U and 0.38/mg of protein respectively (Fig. 4.8). The supernatant obtained after harvesting the cells (extracellular enzymes) showed less chromium reductase activity (Cr(VI) reduction/mg protein/min) compared to CFE. This clearly showed that the Cr (VI) reduction was associated with the soluble fraction of the cells and not with extracellular enzymes.
Chapter 4: Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using Bacillus subtilis P13 and exploring its chromium resistance mechanisms

Fig. 4.8: Reduction of Cr (VI) by different cellular fractions of B. subtilis P13 under static and shaking conditions in presence of Cr(VI) 10ppm.

CB - cell bound; CFE - cell free extract, control is 24 h old culture and treated is the culture grown in presence of 10ppm of Cr (VI).

Chromium reducing capability of the bacterial isolate was checked by adding Cr (VI) at 10ppm concentration in the culture medium and estimating the amount of residual chromium in the CFE (Fig. 4.9). Bacillus subtilis P13 could reduce about 35% in 12h, 65% in 24 h, 82% in 36 h and 90% of chromium from the medium after 96 h. Rehman et al., (2008), reported a Bacillus sp.ev3 capable to reduce Cr(VI) (100 mg/L) at 91% in 96 h.

Fig. 4.9: Time course of Cr (VI) reduction in cell free extract of B. subtilis P13
4.3.7: Biochemical characterization of chromate reductase from *B. subtilis* P13

4.3.7.1: Temperature optima of chromate reductase

Maximum chromium reductase activity was observed at 60°C for a pH of 7.5 (Fig. 4.10). Activity at 50°C was about 50% of that at 60°C and at 40 and 30°C the activity was only 15-20% of the optimum activity. 70°C, enzyme showed only 25% of its optimal activity.

![Fig. 4.10: Optimum temperature of chromate reductase from *B. subtilis*.P13](image)

4.3.7.2: Thermostability of chromate reductase

$t_{1/2}$ of chromate reductase was reached after 12h and 24h at 50-55°C and 60-65°C respectively (Fig. 4.11). But at temperatures lower than 50°C, enzyme retained more than 50% activity even after 24h. At 70°C, thermal inactivation was quiet fast. From the above observations, it can be concluded that the enzyme under present study is probably a thermostable enzyme. Similar studies with purified enzyme will give more insight to the thermal denaturations kinetics of chromate reductase from *B. subtilis* P13.
Chapter 4: Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using Bacillus subtilis P13 and exploring its chromium resistance mechanisms

Fig. 4.11: Thermostability of chromate reductase from *B. subtilis* P13

4.3.7.3: *pH optimum of chromate reductase*

Increase in chromate reductase activity observed from acidic range, decreased drastically at alkaline pH having an optimum at pH 7.5 and about 10% decrease in activity at pH 8.0 (Fig. 4.12).

Fig. 4.12: Optimum pH of chromate reductase from *B. subtilis* P13
4.3.8: Biological degradation of CRS

The biological degradation was assessed on the basis of Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) and bioaccumulation by precipitating as Cr(OH)₃ from the pellet (Table 4.1) and supernatant (Table 4.2) of the media containing 1, 5 and 10% CRS. The efficiency of hydrolysis varied with respect to the amount CRS in both LB and OM. With 1% CRS complete hydrolysis (reduction in weight) achieved in 24h, 5% took 48h and 10% was poorly hydrolysed even after 72h. Reported amount of Cr (III) in chromeshavings varies from 200-300ppm and estimated as 242± 2.1 ppm in the present study. Amount of chromium recovered from 1, 5 and 10 % varied in the range 100 - 1400 and 65 – 350 ppm respectively in the case of supernatant and pellet. Both the parameters increased with increased concentration of CRS. All the parameters studied above were beyond the permissible limits for effluent.

Table 4.1: Effluent parameters, protease activity and Cr (III) recovered from the culture pellet of *B. subtilis* P13

<table>
<thead>
<tr>
<th>Combinations</th>
<th>BOD (ppm)</th>
<th>COD (ppm)</th>
<th>Protease (U/ml)</th>
<th>Cr(III) (µg/g of pellet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB+1% CRS</td>
<td>325±2.87</td>
<td>610±3.031</td>
<td>0.12±0.0056</td>
<td>65±1.028</td>
</tr>
<tr>
<td>PM+1% CRS</td>
<td>356±3.56</td>
<td>520±4.11</td>
<td>0.83±0.0062</td>
<td>80±2.221</td>
</tr>
<tr>
<td>LB+5% CRS</td>
<td>419±2.76</td>
<td>1000±5.63</td>
<td>0.19±0.0073</td>
<td>136±1.32</td>
</tr>
<tr>
<td>PM+5% CRS</td>
<td>429±1.94</td>
<td>890±4.94</td>
<td>0.55±0.0099</td>
<td>158±1.905</td>
</tr>
<tr>
<td>LB+10% CRS</td>
<td>478±1.12</td>
<td>1640±3.11</td>
<td>0.22±0.0027</td>
<td>370±2.280</td>
</tr>
<tr>
<td>PM+10% CRS</td>
<td>491±2.34</td>
<td>1230±2.807</td>
<td>0.33±0.0079</td>
<td>361±4.10</td>
</tr>
</tbody>
</table>
Table 4.2: Chromium recovered, effluent parameters and protease activity from the culture supernatant of *B. subtilis* P13

<table>
<thead>
<tr>
<th>Combinations</th>
<th>BOD (ppm)</th>
<th>COD (ppm)</th>
<th>Protease (U/ml)</th>
<th>Cr(III) (µg/g of pellet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB+1% CRS</td>
<td>130±3.27</td>
<td>450±2.997</td>
<td>0.6 ±0.0054</td>
<td>95±3.028</td>
</tr>
<tr>
<td>PM+1% CRS</td>
<td>112±2.56</td>
<td>370±1.904</td>
<td>1.2±0.0062</td>
<td>108±2.561</td>
</tr>
<tr>
<td>LB+5% CRS</td>
<td>175±3.06</td>
<td>750±3.70</td>
<td>0.54±0.0043</td>
<td>449 ±1642</td>
</tr>
<tr>
<td>PM+5% CRS</td>
<td>150±1.84</td>
<td>520±4.14</td>
<td>1.55±0.0029</td>
<td>503±2.034</td>
</tr>
<tr>
<td>LB+10% CRS</td>
<td>221±4.01</td>
<td>950±1.765</td>
<td>0.11±0.0017</td>
<td>940±2.42</td>
</tr>
<tr>
<td>PM+10% CRS</td>
<td>256±3.46</td>
<td>825±2.37</td>
<td>0.19±0.0067</td>
<td>965±1.990</td>
</tr>
</tbody>
</table>

4.3.9: Solid substratum fermentation of CRS by *B. subtilis* P13

To develop a process for the microbial hydrolysis of CRS with minimum generation of waste, a solid substratum column with minimal addition of nutrients was set up. Fig. 4.13 depicts the visual observation of column during different stages of the study. There was a 54% reduction in column height after 7 days (Fig. 4.13). The remaining 46% of the column height after 7d was occupied by solubilized viscous remnants of CRS containing bacterial biomass. When this was reused as inoculum for a second refill of the column reactor similar performance was achieved confirming reusability of the biomass. The molecular weight of the protein and zymogram nature (Fig. 4.15) confirms that the major protein present in the effluent corresponds to 31 kDa protein which is a dehairing keratinolytic protease. The effluent collected during the entire incubation period was about 10 ml and had protease activity of 4 U/ml (Fig. 4.14) and was not only higher than batch cultures on CRS but also 1.5 folds higher than the protease activity from the optimized medium with soya bean meal. A concentration 0.2U of this preparation could perform dehairing on 5x5 cm of hide (Fig. 4.16) indicating that one batch of the
small reactor with 3g of CRS produced enough enzyme to carry out dehairing of ten pieces of 100x100 cm of hide.

![Image](image-url)

Fig. 4.13: Hydrolysis of chromatshavings by *B. subtilis* P13 from CRS substratum glass column

1, 2 and 3 are control at the begining of the experiment and after 122h and 168h respectively.

![Graph](graph-url)

Fig. 4.14: Correlation of CRS degradation meassured as reduction in column height and protease activity from substratum column hydrolysed by *B. subtilis* P13
Chapter 4: Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using *Bacillus subtilis* P13 and exploring its chromium resistance mechanisms

Fig. 4.15: SDS-PAGE and zymogram of the CRS hydrolysate collected from the column

Lane 1: Mw. marker; 2,3: protein profile and 4,5 are gelatin zymogram of effluent collected after 122 and 168h

Fig. 4.16: Deharing of goat hide using the hydrolysate from CRS column.

Effluent from the column was diluted and applied at a protease activity of 0.2U/g hide
4.4: Discussion

Current study revealed that *Bacillus subtilis* P13, a keratinolytic serine protease producer could hydrolyse CRS by virtue of its tolerance to chromium and capability to utilize thermally processed collagen present in the CRS as a protein source. CRS are small particles, in a variety of shapes, mainly consisting of collagen cross-linked with complexes. Upon chrome tanning the collagen matrix of hides becomes highly stabilized due to the formation of metal ion-mediated coordinated cross links in the protein, involving side-chain carboxyl ions of aspartic and glutamic acids of the collagen (Usha and Ramasami 2000). This has been shown to impart collagen with both hydrothermal stability and stability against enzymatic degradation (Gayatri et al., 2001). The lack of growth of indigenous microorganisms in the waste can be attributed to the recalcitrant nature of the waste. Chrome tanned leather as well as its shavings are therefore not prone to bacterial or enzymatic attack. CRS is recalcitrant to proteolytic action without chemical pretreatment to denature the collagen fibers (Taylor et al., 1998). In this light, the degradation of CRS by *B. subtilis* P13 is remarkable. Autoclaving of CRS was important in rendering it amenable to attack by the bacillus, since no degradation was observed when UV-sterilized CRS used. Thermal denaturation due to autoclaving might result in the conversion of collagen to gelatin, which is known to be efficiently degraded by *B. subtilis* P13. Comparable observations were reported from the studies carried out using *Aspergillus carbonicus*, where it is reported that double sterilization of CRS at 121 °C, 101 kPa for 15 min was essential for the degradation (Katsifas et al., 2004). Barring studies carried out by the latter group no other reports exist on the use of microorganisms directly for the biodegradation of CRS. *B. subtilis* P13 is much more efficient at CRS degradation than *A. carbonicus*, which is reported to liquefy 1% CRS after 12 d whereas *B. subtilis* P13 achieved similar effect in 24h.

Mechanism of metal tolerance or bioremediation is by biosorption, bioaccumulation (Teitzel and Mathew, 2003; Meriah and Tebo, 2002; Katiyar and Katiyar, 1997) or by transformation of heavy metals, entrapment in extracellular capsules, protein DNA adduct formation, induction of stress, transformation of components by oxidation, reduction, methylation and demethylation and by binding cytosolic molecules (Lovely and Coates, 1997; Gadd, 1990; Ksheminska et al.,
Bacillus subtilis P13 exhibited both biosorption and bioaccumulation as mechanisms of Cr tolerance. Srinath et al., 2002 reported two Bacillus strains showing biosorption of 10mg/g, when 100 ppm of Cr (VI) was provided. Rehman et al., (2008) reported a Bacillus sp.ev.3 isolated from metal contaminated waste water which could tolerate 4800 ppm of Cr (VI). Four different species of Bacillus were isolated from Brazil were able to tolerate chromium concentration in the range of 500-2500 mg/l. Apart from Bacillus.sp, Acinetobacter and Ochrobactrum (Francisco et al., 2002), Arthrobacter (Megharaj et al., 2003), Pseudomonas sp (Rajkumar et al., 2005), Serratia marcescens (Campos et al., 2005), Ochrobactrum sp (Thacker and Madamwar, 2005), were also reported for its tolerance to chromium.

In the case of B. subtilis P13 mechanisms like, bioaccumulation, biosorption and the presence of reductase system have been attributed as the mechanism of chromium tolerance. Studies from different cellular fractions confirmed that chromate reductase in B. subtilis P13 present in the intracellular fractions of B. subtilis P13. The temperature optimum of 60°C confirms the thermophilic nature of chromate reductase from the under study. Wang et al., (1990) reported that no chromate reduction was observed at 4 and 60 °C by E. coli. The optimum pH and temperature for chromate reductase extracted from Bacillus sp. ES 29, Bacillus sphaericus 303, E.coli ATCC 33456, Actinomycete, and Arthrobacter crystalllopoietes ES 32 were reported to be between pH 5 to 9 and 30°C, respectively (Camargo et al., 2003; Bae et al., 2005). Chromium recovery studies carried out using B. subtilis P13 indicated the better recovery from the supernatant compared to the pellet and similar observation was reported by Katsifas et al 2004; using A. carbonarius and could recover 25-28µg/g of chromium. COD was higher in the supernatant and BOD in the pellet in our study and also in the previous report (Katsifas et al., 2004).

Reports on hydrolytic degradation of CRS are focused on the use of enzymes or the combination of alkaline treatment and enzymatic hydrolysis (Kanagaraj et al. 2006). A. carbonarius strain reported by Katsifas et al. (2004) to degrade CRS has been envisaged as useful tool in the tanning industries resulting in the degradation of CRS and recovery of valuable Cr. Similarly B. subtilis P13 can also be considered useful for solid waste treatment and Cr recovery. The advantages of this organism
are that it is more efficient and during management of CRS it also exhibits protease production which is of use in pre-tanning processes. Thus the CRS management process described here offers a valuable by product which is useful for the upstream tannery processes. This additional potential of the \textit{B. subtilis} P13 pronounce it as a proficient candidate for in-house waste management and saving the cost for the purchase of a valuable consumable commodity needed for the pretanning processes.

The proposed eco-friendly process could be scaled up to tannery application by designing the projected continuous reactor (Fig. 4.17) where chrome shavings (thermally denatured at 100°C) could be added as feed at 10% in PM in a vessel, followed inoculation with 2% (w/v) of 0.6 OD \textit{B. subtilis} P13. Liquid oozing out (effluent) will have highly concentrated keratinolytic protease enzyme, which could be applied in pre-tanning processes such as soaking, dehairing and bating. Biomass generated can be reused in a continuous manner as the seed for hydrolysis and this process could be a better way of in-house waste management and byproduct recovery.
Chapter 4: Bioprocess development for the management of chrome shavings, a solid waste generated in tanneries using Bacillus subtilis P13 and exploring its chromium resistance mechanisms

Fig. 4.17: Schematic representation of process for the byproduct recovery and inhouse waste management
4.6: Conclusions

*B. subtilis* P13, a hot spring isolate, was able to effectively degrade and grow using chrome shavings as the protein source and the spent medium showed high production of keratinolytic serine protease which is efficient in dehairing of hides. The bacterium was moderately chromium resistant tolerating up to 35 ppm and 350 ppm of Cr (VI) and Cr (III), respectively and showed bioaccumulation and biosorption of Cr (III) and Cr (VI). Growth profile and enzyme production were comparable in basal and production media containing chrome shavings. An efficient waste management process was developed using solid substratum column reactor leading to the liquefaction of the proteinaceous waste, recovery of the dehairing protease as concentrated product. A continuous reactor scheme is proposed, where the biomass can be reused as the seed for hydrolysis for in-house waste management and byproduct recovery for tannery industry. Apart from the capacity to produce a protease which finds application in pre-tanning processes, this additional potential of the *B. subtilis* P13 pronounces it as a potential candidate for in-house waste management and the recovery of a valuable by-product, protease. Findings from the present study can be developed as a sustainable, eco-friendly and cost-effective process in tanneries.