Protein Extraction from Chromium Tanned Leather Waste by *Bacillus Subtilis* Enzymes

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Abstract

Leather industry has been facing with new challenges and the need to improve and optimize processes in order to achieve required quality in their final articles as well as meet the environmental legislation. From each ton of hides, we can estimate that about 20 % will be transformed in chromium tanned wastes. The enzymatic treatment of chromium tanned leather wastes is a promising technology. In this work is described the extraction of proteins from chromium tanned wastes using crude enzymatic extracts of cultures of two new *Bacillus subtilis* strains. The aerobic sludge of a tannery was used as source of the microbial community for screening and selection of microorganisms. The tanned wastes were treated with alkali and then with the crude enzymatic extract. This process permit the obtaining of gelatin and hydrolysate protein, that can be applied at fertilizer, retanning agents and in cosmetics industry, and a sludge chromium concentrated, from which chromium can be recuperated and reused for leather tanning. So, this is a safe alternative for treatment and reutilization of chromium tanned waste.

Keywords: enzymes, hydrolysis, reuse, wastes

1. Introduction

Reduce wastes generation and deposited in landfills are some of the actual leather industry challengers. The demand for leather products is increasing worldwide with rapid growth of the world population in the last century, consequently, the wastes generate during hides and leather manufacture increase also. According to the Center for the Brazilian Tanning Industry (Center for the Brazilian Tanning Industry, 2012), in the beginning of 2012, Brazil exports 36.9 tons of hides and skins per month.

The tanning method most frequently used is the treatment with chromium salts, can be estimate that approximately 700 kg of waste (tanned and untanned) are generated per ton of processed hide. From the total of waste generated approximately 210 kg are tanned wastes and the finished product constitutes only about 26 % of initial hide weight (Industrial Engineering and Technology Institute, 2000).

Among the solid waste from manufacture of chromium tanned leather, trimmings and shavings produced during leather thickness adjustment are the major problem due to the large amount of waste generated and high levels of chromium, classified as hazardous waste according to ABNT NBR 10004:2004 (Brazilian Laws). So it must be stored in specially constructed landfills for hazardous industrial waste; many alternatives for treatment and reutilization has been studied (Dettmer et al. 2010a,b,c; Catalina et al. 2010; Piccin et al. 2012; Ocak et al. 2011; Matyasovsky et al. 2011). The shavings from wet-blue thickness adjustment represent 90 % of solid waste generate in chromium tanning process. The leather thickness adjustment produces on average 4.5 kg of shavings per hide (Daut et al. 2007).

The waste hydrolysis in aqueous medium is an alternative for reutilization of trimmings and shavings; it does not involve high technology or high investments (Ribeiro 2003). Hydrolysis can be defined as a breaking of polypeptide chains into small peptide fragments or amino acids and can be accomplished by heating the protein in acid or alkaline solutions. According IULTCS (2012), the waste hydrolysis process can be conducted: by chemical process (acid or alkali) and by enzymatic process.

In the process of chemical hydrolysis, acid and alkaline processes have one thing in common: phases rich in proteins that can be used as animal feed or fertilizer, due to the low molecular weight protein obtained by these processes (Taylor 1993). In alkaline hydrolysis process can be used: calcium oxide, calcium hydroxide, magnesium oxide, sodium hydroxide, potassium hydroxide. Temperature is an important variable in the process since at higher temperatures the reaction kinetics is favored, but at temperatures around 80 to 90°C the precipitation and filtration of chrome are better. The choice of reagent that is used depends on the end product to be obtained: gelatin, glue, protein, animal feed, among others tanning auxiliaries, cement, plaster, and, ceramic components (Gutterres 2008). Several authors dedicate yours researches to chemical and enzymatic hydrolysis of chromium tanned leather (Taylor et al 1990; 1991; 1992; 1998a,b; Amaral 2008; Gutterres et al 2010; Liu et al. 2012; Aslan et al. 2006).

Enzymatic digestion of chromium tanned wastes results in a protein hydrolysate and gelatin of high quality and also a chrome sludge. The hydrolysate can be used in retanning agents, as foam stabilizers, in plaster industry and agglomerates. The chrome sludge can be reused in the production of chromium sulfate (IULTCS, 2012).

This study aimed to evaluate the efficiency of protein extraction through chemical and enzymatic hydrolysis of chrome solid waste generated during leather thickness adjustment. Analyze the composition of specific products and by-products obtained after treatment and possible use in industries.

2. Materials and Methods

2.1 Analytical Methodologies

The solid chromium tanned wastes used in the study were provided by a local tannery. They were characterized about pH, moisture, ashes, total chromium and total Kjeldahl nitrogen (TKN). Values of samples pH were determined in accordance with ASTM D 2810-72:1996. About 5 g of solid wastes were weighed in triplicate, 100 ml of distilled and deionized water

was added, the triplicates were placed under stirring for 24 hours, and then filtered, and the pH of the solutions was measured potentiometrically.

Moisture was determinate according ABNT NBR 11029:2012; 3 g of solid wastes were weighed into dry and weighed crucibles. Samples were heated to 102 ± 2 °C until constant weight, approximately 12h.

Ashes content was determinate weighed in triplicate 3 g of solid waste in porcelain previously weighed crucibles. Samples were heated to 600 °C until constant mass, according ASTM D2617:1969. For determination of total chromium content of solid waste and hydrolysis products (cake and liquid), the samples were analyzed by atomic absorption spectrometry based on Standard Methods (2012). For the solid chromium waste and the cake obtained after the hydrolysis, an acid digestion was necessary and it was performed according to ASTM D6656:2001. For the determination of total chromium content of the liquid hydrolysis product, acid digestion was not necessary.

In order to determine the content of protein in the hydrolysis products and byproducts were performed analyzes for TKN content, executed according to ASTM D2868-96.

2.2 Chemical hydrolysis

Before enzymatic hydrolysis, the solid chromium tanned wastes were submitted to chemical alkaline hydrolysis; in this step the aim was determine the better chemical for protein extraction before enzymatic hydrolysis. The methodologies were based on the work of Brown et al. (1998).

The chemical alkaline hydrolyses were performed in duplicates and two bases were used; a solution of sodium hydroxide (NaOH, 0.1 M) and magnesium oxide. To perform the alkaline hydrolysis were weighed 50 g of solid chromium tanned wastes and added 250 mL of distilled water. The alkaline solution/reagent was added until the samples reached pH values about 9.0±0.5, were utilized approximately 220 ml of NaOH solution and about 2g of magnesium oxide. The samples were kept under stirring of 60 rpm and 70 °C for 15 hours. Then the samples were filtered using Buchner funnel and the solid and liquid phases were separated and analyzed for chromium content and TKN.

2.3 Chemical and enzymatic hydrolysis

Assays of enzymatic hydrolysis were carried out with two crude proteolytic enzymes extracts (A and B), obtained from cultivation of two new *Bacillus subtilis* strains, previously characterized by Dettmer (2012a, b, c).
a) enzyme A: obtained by cultivation of *Bacillus subtilis* Blbc 11, collagenolytic activity of 61.33 U/ml, pH value for maximum activity 9, thermally stable when exposed for 2 hours at 45 °C, proteolytic activity of 130.5 U/ml;
b) enzyme B: obtained by cultivation of *Bacillus subtilis* Blbc 17, collagenolytic activity of 135.70 U/ml, pH value for maximum activity 10, thermally stable when exposed for 2 hours at 45 °C, proteolytic activity 133.6 U/ml.

The chemical and enzymatic hydrolysis process consisted of two stages: chemical alkaline hydrolysis, with the goal of obtaining gelatin (collagen) and enzymatic hydrolysis step; were amino acids and a chromium cake was obtained.

In the first stage, 50 g of solid chromium tanned wastes were weighed in quadruplicate and added 250 mL of distilled water; magnesium oxide was added until pH value of the solution reached 9.0±0.5. The samples were maintained under stirring of 60 rpm and 70 °C for a period of 6 hours. After this period the samples were filtered under vacuum, the solid and liquid phases were separated and analyzed for their content of chromium and TKN.

For the second stage, the chromium cake obtained from first stage was used. Before the enzymatic hydrolysis was necessary to adjust the medium pH, for better enzymatic action. For the enzyme A pH value was adjusted to 9.0 and for enzyme B pH value was adjusted to 10.0. Based on work of Dettmer et al. (2012a, b, c) were used 300 U of enzymatic activity/g of solid waste. Around 115 ml of crude enzymatic extract of enzymes A and B were added, samples were maintained at 45 °C and stirring of 60 rpm during 15 h. The samples were filtered; the solid and liquid phases were separated and evaluated about their chromium and TKN content.

3. Results and discussion

3.1 Characterization of solid chromium tanned wastes

The results of solid chromium tanned wastes characterization are presented in Table 1.

Table 1 – Characterization of solid chromium tanned wastes

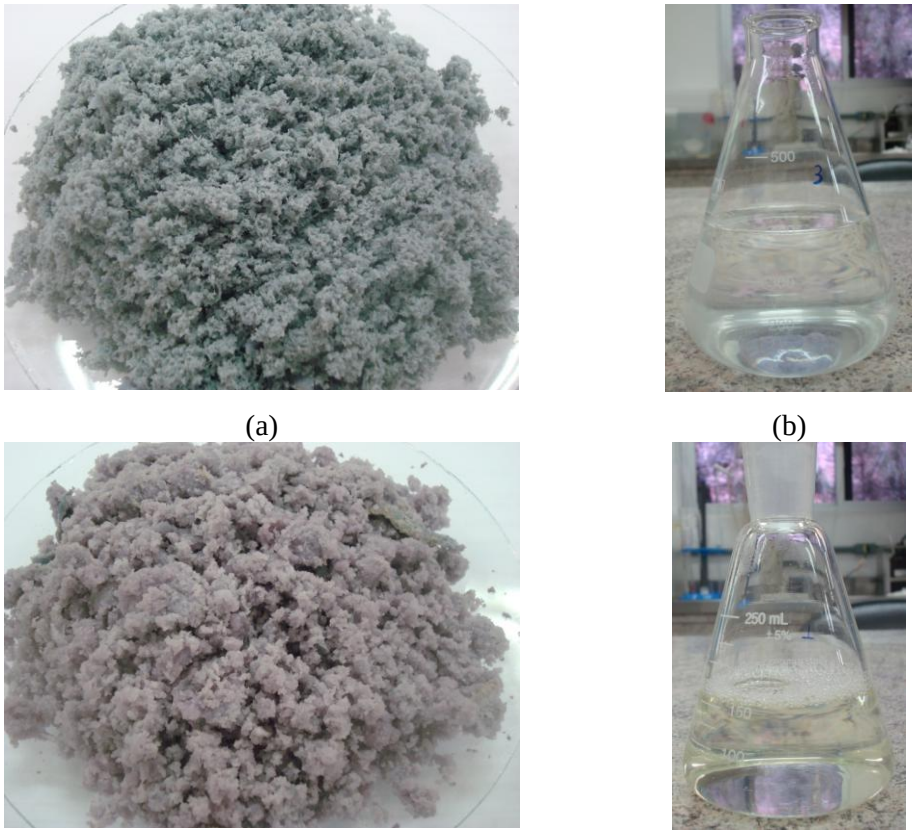
Parameter	Results
Total Cr	2,74 %
Moisture	53,58 ± 0,2 %
pH	3,34
Ashes (d.b.)*	7,38 ± 0,5 %
TKN(d.b.)*	14,47± 3,3 %

Notes: *d.b.= dry basis; Values are means ± standard deviation for three samples.

According to the results presented at Table 1 can be observed that the values for moisture, TKN and chromium are close the values presented by other authors. For the value found for ashes, which characterizes the sample in relation to its content of salts and determine the amount of non-volatile inorganic material, there is a variation with respect to some results of literature (Silva, 2008), this happens because the ash content vary according to the nature of each sample and each tanning process.

3.2 Chemical Hydrolysis

At this stage of the experiment we tried to obtain a comparison between two alkalis in the extraction of protein. The alkalis chosen were sodium hydroxide and magnesium oxide. Figure 1 present the products obtained from alkali hydrolysis.



(a) (b)

(c) (d)

Figure 1 – Products of alkaline hydrolysis (a) chromium cake and (b) gelatin obtained using NaOH 0.1M, (c) chromium cake and (d) gelatin obtained using MgO

The products obtained from basic hydrolysis using NaOH 0.1 M solution, showed different aspects of the hydrolysis products obtained utilizing magnesium oxide. For hydrolysis with magnesium oxide was observed the formation of a gelatinous liquid, slightly yellowish, yellow coloration is characteristic of collagen, but can also be caused by oxidation of Cr^{3+} to Cr^{6+}; according to Dexheimer (2006) this oxidation can occur due high pH values. Under the conditions used in this work the possibility of oxidation is remote, due to the pH not being extremely high and the temperatures used were mild. The liquid obtained by hydrolysis with NaOH 0.1 M showed no gelatinous aspect, this indicate that not occurred protein solubilization. The results obtained for the alkali hydrolysis are shown in Table 2.

Table 2 - Results of chromium and TKN analyzes after alkaline hydrolysis (average of duplicates)

Parameter	**NaOH 0,1M**		**MgO**	
	Solid	Liquid	Solid	Liquid
Total chromium	2,76 %	0,396 mg/L	2,99 %	0,133 mg/L
TKN	15,46 %	0 %	12,27 %	0,35 %

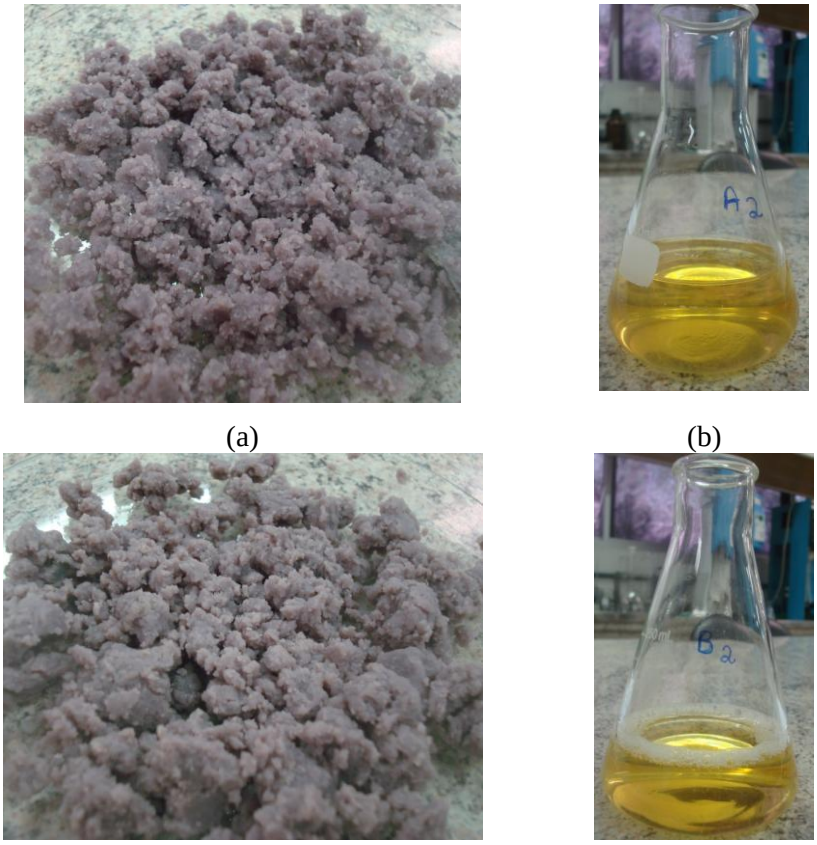
Through the results of Table 2 it possible observes that the extraction with NaOH solution was not effective because there is no TKN in liquid material. Hydrolysis using magnesium oxide method was more efficient, protein solubilization has occurred, the values of TKN found in the liquid product were satisfactory if compared with the results obtained in the study of Brown et al. (2012) that present a value of 11 mg of TKN/l or 0.00011 %.

Mean values for chromium content in liquid, using magnesium oxide for extraction, were relatively low. However, since the product exhibited a certain amount of chromium may not be used as supplement in animal feed, according to ordinance n° 55.871 from March 26, 1965 (Brazilian Law), concerning the rules governing the use of food additives, which regulates the maximum value of chromium allowable is 0.10 mg/l. The products obtained can be used as a fertilizer in soil. The cake containing chromium may be subjected to a chromium recovery process and thus it may be used again in the tanning of hides.

The process of alkaline hydrolysis using magnesium oxide was more efficient, so this reagent was chosen to perform the enzymatic hydrolysis.

3.3 Chemical and enzymatic hydrolysis

To increase the protein extraction, proteolytic enzymes A and B were used. The products obtained by both enzymatic hydrolysis showed similar appearance. The liquids obtained in the first stage (reaction with magnesium oxide) showed gelatinous aspect; liquids obtained in the second stage (reaction with proteolytic enzymes) showed no gelatin aspect to be formed by amino acids which are formed by smaller chains. Figure 2 present the products obtained from enzymatic hydrolysis.



(a) (b)

(c) (d)

Figure 2 – Products of enzymatic hydrolysis (a) chromium cake and (b) amino acids obtained using enzyme A, (c) chromium cake and (d) amino acids obtained using enzyme B

Table 3 present the results for chromium concentration and TKN for the chemical hydrolysis with MgO (first stage) and enzymatic hydrolysis (second stage).

Table 3 - Results for chromium analyzes and TKN for chemical and enzymatic hydrolysis (average of duplicates)

Parameter	Chemical Hydrolysis		Enzymatic hydrolysis			
	MgO		Enzyme A		Enzyme B	
	Solid	Liquid	Solid	Liquid	Solid	Liquid
Total Chromium	2,78 %	0 %	3,66 %	0,171 mg/L	3,82 %	0,359 mg/L
TKN	13,07 %	0,26 %	6,88 %	0,85 %	5,55 %	0,86 %

As can be observed at Table 3, a liquid without chromium was obtained at first stage (chemical hydrolysis), showing that solubilization of chromium into the liquid do not occur. The hydrolysis process using an extraction time of 6 hours was more efficient, comparing this result with the values found in the hydrolysis process performed in a time of 15 hours. Longer extraction time resulted in increase of chromium solubilization, as shown in Table 2.

Due to TKN content and the absence of chromium, the liquid product obtained from the first stage can be used for manufacture of animal feed supplement and also gelatin, results obtained in this work are similar to the values found by Amaral (2008) that found about 0.31 % TKN and absence of chromium.

The results obtained in the second stage (enzymatic hydrolysis) have showed the efficiency of the enzymatic action, it is observed that the protein contained in the chromium cake was solubilized for liquid.

The results from this study are similar to results reported by Brown et al. (1996), the authors suggest that chromium cake obtained in the hydrolysis process can be chemically treated to produce chromium retanning products; another possibility would be use the recovered chromium in cement industry. The liquid obtained from the hydrolysis process may be used as a fertilizer ingredient. It can be observed that the liquid extracted from the hydrolysis carried out by the enzyme A, had lower chromium content. The increase in cake chromium content is due to the solubilization of protein from solid waste to liquid and the consequent decrease of mass. For TKN concentration both hydrolysis products presented similar levels. Thus, the process of hydrolysis using the enzyme A was more efficient because there was a better separation of protein and chromium.

4. Conclusions

From the results obtained in chemical and enzymatic hydrolysis, can be concluded that alkaline hydrolysis with magnesium oxide was more efficiently and solubilized more proteins. TKN values found in the liquid product were satisfactory in hydrolysis with magnesium oxide, but showed a quantity of chromium above the maximum permitted for food additives; therefore these products cannot be used as a supplement for animal feed, but can be used as a fertilizer in soil.

For the chemical and enzymatic hydrolysis in the first stage of the hydrolysis, the liquid product obtained showed no chrome due to the shorter time of extraction, the product obtained can be used for the production of animal feed supplement and also gelatin. Enzyme B had better results in solubilization of the protein but also there was a higher solubilization of chromium to the liquid. The chromium cake obtained in the hydrolysis process can be used as chromium retanning product, and also in the cement industry and the liquid obtained may be used as an ingredient in soil fertilizer.

The best option is chemical and enzymatic hydrolysis, using MgO followed by enzymatic hydrolysis and so a hazardous waste can be transformed into value-added products and reduce the amount of deposited waste landfills.

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