Applications of Biotechnology in Leather

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Abstract: There are any definitions for biotechnology, in according with the convention about biology diversity of ONU: "Biotechnology is the use of knowledge about biologic processes and about living beings proprieties, with the end to resolve problems and create utility products". In recent years the industries have seen a growth in the use of biotechnology, using processes like biocatalyst and biotransformation. The use of biotechnology presents a lot of advantage for industries. Some advantages are: commonly the reaction or biotechnological processes occur at low temperatures and at atmospheric pressure, the process has a high space-time yield, renewable ingredients and in many cases there is reduction of environment impact. In leather production, biotechnology, mostly using enzymes, can be applied in different steps of process: soaking, unhairing, bating, dyeing, degreasing or in effluent and proteinaceous solid wastes treatment. In the present study, two processes of soaking and liming were tested, an enzyme-assisted test, using chemicals and enzymes, and a conventional test using only chemicals. From the analysis of chloride removal and fat removal from the hide and of SEM images, it was possible to realize that the enzyme-assisted is better when the variable in question is the time in the soaking, as well as it is more efficient for the unhairing stage. In other studies, the experiments to remove chromium from leather wastes through enzymatic action, showed reduction of 53.7% of residues mass. Experiments of waste treatment with bacterial *Pseudomonas aeruginosas* obtained reduction of 57.4% of chromium quantity present initially. So, biotechnology can be used in leather production and it will contribute for the reduction of pollution, principally, of soil and water.

Keywords: biotechnology, leather, enzymes.

1. Introduction

The development and disorganized industrial growing have caused serious problems to people worldwide. It is, therefore, necessary the diffusion of ideas of sustainable development, where the natural resources are used rationally, and mostly where it is possible to maintain the ecosystems that now exists, through the reduction of pollution of air, soil and water.

In this context, we can insert biotechnology. The biotechnology has impacts in any productive sector, offering new employ opportunities, providing plants that resist to diseases, biodegradable plastics, biofuels, less pollutant industrial processes.

There are any definitions for biotechnology, in according with the convention about biology diversity of ONU: "Biotechnology is the use of knowledge about biologic processes and about living beings proprieties, with the end to resolve problems and create utility products".

Biotechnology is also the knowledge that permits the utilization of biological agents (organisms, cells, organelles, molecules) to obtain goods or to ensure services. Biotechnology is present in different knowledge areas, like basic science (molecular biology, microbiology, cellular biology, genetics and embryology), applied science (immunological techniques, chemistry and biochemistry) and other technologies (informatics, robotic and process control). The table 1 shows some products with biotechnological origin.

Table 1 – Products with biotechnological origin and it sectors

Sectors	Goods and services
Agriculture	Feed additives, fertilizer, transgenic plants
Food	Bread, cheese, beer, unicellular proteins, enzymes used in food preparation
Chemicals	Buthanol, acetone, glycerol, acids, enzymes, polymers, biocatalysis
Electronics	Biosensors
Energy	Ethanol from biomass, biogas
Environment	Recuperation of petroleum, waste treatment, water purification
Pharmaceutical	Antibiotics, hormone and other products, vaccines, reagents and tests to diagnostic, glycoprotein engineering
Materials	Paper, textile, ***leather treatment***

Biotechnological processes applied in leather production, allowing the reduction of environment impact of this activity. Nowadays, tanneries use high quantities of water and harmful products, like chromium and sulfide, generate a high levels of effluent that must be treated and solid wastes that could be reused or better treated. In leather production, biotechnology, mostly using enzymes, can be applied in different steps of process: soaking, unhairing, bating, dyeing, degreasing or in effluent and proteinaceous solid wastes treatment.

1.1 Enzymes

Enzymes are organic substances, generally proteins, known like biocatalyst to multiples chemicals reactions and are commercially exploited in detergent, food, pharmaceutical, diagnostics and fine chemicals industry. They are between the more notable biomolecules due their extraordinary specificity and catalytic power, which are higher that the catalytic power of catalyst produced by man.

1.1.1 Screening and selection

Most industrial enzymes are obtainable from microorganisms, but they can be obtained too from animals and plants. The advantages of using microorganisms are numerous, in contrast with their production from plants and animals and are as follows:

(a) Plants and animals grow slowly in comparison with microorganisms;

(b) Enzymes form only small portions of the total plant or animal and large tracts of land as well as huge numbers of animals would be necessary for substantial productions. These limitations make plant and animal enzymes expensive. Microbial enzymes on the other hand are not subject to the above constraints and may be produced at will in any desired amount.

(c) By far the greatest attraction for the production of microbial enzymes, however, is the great diversity of enzymes which reflects the diversity of microbial types in nature. Microbial enzymes have been isolated which operate under extreme environmental conditions, for example, at temperatures as high as 110°C and at pH values as high as 11 or as low as 3.

(d) Finally, following from greater understanding of the genetic basis for the control of physiological function in microorganisms it is now possible to manipulate microorganisms to produce virtually any desired metabolic product, including enzymes.

Microorganisms that produce enzymes can be isolated from soil, water or wastes. The primary stage in the isolation and selection of microorganisms is to isolate strain(s) capable of producing the target product. This approach results in intensive screening programs to test a large number of strains to identify high producers having novel properties (Kumar and Takagi, 1999).

The conventional practice with many microbial products is to grow a large number of organisms on agar plate media. Normally, organisms are isolated by surface plating on medium and subsequent screening for the desired characteristics. The organisms are further grown on specific media for estimating proteolytic, amylolytic or lipolytic activities using appropriate substrates such as skim milk

or casein, starch, tributyrin, butter fat. The isolates exhibiting desired level of activity are chosen and maintained on slants for further use.

1.1.2 Enzymes production

Most enzyme production is carried out in deep submerged fermentation; a few are best produced in semi-solid media (Okafor, 2007). Most fermentors used are of the submerged type, because the submerged fermentor saves space and is more amenable to engineering control and design.

Semi solid medium – commonly the organisms used are fungi, which appear amenable to high enzyme production because of the low moisture condition and high degree of aeration of the semi-soluble medium. The temperature is kept at about 30°C by the circulating cool air. The production period is usually 30-40 hours, but could be as long as seven days. The optimum production is determined by withdrawing the growth from time to time and assaying for enzyme.

Submerged production - Most enzyme production is in fact by submerged cultivation in a deep fermentor. The medium must contain all the requirements for growth, including adequate sources of carbon, nitrogen, various metals, trace elements, growth substances, etc. However, a medium adequate for growth may not be satisfactory for enzyme production. Temperature and pH requirements have to be worked out for each organism and each desired product. The temperature and pH requirements for optimum growth, enzyme production, and stability of the enzyme once it is produced are not necessarily the same for all enzymes. The temperature adopted for the fermentation is usually a compromise taking all three requirements into account. The oxygen requirement is usually high as most of the organisms employed in enzyme production are aerobic. Vigorous aeration and agitation are therefore done in the submerged fermentations for enzyme production. Batch fermentation is usually employed in commercial enzyme fermentation and lasts from one to seven days.

1.1.3 Enzyme extraction

In general the initial step of enzyme extraction is to separate solids, mostly cells, from the liquid fraction thereby facilitating further extractive steps. Removal of insoluble material can be done by centrifugation, filtration or decantation. Most enzymes are extracellular in nature. In the case of cell bound enzymes, the cells are disrupted before centrifugation and/or vacuum filtration. The extent of the purification after the clarification depends on the purpose for which the enzyme is to be used. There are many methods for the enzyme purification (fractional precipitation; chromatography; chemical derivatization). The final isolation of the product is done in one of the two ways: (a) processing of crystalline products - crystalline products are free-filtering and non-compressible and therefore may be filtered on thick beds under high pressure. This is usually done on a centrifugal machine capable of developing very high (about 1,000 fold) gravitational force. The crystals are washed to remove adhering mother liquor. After washing they are dried by spinning for further drying or solvent removal. (b) drying of products direct from solution. Drying consists of liquid removal (either organic solvent or water) from wet crystals such as was described above from a solution, or from solids or cells isolated from the very earliest operation. Several methods of drying exist and the one adopted will depend on such factors as the physical nature of the finished product, its heat sensitivity, the form acceptable to the consumer, and the competitiveness of the various methods in relation to the cost of the finished product (Okafor, 2007).

1.1.4 Packaging and finishing

The packing of enzymes has become extremely important since the experience of the allergic effect of enzyme dust inhalation. Nowadays, enzymes are packaged preferably in liquid form but where solids are used, the enzyme is mixed with filler and it is now common practice to coat the particles with wax so that enzyme dusts are not formed.

1.2 Biotechnology and leather process

According to Thanikaivelan *et al*. (2004), biotechnology has been used in the tanning industry for several years. Currently, the majority of enzymatic preparations for the tanning industry do not have sufficient specificity. The authors explains that the principal advantage of an enzyme soak are the shorter wetting time, better fiber opening, solubilization and removal of proteins, fat and carbohydrates.

Enzymes can be used too on unhairing processes, on epidermis and hair removal, removal of residual components, removal/dispersion of adipose components, and reduction on effluent load.

Macedo *et al*. (2005) studied the capacity of removal of hairs from a keratinase obtained from *Bacillus subtilis*. The authors observe that the enzyme does not hydrolyze collagen and has efficiently hair removal, maintaining the same conditions (pH, time) of traditional unhairing process, but without using sulfide.

Dayanandan *et al*. (2003) studied the unhairing of hides using an alkaline protease, isolated from Aspergillus tamarii. The physical proprieties of the experimental leather in comparison with the control sets (traditional process) gave better results with respect to tensile strength and elongation at break.

During the bating process, enzymes can act on removal of degraded hairs and epidermis, promote the removal of non structural proteins and help on carbohydrates removal.

Kanth *et al*. (2008) study the application of a bacterial collagenase in leather dyeing. The authors obtained great results, uptake of dye as high as 99% has been observed by treatment with collagenase. Using the conventional process the exhaustion of dyes was found to be 85%. The utilization of enzymes improved the softness of leather, while the strength characteristics are not significantly altered.

Enzymatic treatment can be used too on wastes generated during the leather process. For not tanned wastes can be used proteolyses enzymes, neutral and alkaline, generating hydrolysates rich in fat and proteins, in temperature about 50°C. To tanned wastes, containing chromium, can be obtained three fractions: the cake containing chromium, proteins and hydrolysated collagen.

Kumar *et al*. (2008) studied the obtaining of an alkaline protease from *Pseudomonas aeruginosa* using proteinaceous wastes (not tanned) from tanneries. The authors say that the microbiological method to hydrolysate proteinaceous waste is an interesting alternative to other methods, like chemical and thermal, used nowadays to treatment of solid waste.

In this work, enzymes were used during soaking and liming operations. Additionally, were studied the bacterial decomposition of tanned leather wastes and the possibility to remove chromium contained in this wastes, using the bacteria, *Pseudomonas aeruginosa.*

2. Methods

2.1 Enzymatic leather process

Two process of soaking and liming were developed, one of them was called enzyme-assisted test (E-A A), using chemicals and enzymes and the other was called conventional test (C) using only chemicals. The tests were performed at laboratory scale in drums. Two kinds of liquid enzymes, called protease A (AP) and lipase A (AL) were provided. More two tests were carried out with only each one of these enzymes. Chemicals used in leather processing and biochemical analysis were of commercial and analytical grade, respectively.

The processing time to the stage of soaking (enzyme-assisted soaking) was four hours and for the conventional soaking was five hours. The liming stage lasted 12 hours for both the enzymatic process as for the conventional process.

The chemicals and enzymes used in both process, conventional and enzyme-assisted, in the soaking stage are described in the table 2, with the percentage of application calculated on the weight of the hide (approximately 250g of each sample). Each test was carried out from the beginning.

Table 2 - Products used for each process in soaking stage

	Conventional	Enzyme-assisted		
	Test C	Test A	Test AP	Test AL
	Weight percentage of application [%]			
Water	200	200	200	200
Sodium carbonate	0.3	0.3	0.3	0.3
Surfactant	0.15	0.02	0.02	0.02
Protease A	0	0.07	0.07	0
Lipase A	0	0.03	0.0	0.03

In soaking stage the chlorides concentrations in the wastewater and the content of extractable substances with dichloromethane from the hides were analyzed.

In the liming stage, the weight percentages of products were added according to table 3. Scanning electron microscopy (SEM) analyses were used in order to show the differences between the results of each process.

Table 3 - Products used for each process in liming stage

	Water	Lime	Sodium sulfide	Sodium carbonate	Mercaptan	Fatty alcohol	Lipase A	Protease A	Surfactant
	Weight percentage of application [%]								
Conventional Test	210	4.1	1.6	0.3	0.6	0.1	0	0	0
Enzyme-assisted Test A	210	4.6	1.1	0.6	0	0	0.04	0.04	0.08

2.2 Chromium removal of leather wastes

Samples of chromed leather waste shavings used in the experiments were taken from a tannery which produces wet-blue leather in a traditional process. Two methods were tested to remove chrome from the waste: enzymatic and bacterial treatment. For the enzymatic hydrolysis, an experimental alkaline protease in liquid solution was provided from a firma. For the bacterial treatment an isolated gram-negative aerobic *Pseudomonas aeruginosa* species was provided from a microbiology laboratory of the university. The identification of the isolated bacteria was done through DNA sequencing.

The chromed leather waste was incubated with the protease enzyme under different conditions in order to determine the most efficient conditions for the hydrolysis. The experiments were carried out as follows: 5 g of leather waste and 100 ml of distilled water were heated and agitated for a specified period of time before basification with calcium oxide. The pH was measured and the enzyme was added under agitation and constant temperature. After a certain period of time, the suspension was filtered. The hydrolysis efficiency was calculated in terms of the quantity of solid (dry basis) which remained in the cake after drying at 102 ° C for at least 16 hours.

The tests with the bacteria were incubated under the appropriate conditions of temperature and agitation required for bacterial growth (incubation time of 7 hours, presence of sucrose and pH adjustment to 7.0). After the incubation period the suspension was filtered, the cake remaining on the filter was washed with sodium lauryl sulfate solution to remove the bacteria adhered to the waste and the sample was filtered again.

3. Results and Discussion

3.1 Enzymatic leather process

The figure 1, presents the chlorides assays results of wastewater; the tests were realized in triplicate and the graph shows the standard deviation.

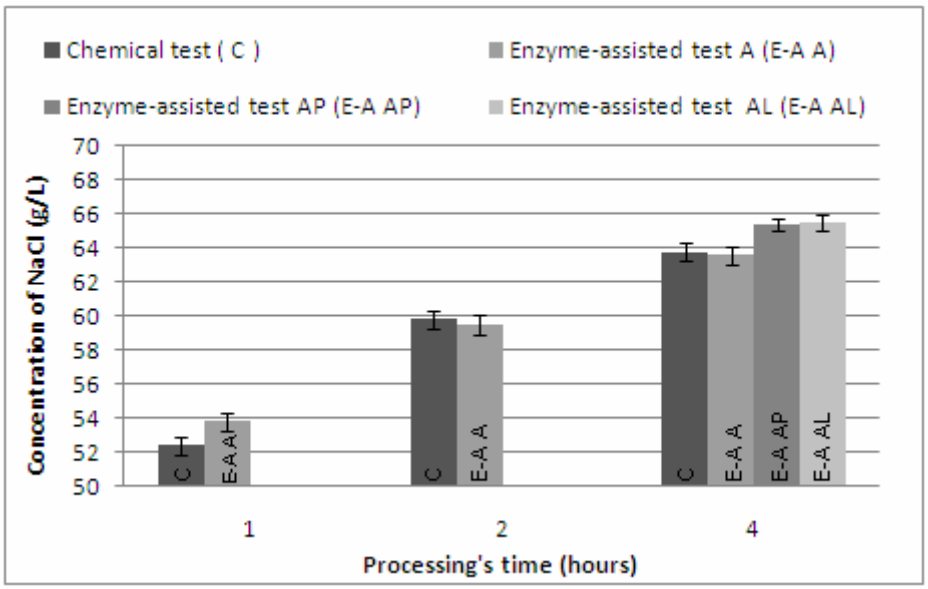

Figure 1 –Chloride concentration of wastewater by soaking

From this figure, it is possible to realize the time influence of soaking over the removal chlorides from the hides. The best results were obtained from 4 hours of soaking. The biggest chlorides removal for the enzyme-assisted process, in the initial times, is explained on the opening collagen fibers capacity which was provided for the hide by the enzymes. However, after a long time the conventional process reaches similar removal results.

The results of the contents of extractable substances with dichloromethane from the hides in the soaking are presented on the figure 2.

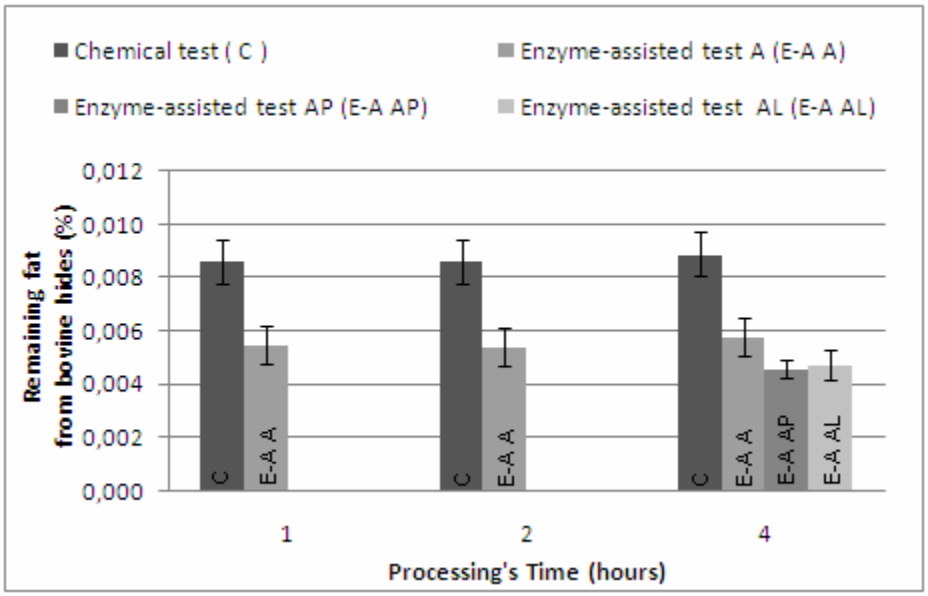

Figure 2: Fat analysis of hide by soaking

Based on this figure, it's clear that the conventional process improve a lower capacity of fat removal, because the hides show higher levels of fat, irrespective of the soaking time. In the enzyme assisted group, the ones that used only protease AP, and only lipase AL showed the best removal percentage. When the protease and the lipase are combined the final result can be reduced, as it is showed. It's possible that when the enzymes connect whit the active site on the substrate, they prevent other enzymes from reaching their active sites.

The results of SEM analysis for soaking and liming are showed in figure 3 for different processing times.

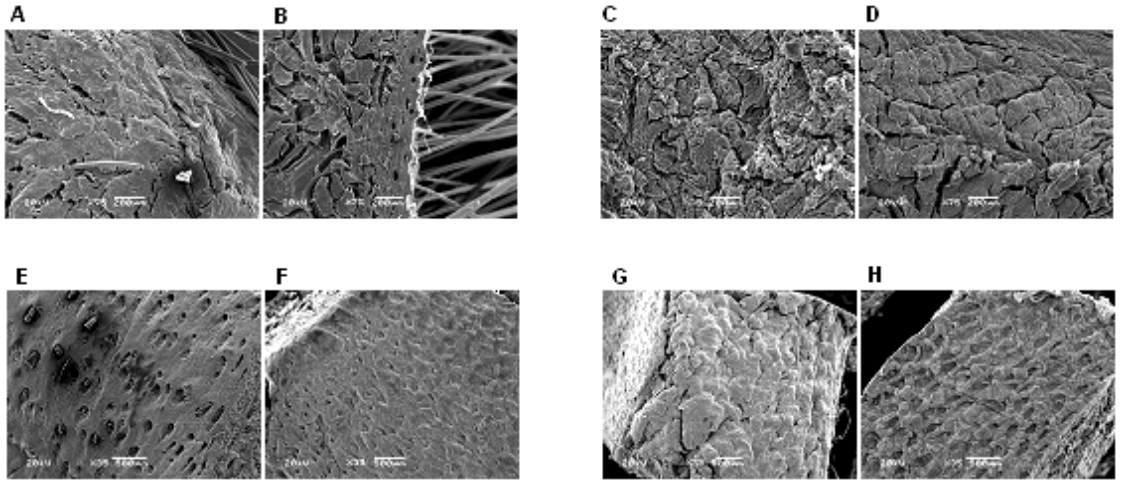

Figure 3 - SEM showing cut surface from: (A) conventional and (B) enzyme-assisted A, 240 minutes after the start of soaking (75X) ; (C) conventional and (D) Enzyme-assisted liming A of bovine hides, 40 minutes after the start of liming (75X). SEM Showing grain surface from: (E) conventional and (F) enzyme-assisted A, 285 minutes after the start of liming; (G) conventional liming and (H) enzyme-assisted A, 465 minutes after the start of liming.

It is possible to infer, based on figures A and B that enzymes provide the effect of opening collagen fibers since the beginning. The images C and D show that there aren't visual differences in cross-section of hides by 40 minutes after the beginning of liming, however, only lipase was added to process by that time. After 285 minutes it is possible to see, in images E and F, the complete unhairing in enzyme-assisted process and the presence of hair´s remains, indicating that the conventional process attacks the chemical structure of hair. The images G and H show the differences of surface by swelling effect provided by lime on skins.

3.2 Chromium removal of leather wastes

The variables investigated in the enzymatic treatment of leather wastes and the results for the mass reduction efficiency obtained via this route are given in Table 4.

The best efficiency was obtained under the conditions of Test 7. On comparing the conditions of the tests it can be verified, that a greater amount of enzyme did not lead to increased efficiency. However as expected, the results did show that an increase in pH improved the hydrolysis efficiency, since the enzyme used in these experiments acts at pH alkaline. The tests revealed a reduction in the efficiency when the temperature is decreased to 30^{o}C, indicating that the enzyme acts better in the hydrolysis at a temperature of 60°C. However, it appears that when the process temperature is increased by 10°C the hydrolysis efficiency reduces. This is due to the fact that the enzyme used decomposes at temperatures higher than 80°C.

It could be inferred that increasing the time from three to five hours resulted in higher hydrolysis efficiency. However, where the basification time was increased and the incubation time was reduced,

the efficiency was higher for the latter, indicating the greater importance of good basification. A comparison between the conditions where the reuse of the filtrate was tested while the other conditions remained the same, reveals no influence on the mass reduction efficiency, but the objective here was principally to concentrate the nitrogen content of the liquid solution.

Table 4 - Conditions for each test by enzymatic treatment of leather waste and efficiency of mass reduction

Test	Temperature [ºC]	Basification time [h]	Incubation time [h]	pH	Enzyme Quantity [% relative to the mass of waste]	Reuse of the liquid filtered	Efficiency of mass reduction [% dry basis]
1	65	1	5	8.00	10	No	61.8
2	65	1	5	8.00	20	No	55.8
3	30	1	3	7.00	50	No	46.9
4	60	0.5	5	9.00	10	No	71.3
5	70	1	3	9.00	10	No	63.8
6	60	1	3	9.00	50	No	70.7
7	60	1	3	9.00	10	No	73.0
8	60	1	3	8.00	10	No	45.6
9	30	1	3	9.00	50	No	44.0
10	60	0.5	3	9.00	10	No	48.7
11	60	1	3	9.00	10	Yes*	35.4
12	30	1	3	9.00	50	Yes*	45.8
13	60	1	3	9.00	50	Yes*	54.8
average value							53.7

* The filtered liquid was reused two times

Table 5 shows the results obtained in the experiments of chrome removal with bacteria. Prior to the analysis the chrome cake was washed with a solution of sodium lauryl sulfate to remove the biofilm. The suspension was filtered again and only then was it possible to analyze the quantity of chrome remaining in the cake, i.e., which was not biologically removed**.** The results obtained from the analysis of the cake after the second filtration are shown in the table. The chrome removed in the bacterial cells is present in the filtrate.

Table 5 - Analysis of the cake of the bacterial treatment of leather wastes after filtration

Test	Concentration of the SLS* [%]	Non volatile material [%]	Chrome content [% Cr_2O_3]	% chrome removed in relation to the blank test
Blank test	-	86.42	4.70	-
Bacterial treatment	1.0	85.24	2.00	57.4

*SLS: Sodium lauryl sulfate

According to previously experiments from Lutckmeier *et al.* (2008), regarding the tested conditions, it was shown that increasing the incubation time led to greater removal of chrome, and the addition of sucrose enhanced the bacterial growth and led to more chrome removal.

4. Conclusions

Based on the chloride removal analysis in soaking wastewater, it's possible to realize that the time is a variable that has a huge importance, mostly to the conventional process, that showed a higher dependence on it to achieve removal values closer to the ones find on the enzyme-assisted process. Besides, it's evident that the enzymes accelerate the hide wet process because it helps to open the collagen fibers structure. The conventional process improves a lower capacity of fat removal in the

soaking, because the hides show higher levels of fat, irrespective of the time. From the analysis of SEM images, it's possible to realize that the liming process, which employs more enzymes is more efficient in unhairing, because the enzymes remove the hair from the root, leading little damage to its structure, while the conventional process promotes the degradation of hair structure.

The experiments of enzymatic hydrolysis of chromed leather shaving waste showed the retention of a solid phase rich in chrome, where the average mass reduction efficiency was 53.7%. From the results of the bacterial treatment it can be concluded that the bacterial species *Pseudomonas aeruginosa* survives in the environment containing the chrome leather waste, with no need for pH adjustment. The bacteria remove the chrome present in the waste; however, the addition of sucrose makes the process more favorable and efficient. The SLS solution was effective in the removal of chrome adhered to the bacterial cells.

References

[1] BRAZILIEN LEATHER GUIDE ABQTIC, 2008.

[2] THANIKAIVELAN, P.; RAO, J. R.; NAIR B.U.; RAMASAMI, T. Progress and recent trends in biotechnological methods for leather processing, Trends in Biotechnology, v. 22, 2004.

[3] MACEDO, A.J.; SILVA, W.O.B.; GAVA, R.; DRIEMEIER, D.; HENRIQUES, J.A.P.; TERMIGNONI, C. Novel keratinase from Bacillus subtilus S14 exhibiting remarkable dehairing capabilities. Applied and Environmental Microbiology, Jan. 2005, p. 594-596.

[4] DAYANANDAN, A.; KANAGARAJ, J.; SOUNDERRAJ, L.; GOVINDARAJU, R.; RAJKUMAR, G. S. Application of an alkaline protease in leather processing: an ecofriendly approach. Journal of Cleaner Production, v. 11, 533-536, 2003.

[5] KANTH, S.V.; VENBA, R.; MADHAN, B.; CHANDRABABU, N.K.; SADULLA, S. Studies on the influence of bacterial collagenase in leather dyeing. Dyes and pigments, v. 76, p. 338-347, 2008.

[6] KUMAR, A.G.; SWARNALATHA, S.; SAIRAM, B.; SEKARAN, G. Production of alkaline protease by Pseudomonas aeruginosa using proteinaceous solid waste generated from leather manufacturing industries. Bioresource Technology, v. 99, p. 1939-1944, 2008.

[7] KUMAR, C.G., TAKAGI, H. Microbial alkaline proteases: from a bioindustrial viewpoint, Biotechnology Advances, v. 17, p. 561-594, 1999.

[8] OKAFOR, N. Modern Industrial Microbiology and Biotechnology, 551p., New Hampshire, 2007, United States of America.

[9] LUTCKMEIER, C.; AMARAL, L.; GUTTERRES, M.; MARCÍLIO, N; Dechroming of wet-blue leather wastes, XVII FLAQTIC Congress, Rio de Janeiro, 2008.