Associated Use of Enzymes and Hydrogen Peroxide for Cowhide Hair Removal

by

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Abstract

This study evaluated the use of enzymatic extract produced by a strain of *Bacillus subtilis* in combination with hydrogen peroxide for hair removal of cowhides. Two concentrations of enzymatic extract (100 and 300 U g-1 of hide), and two concentrations of hydrogen peroxide (4% and 8%) were tested. The hides were evaluated visually, and wastewater was evaluated through analysis of total nitrogen, total, fixed and volatile dissolved solids, glycosaminoglycans, proteoglycans and hydroxyproline. The oxidative-enzymatic unhairing didn't cause destruction of hair and reduced the process time compared to the conventional and the enzymatic unhairing. The results showed that the oxidative-enzymatic unhairing could be a viable alternative to the use of lime and sodium sulfide on hair removal.

Introduction

The unhairing step is a heavy pollution operation in the leather industry. The conventional lime-sulfide process produces a large amount of sulfide, which is toxic to health and difficult to dispose. Moreover, conventional process leads to the destruction of the hair causing increased chemical oxygen demand (COD), biochemical oxygen demand (BOD), and total suspended solids (TSS) loads in the effluent.[1] Therefore, there is the necessity to implement cleaner technology, with the substitution of potentially hazardous reagents, for less aggressive products, and more easily treated in treatment plants. Enzymes are gaining much importance on hair removal process, eliminating the necessity of using sodium sulfide. Enzymes can be used in all stages of leather manufacture, except, perhaps, the current process of tanning.[2] Alkaline proteases produced by bacterium found numerous applications in various industrial sectors. In the leather industry, the enzymatic treatment destroys undesirable pigments, increases the area of hide and produces cleanest hides. Alkaline proteases increase the speed of unhairing because the alkaline conditions allow the swelling of the hair roots, and the subsequent attack of protease on the hair follicle protein, enabling easy removal of the hairs.[3]

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Another alternative to the use of lime and sodium sulfide is the use of oxidizing agents for the unhairing of the hide. The application of oxidative hair removal with recovery of hair contributes for the replacement of sodium sulfide and the reduction of pollutants load in wastewater. Recently, more attention is being given to oxidative unhairing with hydrogen peroxide (H_2O_2) due to the fact that it can remarkably reduce the environmental impact.[4] Both the sodium sulfide and hydrogen peroxide act in highly alkaline conditions: however, the sodium sulfide acts as reducing agent while hydrogen peroxide acts as oxidant one, decomposing and transforming into water, thus generating wastewater less pollutant.[5]

The objective of this work, therefore, is to bring together the benefits of using enzymatic extract and hydrogen peroxide, suggesting the hair removal by an oxidative-enzymatic process. For this purpose were used the enzymatic extract produced by cultivation of a *Bacillus subtilis* strain, named BLBc 17, isolated and selected in previous works of Dettmer and collaborators.[6] The oxidative-enzymatic unhairing proposed in this work was evaluated by performing comparative tests between the proposed method of hair removal and hair removal methods purely enzymatic (using only the enzymatic extract), and conventional (lime and sodium sulfide), through visual analysis of the hide and analysis of pH, total nitrogen, total dissolved, fixed dissolved and volatile dissolved solids, besides quantification of interfibrillar proteins (proteoglycans and glycosaminoglycans) and the determination of hydroxyproline in residual bath.

EXPERIMENTAL

Materials

For this work was used a salted and pre-fleshed cowhide. After removal of the flanks, thighs and head, the hide was cut in two halves, and one center strip, approximately 10 cm, was disregarded since this band from the dorsal line has greater stiffness. Each half has been transversely divided into three parts: upper, middle and lower. The top and bottom portions were then cut into smaller pieces to provide the 10 cm x 10 cm (100 cm^2), and central (middle) gave the larger pieces of 10 cm x 20 cm (200 cm^2). Each test was conducted using three pieces of the hide, from the top, the middle and the bottom.

Hydrogen Peroxide solution (35%) was used as the oxidizing agent. Enzymatic extract (proteases) used in the unhairing tests conducted in this study were produced by a strain of *Bacillus subtilis* isolated and selected by Dettmer et al.[6] The strain identified as BLBc 17 was selected due to its higher activity on *keratin azure*, i. e. greater effect on keratin. For the enzymatic extract production, single colonies of bacterium from plates were inoculated into 100 mL of medium (containing: peptone, 0.1%; soybean meal, 1.4%; yeast extract, 0.2%; K_2HPO_4 , 0.7%; K_2HPO_4 ,0.3%), in Erlenmeyer flasks (250 mL) and incubated at 37°C in a shaker at 80 rpm. After reaching the optical density (OD, 600nm) of 1.0, these cultures were used to inoculate in production flasks at a rate of 10% (v / v) of the final volume to be produced. After inoculation, they were grown, also at 37°C in a shaker at 80 rpm for 48 hours. After this period, the medium is centrifuged to obtain the enzymatic extract.

Proteolytic Activity

The proteolytic activity analyzes using azocasein as substrate was based on Goingo et al.[7] and Dettmer et al.[6] The reaction mixture contains 100 μL of substrate (azocasein 10 mg mL^{-1}), 100 μL of buffer, and 100 μL of enzyme solution. The samples were incubated for 30 minutes at 37°C; the reaction was stopped by adding 500 μL of solution TCA (trichloroacetic acid) 10%. After centrifugation at 10,000 g for 5 minutes, 800 μL of the supernatant was added to 200 μL of a solution of 1.8N NaOH. The reading of sample was performed on spectrophotometer at a wavelength of 420 nm. An enzyme unit is defined as the amount of enzymatic extract required to increase the absorbance of 0.01 at 420 nm under conditions of time and temperature testing. The "blank test" was conducted by adding the same amount of enzyme solution, buffer and substrate to TCA. The analysis was performed in triplicate.

From the enzymatic activity is calculated the amount of enzymatic extract to be used in unhairing testing. This quantity depends on the mass of hide and enzyme concentration desired for each test. The enzymatic extract concentration is thus measured in enzyme unit per gram of hide (U g^{-1}).

Hide Unhairing

The tests were conducted on a (battery) of drums (rotating cylindrical reactor), model OXY-62 of Oxilab, with temperature and rotation controls. The tests were performed on batches according to the sample size and capacity of the equipment. After removal of salt, the hides were properly washed and soaked. The formulations of soaking, as well as the test formulation of enzymatic-oxidative, purely enzymatic and conventional unhairing are shown in Tables I and II. All mass percentages were calculated from the mass of hide.

TABLE I
Formulation used for soaking and washing.

Step	Chemical	Quantity (%)	Time
Washing	Water	200	15 minutes
			Drain
Soaking	Water	200	
	Sodium carbonate	0.30	
	Surfactant	0.15	3 hours
			Drain

TABLE II
Formulation used for unhairing tests.

Test 1	Test 2	Test 3	Test 4	Enzimatic Unhairing	Conventional Unhairing
Enzymatic Extract (BLBc 17): $100\ U\ g^{-1}$ hide	Enzymatic Extract (BLBc 17): $100\ U\ g^{-1}$ hide	Enzymatic Extract (BLBc 17): $300\ U\ g^{-1}$ hide	Enzymatic Extract (BLBc 17): $300\ U\ g^{-1}$ hide	Enzymatic Extract (BLBc 17): $300\ U\ g^{-1}$ hide	50% H_2O
2 hours	2 hours	2 hours	2 hours	6 hours	1% Lime 0.16% surfactant (Eusapon® L-DE, BASF)
200% H_2O	200% H_2O	200% H_2O	200% H_2O		
1% NaOH	1% NaOH	1% NaOH	1% NaOH		60 minutes
10 minutes	10 minutes	10 minutes	10 minutes		1% Sodium sulfide
4% H_2O_2	8% H_2O_2	4% H_2O_2	8% H_2O_2		0.5% Lime
2 hours	2 hours	2 hours	2 hours		45 minutes 1.5% Sodium sulfide 0.5% Lime 60 minutes 2% Lime 0.04% surfactant (Eusapon® L-DE, BASF) 150% H_2O 90 minutes 15 hours

In enzymatic-oxidative tests were used two different concentrations of enzymatic extract ($100\ U\ g^{-1}$ hide and $300\ U\ g^{-1}$ hide) and two concentrations of hydrogen peroxide (4% and 8%). Comparative tests were also performed, with the conventional method of unhairing, using the system lime-sodium sulfide, and the purely enzymatic unhairing, where the enzymatic extract produced by the strain BLBc 17 was applied directly to the hide without any addition of other chemicals. This formulation is based upon the formulations used in tanneries. The operating conditions of the system of drums were kept constant, working with rotation of 13 rpm and at room temperature around 25°C. The hides, subjected to the tests after hair removal were evaluated visually and using a magnifying glass with light, TOYO - TL -1000, which allows resolution increase of 12 times.

Analysis of Wastewater

The wastewater were collected at the end of the unhairing for analysis of pH, Total Dissolved Solids (TDS), Volatile Dissolved Solids (VDS) and Fixed Dissolved Solids (FDS), Total Kjeldahl Nitrogen (TKN), glycosaminoglycans, proteoglycans and hydroxyproline.

The pH was measured immediately at the end of the tests. The determination of total dissolved, fixed dissolved and volatile dissolved solids was performed according to the method based on NBR 14 550 of ABNT.[8] The analysis of total dissolved solids is carried out by adding 25 mL of homogenized and filtered sample, in a porcelain capsule containing calcined sand. To the capsules, previously tared, one should add calcined sand (20 g), and these should be left in the oven at 105ºC for one hour. After, take off, cool in desiccator and record the result, then adding the sample. The capsules are

brought to the oven (105ºC) where they will stay for one day or until constant weight. At the end, the capsules are cooled in a desiccator until reaching room temperature, weighed and the result recorded. For the analysis of fixed dissolved solids, the capsules used in the analysis of TDS are taken into the furnace at a temperature of 550°C – 600°C for two hours. The result of the analysis of volatile dissolved solids is obtained indirectly from the difference between the two previous results.

Analysis of Total Kjeldahl Nitrogen was performed according to the procedure adapted from ASTM D2868 - 10.[9] This method consists in complete digestion of 25 mL sample in 18 mL of concentrated sulfuric acid in the presence of a catalytic mixture consisting of potassium sulphate and copper sulphate (10 g) at high temperature (300ºC). The complete digestion of samples occurs when these become translucent. The samples were digested in the digester model DK20 (Velp Scientific) with capacity for twenty samples. After digested and cooling it follows the distillation of the same with solution of sodium hydroxide at 40% in the distiller model UDK 129 (Velp Scientific). Nitrogen is distilled into a boric acid indicator solution (125 mL) until approximately 200 mL. During distillation this solution turns green and translucent, indicating the presence of nitrogen. The solution is then titrated with dilute sulfuric acid until turning from green to violet.

For analyze of proteoglycans , 1 mL of sample is taken and mixed with 100 mL of fresh solution of periodic acid and incubated at 37°C for 2 hours. Then 100 mL of Schiff's reagent (colorless) are added, mixed and left for at least 30 minutes at room temperature. The standard curve is obtained using mucin as pattern. The absorbance is measured at 555 nm. This method is based on work of Madhan and collaborators.[10]

The glycosaminoglycans analyze is accomplished by testing the modification of dimethylmethylene blue, which is specific for the quantification of sulfated glycosaminoglycans. 100 mL of each sample and 2.5 mL of color reagent of dimethylmethylene blue are mixed and transferred to their respective cuvettes. The absorbance at 525 nm should be immediately read in a spectrophotometer. The standard curve is obtained using chondroitin sulfate as a standard. The method is based on the work of Madhan et al.[10] The hydroxyproline content in the wastewater was determined by the method developed by the Institute FILK (Forschungsinstitut Leder und für Kunststoffbahnen, Freiberg, Germany, 2009 - unpublished), also used in the works of Dettmer et al. [6, 11, 12]

Results and Discussion

By visual assessment of the hides (Figure 1) it is observed partial removal of the hair in all tests, but in the third one carried out with, higher enzymatic extract concentration and lower peroxide concentration, 300 U g^{-1} of hide and 4%, respectively, the removal was more pronounced. However, it was found that also in all these assays, the hair could be physically removed. Thus, the hair was mechanically removed scraping with a blunt knife. Figure 1 also shows the hides after mechanical removal of the hair.

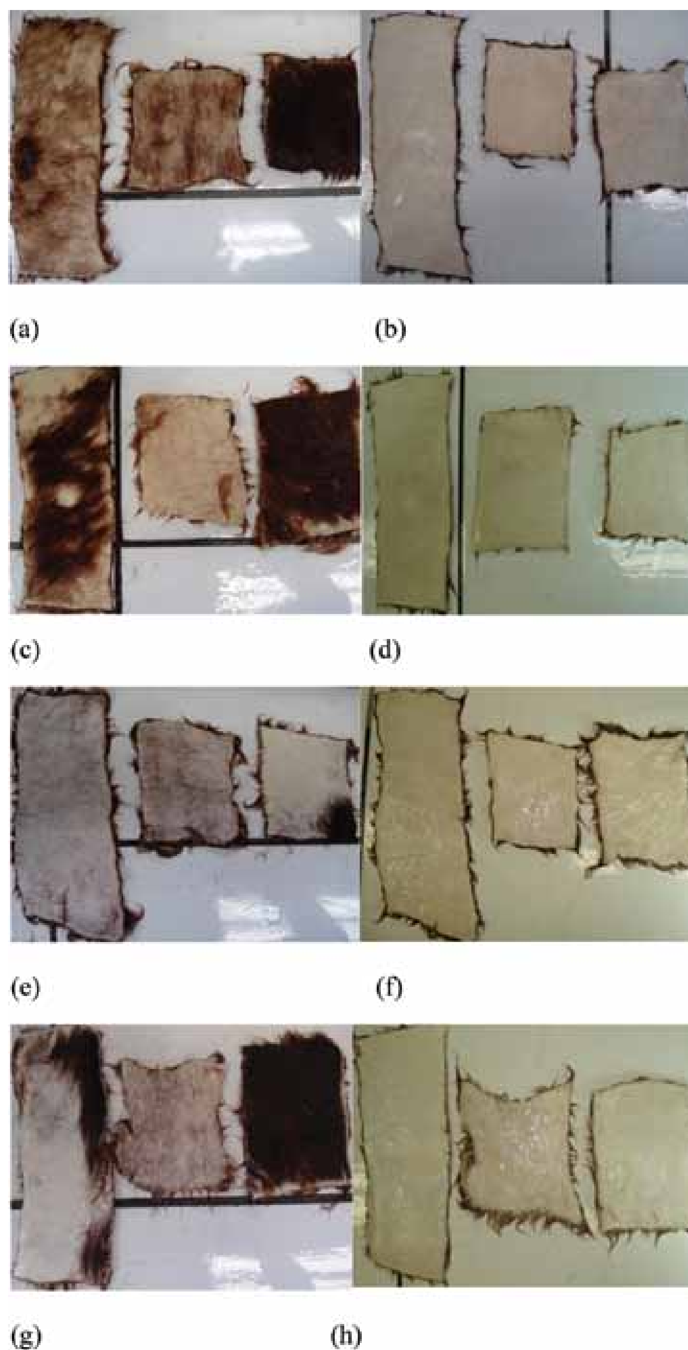

Figure 1. Images of hides after the tests. Right side: after physical removal of hairs. (a) and (b) Test 1: Conc. enzymatic extract 100 U g^{-1} of hide, 4% H_2O_2; (c) and (d) Test 2: Conc. enzymatic extract 100 U g^{-1} of hide, 8% H_2O_2; (e) and (f) Test 3: Conc. enzymatic extract 300 U g^{-1} of hide, 4% H_2O_2; (g) and (h) Test 4: Conc. enzymatic extract 300 U g^{-1} of hide, 8% H_2O_2.

Figure 2 shows magnified view of the hide after physical removal of hairs. It appears that the hairs and epidermis were removed completely, leaving no residue in the pores. The images at right side show the hair removed. It appears that hairs have been entirely removed, i.e. are not broken by chemical attack.

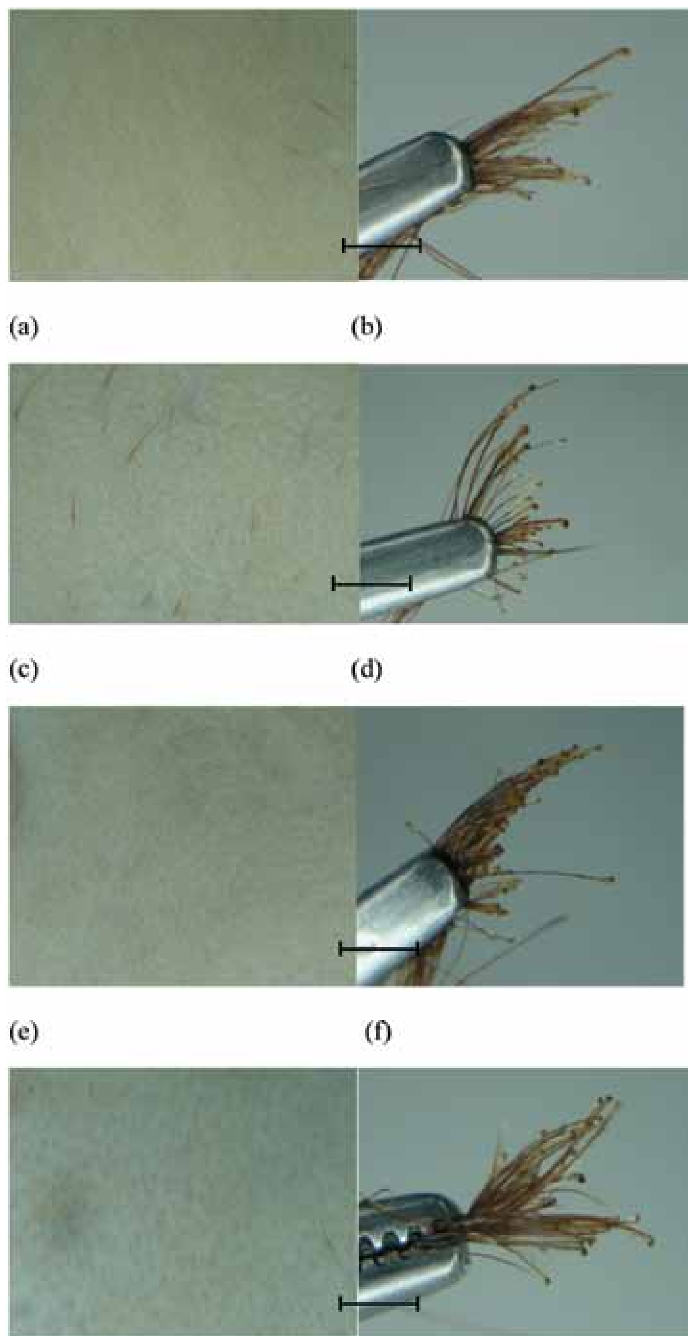

Figure 2. Images of hides after the tests (magnification 12x) and removed hairs. (a) and (b) Test 1: Conc. enzymatic extract 100 U g^{-1} of hide, 4% H_2O_2; (c) and (d) Test 2: Conc. enzymatic extract 100 U g^{-1} of hide, 8% H_2O_2; (e) and (f) Test 3: Conc. enzymatic extract 300 U g^{-1} of hide, 4% H_2O_2; (g) and (h) Test 4: Conc. enzymatic extract 300 U g^{-1} of hide, 8% H_2O_2.

With respect to the concentration of enzymatic extract, it was found that a higher concentration favors the unhairing. However, with respect to the concentration of hydrogen peroxide, different effects were not observed by visual analysis, since the concentration of this reagent did not interfere in the process. The use of peroxide in this case only accelerated the process. Comparing the purely enzymatic process, the addition of hydrogen peroxide at the same concentration of enzymatic extract (300 U g^{-1} of hide) reduced process time from 6 to 4 hours (see formulations at Table II).

The hair removal using lime and sodium sulfide promoted total removal of the hairs, but by degradation of them (Figure 3). There is, however, regions on the hide where the hair root has not been removed, remaining inside the pore. The pores were more open in this unhairing process, when compared to enzymatic-oxidative process. But the hides processed with lime/sodium became more rigid and swollen than the hides processed by enzymatic-oxidative unhairing.

One of the advantages of alternative unhairing proposed in this paper, compared to the use of lime-sulfide, is the total elimination of sodium sulfide in the process, what for environmental and security of employers in tanneries is very beneficial. Apart there is reduction of processing time from 15 to 18 hours performed by the system lime/sodium sulfide to about 4 hours for enzyme-oxidative hair removal. Another great advantage of enzymatic-oxidative unhairing against the conventional process is not the destruction of the hair.

Figure 4 shows the results of visual analysis of the carried out tests only using enzymatic extract. It is found that the hairs were partially removed therefore due to effect of loosening them from the hide they could be physically removed with stripping by blunt knife. The image (b), (c) and (d) show the hides after physical removal of hairs where it can be verified the efficiency of removing and cleaning the pores. The unhaired hide with only enzymatic extract had similar appearance to that of enzymatic-oxidative process. The hair was again not destroyed but the process time was superior, it took around 6 hours against the enzymatic-oxidative one that was accomplished in about 4 hours.

Table III shows the comparative results of the analysis of TDS, FDS, VDS, Total Kjeldahl Nitrogen, glycosaminoglycans, proteoglycans, hydroxyproline and pH.

It is found for enzymatic-oxidative unhairing tests higher concentrations of total solids in wastewater using higher concentration of enzymatic extract (tests 3 and 4). The concentration of total solids in wastewater with lime and sodium sulfide system were slightly lower. This same behavior is also observed for the analysis of fixed and volatile solids. The fixed solids represent inorganic material present in wastewater. The concentration of volatile solids is related to

the removal of organic matter, such as fats and proteins. It is noticed that by the evaluation of these results in the removal of both organic matter as the inorganic material, the enzymatic extract concentration has the main influence. The higher solids concentration in enzymatic-oxidative tests with higher concentration of enzymatic extract used, may outcome to the preparation of this extract, since the extract even after centrifugation it can still have minor leaving solids from the used growth medium.

With respect to the interfibrillar proteins (proteoglycans and glycosaminoglycans), it was observed that the hair removal using lime-sodium sulfide hardly promoted the removal of these proteins. The highest removals of proteoglycans and glycosaminoglycans were observed by enzymatic-oxidative assays, where the enzymatic step was made with a higher concentration of enzymatic extract. With respect to hydroxyproline by hair removal with lime-sodium sulfide it was not detected the presence of this amino acid in wastewater, noting that there was no attack on the structure of collagen. However, for the enzymatic and enzymatic-oxidative tests it was verified the presence of hydroxyproline, and the highest concentrations were found for enzymatic-oxidative unhairing where the enzymatic step was performed with a higher concentration of enzymatic extract. The concentration of hydrogen peroxide again had no effect; however as in other trials it helped to accelerate the process. Regarding purely enzymatic unhairing, the concentration of hydroxyproline showed an intermediate result between the enzymatic-oxidative unhairing using high and low enzymatic extract concentration.

The removal of hydroxyproline, in other words the attack on collagen, was expected in processes where enzymatic extract were used, as these enzymes are not strictly selective to act only on the keratine protein, they exhibit some activity on collagen. However, the concentration of hydroxyproline in wastewater was very low considering it a, slight attack on the structure of collagen. It is noted that these results show the concentration in micrograms per gram of hide, i. e. an order of magnitude smaller than the results of the remaining tests. The results show that the pH performed using lime and sodium sulfide was higher than the other, reaching a value of 14.0. For the enzymatic-oxidative assays, the pH values were slightly lower, around 12.0. The processes purely enzymatic generated wastewater with lower pH values, near 8.0. The better option would be the purely enzymatic unharing, however, despite the alkaline pH of oxidative-enzymatic baths; these not contain sulfides or lime, which is environmentally advantageous. With respect to nitrogen, it is shown that the highest concentration was found for the lime-sodium sulfide system. Excepting test 4 (higher concentrations of enzymatic extract and hydrogen peroxide) in enzymatic-oxidative unhairing, all enzymatic-oxidative assays showed lower nitrogen removal when compared to lime-sulfide system and purely enzymatic unhairing.

The highest concentration of nitrogen present in the wastewater for the lime-sodium sulfide system is due to the fact that in this process the hair is degraded, thus increasing the organic

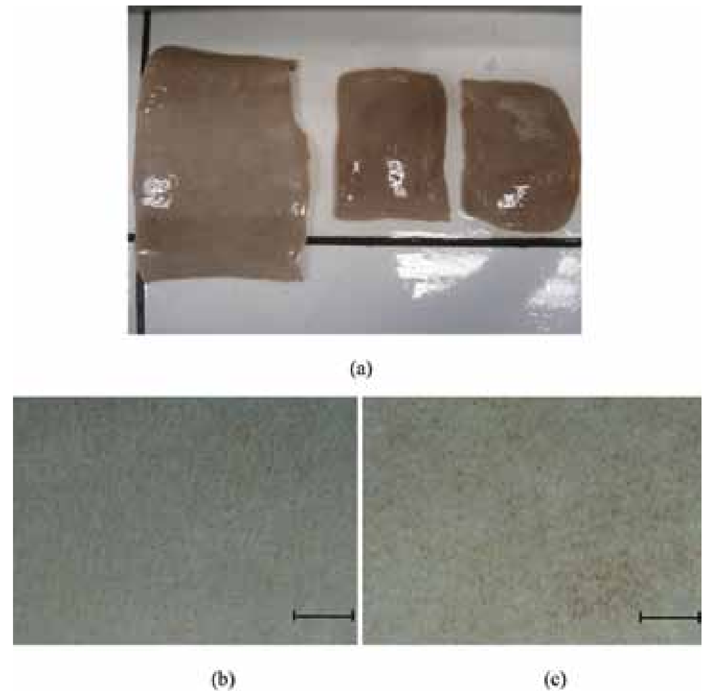

Figure 3. Images of the conventional unhairing (lime / sodium sulfide). (a) hides at the end of the process, (b) and (c) hides visualized with increase of 12x.

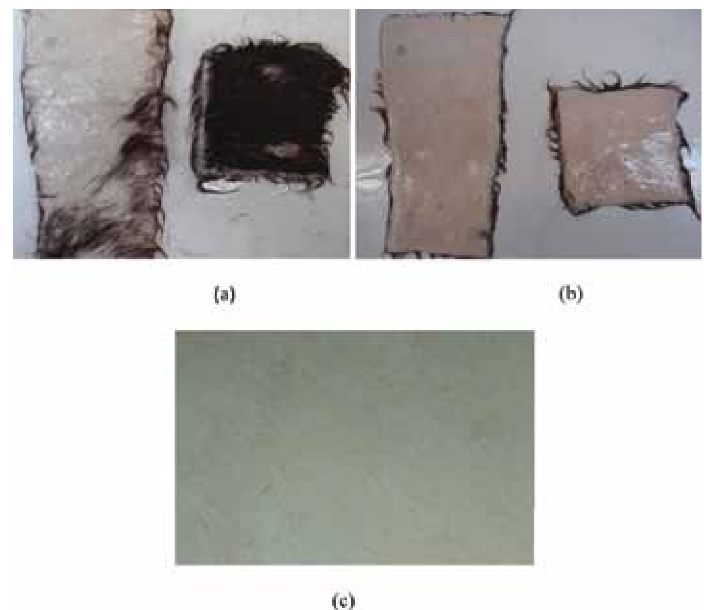

Figure 4. Visual analysis of purely enzymatic unhairing. (a) at the end of the unhairing, (b) after physical removal of hair, (c) evaluation with increase of 12x.

TABLE III
Total Dissolved Solids (TDS), Fixed Dissolved Solids (FDS), Volatile Dissolved Solids (VDS), Total Kjeldahl Nitrogen (TKN), Glycosaminoglycans, Proteoglycans, Hydroxyproline and pH, in wastewater for the unhairing tests.

	TDS mg g^{-1} hide	FDS mg g^{-1} hide	VDS mg g^{-1} hide	Nitrogen (TKN) mg g^{-1} hide	Glycosaminoglycans mg g^{-1} hide	Proteoglycans mg g^{-1} hide	Hydroxyproline µg g^{-1} hide	pH
100 U g^{-1} + 4%	56.98 ± 3.43	40.01 ± 2.27	16.97 ± 1.17	1.56 ± 0.60	0.44 ± 0.02	3.65 ± 0.96	8.02 ± 1.94	12.30 ± 0.51
100 U g^{-1} + 8%	64.38 ± 12.28	46.30 ± 9.00	18.08 ± 3.28	1.70 ± 0.98	0.47 ± 0.06	4.45 ± 0.79	8.23 ± 1.37	12.10 ± 0.88
300 U g^{-1} + 4%	75.67 ± 1.94	55.48 ± 0.84	26.94 ± 1,22	2.08 ± 1.24	0.69 ± 0.02	4.95 ± 0.59	16.45 ± 1.37	11.70 ± 0.39
300 U g^{-1} + 8%	83.54 ± 10.7	56.64 ± 8.70	26.91 ± 2.11	3.78 ± 0.44	0.66 ± 0.04	4.87 ± 1.09	15.10 ± 2.41	11.50 ± 0.66
Lime/ Sodium sulfide	73.46	53.37	20.09	4.72	0	0	0	14.00
Enzymatic extract 300 U g^{-1}	59.98	41.30	18.86	3.10	0.47	4.05	11.09	7.90

residual load. Baur examined the amount of nitrogen present in hair, obtaining a value of 5.37% (dry basis).[13] This indicates that the degradation of keratin favors the increase of nitrogen concentration in the wastewater. While the presence of nitrogen in the wastewater for the enzymatic and enzymatic-oxidative tests is related mainly to the removal of interfibrillar proteins, and other proteins and fats, which are attacked by the used proteolytic enzymes. For tests with enzymes, the nitrogen content in baths may also be related to the presence of organic material in the enzymatic extract used, because this extract, even after centrifugation, may contain traces of reagents used in the composition of the culture medium. However, the presence of nitrogen in enzymatic and enzymatic-oxidative assays was smaller than in test performed with lime and sodium sulfide, showing that these unharing methods are capable to promote the reduction of nitrogen in effluent.

With regard to the quality of the leather, in this work mechanical tests were not carried out (strength, tensile strength, etc.). However it is believed that there are not significant differences in the quality of leather produced by the process both with lime and sodium sulfide and by the enzymatic and enzymatic-oxidative, as several authors have performed these tests and found no significant changes in the results of the resistance tests. Dettmer et al. conducted a comparison between the enzymatic hair removal process using the enzymatic extract produced by a strain of *Bacillus subtilis*, compared to conventional process using sulfides, which were evaluated the mechanical and hydrothermal stability of hides obtained by the two processes. The authors found no significant differences with respect to the chromium content and temperature retraction. There were also no losses in the resistance of the leather obtained by enzymatic removal.[12]

CONCLUSIONS

The enzymatic-oxidative unhairing proposed in this work proved to be a viable alternative to conventional process (lime/ sodium sulfide). The enzymatic-oxidative unhairing, besides do not promote degradation of hair, eliminates the use of lime and sodium sulfide, thereby generating less waste organic load. Furthermore, there is not toxic gases emission, and the not degraded hairs can be used for other purposes like the production of fertilizers or extraction of keratin. Another advantage is the reduction in process time, as while the conventional unhairing lasts 15 to 18 hours, the enzymatic-oxidative process only lasted 4 hours.

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