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Application of Enzymes in Leather Processing: A Comparison between Chemical and Coenzymatic Processes

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Abstract

The use of biotechnology by industries has increased in recent years, including for use in processes, such as biocatalysts and biotransformations. Biotechnology, primarily using enzymes, can be applied during different steps of the leather production process: soaking, dehairing, bating, dyeing, degreasing or in effluent and solid waste treatment. This study aimed to evaluate the performance of five commercial enzymes in soaking and unhairing/liming by comparing the chemical and coenzymatic processes. Tests were conducted in bench drums to evaluate the action of enzymes during each stage of leather production. The conditions of the application, including concentration, processing time and type of enzyme, were varied. The total organic carbon (TOC) and the soluble protein were used to measure the efficiency of the processes. Enzymatic activity assays on collagen, keratin and lipid for enzyme and scanning electron microscopy (SEM) analyses of hides were used to complement the study. Coenzymatic processes generally showed better results in comparison to chemical processes, especially the C enzymes (microbial) for soaking and the A (lipase) and B (protease) enzymes for dehairing/liming. The enzymes showed activity on all substrates, and the test pH was an important variable. SEM analysis of the hides showed a clear difference between the chemical and coenzymatic processes.

Keywords: biotechnology, enzyme, beamhouse, enzymatic activity

1. Introduction

The scarcity of natural resources and the accumulation of pollution caused by human activity have required the development of production technology that is less harmful to the environment. The concept of cleaner production has been used in tanneries in order to mitigate its impact and reduce the loss of chemicals, water and raw materials. According to Rajamani *et al.* (2009), the world capacity of leather processing is 15 million tons of hides and skins per year. The average wastewater discharge is more than 15,000 million liters/day. Solid waste generation from the tannery process is estimated at 6 million tons/year. The disposal of large quantities of sludge is approximately 4.5 million tons/year, and the effluent from treatment plants is a major issue.

The amount and type of waste generated during leather production is variable and depends on numerous factors such as breed, slaughtering procedure, conservation of hides, and the technology used for hair removal and tanning. Lime/sulfide is widely used in hair removal because it is more efficient and cheaper than other currently available technologies. Chromium salts are the most common tanning agents.

The sulfur present in the effluent is released from organic matter (especially hair) and from some compounds used in the processing of hides including surfactants and unhairing agents, such as sodium

sulfide (Na_2S). Sulfur is found in effluents in the form of sulfates and sulfides. The risk of hydrogen sulfide (H_2S) formation during effluent treatment poses a serious environmental problem. To avoid generating hydrogen sulfide from the effluent, the sulfide should be oxidized, which requires an additional step in wastewater treatment. The unhairing process can be performed using bacteria or chemicals to oxidize substances such as hydrogen peroxide and sodium hypochlorite.

Traditionally, tanneries apply enzymes in the bate step to achieve deep cleaning of the hide. However, enzymes were also used in the hair removal process at the beginning of the last century before the development of chemical processes for hair removal. These proteins are gaining more prominence because they are considered environmentally friendly technologies and because of advancements made in the purification, development and improvement of enzymes. Enzymes are currently applied at various stages of leather processing, from beamhouse operations until the final stages, as shown by Dettmer *et al.* (2011), Thanikaivelan *et al.* (2005), Choudhary *et al.* (2004), Alexander (1988), Taylor *et al.* (1987) and Feairheller (1985).

The main enzymes that are of interest to the leather industry are as follows:

- Proteases because they hydrolyze the protein fraction of dermatan sulfate, making the collagen more accessible to water and reducing the attachment of the basal layer of pigment on the hide. In addition, they act in the removal of globular proteins;
- Lipases, which hydrolyze fats, oils and greases present in the hypoderm;
- Keratinases, which hydrolyze keratin of hair and epidermis and break down the disulfide bonds of this molecule.

In the last few decades, research on eco-friendly hair removal has increased substantially with growing environmental awareness. Unhairing enzymatic technologies are interesting because they can preserve the hair and contribute to a reduction in the organic load released in the effluent. These processes eliminate or reduce the dependence on harmful chemicals such as sulfide, lime and amines.

Research by Bhavan *et al.* (2008) demonstrated the technical and economic feasibility of a hair removal coenzymatic process, in which the lime/sulfide system in drums was replaced by the dip and pile method with enzymes and sodium metasilicate. A comparison between the conventional and proposed technologies indicated a significant reduction in environmental parameters, including chemical oxygen demand (COD) and total solid loads by 55% and 25%, respectively. Saravanabhavan *et al.* (2003) proposed a green solution for tanneries using enzymatic unhairing and pickle-free tanning.

One of the most recent findings for the application of enzymes is for use in pre-tanning. This method takes advantage of exhausted tanning systems in which increased cross-linking requires less tanning agent. According to Kanth *et al.* (2009) and Aravindhan *et al.* (2007), the results are promising from an environmental viewpoint.

Targeting bath exhaustion , Yuan *et al.* (2008) and Parvinzadeh (2007) applied proteolytic enzymes in the wet finishing of leather and in the pre-dyeing and dyeing of wool. The authors reported improvements in properties such as affinity to the dye, bath exhaustion, uniformity of color, dye penetration in the hide, wettability of the fiber, dye absorption, reduced shrinkage and improved tension in stretch wool fibers.

In studies related to waste treatment, enzymes were applied in the recovery of chromium and proteins from tanned cuttings (Cantera *et al.* (2002); Kolomaznik *et al.* (2008); Jian *et al.* (2008); Amaral *et al.* (2008); Gutterres *et al.* (2009)) and in the production of collagen hydrolysates from untanned cuttings (Bajza (2001).

In this study, several commercially available enzymes specific for the leather sector were tested in the soaking and unhairing/liming processes. Eleven soaking tests (3 control and 8 experimental tests) and six unhairing/liming tests (2 control and 4 experimental tests) were carried out to evaluate the enzymatic action of five enzymes and to quantify the levels of the total organic carbon and soluble protein in residual baths. In addition, the enzymatic activity on collagen, keratin and lipids was determined. Finally, SEM images of the hides were acquired for the coenzymatic and control tests.

2. Materials and Methods

2.1. Materials

The following five enzymes used in this study are commercially available by specialized companies for the leather sector: (A) a lipolytic enzyme recommended for soaking and unhairing/liming; (B) a combination of proteolytic enzyme recommended for soaking and unhairing/liming; (C) a microbial enzyme recommended for soaking; (D) a microbial enzyme recommended for unhairing/liming and (E) a lipolytic microbial enzyme recommended for soaking, unhairing/liming and bate (information according to the manufacturers). The percentages of the enzymes applied in each test were suggested by the companies.

Sodium carbonate (Na_2CO_3) was used for soaking, and calcium hydroxide ($Ca(OH)_2$) and sodium sulfide (Na_2S) were applied in the unhairing/liming stage. Surfactants were used throughout all of these processes. The hide was supplied by a local tannery and was previously salted and fleshed. Each test used samples (approximately 200 g) obtained from different regions of the hide. The samples were processed in bench reactors (drums) with controlled rotation speed and temperature. The chemicals and enzymes used for leather processing were of commercial grade, and the chemicals used for analytical techniques were of laboratory grade.

2.2. Analyses and Enzyme Characterization

The concentration of total organic carbon (TOC) and soluble protein were analyzed in the residual bath (wastewater) with a total organic carbon analyzer (Model V CSH, Shimadzu) and by the method of Lowry *et al.* (1951), respectively. The objective of these assays was to observe the efficiency of the processes related to the removal of organic matter (lipids, keratin, collagen and proteins from the extracellular matrix) from the hide and to identify a more efficient process for hide cleaning (the focus of the beamhouse stages). The SEM analyses (JSM 6060 – JEOL) of the hides were carried out to verify the opening of the structure after soaking and to verify the opening and hair removal after the unhairing/liming step.

The enzymatic activity on keratin was determined according to the methodology described by Ionata *et al.* (2008). The enzyme solution (500 µl) was incubated with 4 mg of keratin azure (Sigma) and 500 µl of buffer. The reaction mixture was incubated at 55 °C for 30 min under agitation. After centrifugation at 10,000 rpm for 5 min, the absorbance was determined at 595 nm. One unit of enzyme activity (U) corresponds to the amount of enzyme causing a change of absorbance of 0.01 at 595 nm for 30 min at 55 °C. The control was prepared by adding the substrate and buffer without the enzymatic solution.

The methodology described by Adigüzel *et al.* (2009) was used to measure the enzymatic activity on collagen. The collagenolytic activity of the enzymes was determined with the same procedures as those for the keratinolytic activity. Azocoll (Sigma) was used as the substrate, and the absorbance was determined at 545 nm. One unit (U) of enzyme activity corresponds to the amount of enzyme causing a change in absorbance of 0.01 at 545 nm for 30 min at 55 °C. The control was prepared in the same way as previously described.

The measurement of the enzymatic activities on lipids followed the method described by Winkler and Stuckmann (1979). Lipolytic activity was determined by incubating 1.35 ml of the substrate/buffer solution with 0.15 ml of the enzymatic solution. The reaction mixture was incubated at 37 °C for 30 minutes under agitation. Immediately after centrifugation at 12,000 rpm for 10 minutes, the absorbance was determined at 410 nm. One unit (U) of enzyme activity was determined as the amount of enzyme necessary to cause a change of absorbance of 0.01 at 410 nm with the experimental conditions of time and temperature. The control was prepared by adding the substrate/buffer solution with the enzymatic solution and pre-incubating at 100 °C for 30 minutes.

2.3. Soaking Tests

Soaking tests were processed at 28 ° C and agitated at 20 rpm. Six different variations of the formulations were used in the 11 tests to evaluate the processing time and enzyme type (Table 1). The percentages of inputs were calculated based on the mass of the hide. Tests 1, 2 and 3 (control) used a conventional (chemical) soaking formulation. Tests 4-11 (coenzymatic) used enzymes and lower percentages of surfactant to reduce the environmental impact of the wastewater.

Table 1. Experimental conditions of the soaking tests

		Weight Percentage of Application (%)			
Test	***Time***	***Water***	***Enzyme***	***Surfactant***	***Na_2CO_3***
1	1 h	200	0	0.15	0.03
2	2 h	200	0	0.15	0.03
3	4 h	200	0	0.15	0.03
4	1 h	200	A - 0.03/B - 0.07	0.02	0.03
5	2 h	200	A - 0.03/B - 0.07	0.02	0.03
6	4 h	200	A - 0.03/B - 0.07	0.02	0.03
7	1 h	200	C - 0.3	0.02	0.03
8	2 h	200	C - 0.3	0.02	0.03
9	4 h	200	C - 0.3	0.02	0.03
10	4 h	200	B - 0.07	0.02	0.03
11	4 h	200	A - 0.03	0.02	0.03

2.4. Unhairing and Liming Tests

Unhairing/liming tests were processed at 28 °C and agitated at 20 rpm. The formulations were varied in 6 tests to evaluate the type and concentration of enzyme (Table 2). The addition of water, chemicals and enzymes occurred during the first 4 hours and 15 minutes. The rotation was changed to 10 rpm for the following 12 hours. Test 1 (control) contained a conventional formulation. Test 2 had the same percentage of chemicals but contained reduced water input compared to the coenzymatic tests (90%). Tests 3 to 6 (coenzymatic) had reduced volume of water, lime and sulfides and contained enzymes.

3. Results and discussion

The analyses of soluble protein and TOC in the residual baths were performed in triplicate. The values shown in the graphs are averages and standard deviations. Because both analyses identify substances of organic origin, a similar pattern at each stage was expected. Soaking tests 1-3 used a chemical formulation without enzymes (conventional process) with variable processing times. Tests 4-6 used a combination of A/B enzymes and tests 7-9 used C enzymes. Tests 10-11 were designed to compare the

use of single enzymes with the combinations of enzymes in tests 4-6 and their effects on organic matter removal. Figure 1 and 2 show the results of the TOC and soluble protein for the soaking tests.

Table 2. Experimental conditions of unhairing/liming tests

Test	*Time (h)*	*Weight Percentage of Application (%)*				
		Water	*Lime*	*Na_2S*	*Surfactant*	*Enzyme*
1	16.4	200	4	2.5	0.1	0
2	16.4	90	2.6	1	0.1	0
3	16.4	90	2.6	1	0.1	A - 0.04/ B - 0.1
4	16.4	90	2.6	1	0.1	A - 0.08/ B - 0.2
5	16.4	90	2.6	1	0.1	D - 0.20/ E - 0.03
6	16.4	90	2.6	1	0.1	D - 0.40/ E - 0.06

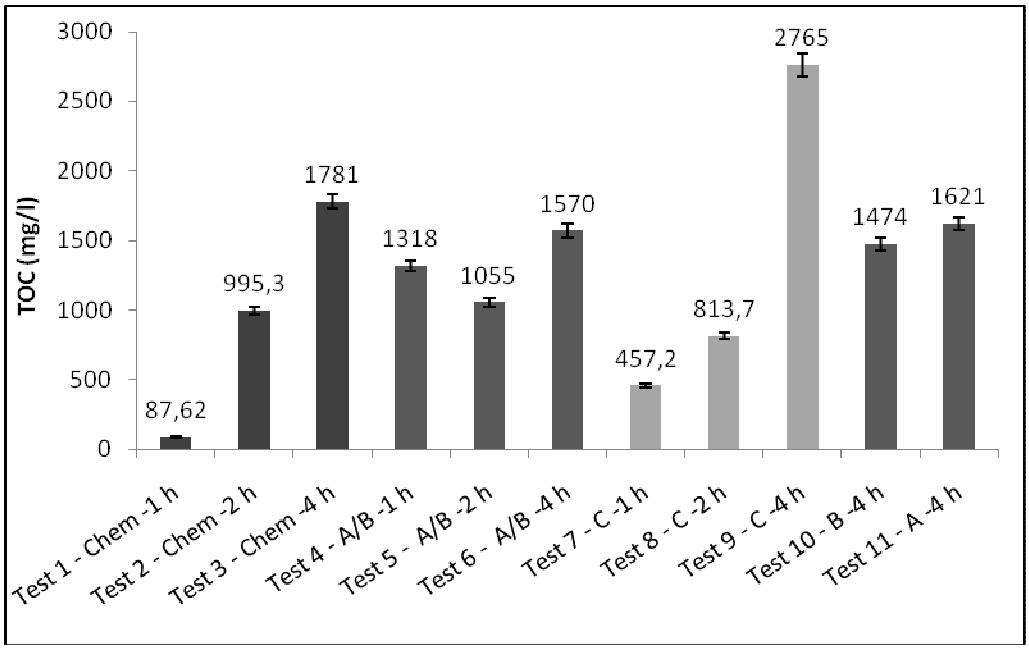

Figure 1: Analysis of TOC in the residual baths of the soaking tests

Differences in organic matter removal were observed using the same formulation conditions (such as tests 1 to 3). The analysis of soluble protein (Figure 2) confirmed this profile, indicating a relationship between the processing time and the removal of soluble protein. At the beginning (1 h), the coenzymatic treatments showed superior performances to the chemical processes.

In both the TOC and soluble protein assays, test 9 exhibited the highest performance at the end of soaking (4 h). The C enzyme used in this test was applied on the hide with a mass percentage of 0.3%, which is three times higher than the percentage of the A/B enzymes applied (tests 4-6). However, the efficiency when using only enzyme A was as good as the enzyme combination (test 6). These results indicate competition for the substrate between the enzymes.

The results of material removal for the unhairing/liming step were similar to the TOC and soluble protein analyses (Figures 3 and 4). As shown in Table 2, test 1 cannot be compared graphically to the other tests because of the dilution of chemicals due to the added water. The coenzymatic processes were more efficient than the chemical methods (tests 3-6 compared to test 2). Although the A/B enzymes were applied at a lower concentration than the D/E enzymes, they showed better results (tests

3 and 4). Increasing the concentrations of both the A/B and D/E combinations led to increased organic matter content in the bath, but not in the same proportion (doubling the concentration does not correspond to twice the TOC).

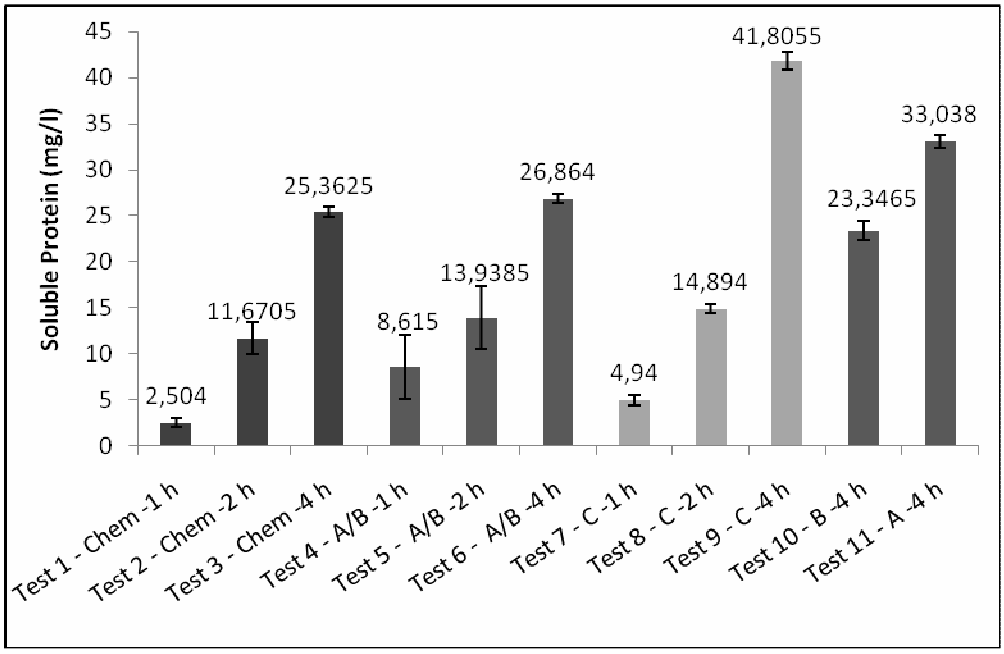

Figure 2: Analysis of soluble protein in the residual baths of the soaking tests

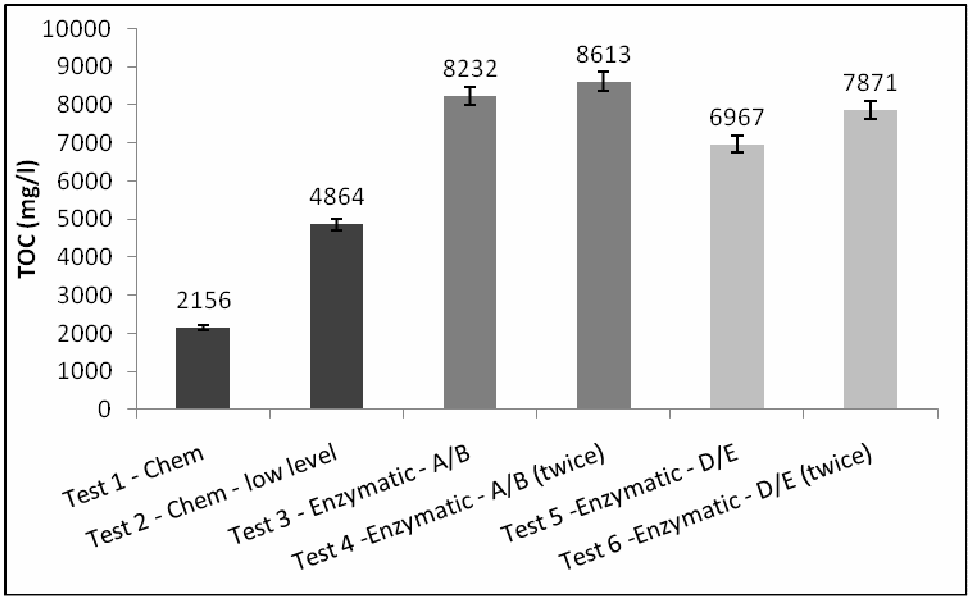

Figure 3: Analysis of the TOC in the residual baths in the unhairing/liming tests

The results of the enzymatic activity assays are summarized in Table 3, which presents the average values of the specific activity and the corresponding standard deviation for each characterization. The activities were determined at pH 8 (according to the analytical procedure) and at the pH of the processes (pH 8 for soaking and pH 12 for unhairing/liming) to observe the susceptibility of the enzymes to variable conditions and, in particular, to observe their activity during the hide processing stage. The specific activities (Table 3) were obtained from the measurements of enzymatic activity (U) divided by the mass in grams of enzyme used (as previously described in the methods).

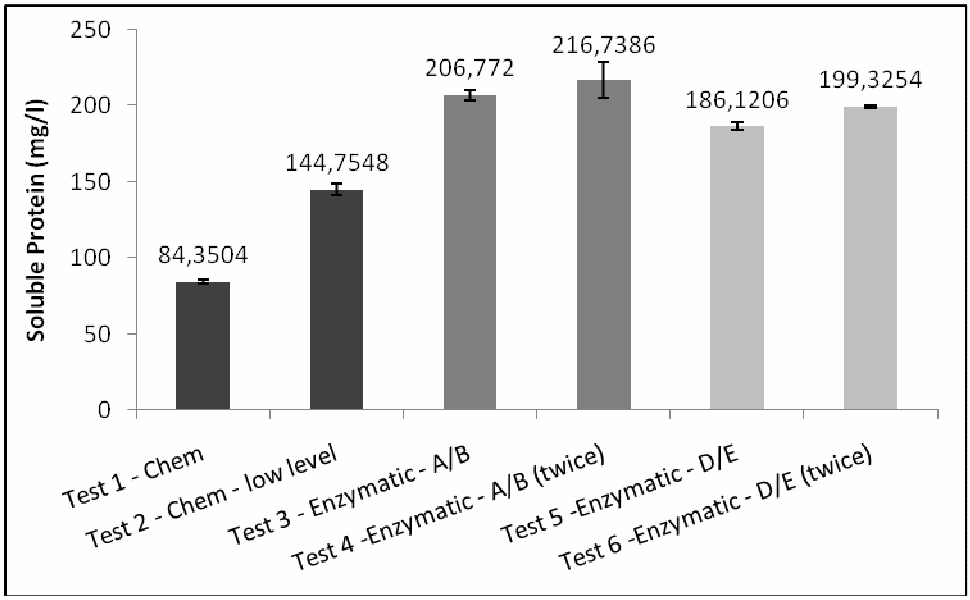

Figure 4: Analysis of soluble protein in the baths for the unhairing/liming tests

The highest specific activity values at pH 9 (soaking) were observed for enzymes A on keratin and lipid and B on collagen substrates (Table 3). For pH 12 (unhairing/liming), the best results were obtained for enzyme A on keratin, B on collagen and E on lipid. The enzymes showed residual (low) activity on all of the tested substrates except for enzyme A (lipase), which exhibited high activity not only on lipids but also on keratin. Low activity on collagen and high activity on lipid are desirable for soaking to promote opening of the collagen fibers and consequent removal of proteins, moisturizing of the hides and emulsifying of the fat in the bath. In the unhairing/liming stage, the action of keratinase is important for eliminating hair from the hide. In addition, high collagenase activity is not desirable because these enzymes can damage the hide (leather) grain and the physical-mechanical characteristics of the hides.

Table 3. Enzymatic specific activity on different substrates and at various pH values

Enzyme	*Ph*	*Specific Activity (U/g enzyme protein)*		
		Keratinolytic Activity	*Collagenolytic Activity*	*Lipolytic Activity*
A	8	299565 ± 9571	1941 ± 108	233358 ± 9571
	9	327415 ± 2530	0	261867 ± 2530
	12	1485 ± 228	0	0
B	8	21060 ± 7134	56549 ± 1724	5453 ± 128
	9	12480 ± 1419	63186 ± 6206	34205 ± 14196
	12	1481 ± 414	4697 ± 880	0
C	8	37752 ± 187	2321 ± 297	18889 ± 187
	9	36624 ± 192	282 ± 21	16800 ± 192
D	8	4875 ± 212	11777 ± 1171	12194 ± 367
	12	0	2476 ± 336	1005 ± 167
E	8	2318 ± 555	0	14948 ± 3043
	12	0	504 ± 42	6518 ± 2902

The C enzyme was only applied during soaking, and the D enzyme is indicated only for unhairing/liming. All of the other enzymes can be applied in both stages. The A and E enzymes are recommended to attack lipids, and B, C and D are suggested for proteins. Some enzymes showed no activity at certain pH values, and this variable proved to be relevant for the enzymatic activity. At pH 12 (unhairing/liming), the following enzymes exhibited no activity: D and E on keratin, A on collagen and lipids and B on lipids. At pH 9 (soaking), A exhibited no activity on collagen, and at pH 8 (assays pattern), E showed no activity on collagen.

Figure 5 presents the results of the SEM analyses. Images of the hides processed by the conventional (control) and coenzymatic methods are shown. The SEM analysis demonstrated no visual differences between the hides using the coenzymatic tests. However, differences were observed when comparing the chemical and coenzymatic tests. The images of the soaking tests Ia (control experiment) and IIa (coenzymatic experiment) demonstrated the efficiency of the coenzymatic test in fiber opening. The images of the unhairing/liming tests Ib (experiment 1) and IIb (coenzymatic experiment) indicated that enzymes do not dissolve hair but act at the basal membrane of hair roots and promote its complete removal. The presence of the hair remaining in image Ib verified that the hair was not fully dissolved by sodium sulfide. Images Ic and IIc confirm the hair removal assumption that the hair was entirely removed from the root. A hair in the decomposition process due to the action of sulfide is shown in image Ic. By contrast, image IIc shows the action of the enzymes at the base of the hair follicle.

4. Conclusions

The coenzymatic processes performed better than the conventional method in the studied beamhouse steps (soaking and unhairing/liming). No differences in the SEM images of the hide were observed between the various types of enzymes applied. However, differences were observed in the analysis of the TOC and soluble proteins in the residual baths. Enzyme C exhibited a higher removal of organic matter during soaking, and the A and B enzymes performed better in the unhairing/liming steps. However, the percentages of application in formulations of tests for enzymes C, D and E were always higher than for enzymes A and B.

The coenzymatic processes operate faster than the chemical processes. The enzyme concentration in the tests does not exhibit a linear relationship with the organic matter removal, as excess enzymes did not lead to a greater removal of this material.

The results of the enzymatic activity confirmed the assumption that the B enzymes have higher specific activities than the other enzymes. However, the behavior of the enzymes with respect to pH is very sensitive and is not linear. The enzymes showed affinity for all of the substrates (keratin, collagen and lipid). The enzymes recommended to remove fat (A and E) exhibited more activity on lipids, and the enzymes for removing protein had a higher affinity to azocollagen.

An assessment of the costs and knowledge about the specific activity of enzymes are essential for enzyme selection. In addition to cost, the mass percentage of application recommended by the manufacturer and the specific activity of enzyme for the main substrates at the pH of the process should also be considered.

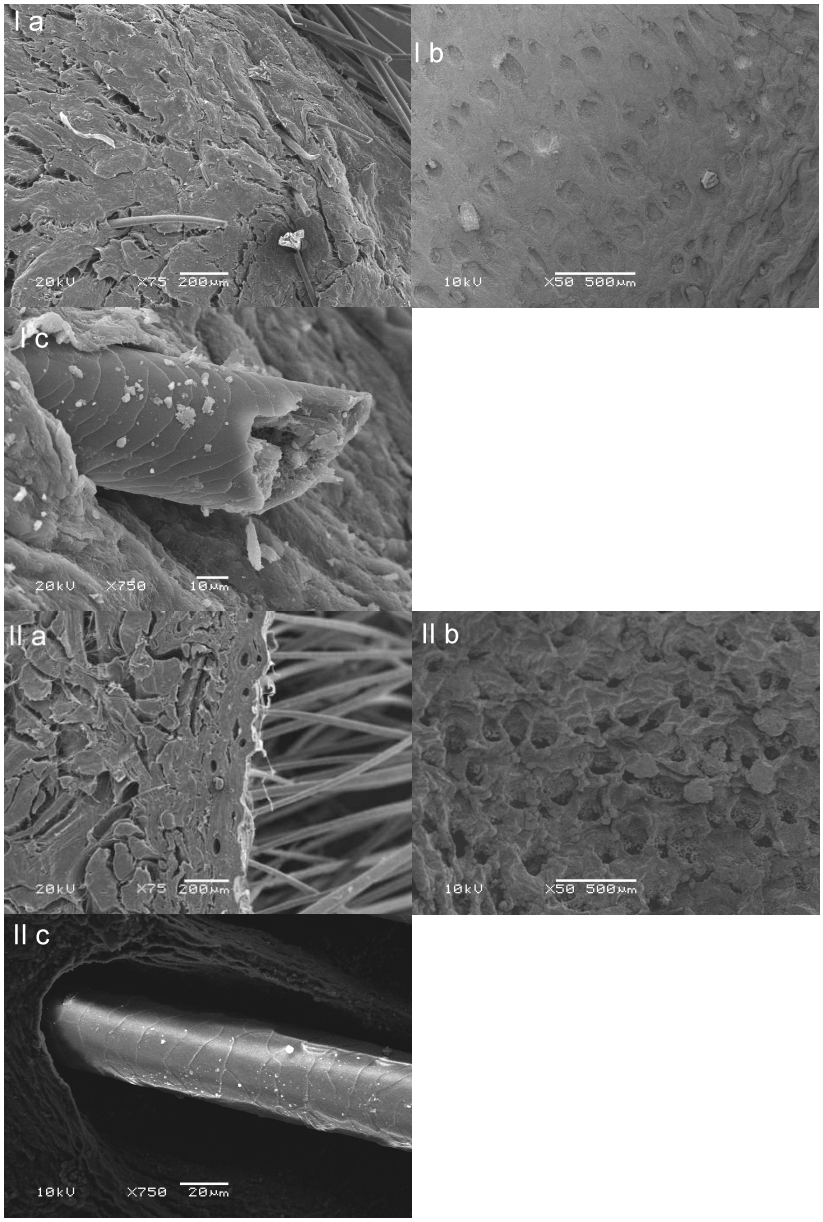

Figure 5: SEM analysis of soaking (a) X 75, unhairing/liming (b) X 50 and hair (C) X 750 of control (I) and experimental (II) tests

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