APPLICATION OF ENZYMES IN LEATHER PROCESSING: A COMPARISON BETWEEN CHEMICAL AND COENZYMATIC PROCESSES

F. R. de Souza and M. Gutterres[*]

Laboratory of Leather and Environmental Studies (LACOURO), Chemical
Engineering Department, Phone: + (55) (51) 33083954, Fax: + (55) (51) 33083277,
Federal University of Rio Grande do Sul, Porto Alegre - RS, Brazil.
E-mail: franck@enq.ufrgs.br; mariliz@enq.ufrgs.br

Abstract - The use of biotechnology by tanneries has increased in recent years. 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 evaluated 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. Concentration, processing time and type of enzyme were varied. Total organic carbon and soluble protein were used to measure the efficiency of the processes. Enzymatic activity assays on collagen, keratin and lipid and scanning electron microscopic (SEM) analyses of hides were used to complement the study. Coenzymatic processes generally showed better results in comparison to chemical processes. The enzymes showed activity on all substrates, and the SEM analyses of the hides showed a clear difference between the chemical and coenzymatic processes.
Keywords: Leather processing; Beamhouse; Biotechnology; Enzyme; Enzymatic activity.

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 their 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, approximately 4.5 million tons/year, and

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 comes from organic matter (especially hair) and from 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 such as hydrogen peroxide and sodium hypochlorite to oxidize substances.

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 to be 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. In addition, they act in the removal of globular proteins;

▪ Lipases, which hydrolyze fats, oils and greases present in the hypoderm;

▪ Keratinases, which hydrolyze the 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 the growing environmental awareness. Enzymatic unhairing technologies are interesting because they can preserve the hair and contribute to a reduction in the organic load released into 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 coenzymatic hair removal 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 and Valeika *et al.* (2012) suggests utilization of enzymes in unhairing step at low pH to avoid the usage of lime that causes a strongly alkaline pH.

One of the most recent applications 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 the affinity to the dye, bath exhaustion, uniformity of color, dye penetration into 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 total organic carbon and soluble protein in the residual baths. The formulations used in the tests were based on the work of Souza *et al.* (2010). 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.

MATERIALS AND METHODS

Materials

The following five enzymes used in this study are commercially available from specialized companies for the leather sector: (A) a lipolytic enzyme recommended for soaking and unhairing/liming – Buzyme 2103, Buckman Laboratories; (B) a combination of proteolytic enzyme recommended for soaking and unhairing/liming – Buzyme 7703,

Buckman Laboratories; (C) a microbial enzyme recommended for soaking – Tanzyme RD04, Tanquímica; (D) a microbial enzyme recommended for unhairing/liming – Tanzyme CD05, Tanquímica; and (E) a lipolytic microbial enzyme recommended for soaking, unhairing/liming and bate – Tanzyme DG, Tanquímica (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. The pH at each stage was around 9 and 12 for soaking and unhairing/liming, respectively. Surfactant (Eusapon LDE, Basf) was used throughout all of these processes. One Brazilian zebu bovine hide was supplied by a local tannery (Krumenauer S/A, Rio Grande do Sul) and was previously salted and fleshed. Each test used samples (approximately 200 g) obtained from different regions of the hide, excluding the belly, flank and head. The full-grain 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.

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 by the same procedures as 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 (p-nitrophenyl palmitate, Sigma)/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 under the experimental conditions of time and temperature. The control was prepared by adding the substrate/buffer solution to the enzymatic solution and pre-incubating at 100 °C for 30 minutes.

The assays of enzymatic activity were developed at pH 8 according to the methodology and pH 9 and 12 to simule soaking and unhairing/liming conditions, respectively. At pH 8 and pH 9 the buffer Tris-HCl was employed, at pH 12 the buffer KCl-NaOH was used.

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.

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.

Table 1: Experimental conditions of the soaking tests

Test	Time	Weight Percentage of Application (%)			
		Water	Enzyme	Surfactant	Na₂CO₃
1	1h	200	0	0.15	0.03
2	2h	200	0	0.15	0.03
3	4h	200	0	0.15	0.03
4	1h	200	A - 0.03/B - 0.07	0.02	0.03
5	2h	200	A - 0.03/B - 0.07	0.02	0.03
6	4h	200	A - 0.03/B - 0.07	0.02	0.03
7	1h	200	C - 0.3	0.02	0.03
8	2h	200	C - 0.3	0.02	0.03
9	4h	200	C - 0.3	0.02	0.03
10	4h	200	B - 0.07	0.02	0.03
11	4h	200	A - 0.03	0.02	0.03

Table 2: Experimental conditions of the unhairing/liming tests

Test	Time (h)	Weight Percentage of Application (%)				
		Water	Lime	Na₂S	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

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. Figures 1 and 2 show the results of the TOC and soluble protein for 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 performance 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.
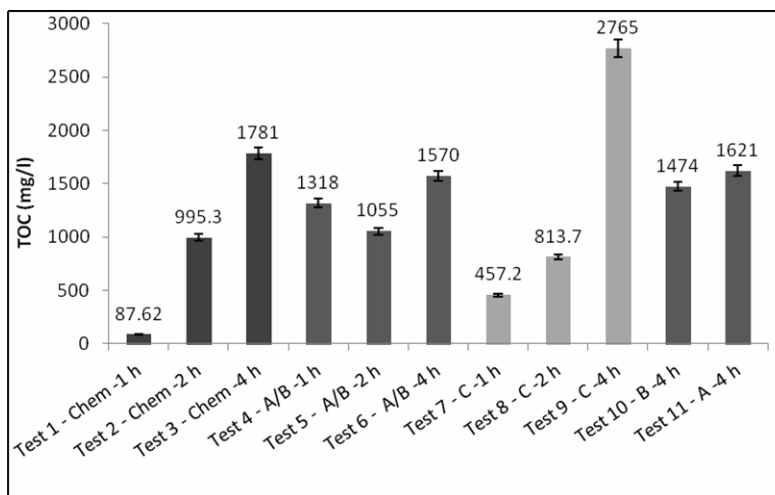

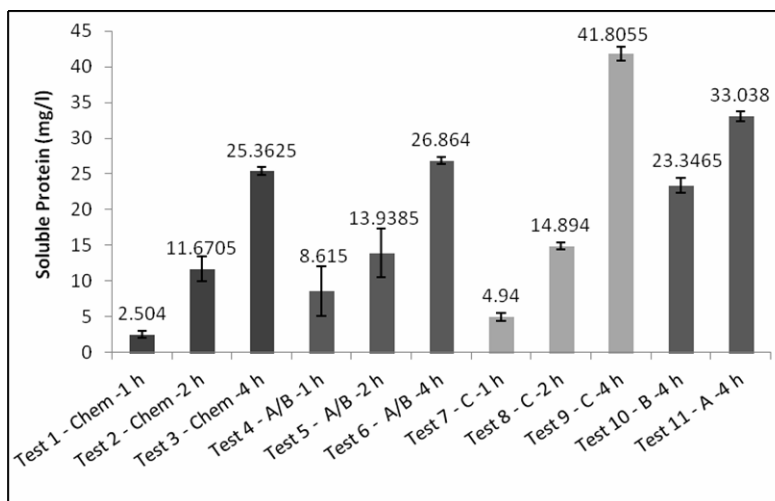**Figure 1:** Analysis of TOC in the residual baths of the soaking tests

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

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 an insignificant increase of organic matter content in the bath. This means that the excess of enzyme does not improve the release of organic matter.

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 9 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).
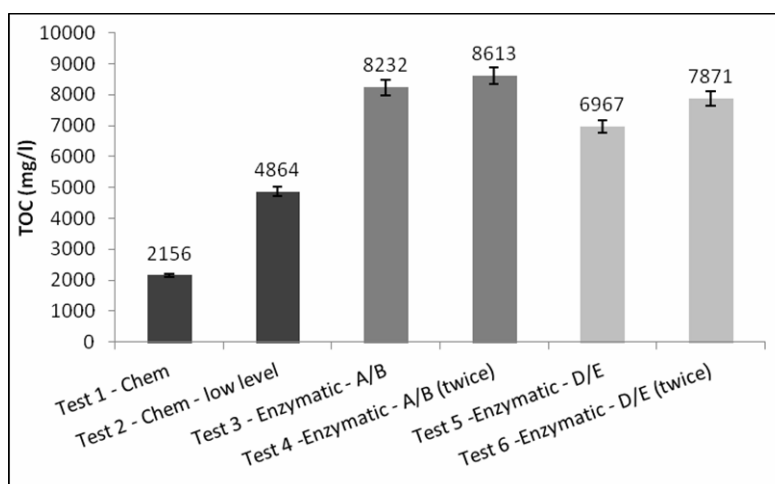

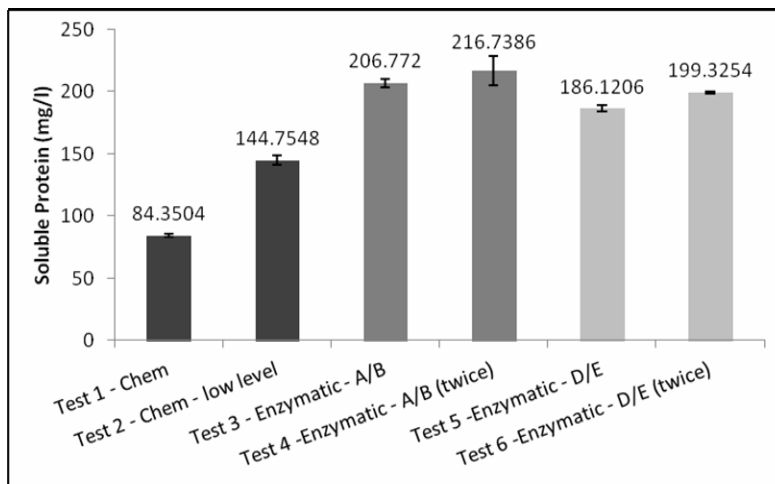**Figure 3:** Analysis of the TOC in the residual baths in the unhairing/liming tests.

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

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 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 substrates tested 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 the consequent removal of proteins, moisturizing of the hides and emulsification 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.

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.

Table 4 shows the specific activity in the experimental tests considering the mass of enzyme applied. It was verified, according to Tables 1 and 2, that the enzymes A and B were applied in quantities lower than the enzymes C and D because they have higher specific activity (Table 3). Therefore, the results of specific activity justify the differences between the amounts of enzymes applied in the formulations. These results are consistent with the

observed superior performance of the A/B enzymes in tests of unhairing/liming through the analysis of TOC and TKN in residual baths.

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 analyses 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 assumption that the hair was entirely removed from the root. A hair in the process of decomposition due to the action of sulfide is shown in image Ic. In contrast, image IIc shows the action of the enzymes at the base of the hair follicle.

Table 4: Enzymatic specific activity in the tests conditions

Enzyme	Stage	Specific Activity (U)*		
		Keratinolytic Activity	Collagenolytic Activity	Lipolytic Activity
A	Soaking	19644,90	0	15712,02
	Unhairing	118,80	0	0
	Unhairing 2X	237,6	0	0
B	Soaking	1747,20	8846,04	4788,70
	Unhairing	296,20	939,40	0
	Unhairing 2X	592,4	1878,8	0
C	Soaking	21974,40	169,20	10080
D	Unhairing	0	990,40	402
	Unhairing 2X	0	1980,8	804
E	Unhairing	0	30,24	391,08
	Unhairing 2X	0	60,48	782,16

*The values of specific activity were multiplied by the respective mass of enzyme added in each test
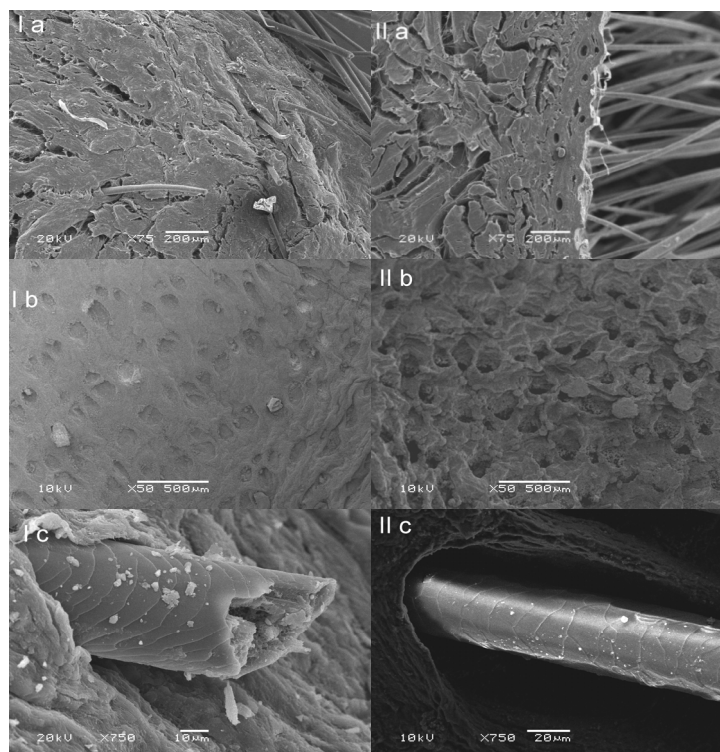

Figure 5: SEM analysis of soaking: (a) X 75, unhairing/liming; (b) X 50 and hair; (c) X 750 of the control (I) and experimental (II) tests

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 the formulations of the 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 enzyme did not lead to a greater removal of this material.

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 recommended for application by the manufacturer and the specific activity of the enzyme for the main substrates at the pH of the process should also be considered.

REFERENCES

Adigüzel, A. C., Bitlisli, B. O., Yaåÿa, I. and Eriksen, N. T., Sequential secretion of collagenolytic, elastolytic, and keratinolytic proteases in peptide-limited cultures of two *Bacillus cereus* strains isolated from wool. Journal of Applied Microbiology, v. 107, n. 1, p. 226-234 (2009).

Alexander, K. T. W., Enzymes in the Tannery - Catalysts for Progress? Journal of the American Leather Chemists Association, v. 83, n. 9, p. 287-316 (1988).

Amaral, L. A., Lutckmeier, C. V., Gutterres, M., Marcílio, N. R., Descromagem de resíduos de couro wet blue através de hidrólise enzimática. XVII Congresso Brasileiro de Engenharia Química. Recife: p. 8 (2008). (In Portuguese).

Aravindan, R., Saravanabhavan, S., Thanikaivelan, P., Rao, J. R., Nair, B. U., A chemo-enzymatic pathway leads towards zero discharge tanning. Journal of Cleaner Production, v. 15, n. 13-14, p. 1217-1227 (2007).

Bajza, Z., Thermal and enzymatic recovering of proteins from untanned leather waste. Waste Management, v. 21, n. 1, p. 79-84 (2001).

Bhavan, S., Rao, J. R. and Nair, B. U., A potential new commercial method for processing leather to reduce environmental impact. Environmental Science and Pollution Research, v. 15, n. 4, p. 293-295 (2008).

Cantera, C., Martegani, J., Esterelles, G., Vergara, J., Collagen hydrolysate: 'soluble skin' applied in post-tanning processes - Part 2. Interaction with acrylic retanning agents. Journal of the Society of Leather Technologies and Chemists, v. 86, n. 5, p. 195-202 (2002).

Choudhary, R, B., Jana, A. K. and Jha, M. K., Enzyme technology applications in leather processing. Indian Journal of Chemical Technology, v. 11, n. 5, p. 659-671 (2004).

Dettmer, A., Ayub, M. A. Z. and Gutterres, M., Hide Unhairing and characterization of commercial enzymes used in leather process. Brazilian Journal of Chemical Engineering, v. 28, n. 3, p. 373-380 (2011).

Feairheller, S. H., Next major change in leather manufacturing technology - What is it likely to be and how close are we to it? Journal of the American Leather Chemists Association, v. 80, n. 12, p. 312-323 (1985).

Gutterres, M., Dettmer, A., Souza, F. R., Amaral, L. A. and Sousa, M. F., Applications of biotechnology in leather. Lederpiel, v. 80, 2nd trimester, p. 36-42 (2010).

Ionata, E., Canganella, F., Bianconi, G., Benno, Y., Sakamoto, M., Capasso, A., Rossi, M. and La Cara, F., A novel keratinase from *Clostridium sporogenes* bv. Pennavorans bv. nov., a thermotolerant organism isolated from solfataric muds. Microbiological Research, v. 163, n. 1, p. 105-112 (2008).

Jian, S., Wenyi, T. and Wuyong, C., Ultrasound-accelerated enzymatic hydrolysis of solid leather

waste. Journal of Cleaner Production, v. 16, n. 5, p. 591-597 (2008).

Kanth, S. V., Venba, R., Jayakumar, G. C. and Chandrababu, N. K., Kinetics of leather dyeing pretreated with enzymes: Role of acid protease. Bioresource Technology, v. 100, n. 8, p. 2430-2435 (2009).

Kolomaznik, K., Adamek, M., Andel, I. and Uhlirova, M., Leather waste-potential threat to human health, and a new technology of its treatment. Journal of Hazardous Materials, v. 160, n. 2-3, p. 514-520 (2008).

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with the folin phenol reagent. J. Biol. Chem., v. 193, p. 10 (1951).

Parvinzadeh, M., Effect of proteolytic enzyme on dyeing of wool with madder. Enzyme and Microbial Technology, v. 40, p. 4 (2007).

Rajamani, S., Chen, Z., Zhang, S. and Su, C., Recent Developments in Cleaner Production and Environment Protection in World Leather Sector XXX IULTCS Congress. Beijing China: p. 5 (2009).

Saravanabhavan, S., Aravindhan, R., Thanikaivelan, P., Rao, J. R. and Nair, B. U., Green solution for tannery pollution: Effect of enzyme based lime-free unhairing and fibre opening in combination with pickle-free chrome tanning. Green Chemistry, v. 5, n. 6, p. 707-714 (2003).

Souza, F., Alencar, M. I. F., Silva, E. M., Guterres, M., Aplicação de Enzimas no Processamento de Couros: Comparação entre Processos Químicos e Coenzimáticos. XVIII COBEQ. Foz do Iguaçú, Brazil, p. 6 (2010). (In Portuguese).

Taylor, M. M., Bailey, D. G. and Feairheller, S. H., Review of the uses of enzymes in the tannery. Journal of the American Leather Chemists Association, v. 82, n. 6, p. 153-165 (1987).

Thanikaivelan, P., Rao, J. R., Nair, B. U. and Ramasami, T., Recent trends in leather making: Processes, problems, and pathways. Critical Reviews in Environmental Science and Technology, v. 35, n. 1, p. 37-79 (2005).

Valeika, V., Beleska, K. and Sirvaityte, J., Alkali-free method of hide preparation for tanning. Braz. J. Chem. Eng., vol. 29, n. 2, p. 315-323 (2012).

Winkler, U. K. and Stuckmann, M., Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescen*s. J. Bacteril., v. 138, p. 8 (1979).

Yuan, J., Fan, X., Wang, Q., Wang, P. and Cui, L., Modification of wool fiber with protease - 1. Effect of ionic liquid pretreatment. Journal of Biotechnology, v. 6, p. 3 (2008).