Advances in Understanding of Enzymatic Unhairing of Bovine Hides

by

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Abstract

The unhairing process is the most significant contributor to pollution load in the beamhouse. This has made unhairing one of most investigated areas over the past few decades, where the focus has been to reduce the amount of sulfur-based chemistry used during processing. In a country like Brazil, with 36 million hides processed every year, the amount of sodium sulfide applied during unhairing is around 10.800 tons/year, which represents approximately 2.200* ton of sulfur added to the process.

A conventional unhairing process for bovine hides normally uses sulfide, sulfhydrate, lime, surfactants, and one or more auxiliaries based on mercaptans, thioglycolate, amines, urea, enzymes, or combination of these. The use of such auxiliaries was introduced many years ago with the purpose of reducing the amount of sulfide/sulfhydrate applied, while improving the efficiency of hair removal and helping to control swelling. Since then, many types of unhairing auxiliaries have come to the market, with different chemical compositions, for different purposes and with different efficiencies. Nevertheless, when the amount of sulfide/sulfhydrate needs to be reduced to very low levels without diminishing the quality of the unhairing and at reasonable cost, enzymatic auxiliaries are the preferred and logical choices.

In this paper we will present the results from an enzymatic unhairing process developed by Buckman that allowed the reduction of Na_2S offer to half of the normal levels. In addition to reducing the environmental impact of unhairing - and the whole beamhouse operation - the process also produced significant benefits in terms of increased area yield (from raw hide to wetblue), improved flatness, and less drawing while maintaining the same characteristics of grain integrity and tightness.

*Considering 25kg/hide and an offer of 1.2% of commercial Na2S with 50% concentration.

Introduction

Despite much investigation, unhairing in an environmentally and economically feasible manner is challenging. Many critical questions remain unanswered. An important approach in answering these questions is to understand the substrate you are working with and review different routes of unhairing. Another relevant factor is consideration of the structural conformation of the skin. In particular, physical obstacles (steric hindrance) to the access and action of chemicals and to the removal of unwanted substances/components are poorly understood.

Hair and epidermis, composed mainly of keratins, are the main targets of the unhairing process. Efficiency is measured by the removal of such components. Therefore, it is understandable that the majority of the unhairing auxiliaries on the market are active against keratins. However, a more careful look at the skin structure reveals other relevant substrates, which need to be addressed when the goal is to reduce the offer of sulfide/sulfhydrate during the unhairing process.

Understanding such substrates, especially the interface zone between dermis and epidermis - the Basement Membrane - is of great importance for the development of innovative, environmentally, and economically advantageous solutions. The review of the composition and morphology of the basement membrane presented in this paper is based mainly on human, porcine and mouse skin. Although some differences are expected, most of the components and distribution of them are similar enough to use this information to better understand the bovine hide basement membrane.

The Basement Membrane

The animal skin presents layers (zones) with specific morphology and composition. The epidermis and dermis are well known by the tanner and between these layers lies the Basement Membrane.

In a very simple way, the basement membrane can be described as an intermediate layer between dermis and epidermis, formed by several proteins that interconnect with each other, creating anchors and providing stability to the membrane (Figure 1). One of the main functions of the basement membrane is to promote adhesion between the dermis and epidermis.[1,2,3]

The basement membrane is a very complex structure that can be divided into 2 sub-layers (*lamina lucida* and *lamina dense*), formed by a variety of protein families (Figure 2).

The "outer" layer of the basement membrane (*lamina lucida*) is made up of different types of glycoproteins, among which the Laminin[2] family is the most abundant. The adhesion of the basement membrane to the epidermis and to the hair bulb is a result of links between laminins and other proteins from the epidermis (mainly *Collagen XVII* and *α6β4-Integrin)*. The "inner" layer of the basement membrane (*lamina dense*) is a protein network composed basically of *Collagen IV*, which confers structure to the membrane. It's in the *lamina dense* that the anchoring filaments of laminins and integrins are attached, and it's also in this layer that the anchoring fibrils – made up of *Collagen VII* - are "launched" into the papillary layer of the dermis, keeping both structures attached to each other.

Laminins, Collagen IV and Collagen VII

Of all the proteins that make up the basement membrane, 3 classes must be highlighted for either their structural or anchoring roles. These are the Laminins, Collagen IV and Collagen VII. Laminins[2, 3] are a family of gylcoproteins that probably comprises more than 50 members (12 types already documented). From these, laminins 5, 6, 7 and 10 are the most commonly found in the basement membrane of the dermal-epidermal junction of mammals, forming anchoring filaments with other proteins, such as integrins, collagen XVII (BP 180) and nidogen. These anchoring filaments keep the epidermis adhered to the basement membrane.

Collagen IV[3,4,5] presents a molecular and macromolecular structure that differs from all other collagen types. Currently 6 polypeptide chains have been documented for collagen IV, with $\alpha1(IV)$, $\alpha2(IV)$, $\alpha5(IV)$ and $\alpha6(IV)$ being the most commonly found in the basement membrane between the dermal-epidermal junction. Collagen IV makes up a network that gives structural form to the *lamina dense* of the basement membrane (Figure 4). It is in this network that the anchoring
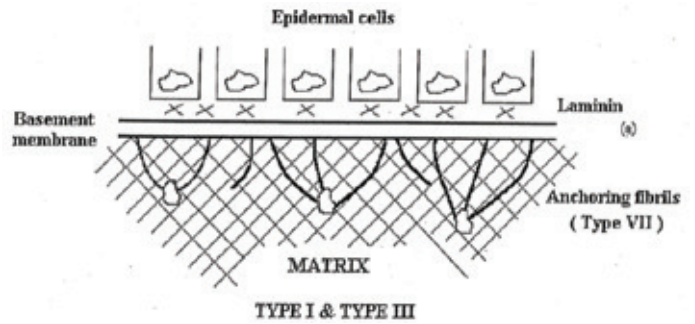

Figure 1. Schematic drawing of the anchoring structure between dermis and epidermis through the basement membrane *Extracted from "Cantera, C.S.: Hair saving unhairing process. Part 3. "Cementing Substances" and the basement membrane. J. Soc. Leather Tech. Chem., vol. 85, p.93."*
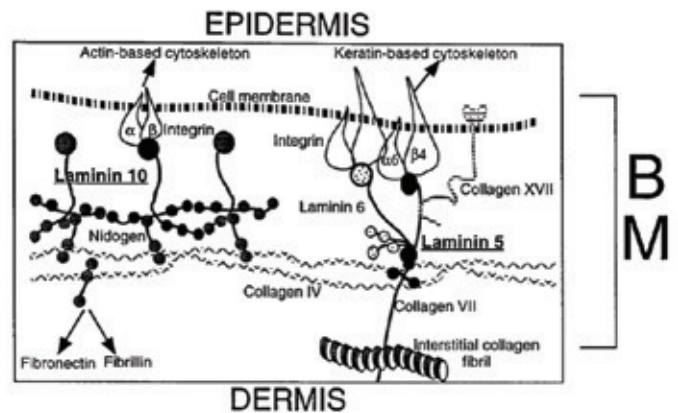

Figure 2. Schematic representation of the protein links within the basement membrane, creating anchoring filaments and fibrils, holding epidermis adhered to the dermis. *Extracted from "Aumailley, M. and Rousselle, P.: Laminins of the demo-epidermal junction. Matrix Biology 18, (1999), 19-28."*
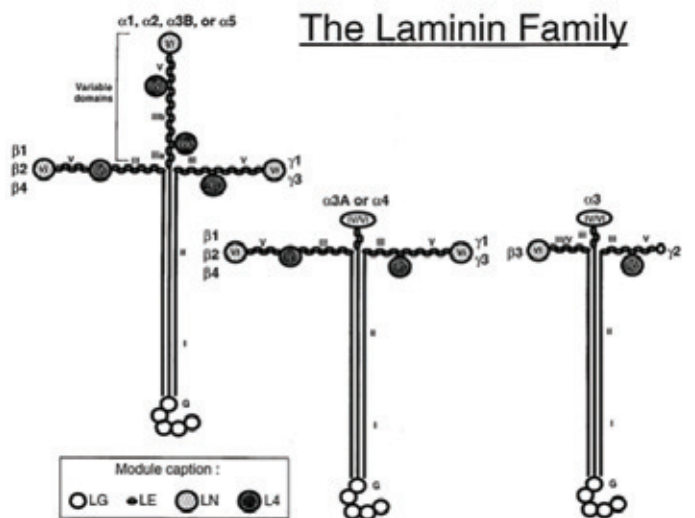

Figure 3. Schematic representation of laminins: linking proteins of the basement membrane. *Extracted from "Aumailley, M. and Rousselle, P.: Laminins of the demo-epidermal junction. Matrix Biology 18, (1999), 19-28."*

filaments described above and the anchoring fibrils of collagen VII attach themselves.

Collagen VII[3,6,7] is the main structural component of the anchoring fibrils, critical for the adhesion of dermis and epidermis. The molecule of collagen VII presents a triple-helical region 50% longer than all other collagen types. It has 2 non-collagenous regions (NC-1 and NC-2) and several cysteine residues that create intra- and inter-molecular disulfide bridges. Two chains of collagen VII assemble to create a dimer, which in turn aggregates with another dimer to form a tetramer, stabilized by disulfide bridges. This tetramer is the basic structure of the anchoring fibrils that attach to the collagen IV in the basement membrane and then extend themselves to the outer layers of the papillary dermis. There, the anchoring fibrils condense with the terminals of other fibrils or return to the basement membrane in a loop[6,7] (Figure 4).
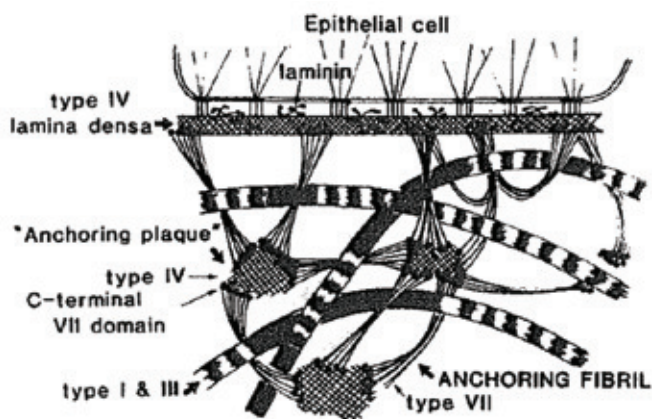

Figure 4 . Illustrative drawing of one of the accepted models of the distribution of anchoring fibrils. *Extracted from "Burgeson, R .E.: Type VII collagen, anchoring fibrils and epydermolysis bullosa. J. Invest. Dermatology, vol. 101, No. 3, Sep 1993."*

From the perspective of unhairing process, the breaking down of the anchoring systems in the above described structures may weaken the adherence of epidermis and hair bulb to the dermis. Since these systems are essentially composed of laminins, integrins and non-fibrous collagens, the use of selected proteolytic enzymes could present an efficient solution. The great challenge in this case is the selection of enzymes that would attack the basement membrane and epidermis proteins, while presenting no significant activity against dermis Collagen Types I and III.

Enzymatic Assisted Unhairing
The application of proteolytic enzymes as unhairing auxiliaries has been practiced for a long time and is well known by the leather industry. There are a number of benefits from such use:

- reduction in environmental impact and of safety issues related to handling hazardous chemicals;

- improvement in the quality of the pelts in terms of cleanliness and openness;

- significant increase in the wet blue area yield.

Nevertheless, the use of proteolytic enzymes still remains controversial. One of the main reasons is probably the lack of general knowledge of the action mechanism and control of enzymatic processes.

Enzymes are functional proteins that act as catalysts for specific chemical and biochemical reactions. There are different classes of enzymes and in the case of leather processing, the most commonly used are the hydrolases: enzymes that catalyze the hydrolysis of other molecules. Amylases, lipases, proteases, cellulases, glucanases, among others, are examples of hydrolases. In the case of the proteases, the catalyzed reaction is the hydrolysis of the peptide bond between amino acids in a protein, resulting in the breakdown of the protein backbone into smaller fractions (peptides or peptide chains). Each enzyme presents a certain level of specificity and it may be strictly specific to a single substrate or amino acid pair. In many industrial applications, the degree of hydrolysis is defined not only by the specificity of the enzyme, but also by process conditions, such as pH, temperature, contact time, presence of inhibitors or activators, ionic strength of the system, and the type of substrates present, among others.

In the animal skin *in natura* one will find substrates that could be hydrolyzed by different enzymes, such as lipases, proteases or carbohydrases (cellulases, glucanases, amylases, etc.). Among them, the proteases are the most commonly used by the tanner. Each protease presents a distinct hydrolysis profile or pattern, which is determined and controlled by its intrinsic specificity and by process conditions. The selection of the most appropriate protease to be used in each step in the beamhouse and the identification of the conditions that will lead to the best result from its action, is a crucial part of the process.

In the case of the unhairing process, considering the target substrates to be removed or hydrolyzed (basement membrane proteins, anchoring fibrils, keratins and pre-keratins, proteoglycans, glycosaminoglycans) and those that need to be totally or partially preserved (collagens I, III and VI, elastin), the choice of the appropriate enzyme is not an easy task!

This paper presents the results from the application of 2 different proteases (A and B), both alkaline serines of bacterial origin. Besides their proven efficiency in soaking and unhairing, another determining factor that led to the selection of these proteases was their negligible activity over collagen I or III under regular process conditions. The results confirmed that is possible and feasible to use proteases with no activity

over collagen to dehair different types of hides, while lowering the regular offer of sulfur-based chemicals and improving the quality and productivity of the process.

Another conclusion is that further investigations about the mechanism of basement membrane disruption during unhairing may reveal new ways to process leather in the beamhouse.

MATERIALS AND METHODS

The practical work presented in this paper is part of the ongoing screening used by Buckman to ensure efficacy during initial evaluations of every new unhairing chemical, especially enzyme-based ones. It was not the purpose of this paper to evaluate the action of enzymes on any particular component of the basement membrane, but to evaluate the overall activity in real trials. Future work should be carried out to further investigate the effect of enzymes or other unhairing auxiliaries on specific basement membrane substances, hair, and epidermal structures.

The proteases used in this study were formulated, stabilized and then evaluated in soaking and unhairing on pilot and industrial scale trials, using bovine hides of different origins (Zebuine, mixed breeds, and Angus).

The enzymatic preparations – stabilized formulations of proteases A and B – were applied during soaking, unhairing and liming. Besides the formulated proteases, regular chemicals were also used, such as sodium sulfide and/or sulfhydrate, lime, sodium carbonate, NPE-free surfactants, bactericides, and lipases.

To select the enzymes used in this work, the main factors considered were:

1. Enzyme efficiency on the substrates that need to be removed or hydrolyzed.

2. Low or negligible activity of the enzyme on substrates that need to be partially or completely preserved.

3. Commercial availability of the enzyme for large scale application.

4. Safe handling and storage.

Safe handling and storage of an enzyme is related to its stability and potential exposure of the workers. The most direct route of exposure to enzymes is by inhalation. This risk is minimized by employing enzymes in liquid form. Ensuring that the enzymes remain stable in liquid form requires proprietary technology and a high degree of formulation expertise. The enzymes used in this work were formulated and stabilized using proprietary technology to meet these safety objectives.

To assess the efficiency of each enzyme as a soaking or unhairing auxiliary, the only reliable method is the practical evaluation under optimal conditions. Analytical assays help to identify which enzyme presents higher activity against specific substrate under pre-defined conditions. However, since the performance on the leather also involves the morphology, physical structure, availability of substrates, and interaction among the chemicals used, such assays will only point in the right direction and help define process efficiency.

The activity of enzymes against substrates that need to be preserved is a more complex matter. Fortunately, enzyme activity can be addressed using analytical assays. The main structural components of the dermis are collagen type I and III. Obviously, these should not be removed. As collagen is the only protein in the animal skin to contain hydroxyproline in its molecule, it is common practice to estimate the amount of collagen in a sample by analyzing the amount of hydroxyproline. Thus, bench-scale soaking and unhairing trials[8] were conducted with bovine hides, using the proteases A and B (applied at 0.01% of the hide weight). The temperature was kept constant at 30°C and the pH was adjusted with Na_2CO_3 or NaOH. A "Control" (no enzyme, same pH, time and temperature conditions) and a "Positive Test" (thermal denaturation of the hide followed by extreme enzyme dosage) were carried out in parallel with the enzymatic tests.

Table I shows the hydroxyproline found in the float after 3h and 6h in each test, analyzed according to the method "FILK-AA-751.33 - Determination of hydroxyproline content". The results show that proteases A and B have no hydrolysis activity over collagen under the conditions evaluated.

Pilot scale Evaluations
Fresh bovine hides were sourced in the Southeast Region of Brazil and processed at Buckman Development and Application Laboratory, in the city of Franca. Each hide was cut in half (matched sides) and processed in identical drums with load capacity of 50kg each. One drum used a conventional soaking/liming recipe (control) and the other an enzymatic assisted unhairing recipe (Table II). After liming and tanning each side was visually assessed against its respective twin. Float samples were collected at the end of liming for analysis of COD, BOD, TSS, TKN, N-NH$_3$ and Sulfide

Industrial Scale Evaluations
Several trials were carried out in tanneries that produce and export wetblue, starting from fresh, wet salted, or brine cured bovine hides. Some hides were pre-fleshed and some were not, depending on the tannery. Each trial drum was processed against a control drum containing hides from the same source and initiated the same day. The visual assessment of pelts and WB was done comparing control and test drums.

TABLE I
Hydroxyproline analysis (mg/l) in the process float after treatment of bovine fresh hides with proteases A or B (0.01% over hide weight) at different pH values and contact times.[8]

Enzyme	pH 8 / 30°C		pH 10 / 30°C		pH 12 / 30°C	
	3h	6h	3h	6h	3h	6h
Protease A – 0.01%	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Protease B – 0.01%	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Control*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Positive test**	---	---	---	---	---	**662 mg/l**

N.D.: not detected; detection limit = 20 mg/l. *Control: no addition of enzyme. **Positive test: thermal denaturation of the hide at 95°C for 30min, then cool down to 30°C and treat with 0.10% of protease A for 6h at pH 12.

In order to evaluate potential area yield increase, more than 74.000 hides were selected and separated according to their average weight and source. Then, 27.171 hides were processed using enzymatic soaking and liming and the remaining ones using a control recipe. Tanning of all the pelts were carried out using the same recipe and the area of each Wet Blue was measured and compared to the expected area considering its raw hide weight. These data were used to calculate the area yield increase/loss achieved for each process batch (Table VI). The evaluation of crust leather was done taking leathers from 15 different tanning batches; all produced using enzymatic soaking and liming recipes, and comparing them to regular production crust leather. They were all retanned as shoe upper leathers.

Results and Discussions

The pilot scale evaluations followed the recipes presented in Table II. The sodium sulfide offer was reduced by 50% compared to the control recipe. Visual assessment of the matched sides was done during unhairing, at the end of liming, at the end of tanning, and after processing to crust. The main quality control parameters evaluated were: cleanliness, presence of hair roots, intensity of wrinkle, flatness/openness, color uniformity in wet blue, and grain tightness in crust. In addition, the environmental impact of each process was compared by analyzing the liming float for BOD, COD, TKN, N-NH$_3$, TSS and Sulfides.

The enzymatic assisted process presented similar or better results than the control for each parameter evaluated, including quality (Table III) and environmental (Table IV) attributes. The photographic record of the limed pelts also showed clear evidence of the superior performance of the enzymatic process (Figure 5).

Industrial scale evaluations followed the same basic soaking and liming recipe as used in the pilot trials, with minor adjustments were applied to match the operating requirements of each tannery where the trials were carried out. Table V summarizes the soaking and liming recipes used in the industrial scale trials. The leather was evaluated according to the same parameters used in the pilot tests, including the performance of the crust leather retanned for shoe uppers. Additionally, the efficiency of lime-fleshing (visual assessment) was evaluated for each production run and the area yield was measured through the wetblue stage.Besides confirming the superior results from the enzymatic assisted process in terms of cleanliness, openness, color uniformity and grain tightness, the leather processed with enzymes A and B presented:

- more complete removal of the flesh after liming,

- an average increase of 7.9% on area yield up to wetblue (see Table VI),

- reduction of up to 50% of sodium sulfide/sulfhydrate offer, and

- elimination of sulfur-containing auxiliaries.

The evaluations of crust leather also showed positive results: all 15 batches produced with enzymatic assisted process performed similar to regular production leather (data not shown).

Final Considerations

The results found in this work are in complete accordance with the commonly accepted theory of enzymatic assisted unhairing and can be summarized as follows:

- The potential action of the proteases evaluated in this paper over the basement membrane (intermediate layer to which the dermis and epidermis are attached) and on the basal layers of hair and epidermis, may have an important role in contributing to a deeper and more efficient removal of keratin-based compounds, resulting in a better cleanness.

TABLE II
Soaking and liming recipes used in pilot trials. Chemical dosage calculated based on fresh hide weight (not fleshed). *Protease expressed as concentrated version.

Process step/chemicals	Enzymatic process	Control process	Time
Soaking			
Water	100%	100%	
Sodium Carbonate	0,30%	0,30%	
Bactericide	0,10%	0,10%	
Lipase	0,05%	0,05%	
Protease A	**0,005%**	----	120 min.
Drain			20 min
Unhairing/Liming			
Water	40%	40%	
Lime	0,5%	0,5%	
NPE-free surfactant	0,10%	0,10%	
Dispersant/penetration aux.	0,05%	0,05%	30 min.
Lime	0,5%	----	
Protease A*	**0,0035%**	----	45 min.
Sodium sulfide (50%)	0,3%	0,6% (+ 0,5% lime)	
NPE-free surfactant	0,15%	0,15%	90 min
Lime	0,5%	0,5%	
Sodium sulfide (50%)	0,3%	0,6%	60 min.
Lime	0,5%	----	
Protease A*	**0,0015%**	----	60 min.
Water	Complete float	Complete float	
Lime	2,0%	2,5%	
NPE-free surfactant	0,10%	0,10%	30 min.
			Automatic o/n
			Wash / unload

TABLE III
Assessment of twin sides from pilot scale trials (pelt, wet blue and crust). Grades from 1 (worst) to 5 (best).

Parameter	Enzymatic process	Control process
Cleanness	5	4
Hair root / scud presence	5	3
Draw	4	3
Openness	4	3
WB color uniformity	4	4
Grain tigthness (Crust)	5	5

TABLE IV
Variation on pollution load of liming final floats from pilot scale trials: enzymatic process compared to control.

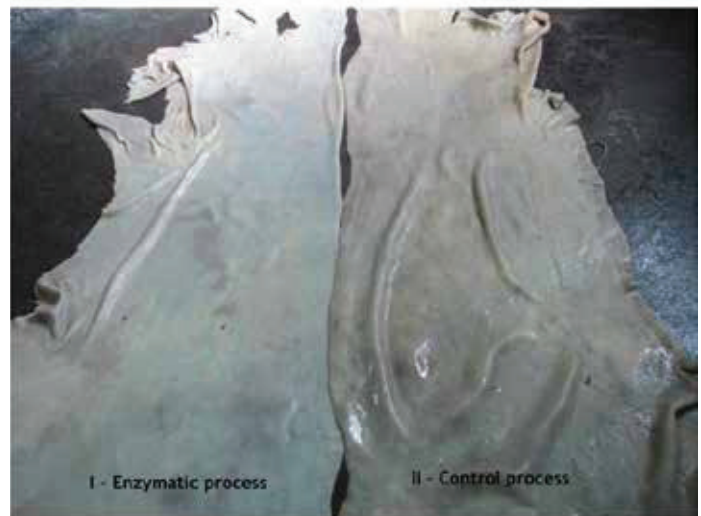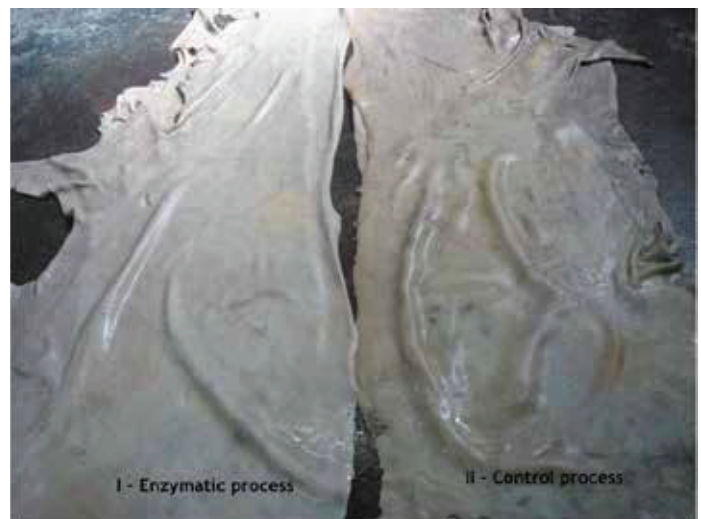| Parameter	Enzymatic process	Non Enzymatic (Control)	Difference
BOD	3.615 mg O_2/l	4.488 mg O_2/l	Reduction of 19,5%
COD	13.725 mg O_2/l	14.706 mg O_2/l	Reduction of 6,7%
N-NH$_3$	41,08 mg/l	49,14 mg/l	Reduction of 16,4%
TKN	1.083 mg/l	1.043 mg/l	Increase of 3,7%
TSS	3.000 mg/l	3.500 mg/l	Reduction of 14,3%
Sulfide offer	0.6-0.7% of Na$_2$S (50%)	1.2-1.4% of Na$_2$S (50%)	Reduction of 50%

Figure 5. Photographs of limed pelts from enzymatic (I) and control (II) processes on pilot trials.

TABLE V
Main differences between sulfide/sulfhydrate offer and unhairing auxiliaries during industrial scale trials. *Protease expressed as concentrated version.

		Tannery 1 – BR fresh, pre-fleshed hides		Tannery 2 – US fresh, pre-fleshed hides	
		Enzymatic	**Control**	**Enzymatic**	**Control**
Soaking	Protease A	0,0015%	----	0,0035%	----
Unhairing/Liming	Protease A	0.005%	----	0.0015%	----
	Protease B	----	----	0,003%	----
	Na$_2$S (50%)	0,60%	1,2%	0,15% (60% conc)	0,30% (60% conc)
	NaSH (70%)	----	----	1,30%	2,70%
	Sulfur-based unhairing auxiliary	----	1,0%	----	0,80%
	Lime	2,2%	2,2%	2,0%	2,0%

- The potential action of the proteases used in this paper on the basement membrane proteins and the pre-keratins for hair bulb and epidermis, weaken the adherence of epidermis and hair to the dermis, making their removal easier. As a practical result, much less sulfide/sulfhydrate and no other auxiliary is required for a clean and thorough unhairing process. There is also a significant reduction on BOD, Total Solids, N-NH$_3$ and Sulfide content in the wastewater.

- The removal of interfibrillar materials (non-structural proteins), initiated by the proteases tested during soaking and continued during unhairing, allows a higher relaxation of the dermis fiber structure. As a result, limed pelts and Wet Blue leather become more open, with less pronounced draw and a significant increase in Wet Blue area yield has been observed.

- Using the hydroxyproline content on the float as indicative for collagen hydrolysis, it is reasonable to conclude that the proteases evaluated in this paper do not demonstrate measurable activity on collagen under regular beamhouse conditions. Using these proteases, it would be possible for the tanner to benefit from the technical, productivity and environmental advantages already described, without the risk of damage to the final leather.

The results from pilot and industrial scale trials presented in this paper demonstrate that it is possible to reduce up to 50% the offer of sulfide/sulfhydrate chemicals during unhairing. At the same time, quality and productivity are improved, area yield is significantly increased and environmental impacts are reduced.

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TABLE VI
Comparative results of Wet Blue area yield (m² WB / kg of raw hide) from production scale trials. Control = regular soaking/unhairing recipe. Buckman = enzymatic soaking/unhairing recipe.

Raw hide supplier	Type of Soaking/ Unhairing Process	# of hides evaluated	Hides average weight (kg)	Wet Blue average area (m²)	WB expected area (m²)	Area Yield per drum (m² WB / kg of raw hide)	Difference from control
A	Control	2441	39,51	4,273	4,454	0,1081	
A	BUCKMAN	601	40,8	4,654	4,535	0,1141	+5.47%
B	Control	4676	40,68	4,342	4,529	0,1067	
B	BUCKMAN	3011	40,04	4,621	4,485	0,1154	+8.12%
C	Control	4387	41,34	4,370	4,562	0,1057	
C	BUCKMAN	3518	39,42	4,518	4,454	0,1146	+8.42%
D	Control	8803	47,7	4,457	4,789	0,0934	
D	BUCKMAN	3815	46	4,860	4,716	0,1057	+13.1%
E	Control	2198	32,85	4,093	4,155	0,1246	
E	BUCKMAN	1599	31,46	4,313	4,095	0,1371	+10.0%
F	Control	7255	43,49	4,512	4,651	0,1037	
F	BUCKMAN	1591	42,49	4,671	4,609	0,1099	+5.97%
G	Control	3294	32,39	4,058	4,143	0,1253	
G	BUCKMAN	1844	32,7	4,285	4,15	0,1310	+4.59%
H	Control	2670	32,28	4,017	4,141	0,1244	
H	BUCKMAN	1560	32,2	4,243	4,138	0,1318	+5.89%
I	Control	11301	44,28	4,268	4,69	0,0964	
I	BUCKMAN	9632	41,26	4,371	4,558	0,1059	+9.92%

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LIFE LINE

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