From a problem of solid waste to an useful product in beamhouse process

Galarza Betina1, Garro María Laura1, Gortari Cecilia2, Bonfranceschi Alfonsina1, Hours Roque2, Cantera Carlos1
1- Inti-Cueros (Citec-CICPBA), Camino Centenario e/505 y 508, Gonnet (1897), Argentina (54-0221-4840244)
2- CINDEFI (CONICET-UNLP), 50 y 115, La Plata (1900), Argentina (54-0221-4833794).
Biochemist Betina Claudia Galarza: e-mail: betinagal@hotmail.com

Abstract

In hair-saving unhairing process, almost 10% of the weight of salted bovine skin becomes hair waste. A strain of fungus Trichophyton ajelloi was isolated in samples from local soil using "Vanbreuseghem´s hair baiting technique". This strain was capable of growing in a liquid mineral medium, added with 10g/l of glucose and 5 mM of thioglycolic acid, using hair waste as only source of N and C. After optimizing different variables of culture, it was possible to obtain a crude extract of maximun performance in proteolytic activity, specially keratinolytic, ammonium and SH-generation.
When it was applied in beamhouse process in optimal conditions to reaction, along with commercial tensioactives and biocide (soaking, unhairing and bating process) at laboratory scale, changes at histological level and hair release were observed by optical microscopy. It is possible to suggest that this extract could be a useful by-product in leather technology.

Keywords: fungus Trichophyton, bovine skin, hair waste .

1. Introduction

Leather industry produces potentially hazardous waste, which depends of the unhairing process employed by the tanner. To diminish pollution, modified unhairing systems had been developed, enzymatic among them. The replacement of the conventional hair burning unhairing process breaking down the hair structure with lime and sodium sulphide by saving unhairing enzyme assisted can reduce pollution and the costs treatment of the associated effluent (Galarza et al.2012; Crispim et al. 2003; Schraeder et al.1996).
The enzyme activity must be directed to epidermis, hair follicle and basal lamina. In this procedure proteolysis needs to be controlled to prevent collagen damage (Cantera 2001, Cantera et al. 2004, Garro et al. 2008). Collagen is the principal protein related with the quality of leather. The potential for commercial use of enzymes in leather industry has an important economic and environmental impact (Riffel et al. 2003).

Keratinases, enzymes for proteolytic depilation and keratin degradation, have been purified and characterized from pancreas and from different microorganisms such as fungi, actinomycetes and bacteria.

Some fungi from Dermatophytes and fungi imperfecti genera (Kunert 2000; Simpanya 2000) have been reported high keratinolytic enzyme production (Friedrich et al 1999). These extracellular enzymes are capable to degrade proteins in bovine skin: keratins, albumins, globulins (Lollar1956) and proteoglycans (Cantera 2001).

The processes of keratin degradation in Dermatophytes involve two complementary mechanisms: sulphitolysis and the breaking of the intra-chain peptide bond. During the first mechanism, rupture of disulphide bond of keratin provides the sulphite ion that would act on the same kind of bond to generate thiosulphuric esters like S-sulfocysteine and thiols (Ruffin et al. 1976). In the second mechanism protein proteolysis would take place.

In hair-saving unhairing process, it is possible to recover hair at the level of about 10% of the weight of salted bovine hair. In this paper hair waste was the source of C and N to the growing fungus Trichophyton ajelloi. This strain was isolated in samples from local soil using "Vanbreuseghem´s hair baiting technique" (Galarza et al. 2004).
Enzymatic pool from Trichophyton ajelloi was applied in different stages of the beamhouse process at laboratory scale, as depilation agent principally. Changes at histological level were determined by optical microscopy.

Material and Methods

Culture conditions

The innoculum was prepared as follows: Trichophyton ajelloi was cultured seven days in Sabouraud's Agar in 250 ml Erlenmeyers. Then, the conidia (fungal spore) were resuspended with magnetic agitation in 10 ml of destilled water with Tween 80. Submerged cultures were carried out in 500 ml Erlenmeyers flaskes using 200 ml of minimal mineral medium (MMM): buffer NaH2PO4-K2HPO4 pH 7,2, chloranphenicol 0,5 g/l and trace amounts Cl3Fe, Cl2Zn and Cl2Ca with 1% (w/v) of hair waste from hair-saving unhairing process, cleaned ,ground and autoclaved at 121 ℃ for 15 min (Galarza et al. 2007). MMM was added in a final concentration 5 mM of sodium thioglycolate.
Flasks were inoculated with 10 ml conidia suspension whose viable-count was an average of 105ufc/ml and then incubated at 28°C, 200 rpm for 28 days. Crude extract /CE) was separated by cold centrifugal pump at 3000 x g, 5°C for 15 min and was vacuum filtered through 0,45 µm cellulose membrane in an ice bath and kept at -20°C until later analysis of the variables.

Assay conditions

Keratinolytic activity, protein concentration and pH were determined in initial CE and in process baths (remanent activity). Optimal conditions of the enzyme when it was applied along with biocide, different tensioactives and Ca+2 were sought (Matikevičienė et al. 2011).
For evaluate the enzymatic stability, residual final keratinolytic activity was measured when crude extract was incubated for 4 h 30 min at 37°C with tensioactives and biocide.
Enzymatic extract was used in the following stages of the beamhouse area: soaking, unhairing, deliming, and bating. Controls: skin whitout any treatment and skin with conventional unhairing process based in sodium sulphide and lime.

Previous trials

Activation or inactivation of the added compounds at different process over keratinolytic enzymes in CE was measured at the followings finals relationship (Cantera 2003).
A. biocide/enzyme (w/w): 100/1
B. Ca+2/enzyme: 1 mg/ml CE
C. biocide/enzyme (w/w): 100/1; 1 mg Ca+2/ml CE

Initial CE conditions: 1,32 Uk/ml, 0,42 Uazo/ml, 0,136 mg/ml de prot.
Ca +2as (OH)2Ca: 1 mg/ml in 0,1 M Tris-HCl buffer pH 9.
Biocide: TCMTB 27%, 10% N,N-Dimethylethylamine (penetrating/emulsify), 63% cresol (FUBA chem).

Assessment of protein content and proteolytic activity

Protein concentration was determined by Bradford's method (Bradford 1976).

Assay of proteolytic-keratinolytic activities

Azocaseinolytic activity was determined using azocasein as substrate (sulphamide azocasein, Sigma Chem.Co., St. Louis, MO). Reaction mixture containing 100 µl of crude extract and 250 µl of the 1% (w/v) substrate solution in 0,1 M Tris-HCl buffer pH 9 was incubated for 30 min a 37°C and then stopped by the addition of 1 ml of trichloroacetic acid (TCA) 10% (w/v) and centrifuged (3000xg, 15 min). One ml of NaOH was added to 0,9 ml of the supernatant, agitated and measured at 440 nm (triplicate). Blank was performed using enzyme solution inactivated for heating at 100°C, 5 min (Liggieri et al 2004).
Azocasein activity unit (Uazoc) is defined as the amount of enzyme that, under the test conditions, causes an increase of 0,1 Abs440nm per minute.
Keratinolytic activity was assayed by using hair waste as substrate, washing with tensioactives, dried at 45°C, ground, autoclaved at 121°C and retained with 850 µ sieve, USA Standard ASTM E 11-61.
Reaction mixture containing 150 µl of CE and the 1%(w/v) substrate in buffer Tris-HCl 0,1 M pH 9 (Yamamura et al 2002) was incubated at 37°C in agitation (100 rpm) for 1 h, then stopped by the addition of 1 ml of trichloroacetic acid (TCA) 10% (w/v), centrifuged (5000x g 15 min) and the supernatant was measured at 280 nm. Blank was performed using CE inactived with TCA 10% (w/v) at the beginning of the incubation.
Keratinolytic activity unit (Uk) was defined as the amount of enzyme that, under the test

condition, causes an increase of 0,01 Abs280nm per minute.

Experimental procedure for skin observations

Pieces of fresh skin of 3 cm x 2,7 cm and aproximately 3 gr each were placed one each beakers with 30 ml of liquid, according to the following treatment:
I)Soaking stage Treatment 1(T1): enzymes (CE) (from a lyophilisate dissolved in buffer Tris-HCl 0,1 M, pH9), isogras AN 0,1%: S1
Treatment 2 (T2): without enzymes, same conditions as 1: S2 Treatment 3 (T3): enzymes (dissolved in similar conditions), isogras AN 0,1%, pH 9, biocide FUBAC 0,2% of wet skin: S3 Treatment 4 (T4): without enzymes, same conditions as 3: S4 Treatments 5 y 6 (T5 y T6): with conventional procedure: without enzymes,buffer Tris-HCl 0,1 M, isogras AN 0,1%, pH 9, biocide 0,2% of wet skin: S5 y S6.

Length and conditions of soaking: beakers were agitated at 40 rpm in thermostatic bath at 37°C for 4 h.
II) In unhairing stage, 0,5 % Baymol surfactant was added to T1: U1, T2:U2, T3:U3, T4:U4, which increases azocaseinolitic activity by 117% and keratinolytic activity by 331%. Conventional unhairing with immunization was applied in T5:U5 and T6:U6 processes. Lime 3% was added, then sodium hidrosulfide 1%; 30 min later, sodium sulphide 2%. The proportions used were related to wet skin weight.

Length and conditions of unhairing: the beakers were agitated in thermostatic bath at 40 rpm at 37°C for 24- 48 h.
III) Deliming stage: only in T5 y T6. The aims are removing the residual lime of the skin, decreasing its pH for subsequent enzymatic action and reducing the skin swelling. In this step ammonium sulphate 2% and sodium metabisulphite 1% were added.

Length and conditions of deliming: thermostatic bath at 37°C for 60-120 min.
Control pH was performed in bath with pH meter and in skin interior with phenolphtalein.

IV) Bating stage: to remove the unwanted skin remains (epidermis, hair roots, glands, lipids) enzymatic solution (CE) was used only in T5: B5. In T6: B6 was only washed, and after buffer Tris-HCl 0,1 M pH 9 was added.
Length and conditions of bating: the beakers were agitated in thermostatic bath at 40 rpm at 37°C for 40 min.

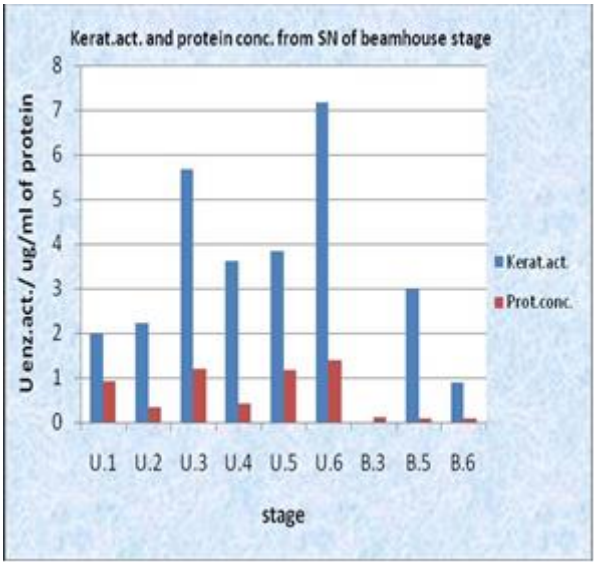
Histological techniques

Pieces of bovine skin from each stage were fixed in formaldehyde 10% at room temperature, washed, dehydrated in alcohols 100, 96 and
70%, cleared in two changes of xylenes and embedded in paraffin. Paraffin blocks were cut in sections approximately 6 µm thick and mounted on alcohol cleaned slides. After that, deparaffinated in xylene and passed from 100, 96, 70% alcohol and distilled water. Rehydrated sections were stained with hematoxylin eosin (Prophet et al.1992) and observed in an Olympus CHBS microscope.

Results and Discussion

In previous trials, in activation/inactivation assays, it was observed activation in experiments A (thirty times) and C (seventeen times), after enzyme incubation with biocide only and with biocide and Ca+2 respectively along 4h 30 min at 37°C in agitation(100 rpm).

Assays in process baths (remanent activity).



U: unhairing process
B: bating process
SN: supernatant

In U2 and U4 it was observed keratin hydrolysis, therefore absorbance at λ 280 nm

(free aminoacides and polypeptides absorption) and protein concentration increased even if enzyme was not present.

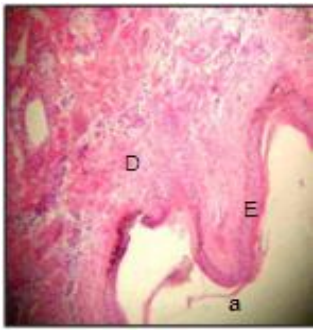
When conventional unhairing process is applied (U6, U5) maximum destruction of hair was measured, while in B5 greater dissolution of protein from dermis, epidermis and hair was continued.
Bating process have made only in T3 (B3) because in U1, U2, U4 unhairing was not evident.

Microscopic observations

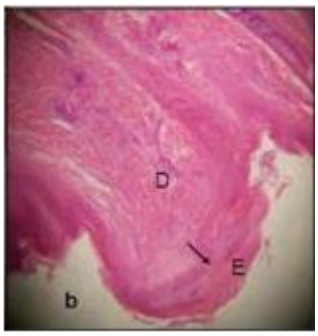
By means of histological techniques, changes at dermis, epidermis and annexes subject to different treatments were found.
In control without treatment (Figures 1a-2a), the skin preserved intact dermis and epidermis structure with normal distribution of collagen fiber and epidermal layers visible with the employed stain. Hair follicle sheets were clearly observed.

In samples after 48 h treatment (T3, U3) with enzyme, biocide and tensioactive, dermis, epidermis and hair follicle were altered with dermal collagen fibres disorganized and loss of epidermal layers. The combination of tensioactives with enzyme (T1,S1) after 48h treatment caused collagen disorganization, hair follicles without epithelial sheets and epidermis detached from dermis (Figure 3b). Tensioactives as sodium dodecylsulphate (SDS) are capable of bound to fibrous and globular proteins, when applied in a solution produces the disorganization of collagen structure (Cantera et al. 2004). In figure 3a treated 48h with tensioactive (T2, S2) the collagen bundles of the superficial dermis appear extremely closed and compact (Sivasubramanian et al. 2008).

Figure1. Hematoxylin Eosin



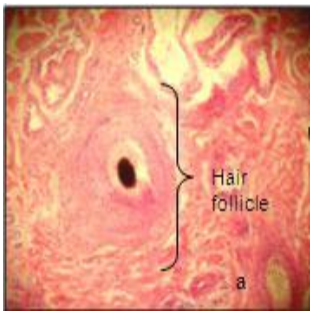
a. Control without treatment



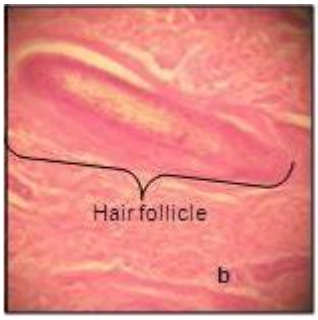
b. Enzyme-tensioactive-biocide
48 h treatment (10 x)

(E: epidermis D: dermis)

Figure 2. Hematoxylin Eosin

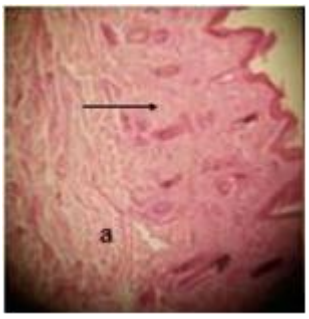


a: control without treatment
(10 x)

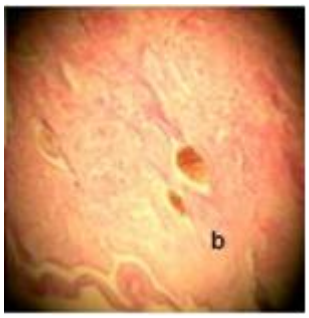


b.enzyme-tensioactive-biocide
48 h treatment (10 x)

Figure 3. Hematoxylin Eosin



Tensioactive
48 h treatment (4x)



b.Tensioactive-Enzyme
48 h treatment (10 x)

Figure	Treatment	Microscopic observation
1a	none	Dermal collagen fibres with typical distribution. Epidermis withvisible layers. Epitelial sheets of hair follicle without changes
1b	Enzyme-biocide-tensioactive (48 h)	Dermal collagen fibres desorganized and loss of epidermal layers
2a	none	Hair follicle cross section with undamaged epithelial sheets
2b	Enzyme-biocide-tensioactive (48 h)	Empty hair follicle without epithelial sheets.
3a	Tensioactive (48 h)	Compact collagen bundles in superficial dermis
3b	Enzyme-tensioactive (48 h)	Hair follicle without epithelial sheets. Collagen disaggregation. Epidermis detached from dermis

Conclusions

The treatments with enzymes combined with tensioactive and biocide, enzymes and tensioactive as only with tensioactive altered collagen, hair follicle and epidermis of bovine skin. These changes were clearer when enzymes were added. The time of contact between substances with skin could influence the alteration and must be considered in future assays.
The optimal conditions for the use of fungal extract must be analyzed as well as the stage of the leather process in which utilize it for application as by-product in leather technology.

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References

Bradford M, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72 (248-254p).

Cantera C, 2001. Hair-saving unhairing process Part 3. Cementing substances and the basement membrane. Journal of the Society of Leather Technologists and Chemists, 85 (93-98 p).

Cantera C, 2001. Hair saving unhairing process Part 4.Remarks on the evolution of the investigations on enzyme unhairing. Journal of the Society of Leather Technologists and Chemists, 85 (125-132 p).

Cantera C, Goya L, Galarza B, López LM, Garro ML, 2003. Characterization of enzymatic preparations applied in soaking and unhairing process, Part 5. Journal of the Society of Leather Technologists and Chemists, 87 (89-90 p).

Cantera C, Garro ML, Goya L, Barbeito C, Galarza B, 2004. Hair-saving unhairing process. Part 6. Stratum corneum as a diffusion barrier. Chemical-mechanical injury of epidermis. Journal of the Society of Leather Technologists and Chemists, 88 (121-131p).

Crispim A, Mota M, 2003. Unhairing with enzymes. Journal of the Society of Leather Technologists and Chemists, 87 (198-202 p).

Friedrich J, Gradisar H, Mandin D, Chaumont JP, 1999. Screening fungi for synthesis of keratinolytic enzymes. Letters in Applied Microbiology, 28, (127-130 p).

Galarza B, Goya L, Cantera C, Garro ML, Reinoso E, López LM, 2004. Fungal transformation of bovine hair. Part 1: Isolation of fungus with keratinolytic activity. Journal of the Society of Leather Technologists and Chemists , 88 (93-98 p).

Galarza B, Garro M, Cavello I, Cazau M, Hours R, Cantera C, 2007. Fungal biotransformation of bovine hair: assessment of structural changes. Journal of the Society of Leather Technologists and Chemists 91 (229-232p).

Galarza B, Cavello I, Garro ML, Gortari M, 2012. Evaluation of increase at the production of keratinolytics enzymes. Journal of Aqeic, 63 (70-74p).

Garro ML, Barbeito C, Mario R, Galarza B, Cantera C, 2008. Evaluación histológica del efecto de enzimas con actividad depilatoria sobre piel vacuna. Un estudio preliminar. InVet Investigación Veterinaria, 10, nº1 (103-110p).

Kunert J, 2000. Physiology of keratinophilic fungi. Ed. Revista Iberoamericana de Micología, Ed. RKS Kushwaha & Guarro J, Bilbao. ISBN 84-607-0711-3, cap 10 (77-85 p).

Liggieri C, Arribère MC, Trejo S, Canals F, Avilés F, Priolo N, 2004. Purification and biochemical characterization of Asclepain c I from the latex of Asclepias curassavica. The Protein Journal, 23 (403-411p).

Lollar R,1956. The Chemistry and Technology of Leather, Ed O´Flaherty F, Roddy W y Lollar R, Cap I (303 p).

Matikevičienė V, Grigiškis S, Levišauskas D, Sirvydytė K, Dižavičienė O, Masiliūnienė D, Ančenko O, 2011. Optimization of keratinase production by actinomyces fradiae 119 and its application in degradation of keratin containing wastes. Environment, Technology, Resources. Proceedings of the 8th International Scientific and Practical Conference, Rēzeknes Augstskola, Rēzekne, RA Izdevniecība, vol. 1 (294-300 p).

Prophet E, Mills B, Arrington J, Sobin L, 1992. Laboratory Methods in Histotechnology. Armed Forces Institute of Pathology (AFIP), United States of America Washington DC, American Registry of Pathology.

Riffel A, Ortolan S, Brandelli A,2003. De-hairing activity of extracellular proteases produced by keratinolytic bacteria. Journal Chemical Technology and Biotechnology, 78 (855-859 p).

Ruffin P., Andrieu S., Biserte G ,1976. Sulphitolysis in keratinolysis. Biochemical proof. Sabouraudia. 14 (181-184 p).

Schraeder C, Ervin R, and Eberspacher J, 1998. Economic analysis of the feasibility of using enzymes in the unhairing process, Journal of the American Leather Chemists Association, 93 (265-271).

Simpanya M, 2000. Dermatophytes: Their taxonomy, ecology and pathogenicity. Revista Iberoamericana de Micología, (1-12 p).

Sivasubramanian S, Manohar M, 2008. Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. Chemosphere, 70 (1025-1034 p).

Yamamura S, Morita Y, Hasan Q, Yokoyama K, Tamiya E,2002. Keratin degradation: a cooperative action of two enzymes from Stenotrophomonas sp. Biochemical and Biophysical Research Communications, 294 (1138-1143 p).