From A Problem of Solid Waste to an Useful Product in Beamhouse Process

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Abstract

In hair-saving unhairing process it is posible to recover hair at the level of about 10%of the weight of salted bovine hair. 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, aditionned with 10g/l of glucose and 5 mM of thioglycolic acid, using hair wasre as only source of N and C. After optimizing different variables of culture, it was posible 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 comercial tensioactives and biocide (soaking, unhairing and bating process) at laboratory scale, changes at histological level and hair release were observed by optical microscope and SEM. It is possible to suggest that this extract could be an useful by-product in leather technologies.

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 associated effluent treatment costs (Galarza et al.2012; Crispim et al. 2003; Schraeder 1996).

The enzyme activity should be directed to epidermis, hair follicle and basal lamina. In this procedure, difficulties are originated because proteolysis needs to be controlled to prevent the extension of its activity to collagen (Cantera 2001, Cantera et al. 2004). Collagen must keep its optimal conditions for tannage process and to give high quality leather. The potential for commercial use of enzymes in leather industry has an important economic and environmental impact (Riffel 2003).

Enzymes for proteolytic depilation and keratin degradation, the keratinases, have been purified and characterized from pancreas and different microorganisms such as some fungi, actinomycetes and bacteriagenera. Some fungi, especially that belonging to Dermatophytes genera (Kunert 2000; Simpanya 2000) and others from fungi imperfecti have been reported as a good producers of keratinolytic enzymes (Friedrich et al 1999).

These microorganisms have showed to produce extracelular enzymes ables for degradation of different types of proteins presents in bovine skin: keratins, albumines, globulines ,Lollar(1956) and proteoglicanes (Cantera 2001).

Among dermatophytes , keratin degradation process involve two complementary mechanisms: sulphitolysis process and inter e intrachains bonds peptidics breaking. During the first mechanism ruptura of disulphide bond of the keratin providing the sulphite ion that would

act on the same kind of bond to generate thiosulphuric esters like S-sulfocysteine and thiols (Ruffin 1976).

In hair-saving unhairing process it is posible to recover hair at the level of about 10%of the weight of salted bovine hair. In this paper hair waste was the source 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). Crude extract was utilisated as depilation agent.
In this paper, enzymatic pool from fungus *Trichophyton ajelloi* was applied in different stages of beamhouse process at laboratory scale for determine changes at histological level by optical microscope.

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 conidia was resuspended whit stir agitation in 10 ml water destilated aditionated with Tween 80. Submerged cultures were carried out in 500 ml Erlenmeyers flaskes using 200 ml of minimal mineral médium (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 ºC for 15 min (Galarza et al 2007). MMM was aditionated in final concentration 5 mM of sodium thioglycolate. Flasks were inoculated with 10 ml conidia suspension (fungal spore) whose viable-count was an average of 10^5 ufc/ml and then incubated at 28ºC, 200 rpm for 28 days. Crude extract was separated by cold centrifugal pump at 3000x g, 5ºC for 15 min and was vacuum filtered through 0,45 µm cellulose membrane in an ice bath and kept at -20ºC until protein and enzymatic activity determination.

Assay conditions

The enzymatic activity was evaluated in every tanning stage, where enzymes are used. Optimal conditions of the enzyme and the necessary compounds that facilitate its action were sought (Matikevičienė 2011).

Enzymatic extract was used in the following stages of the beamhouse area: soaking, unhairing, deliming, and bating. Controls: Skin whitout treatment and skin with conventional hair burning unhairing process

Keratinolityc activity , protein concentration and pH were determined in initial crude extract , and in process baths(remanent activity) Besides residual final keratinolytic activity was measured when crude extract was incubated for 4h30 min at 37ºC with tensiaoactives and biocides utilized.

Previous trials:

Activation or inactivation of the aditionated compounds at differents process over keratinolytic enzymes in crude extract (CE) was measured at the followings relationship (w/w) (Cantera 2003).

A. enzyme/biocide: 100/1

B enzyme/Ca^{+2}: 1 mg/ml CE

C enzyme/biocide/Ca^{+2}: 100/1/ 1mg/ml CE

Initial crude extract conditions: 1,32 $U_{k./ml}$, 0,42 U_{azoc}./ml, 0,136 mg/ml de prot.

Ca^{+2} as $(OH)_2Ca$: 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 (sulphamide azocasein) as substrate from 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 37ºC and then stopped by the addition of 1 ml of tricloroacetic 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 100ºC 5 min. (Liggieri et al 2004)

Azocasein activity unit (U_{azoc}) is defined as the amount of enzyme that, under the test conditions causes an increase of 0,1 Abs_{440nm} per minute.

Keratinolytic activity was assayed by using hair waste as substrate, washing with tensioactives, dried at 45ºC, ground, autoclaved at 121ºC and retained with 850 µ sieve, USA Standard ASTM E 11-61

Reactions mixture containing 150 µl of the 1%(w/v) (Yamamura 2002) substrate solution in buffer Tris-HCl 0,1 M pH 9 was incubated at 37ºC in agitation (100 rpm) for 1 h and then stopped by the addition of 1 ml of tricoloroacetic acid (TCA) 10% (w/v) and centrifuged (5000 x g, 15 min) and the supernatant was measured at 280 nm. Blank was performed using enzyme solution inactived with TCA at the begining of the incubation.

Keratinolytic activity unit (Uk) was defined as the amount of enzyme that, under the test conditions causes an increase of 0,01 Abs_{280nm} per minute.

Experimental procedure for skin observations:

In soaking stage, six 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

1) Enzyme (EC) (from a lyophilisate dissolved in buffer Tris-HCl 0,1 M, pH9), isogras 0,1%.

2) Without enzyme, same conditions as 1)

3) Enzyme (dissolved in similar conditions), isogras 0,1%, pH 9, biocide FUBAC al 0,2% of wet skin

4) Without enzyme, same conditions as 3)

5) y 6) With conventional procedure: without enzyme, buffer Tris-HCl 0,1 M, isogras 0,1%, pH 9, biocide 0,2% wet skin.

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 1): U1, 2):U2, 3):U3, 4):U4, which increases azocaseinolitic activity by 117% and keratinolytic activity by 331%. . Conventional unhairing with immunization was applied in 5):U5 and 6):U6 processes: lime 3% was added, then sodium hidrosulfide 1%; 30 min later,sodium sulphide2%. The proportions used are in relation with wet skin weight.

Length and conditions of unhairing: the beakers were agitated in thermostatic bath at 40 rpm at 37°C for 4 h.

III) Deliming stage: The aims are removing the residual lime of the skin, decreasing its pH (for subsequent enzymatic action and reducing the skin swalling.In this step ammonium sulphate 2%was added to 5) and 6), and sodium metabisulfite 1%.

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: skin remains: epidermis, hair roots, glands, lipids. Were remove with enzymatic solution (EC) was used only in 5):B5; Beaker 6): B6 was washed, and after buffer Tris-HCl 0,1 M pH 9 was added.

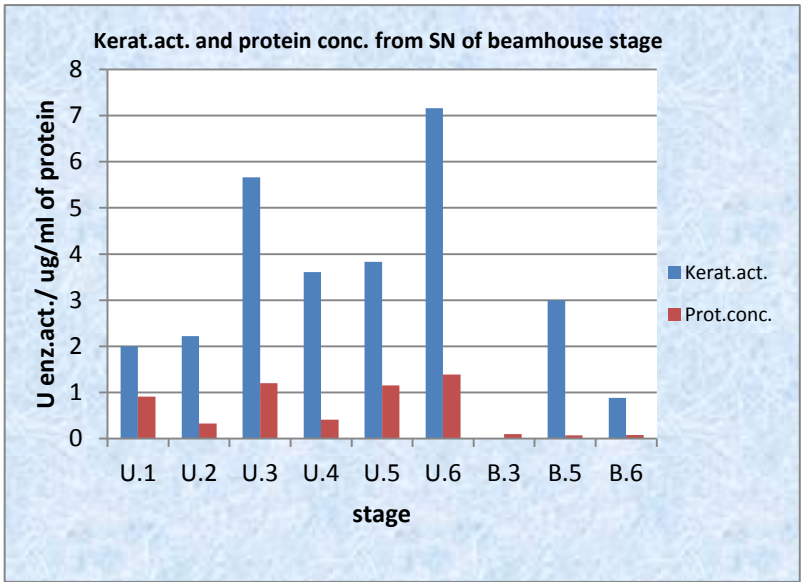
Length and conditions of bating: the beakers were agitated in thermostatic bath at 40 rpm at 37°C for 40 min.

Pieces of bovine skin from each stage were set aside for observation and microscoping staining, stored in formaldehyde 10% at room temperatura,washed, dehydrated in alcohols 100, 96 and 70%, cleared in wo 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 destilled 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, activation/inactivation assays it was observed activation in experiments A (thirty times) and C (seventeen times), after incubation 4 h 30 min at 37ºC in agitation(100 rpm)

Assays in process baths (remanent activity)



U: unhairing process
B: bating process
SN: supernatant
In U.2 and U.4 it observed hair hydrolysis, therefore $A_{280\ nm}$ (free aminoacides and polypeptides) and protein concentration increased even if enzyme was not present.

When conventional unhairing process is applied (U.6, U.5), maximum destruction of hair was measured, while in B.5 greater dissolution of protein from dermis, epidermis and hair was continued. Bating process have made only in 3 treatment (B.3) because in U.1, U.2, U.4 unhairing was not evident.

Microscopic observations

With histological techniques (Figures 1a-2a) we observed that in control without treatment 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 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, after 48h treatment caused collagen disgregation, 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 2004). In figure 3a treated 48h with tensioactive the collagen bundles of the superficial dermis appear extremely closed and compact (Sivasubramanian 2008).

Figure 1. Hematoxylin Eosin-10X. . E: epidermis D: dermis **a.** Control without treatment. **b.** Enzyme-tensioactive-biocide 48 h treatment

Figure 2. Hematoxylin Eosin 10X.**a:** control without treatment **b.**enzyme-tensioactive-biocide 48 h treatment

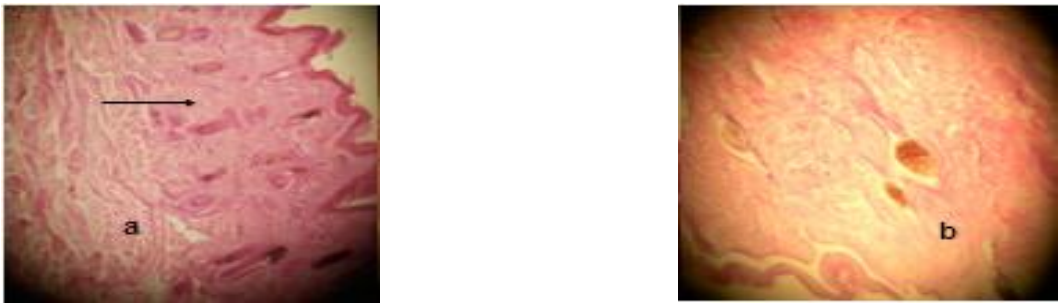


Figure 3. Hematoxylin Eosin **a.**.4X Tensioactive 48h . **b**. 10X-Tensioactive-Enzyme, 48h.

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

With scanning electron microscope (SEM) the dehydration and fixation of bovine skin failed so it will be necessary to adjust the procedure required for bovine skin specimens in a future work.

Conclusions

The treatment of the enzyme combined with tensioactive and biocide, enzyme and tensioactive as only with tensioactive altered collagen, hair follicle and epidermis of bovine skin. These changes were clearer when enzyme was 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.

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