A New Fungal Isolates Applicated To Bovine Skin In Beamhouse Processes

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Abstract. Some new fungal enzyme extracts were applied on submerged bovine skin in the soaking and unhairing steps. *Clonostachys rosea* (CR), *Emericellopsis minima* (EM), *Purpureocillium lilacinum* (PL), *Penicillium* sp (Psp), *Fusarium oxysporum* (FO), *Acremonium* sp (A) and an unidentified filamentous fungal strain with sterile yellow mycelium (SYM) enzymatic extracts from submerged culture demonstrated a different unhairing capacity observed by SEM.

1 Introduction

Beamhouse area is an important step in leather technology, either in the final quality of the leather or in the contribution to the contamination of effluents. The process of unhairing assisted with enzymes compared to the traditional method brings a decrease of 50% in the effluent sulfide content as well as the suspended solids by 40%, reducing the emission of odors and allowing by oxidation to convert sulfur to sulfate in concentrations that meet established specifications and reduce the cost of effluent treatment (1). The hydrogen sulfide gas generated in the traditional unhairing process can reach maximum values of 2000 ppm after deliming (both at acid and alkaline pH and with organic or inorganic acids, ammonium salts or combinations), while concentrations close to 10-40 ppm were detected in the area near the drum (2). For example, at low concentrations of sodium sulfide, at pH 9 the concentration of gaseous H_2S is higher than the allowed values in work environments (Table 1).

Table 1. Allowed values of SH_2 in effluents and work environments.

	Allowed values in the effluents	Allowed values in work environments			Traditional unhairing
		MAC	VLA-ED	VLA-EC	
Liquid effluent	1 mg/l as S^{-2} [1]				50-120 mg/l de S^{-2} [7,8]
Gaseous effluent	0.008 ppm SH_2 [2]				20-40 ppm SH_2 [7,8] 2000 ppm SH_2 [9]
Solid waste	500 mg SH_2/kg [3]	10 ppm [4]	5 ppm [5]	10 ppm [6]	500-1500 mg SH_2/kg [7,8]

[1](3); [2]Ministry of the Argentina Nation, National Law N°24.051, decree N°831/1993, (1993) Hazardous Waste Law, Annex II, table 10, Air quality guide levels; [3]Annex V, 1-1.7, Physical parameters of sludges; [4]MAC (Maximum permissible concentration to which a worker can be exposed 8 h a day, 5 days a week); [5]VLA-ED (Environmental Limit Value, for an exposure for 40 h per week); [6]VLA-EC (Environmental Limit Value for a short-term exposure) (4);[7](5);[8](6);[9](2).

Enzyme use brings advantages for the environment but its application is limited. Sometimes the mechanism of proteolysis cannot be controlled totally because the enzymatic action on the structure of the collagen reticular layer lasts, influencing remarkably the properties of the finished leather.

The enzymatic unhairing mechanism consists of two simultaneous processes: sulfitolysis mediated by keratinolytic enzymes and proteolysis by proteases, especially of the endopeptidase type. The characterization of enzymatic extracts allows controlling the proteolysis mechanism so that its action does not attack the reticular structure.

However, keratinases would be the specific enzymes capable of acting as proteolytic depilating agents and degrading keratin. They have been isolated, characterized and purified from different microorganisms such as fungi, actinomycetes and other bacteria.

Especially the fungi belonging to the three types of dermatophyte group: *Microsporum*, *Trichophyton* and *Epidermophyton* and other genera belonging to the fungi imperfecti genre (*Chrysosporium*, *Aspergillus*, *Alternaria, Trichuris*, *Curvularia*, *Cladosporium*, *Fusarium*, *Geomyces*, *Gliomastix*, *Paecilomyces*, *Scopulariopsis*, *Penicillium* y *Doratomyces*) have been reported as good producers of keratinolytic enzymes (7; 8).

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2 Objectives

New fungal species with potential industrial application isolated from soil with high keratinolytic activity *in vitro*, were applied in bovine skin in the soaking and unhairing stages. Morphological changes were observed by SEM.

3 Materials and methods

3.1 Isolation, culture and characterization of fungal extracts

Fungal strains were isolated from crabs dwell at alkaline soils of coast of Buenos Aires province by soil washing method (9) and further drying in filter paper (10). Strains were isolated in malt extract agar with antibiotics.

Acremonium sp Link 1809: (A), *Clonostachys rosea* (Preuss) Mussat 1901*:* (CR), *Emericellopsis minima* Stolk 1955*:* (EM), *Purpureocillium lilacinum* (Thom) Samson (Luangsa-ard et al.)*:* (PL), *Penicillium* sp Link 1809: (Psp)*, Fusarium oxysporum* Schltdl. 1824: (FO) and an unidentified filamentous fungus strain with sterile yellow mycelium (SYM) were the selected fungal strains from keratinolytic screening between other twenty isolated.

Fungal crude enzyme extracts (EE) were obtained by submerged liquid culture (batch) with 1% hair waste substrate from the hair-saving unhairing process in Sabouraud breeding ground. Cultures

were incubated 8 days at 30±1°C in orbital agitation at 180 rpm and biomass was separated by filtration through a 0.45 µm membrane.

3.2 Assay of keratinolytic activity

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 µm sieve, USA Standard ASTM E 11-61).

Reaction mixture containing 150 µl of EE and the 1% (w/v) substrate in buffer Tris-HCl 0.1 M, pH 9 (11), Isogras AN 0.1% 50 µl , Baymol AZ 0.5% 50 µl and biocide TCMTB (relation biocide/enzyme (w/w): 100 µg biocide/1 µg CE protein) was incubated at 37°C with agitation (100 rpm) for 60 min. Reaction was stopped by addition of 1 ml of trichloroacetic acid (TCA) 10% (w/v), centrifuged (5000 x g, 15 min) and the supernatant was measured at 280 nm (triplicate). Reaction blanks were performed by incubation 60 min: substrate, tensioactives and buffer. After that EE, biocide and TCA 10% (w/v) were added and procedure was the same as before. Keratinolytic activity unit (U_{ker}) was defined as the amount of enzyme that, under the test condition, causes an increase of 0.01 Abs_{280nm} per minute.

3.3 Assay with submerged skin bovine in fungal extracts

Pieces of fresh bovine skin from the butt of 1 cm by 1 cm were placed in glass tubes of 11 cm length and 2.5 cm width with 5 ml of different solutions and reagents. In the soaking step, EE of different fungal cultures with 0.1% Isogras AN and the biocide TCMTB (0.2% w/w wet skin) were incubated for 4 h at 25°C with agitation (40 rpm). In the unhairing step, after soaking, 0.5% Baymol AZ was added in each reaction mixture, incubated for 48 h at 25°C with agitation (40 rpm).

Controls: a. 5 ml Buffer 0.1 M Tris-HCl, biocide TCMTB (0.2% w/w wet skin); b. 5 ml Buffer Tris-HCl (0.1 M, pH 9), 0.1% p/v Isogras AN, 0.5% Baymol AZ and biocide (0.2% w/w wet skin); a and b incubation was done during 48 h at 25°C with agitation (40 rpm); c. fresh skin without treatment and incubation.

3.4 SEM observation

After completion of the treatments, samples of skin were fixed, post-fixed in 4% formaldehyde and dehydrated in ethanol 30%, 50%, 70% and 100% (12). After critical point drying and metalized, samples were observed by Scanning Electron Microscope (FEI-Quanta 200, LIMF Research Laboratory on Physical Metallurgy, School of Engineering, National University of La Plata, Argentina).

4 Results and discussion

4.1 Assay of keratinolytic activity

The behavior of the selected fungal extracts in relation to its keratinolytic activity is shown in Figure 1. Enzymatic activities decreased in the following order: SYM>FO>CR>>Psp>PL>A. SYM produced an EE with the highest enzymatic activity: 9.06 U_{ker} while EM produced the less (1.5 U_{ker}). The other strains, A, FO, CR, Psp, PL, presented intermediate keratinolytic activities, in decreasing order, respectively. These last strains have been reported as keratinolytic strains (13; 14; 15; 16; 17).

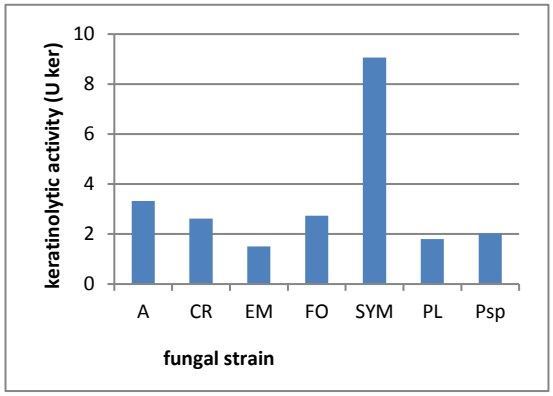

Fig. 1. Keratinolytic activity (U_{ker}) of different fungal enzyme extract (see text for details).

4.2 SEM observation

In **Fig. 2** skin control with buffer and biocide, epidermis and hair without modification is observed. Changes in the skin were observed depending on the fungal strain. FO EE showed the greatest effect. In **Fig. 3** the absence of epidermis, visible dermal papilla and empty hair follicles are appreciated. In **Fig. 4 y 5**, *Fusarium oxysporum* EE and *Acremonium* sp EE, respectively, caused strong changes: epidermis removed, papillary layer exposed and hair layers detached.

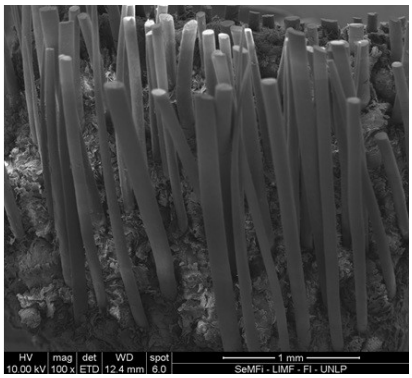

Fig. 2. Control 100 X

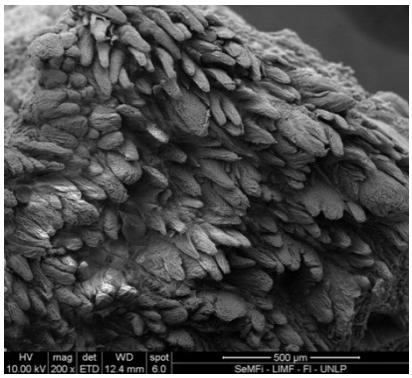

Fig. 3. *Fusarium oxysporum* EE 200X

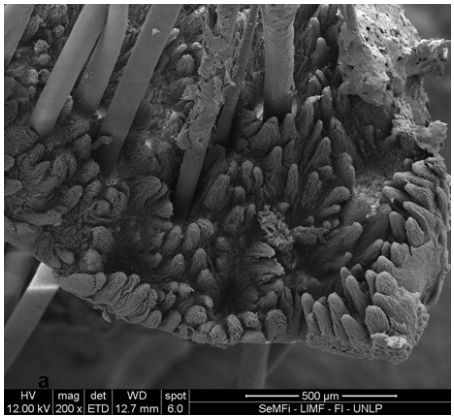

Fig. 4. *Fusarium oxysporum* EE 200 X

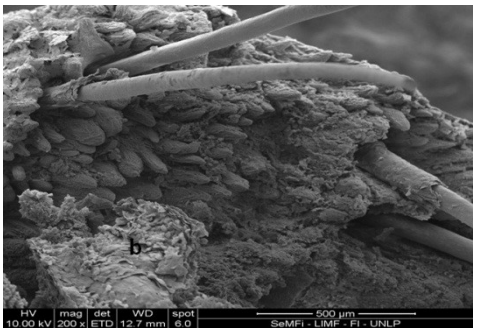

Fig. 5. *Acremonium* sp EE 200 X

In **Fig. 6** and **7**, PL EE has not produced changes in the skin: hair and epidermis remained intact, while with SYM EE, detachment of epidermis and removal of hairs.

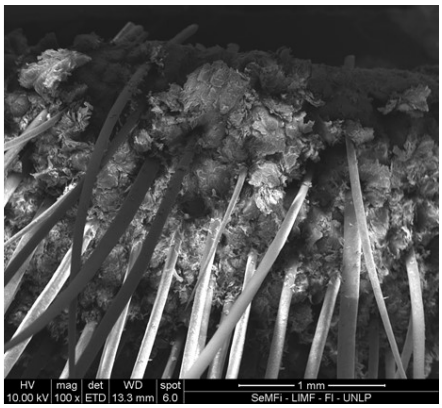

Fig. 6. *Purpureocillium lilacinum* EE 100 X

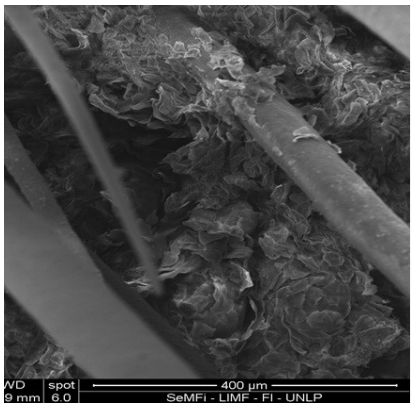

Fig.7. Sterile yellow mycelium strain EE 400 X

Psp EE (**Fig. 8**) and CR EE, skin presented normal characteristics, hair and epidermis with normal patterns. In **Fig. 9**, EM EE produced a detachment of hair follicle sheath.

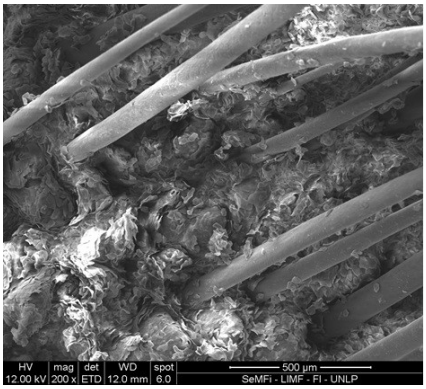

Fig. 8. *Penicillium* sp EE 200 X

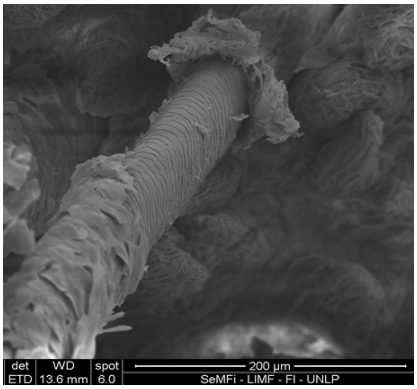

Fig. 9. *Emercicellopsis minima* 800 X

5 Conclusions

Submerged liquid culture with keratin as inductor produced keratinolytic enzymes useful for unhairing step. *Fusarium oxysporum* enzymatic extract showed the greatest effect on the skin, thus the relationship between keratinolytic activity and depilatory effect was found. However, it is necessary to find the optimal conditions to avoid the damage of collagen and enable its application as a sustainable technology.

References

1. *Crispim, A. & Mota, M.: Unhairing with enzymes, Journal of the Society of Leather Technologists and Chemists, 87 (5), 198-202, 2003.*
2. *Skrabs, R.: Über die Gefarhren des Schwefelwasserstofs bei der Lederherstellun, Das Leder, 27, 153-157, 1976.*
3. *ACUMAR (Cuenca Authority Matanza Riachuelo): Resolutions: 001/2007-336/2003, Entity for the environmental sanitation plan, Government of the Nation, Argentina, 2003.*
4. *Instituto Nacional de Seguridad e Higiene en el Trabajo (INSHT). (2015). Professional exposure limits for Chemical Agents in Spain. doi: www.insht.es/Inshtweb/contenidos/documentacion, 2015.*
5. *Frendrup, W. & Buljan, J. (2000). Hair-save-unhairing methods in leather processing. United Nations Industrial Development Organization (UNIDO), US/RAS/92/120, 1-37, 2000.*

6. *Galarza, B., Garro, M., Martegani, J. & Hours, R.: Characterization and Evaluation of Fungal Enzymatic Pool with Unhairing Activity. Journal of the Society of Leather Technologists and Chemists. 100 (5), 257-262, 2016.*
7. *Friedrich, J., Gradišar, H., Mandin, D. & Chaumont, J.: Screening fungi for synthesis of keratinolytic enzymes. Letters of Applied Microbiology, 28, 127-130, 1999.*
8. *Galarza, B., Garro. M., Gortari, C., Bonfranceschi, A., Hours, R. & Cantera, C.: From a problem of solid waste to an useful product in beamhouse process, Journal of AQEIC, 65 (2), 49-55, 2014.*
9. *Parkinson, D. & Williams, S.: A method for isolating fungi from soil microhabitats, Plant and Soil, 13, 347-355, 1961.*
10. *Widden, P. & Parkinson, D.: Fungi from coniferous forest soils. Canadian Journal of Botany, 51(12), 2275-2290, 1973.*
11. *Yamamura, S., Morita, Y., Hasan, Q., Yokohama, K. & Tamiya E.: Keratin degradation: a cooperative action of two enzymes from Stenotrophomonas sp., Biochemical and Biophysical Research Communications, 294, 1138-1143, 2002.*
12. *Garro, L., Galarza, B., Greco, C. & Hours, R.: Unhairing of Bovine Skin with Fungal Enzymes by Immersion and Spread Throughout the Epidermis, Journal of the Society of Leather Technologists and Chemists, 103, 28-34, 2019.*
13. *Singh, I.: Extracellular keratinase of some dermatophytes, their teleomorphs and related keratinolytic fungi, European Journal of Experimental Biology 4(4):57-60, 2014.*
14. *Kumar, J. & Kushwaha, R.: Screening of fungi efficient in feather degradation and keratinase production, Archives of Applied Science Research 6 (1):73-78, 2014.*
15. *Dalee, A., Chehama, M., Sali, K., Hayeeyusoh, N. & Hayeewangoh, Z.: Keratinase-producing fungi from local environmental samples of Far South Thailand and their efficiency in hydrolyzing keratinous wastes, IOP Conf. Series: Journal of Physics: Conf. Series 1097, 012036, doi :10.1088/1742-6596/1097/1/012036, 2018.*
16. *Wang, Q. & Liao, M.: Biodegradation of feather wastes and the purification and characterization of a concomitant keratinase from Paecilomyces lilacinus, Applied Biochemistry and Microbiology, 50(3), 279–285, 2014.*
17. *Eliades, L. Cabello, M., Voget, C., Galarza, B. & Saparrat, M.: Screening for alkaline keratinolytic activity in fungi isolated from soils of the biosphere reserve "Parque Costero del Sur" (Argentina), World Journal of Microbiology and Biotechnology 26(11), 2105-2111, 2010.*