

## STUDIES ON BIODEGRADATION OF TANNED LEATHER

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### Abstract

Wastes from the leather industry as well as insoluble and hard-to-degrade animal proteins are currently eliminated by incineration, method with high ecological disadvantages. In order to avoid these inconvenients, microbiological and/or enzymatic methods are examined. Leather degradation was performed in two trials: one enzymatic-assisted and the other microbial-assisted. Enzymatic hydrolysis of collagen, the representative protein of leather, was performed with collagenase type IA, with six enzyme concentrations, for up to 10 days. The hydrolysis yield was evaluated by free amino acids determination. Microbial hydrolysis involved 35 bacterial strains belonging to various genera (*Streptomyces*, *Bacillus*, *Pseudomonas*, *Serratia* etc), screened for their ability of growth in minimal medium containing bovine leather, ovine leather or wool as unique carbon or nitrogen sources. Three of these strains: *Bacillus licheniformis* ATCC 14580, *Pseudomonas fluorescens* ATCC 13525 and a new bacterial isolate, designated BN7, were able to grow in the presence of treated leather or wool, visible differences being observed after 10 days of incubation. The best results were obtained with the strain BN7, the level of free amino acids and of the extracellular proteins (as a measure of organic substrate degradation) was rather high (2.22  $\mu$ moles amino acids and 61  $\mu$ g protein respectively). Amino acids release and extracellular protein synthesis indicates both collagenase and keratinase activity. The degradation of tanned leather was examined microscopically: significant disorganization of leather fibres was observed.

**Key words:** bacterial strains, collagenase, keratinase, leather degradation, wool

### INTRODUCTION

Leather industries as well as meat industries are important generators of insoluble and hard-to-degrade animal proteins, which are converted in waste with high potential of environmental pollution. Usually, waste from these industries are eliminated by incineration, method with high ecological and sanitary disadvantages [Suzuki et al., 2006]. In order to avoid these inconvenients, microbiological and/or enzymatic methods are examined. It is well known that proteolytic enzymes constitute an important group of commercial enzymes; nevertheless their use in experiments for bioprocessing industrial waste containing animal proteins is well documented. Several experiments were carried out on enzymatic hydrolysis of collagen, the most representative protein of leather waste: the hydrolysis was carried out by collagenases, enzymes that can hydrolyze both native and denaturated collagens [Olde Damnic et al.,

1995, Zerdani et al., 2004]. Other experiments are focused on keratinolytic enzymes useful for degradation of fibrous insoluble proteins in the form of feathers, hair, nails, horn etc, available as agroindustrial by-products [Brandelli, 2008]. In the last ten years, a lot of articles regarding microorganisms able to degrade various types of animal proteins have been published [Brandelli, 2008; Suzuki et al., 2006; Kansoh et al., 2009; Riffel et al., 2003; Gousterova et al., 2005]. It was shown that strains of *Streptomyces* isolated from keratinaceous materials enriched soil were able to degrade human hair, feathers, silk and wool [(Riffel et al., 2003; Kansoh et al., 2009]. Moreover, among *Bacillus* and *Pseudomonas* genera various strains with highly proteolytic activities were identified, potentially useful for biodegradation of insoluble animal proteins [Cai et al., 2008; Tork et al., 2010; Agrahari and Wadhwa, 2010]. Most of the microorganisms isolated from soil presented reduced collagenase activity [Zerdani et al.,

2004] but significant keratinolytic action was detected among some bacterial isolates [Riffel and Brandelli, 2006; Agrahari and Wadhwa, 2010].

This study presents the action of collagenase type IA against sheep and bovine leather, as well as the capability of some mesophilic bacterial strains to synthesize hydrolytic enzymes with collagenolytic and/or keratinolytic activities.

## MATERIAL AND METHODS

**Culture medium** for collagenolytic/keratinolytic enzymes production contained the following constituents (g/L): NaCl 1.0; CaCl<sub>2</sub> 0.05; KH<sub>2</sub>PO<sub>4</sub> 0.7; sucrose 3; MgSO<sub>4</sub> 0.91; K<sub>2</sub>HPO<sub>4</sub> 2.38, and sheep or bovine leather, and wool 6.0; pH 7.2 [Cai and Zheng, 2009].

**Microorganism.** 27 strains of *Streptomyces* isolated from compost and seven bacterial strains (*Bacillus subtilis* ATCC6633, *Bacillus licheniformis* ATCC 14580, *Bacillus amyloliquefaciens* BW, *Bacillus spp.* OS15, *Bacillus spp.* OS17, *Pseudomonas fluorescens* ATCC 13525, *Serratia spp.*S1) from the collection of Faculty of Biotechnology, Bucharest, Romania, and a new isolate from soil (designated BN7) were used in experiments.

**Free amino acids** released by the extent of collagen breakdown were determined using a modification of the Moore and Stein (1948) colorimetric ninhydrin method, by transferring 0.2 ml of sample to test tubes containing 2.0 ml of ninhydrin-ethylene glycol monoethyl ether mixture. After boiling for 30 minutes in a water bath and after cooling, the samples were diluted with 10 ml of 50% n-propanol. The absorbance was determined at 600 nm 15 minutes later. An L-leucine standard curve was used to determine micromoles amino acid equivalent to leucine liberated.

**Total soluble protein** concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

**Enzyme-assisted leather degradation test** was performed using a 2 mg/ml stock solution of bacterial collagenase type IA (Sigma-

Aldrich Chemicals Company). The experiments were carried out with 2 cm<sup>2</sup> sheep leather samples in 50 mM Tris-HCl buffer pH 7.4 and six IA collagenase concentration levels ranging from 0.05 to 0.6 mg/ml, at 37°C, with shaking (150 rpm), for 10 days. Released amino acids determination was carried out at 48, 72, 96 and 240 hours of reaction.

**Microbial-assisted leather degradation test** was performed by cultivation in submerged conditions, at 28°C, with shaking (120 rpm) for 10 days. Preliminary screening of microorganisms for proteolytic activity was performed on milk agar plates [Riffel and Brandelli, 2006]. Biochemical determinations consisted in total soluble protein and released amino acids assays carried out at the end of cultivation period.

## RESULTS AND DISCUSSIONS

*In vitro* degradation trial using collagenase (type IA) solution proved the hydrolytic potential when breaking down the collagen from the tanned leather. Because the degradation behaviour is depending on enzyme concentration, six collagenase concentration levels (0.05, 0.1, 0.175, 0.25, 0.3 and 0.6 mg/ml) were tested in order to find out the minimum necessary amount for relevant digestion of the tanned leather. For a longer reaction time (ten days) significant reduction in the degradation of collagen was observed when using 0.1 mg/ml, but for shorter evaluation period (four days) an increased enzyme concentration was needed (at least 0.25 mg/ml) (Fig. 1).

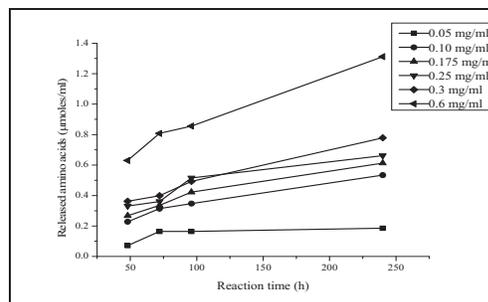


Fig. 1. Leather degradation using six IA collagenase concentrations

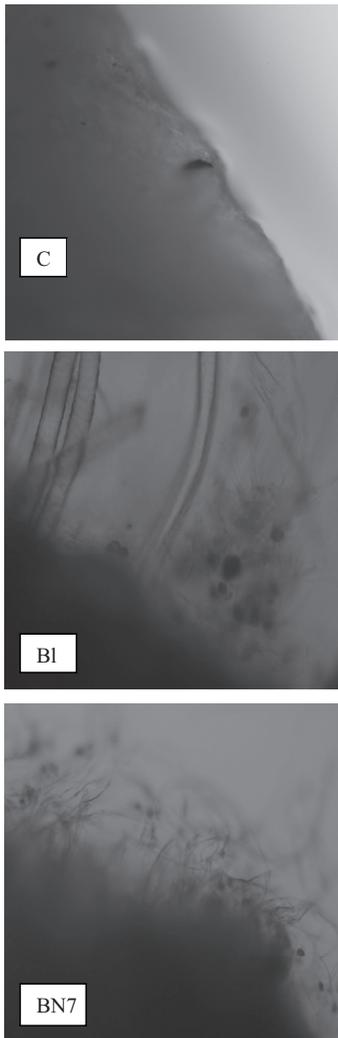


Photo 1. Microscopic examination of sheep pelts: C = control (untreated pelt); B1 = incubation with *B.licheniformis* ATCC14580 for 10 days; BN7 = incubation with the bacterial isolate BN7 for 10 days.

The screening test showed that from the seven bacterial tested strains only the two collection bacteria (*Bacillus licheniformis* ATCC 14580 and *Pseudomonas fluorescens* ATCC 13525) and the new isolate BN7 were able to grow in minimal medium containing leather as sole carbon and nitrogen source. Among streptomycetes, only five strains presented a relatively poor growth in the same medium, but their collagenolytic activity was much reduced. On milk agar plates, the strain

designated BN7 produced clear zone of hydrolysis.

The microscopic examination of sheep or bovine leather fragments incubated with selected bacteria showed significant differences from control. In the control, the edges of the leather fragments were intact, and the aspect was compact (photo 1). After 10 days of incubation with the selected bacteria, the margins structure of sheep leather was modified: it has become more disorganized and both globular and fibrous fragments were released in medium.

The highest degradation, both of sheep leather and of wool samples was observed when the new isolated strain BN7 was used, suggesting the biosynthesis of collagenolytic as well as of keratinolytic enzymes (photo 2).

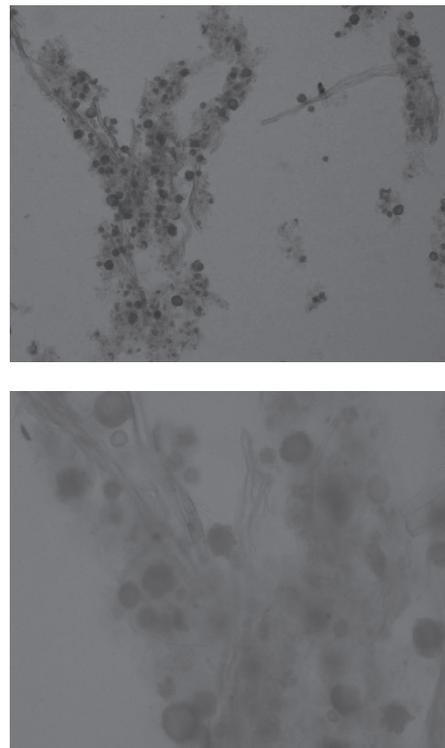


Photo 2. Microscopic aspect (10x and 40x) of degradation fragments resulted after incubation of sheep leather fragments with the bacterial isolate BN7 for 10 days

Similar aspect were observed on bovine pelts treated with the same bacterial strains: the disorganization of the margins and the release

of small fragments are the main actions of the degradative bacteria (photo 3).

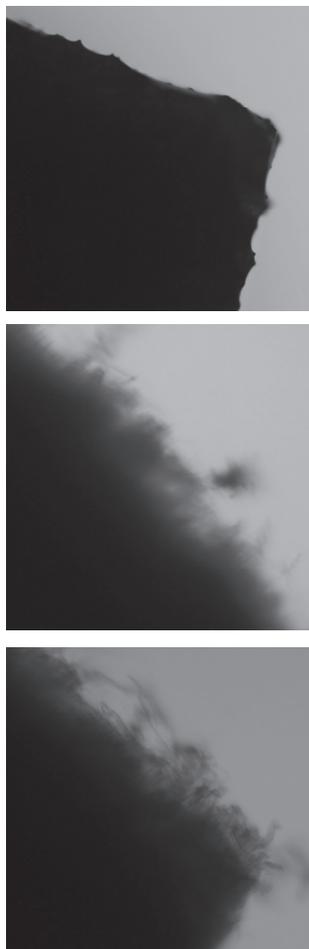


Photo 3. Microscopic aspects of bovine pelts incubated for 10 days with the bacterial isolate BN7 (middle and down) comparative with the untreated sample (up)

Biochemical analysis confirmed the microscopic aspects showing high amount of released amino acids after 10 days of cultivation for all the three tested strains, with higher values for bacterial isolate BN7 (Fig. 2). Degradation of sheep leather was, at least, more than twice accelerated than the degradation of bovine leather.

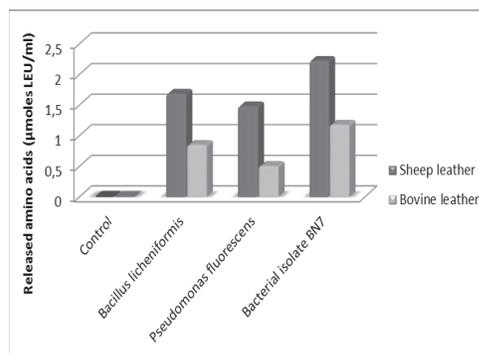


Fig. 2. Degradation of sheep and bovine leather expressed as released amino acids after 10 days of cultivation

Total soluble protein concentration was almost similar for all the tested strains, when acting both on sheep and bovine leather, meaning that enzyme synthesis was quite close (Fig. 3). Though the results obtained for the amino acids released by the action of proteolytic enzymes were totally different. An explanation might be the inhibition produced by the presence of several compounds used for leather processing and acting different, according to the enzyme microbial source.

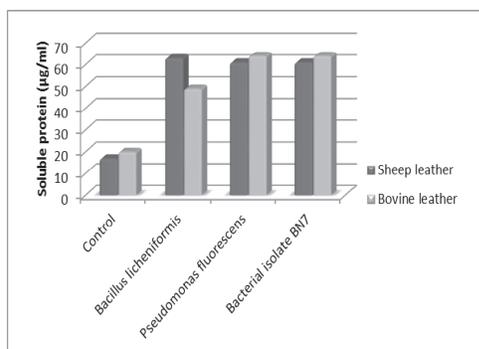


Fig. 3. Degradation of sheep and bovine leather expressed as total soluble protein concentration after 10 days of cultivation

These results suggest that the selected bacteria, and especially *Bacillus licheniformis* ATCC14580 and the new isolated strain BN7 are able to produce increased levels of hydrolytic enzymes, active both on leather and wool.

## CONCLUSIONS

The tested strains *Bacillus licheniformis* ATCC 14580, *Pseudomonas fluorescens* ATCC 13525 and the new isolate BN7 can be used for improvement of waste leather biodegradation process. Collagenase activity may be considered as a measure of this degradation process.

## ACKNOWLEDGMENTS

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## REFERENCES

- [1] Agrahari, S., Wadhwa, N., *Degradation of chicken feather a poultry waste product by keratinolytic bacteria isolated from dumping site at Gahzipur poultry processing plant*, *Internat. J. Poultry Science*, 2010, 9, p.482-489
- [2] Brandelli, A., *Bacterial keratinases: useful enzymes for bioprocessing agroindustrial wastes and beyond*, *Food Bioprocess Technol.*, 2008, 1, p.105-116.
- [3] Cai, C., Lou, B., Zheng, X., *Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis**, *J. Zhejiang Univ. Sci. B.*, 2008, 9, p.60-67
- [4] Cai, C., Zheng, X., *Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology*, *J. Ind. Microbiol. Biotechnol.*, 2009, 36, p.875-883
- [5] Gousterova, A., Braikova, D., Goshev, I., Christov, P., Tishinov, K., Vasileva-Tonkova, E., Haertle, T., Nedkov, P., *Degradation of keratin and collagen containing wastes by newly isolated thermoactinomycetes or by alkaline hydrolysis*, *Letts. Appl. Microbiol.*, 2005, 40, p.335-340
- [6] Kansoh, A. L., Hossiny, E. N., El-Hameed, E. K. A., *Keratinase production from feathers wastes using local *Streptomyces* isolates*, *Australian J. Basic Appl. Sci.*, 2009, 3, p.561-571.
- [7] Lowry, O. H., Rosebrough, N.J., Farr, A. L., Randall, R. J. *Protein measurement with the Folin phenol reagent*, *J. Biol. Chem.* 1951, 193 (1), p. 265–275.
- [8] Moore, S. and Stein, W. H. *A modified ninhydrin reagent for photometric determination of amino acids and related compounds*, *J. Biol. Chem.* 1954, 211, p.907-913.
- [9] Olde Damink, L. H. H., Dijkstra, P. J., Van Luyn, M. J. A., Van Wachem, P. B., Nieuwenhuis, Feijen, P., *J. Changes in the mechanical properties of dermal sheep collagen during in vitro degradation*, *J. Biomed. Mat. Research*, 1995, Vol. 29, p.139-147.
- [10] Riffel, A., Brandelli, A., *Keratinolytic bacteria isolated from feather waste*, *Braz. J. Microbiol.*, 2006, 37, p.395-399.
- [11] Riffel, A., Ortola, S., Brandelli, A., *De-hairing activity of extracellular proteases produced by keratinolytic bacteria*, *J. Chem. Technol. Biotechnol.*, 2003, 78, p.855-859.
- [12] Suzuki, Y., Tsujimoto, Y., Matsui, H., Watanabe, K., *Decomposition of extremely hard-to-degrade animal proteins by thermophilic bacteria*, *J. Biosci. Bioeng.*, 2006, 102, p.73-81.
- [13] Tork, S., Aly, M. M., Nawar, L., *Biochemical and molecular characterization of a new local keratinase producing *Pseudomonas* sp.MS21*, *Asian J. Biotechnol.*, 2010, 2, p.1-13.
- [14] Zerdani, I., Faid, M., Malki, A., *Digestion of solid tannery wates by strains of *Bacillus* spp.isolated from compost in Morocco*, *Internat. J. Agric. Biol.*, 2004, 6, p.758-761.