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BACTERIAL PROTEOLYTIC ENZYMES TESTED ON KERATIN AND COLLAGEN BASED MATERIAL

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Abstract

Biodegradation of fibrous protein is a challenge due to the resistance of the raw material. Enzymes based processes are an alternative to conventional chemical ones because they are environmental friendly, but their efficiency is still limited. They are used mainly in the process of leather obtaining. In the last decade they gain a lot of credit for waste degradation purposes. The main enzymes involved in leather and fur degradation are collagenases and keratinases. The aim of the reported researches is to test a proteolytic complex for its capacity of destroying leather and fur wastes. Four bacterial strains were tested regarding their capacity of hydrolyzing collagen and keratin from bovine leather and sheep fur. For comparison synthetic fur was considered too. Best results were obtained when using sheep leather. The influence of leather dyes was also investigated. Three types of sheep fur were tested: no painted, white painted and black painted. For the painted materials the degradation was less than half, especially when assessing collagenases activity.

Ke words*: collagenase, fur, keratinase, leather, wastes.*

INTRODUCTION

Leather and leather-based industries represent one of the most important economic sectors, but each year large amounts of solid wastes is produced, mainly collagen and keratin proteins. These wastes may be a source of valuable products, with many applications, so a degradation of the rigid structure of collagen and keratin is necessary.

Destroying of these proteins is usually made by thermal hydrolysis with acid or alkaline solutions, or by enzymatic digestion with specific proteases (collagenases and keratinases). Enzyme based processes are an alternative to conventional chemical ones because they are environmental friendly.

The protein products that result from these treatments have potential as a fertilizer and as an animal feed additive (Aftab et al., 2006). Also, the collagen degradation produced peptides, which were shown to have some biological activities with industrial and medical uses (Braikova et al., 2007), such as immunotherapeutic agent, moisturizer in cosmetic or food additives with preservative and seasoning role (Ku, 1993; Honda, 1998; Ravanti, 2000).

Microorganisms are an attractive source of proteases owing to the limited space required for their cultivation and their ready susceptibility to genetic manipulation (Rao et al., 1998). Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey, 1996). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

Collagenase was first discovered in the broth of the anaerobic bacterium *Clostridium hystolyticum* as a component of toxic products. Later, it was found to be produced by the aerobic bacterium *Achromobacter iophagus* and other microorganisms including fungi (Godfrey, 1996).

Different authors reported as collagenase-producing strains *Bacillus* and *Aspergillus*: *B. subtilis* (Okamoto, 2001; Tran and Nagano, 2002; Rui et al., 2009), *B. licheniformis* (Baehaki et al., 2012), *A. fumigatus* (Reichard et al., 1990).
Also, keratinases have been isolated from various microorganisms and introduced into a wide range of biotechnological applications, including those in the feed, fertilizer, detergent, leather and pharmaceutical industries (Gupta et al., 2006). They have been purified from diverse microorganisms, including fungi, such as *Purpureocillium lilacinum* (Cavello et al., 2013) and *Chryseobacterium gelum* (Chaudhari et al., 2013), and bacteria, such as *Streptomyces* (Jaouadi et al., 2010) and *Bacillus subtilis* (Pillai et al., 2008).
The aim of the reported researches was to test a proteolytic complex for its capacity of destroying leather and fur wastes. Four bacterial strains were tested regarding their capacity of hydrolyzing collagen and keratine from bovine leather and sheep fur. For comparison synthetic fur was considered too. The influence of leather dye was also investigated. Three types of sheep fur were tested: not dyed, white dyed and black dyed. Enzymatic activity and protein content were determined using spectrophotometrical methods.

MATERIALS AND METHODS

Microorganisms and growth conditions

Four bacterial strains were used: three *Bacillus amyloliquefaciens* strains (7.2, BN7 and OMF) were recently isolated and belong to the collection of Faculty of Biotechnology, University of Agronomical Sciences and Veterinary Medicine Bucharest, while Bl strain is a collection strain (*Bacillus licheniformis* ATCC 14580).
The inoculation was done in a basal salt medium supplemented with 0.3% sucrose and 0.6% bovine leather or sheep fur meal. Synthetic fur was also tested for comparison. Proteolytic enzymes were produced in 500 ml flasks, kept at 30 - 32^{o}C, agitated at 140 rpm, for 10 days. The culture was centrifuged at 5000 rpm, at 4^{o}C, for 30 minutes.
White sheep fur, black sheep fur and no dyed fur were used in order to study the effect of fur dyes on the enzymatic activities of proteolytic complex.

Enzymatic assay of collagenase, based on some classical refferences (Moore and Stein, 1948; Mandl et al., 1953), was made as following: after collagenase catalytically promote hydrolysis of collagen, the degree of proteolysis is measured by color development with ninhydrin (absorbance was measured at 570 nm).
The enzymatic activity of collagenase was measured in U/ml (*unit definition*: one unit liberates peptides from collagen equivalent in ninhydrin colour to 1 μmol of leucine in 2 hours at pH 7.4 and at 37 ^{O}C).

Enzymatic assay of keratinase was performed using 4 mg keratin azure as substrate. One unit of keratinase activity was defined as the amount that caused an increase in absorbance of 0.01 at 595 nm within 60 min reaction at 60°C.

RESULTS AND DISCUSSIONS

Enzymatic activity of proteolytic complex on synthetic and natural leather and fur

The four bacterial strains: 7.2, BN7, OMF, and Bl were tested regarding the collagenolytic and keratinolytic activity on different materials. Bl strain cultivated on medium containing sheep fur registered the highest collagenase activity (23.7 U/ml) (fig. 1). Also the BN7 strain manifested a remarkable collagenase activity both in the samples with bovine leather (18.2 U/ml) and in the ones with sheep fur (18.07 U/ml).
As we expected, the keratinolytic activity was higher at the strains cultivated in medium with sheep fur, which contained both leather and hair, last one being the specific substrate for keratinases. The best results were again obtained with *Bacillus licheniformis* strain Bl which showed the highest activity (4.44 U/ml) (fig. 2). When using the bovine leather the keratinases synthesized by 7.2 and BN7 strains registered a better activity (1.25 U/ml, respectively 1.15 U/ml).

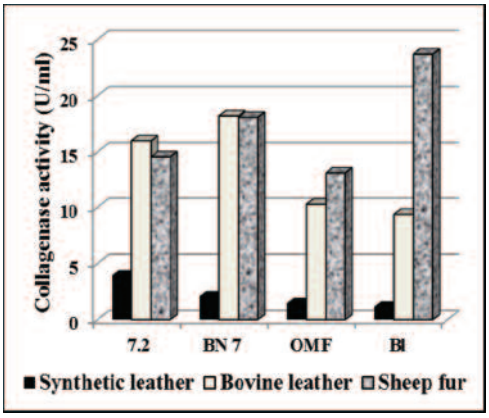

Figure 1. The collagenase activity of enzymatic complex on different materials

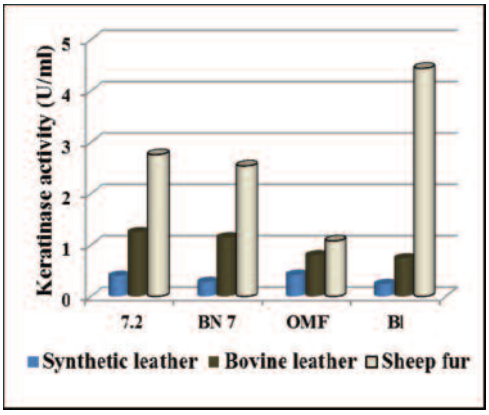

Figure 2. The keratinase activity (of enzymatic complex) on different materials

The obtained results indicated a slightly enzymatic activity also in the samples containing synthetic leather, which may be a residual activity derived from inoculum (bacterial suspension cultivated on natural fur).

Effect of fur dyes on the activity of proteolytic complex

In order to emphasize the effect of fur dyes on the enzymatic activity of the proteolytic complex, an experiment was performed: white and black painted samples of sheep fur were added in the cultivation medium used for the bacterial strains (Figure 3) and then the enzymatic activities were measured. Unpainted white fur was used in the same conditions and considered as control.

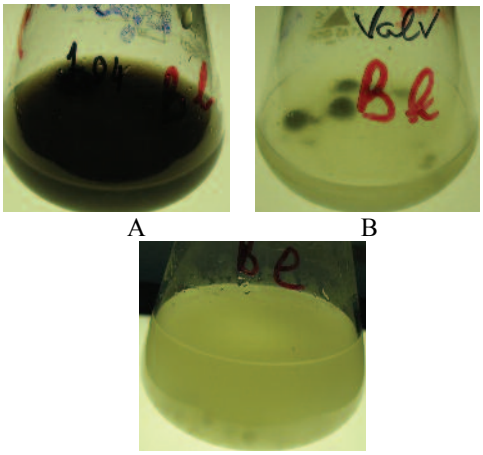

Figure 3. Degradation degree of some samples of natural sheep fur under action of *Bacillus licheniformis* ATCC 14580 strain (Bl). A = black painted fur; B = white painted fur; C= no painted fur

Table 1. Collagenase activity of enzymatic complex on different types of fur

Fur type	Bacterial strain	Collagenase activity (U/ml)
no painted fur	7.2	5.81
	BN7	18,55
	Bl	21.59
white painted fur	7.2	1,7
	BN7	6.13
	Bl	10.44
black painted fur	7.2	0
	BN7	5.81
	Bl	9.81

The obtained results indicated that the enzymatic activity of proteolytic complex decreased in the presence of specific dyes used for painting the natural furs. Bl strain manifested the highest collagenase activity (21.59 U/ml) followed by BN7 strain. During this test 7.2 strain showed very low collagenase activity (Table 1).

This trial showed lower results for keratinase, probably due to the fur biochemical compositon. Anyhow, the highest values were obtained for Bl strain (Table 2). Poor keratinase activity was registered for 7.2 bacterial strain when using undyed sample, while the enzyme activity was absent for dyed samples.

Table 2. Keratinase activity of enzymatic complex on different types of fur

Fur type	Bacterial strain	Keratinase activity (U/ml)
no painted fur	7.2	0.38
	BN7	1.33
	Bl	3.29
white painted fur	7.2	0
	BN7	1.12
	Bl	1.61
black painted fur	7.2	0
	BN7	1.05
	Bl	1.22

The activity of the proteolytic complex was highly affected by the dying process. Regarding collagenase activity, it is obviously that the values registered in the samples with painted fur are at least 50% lower than the ones measured in the control samples (with no painted fur) (Figure 3). When using black painted fur, 7.2 strain showed no collagenase activity.

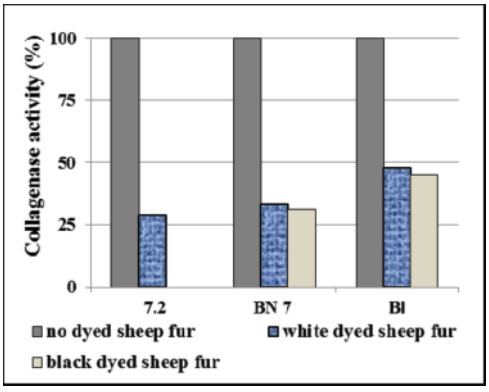

Figure 3. The percentage decreasing of collagenolytic activity under dyes effect

The keratinase activity of BN7 and Bl cultivated with dyed fur samples was not so deeply affected, especially for the first one. For these strains the enzymatic activity was 15-60% lower for the painted samples comparing with no painted fur, while for 7.2 bacterial strain the activity was absent for both dyed samples (Figure 4).

Considering collagenase activity, Bl strain was the most resistant to dyes effect, while BN7 strain showed higher resistance regarding keratinase activity. Unlike, the 7.2 strain was significant affected by the presence of dyes, considering that the collagenase activity was 75% lower only for white dyed fur and the keratinase activity was totally inhibited in the sample containing painted fur.

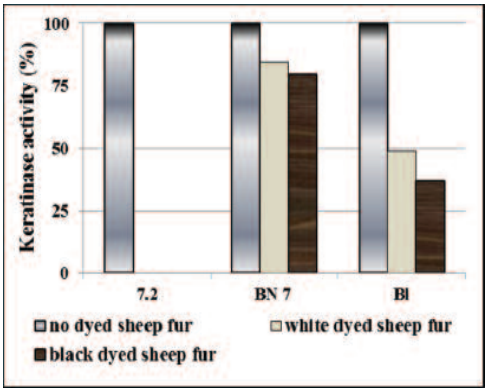

Figure 4. The percentage decreasing of keratinolytic activity under dyes effect

Moreover, the colour of the used dyes influenced differently the enzymatic activity of proteolytic complex: lower values were measured in the samples containing white painted fur than in the ones with black painted ones. It is not sure if the dye itself is the single cause of the enzyme inhibition or it can be also the result of the chromium oxide content in the tested samples. White painted fur contained 1.6 up to 2.06% chromium oxide, comparing with black painted fur with 2.32 up to 3.09%. This significant difference in chromium oxide concentration (used in the tanning process) may influence in a different measure the enzymatic activity of the proteolitic complex.

CONCLUSIONS

Enzymatic activity of proteolytic complex was higher in the samples containing sheep fur added in cultivation medium than in the ones with bovine leather, both for collagenase and for keratinase.

Bl and BN7 strains registered the best results regarding the enzymatic activity of the proteolytic complex.

The use of fur dyes determined a 50-70% decreasing of the collagenase activity and 15-60% decreasing of the keratinase activity. Both enzymatic activities were less affected when using white fur than black painted ones.

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