Paper D2

Screening of a new bacterium that produces enzymes with application on leather processing

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Abstract

Leather industry has an immense environmental impact. Currently, it uses large quantities of water and toxic chemicals and generates high amounts of effluent as well as solid wastes. The enzymatic treatment of leather is a promising technology. In this work is described the screening and isolation of a new *Bacillus subtilis* strain, producer of proteases to be used in leather unhairing to replace sodium sulfide, a toxic chemical. The aerobic sludge of a tannery was used as source of the microbial community for screening and one bacterium was selected showing good proteolytic activities and was identified as *Bacillus subtilis*. Crude enzymatic extracts of cultures of *B. subtilis* were used for the unhairing process of hides showing excellent results, suggesting that this safe enzymatic preparation can be used for the replacement of the toxic sodium sulfide in this process.

1. Introduction

Concern about the negative impacts caused by industrial development has gained more importance, especially since last decades. Throughout this period many changes have occurred resulting from a new perception of society regarding the importance of protect the environment and have been influencing the actions of public and private institutions.

During the hide unhairing process large quantities of water and toxic chemicals are employed, such as sulfide, generating huge amounts of effluent that must be treated, as well as solid wastes, which could be reused or better treated to avoid soil and water contaminations [1-3].

The application of biotechnology in leather manufacturing, with the replacement of chemicals for enzymes, is an alternative for the reduction of the environmental impact. Several researchers have addressed their efforts towards studying new sources of microorganisms capable of producing proteases for many different applications, including leather production. For this industry, the most important proteases are from the keratinase family, which can be applied for the unhairing of hides [4-7]. Microorganisms that produce proteases can be isolated from several environments [7-11].

Usually, enzymes used in leather manufacturing are non-specific, so some authors characterized commercial enzymes. Foroughi et al. (2006) characterized commercial enzymes and compared the results for these enzymes with results of activity from enzymes from bacterial and fungal isolated.

In the work of Dettmer et al. (2011), five commercial enzymes were characterized. Based on the results of this work, new enzymes were screened and the results are present in this work. Plates containing skimmed milk were used for microorganism selection. After the selection of the microorganism the optimization of medium composition was done and the enzymes were also characterized for their best pH, temperature, thermal stability, and the influence of inhibitors. Attempts

to purification of this enzyme were also presented. Furthermore, due to the possibility of eliminating the use of lime and sulfide, these enzymes were also tested in hide unhairing process. The unhairing capability was evaluated by scanning electron microscopy (SEM) and by optical microscopy.

2. Materials and Methods

2.1 Isolation of proteolytic enzyme producing bacteria

Screening of new microorganisms for application on leather unhairing was carried out using sludge from a local tannery. Samples were directly plated on to Luria-Bertani agar plates with 1% (w/v) skimmed milk after a 1:10 dilution in 0.9 % sterile saline solution [11]. Plates were incubated for 48 h at 37°C. Proteolytic activity was detected by the formation of translucent halos around the colonies, which were selected and isolated for more detailed studies.

For the quantitative estimation of extracellular protease activity by the selected bacteria, single colonies were picked-up and grown in medium (M) containing (in g/l): K_2HPO_4, 7; KH_2PO_4, 3; $MgSO_4$, 0.1; yeast extract, 5; soybean meal, 10, [11] at 37°C, pH 7 and 80 rpm of agitation in a rotatory shaker.

2.2 Proteolytic assays

Microorganisms were investigated for their activities over azocasein, keratin azure, and azocoll. The methodology using azocasein as substrate was based on the method described by Giongo et al. [14]. The cultivation broth was centrifuged at 10,000 g for 10 min and the supernatant was used for the enzymatic assay. The reaction mixture contained 100 µl of substrate (azocasein 10 mg/ml), 100 µl of buffer and 100 µl of supernatant with the enzyme. The sample was incubated at 37°C for 30 min, and the reaction was stopped using 500 µl of 10% TCA (trichloroacetic acid). After centrifugation at 10,000 g for 5 min, 800 µl of the supernatant were added to 200 µl of 1.8N NaOH and the absorbance was determined at 420 nm. One unit of enzyme activity was defined as the amount of enzyme causing a change of absorbance of 0.01 at 420 nm for 30 min at 37°C. A control was prepared adding buffer and substrate solution to TCA.

Enzymatic activity over keratin and collagen was determinate using keratin azure and azocoll as substrates, respectively. The methodology was adapted from Adigüzel et al. [15] and Ionata et al. [5]. Both keratinolytic and collagenolytic activities were determined by incubating 500 µl of the enzymatic supernatant with 4 mg of either keratin azure or azocoll and 500 µl of buffer. The reaction mixture was incubated at 55°C, for 30 min under agitation. After centrifugation at 10,000 g for 5 min, the absorbance was measured at 595 nm. One unit of enzyme activity was the amount of enzyme causing a change of absorbance of 0.01 at 595 nm for 30 min at 55°C. A blank was prepared adding enzymatic solution and buffer, without substrate.

2.3 Enzymatic characterization

The enzyme obtained from optimized medium cultivation was characterized for their best pH, temperature, and thermal stability. The activities were evaluated in sodium phosphate 0.1M (pH 6 – 8), or in sodium bicarbonate 0.1M (pH 9 – 13) buffers, at 37°C. The effect of temperature on enzymatic activities was tested between 28 and 75°C, with the pH fixed in its best value, previously determined. The thermal stability of the enzymes were determined at their optimal pH, with enzymatic solutions being exposed to temperatures of 37, 45, and 55°C for 15, 30, 60, and 120 min. The residual enzymatic activity was determined as described above at 37°C and using azocasein as substrate. The effects of some chemicals on the activity of enzyme were tested. The chemicals were: EDTA, 5 mM; surfactant (Eusapon, Basf), 0.1% (w/v); fatty alcohols (Busperse 7769, Buckman Laboratories), 0.1% (v/v); salts (calcium carbonate, 0.5 and 1% w/v, sodium carbonate, 0.3% w/v); and sulfides, 1% (w/v); which are normally used during the leather production process. Enzymes were preincubated for 15

min at room temperature with the tested chemicals at room temperature. The residual enzymatic activities were determined as described above at 37°C and using azocasein as substrate.

2.4 Enzyme purification

For the purification of enzyme from isolated bacteria were used acetone, ethanol and ammonium sulfate at different concentrations (25, 35, 50 and 60%). After 48 h of microorganism cultivations, the culture medium was centrifuged for 10 min at 10,000 g and the resulting supernatant was used for the purification of enzymes. For the purification were used 10 ml of liquid [supernatant + reagent(ethanol or acetone)], for the case of ammonium sulfate utilization were used 10 ml of supernatant and 2.5 g of reagent for the case of 25% ammonium sulfate concentration. Acetone, ethanol and ammonium sulfate were added slowly; then the samples were stored in a refrigerator (0 – 4°C) during 12h for protein (enzyme) precipitation.

After the precipitation time, the samples were centrifuged per 20 min at 4,800g. The precipitate was resuspended with 5 mL of distilled water and analyses of soluble protein and enzymatic activity were made.

Enzymatic activity was determinated according to described above. Soluble protein was determinate according Lowry method. Specific activity was defined as a ratio between enzymatic activity and the resuspended protein.

2.5 Unhairing activity

The unhairing process was devised in order to verify whether the proteases of *B. subtilis* were suitable for this process. Bovine hide pieces weighing approximately 40 g were immersed in 80 ml of crude enzyme extract obtained from 48 h cultivation, standardized to have a proteolytic activity of 90 U/ml. The hide samples were incubated for 18 h at room temperature and compared with a control (treated only with water) and a hide submitted to the conventional process. The tests were conducted in a laboratory drum (cylindrical rotating reactor, used for hide and leather processing), at 24 rpm. The hides were microscopically evaluated.

3 Results and discussion

3.1 Isolation and identification of proteolytic enzyme producing microorganism

Colonies that form clear halos indicating proteolytic activity were isolated and identified. According to *16S rRNA* gene sequencing the isolated colonies belong to the genus *Bacillus,* all of them with more than 98% of genetic identity. The microorganism studied in this work presented, moderate activity on keratin azure, good activity on azocasein and high activity on azocoll. The results are presented in Table 1. Despite its high activity on azocoll, this microorganism was selected for carrying out further studies on enzyme production for determination of best time and the consequences of its application on leather quality.

Table 1 - Activity of the isolated bacteria on different substrates

Substrate	**Enzymatic activity (U/ml)**
Azocasein	103.73
Keratin azure	13.45
Azocoll	135.70

3.2 Enzyme characterization

The effects of pH on the enzymatic activity are shown in Figure 1A. As can be seen, proteolytic activities of the enzymes form a plateau, presenting constant values of enzymatic activity for pH ranges from 6.0 to 12, presenting a decrease in its activities for pH values above 12. This can be possible due the not purification of the enzymatic crude and the presence of any enzymatic forms.

The profile of temperature influence over proteolytic activities can be observed in Figure 1B, with maximal activities between 37 and 55°C. Above 60°C the enzyme presented decreased activities. At 28°C the enzyme showed lower activity, but still satisfactory.

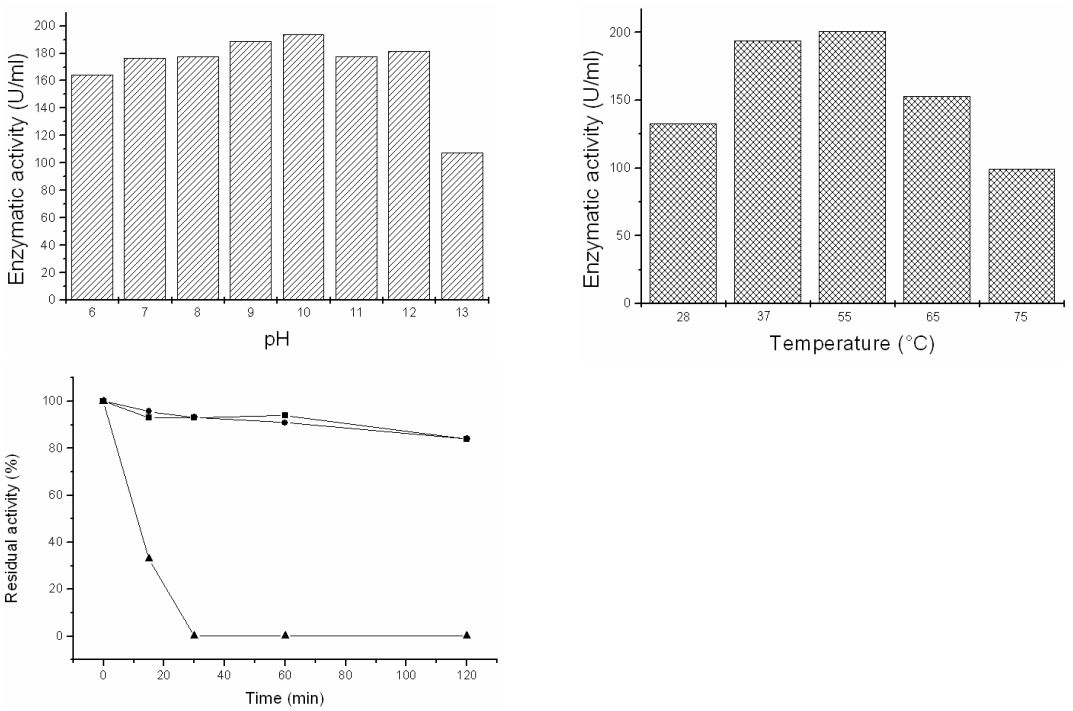


Figure 1 - Effect of pH (A); temperature (B) on the enzymatic activity, and thermal stability of enzyme at - 37°C (–■–), 45°C (–●–), 55°C (–▲–) (C), using azocasein as substrate

In Figure 1C, the thermal stability of the enzyme is presented. The enzyme was stable at 37 and 45°C, maintaining about 90 % of its initial activity after 120 minutes, although at 55°C even 30 min of incubation were sufficient for its inactivation.

The presence of salts and other chemicals present in industrial solutions may interfere with enzyme activity and therefore must be tested in order to prevent process losses. In Table 2 are presented the results of the effects of inhibitors and other chemical products on the enzymatic activity. The enzyme was also partially inhibited by sodium sulfide and surfactant. EDTA, fatty alcohol, which is an organic solvent, sodium carbonate, and the calcium carbonate, caused no significant variations on enzymes activities.

Table 2 - Effect of some inhibitors and chemical products on the enzymatic activity

Chemical	Concentration	Residual activity (%)
EDTA	0.15% (w/v)	90.59
Fatty alcohol (Busperse 7769)	0.1% (v/v)	95.55
Sodium carbonate	0.3% (w/v)	94.84
Calcium carbonate	0.5% (w/v)	98.58
	1% (w/v)	97.94
Surfactant (Eusapon)	0.1% (w/v)	79.52
Sodium sulfide	1% (w/v)	61.12

Riffel et al. (2007), reported the enzyme inhibition after exposure to reducing agent mercaptoethnol, according to the authors this may be an indication that intact disulfide bonds are necessary to perfect

action of the enzyme. Thus this can explain the residual activity of around 65%, obtained in this work, after contact with sodium sulfide, which is also a reducing agent.

3.3 Enzyme purification

To evaluate the possibility of protein recuperation and enzymatic activities increasing, enzyme purification was carryed out using acetone, ethanol and ammonium sulfate. Results are showing in Table 3.

Table 3 - Results for enzyme purification

Purification	[C]	Enzymatic activity (U/ml)	Protein (mg)	Specific activity (U/mg protein)	Purification*	Protein recuperation ** (%)
Crude extract		131.07	1.44	91.20	1	100
Ethanol	0.25	28.95	0.30	95.26	1.04	21.10
	0.35	27.03	0.30	90.28	0.99	20.79
	0.50	8.80	0.30	29.36	0.32	20.81
	0.65	14.20	0.25	56.44	0.62	17.47
Acetone	0.25	48.17	0.18	268.91	2.95	12.44
	0.35	32.27	0.19	276.53	3.03	13.14
	0.50	51.23	0.19	170.55	1.87	12.87
	0.65	36.27	0.14	263.89	2.89	9.54
Ammonium sulfate	0.25	30.70	0.25	120.95	1.33	17.62
	0.35	100.97	0.29	342.32	3.75	20.48
	0.50	143.85	0.37	393.32	4.31	25.40
	0.65	77.80	0.43	181.25	1.99	29.80

* Ratio between specific activity after purification with different reagents and the specific activity of crude extract
** Ratio between the quantity of protein after purification and protein quantity in crude extract

The purification with ethanol and ammonium sulfate presented better results for protein recuperation, but the tests made with ammonium sulfate presented higher specific enzymatic activity, until 4 fold crude extract, not purified. The tests with application of purified enzymes will be made and evaluate.

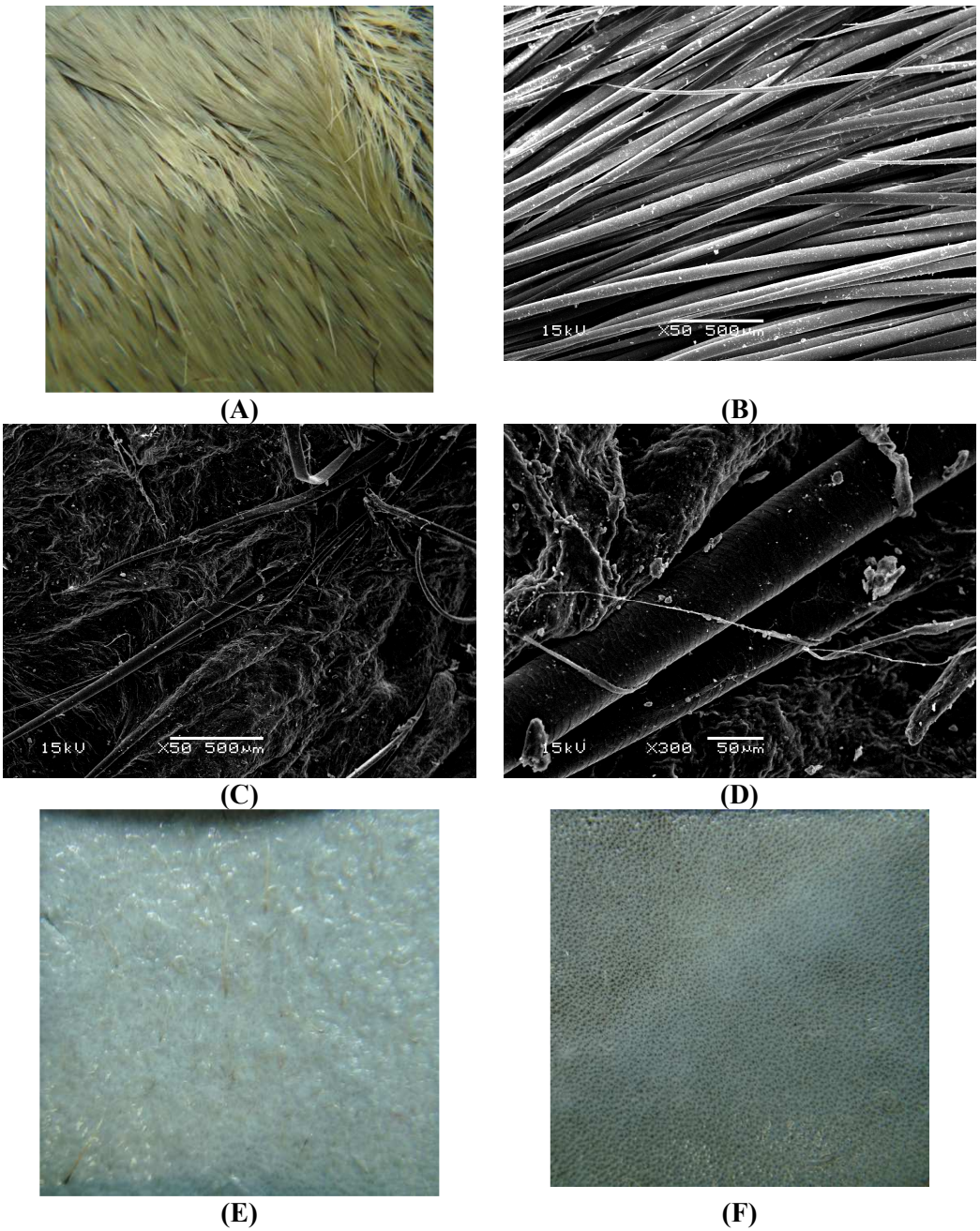
3.4 Hide unhairing

The enzymatic crude extracts from cultures of *B. subtilis* were applied on hide samples to test their capability for the hide unhairing process. Figure 2 shows the results. The enzymatic treated hides and the control show significant differences. Hides treated with the enzyme showed less hair as can be observed at Figure 2 (C, D, E), compared to controls treated just with water (Figure 2A, B). Figure 2E and 2F shows the samples submitted to the enzymatic process (after 18h) and to the conventional process, respectively. As can be seen the samples treated with the enzymatic crude extract, presented damages on its grain. This fact was can be explained through its collagenolytic activity, presented at section 3.1. Collagen is a key component of animal hides, which should not be hydrolyzed in bioprocess so as to maintain leather qualify. It is desired that the protease used for this purpose should be with less or without activity on collagen [8, 17]. Another way, according to Gupta and Ramnani, (2006) proteases with mild collagenolytic and elastolytic activities might be particularly suitable enhancing dehairing process without harming the tensile strength of leather.

4 Conclusions

The results obtained in this study have shown a possibility to isolate a new strain of *B. subtilis* that presented a good unhairing capability. The characterization of this enzyme help to elucidate some properties of enzymes, including the determination of the ideal pH and temperature in which the enzymes will show best stabilities and activities and also the effects of some salts and other chemical products on its activity. The application of the crude extract of cultures directly on hides were

effective in the process of unhairing. Further studies are granted in order to improve the production and use of protease from *B. subtilis* in the unhairing process.



(A) **(B)** **(C)** **(D)** **(E)** **(F)**

Figure 2 - 1 – Optical (A) and scanning electronic microscopy (SEM) 50x magnification (B) of control samples, without enzyme; SEM of hides after application of crude enzymatic extract of *B. subtilis* 50x magnification (C); 300x magnification (D); optical microscopy of a tanned leather after unhairing with crude enzymatic extract of *B. subtilis* (E) and tanned leather after conventional unhairing process (F)

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