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A Thermostable Serine Protease By *Aspergillus parasiticus* TEM Have Promising Activity In Enzymatic Dehairing Process

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Abstract- In the present study, *Aspergillus parasiticus* TEM obtained from our culture collection produced appreciable quantities (2955.7±39.4 U/gds) of extracellular protease, but negligible quantities of keratinase, when grown on wheat bran moistened to 75% with Czapex Dox solution containing 1% $NaNO_3$ by means of solid-state fermentation (SSF). After incubation at 27°C for 3 days, *A. parasiticus* TEM reached to a maximum protease activity. The optimum pH of purified enzyme was around pH 8.0 at 30°C, but substantial activity was recorded at the pH 7.0-11.0 and it is stable pH values ranging from 7.0-9.0 for 8 hours. Temperature optimum of the enzyme was found to be at 50 ^{o}C without Ca^{++} and to be highly stable (90%) at 30^{o}C for 8 hours. When the protease from *A. parasiticus* TEM was used in dehairing process of cattle hide, the mixture of 100% crude enzyme, 1.5% lime and 1% sodium sulphide based on hide weight was completely dehaired hides at 30^{o}C for 6 hours, but crude enzyme 200% alone carried out this process for 8 hours.

Keywords- *Protease, Leather Industry, Dehairing, Solid State Fermentation*

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1. Introduction

The global supply of cattle hides for leather production was about 8.8 million tons in 2005 [1]. Among all processes in leather manufacturing, it is the beamhouse processes such as dehairing and liming that generate the most waste [2]. The sodium sulphide blended with lime is traditionally used for dehairing of hides and skins [3,4]. These chemicals dissolve the hair and open up the fiber structure. Major pollutants from the leather industry that have significant environmental impact include gas wastes with hydrogen sulphide and solid wastes with hair, lime and organic matter forming sludge [5]. The wastes generated from beamhouse processes are responsible for 84% of BOD (biological oxygen demand), 75% of the COD (chemical oxygen demand), 92% of the suspended solids and 100% of the toxicity of the total pollution from a tannery [6,7].

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The ideal dehairing is still the objective but the needs of the leather industry and the requirements for protecting the environment must be considered [8]. In leather industry, one of the ways of solving the industrial pollution problems resulting from tannery effluents is the substitution of chemical dehairing agents by proteolytic enzymes produced with microorganisms [9,10,11]. However, nowadays, less than 10% of cattle hides are soaked and dehaired in enzyme-assisted process.

The use of proteases in the dehairing step has proved very successful both in improving the leather quality and reducing the pollution [12]. Reduction in the use of sulfides by 40% is possible by the use of a protease during the liming process used in dehairing and dewooling step [13] When enzymes are used in the dehairing process, the hair is not dissolved and can be filtered out from the liming float. In this way, it is possible to reduce COD by 50% and BOD by 35% in waste discharges [14].

Proteases accounting for about 60% of the total worldwide sale of enzymes are one of the three largest groups of industrial enzymes, which are used in different industrial processes such as detergent, food, pharmaceutical, leather and silk industries and for recovery of silver from used X-ray [15,16,17,18,19]. Bacteria and fungi are used for protease production in submerged and SSF. Protease sources used in leather industry for dehairing process are in *Aspergillus, Penicillum, Rhizopus, Streptomyces* and *Bacillus* genus [20]. In recent years the shift has been observed the protease production in SSF system and wheat bran has been the preferred choice in most of the studies although many agro-industrial substrates have been employed for cultivating different microorganisms [17].

Therefore, it was of interest to study the production of protease which is useful for dehairing and also able to hyper-produce on low-cost agricultural residues. In this work, we aimed to describe the production of protease by *A. parasiticus* TEM grown on wheat bran, some factors affecting the activity of partially purified enzyme and its application to dehairing.

2. Materials and Methods

2.1. Materials

2.1.1. Raw Cattle Hides

In this study, 4 pieces of wet-salted hides obtained from a tannery located in Izmir, Aegean Region of Turkey were utilized in the dehairing process.

2.1.2. Crude Protease Extract

Crude protease extract, produced from *A. parasiticus* TEM was used in the dehairing process.

2.2. Methods

2.2.1. Microorganism

A. parasiticus TEM, a potent protease producer, was kindly provided from Department of Biology, Basic and Industrial Microbiology Section, Faculty of Science, Ege University, Bornova, Izmir, Turkey. The fungus was cultivated in PDA slants at 27˚C for 4 days and maintained at +4˚C.

2.2.2. Protease Production

A. parasiticus TEM was inoculated in 50 ml PDA medium prepared in 250 ml flasks. After sporulation at 27^{o}C for 3 days, 10 ml of 0.5% sodium lauryl sulphate was added to flasks to prepare spore suspension. The enzyme used in dehairing process was produced by SSF on wheat bran. For protease production, 5 g wheat bran that moistened to 75% by Czapex Dox solution (10 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl) was autoclaved at 121˚C for 45 min [16]. Each flask was inoculated with 10^7 spores.ml^{-1} and incubated at 27°C for 4 days.

2.2.3. Effect of Nitrogen Sources on Protease Production

To determine the effect of organic and inorganic nitrogen sources on protease production, 1% of casein, yeast extract, peptone, corn steep liquor (CSL), NH_4NO_3 and $NaNO_3$ was added to 75% moistened wheat bran with Czapex Dox solution and incubated at 27^{o}C for 4 days. Extracellular protease activity was measured during 4 successive days.

2.2.4. Extraction and Partial Purification of Protease

The enzyme was extracted from fermentation medium with 50 ml distilled water by shaking them at room temperature for 20 min at 100 rpm. Then, cultures were completely homogenized at warring blender for one min low speed. After filtration through nylon mesh, the culture was centrifuged at 10000 rpm for 15 min to remove cells and insoluble residues. Supernatant was used as enzyme source both partially purification and dehairing process [17].

Supernatant obtained from fermentation medium were precipitated overnight by adding 75% solid $(NH_4)_2SO_4$ (w/v). Precipitate was suspended in 20 mM Na-Borate buffer (pH 8.0) and recollected by centrifugation at 10.000 rpm for 10 min. Precipitate was dialyzed against the same buffer [21]. All partial-purification procedures were accomplished at +4 ^{o}C.

2.2.5. Determination of Protease Activity

Protease activity was determined by Anson method with modifications [22]. A volume of 2.5 ml of substrate solution containing 20 mM Na-Borate buffer (pH 8.0) and 0.6% Hammerstein casein as substrate was pre-incubated at 30 ^{o}C for 10 min. A volume of 0.5 ml of appropriately diluted supernatant as an enzyme source was added to substrate solution and the incubation continued for further 20 min at 30 ^{o}C. 2.5 ml trichloroacetic acid (TCA) solution (0.11 M TCA, 0.22 M sodium acetate and 0.33 M acetic acid) was used as reaction stopper and incubated 30 min at room temperature further. After centrifuging at 6000 rpm for 10 min released tyrosine concentration was measured spectrophotometrically at 280 nm. One unit of the protease activity was defined as the amount of enzyme that released 1 μg of amino acid equivalent to tyrosine per min under the standard assay conditions [23].

2.2.6. Effects of pH, Temperature and Inhibitors on Protease Activity and Stability

The optimal temperature for enzyme activity was determined by incubating the reaction mixture between 20-70°C in 20 mM Na-Borate buffer (pH 8.0). The thermostability of the protease at 30°C was investigated by measuring the residual activities with the standard assay after the enzyme was incubated at appropriate time.

The optimum pH of the enzyme was assayed in a pH range of 5.0-11.0 using the following buffers at the concentration of 20 mM; phosphate buffer (pH 5.0-7.0), Na-Borate buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0-11.0). The effect of pH on protease stability was measured with the enzyme previously incubated at pH 8.0 for 8 hours at 30°C and residual activity was assayed using the standard procedure as described above.

To determine the effects of inhibitors on the enzyme activity, 0.5 ml appropriate diluted enzyme was pre-incubated with the following protease inhibitors: 5.0 mM phenylmethylsulphonyl floride (PMSF) dissolved in isopropyl alcohol, (10 % w/v) 5.0 mM EDTA and 0-1% Na_2S (w/v); dissolved in 20 mM Na-Borate buffer (pH 8.0) at 30°C for 20 min [24]. After incubation, residual activity of the enzyme was measured as described above.

2.2.7. Keratinase Assay

Keratinase activity was assayed using the method of Aslan et.al.[25] at 50 ^{o}C using azo-keratin (4 mg/ml) as the substrate, which was dissolved in 20 mM Na-Borate buffer, pH 8.0. One unit (U) of keratinase activity was defined as the amount of enzyme causing 0.01 absorbance increase between sample and control at reaction conditions [26,27].

2.2.8. Assays for Protein Content

Protein concentration was measured by the method of Bradford with bovine serum albumin (Sigma P0914) as a standard [28].

2.2.9. Application of Crude Protease Extract in Dehairing Process

Firstly, the hides were soaked, fleshed and cut from backbone into two sides, and then the samples (15 cm x 15 cm) were taken from sides to parallel to backbone. Crude protease extract is applied to the cattle hide samplesto determination of the effect of crude protease extract in the dehairing process. Experiments were done in stainless steel drums, 35 cm diameter and 20 cm wide and have 10-11 rev/min rotating speed. Three different ratios that 50%, 100% and 200% crude protease extract was used in the dehairing trials based on hide weight. In addition, 100% crude protease extract combined with 1% sodium sulphide and 1.5% lime was used in the trials based on hide weight. Combination of 2% sodium sulphide with 3% lime was used in control specimen. The efficacies of the enzyme on dehairing with different enzyme concentrations were studied for both dehairing by sole enzyme and enzyme with reduced concentration of sodium sulphide and lime mixture. Samples were removed at definite time intervals and visually analyzed for some properties such as presence or absence of depilated areas, resistance to dehairing when the skin pieces were gently scraped with fingers, and color effects after incubation period.

3. Results and Discussions

Fungal extracellular protease are produced with a wide range of activities from 58,4-11000 gds using various substrates in solid state fermentation (SSF) processes [20]. Although a number of protease productions were performed using submerged systems, solid state fermentation was found to be more economical mainly due to the cheap and abundant availability of agricultural wastes which can be used as substrates. *A. parasiticus* TEM was capable of exhibiting proteolytic activity on potato dextrose agar the diameter of the clear

zones ranging from 4.0-4.5.5 cm and this fungus was selected as the potential producer of protease in this study.

3.1. Effect of Nitrogen Sources on Protease Production

As seen Figure I, *A. parasiticus* TEM grew well on all nitrogen sources and produced protease at a high rate. Among these, Czapex Dox solution containing 1% $NaNO_3$ (2955.7±39.9 U/gds) and then $NaNO_3$ –free wheat bran (2897.4±79.9 U/gds) seem to be good nitrogen sources for enzyme production and secretion. Conflicting reports regarding the effects of nitrogen sources on protease production by fungi are available.

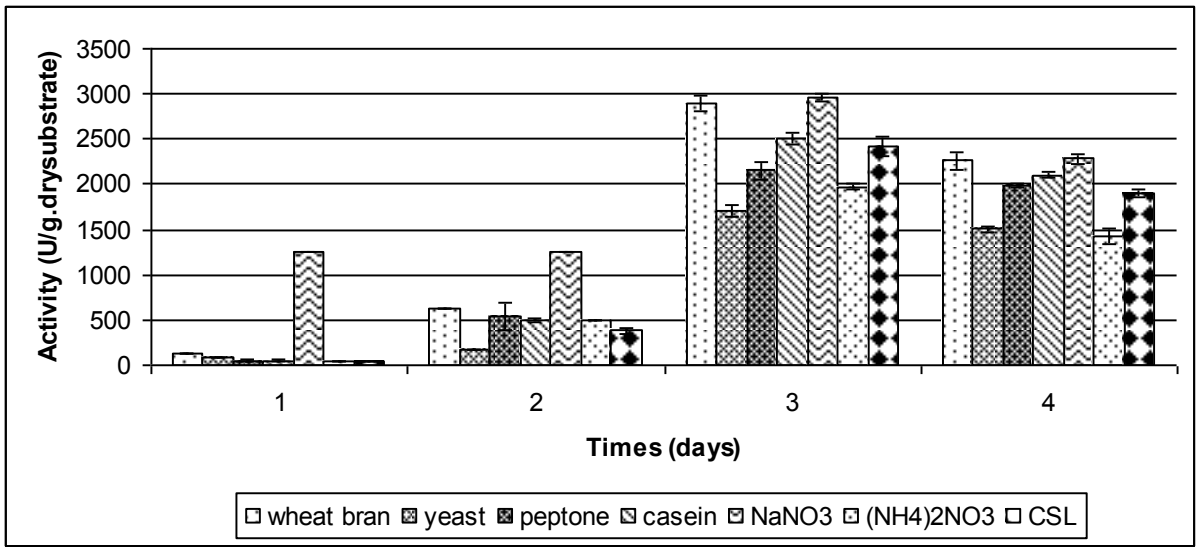


Figure 1. Effect of different nitrogen sources on production of extracellular protease from *A. parasiticus* TEM.

Chakraborty et al. [29] used a strain of *A. niger* and Malathi and Chakraborty [30] used *A. flavus* IMI 327634 for protease production were reported that wheat bran was as the sole carbon sources when grown it in SSF. The results obtained from using other *Aspergillus* sp for protease production are somewhat different from *A. tamari* [31] and *A. niger* [32], the most effective nitrogen sources of which were soy bean and casein, respectively. Protease activity of *A. parasiticus* TEM grown in medium containing wheat bran plus 1% $NaNO_3$ was found to be 200 times higher than that of *A. flavus* IMI 327634 [30], protease of which was used as depilation agent.

3.2. Partial Purification and Characteristics of Protease

Regarding the application of the enzyme for dehairing of cattle hide, various factors like the effect of pH, temperature, sodium sulphide concentration were investigated in partially purified protease of *A. parasiticus* TEM (Table 1). Ammonium sulphate precipitation for the partiall purification of protease was found to be most effective. About 91.4 % of the total activity was retained, while only 7.32% of the original total protein remained.

Table 1. Partial purification of *A. parasiticus* TEM extracellular protease.

Purification Steps	Total Volume (ml)	Total Activity (U)	Total Protein	Specific Activity (U/mg protein)	Purification Fold	Recovery (%)
Culture Filtrate	100	29557,14	169,5	174,4	1	100
$(NH_4)_2SO_4$ Precipitation	11	27008,14	12,4	2182,9	12,5	91,4

Depending on microorganism; microbial proteases can be produced acidic, alkaline and neutral protease or their mixtures [32, 33]. The partially purified *A. parasiticus* TEM enzyme has an activity maximum at pH 8.0 (Figure 2), similar to other extracellular alkaline proteases of microbial origin [10]. pH stability of the enzyme was detected at pH 8.0 in 20 mM Na-Borate buffer for 8 hours and at the end of the time enzyme was also stable with a percentage of 88.91. (Figure 3). This is compatible with the pH stability of *A. tamarii* protease and its enzyme was reported as suitable for dehairing process [30].

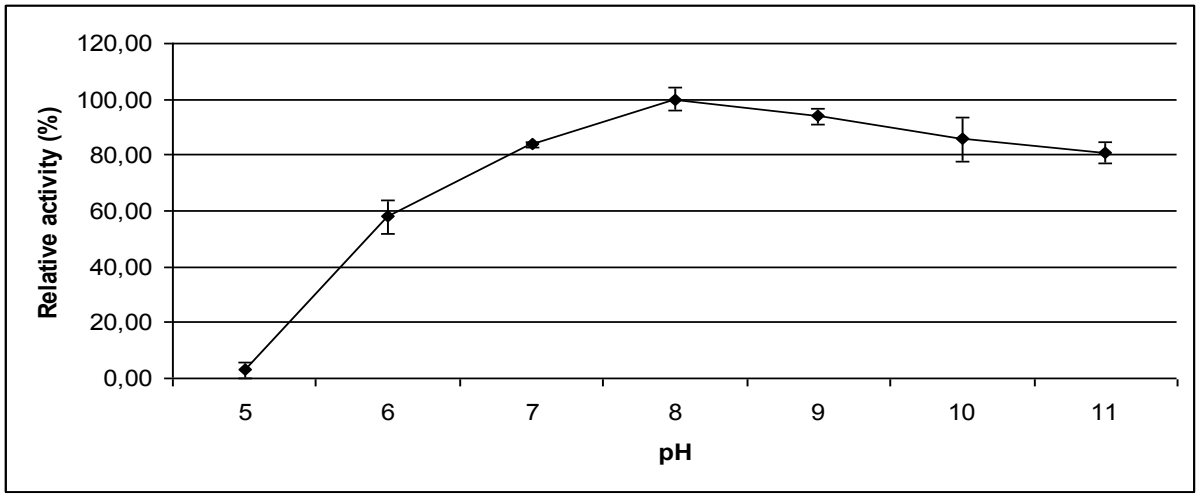


Figure 2. Effect of pH on proteolytic activity of partially purified protease from *A. parasiticus* TEM. Activities are expressed relative to that of the most active sample.

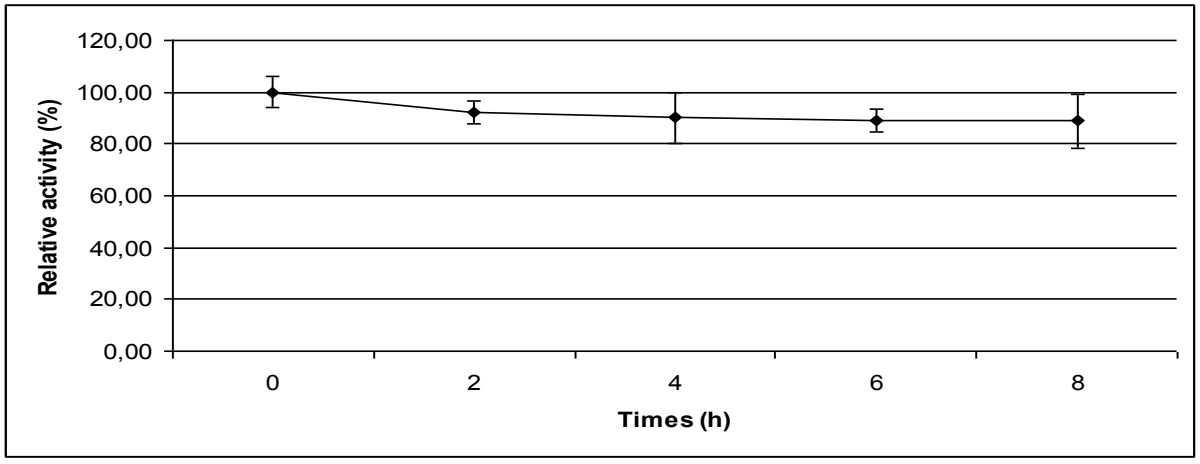


Figure 3. pH stability of partially purified protease from *A. parasiticus* TEM at 30°C for 8 hours. Activities are expressed relative to that of the most active sample.

In enzymatic dehairing, apart from pH, temperature also plays a very important role. Enzymatic dehairing is carried out at 30ºC, so that the collagen structure would not be denaturated and this condition is also most prevalent in many tanneries [34]. Although the enzyme extracted from *A. parasiticus* TEM has an optimum temperature at 50ºC (Figure 4), thermostability studies and dehairing process was accomplished at 30ºC. After 8 hours incubation period, partially purified protease from *A. parasiticus* TEM retained about 90% of its initial activity (Figure 5).

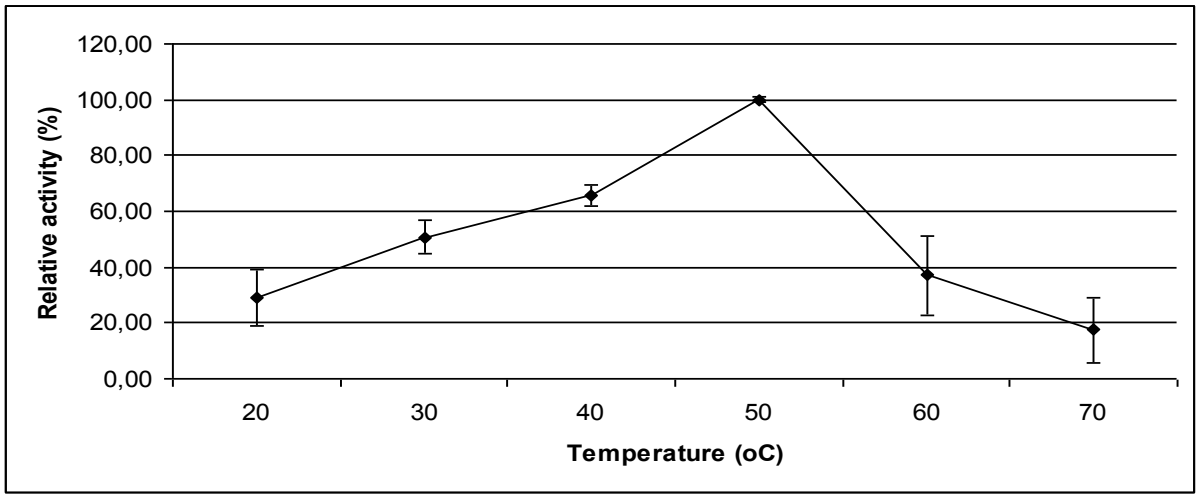


Figure 4: Effect of temperature on *A. parasiticus* TEM protease activity. Activities are expressed relative to that of the most active sample.

Thermostability studies of the fungal proteases indicated that protease activity was stabile with 90%, at 70^{o}C for 1.5 hours [35] and with 95%, at 40^{o}C for an hour [17]. The proteases from *Bacillus* strains were thermostable and active above 80^{o}C [36].

The enzyme produced from *A. parasiticus* TEM showed maximal protease activity at 50^{o}C (Figure 4). Protease produced *A. parasiticus* TEM was also remarkably thermostabil. The partially purified enzyme retained about 90% of its original proteolytic activity for 8 hours at pH 8.0 and 30^{o}C (Figure 5).

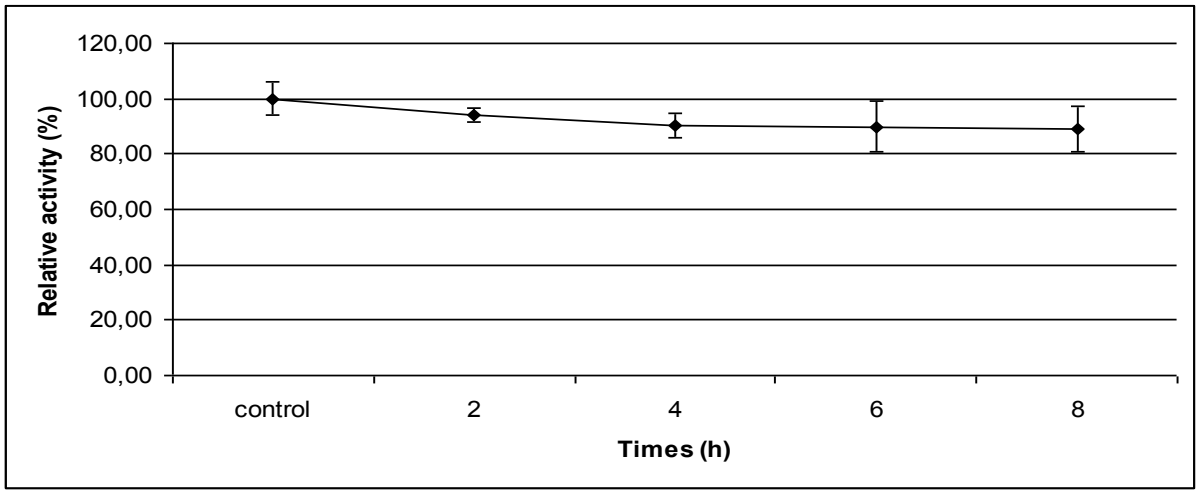


Figure 5. Thermal stability of *A. parasiticus* TEM protease at 30°C in 20 mM Na-Borate buffer (pH 8.0). Activities are expressed relative to that of the most active sample.

3.3. Keratinase Activity

Keratinases are proteolytic enzymes able to hydrolyze both native and denatured keratin [37]. These enzymes have also been studied for de-hairing processes in the leather industry [34]. With this purpose, keratinase activity was investigated in partially purified protease preparate and found to be negligible (less than 0.002 U/gds). Keratinases are mostly isolated from pathogenic dermatophytes, and the ability of filamentous fungi to degrade keratin seems to be rare. Thus, Santos et al. [35] cultivated *A. fumigatus* in mineral liquid medium containing 1% glucose and 0.6% $NaNO_3$ (w/w) and reported these supplements supported mycelial growth but not keratinase production, which is similar to our findings.

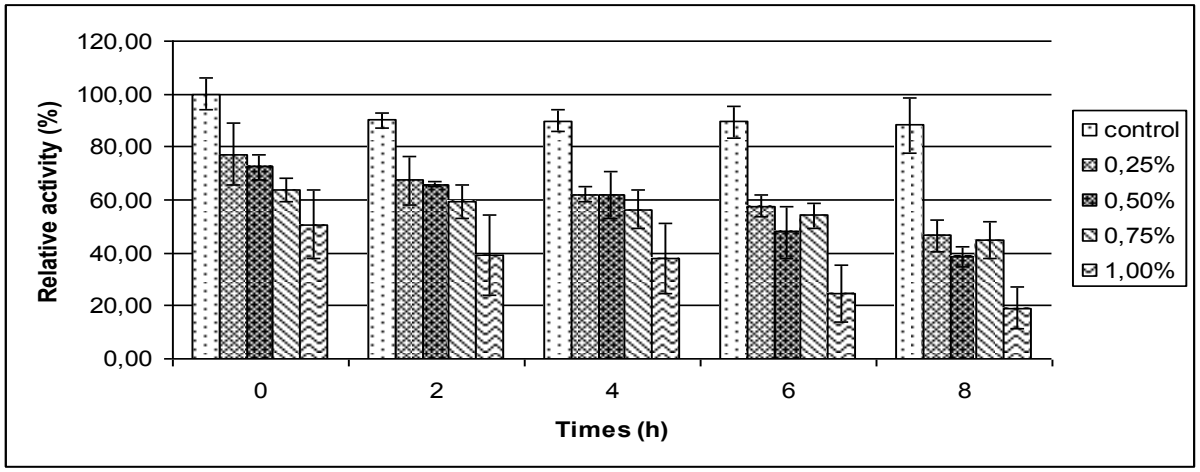
Phenylmethylsulfonyl fluoride (PMSF) and Ethylenediaminetetraacetic acid (EDTA) are specific protease inhibitor and inhibits serine and metallo-protease, respectively [38, 39]. PMSF is inhibited the *A. parasiticus* TEM protease in a percentage of 95.6% (Table 2). This inhibition profile suggested that the protease produced from *A. parasiticus* TEM belongs to the family of serine proteases. Most proteases including those with dehairing activity are serine proteases.

Table 2. Influence of inhibitors on protease activity.

Inhibitors	Inhibition Rate (%)
None	0,00
PMSF	95,60
EDTA	0,38

Although mixture of sodium sulphide and lime used conventionally in dehairing process, nowadays eco-friendly techniques are highly essential. Moreover, half-decreased concentrations of sodium sulphide in enzyme-sodium sulphide and lime mixtures have important reductions in BOD and COD values of leather industrial wastes [10]. Approximately 45% of the enzyme activity was retained in the mixture with 0.25-0.75% sodium sulphide (w/v) at 20 mM Na-Borate buffer (pH 8.0) for 8 hours at 30°C (Figure 6). In addition, the high stability of the enzyme in presence of sodium sulphide (0,25% w/v) is advantageous for its use in dehairing process.

Figure 6. Effect of sodium sulphide concentration on protease produced *A. parasiticus* TEM in 20 mM Na-Borate buffer pH 8.0 at 30°C).



3.4. Enzymatic and Enzyme-assisted Dehairing Process

The activity and performance of the crude enzyme extract have been found satisfying so, the affectivity of crude enzyme extract in dehairing process have been examined. However, it was concluded that passing time for dehairing process according to leather processed as control was longer. But in investigations combined the crude extract with sodium sulphide and lime, process time was shorter than sole enzyme used processes as seen in Table 3.

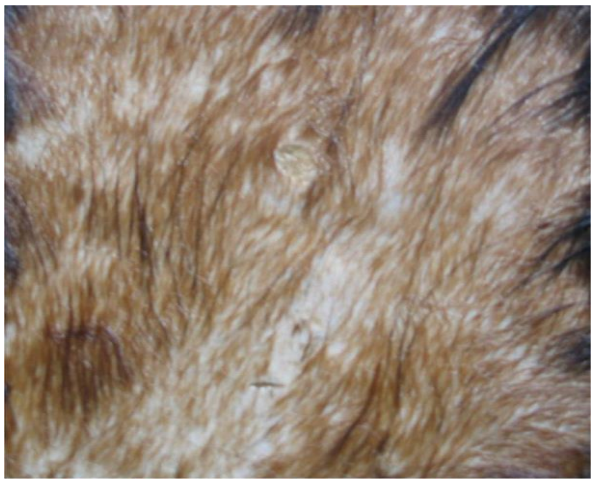
Table 3. Dehairing effect of *A. parasiticus* TEM extracellular protease.

Assays	**1 hour**	**2 hours**	**4 hours**	**6 hours**	**8 hours**	**10 hours**	**12 hours**
2% Sodium sulphide 3% Lime	X	XX	XXX	XXX	XXX	XXX	XXX
50% Enzyme	**—**	**—**	**X**	**X**	**X**	**XX**	**XX**
100% Enzyme	—	X	X	XX	XX	XX	XXX
200% Enzyme	**—**	**X**	**XX**	**XX**	**XXX**	**XXX**	**XXX**
100% Enzyme 1% Sodium sulphide 1.5% Lime	X	XX	XX	XXX	XXX	XXX	XXX

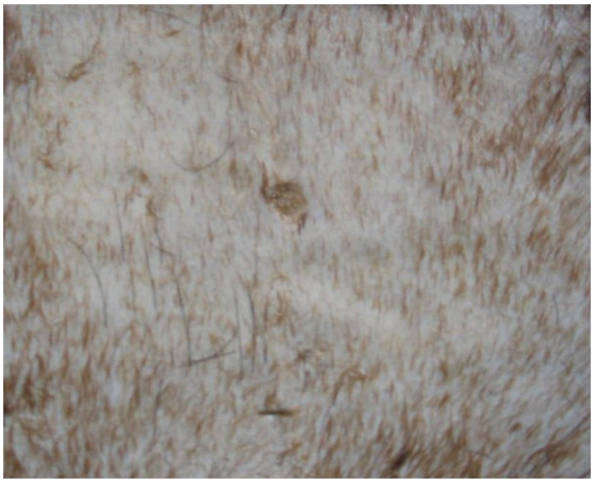
The scale of unhairing was observed as follows: —; no unhairing, X; slightly unhairing, XX; moderate unhairing, XXX; complete unhairing [10, 40].

It has been detected that the swelling of the hides has reached the maximum level in the control specimen where sodium sulphide and lime have been used on their own. On the other hand, when the enzyme is used with 1% sodium sulphide (w/w) and 1.5% lime (w/w), it has been detected that the swelling has decreased due to the decreasing level of sodium sulphide and lime. When the enzyme is used on its own, it is seen that the leathers do not swell, has a light colour close to white and has a clear skin appearance. No hair follicle has been found in grain surface of hides (Picture 1-4).

Picture 1: Pre-process leather sample



Picture 2: 2h after treatment with 200% enzyme



Picture 3: 4-6 h after treatment with %200 enzyme

Picture 4: 8 h after treatment with %200 enzyme

Some characteristics of *A. parasiticus TEM* protease described in this paper, such as easy cultivation, production of protease on simple and cheap medium and stability of the enzyme at a slightly alkaline range of pH make this fungus a potential source of protease for dehairing applications.

When protease from *A. parasiticus TEM* was used with combination of less than 50% sodium sulphide and lime in dehairing experiments, there is a reduction in the process time. Lime-sulfide free or diminished concentrations of lime-sulfide applications in enzymatic dehairing process would lower the use of chemicals and reduce the effluent load. Other benefits can include increases area yield and improved quality of the final product. In the further steps of our research, the effect of the produced enzyme on the quality of the leathers will be examined in detail.

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