

## Wool Quality Improvement Using Thermophilic Crude Proteolytic Microbial Enzymes

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**Abstract:** Wool textile industry is one of the most promising areas for many countries, but there is no real improvement in the Middle East. Regarding that most of the chemicals used in wool treatment are patently protected and environmentally unfriendly; enzymatic treatment will be the best solution. Proteases are being used to decrease the felting tendency of wool and to improve the feel of the fabrics by imparting soft and smooth handle. Simple methods for wool treatment using proteases from thermophilic *Bacillus* strains isolated from Egyptian ecosystem were used. The wool quality improvement process has been proved by both enzyme activity and scanning electron microscope study. The scanning electron microscope proved perfectly the improving quality of the wool fiber surface. Using procedures involved in this study is recommended to improve the wool quality and to substitute chemical based treatments specially to save environmental pollutions and to increase national economy resources.

**Key word:** Wool • Treatment • Protease

### INTRODUCTION

Wool is a fabric made from the hair of sheep, being one of the oldest textile fibers known, has unique properties and considers being a masterpiece of design. Wool absorb moisture vapor, result in superior comfort in both hot and cold weather[1]. Wool has the ability to insulate against heat and cold which protect body against sudden change of temperature [1]. Wool fiber is so resilient and elastic that it can be bent 30,000 times without danger of breaking or damage. Every wool fibers have a natural elasticity and wave or crimp that allows it to be stretched as much as one third and then spring back into place. Its complex cellular structure also enables it to absorb moisture vapor but repel liquid-try and soak up water with wool clothe. No synthetic fibers have been able to combine all these characteristics and science has not been able to produce other fibers containing all the natural properties of wool [1].

“Facing up” is the trade term for the ruffling up of the surface of wool garments by abrasive action during dyeing. Enzymatic treatment reduces facing up, which significantly improves the pilling performance of garments and increases softness [2-8].

Other contributors to environmental pollution is the finishing processes of wool that involves oxidation of the wool surface by means of chlorination or application of

softening agents to modify the handle and improve these properties. Hand and dyeing behavior are important quality aspects for wool. These processes have disadvantages as they are sources of environmental pollution. For this reason, attention of tanners is focused towards revamping the processing methods, recovery system and effluent treatment techniques in order to develop environmentally friendly alternative processes and to make processing eco-friendly. Enzymes can be used as an alternative technology for pre-tanning processes [9].

The major building proteins, in epithelial cells of vertebrates, are keratin and represent the major components of skin and appendages (nail, hair, feather and wool). Keratinase is a specific protease hydrolyzing keratin. These proteases have been found in some species of *Bacillus* [10-12], saprophytic and parasitic fungi [13] and actinomycetes [14-16]. Both the cuticle and cortex of the fibers are modified by proteolytic enzymes, Moreover, the handle of wool top and yarn can be improved by the reduction of binding modulus as a result of a partial hydrolysis caused by proteases[5].

This study aims to investigate the ability of proteases isolated from Egyptian thermophilic *Bacillus* strains to improve the wool quality as well as introducing simple protocol for enzyme activity evaluation.

## MATERIALS AND METHODS

**Bacillus strains and growth conditions:** Two thermophilic *Bacillus* strains were isolated out of twelve strains able to degrade wool at temperature higher than 50°C are identified by standard criteria as *Bacillus licheniformis* and *Geobacillus sp.* These strains grown on LB medium (Luria-Bertani) [17] at 50°C and maintained at -70°C by adding 300 µl glycerol to each 1 ml culture in suitable plastic container.

**Detection of the proteolytic activity on plates:** *Bacillus* strains cultivated on 0.5 gm wool autoclaved in Erlenmeyer flask contain 200 ml tap water, incubated in shaker incubator at 50°C and 200 rpm (Innova 4230 – New Bruaswick Scientific). The different *Bacillus* strains screened for their proteolytic activity using Agar well diffusion plate method, where 3 gm skim milk suspended in 100 ml water and autoclaved. After autoclavation the soluble component added to sterile water agar (16 gm agar/L). The suspension then stirred gently and distributed in Petri dishes (25 ml/plate). After complete solidification of the agar on plates, wells were punched out of the agar, by using a clean sterile cork borer (6 mm in diameter). The base of each hole was sealed with a drop of melted sterile water agar (15 g agar per liter H<sub>2</sub>O) using sterile Pasteur pipette. 75 µl of each bacterial supernatant added to each well and preincubated at -4°C for 30 min and then overnight incubated at different temperatures.

**Visualization of the enzyme clear zone:** Coomassie blue (0.25%, w/v) in methanol-acetic acid-water 5:1:4 (v/v/v) was used in plates staining to visualize the skim milk degradation where 10 ml was added to each plate and incubated in room temperature for 15 min followed by removing the staining solution from the plates surface and washing gently by distilled water. Then the plates de-stained using destaining solution (66 ml methanol, 20 ml acetic acid and 114 ml H<sub>2</sub>O<sub>bidest</sub>) for a suitable time [18].

**In flask enzyme activity determination:** The enzyme activity was determined directly by determining the wool degradation rate/h. Two gram of crude wool autoclaved in Erlenmeyer conical flasks each contains 200 ml distilled water. One inocula of each *Bacillus* strain was added to each flask and incubated in shaker at 200 rpm (Innova 4230 – New Bruaswick Scientific) and 50°C. After complete degradation of the wool the culture centrifuged at 10000 rpm (Biofuge 15 - Heraeus Sepatech)

at 4°C for 10 min. The protein content/ml determined spectrophotometrically at 280 nm (PerkinElmer-.UV/VIS Spectrometer Lambda). The activity of the aliquot calculated as Units/ml. One unit enzyme identified for their ability to degrade one mg of the crude wool per minute at the experiment conditions.

**Preparation of L-tyrosine standard curve:** 1.1 mM L-tyrosine was dissolved 100 ml deionized water and heated gently (without boiling). The standard curve generated by read the absorbency at 280 nm (PerkinElmer-.UV/VIS Spectrometer Lambda) for 0, 12.5, 25, 50, 100, 200, 250 and 500 µl from L-tyrosine solution completed to 1 ml by adding deionized water. The relationship between the absorbency and the mM L-tyrosine then plotted as y/x plot.

**Preparation of Casein-Universal buffer for different pH enzyme activity:** Universal buffer was prepared according to Britton and Robinson [19] which consist of 40 mM H<sub>3</sub>PO<sub>4</sub>, 40 mM acetic acid, 40 mM H<sub>3</sub>BO<sub>3</sub>. The different pHs were adjusted by adding different volume of 0.2 M NaOH. 0.325 mg casein weighted and dissolved in 50 ml Universal buffer (pH 5, 6, 7, 8 and 9). The mixture dissolved by heating gently to 80-90°C without boiling.

**Enzyme activity:** 300 µl of each supernatant which contain the enzyme added to the same volume of the Casein-Universal buffer solution (pH 5, 6, 7, 8 and 9). The enzymes-substrate mixture for each pH incubated at 50, 60, 70 and 80°C for 30 min. After the incubation period the enzyme reaction stopped by adding 600 µl of 10% trichloroacetic acid. The mixture allowed to stand at room temperature for 15 min then centrifuged at 10000 rpm for 10 min (Biofuge 15- Heraeus Sepatech). The absorbance of each sample determined spectrophotometrically at 280 nm (PerkinElmer-.UV/VIS Spectrometer Lambda) and their tyrosine content derived from the tyrosine standard curve and the enzyme activity determined as Units/ml.

**Enzyme stability:** The enzyme stability determined by incubating supernatant which contain wool degrading enzymes at time interval 30, 90, 120, 180, 210 min and 2 h. at 37 and 70°C. After each time interval (30, 90, 120, 180, 210 min and 24 h), 300 µl of the incubated enzymes added to 300 µl from the 1.1 mM Casein-Universal buffer pH 9 and incubated at 37 and 70°C. After 30 min the reaction stopped by adding 600 µl 10% TCA followed by incubation at room temperature for 15 min. The mixture then centrifuged at 10000 rpm for 15 min (Biofuge 15-

Heraeus Sepatech). The absorbency of each sample detected spectrophotometrically at 280 nm (PerkinElmer .UV/VIS Spectrometer Lambda) and the different enzyme activates determined as Units/ml.

**Sample preparation for electron microscopy:** To study the keratinase activity effect on the wool surface, electron microscope was used to scan the surface of the wild and treated wool. Wild and treated wool fibers after 4 and 12 h, one fiber of each treatment fixed in the surface of flat glass slid and washed gently by distilled water for 3 sec then allowed to dry at 37 °C. The dry wool surface then coated with approximately 15 nm gold (SPI-Module™ sputter Coater).

**Scanning of the wool surface:** The golden coated sample then subjected to be scan by analytical scanning electron microscope (Jeal JSM-6360LA) with secondary element at 20 KV acceleration voltages at room temperature. The digital image were adjusted and saved.

## RESULTS

**Detection of the proteolytic activity on plates:** Thermophilic proteolytic activity of the cell free supernatant was determined on skim milk agar plate using Agar well diffusion method. The clear zones appear clearly after visualization using staining method as in Fig. 1.



Fig. 1: Skim milk-Agar well diffusion method for study the proteolytic activity of the wool degrading enzymes



Fig. 2: Two flasks represent Bacillus inoculums growing with and without wool

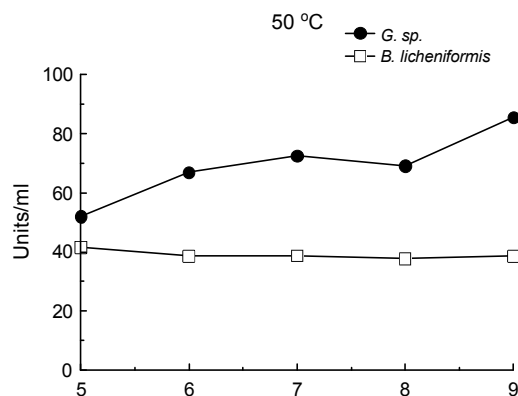


Fig. 3: Enzyme activity of *B. licheniformis* and *G. sp.* enzymes calculated as Units/ml at 50°C and pH from 5-9

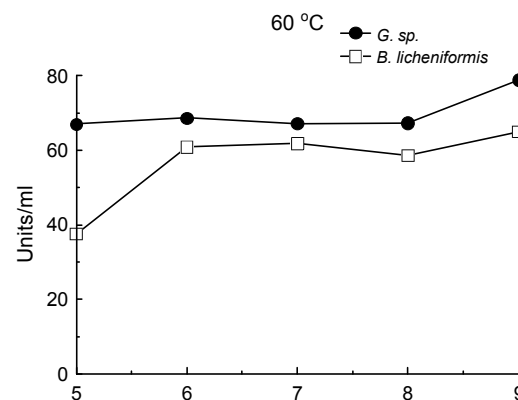


Fig. 4: Enzyme activity of *B. licheniformis* and *G. sp.* enzymes calculated as Units/ml at 60°C and pH from 5-9

This technique gives preliminary fast idea about the proteolytic activity of the crude enzymes as well as can be used to compare different samples.

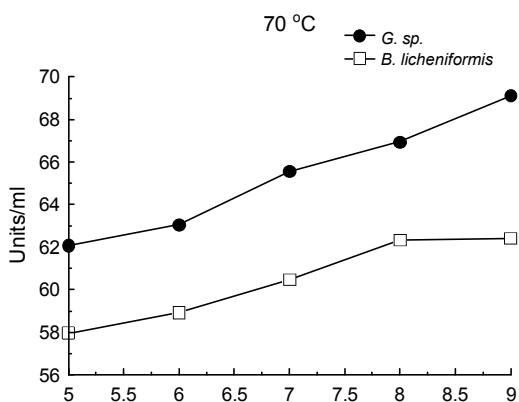


Fig. 5: Enzyme activity of *B. licheniformis* and *G. sp.* enzymes calculated as Units/ml at 70°C and pH from 5-9

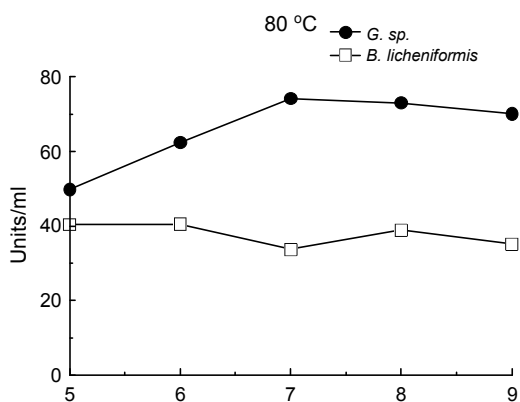


Fig. 6: Enzyme activity of *B. licheniformis* and *G. sp.* crud Protease growing on wool and calculated as Units/ml at 80°C and pH from 5-9

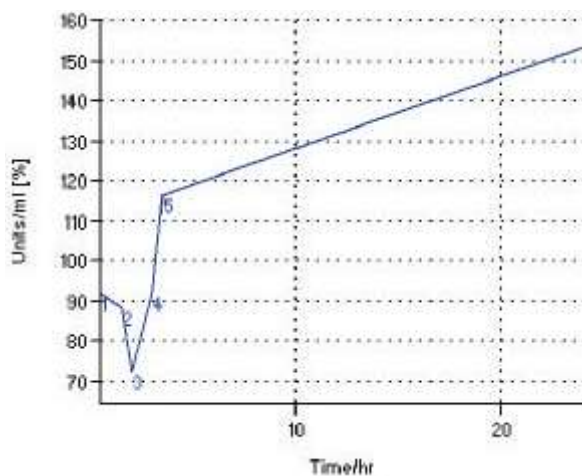


Fig. 7: Enzyme stability of *G. sp.* crude proteases growing on wool and calculated as Units/ml at pH 9 during 24 h incubation at 70°C

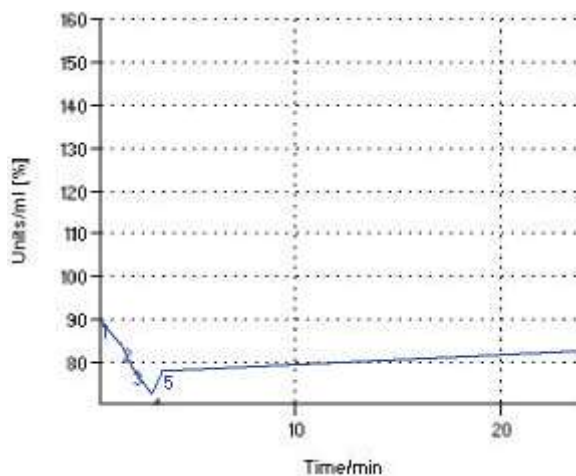


Fig. 8: Enzyme stability of *G. sp.*, crude proteases growing on wool and calculated as Units/ml at pH 9 during 24 h incubation at 37°C

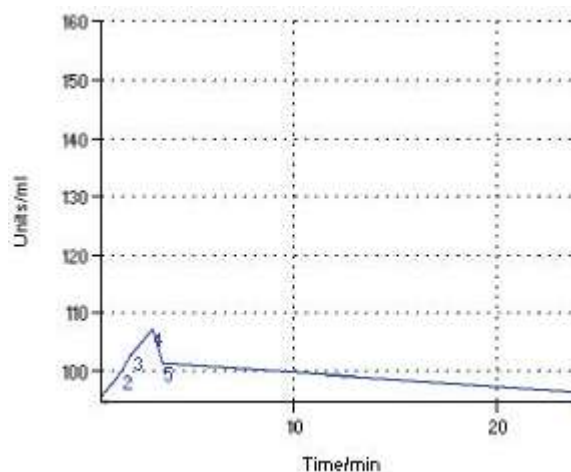


Fig. 9: Enzyme stability *B. licheniformis* crude proteases growing on wool and calculated as Units/ml at pH 9 during 24 h incubation at 70°C

**In-flask enzyme activity determination:** The keratinolytic activity determined directly in flask as in Fig. 2 by measuring the degradation of 2 gm wool against time and the activity of each sample determined as Units/ml after comparing with blank and control. *G. sp.*, activity was 4.18 Units/ml while for *B. licheniformis* was 5.1 Units/ml.

**Optimization of pH and Temperature:** The enzymes activities were optimized at different pH (5, 6, 7, 8 and 9) at 37 and 70°C. The results showed that the enzymes are thermostable have activities as in Fig. 3-6. The optimum activity of *G. sp.* was at 60°C and pH 9 while for *B. licheniformis* was at 50°C and pH 9 as in Fig. 3.

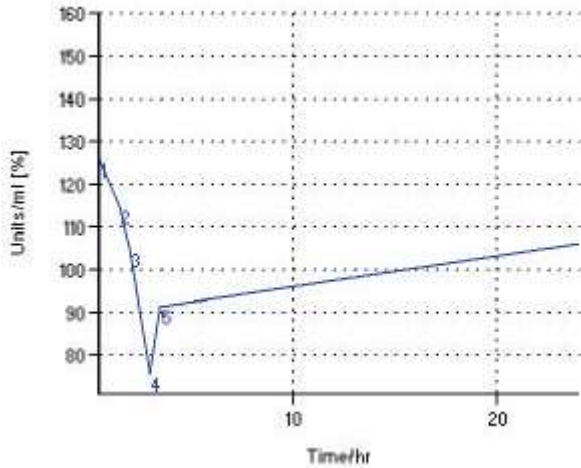


Fig. 10: Enzyme stability of *B. licheniformis* crude Proteases growing on wool and calculated as Units/ml at pH 9 during 24 h incubation at 37°C

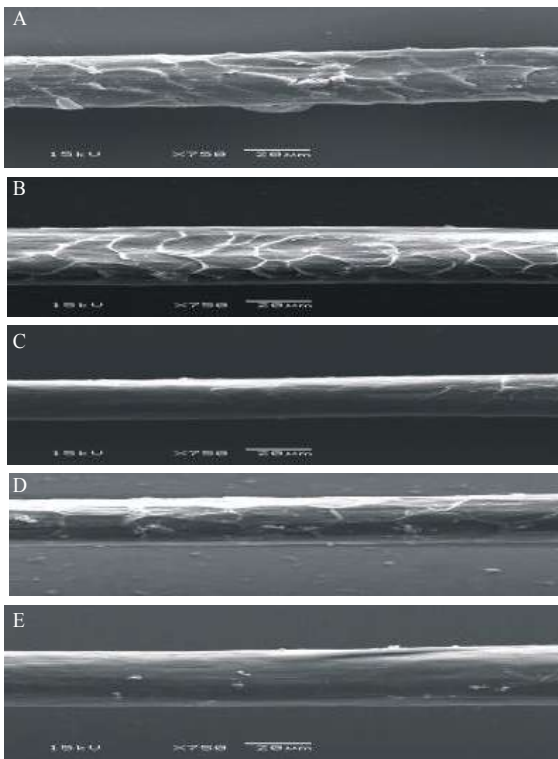


Fig. 11: Scan Electron Microscope for wild wool fiber without treatment (x 750) (A); wool fibers treated with *B. licheniformis* proteases (x 750): (B: 4 h and C: 12 h); wool fibers treatment with *G. sp.* proteases (x 750) (D: 4 h and E: 12 h) (x 750)

**Enzyme stability:** The enzyme stability at pH 9 at 37 and 70°C proved that both enzymes are stable in both temperatures. Slight decrease in the enzymes stability

was shown by increasing time (Fig. 7,9-10) except *G. sp.* at 37°C where the activity increased by increasing the treatment time (Fig. 8).

**Scanning Electron Microscope study:** The study of wool fibers surface, using electron microscope, represent the effect of the proteolytic enzyme on the wool fibers (Fig. 11). Using time interval 0, 4 and 12 h, the treated sample clearly show the effect of the proteolytic enzymes on the wool fibers surface. The proteolytic enzymes smooth the surface of the wool by increasing the treatment time. After 12 h. treatment, the scan electron microscope results illustrated that the wool fibers become very smooth without any damaging.

## DISCUSSION

Wool textile industry is one economic missing factor which could help in improve the live of recognizable populations sector in developmental countries including Egypt. Wool as textile is very useful and still preferable regarding its unique properties and comfort when use. The global wool price in the market still high based on their high demand, so far it will be no competitions between the international and national production of wool textile [1].

The most limiting factor affect on Egyptian wool textile production is their low quality comparable with the other varieties of the wool exported from countries like Australia and Newzland. The fact that trying to improve wool quality using chemical treatment will be cost effective as well as a source of pollutants, so fare enzymatic treatment will be the solution [2-8]. The study described the use of two thermophilic *Bacillus* strains produce thermophilic proteases to improve the wool fibers quality as shown in the electron microscope studies. The proteolytic activity of wool degrading enzymes determined using Agar well diffusion method on water agar containing skim milk as a substrate while the keratinolytic activities were determined directly using In-flask method on the crude wool. The proteolytic activities on plates visualized using coomassie blue staining method while the wool degradation rate was determined using degradation of 2 gm of crude wool against time. The big amount of wool reduces the error factor. The enzyme activity determined as Units/ml and estimated to be 5.1 for *G. sp.* and 4.18 for *B. licheniformis*.

In the degradation process, only tap water was used which reduce the cost effective tremendously. In fact using crud wool give the advantage for the bacillus stains

to utilize the wastes between the fibers as well as the trace elements in the tap water to initiate the degradation processes.

Enzyme optimization studies for the wool proteolytic enzymes (derived from wool degradation) on the casein as a substrate were performed using different pH values include 5, 6, 7, 8 and 9 as well as different temperature values include 50, 60, 70 and 80°C.

The enzymes proved to be thermostable and able to perform activity in all experiments and different conditions. pH 9 show the maximum performances in most cases and used in further stability studies. *G. sp.*, shows maximum activity at 60°C while *B. licheniformis* shows maximum activity at 50°C. 70° C was used to study the enzymatic stability of both strains because enzymes show remarkable constant linear activity in all pH at 70°C (Fig. 5).

The stability studies show that the enzymes are stable at 37 and 70°C for 24 h examination which prove their ability to use directly in industrial applications.

After proving the enzymes activity on different substrates (skim milk, wool and casein), study their wool degradation ability, optimize their pH and temperature, prove their thermostability and their minimum requirement to be produced where the organism able to produce enzymes only by using crude wool and tap water; electron microscopic studies were performed to test their real effect on the wool fibers surface.

The scanning electron microscope studies showed that the enzyme perfectly improves the wool surface quality by smoothing the wool fibers outer layer. The smoothing of the wool fibers process against time gives the best results. While we using only tap water and regarding the fact that the enzyme work perfectly at these condition, optimization of factors other than pH and temperature have been neglected to reduce the research time and cost. Inventing In-flask method for detecting direct keratinolytic activity on crude wool in our point of view is more practical, cost and time effective and match our requirement to investigate the keratinolytic activities on the keratin we need which in our case is the wool. This study has been designed to be rather simple and effective to be used as a model for wool quality improvement. While the experiments in this study were done in small lab scale laboratories, but further experiments and analysis should be performed to scaling up the wool treatment in industrial scale which will be covered in future. The wool improving will add a big value to the national economy. The tools for wool quality improving included in this study can be used also to invent new proteolytic

applications by reusing the proteases produced during (and involved) in the wool improvement processes.

Regarding to the cost and profit issues of this study the enzymes used in wool quality improvement cost minimum and profit maximum. The substrate is the wool it self, the media is tap water plus the impurities around the wool fibers. No need for excessive washing process. The enzyme which used come spontaneously during the processes as well as these enzymes in fact can be collected and reused in another kind of biotechnological processes; studies will be done in future.

We recommended using and following the protocols included in our study to perform more tools and to detect more strains able to improve wool quality.

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