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Biotechnological Potential of a Thermostable and pH-Stable Protease from Bacillus sp. AM12: An Innovative Approach for Wool Biodegradation and Industrial Applications

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Abstract

Biodegradation of wool fibers using microbial proteases is an environmentally friendly sustainable biotechnology used as a substitute for regular chemical treatments. This study isolated and identified a potent wool-degrading Bacillus sp. AM12 from soil and water samples in Egypt. The isolated bacterium's maximum strong proteolytic activity (180.7 U/ml) was detected upon growth on the APPB medium, confirming wool as an inducer. The crude enzyme was identified as an alkaline protease enzyme with optimal activity at pH 10 and 60°C. Wool degradation by crude enzyme was confirmed by a decrease of weight without shrinkage, while SEM imaging revealed surface smoothing and modifications. Anion exchange and gel filtration chromatography were used for enzyme purification, resulting in a 2.66-fold increase in purity and a specific activity of 5.6 U/mg. SDS-PAGE analysis of the purified enzyme predicted its molecular weight at 24 kDa. Moreover, the enzyme retained its thermostability, pH stability and over 80% activity in commercial detergents such as SDS and Tween-80, confirming its industrial biotechnological compatibility. These findings showed the potential of Bacillus sp. AM12 protease as a sustainable and effective alternative in industrial wool treatment.

Keywords: Protease; Thermostable**;** Industrial Biotechnology.

1. Introduction

Wool is the second most eco-friendly used fibers after plant fibers [1]. Wool fiber is durable, flexible and elastic and it can be bent 30,000 times without risk of breaking or damage. Recently, wool has been used as a reinforcing material for additive manufacturing in some high-tech industrial sectors [2,3]. Every wool fiber has a natural elasticity and wave or crimp that allows it to be stretched as much as one-third and then spring back into place [4]. Its complex cellular structure also enables it to absorb moisture up to 30% of its weight [5].

Wool consists principally of one member of a group of proteins called keratins [6]. Keratin fibers are not chemically homogeneous; they consist of a complex mixture of widely different polypeptides. Despite the classification of wool as a keratin, clean wool contains only approximately 82% of the keratinous proteins, characterized by a high concentration of cysteine [7]. Approximately 17% of wool is composed of proteins which have been termed non-keratinous, because of their relatively low cysteine content. The wool fiber also contains approximately 1% by mass of non proteinaceous material consisting mainly of waxy lipids plus a small amount of polysaccharide material [8]. The non-keratinous proteins and lipids are not uniformly distributed throughout the fiber but are concentrated in specific regions of the structure [9]. Thus, wool is a complex natural fiber composed mainly of proteins (97%) and lipids (1%), with a heterogeneous morphological structure [10,11].

Wool tendency to felting and shrinkage limit the

use of untreated wool materials as machine-washable textiles [11]. The hydrophobic character and the scaly structure of the wool surface are the main factors causing the differential frictional effect (DFE), resulting in all fibers to move to their root end, when mechanical action (such as moisture, heat, and pressure) is applied in the wet state [12,13]. The felting changes affect not only the garment's dimensions but also its look; the woven or knitted structure becomes less visible, and the garment becomes thicker and less elastic [11].

The most successful industrial shrink-proofing processes are based on a modification of the fiber surface either by oxidative or reductive methods and/or by applying a polymer resin onto the surface [14]. The most frequently used industrial wool modification process is the chlorine/Hercosett process which consists of a chlorination step followed by a de-chlorination step and polymer application [15]. Although this process is highly efficient in reducing shrinking, chlorination produces adsorbable organohalogens (AOX) which appear in the effluent and generate toxicity. With the increasing ecological and economic restrictions imposed on the textile industry, industries are required to find environmentally favorable alternatives in wool treatment processes [16]. Research on the use of proteases for decreasing the felting tendency of wool has been carried out since the beginning of the 1900s, but the results obtained so far present a high variance and no great achievements compared to the classical chemical method [17]. The use of specific microbial or enzymatic processing is needed, in order to develop environmentally friendly alternative processes in wool treatment technology [11,18].

Microbial proteases are preferred to enzymes from plant and animal sources, since they possess almost all the characteristics desired for biotechnological applications [19]. Commercial proteases are mostly produced from various bacteria and it was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria sources [20,21]. Bacillus species are well-recognized as commercial producers because they secrete substantial amounts of extracellular enzymes [22].

A new approach has attempted the modification of proteases to increase their molecular sizes to limit the enzymatic degradation of wool fiber to their cuticle scales. The increase in molecular size of proteases can be achieved by covalently attaching proteases onto soluble polymer. Laboratory results have shown that fiber degradation is reduced by modified proteases to achieve machine washable wool by enzymatic treatment with modified proteases [23].

Previous reports have established new enzymatic wool finishing processes, since they allowed for control enzyme hydrolysis [11], which was the major drawback of this environmentally friendly option to the conventional chlorine treatments [24]. They studied the diffusion of serine proteases into wool fabrics and yarns. The proteases used were free subtilisin and subtilisin-PEG (the same enzyme that was covalently linked to polyethylene glycol). The enzyme adsorption and diffusion were facilitated by the pretreatment performed, the alkaline surfactant washing and bleaching, the most infective concerning enzyme adsorption. Their study suggested that the diffusion of proteases into wool is dependent on the size of the protease. The free enzyme penetrates the wool fiber cortex while the modified bigger enzyme is retained only at the surface, in the cuticle layer. Also, proteins without proteolytic activity do not adsorb considerably on wool due to their hydrophobic nature, therefore the diffusion is facilitated by hydrolytic action [25].

Furthermore, Heine and Höcker (1995)[10] as well as Silva et al., (2005)[11] have suggested that either the enzyme has to be controlled (for example, diffusion control by enzyme immobilization) or the enzyme has to be specially “designed” (for example, by genetic engineering) in such a way that only a distinct part of the substrate is altered.

The high content of cystine in the protein chains of wool keratins contributes to its mechanical stability through the formation of covalent cross-links between polypeptide chains [26]. Despite its recalcitrance, wool keratins can be proteolytically degraded by many Microorganisms (bacteria, actinomycetes, and fungi) via keratinolytic and proteolytic enzymes [27]. In addition, by breaking disulfide bonds in the α-keratin structure, protein fractions become more soluble and available for the growth of microorganisms [28].

Using protease technology in the woolen Egyptian textile industries would contribute to decreasing the felting tendency of wool and improve the feel of the fabrics by imparting a soft and smooth handle. So, the scientific outcome of this study would contribute to the development of new biotechnological enzymatic processes for the wool textile industry resulting

in more environmentally friendly production technologies, lower manufacturing costs, less hazardous waste and reduced energy consumption, as well as higher product quality.

2. Materials and Methods

2.1 The Extraction Process of Collagen from Fish Skin

2.1. Microorganisms

Microorganisms used in this study were isolated from different Egyptian habitats. Bacteria which showed proteolytic activity and high degradability rate to the wool fibers were identified as Bacillus sp. and were chosen for this study.

2.2 Bacterial Growth Medium

Luria Bertani (LB) medium was prepared following the composition described by Sambrook et al. (1989)[29], consisting of tryptone (10 g/L), NaCl (10 g/L), and yeast extract (5 g/L). For solid LB medium, agar (15 g/L) was added to the mixture, and the pH was adjusted to 7.0 before sterilization. Skimmed milk agar medium was prepared by suspending 1 g of skimmed milk in 100 mL of distilled water, followed by autoclaving. After sterilization, the soluble component was added to sterile water agar (15 g/L), stirred gently, and distributed into Petri dishes (25 mL/plate) as per Amara and Serour (2008)[30].

For screening proteolytic activity and high wool degradation, different media were utilized: (a) tap water (100 mL) containing 1 g of wool fibers, (b) distilled water (100 mL) containing 1 g of wool fibers, and (c) a minimal medium containing wool fibers (MMF), composed of wool fiber (1.0 g/100 mL), NaCl (0.05 g/100 mL), K_2HPO_4 (0.03 g/100 mL), and KH_2PO_4 (0.04 g/100 mL), with the pH adjusted to 7 before sterilization [34]. Additionally, an alkaline protease-producing broth (APPB) was prepared according to Abu Sayem et al. (2006) [35], containing glucose (1.0 g/100 mL), peptone (0.5 g/100 mL), yeast extract (0.5 g/100 mL), K_2HPO_4 (0.5 g/100 mL), and $MgSO_4 \cdot 7H_2O$ (0.01 g/100 mL), with the pH adjusted to 7 before sterilization.

For the cultivation of alkaline protease-producing bacteria, a modified APPB medium was prepared, containing yeast extract (0.14 g/100 mL), $MgSO_4 \cdot 7H_2O$ (0.07 g/100 mL), NaCl (0.9 g/100 mL), K_2HPO_4 (0.7 g/100 mL), and wool (2.0 g/100 mL). The pH was adjusted to 10 before sterilization to optimize bacterial growth conditions.

2.3. Wool fibers collection

Wool fibers were obtained whether from sheep grazing waste of farms, slaughterhouses or textile processing industries in Egypt then cleaned with water before sterilization and treatment.

2.4. Isolation process of alkaline protease producing bacteria

Samples were collected from different Egyptian habitats; sand samples from Borg El-Arab, Matrouh and Sohag cities, soil and sea water samples from Alexandria city. Aliquots of different dilutions of soil and sand suspensions and water samples were plated onto LB agar medium and incubated at 37°C for two days, then different colonies of the isolates were cultivated on LB broth medium for 24 h at 37ºC and screened for their proteolytic activity using agar well diffusion plate method. After preparation of skimmed milk agar medium and complete solidification of agar, wells were punched out 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/l) using sterile Pasteur pipette. 1 ml of culture of each isolate was centrifuged at 13000 rpm, then 75 µl of each supernatant was transferred to each well, pre-incubated at 4ºC for 30 min. then incubated overnight at 37ºC [30,31,32].

To reveal the degradation of skimmed milk, staining buffer was used, where 10 ml was added to each plate and incubated at room temperature for 15 min. followed by removing the staining solution from the plate's surface and washing gently by distilled water. Plates were then de-stained using de-staining solution, for a suitable time; till a clear contrast between plates background and the degradation zone around wells appears [30,31,32].

2.5.1. Identification of alkaline protease producing bacteria

2.5.1.1. DNA extraction

The genomic DNA was isolated by modified method of Sambrook et al. (1989)[29]. Aliquot of overnight culture (2 ml) was centrifuged at 6,000 rpm for 10 min. at 4oC. Cells were re-suspended in 500 µl TEN buffer containing 10 mg/ml lysozyme then incubated at 37°C for 30 min, 75 µl of 10 % SDS were then added and mixed gently till complete lysis. Proteinase k (20 mg/ml) were then added (3 µl) and the tubes were incubated at 37°C for one hour. The mixture was then extracted several times with phenol: chloroform: isoamyl alcohol (12:12:1) to remove protein and once with chloroform to

remove phenol traces. Then the extracted DNA was precipitated using 700 µl of isopropanol and washed with 70% ethanol. The isolated DNA was dried and dissolved in sterile distilled water then stored at -20 °C.

2.5.1.2. Polymerase Chain Reaction (PCR)

The reaction mixture of PCR contained 10-50 ng of DNA, 5 µl 10X DNA polymerase buffer, 10-30 Pmol of each primer; 25 or 50 µM deoxynucleoside triphosphate, 1 unit of Taq DNA polymerase and the volume was completed to 50 µl with sterile distilled water in 200 µl Eppendorf tubes. The primer used in PCR method to identify 16S rDNA sequence of the strain under investigation, forward sequence

(5' GAGTTTGATCCTGGCTCAG3') and reverse sequence

(5' GGTTACCTTGTTACCACTT3').

Amplification of a conserved region of the gene was set in thermal cycler as follows: initial denaturing step 95ºC for 5 min., then (30 cycles) comprised a denaturing step at 95ºC for 1 min., annealing at 51ºC for 1 min. and extension at 72ºC for 1.5 min. followed by final extension 72ºC for 10 min. with forward and reverse primers.

Identification of the isolate was done by 16S rDNA amplification and sequence analysis [33]. Sequence similarity search was done using NCBI server. The organism was then followed by gram staining method.

2.8. Cultivation of alkaline protease producing bacteria

The isolate that exhibited high proteolytic activity on skimmed milk agar plate expressed as diameter of clear zone in mm was selected for its ability to degrade or grow on wool. In this study, 4 types of wool containing media. were used to select high levels of proteolytic activity by the selected isolate. 5 ml of LB broth medium was inoculated by the desired isolate and incubated overnight at 37ºC. The resulting culture was then transferred to 95 ml of the selected media; minimal medium containing wool fibers MMF [34] alkaline protease-producing broth APPB [35]; tap and distilled water [30] and incubated in a rotary shaker at 200 rpm and 37ºC.

The enzyme activity was determined directly by determining the wool degradation period. After complete degradation of the wool, the culture was centrifuged at 13000 rpm at 4oC for 20 min. and the protein (mg/ml) was determined spectrophotometrically at 280 nm. The activity of the aliquot is calculated as U/ml. One unit enzyme is identified for its ability to degrade one mg of the crude wool per minute at the experiment conditions [30,32].

2.9. Optimization of the cultivation conditions for the production of alkaline protease by the potent isolate

2.9.1. Determination of optimum incubation period for enzyme production

To determine the optimum incubation period for alkaline protease production, the cultivation conditions were carried out at 200 rpm, pH 7 and 37°C and the samples were collected at different incubation intervals (24, 48, 72, 96 and 120 h). The supernatant was separated by centrifugation for 15 min. at 13000 rpm for the determination of alkaline protease activity [19].

2.9.2. Effect of culture components on enzyme production

To determine the optimum compositions of the APPB [35]medium that enhanced alkaline protease production by tested strain, the amount of each medium constituent was tested at variable levels as described in Table 2, and cultivation was carried out at 200 rpm, pH 7 and 37°C for 72 h.

Different concentrations of APPB medium components were tested. Yeast extract was varied from 0.04 g/100 mL to 0.20 g/100 mL in increments of 0.02 g, while NaCl and K_2HPO_4 were each tested at concentrations ranging from 0.10 g/100 mL to 1.00 g/100 mL in increments of 0.10 g. $MgSO_4 \cdot 7H_2O$ was adjusted from 0.01 g/100 mL to 0.10 g/100 mL in increments of 0.01 g. Wool, an essential substrate for enzyme induction, was tested at concentrations ranging from 0.25 g/100 mL to 2.00 g/100 mL. These variations aimed to identify the optimal composition for maximizing enzyme yield by determining the most effective balance of nutrients and inducers.

2.9.3. Effect of inoculum size on enzyme production

To standardize the inoculum amount for alkaline protease production, different volumes (1 to 10%, v/v) of homogenous inoculum have been used to inoculate the 100 ml media [19,36,37]. The cultivation was also carried out at 200 rpm, pH 7 and 37°C for 72 h.

2.9.4. Effect of pH on enzyme production

To detect the effect of different initial pH on the production of alkaline protease, pH of the cultivation broth was adjusted to 4, 5, 6, 7, 8, 9, 9.5, 10 and 11 separately with HCl or NaOH in

250 ml Erlenmeyer flasks containing 100 ml broth. The fermentation was carried out at 200 rpm and 37°C for 72 h [19].

2.9.5. Effect of incubation temperature on enzyme production

To investigate the effect of different temperatures on the production of alkaline protease, cultivation was carried out in 250 ml Erlenmeyer flasks containing 100 ml medium at different temperatures (20, 25, 30, 35, 37, 40, 45, and 50°C) in an orbital shaker at 200 rpm [19,36].

2.9.6. Effect of agitation speed on enzyme production

To determine the optimum shaking rate for the production of alkaline protease, cultivation was carried out at different shaking rates in 250 ml Erlenmeyer- flask containing 100 ml media (100, 150, 200, and 250 rpm) [19].

2.10. Analytical methods

2.10.2. Protease assay

To determine the proteolytic activity of the wool degrading isolate, 30 µl of the cell free supernatant, which contains the crude enzymes was added to 470 µl of the Casein-0.1 M phosphate buffer solution of a certain pH (e.g. 7). The enzyme- substrate mixture was incubated at a certain temperature (e.g. 37°C) for 30 min. After the incubation period, enzyme reaction was stopped by adding 500 µl of 10% Trichloro acetic acid. The mixture was then centrifuged at 13000 rpm for 15 min. The absorbance of the supernatant was determined spectrophotometrically at 280 nm, its tyrosine content derived from the tyrosine standard curve and enzyme activity expressed as U/ml. One unit of the enzyme activity was defined as the amount of enzyme, which produces one µg tyrosine/ min under the assay condition [30].

2.10.3. Effect of pH and temperature on crude enzyme activity

The optimum pH was determined at 37°C for 30 min. The substrate (0.65 %, w/v, casein) was prepared over a range of pH values (6, 7, 8, 9, 10, 11 and 12) in 0.1 M phosphate buffer solution (3.5.4.). Protease activity was measured at different pH values under standard assay conditions. The optimum temperature was determined at different temperatures ranging from 20 to 70°C, using a standard reaction mixture for casein [30].

2.10.4. Determination of total protein content

The total protein contents of the samples were determined according to the method described by Bradford (1976) [37]; the protein standard used was Bovine Serum Albumin (BSA) and Bradford solutions were prepared as described under.

Protein standard concentrations, in the range of 0-20 µg/100µl distilled water were prepared to obtain a standard curve. Samples (cell-free supernatant) were diluted to 500 µl with distilled water so that the protein content would be within the range of the standards. 1 ml Bradford reagent solution was added to 100 µl of sample within each tube and mixed well. The solutions were kept 2 minutes then absorbance was determined at 595 nm.

2.10.5. Purification of alkaline protease enzyme produced by the selected isolate

Purification of the protease enzyme was performed through ÄKTA fast protein liquid chromatography (FPLC) by Amersham Pharmacia Biotech. The HiPrep 16/10 FF-DEAE-Sepharose CL 6B column as an anion-exchange liquid chromatography technique was used for separation and purification of the protease with running buffer 20 mM glycine buffer (pH 9) and the elution buffer was 1.0 M NaCl in 20 mM glycine (pH 9). The flow rate was 1 ml/min with elution rate of 0 to 100%. The fraction size was 5.0 ml and the sample volume was 5 ml. The HiPrep 16/60 Sephacryl S-200 high resolution column as a gel filtration liquid chromatography technique was used for the separation and purification of the protease that pooled from the anion-exchange separation. The running buffer used was 20 mM phosphate buffer (pH 8.0) with 200 mM sodium chloride and the flow rate was1.0 ml/min with collecting fraction size of 5.0 ml [38]. The denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE) has been used according to the method of Laemmli (1970), with 12% polyacrylamide concentration. This technique was used for detecting the protease enzyme homogeneity and estimation of its molecular mass using protein molecular weight marker [38,39].

2.10.7. Characterization of the purified protease enzyme

The kinetic studies on protease obtained after gel filtration column chromatography was carried out in terms of optimum temperature, thermo-stability, optimum pH and pH stability, metal ions, detergents, organic solvents, inhibitors and chelating agents.

2.10.7.1. Effect of pH and temperature on enzyme activity

The effect of pH was determined at 37°C for 30 min. The casein hammarstein was prepared (0.65%, w/v) in over a range of pH values (6, 7, 8, 9, 10, 11 and 12) in 0.1M phosphate buffer solution (3.5.4.). The temperature effect was determined at different temperatures ranging (20 - 70°C), using a standard reaction mixture for casein by the crude enzyme. The absorbance was determined and relative activity was calculated under standard assay conditions [21].

2.10.7.2. Enzyme thermo-stability

The thermo-stability was determined by pre-incubating the purified enzyme at time intervals of 15, 30, 45 and 60 min. at 30, 40, 50 and 60oC and then quickly cooled. After each time intervals, the residual activity was determined under standard assay conditions (Casein- 0.1 M phosphate buffer solution pH 10 and 60oC) [19].

2.10.7.3. pH stability profile

The pH stability was determined by incubating equal volumes of the purified enzyme with 0.1 M phosphate buffer in the pH range 7-12 for 2 and 4 h and the residual activity (%) was determined by standard assay procedure (pH 10, 60oC) [40].

2.10.7.4. Effect of metal ions on enzyme activity

The effect of different metal ions (5 mM) on protease activity was determined by the addition of the corresponding ion at a final concentration of 1.0 mM to the reaction mixture, and the assay was performed under standard conditions. The tested ions included the following corresponding salts: NaCl, CaCl2, MgSO4, FeCl3, CoCl2, MnSO4, CdCl2, CuSO4, and ZnSO4. The activity is expressed as the percentage of the activity level in the absence of metal ion. The enzyme was pre-incubated with metal ion for 20 min. at room temperature [41,42].

2.10.7.5. Effect of inhibitors on enzyme activity

The effect of various protease inhibitors (5 mM) such as serine inhibitors; phenylmethylsulphonyl fluoride (PMSF) and β- mercaptoethanol and a chelator of divalent cations; ethylenediaminetetraacetic acid (EDTA) were determined by the addition of the corresponding inhibitors and chelators at a final concentration of 1.0 mM to the reaction mixture with 20 min. pre-incubation time and assayed under standard conditions [42]. Then, relative protease activity was measured.

2.10.7.6. Effect of detergents and solvents on enzyme activity

The effect of detergents on enzyme activity was studied using ionic and non ionic detergents (0.5 %) such as Tween 20, SDS and Triton 100. The effect of various solvents on enzyme activity was studied in order to find some valuable information about catalytic site of enzyme. The following solvents were added with reaction mixture at 0.5 % (v/v). The solvents used were ethanol, methanol, isopropanol, dimethyl sulfoxide (DMSO) and chloroform. The enzyme was pre-incubated with the detergent or the solvent for 20 min. at room temperature. The relative activity was measured. The activity of enzyme without any surfactants or organic solvents was taken as 100 % [43].

2.10.8. Effect of bacterial growth on wool-fibers

2.10.8.1. Evaluation of the physical properties of wool fibers

The selected strain was grown in the optimized modified APPB medium at 37oC with constant shaking 200 rpm, using wool fiber as carbon and nitrogen source. The supernatant as crude enzymes was used for wool treatment where wool fiber samples (5 cm long and 0.25 g weight) were incubated with the supernatant at 40, 50 and 60oC. Every 20 h, wool fibers were harvested, thoroughly washed with water and dried at 60oC to a constant weight. The physical properties of wool fiber were evaluated by the percentage of wool degradation calculated from the differences in residual dry weight before and after treatment [34]. The degree of shrinkage was calculated based on the original diameter of the fiber, prior to microbial treatment [44].

2.10.8.2. Electron microscopy study of wool surface

To study the effect of protease activity on wool surface, electron microscope was used to scan the surface of wild and treated wool (sample of 80 h, at 50°C and 60°C). Wild and treated wool fibers were fixed in the surface of flat glass slide and washed gently by distilled water for 3 sec. then allowed to dry at 37°C. The dry wool surface then was coated with approximately 15 nm gold (JFC- 1100E ion sputtering device). The golden coated sample was then subjected to be scanned by analytical scanning electron microscope (Jeol JSM-5300) with secondary element at 25 KV acceleration voltages at room temperature. The digital image were adjusted and saved [11,30].

3. Results and Discussion

3.1. Isolation of wool degrading bacteria and its proteolytic activity

Many bacterial strains were isolated from soil, sand and water samples from different Egyptian localities (from Borg El-Arab, Matrouh, Sohag and Alexandria cities). Some of the isolated bacteria were able to grow on wool fibers as a sole carbon and nitrogen source as reported in earlier studies by Amara and Serour (2008) [30]and Infante et al., (2010) [34]. Among these isolates, the one that exhibited high proteolytic activity on skimmed milk agar (SMA) plates, expressed as a qualitative diameter of clear zone (Figure 1), was selected. The staining method causes an appearance of clear contrast between the plate's background and the degradation zone around the wells. This technique gives a preliminary fast image of the ability of microorganisms to produce proteolytic enzymes [45].

Figure 1. Skimmed milk-agar well diffusion plate for studying the proteolytic activity of wool degrading enzymes.

The selected isolate growth was tested in Tap and Distilled water as compared to various types of wool-containing media. Extracellular protease activity levels increased in the case of APPB medium compared to other types of media; 180.7 U/ml (Table 1), indicating that wool was used as an inducer for protease production. Furthermore, low levels of proteolytic activity and an elongated period of wool degradation were observed when using tap and distilled water as compared to APPB medium (Table 1). The cost of the enzyme production from the minimal media (tap and distilled water) used in wool quality improvement is remarkably reduced. Furthermore, no excessive washing process is needed to remove the impurities of growth medium.

Table 1: Screening for a medium that promotes high levels of proteolytic activity by the selected isolate.

Media	Enzyme activity (U/ml)	Degradation period (day)
Tap water	125.27	4
Distilled water	75.05	5
APPB	**180.77**	**3**
MMF	91.97	5

3.2. Identification of the potent wool degrading bacterial isolate

Identification of the selected isolate AM12 was firstly confirmed using Gram staining method (Figure 2), amplification and sequence analysis methods [33,34]. Sequence similarities were accomplished using the NCBI database. The isolation of wool-degrading bacteria belonging to the genus Bacillus has been reported previously [46].

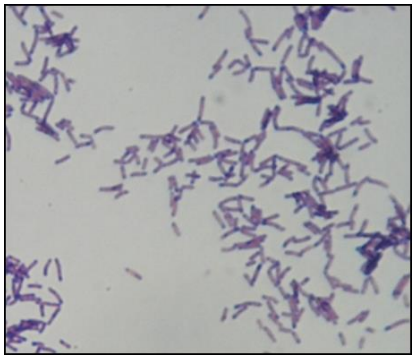

Figure 2. Gram staining for the wool degrading isolate AM12. Strain

The isolated strain was identified as Bacillus sp. AM12 with accession number on Genbank JX416707. Furthermore, molecular phylogenetic studies showed that the strain AM12 was a member of Bacillus group. A comparison of the DNA sequence with sequences in the NCBI database with BLAST software showed 100%

sequence identity with the published 16S rRNA gene sequence of closely related Bacillus species (Figure 3).

Bacteria belonging to the genus Bacillus are widely distributed and known for their ability to produce and secrete large amounts of industrially useful proteins [47].

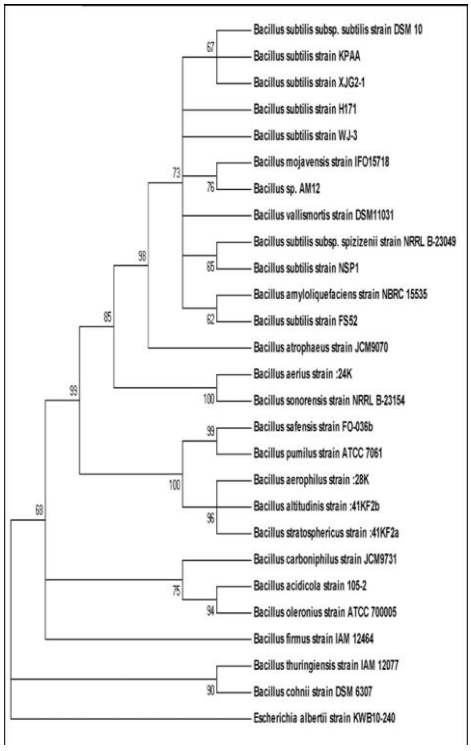

Figure 3. Phylogenetic position of Bacillus sp. AM12 within the genus Bacillus.

3.3. Effect of Temperature and pH on protease crude enzyme activity

The crude enzyme activity was optimized at different pH values and temperatures. The results showed that the enzyme is alkaline. The enzyme isolated from Bacillus sp. AM12 was identified as a typical alkaline protease, displaying its activity for casein predominantly in the alkaline pH 8.0 - 11.0 with an optimum pH 10 (Figure 4a) [48]. The enzyme has a temperature optimum at 60o C (Figure 4b). These results are in agreement with a previously reported strain of Bacillus sp. with an alkaline protease optimum temperature of 60º C [49]. These findings are also in accordance with several earlier reports showing pH and temperature optima 11 and 60ºC for protease of Bacillus sp. L21 [19], 9 and 60ºC for protease of Geobacillus sp. [30,31].

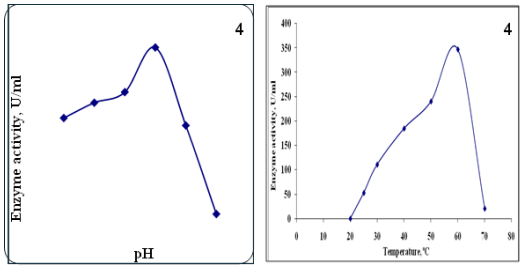

Figure 4. Effect of different pH values on crude protease enzyme activity at 60˚C in 0.1 M Casein-phosphate buffer solution after 30 min. of reaction (4a). Effect of different temperature values on crude protease enzyme activity at pH 7 in 0.1 M Casein- phosphate buffer solution after 30 min of reaction (4b).

3.4. Evaluation of effect of bacterial growth on wool- fibers

The effect of Bacillus sp. AM12 on wool-fiber shrinkage was evaluated after examining the enzymes activity on different substrates (skimmed milk, wool and casein), studying their wool degradation ability and optimizing their pH and temperature. Results showed that the effect of growth of Bacillus sp. AM12 on wool fibers at various temperatures (40, 50 and 60°C) results in loss of weight calculated by subtracting weight before treatment – weight after treatment but the fiber diameter did not change referring to the decrease of the shrinkage behavior of wool fibers as shown in Table 2.

Results showed that during the incubation of wool fibers with crude protease containing supernatant derived from wool degradation medium (modified APPB), the wool fibers lost weight, whereas no change in diameters and shrinkage at different temperatures after 80 h treatment was observed. Similar studies reported the same results of losing weight and decreasing the shrinkage behavior of wool fibers whereas no change in wool diameter could be detected during growth of B. thuringiensis L11 on minimal medium using wool as energy source [34].

Table 2: Effect of crude protease enzyme produced by Bacillus sp. AM12 on wool fibers degradation incubated at 40, 50 and 60°C.

Incubation Period (h)	Loss weight (g)			Wool degradation (%, w/w)		
	40°C	50°C	60°C	40°C	50°C	60°C
20	0.005	0.009	0.018	2	3.6	7.2
40	0.011	0.013	0.03	4.4	5.2	12
60	0.019	0.022	0.046	7.6	8.8	18.4
80	0.023	0.027	0.054	9.2	10.8	21.6

Scanning electron microscope analysis showed that protease of Bacillus sp. AM12 hydrolyzed the cuticles on the wool surface and thus perfectly improved the wool surface quality by smoothing the wool fibers' outer layer. The SEM micrographs of untreated and different treated wool fibers presented in (Figure 5) confirmed significant differences in the effectiveness of temperature effect on the enzyme. The smoothing of the wool fibers process against time and different temperatures gives the best results. An interesting effect is that somehow the enzyme "cracked" the wool surface [50].

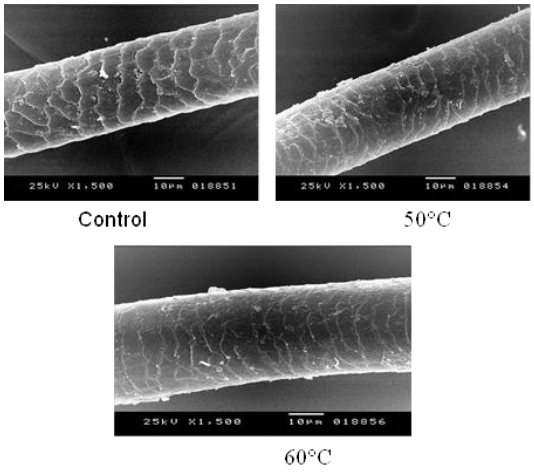

Figure 5. SEM micrographs of native and treated wool fibers using crude proteases of Bacillus sp. AM12 at 50°C and 60°C.

3.5. Purification of protease enzyme by liquid chromatography

Protein content and protease activity measured in the culture filtrate of Bacillus sp. AM12 isolate grown on the modified APPB medium were 322.4 mg and 678.60 U/ml, respectively, with a specific activity of 2.10 U/mg.

Using the DEAE anion-exchange technique for protein purification there were protein and protease activity peak I (Figure 6) with an estimation of the protein content and protease activity in the pooled fractions (12, 13, 14 and 15) found to be 12.8 mg and 48.3 U/ml, respectively. The fractions of peak I on the DEAE-Sepharose column were collected and applied on a Sephacryl S- 200 column. Only one strong protease activity peak was detected with protein content and protease activity 29.6 U/ml and 12.8 mg/ml respectively (Figure 6). Thus, continuing purification using gel-filtration technique resulted in a good purification fold of the enzyme compared to the separation process by anion-exchange technique as shown in (Table 3). The purification-fold rises to 2.66 fold which shows that such separation technique was an excellent method for protease purification.

Table 3: Purification of alkaline protease enzyme from Bacillus sp. AM12.

Fraction	Activity (U/ml)	Protein (mg/ml)	Spec activity (U/mg)	Purification (fold)	Recovery (%)
Cultural supernatant (filtrate)	678.6	322.4	2.1	1	**100**
Anion exchange chromatography	48.3	12.8	3.7	1.76	**7**
Gel filtration chromatography	**29.6**	**5.3**	**5.6**	**2.66**	**4.3**

The final purification step presented 2.66-fold enzyme purification with a specific activity of 5.6 U/mg. These results indicated the effectiveness of the purification method. Guangrong et al., (2006)[21] reported similar results of purification folds and yields in the purification chromatography steps (Sepharose: 3.18-fold and 12.7% yield, while Sephacryl: 4.25-fold and 5.1% yield, respectively).

The fractions of the protease activity peaks were collected and run in an SDS-PAGE gel. A single band was obtained with a molecular mass of approximately 24 kDa (Figure 7). SDS-PAGE was conducted to determine the molecular weight of the protease enzyme. By comparing the protease molecular weight of the organism under investigation with the protein marker, an estimation molecular weight was found to be 24 kDa as shown in Figure 7.

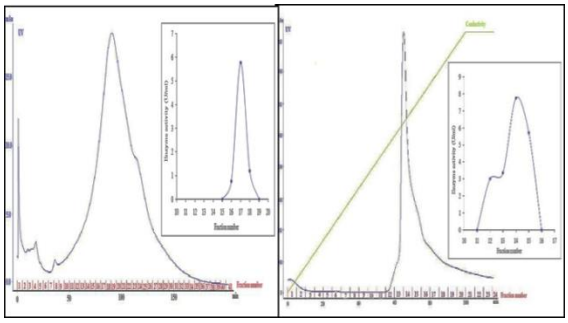

Figure 6. Profile of FPLC- anion exchange chromatography of crude supernatant of strain AM12. Profile of FPLC- gel filtration chromatography of pools fractions (12, 13, 14 and 15) from the anion exchange FPLC.

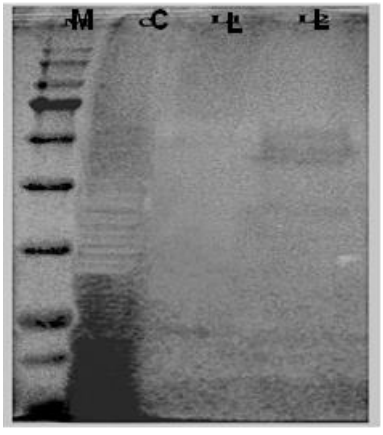

Figure 7. Protein profile of each purification step on SDS-PAGE (12% gel gradient). Lane M: protein Marker; lane C: crude supernatant of the strain AM12; Lane L1: purified protease from FPLC- HiPrep 16/60 Sephacryl S-200 high resolution column; lane L2: pool of fractions 12, 13, 14 and 15 of FPLC- HiPrep 16/10 FF-DEAE-Sepharose CL 6B column.

3.6. Characterization of the purified protease enzyme

The kinetic studies on protease obtained after gel filtration column chromatography was carried out in terms of optimum temperature, thermo-stability, optimum pH and pH stability, metal ions, detergents, organic solvents, inhibitors and chelating agents.

The protease from Bacillus sp. AM12 was active over a broad temperature range of 25-60°C (Figure 8). The optimum temperature for protease activity was found to be 60°C and retained about 5-8% of the original activity at 70°C. The heat stability of protease enzymes is affected by at least two factors individually or in combination. The first one is the primary structure of the enzyme. A high content of hydrophobic amino acids in the enzyme molecule provides a compact structure, which is not denatured easily by a change in the external environment [51]. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation [4]. Results also showed that the enzyme is active over a broad pH range of 7-11 (Figure 8). The optimum pH for protease activity was found to be 10 and retained about 4.5% of the original activity at pH 12. As shown in Figure 8, after 2 h, the enzyme was very stable at a broad pH range, maintaining over 70-87% of its original activity (between pH 7 and 10).

This activity decrease with about 22% of the initial activity being retained at pH 11, and then declined sharply at pH 12. After 4 h, the enzyme was stable maintaining 34-48% of its original activity (between pH 7 and 10), but declined sharply with about 5% activity remaining at pH 11, then no more activity was remarked at pH 12. The optimum pH range of Bacillus alkaline proteases is generally between pH 9 and 11, with a few exceptions of higher pH optima of 11.5 [52]. The protease activity was enhanced with an addition of 1.0mM $CaCl_2$ resulting in the relative activity of 103.70% and slightly inhibited by the addition of 1.0mM $ZnSO_4$, $CdCl_2$, $MnSO_4$, $CuSO_4$, NaCl and $MgSO_4$ resulting in the relative activity of 97.30, 93.90, 93.34, 91.40, 90.70 and 90.42%, respectively. These results suggest that metal ions (Ca^{+2}) had a capability to protect enzyme against denaturation [53]. The effect of different inhibitors on the protease enzyme activity was studied (Figure 8). Of the inhibitors tested (at 1.0 mM concentration), PMSF was able to inhibit the protease considerably, while ß-mercaptoethanol and EDTA exhibited 84 and 21% inhibition, respectively. The enzyme activity was (109.0 %) with Triton x 100 and SDS and (105.5%) with Tween 20 (Figure 8). The extracellular production of the enzyme, its thermostable nature and compatibility with most commercial detergents are features that suggest its application in detergent industry [54].

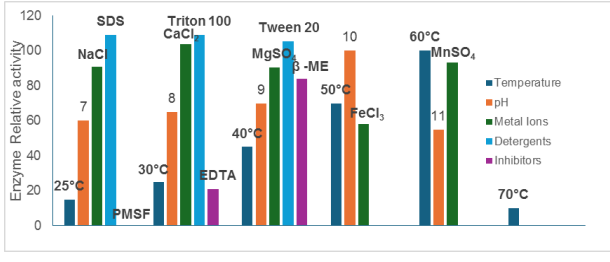

Figure 8. Characterization of the Purified enzyme.

4. Conclusion

This study successfully isolated and characterized a thermostable protease from Bacillus sp. AM12. The enzyme demonstrated its efficacy in wool degradation with optimal activity at pH 10 and 60°C and stability across a broad pH range (7–11) providing more than 80% activity at temperatures between 30–70°C. The purified enzyme maintained a specific activity of 5.6 U/mg, with a molecular weight of 24 kDa. Furthermore, the enzyme showed strong compatibility with commercial detergents, reinforcing its potential for industrial applications. These findings highlight Bacillus sp. AM12 protease as a promising biocatalyst for eco-friendly wool processing and detergent formulations, offering a sustainable alternative to harsh chemical treatments.

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